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# A role for Mlh3 in somatic hypermutation

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ABSTRACT

Somatic hypermutation (SHM) and class switch recombination (CSR) allow B cells to make high affinity antibodies of various isotypes. Both processes are initiated by activation-induced cytidine deaminase (AID) to generate dG:dU mismatches in the immunoglobulin genes that are resolved differently in SHM and CSR to introduce point mutations and recombination, respectively. The MutL homolog MLH3 has been implicated in meiosis and DNA mismatch repair (MMR). Since it interacts with MLH1, which plays a role in SHM and CSR, we examined these processes in *Mlh3*-deficient mice. Although deficiencies in other MMR proteins result in defects in SHM, *Mlh3*$^{-/-}$ mice exhibited an increased frequency of mutations in their immunoglobulin variable regions, compared to wild type littermates. Alterations of mutation spectra were observed in the Jh4 flanking region in *Mlh3*$^{-/-}$ mice. Nevertheless, *Mlh3*$^{-/-}$ mice were able to switch to IgG3 or IgG1 with similar frequencies to control mice. This is the first instance where a loss of a DNA repair protein has a positive impact on the rate of SHM, suggesting that Mlh3 normally inhibits the accumulation of mutations in SHM.


## 1. Introduction

Somatic hypermutation (SHM) and class switch recombination (CSR) generate antibodies that have the affinity and range of effector functions required to protect us from bacteria, viruses, and toxins. In SHM, the variable (V) regions of heavy and light chain genes encoding the antigen-binding site are heavily mutated (reviewed in [1–3]). In CSR, the $\mu$ constant region of the heavy chain is replaced by one of the downstream $\gamma$, $\varepsilon$, and $\alpha$ constant regions via intrachromosomal recombination between the donor and recipient switch regions [2,4]. This allows each V region to be expressed with many different constant regions and to carry out different effector functions.

Activation-induced cytidine deaminase (AID), an essential molecule for both SHM and CSR [5,6], initiates these two processes by deaminating deoxycytidine (dC) in single stranded DNA [7–11] to generate a dG:dU (G:U) mismatch. AID itself [10], or in association with replication protein A (RPA) [12] and perhaps other proteins, has been shown in biochemical assays to preferentially deaminate dC in WRC (W=A/T, R=A/G)

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motifs [10,12]. Once a G:U mismatch is generated, uracil-N-glycosylase (UNG) and mismatch repair (MMR) proteins, along with other DNA repair proteins such as non-homologous end joining (NHEJ) proteins and error-prone polymerases, compete to repair the mismatch [13]. For example, the resulting dU can be fixed by replication to produce transition mutations (C–T or G–A), or removed by UNG to create abasic sites that can then be bypassed by error-prone DNA polymerases to generate transition or transversion mutations at G:C bases. These paths have been called the first phase of SHM. Alternatively, the G:U mismatches are recognized by a heterodimer of the MutS homologues Msh2 and Msh6 to initiate repair with the involvement of MutL homologues Mlh1 and Pms2 [2]. The DNA strand containing the G:U mismatch is thought to be incised by an unknown endonuclease, digested by exonuclease Exo1 [14], and re-synthesized by the error-prone translesional polymerase eta (pol η) [15–19] and probably by other error-prone polymerases [20,21]. This results in the introduction of mutations at A:T bases that in vivo constitute approximately half of the mutations found in Ig V regions and this has been called the second phase of SHM [22]. UNG and MMR proteins may also use alternative pathways to process G:U mismatches in CSR [13], although the precise molecular mechanisms are not well understood. In particular, although MMR deficiency leads to decreases in CSR and changes in the sites of recombination, the exact biochemical roles of each of the MMR proteins in CSR are still unclear (reviewed in [2]). It is conceivable that MMR proteins can generate double stranded breaks after excising the G:U mismatches, or play a role in resolving the breaks, or both. In addition, the Msh2–Msh3 heterodimer has been suggested to play a role in processing the breaks [23,24].

