Spt5 accumulation at variable genes distinguishes somatic hypermutation in germinal center B cells from ex vivo–activated cells

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Variable (V) genes of immunoglobulins undergo somatic hypermutation by activation-induced deaminase (AID) to generate amino acid substitutions that encode antibodies with increased affinity for antigen. Hypermutation is restricted to germinal center B cells and cannot be recapitulated in ex vivo–activated splenic cells, even though the latter express high levels of AID. This suggests that there is a specific feature of antigen activation in germinal centers that recruits AID to V genes which is absent in mitogen-activated cultured cells. Using two *Igh* knock-in mouse models, we found that RNA polymerase II accumulates in V regions in B cells after both types of stimulation for an extended distance of 1.2 kb from the TATA box. The paused polymerases generate abundant single-strand DNA targets for AID. However, there is a distinct accumulation of the initiating form of polymerase, along with the transcription cofactor Spt5 and AID, in the V region from germinal center cells, which is totally absent in cultured cells. These data support a model where mutations are prevalent in germinal center cells, but not in ex vivo cells, because the initiating form of polymerase is retained, which affects Spt5 and AID recruitment.

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Abbreviations used: 3′RR, 3′ regulatory region; AID, activation-induced deaminase; ChIP, chromatin immunoprecipitation; Eμ, intron enhancer; pol II, RNA polymerase II; UNG, uracil DNA glycosylase.

Somatic hypermutation is initiated by the activation-induced deaminase (AID) protein, which is expressed in activated B lymphocytes. AID functions by deaminating cytosine to uracil in DNA (Maul et al., 2011), and the U:G mismatch produces a mutational storm to generate extreme diversity in the immunoglobulin (Ig) loci. Proteins are drawn in from base excision and mismatch repair pathways (Rada et al., 2004), as well as low-fidelity DNA polymerases (Saribasak et al., 2012), to produce nucleotide substitutions and single-strand breaks. Peaks of mutation are found overV regions on the heavy (H) and light chain loci, and over switch (S) regions preceding constant (C) genes on the H chain locus (Maul and Gearhart, 2010). Mutations occur downstream of promoters, which implicates transcription in the process (Lebecque and Gearhart, 1990; Peters and Storb, 1996; Xue et al., 2006). However, the mechanism of how transcription focuses AID to these two regions is unclear.

For S regions, recent findings have revealed that the DNA sequence is important for recruiting AID. These 2–8 kb regions of intronic DNA are composed of repeats of 3–4 G clusters, which form stable RNA-DNA hybrids (R-loops) when transcribed (Huang et al., 2007), and WGC (W = A or T) motifs, which bind AID (Kohli et al., 2009; Wang et al., 2010). RNA polymerase II (pol II) accumulates as it transcribes the repetitive region (Rajagopal et al., 2009; Wang et al., 2009), leading to recruitment of AID via interaction with Spt5 (Pavri et al., 2010) and the RNA exosome (Basu et al., 2011). AID then deaminates C on both nontranscribed and transcribed

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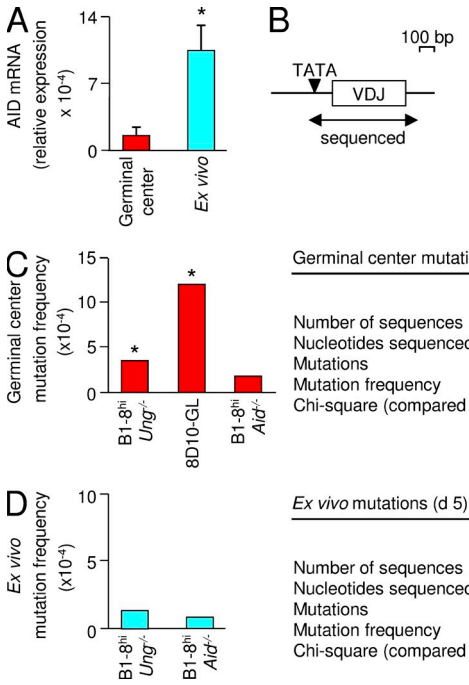


