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Antibody diversification caused by disrupted mismatch repair and promiscuous DNA polymerases

Kimberly J. Zanotti and **Patricia J. Gearhart***

Laboratory of Molecular Biology and Immunology, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224, USA

Abstract

The enzyme activation-induced deaminase (AID) targets the immunoglobulin loci in activated B cells and creates DNA mutations in the antigen-binding variable region and DNA breaks in the switch region through processes known, respectively, as somatic hypermutation and class switch recombination. AID deaminates cytosine to uracil in DNA to create a U:G mismatch. During somatic hypermutation, the MutSα complex binds to the mismatch, and the error-prone DNA polymerase η generates mutations at A and T bases. During class switch recombination, both MutSα and MutLα complexes bind to the mismatch, resulting in double-strand break formation and end-joining. This review is centered on the mechanisms of how the MMR pathway is commandeered by B cells to generate antibody diversity.

Keywords

activation-induced deaminase; class switch recombination; DNA polymerase η; mismatch repair; somatic hypermutation

1. Introduction to AID and canonical DNA repair

Cells have evolved multiple pathways to maintain genomic integrity. These pathways include mismatch repair (MMR) to correct DNA replication errors, base excision repair (BER) and nucleotide excision repair to mend base damage from genotoxic agents, and translesion synthesis to bypass lesions. In most cells, these pathways work to efficiently remove DNA mispairs and damaged bases, and faithfully restore DNA to its original sequence. However, B cells use the MMR and BER pathways to generate DNA mutations as part of the antibody diversification process. Initially, the antibody repertoire is created in pre-B cells by the recombination of immunoglobulin (Ig) V(D)J (variable, diversity, joining) gene segments, and by the pairing of heavy and kappa or lambda light chains [1]. The antibody pool is subsequently expanded in mature B cells upon antigen exposure. These

* Corresponding author: Dr. Patricia Gearhart; Laboratory of Molecular Biology and Immunology, National Institute on Aging, National Institutes of Health, 251 Bayview Boulevard, Baltimore, MD 21224, USA. Tel.:+1 410 558 8561; fax: +1 410 558 8386. gearhartp@mail.nih.gov.

antigen-activated B cells undergo further diversification through somatic hypermutation (SHM) of rearranged variable genes and class switch recombination (CSR) of heavy chain constant genes, both of which are initiated by the enzyme activation-induced deaminase (AID) [2].

AID was discovered by Honjo and colleagues in 1999 [3], and was shown to be a member of the mRNA-editing APOBEC protein family [4]. However, further work by Neuberger and others revealed that AID acts upon DNA [5-7], where it deaminates cytosine to uracil in single-strand regions of DNA formed during transcription [8]. The protein is highly expressed in germinal centers from spleen, lymph nodes, and Peyer's patches [3, 9]. AID features an 11 amino acid C-terminal recognition loop, LYFCEDRKAEP, that favors binding to the C in WGCW (where W = A or T) sequence hotspots and deaminates both DNA strands [10-14]. The complete mechanism behind the upregulation and targeting of AID activity exclusively to the Ig loci is currently unknown, although enhancer regions and RNA polymerase II pausing are believed to play major roles [15-18]. AID targeting must be strictly regulated, because deaminations in non-Ig genes can generate translocations that lead to the development of diseases such as B-cell lymphomas [19]. The AID-induced U:G mismatch will mimic T:G, resulting in a C:G to T:A transition following DNA replication of the uracil. Alternatively, the improper uracil either can be repaired via canonical repair pathways, or can employ disrupted repair and translesion polymerases (pol) to generate antibody diversification by SHM and CSR.