In *Saccharomyces cerevisiae*, the Mlh1-Mlh3 heterodimer was initially implicated in the repair of a proportion of insertion/deletion mismatches [25,26]. Later, it was shown that Mlh3 combines with Mlh1 to promote meiotic crossing-over [27]. In mammals, Mlh3 was found to interact with the conserved carboxy-terminal of Mlh1, and cells in culture stably expressing a dominant-negative Mlh3 protein exhibit microsatellite instability [28]. Even though the role of MLH3 in hereditary non-polyposis colon cancer has been controversial [29–33], it is well established that the Mlh3 protein is required for Mlh1 association with meiotic chromosomes during prophase I in mammalian meiosis [34]. Because both Mlh1 and Mlh3 are involved in meiosis and MMR, and Mlh1 plays a role in CSR [23,35] and SHM [36,37], we wondered whether loss of Mlh3 would have any impact on SHM and CSR.

Here we examined three different regions of the immunoglobulin heavy chain in *Mlh3*$^{-/-}$ mice and discovered that all three showed an increased rate of SHM, compared to the wild type littermates. In the intronic region between Jh2–Jh4 and in the upstream of Sμ region, the percentage of mutations at G:C bases was similar in *Mlh3*$^{-/-}$ mice and wild type mice. However, there was a significant increase of mutations at A:T bases in *Mlh3*$^{-/-}$ mice in the Jh4 flanking region of VhJ558-Jh4 rearranged genes. In all three regions, the percentages of mutations at RGYW/WRCY hotspot motifs and transition mutations at G:C bases in *Mlh3*$^{-/-}$ mice were not different from wild type. In addition, *Mlh3*$^{-/-}$ mice were able to carry out CSR to IgG3 or to IgG1 at levels similar to wild type mice.

## 2. Materials and methods

### 2.1. *Mlh3*$^{-/-}$ mice

The *Mlh3*$^{-/-}$ mouse line was generated and reported previously [34]. The *Mlh3*$^{-/-}$ mice used in this study were backcrossed to C57BL/6 mice for at least five generations and were housed in a pathogen-free facility. All of the procedures described here have been approved by the Animal Use Committee of the Albert Einstein College of Medicine.

### 2.2. *SHM assay*

For SHM, two pairs of approximately 9-month-old wild type and mutant mice were sacrificed. PNA$^{high}$ B220$^{+}$ germinal center B cells from Peyer's Patches were stained and FACS sorted. Various regions in the immunoglobulin heavy locus were amplified from DNA extracted from these sorted cells. The Jh2–Jh4 intronic region was amplified as described [38]. The VhJ558-Jh4 rearrangements were amplified using the primers as described [39]. PCR was carried out at 95 °C for 30 s, 67 °C for 1 min 30 s, and 72 °C for 2 min with a hot-start for 34 cycles. The upstream Sμ region was amplified using the primers and conditions as reported previously [40]. All PCR reactions were carried out using Pfu-turbo polymerase and PCR products were gel purified, cloned, and sequenced.

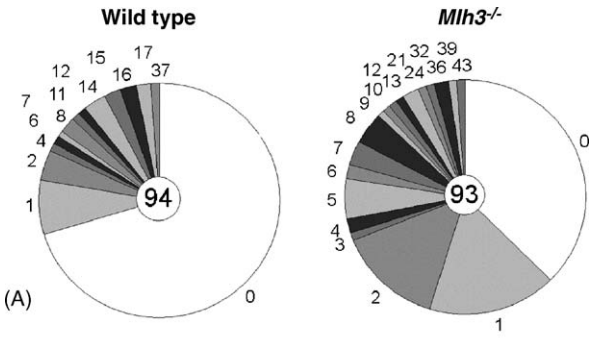
### 2.3. *CSR assay*

For CSR, approximately 8-week-old mice that included two heterozygous mice and their three homozygous littermates were used. Splenic B cells were obtained and then stimulated with 50 μg/ml LPS (Sigma) or 50 μg/ml LPS and 50 ng/ml rIL-4 (R&D) for 4 days as described [24,38]. Surface antibody IgM and IgG were stained and analyzed with a FACSCalibur (Becton–Dickinson). FACS data were analyzed using the FlowJo software package (Treestar), as described previously [24,38].