Figure 1. AID expression and somatic hypermutation in germinal center– and ex vivo–stimulated B cells. (A) AID expression. mRNA levels were measured relative to 18S rRNA levels in B1-8^{hi} mice 7 d after immunization (4 independent experiments with 1 mouse per experiment) or 3 d after LPS and IL-4 stimulation (6 independent experiments with 1 mouse per experiment). Error bars represent SD. *, $P < 0.0002$ (unpaired two-tailed Student's *t* test) compared with germinal center. (B) Region sequenced for mutations. (C) Mutation analysis in germinal center cells. Mutation frequencies were measured in the V region of B1-8^{hi} *Ung*$^{-/-}$, 8D10-GL, and B1-8^{hi} *Aid*$^{-/-}$ mice (3–5 independent experiments with one mouse per experiment). *, P-values compared with the B1-8^{hi} *Aid*$^{-/-}$ control. (D) Mutation analysis in ex vivo cells. Mutation frequencies were measured in B1-8^{hi} *Ung*$^{-/-}$ and B1-8^{hi} *Aid*$^{-/-}$ mice (3–4 independent experiments with 1 mouse per experiment).

strands, and subsequent processing produces double-strand breaks for class switch recombination. Thus, in S regions, R-loops slow down pol II progression, which then magnifies AID activity.

In contrast, V regions do not form R-loops, and it is not known what directs AID to these regions. Furthermore, a long-term conundrum has been why cells stimulated with antigen in germinal centers from mice have mutations in both V and S regions, whereas cells stimulated ex vivo with LPS mitogen or anti-CD40 have mutations only in S regions. Why don't mutations occur in the nearby *Igh* V regions in cultured cells? We reasoned that V region targeting would require additional features specific to activation in germinal centers and sought to identify these factors.

RESULTS

Robust somatic hypermutation in germinal center cells but not in ex vivo–activated cells

To study mutation in V regions on the *Igh* locus, we used two independent knock-in mice that contained a rearranged V–diversity (D)–joining (J) gene on both alleles: the V_H186.2 gene from the J558 V_H family rearranged to D and J_H2 segments, and cloned into the J_H4 intron (B1-8^{hi} mice; Shih et al., 2002); and the VGK7 gene from the VGAM3.8 V_H family rearranged to D and J_H2 segments, and cloned into the J_H4 intron (8D10-GL mice, this work). For germinal center cells, mice were immunized with phycoerythrin, an antigen which has broad specificity for many V genes—including V_H186.2 (Pape et al., 2011)—and GL7$^+$ splenic B cells were isolated on day 7. For ex vivo activation, naive spleen cells from B1-8^{hi} mice were stimulated with LPS and IL-4 for 2–5 d in culture. We first determined the level of expression of AID in cells under both conditions of activation. AID mRNA was measured by qPCR relative to 18S ribosomal RNA; there was fivefold more AID expressed after ex vivo activation compared with germinal center activation (Fig. 1 A). Thus, the lack of mutation in cultured cells is not due to deficient AID expression.

We then measured mutation frequencies in V regions by sequencing ~600 bp around the VDJ exon (Fig. 1 B) in cells from both types of activation. For the B1-8 gene, we used B1-8^{hi} *Ung*$^{-/-}$ cells, which are compromised for base excision repair due to the lack of uracil DNA glycosylase (UNG), to increase the mutation frequency. In germinal center cells 7 d after immunization, both B1-8^{hi} *Ung*$^{-/-}$ and 8D10-GL mice had significant levels of mutation compared with B1-8^{hi} *Aid*$^{-/-}$ control mice (Fig. 1 C). In ex vivo cells 5 d after LPS and IL-4 activation, B1-8^{hi} *Ung*$^{-/-}$ cells had no significant increase in mutation compared with B1-8^{hi} *Aid*$^{-/-}$ cells (Fig. 1 D), which confirms previous reports (Manser, 1987; Reina-San-Martin et al., 2003). Thus, hypermutation of V genes is robust in germinal center cells but debilitated in cells stimulated in culture. Other attempts to establish ex vivo systems to study somatic hypermutation have been elusive as well (McHeyzer-Williams et al., 1991; Decker et al., 1995; Källberg et al., 1996; Nojima et al., 2011; Matthews et al., 2014).