As discussed in more detail elsewhere in this issue [20], DNA repair of base damages, including mismatched uracils, relies on the MMR and BER pathways [21, 22]. Canonical MMR uses a heterodimer complex formed by either MutSα, consisting of MSH2 and MSH6, or MutSβ, formed by MSH2 and MSH3, to recognize and bind to mismatches. MutSα targets single nucleotide mismatches, while MutSβ targets loops formed by inserts, deletions, and multi-base mispairs. A MutL heterodimer, containing either MLH1 and PMS2 (MutLα), MLH1 and PMS1 (MutLβ), or MLH1 and MLH3 (MutLγ) is then recruited to the mismatch. This review will emphasize MutLα, as the other MutL complexes may not be involved in the immune response [23]. MutLα introduces a nearby nick that acts as an exonuclease entry point. Exonuclease 1 (EXO1) removes the mismatch and adjacent bases, creating a single-strand gap. The PCNA sliding clamp recruits a high-fidelity DNA pol, such as pol δ or ε, to accurately resynthesize the gap, followed by DNA ligase I to seal the freshly-repaired strand. Alternatively, BER uses uracil DNA glycosylase (UNG) to remove rogue uracils. This leaves behind an abasic site, which is then cleaved by an apurinic/apyrimidinic endonuclease (APE), producing a single-strand break. Pol β excises the 5’ deoxyribose phosphate group and inserts the correct base, and DNA ligase III closes the nick.

Although canonical DNA repair is desirable under most circumstances, a significantly altered process ensues in B cells during antibody development. In a mechanism initially proposed by Neuberger [24], adjustments to the MMR pathway can introduce mutations at A and T bases, while a modified BER pathway is responsible for generating mutations at C and G bases. MMR and BER proteins also participate in switching between constant genes

during CSR. This review focuses on how the MMR pathway is manipulated by B cells to generate antibody diversity.

2. MutSα complex generates A:T mutations during SHM

2.1. Pathways responsible for creating Ig variable region diversity

Antibodies contain both a rearranged V(D)J gene, which regulates antigen binding, and a constant gene, which determines isotype. Upon antigen exposure, B cells in germinal centers undergo successive rounds of SHM [25]. Mutations occur in two regions of DNA: (1) the variable region containing the rearranged VDJ or VJ gene, and (2) the switch region preceding each constant gene. Mutations start just downstream of transcription start sites in the promoter (variable region) and intronic enhancer (switch region), indicating that transcription is necessary for AID activity [26, 27]. These mutations typically occur as single base substitutions and form at an elevated frequency of 10^{-2} mutations/bp, compared to spontaneous mutation in other loci, which occurs at a frequency of 10^{-8} mutations/bp [28]. The result of SHM in variable regions is increased affinity of the antibody for antigen, and the result of SHM in the switch region is increased double-strand breaks for CSR from IgM to IgG, IgA, and IgE. The absence of AID in humans leads to type II hyper-IgM syndrome [29], where individuals are at increased risk of disease because they can only produce low affinity antibodies of the IgM isotype. Contrary to intuition, deficiencies in MMR proteins actually lead to decreased mutagenesis in variable and switch regions [30, 31]. This occurs because SHM relies on a hijacked version of the MMR pathway to create mutations at A and T residues [32] (Figure 1).

The MutSα heterodimer binds to an AID-induced U:G mismatch and recruits a nick-creating nuclease. The identity of the nuclease is unknown, but it is unlikely to be the MutLα complex employed in canonical repair; possible suspects are examined in more detail in *2.2*. This nuclease acts along with EXO1 to remove the mismatch and adjacent bases. Monoubiquitinated PCNA then encircles the gap and binds error-prone DNA pol η, which favors synthesis of mutations opposite A and T nucleotides, to fill in the gap. It is not known what cellular signals allow SHM to proceed in place of canonical MMR.

An analogous SHM process removes uracils in DNA via a distorted BER pathway [2]. UNG recognizes the uracil and employs its glycosylase activity to create an abasic site. APE creates a nick at the abasic site, which is filled in by Rev1 to create mutations at C and G bases. *Ung*$^{-/-}$ mice have a normal mutation frequency but decreased C:G transversions, while A:T mutagenesis is unchanged [33]. Other uracil glycosylases such as single-strand-selective monofunctional uracil-DNA glycosylase (SMUG1) [34, 35] and methyl-CpG binding domain 4 [36, 37] are not involved in generating abasic sites for SHM under normal physiological conditions.