## 3. Results

### 3.1. *Increased rate of SHM in the Jh2–Jh4 region in Mlh3*$^{-/-}$ *mice*

To examine the effect of loss of Mlh3 on SHM, we first sequenced the intronic Jh2–Jh4 region from the Peyer's patches of 9-month-old mice that had been backcrossed to C57BL/6. This region does not encode any protein and presumably is not subject to selective pressure during the immune response. As shown in Fig. 1A and Table 1A, the percentage of mutated sequences was much higher in *Mlh3*$^{-/-}$ mice than in *Mlh3*$^{+/+}$ mice (62.4% versus 29.8%, $p < 0.0001$). In addition, there were many more sequences with over 30 mutations in *Mlh3*$^{-/-}$ mice (Fig. 1A), and the overall mutation frequency was higher in *Mlh3*$^{-/-}$ mice than in *Mlh3*$^{+/+}$ mice ($7.5 \times 10^{-3}$ versus $4.3 \times 10^{-3}$, $p < 0.0001$). As shown in Fig. 1B, the distribution of mutations among the four different bases was not different between wild type and *Mlh3*$^{-/-}$ mice. The relative percentage of mutations




| Substitution | | Wild type (163 mut) % | Mlh3-/- (275 mut) % |
|---|---|---|---|
| A to: | G | 13 | 16 |
| | T | 9 | 8 |
| | C | 10 | 9 |
| T to: | C | 8 | 8 |
| | A | 7 | 8 |
| | G | 5 | 5 |
| G to: | A | 14 | 14 |
| | T | 3 | 6 |
| | C | 4 | 6 |
| C to: | T | 16 | 12 |
| | A | 6 | 5 |
| | G | 5 | 3 |

(B)

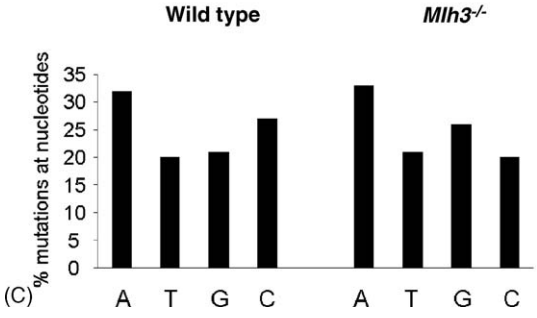
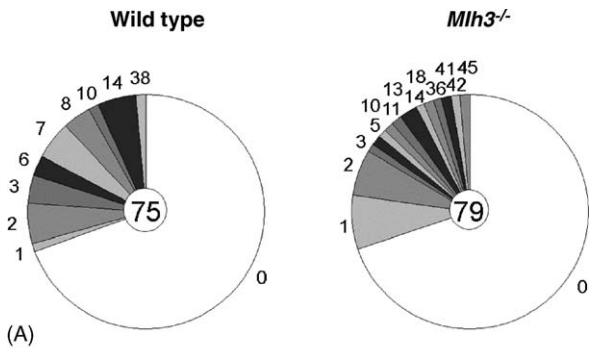



**Fig. 1 – Mutation analyses of the Jh2–Jh4 intron region in *Mlh3*$^{-/-}$ mice. (A) Pie charts depict the percentage of mutated sequences with the indicated number of mutations. The number of sequences analyzed is shown in the center of each circle. (B) The detailed mutation spectrum at each nucleotide is presented as a percentage. Identical mutations at the same position were only counted once. (C) The percentage of total mutations for each nucleotide is grouped.**




| Substitution | | Wild type (101 mut) % | Mlh3-/- (164 mut) % |
|---|---|---|---|
| A to: | G | 14 | 13 |
| | T | 11 | 12 |
| | C | 6 | 8 |
| T to: | C | 6 | 16 |
| | A | 1 | 8 |
| | G | 7 | 5 |
| G to: | A | 19 | 8 |
| | T | 6 | 4 |
| | C | 10 | 8 |
| C to: | T | 11 | 8 |
| | A | 3 | 5 |
| | G | 7 | 4 |

(B)

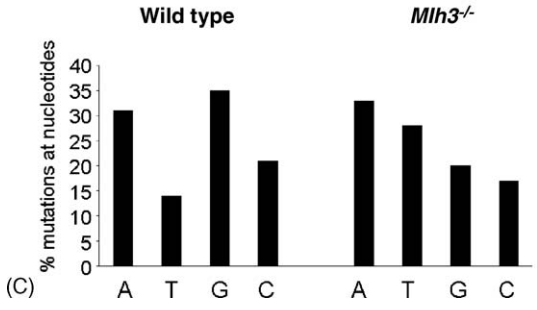



**Fig. 2 – Increased mutations at T nucleotides and decreased mutations at G nucleotides from the VhJ558-Jh4 rearranged Jh4 flanking region in *Mlh3*$^{-/-}$ mice. (A) Pie charts depict the proportion of sequences containing mutations. (B) The detailed mutation spectrum at each nucleotide is presented as a percentage. (C) The percentage of total mutations for each nucleotide is grouped.**