Pol II accumulates for an extended distance in V regions from both germinal center and ex vivo cells

We then analyzed RNA pol II activity by nuclear run-on experiments—which measure polymerase activity in specific regions—in B1-8^{hi} cells activated ex vivo for 2 d. Transcription in isolated nuclei was initiated in vitro, and transcripts were hybridized to 500-bp DNA probes spanning a distance of 1.5 kb from the TATA box to the intron enhancer (Eμ; Fig. 2 A, green probes V1-5), and a control probe in Cμ1. Data were corrected for dTTP content between the different probes because

radiolabeled UTP was incorporated. Compared with the C probe, polymerase loading was significantly higher over the V2, V3, and V4 probes located in the VDJ exon and J_H intron in day 2 cells relative to day 0 (Fig. 2 B). To examine if the increase is a common feature of highly transcribed genes, the *γ-actin* gene was also analyzed with probes similarly located downstream of the TATA box (Fig. 2 A, green probes G1-5). In contrast to the V region, there was no increase in the *γ-actin* gene on day 2 and no difference in levels between the different probes (Fig. 2 B). For a negative control, a probe located in the promoter region of the nontranscribed *CD3* gene showed no transcription. Thus, pol II uniquely accumulated over V regions in ex vivo–activated B cells.

To determine whether pol II also accumulated in V regions from germinal center cells, we performed chromatin immunoprecipitation (ChIP) assays. For B1-8^{hi} and 8D10-GL mice, V and I primers were located 0.6 and 1.2 kb downstream of the TATA box, and E and C primers were placed 1.6 and 6.4 kb downstream in the Eμ and Cμ regions, respectively (Fig. 2 A, red primers). For the *γ-actin* locus, primer *a* was located in the TATA box, and primers *b* and *c* were placed 0.6 and 1.6 kb downstream of the TATA box. For a negative control, a primer was located over the TATA box of the nontranscribed *β-globin* gene. As shown in Fig. 2 C, total pol II levels from germinal center and ex vivo cells were significantly higher over the V and I primers compared with primers for E, C, γ-actin, and β-globin from both B1-8^{hi} and 8D10-GL mice. Pol II levels differed slightly at probes and primers when comparing run-on and ChIP data, which is likely due to the different techniques. For run-on, signals were received directly at the probe location. For ChIP, signals were obtained from large shear sizes of DNA, which could occur ∼500 bp on either side of the primer location. In summary, both germinal center and ex vivo activation produced significant accumulation of pol II for an extended distance of 1.2 kb from the TATA box.

Pol II abundance correlates with single-strand DNA and hypermutation in germinal center cells

To see if the increased pol II density exposed more single-strand DNA, which is the substrate for AID deamination, we measured its frequency in germinal center cells. To eliminate AID-dependent background deaminations, spleens from B1-8^{hi} *Aid*$^{-/-}$ mice were used 7 d after immunization with phycoerythrin. Nuclei were isolated and treated with sodium bisulfite, which deaminates single-stranded cytosine to uracil, similar to AID. The products were sequenced for C:G to T:A transitions to distinguish deamination on the nontranscribed (C to T) and transcribed (G to A) strands in three 700-bp segments of DNA (Fig. 3 A). As seen in Fig. 3 B (top half), activated cells had substantially increased single-strand DNA compared with naive cells (dotted lines). Because pol II could accumulate on the transcribed strand, single-strand cytosines might be more frequent on the nontranscribed strand. However, the frequency was similar on both DNA strands, which indicates that both strands are accessible during transcription for deamination by bisulfite, and presumably AID. It has been suggested that the RNA exosome removes nascent RNA from the transcribed strand in S regions (Basu et al., 2011), and this may occur in V regions as well (Milstein et al., 1998; Rada et al., 2004; Ronai et al., 2007; Parsa et al., 2012). To compare frequencies, the deamination events were grouped into V, I, and E segments and corrected for the nucleotide composition of C:G bases. The single-strand DNA frequency