Recent studies have also examined the roles of APE1 and APE2 endonucleases [38]. APE1 is highly expressed in resting or in vitro activated B cells, but is poorly expressed in germinal centers. APE1-haploinsufficient mice do not have an appreciably altered SHM frequency or spectrum. In contrast, APE2 is highly expressed in germinal centers, and APE2-deficient mice have a 50% decline in overall mutagenesis and a slight decline in

mutations at A:T bases [38, 39]. Mice that are doubly-deficient for UNG and APE2 exhibit an additional 2-fold decline in A:T mutations. These unexpected results suggest that APE2 may have a role outside of the BER pathway, and could interact with additional glycosylases or MMR components. Alternatively, the doubly-deficient cells may have severely impaired cell cycle progression [40], which provides additional time to undergo faithful DNA repair.

2.2. Contribution of individual MMR components to SHM

Many details of the MMR-SHM pathway have been elucidated in knockout or mutant mouse models [32, 41] (Table 1). *Msh2*$^{-/-}$ [42, 43] and *Msh6*$^{-/-}$ [44, 45] mice have reduced mutation frequencies in variable regions relative to wild type mice, and their counterparts containing an inactivated ATPase domain feature a similar but less severe phenotype [46, 47]. The MutSα-deficient mice exhibit an altered mutation spectrum, with a 75-90% decrease in mutations at A:T bp [44, 45, 48]. A marked decline in A:T mutagenesis has also been observed in MSH6-deficient humans [49]. *Exo1*$^{-/-}$ mice have decreased SHM and mutations at A:T bp [50, 51]. An initial report indicated that an *Exo1*$^{E109K/E109K}$ knock-in mouse was catalytically inactive but underwent standard SHM, suggesting that separate exonuclease structural and functional roles exist [51]. However, recent studies have countered that EXO1-E109K is a fully functional protein *in vitro*, and that EXO1 enzymatic function is required for its interactions with MMR proteins [52, 53].-

Mice that are doubly-deficient for MSH2 and MSH6 still exhibit the signature A:T mutation decline but have a large increase in C:G transitions, leading to an overall mutation frequency similar to wild type mice [54]. This may occur due to unrepaired uracils persisting in the DNA and being replicated directly across as C:G transitions. In contrast, deficiencies in MLH1 [55], MSH3 [44, 45], or PMS2 [42, 56] do not alter mutation spectrum, indicating that they are not essential for SHM via the MMR pathway. This stands in contrast to canonical MMR, where the MutL heterodimer is mandatory for DNA repair. Triple knockout *Msh2*$^{-/-}$*Msh3*$^{-/-}$*Msh6*$^{-/-}$ mice likewise do not show additional changes in mutation frequency or spectrum when compared to respective double knockouts [54].

Interactions of other MMR factors with the MutSα complex can have substantial effects on SHM. Post-translational modification of the PCNA sliding clamp is of special interest because it helps regulate the choice between error-free repair and error-prone SHM. PCNA that is polyubiquitinated at lysine 63 elicits a high-fidelity repair pathway [57, 58], while monoubiquitination at lysine 164 triggers SHM [59, 60]. Although the *Pcna*$^{-/-}$ genotype is embryonic lethal in mice, a *Pcna*^{K164R} model was generated that cannot undergo monoubiquitination, and it exhibited a 90% decrease in A:T mutations [61, 62]. The remaining A:T mutations may be introduced through the activity of UNG and/or pol ζ, as discussed later in this manuscript. PCNA deubiquitination by USP1 [63, 64] also likely regulates SHM, although its role has not been extensively analyzed.