at nucleotides (A/T/G/C) was similar between wild type and *Mlh3*$^{-/-}$ (Fig. 1C) and the percentage of mutations at G/C bases was similar (Table 1A). In addition, the percentage of mutations at RGYW/WRCY hotspot motifs in *Mlh3*$^{-/-}$ mice was the same as in wild type mice (Table 1A). The frequency of transition mutations at G/C bases was slightly lower in *Mlh3*$^{-/-}$ mice than in wild type mice, although this was not statistically significant.

### 3.2. *Increased rate of SHM in the Jh4 flanking region of VhJ558-Jh4 rearrangements in Mlh3*$^{-/-}$ *mice*

Since most other MMR deficient mice had shown increased mutations at G/C bases (reviewed in [2]), we analyzed this further by sequencing the Jh4 flanking region from B cells harboring VhJ558-Jh4 rearrangements, using the same DNA samples. In contrast to the data derived from the Jh2–Jh4 region, the percentage of mutated sequences was similar in *Mlh3*$^{-/-}$ mice and *Mlh3*$^{+/+}$ mice (30.7% versus 30.4%, $p = 0.89$) (Fig. 2A and Table 1B). However, as in the Jh2–Jh4 region (Fig. 1A), there were more sequences with a high number of mutations in

**Table 1 – Somatic hypermutation from Peyer's patch B cells in *Mlh3*$^{+/+}$ mice, and *Mlh3*$^{-/-}$ mice**

| | *Mlh3*$^{+/+}$ | *Mlh3*$^{-/-}$ | P-value[a] |
|---|---|---|---|
| **A** Jh2–Jh4 region | | | |
| Mutated sequences[b] | 28/94 (**29.8%**) | 58/93 (**62.4%**) | <**0.0001** |
| Mutation frequency ($\times 10^{-3}$)[c] | 246/57569 (**4.3**) | 439/58260 (**7.5**) | <**0.0001** |
| GC mutations/total | 78/163 (47.8%) | 126/275 (45.8%) | 0.75 |
| RGYW and WRCY/total | 26/163 (15.9%) | 39/275 (14.2%) | 0.71 |
| Transition mutations at G/C | 52/78 (66.7%) | 72/126 (57.1%) | 0.23 |
| Transition mutations at A/T | 34/85 (40.0%) | 68/149 (45.6%) | 0.48 |
| **B** Jh4 flanking region | | | |
| Mutated sequences[b] | 23/75 (30.7%) | 24/79 (30.4%) | 0.89 |
| Mutation frequency ($\times 10^{-3}$)[c] | 186/43800 (**4.2**) | 271/46143 (**5.9**) | <**0.001** |
| GC mutations/total | 56/101 (**55.4%**) | 62/164 (**37.8%**) | <**0.001** |
| RGYW and WRCY/total | 22/101 (21.8%) | 25/164 (15.2%) | 0.23 |
| Transition mutations at G/C | 30/56 (53.6%) | 27/62 (43.5%) | 0.37 |
| Transition mutations at A/T | 20/45 (44.4%) | 48/102 (47.0%) | 0.91 |
| **C** Upstream Sμ region | | | |
| Mutated sequences[b] | 14/76 (**18.4%**) | 36/109 (**33.0%**) | <**0.05** |
| Mutation frequency ($\times 10^{-3}$)[c] | 45/41760 (**1.1**) | 160/58698 (**2.7**) | <**0.0001** |
| GC mutations/total | 20/40 (50.0%) | 36/65 (55.4%) | 0.74 |
| RGYW and WRCY/total | 10/40 (25.0%) | 13/65 (20.0%) | 0.72 |
| Transition mutations at G/C | 10/20 (50.0%) | 19/36 (52.8%) | 0.94 |
| Transition mutations at A/T | 11/20 (55.0%) | 15/29 (51.7%) | 0.95 |

[a] $\chi^2$ test was used to compare two sets of data using the real numbers. Statistical significance (bolded) was defined as $p < 0.05$.
[b] Mutated sequences was defined as the total number of sequences containing mutations divided by the total number of analyzed sequences.
[c] Mutation frequency was defined as the total number of mutations observed divided by the total number of sequenced nucleotides.