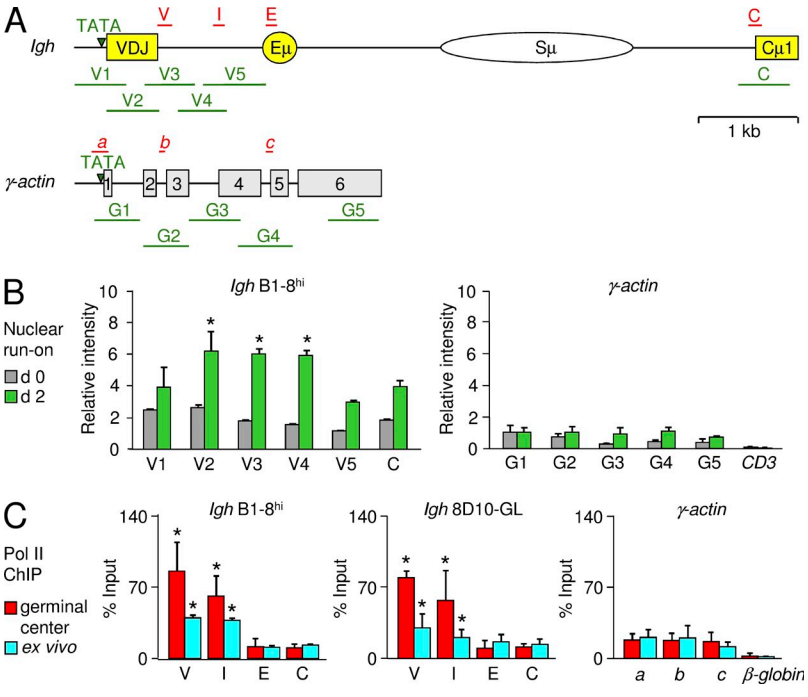
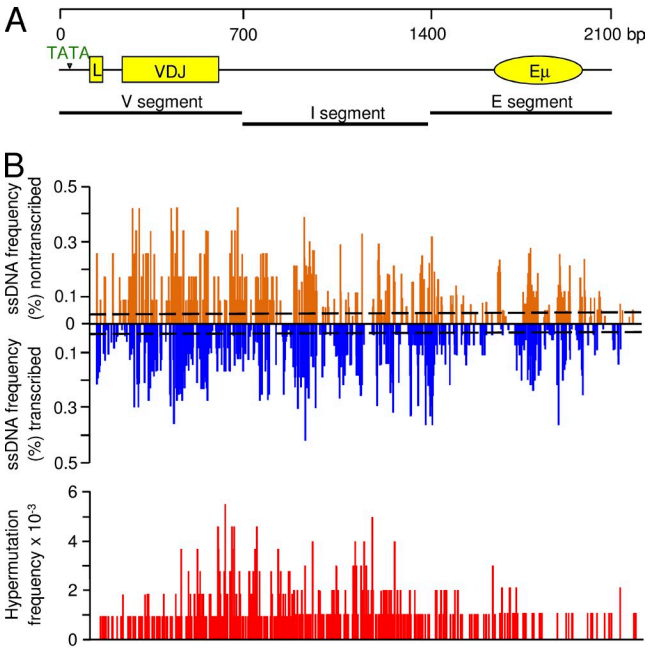