A major question that still remains concerning the MMR pathway in SHM is the identity of the nuclease responsible for providing the nick required prior to EXO1 activity. Nucleases that have been hypothesized to cause nick formation include PMS2 and APE. The MutLα complex may seem like a logical choice, as it interacts with MutSα during canonical MMR and its PMS2 subunit contains latent endonuclease activity [65, 66]. However, *Pms2*$^{-/-}$ mice

have a normal SHM frequency [42, 56, 67], indicating that either PMS2 is not the major nuclease or that compensatory nucleases can act efficiently in its absence. Neuberger and Rada proposed that the glycosylase component of SMUG1 could serve as the nick instigator by creating an abasic site that is then cleaved by APE [34, 68]. Although SHM is unaltered in SMUG1-deficient mice, a small but consistent decrease in A:T mutations occurs in an *Ung*$^{-/-}$*Smug1*$^{-/-}$ background. Additional studies will be necessary to identify the endonuclease involved in making nicks for MMR-SHM.

2.3. Generation of A:T mutations by pol η

Pol η is responsible for the majority of mutations at A:T bp during SHM [69, 70]. Pol η is a γ family translesion polymerase encoded by the *Polh* gene. It inserts nucleotides opposite adducts, including UV-caused cyclobutane pyrimidine dimers and cisplatin-generated crosslinks, via short-patch synthesis [71]. However, during SHM, pol η exhibits promiscuous fidelity when copying A and T bases on undamaged DNA [72]. Pol η has been shown to be catalytically activated upon binding to the MutSα heterodimer *in vitro* [73]. Pol η prefers to insert G opposite T on the transcribed strand, leading to A bases being mutated twice as frequently as T in variable and switch regions [74]. *Polh*$^{-/-}$ mice display an 85% reduction in A:T mutations, although the overall SHM frequency remains constant due to overcompensation by C:G mutations generated in the UNG pathway [75]. A similar alteration in mutation spectrum occurs in humans diagnosed with xeroderma pigmentosum variant syndrome, which is caused by defective pol η and results in a 4-fold decrease in A:T mutations [74, 76-78].

Although both *Polh*$^{-/-}$ and *Msh2*$^{-/-}$ or *Msh6*$^{-/-}$ mice display reduced A:T mutations, a distinct difference occurs in their respective mutation locations. *Msh2*$^{-/-}$ or *Msh6*$^{-/-}$ mice exhibit mutations targeted to AID-favored WGCW sequence hotspots, while *Polh*$^{-/-}$ mice have mutations distributed across the entire variable region [24, 69, 72, 75, 79]. MutSα is responsible for the recruitment of downstream proteins that introduce a single strand gap at the mutation site; in the absence of either of its components, mutations are targeted directly at the uracil site. Meanwhile, a deficiency of pol η does not interfere with the formation of a multi-base gap, allowing for mutations to be spread across a wider region and not focused on sequence hotspots.

The background A:T mutations present in *Polh*$^{-/-}$ individuals must be attributable to an additional promiscuous polymerase(s). Deficiencies in the non-replicative pols ι [80, 81], β [82, 83], λ [83, 84], μ [84], or θ [85] result in unchanged SHM. Pol ζ tends to insert tandem mutations [86, 87], while Rev1 undertakes the majority of G:C to C:G transversions via a distorted BER pathway [88-90]. The polymerase that creates G:C to T:A transversions is currently undefined. The remaining A:T mutations in *Polh*$^{-/-}$ mice typically consist of 50% T to G and A to C transversions, which matches the mutational spectrum of pol κ [69, 91, 92], although *Polk*$^{-/-}$ mice exhibit a normal SHM frequency and spectrum [93].

The polymerase responsible for generating the residual A:T mutations may be masked in the presence of pol η, so doubly-deficient mice were bred. *Polh*$^{-/-}$*Polk*$^{-/-}$ mice showed a 93% reduction in A:T mutations relative to wild type, indicating that pol κ could contribute to mutagenesis in the absence of η [94]. It remains unclear what other polymerase(s) is

responsible for the lingering A:T mutations in *Polh*$^{-/-}$ *Polk*$^{-/-}$ mice, although recent work suggests pol ζ may be involved. *Msh2*$^{-/-}$ and *Msh6*$^{-/-}$ mice both exhibit a decrease in ζ-induced tandem mutations, whereas the frequency of contiguous tandem mutations was unaffected in *Ung*$^{-/-}$ mice [86]. Pol ζ does not have a preferred mutation pattern and may be able to insert mutations at A:T in a *Polh*$^{-/-}$*Polk*$^{-/-}$ environment.