*Mlh3*$^{-/-}$ mice (Fig. 2A) and the overall mutation frequency was higher in *Mlh3*$^{-/-}$ mice than in *Mlh3*$^{+/+}$ mice ($5.9 \times 10^{-3}$ versus $4.2 \times 10^{-3}$, $p < 0.001$) (Table 1B). Even though the distribution of mutations at A nucleotides was similar between wild type and *Mlh3*$^{-/-}$ mice, there were more mutations at T nucleotides in the mutant mice, especially T–C and T–A mutations (Fig. 2B). In addition, G–A transition mutations were reduced in *Mlh3*$^{-/-}$ mice, although there was little change in the detailed mutation distribution at C bases (Fig. 2B). Overall, there were more mutations at T nucleotides and fewer mutations at G nucleotides in *Mlh3*$^{-/-}$ mice (Fig. 2C) and the percentage of mutations at G/C bases was decreased in *Mlh3*$^{-/-}$ mice (Table 1B). As in the Jh2–Jh4 region, the percentage of mutations at RGYW/WRCY hotspot motifs in *Mlh3*$^{-/-}$ mice was not statistically significantly different from the wild type mice, although both the mutations at hot spots and the transition mutations at G/C bases were slightly lower in *Mlh3*$^{-/-}$ mice than in wild type mice (Table 1B).

To confirm the increased mutations at T nucleotides in the Jh4 flanking region in *Mlh3*$^{-/-}$ mice, we repeated these experiments in one *Mlh3*$^{+/+}$ mouse and two littermate *Mlh3*$^{-/-}$ mice that were 6-months old. The data derived from additional *Mlh3*$^{+/+}$ and *Mlh3*$^{-/-}$ mice are consistent with the data mentioned above. Specifically, one of the *Mlh3*$^{-/-}$ mice exhibited 31% mutations at T bases and the other exhibited 33.3% mutations at T bases, in contrast to 12.5% in the wild type littermate mouse (data not shown).

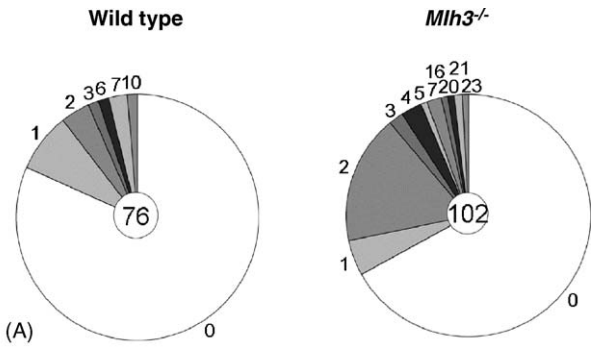
### 3.3. *Increased rate of SHM in the upstream of Sμ region in Mlh3*$^{-/-}$ *mice*

The data presented above show that the loss of Mlh3 increased the rate of SHM in both the Jh2–Jh4 and Jh4 flanking regions. While there was no change in the spectrum of mutation in the Jh2–Jh4 intronic region, there were more mutations at T nucleotides and fewer mutations at G bases in the Jh4 flanking region.

To address whether the mutation spectrum change observed in *Mlh3*$^{-/-}$ mice was specific to the Jh4 flanking region, we additionally sequenced the upstream of Sμ region [40] from the same DNA samples used for sequencing the Jh2–Jh4 and Jh4 flanking regions. The upstream of Sμ region could be more representative for mutation analysis because every B cell should retain that region, whereas only a portion of B cells have the Jh2–Jh4 region and Jh4 flanking region from VhJ558-Jh4 rearrangements. We discovered that, even though the overall mutation frequency was lower, the effect of loss of Mlh3 in this region was very similar to that in the intronic Jh2–Jh4 region. Specifically, the percentages of mutations at G/C bases or A/T bases, of hotspot mutations, and of transition mutations at G/C bases in *Mlh3*$^{-/-}$ mice were similar to those in wild type mice (Table 1C; Fig. 3B and C). However, the percentage of mutated sequences in *Mlh3*$^{-/-}$ mice was 33.0%, much higher than 18.4% in wild type mice (Table 1C). In addition, there were more sequences with a large number of mutations in *Mlh3*$^{-/-}$ mice (Fig. 3A). Moreover, the frequency of SHM in *Mlh3*$^{-/-}$ mice was more than two-fold higher in *Mlh3*$^{-/-}$ mice than in *Mlh3*$^{+/+}$ mice ($2.7 \times 10^{-3}$ versus $1.1 \times 10^{-3}$, $p < 0.0001$) (Table 1C), consistent with the other two regions mentioned above.