Figure 2. Transcription analyses of the V region and *γ-actin* loci from activated B cells. (A) Location of run-on probes (green) in the *Igh* (V1-5 and C) and *γ-actin* (G1-5) regions are shown below the loci. Location of ChIP primer sets (red) in *Igh* (V, I, E, and C) and *γ-actin* (*a*, *b*, and *c*) are shown above the loci. For negative controls, nontranscribed genes *CD3* and *β-globin* had probes and primers located within the promoter regions. (B) Nuclear run-on in ex vivo cells. Hybridization of days 0 (gray) and 2 (green) nascent RNA to DNA probes is graphed relative to a non-DNA control. Error bars represent the SD of values from triplicate blots from 3 experiments with 1 mouse per experiment. *, $P < 0.05$ compared with the C probe (unpaired two-tailed Student's *t* test). (C) Pol II ChIP from day 7 germinal center (red) and day 2 ex vivo (blue) activation. Error bars represent the SD of values from 9 independent experiments for B1-8^{hi}, and 6 experiments for 8D10-GL, with 1 mouse per experiment. For the *Igh* genes, *, $P < 0.05$ compared with values for C (unpaired two-tailed Student's *t* test). For the *γ-actin* gene, results were averaged between B1-8^{hi} and 8D10-GL cells.



C Single-strand DNA B1-8^{hi} *Aid*$^{-/-}$ germinal center (spleen, d 7)

	V segment	I segment	E segment
Nontranscribed DNA strand			
C:G nucleotides sequenced	2160	3434	4536
Single-strand C:G	257	418	325
Single-strand DNA frequency	0.119	0.122	0.072
Chi-square (compared to E)	$P < 10^{-4}$	$P < 10^{-4}$	
Transcribed DNA strand			
C:G nucleotides sequenced	8596	5765	6538
Single-strand C:G	1063	684	498
Single-strand DNA frequency	0.124	0.119	0.076
Chi-square (compared to E)	$P < 10^{-4}$	$P < 10^{-4}$	

D Hypermutation B1-8^{hi} *Ung*$^{-/-}$ germinal center (Peyer's patches)

	V segment	I segment	E segment
Nucleotides sequenced	100,800	92,597	117,488
Mutations	288	283	64
Mutation frequency	2.86×10^{-3}	3.06×10^{-3}	0.54×10^{-3}
Chi-square (compared to E)	$P < 10^{-4}$	$P < 10^{-4}$	

Figure 3. Single-strand DNA and hypermutation frequencies in germinal center B cells. (A) Location of V, I, and E segments used to analyze frequencies. L, leader exon. (B) Position of single-strand nucleotides (top half) and mutations (bottom half) relative to the map in A. Each vertical line represents the mutational frequency for individual nucleotides for a total of 3 independent experiments with 1–2 mice per experiment. Top half: single-strand (ss) deamination events from day 7 germinal centers on the nontranscribed strand (orange) are shown above the x-axis, and events on the transcribed strand (blue) are displayed below. Dotted horizontal black lines show the level of deaminations on day 0. Bottom half: mutations from Peyer's patches on both strands (red) are displayed. Data are from 4 experiments with 1 mouse per experiment. (C and D) Frequencies were analyzed in three 700 bp segments: V, I, and E. χ^2 tests compared the frequency in the V and I segments to the E segment. For single-strand DNA frequency, no significant difference was found between the nontranscribed and transcribed strands.

was significantly higher in the V and I segments compared with the E segment (Fig. 3 C). A similar analysis of single-strand DNA from ex vivo–activated cells also revealed a higher frequency in V and I regions compared with E (unpublished data).

To confirm that somatic hypermutation does indeed mirror RNA pol II accumulation and single-strand DNA density, we collected extensive data from sequencing the B1-8^{hi} region from germinal center B cells from Peyer's patches. As shown in Fig. 3 B (bottom half) and Fig. 3 D, the frequency of somatic hypermutation was significantly higher in the V and I segments compared with E. Although the E segment was not associated with many mutations or RNA pol II accumulation, the single-strand DNA detected there may be due to activity of the intronic promoter (Lennon and Perry, 1985). Collectively, elevated pol II levels correlate with increased single-strand DNA and mutations in the VDJ and J_H intron regions in germinal center cells. However, the lack of mutations after ex vivo activation indicates that pol II density and single-strand DNA alone are not sufficient to generate mutations.