Interestingly, Reynaud and colleagues showed that *Msh2*$^{-/-}$*Polh*$^{-/-}$ mice have completely abolished A:T mutations [94]. This strongly suggests that the residual mutations present in the *Msh2*$^{-/-}$ or *Msh6*$^{-/-}$ mice are created through the activity of pol η in the UNG pathway, perhaps through a long-patch BER mechanism. This hypothesis is further supported by the mutational spectrum of *Ung*$^{-/-}$*Msh2*$^{-/-}$ or *Ung*$^{-/-}$*Msh6*$^{-/-}$ mice, which likewise lack mutations at A:T bases [24, 95, 96]. In summary, Pol η is clearly required for A:T mutagenesis in a normal physiological context.

3. MutSα and MutLα complexes assist in formation of switch junctions during CSR

An antibody's heavy chain constant region is directly involved in regulating its trafficking and binding to cellular receptors. Eight different isotypes are encoded in the murine *Igh* locus. Germline antibodies are exclusively IgM or IgD, but other isotypes are expressed through CSR in activated B cells [97]. In CSR, two switch region double-strand breaks are recombined, and this results in a change in antibody isotype, e.g., from IgM to IgG1. Switch regions are 3-9 kb long, and contain abundant WGCW hotspots alongside clusters of C bases on the transcribed strand, which allow for the formation of stable RNA-DNA hybrid structures during transcription. Thus, the DNA sequence of the switch regions promotes single-strand DNA for AID to bind the multiple hotspots and initiate deamination. This generates a profusion of uracils, which can be processed to create double-strand breaks for CSR.

BER proteins produce the majority of double-strand breaks. *Ung*$^{-/-}$ mice have a 95% reduction in CSR [33], while APE deficiency has a less prominent effect [39, 98]. Nearby BER-induced nicks on opposite strands simulate a double-strand break, which catalyzes recombination with double-strand breaks in other switch regions for CSR [99].

Alternatively, uracils may be spaced too far apart to permit spontaneous double-strand break formation, and instead require the assistance of MMR proteins (Figure 2). This idea is supported by data demonstrating significantly reduced double-strand breaks in the switch regions of MMR-deficient B cells [100]. Additionally, the isotype most severely affected by the deletion of MMR proteins is IgG2a, which contains less AID hotspots than the other isotypes [101].

Whenever MMR and BER proteins are both involved in CSR [99, 102], one AID-induced uracil is processed conventionally by UNG and APE, resulting in a single-strand nick. A second, non-proximal uracil on the opposite strand is recognized by MutSα and MutLα. These proteins recruit EXO1 and PCNA, which bind to a nick generated by an undefined nuclease. EXO1 will excise one strand of DNA between the two nicks, and can act in either

the 5’ → 3’ or 3’ → 5’ direction depending on the relative position of the nicks. The resulting overhang can be filled in by a PCNA-translesion polymerase complex or removed by a flap endonuclease or EXO1. This creates double-strand break substrates for CSR.

These double-strand DNA breaks are usually repaired through non-homologous end joining (NHEJ), which uses either blunt ends or ends with short microhomology [103]. Alternative-end joining featuring longer microhomologies at the site of joining can also occur, but is commonly associated with nonproductive intra-switch recombination [104, 105]. The cell cycle phase also regulates break resolution by CSR. NHEJ is active throughout the cell cycle, while alternative-end joining favors late S or G2 phase [106, 107]. Since AID-induced double-strand breaks occur mainly during the G1 phase [100], break resolution generally proceeds through NHEJ.