### 3.4. *Proficient CSR in Mlh3*$^{-/-}$ *mice*

Having observed an increased efficiency of SHM in *Mlh3*$^{-/-}$ mice, we wondered whether the frequency of CSR would also be increased. Splenic B cells were purified from 8-week-old




| Substitution | | Wild type (40 mut) % | *Mlh3*$^{-/-}$ (65 mut) % |
|---|---|---|---|
| A to: | G | 17 | 12 |
| | T | 3 | 5 |
| | C | 7 | 3 |
| T to: | C | 10 | 11 |
| | A | 10 | 9 |
| | G | 3 | 5 |
| G to: | A | 10 | 18 |
| | T | 7 | 5 |
| | C | 8 | 8 |
| C to: | T | 15 | 11 |
| | A | 7 | 8 |
| | G | 3 | 6 |

(B)

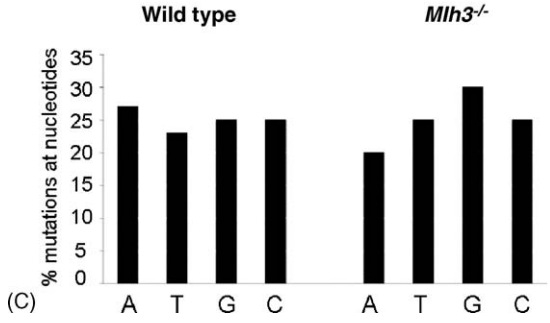



**Fig. 3 – Mutation analyses of the upstream Sμ region in *Mlh3*$^{-/-}$ mice. (A) Pie charts depicting the percentage of mutated sequences with the indicated number of mutations. The number of sequences analyzed is shown in the center of each circle. (B) The detailed mutation spectrum at each nucleotide is presented as percentage. (C) The percentage of total mutations for each nucleotide is grouped.**

naïve mice and stimulated with LPS or LPS and IL4, which activates switching to IgG3 and IgG1, respectively. We observed that after LPS stimulation, *Mlh3*$^{-/-}$ B cells exhibited a similar frequency of switching to IgG3, compared to the *Mlh3*$^{+/-}$ B cells (Fig. 4A and B). Specifically, we observed 16–17% IgG3 positive cells in *Mlh3*$^{+/-}$ mice and 18–19% IgG3 positive cells in *Mlh3*$^{-/-}$ mice. Likewise, after LPS and IL4 stimulation, the rate of switching to IgG1 in *Mlh3*$^{-/-}$ B cells was similar to that of *Mlh3*$^{+/-}$ B cells. Overall, even though switching to IgG3 in *Mlh3*$^{-/-}$ B cells exhibited a very subtle increase, we did not observe significant increase of CSR in *Mlh3*$^{-/-}$ mice. These results suggest that Mlh3 does not play a major role in class switching.

## 4. Discussion

The increased rate of SHM in *Mlh3*$^{-/-}$ mice suggests that Mlh3 may normally play an inhibitory role in SHM. Given that the deficiency of Msh2, Msh6, Mlh1 or Exo1 results in defects in SHM (reviewed in [2]), it is unlikely that Mlh3 is directly collaborating with Mlh1 to promote MMR-mediated patch repair in the process of SHM. While Mlh3 clearly interacts with Mlh1 to form a heterodimer that plays an important role in meiosis [34], it has also been reported to coordinate with Msh3 to correct frameshift mutations in yeast [25] and to colocalize with RPA on meiotic chromosomes [34]. Msh3 is not required for either SHM or CSR but we have previously observed a subtle increase of mutations at A/T bases in the Jh2–Jh4 region in *Msh3*$^{-/-}$ mice [24], which is similar to the increase in the Jh4 flanking region in *Mlh3*$^{-/-}$ mice. The potential interaction of Mlh3 with RPA is interesting since it has been suggested that RPA may bind to and maintain single stranded DNA in the V region and then recruit AID, with which it has been shown to interact [12]. If these interactions are occurring at the V region, it is possible that Mlh3 is a part of the protein complexes that participate in SHM. In this scenario, Mlh3 would not be required to function in SHM but could affect it by changing the composition of the mutation complex.

Another possibility is that Mlh3 plays a role in suppressing MMR by interfering the interaction of Mlh1 with Pms2 in the patch repair of the second phase of SHM. Because Mlh3 interacts with Mlh1 [28], loss of Mlh3 could potentially enhance the stability of the Mlh1–Pms2 complex and consequently enhance the second phase of SHM. In fact, this appears to be the case in meiotic recombination [41] and it could also be true in B cells in the process of SHM. The reduction of transition mutations at G/C bases in *Mlh3*$^{-/-}$ mice (Table 1A and B) also supports the more active second phase of SHM so that fewer G:U mismatches are replicated directly or processed by UNG. It is equally possible that loss of Mlh3 may affect the activity or recruitment of error-prone DNA polymerases that are involved in the second phase of SHM, which in turn may result in a fidelity change when polymerases copy bases at T or G positions. Alternatively, Mlh3 could directly or indirectly inhibit the initial G:U mismatch binding to slow down the initiation of patch repair in the second phase of SHM.

The increased frequency of mutations in *Mlh3*$^{-/-}$ mice could have been due to a defect in the apoptosis or an increase in the rate of division in the germinal center reaction. To investigate these possibilities, cell apoptosis and proliferation were analyzed in paraffin-embedded cross sections of spleens from one *Mlh3*$^{+/+}$ mouse, one *Mlh3*$^{+/-}$ mouse, and two littermate *Mlh3*$^{-/-}$ mice that were immunized with sheep red blood cells. Germinal centers were identified by staining with the lectin PNA. There was no obvious difference in the frequency, nor in the distribution of germinal center size among these mice as measured by surface area (data not shown).