A role for many MMR factors in CSR has been elucidated by examining changes in knockout mouse models (Table 1). Mice that are deficient for MSH2 [43, 101, 108, 109], MSH6 [45, 110], MLH1 [101, 109], or PMS2 [56, 101] all exhibit a 50% or greater reduction in switching in spleen cells stimulated *ex vivo*, or in serum *in vivo*. The ATPase domain of MSH2 [46], MSH6 [47], or MLH1 [111] is likewise utilized; mice with inactivated ATPase sites experienced decreased CSR. The MSH2 and MLH1 ATPase mutant mice possessed additional changes in microhomologies, suggesting that their ATPase activity is a prerequisite for efficient CSR. MSH3 has also been examined; deletion of the protein does not alter CSR, similar to its noninvolvement in SHM [45, 110].

Many other factors that interact with the MMR proteins have been tested for their involvement in CSR. $Exo1^{-/-}$ mice show a 70% decline in CSR and altered microhomology similar to $Msh2^{-/-}$ [50], implying that both proteins are involved in break formation. Monoubiquitinated PCNA may play a role, but conflicting results have been reported in independently derived $Pcna^{K164R}$ mouse models [60, 61]. Although pol η is required for the MMR pathway during SHM, it is not involved in CSR [75]. Future studies will help to extract more information concerning what other cellular interactions oversee CSR.

4. Conclusion and future directions

SHM and CSR are unique in that they commandeer seemingly faithful repair pathways and instead use them to create mutations and strand breaks. Much work remains to be done to determine how B cells choose to respond to AID-induced uracils. Recently published studies suggest that AID is targeted to the Ig locus through its interactions with a combination of transcription complexes and specific sequence elements [112, 113]. Once the uracil is formed, cells must decide whether to undergo repair or mutagenesis using either BER or MMR components. It is unclear whether MutSα and UNG work together or compete to manage uracils. Other poorly understood issues include why mutagenesis occurs in both variable and switch regions, while double-strand breaks are exclusive to switch regions, and why certain proteins participate in one process but not the other.

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Abbreviations

AID	activation-induced deaminase
APE	apurinic/apyrimidinic endonuclease
BER	base excision repair
bp	base pair
CSR	class switch recombination
D	diversity gene segment
EXO1	exonuclease 1
Ig	immunoglobulin
J	joining gene segment
pol	polymerase
MMR	mismatch repair
NHEJ	nonhomologous end joining
SHM	somatic hypermutation
SMUG1	single-strand-selective monofunctional uracil-DNA glycosylase
UNG	uracil DNA glycosylase
V	variable gene segment

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Highlights

- Immunoglobulin diversification utilizes mismatch repair proteins at U:G mismatches.
- MutSα and DNA polymerase η generate mutations during somatic hypermutation.
- MutSα and MutLα regulate class switch recombination.

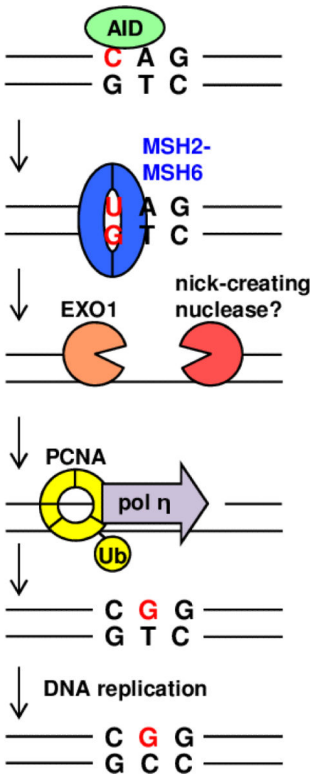

Fig. 1. MMR proteins create mutations at A:T bp during SHM

AID deaminates cytosine to uracil in immunoglobulin variable region DNA and generates a U:G mismatch that is recognized by the MSH2-MSH6 heterodimer. A single-strand DNA gap is produced at the mismatch by EXO1 and an unknown nick instigator. Monoubiquitinated PCNA homotrimer recruits the error-prone DNA pol η to fill in the gap, copying the original T with a G instead of an A. DNA replication results in the G:T mismatch being permanently affixed in one of the two daughter cells as a mutation to G:C.