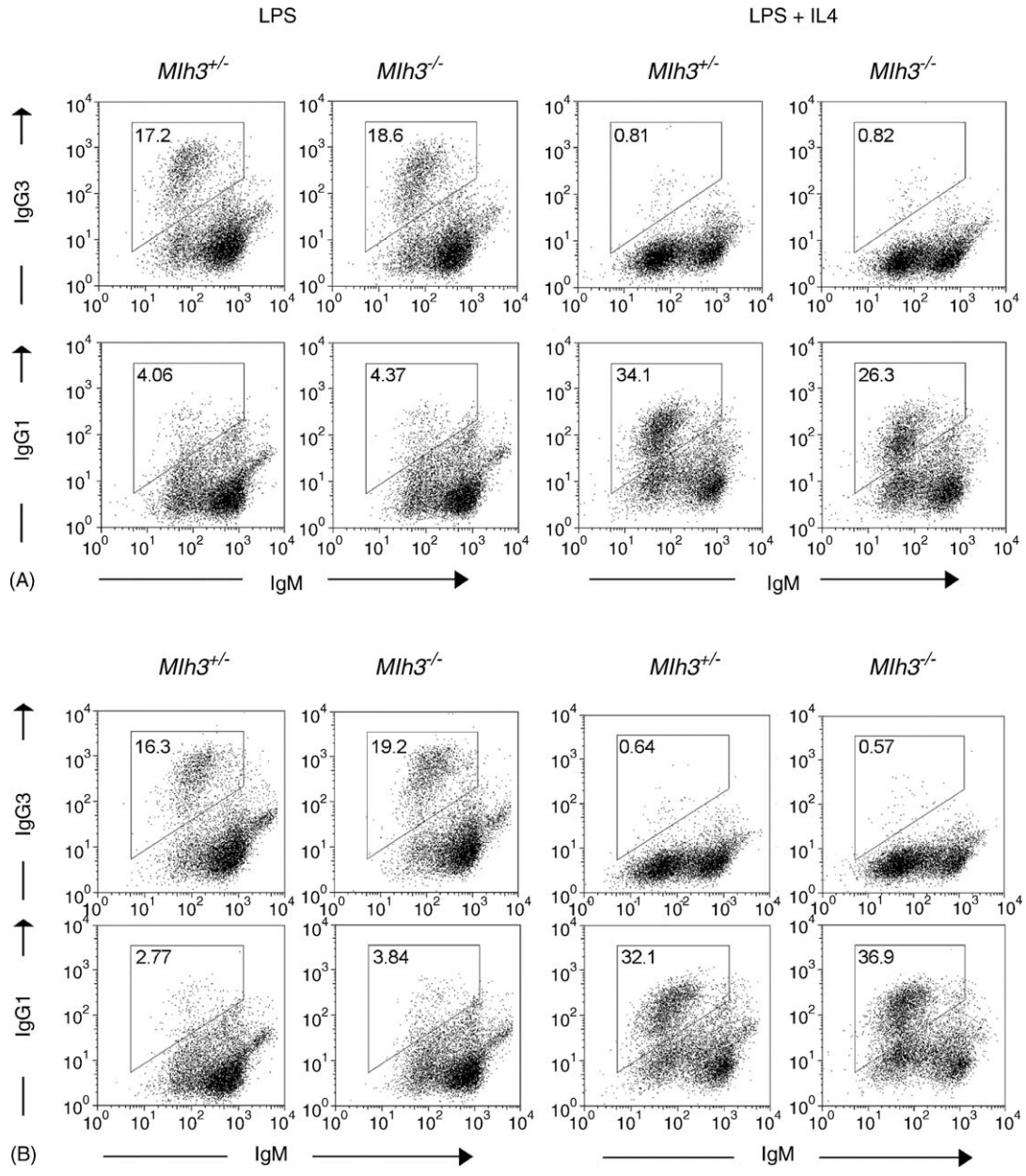



**Fig. 4 – Proficient class switching in *Mlh3*$^{-/-}$ mice. Data were obtained from two heterozygous and three *Mlh3*$^{-/-}$ littermate mice. (A) The effect on IgG3 or IgG1 switching in the first pair of mice. The percentage of IgG positive cells is shown in each box. Under LPS stimulation, the background switching to IgG1 is also shown. Under LPS plus IL4 stimulation, the background switching to IgG3 is also shown. (B) One heterozygous mouse and two *Mlh3*$^{-/-}$ littermate mice were used, but only one *Mlh3*$^{-/-}$ mouse is shown. The data of the second *Mlh3*$^{-/-}$ mouse are similar to those shown here.**

In addition, we performed TUNEL staining and discovered that the proportion of apoptotic cells in the germinal centers of *Mlh3*$^{+/+}$, *Mlh3*$^{+/-}$, and *Mlh3*$^{-/-}$ mice was similar (data not shown), suggesting that loss of Mlh3 does not cause a defect in the apoptosis of germinal center B cells. Furthermore, we did Ki67 staining, which is a proliferation maker, on these sections. We did not find any obvious difference in Ki67 staining of the germinal center from *Mlh3*$^{+/+}$, *Mlh3*$^{+/-}$, and *Mlh3*$^{-/-}$ mice (data not shown), suggesting that loss of Mlh3 does not cause an increase in the rate of division in the germinal center B cells.

The spectra of mutations observed in the Jh2–Jh4 regions and the upstream Sμ region in *Mlh3*$^{-/-}$ mice were similar to those observed in the wild type mice. However, in the Jh4 flanking region of B cells harboring VhJ558-Jh4 rearrangements, there were more mutations at A/T bases in *Mlh3*$^{-/-}$ mice. The different effects of loss of Mlh3 on the mutation spectrum suggest that Mlh3 may have distinct functions in different parts of the immunoglobulin region. It is possible that Mlh3 interacts with a different set of enzymes to compete for SHM or it participates in a different complex of proteins in different regions.

There is little doubt that the cytidine deaminase activity of AID initiates the process of SHM and CSR. The involvement of UNG, MMR proteins, and polymerases in these two processes is also supported by animal models (reviewed in [1,2,4]), even though the precise molecular mechanism and interactions of all of these factors are not yet fully understood. Here we showed that another MutL homolog, MLH3, plays a negative role in SHM, suggesting that there is a queue of proteins involved in these complex processes. To our knowledge, Mlh3 is the first example where loss of a DNA repair protein has a positive effect in SHM, as opposed to the defects observed in mice deficient in AID, other MMR proteins, or error-prone polymerases.

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