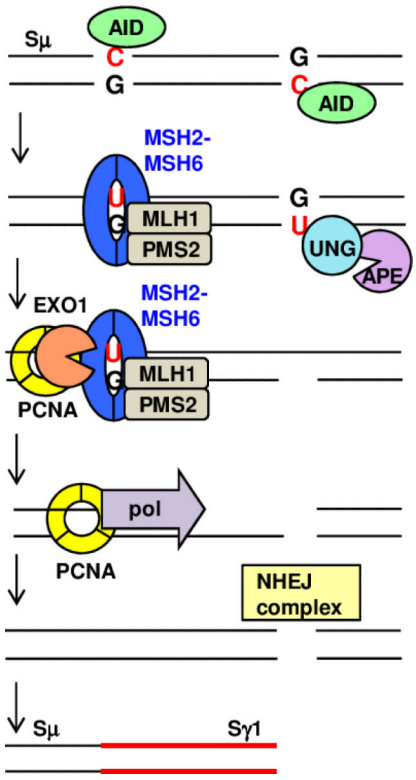

Fig. 2. MMR proteins assist in the conversion of BER-induced single-strand nicks to double-strand break substrates for CSR

AID-induced uracils in immunoglobulin switch region DNA can be bound by either BER or MMR proteins. In BER, UNG and APE remove the uracil and create a single-strand nick. MMR proteins MSH2-MSH6 and MLH1-PMS2 encounter a uracil on the opposite strand and attract EXO1 and PCNA to an adjacent nick. EXO1 excises the sequence between the nicks, leading to a 5' overhang that is processed by PCNA and a translesion polymerase to create a blunt double-strand break. Alternatively, the overhang can be deleted by EXO1 or a 5' flap endonuclease. An analogous process can occur with MMR proteins acting on a uracil located 3' to the BER-induced nick (not shown). When double-strand breaks occur simultaneously in different switch regions, recombination produces a new switch junction mediated by NHEJ factors, as illustrated here between Sμ and Sγ1.

Table 1

MMR mutant mouse models in antibody diversification. Y, yes; N, no.

MMR defect	Decreased SHM?	Decreased mutations at A:T?	Decreased CSR?	Notes	References
Msh2$^{-/-}$	Y	Y	Y		[42, 43, 48, 101, 108]
Msh2^{G674A}	Y	Y	Y	inactive ATPase	[46]
Msh6$^{-/-}$	Y	Y	Y		[44, 45, 110]
Msh6^{T1217D}	Y	Y	Y	inactive ATPase	[47]
Mlh1$^{-/-}$	N	N	Y		[55, 101, 109]
Mlh1^{G67R}	N	N	Y	inactive ATPase	[111]
Pms2$^{-/-}$	N	N	Y		[42, 56, 67, 101, 114]
Pms2^{E702K}	N	N	Y	inactive and unstable endonuclease	[66]
Msh3$^{-/-}$	N	N	N		[44, 45, 110]
Exo1$^{-/-}$	Y	Y	Y		[50, 51]
Exo1^{EK}	N	N	N	conflicting enzymatic activity	[51][52, 53]
Ape1$^{+/-}$	N	N	Y		[38, 98]
Ape2$^{y/m}$	Y	Y	Y?	conflicting CSR	[38, 39, 98]
Pcna^{K164R}	Y	Y	Y?	no monoubiquitination, conflicting CSR	[60-62, 115]
Polh$^{-/-}$	N	Y	N		[75-77, 94, 116]
Polk$^{-/-}$	N	N	N	backup for pol η	[93, 94, 117]
Ung$^{-/-}$	N	N	Y	base excision repair	[33], [118]