Initiating Pol II, Spt5, and AID are present in V regions from germinal center cells but absent in ex vivo cells

To identify what is missing in cultured cells, we first characterized the type of RNA pol II accumulation. Initiating polymerases are marked by having the C-terminal domain phosphorylated at Ser5 (Ser5P; Buratowski, 2009). Using an antibody for Ser5P, ChIP analysis showed that this form of pol II was abundant at the V and I primers in both B1-8^{hi} and 8D10-GL germinal center B cells but not in ex vivo–activated cells (Fig. 4, A and B). Furthermore, Spt5, which is a member of the DSIF complex associated with the initiating form of pol II, was associated with the V and I primers in germinal center cells but was missing in ex vivo cells (Fig. 4 C). Concomitantly, AID was abundant at the V and I locations in germinal center cells but was nonexistent in ex vivo cells (Fig. 4 D). For controls, the transcribed *γ-actin* gene had significant amounts of the Ser5P form of pol II and Spt5 at primer *a* over the promoter in both germinal center and ex vivo cells, whereas the promoter of the nontranscribed *β-globin* gene was negative for these two proteins. AID was not present in the off-target *γ-actin* and *β-globin* genes. Thus, there is good correlation between the Ser5P form of RNA pol II, Spt5, and AID proteins bound to V and I sequences after in vivo immunization. These proteins are totally absent in ex vivo–stimulated cells.

Initiating Pol II, Spt5, and AID are present in Sμ regions from both germinal center and ex vivo cells

Proteins bound to the Sμ region have been well-characterized in ex vivo–stimulated cells, but little is known about their participation in germinal center cells. Because the Sμ region undergoes somatic hypermutation after both types of activation, we determined if recruitment of transcription and AID proteins was identical by ChIP. As shown in Fig. 5 A, there is an intronic promoter located downstream of the Eμ enhancer that is constitutively activated and initiates transcription through the Sμ region. We used E, S, and C primers located before the promoter, in the 5′ Sμ region, and in Cμ, respectively, to measure proteins in germinal center– and ex vivo–activated cells from B1-8^{hi} *Ung*$^{-/-}$ mice. In Fig. 5 B, pol II

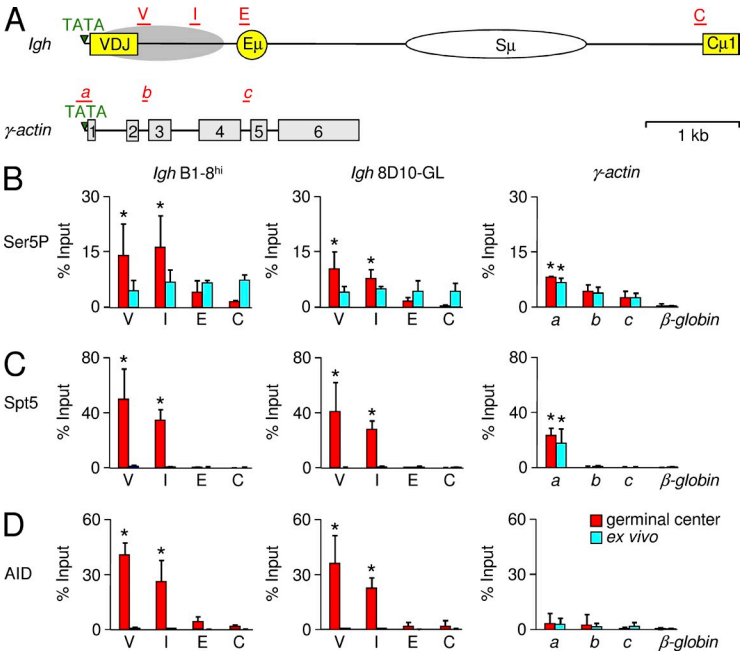
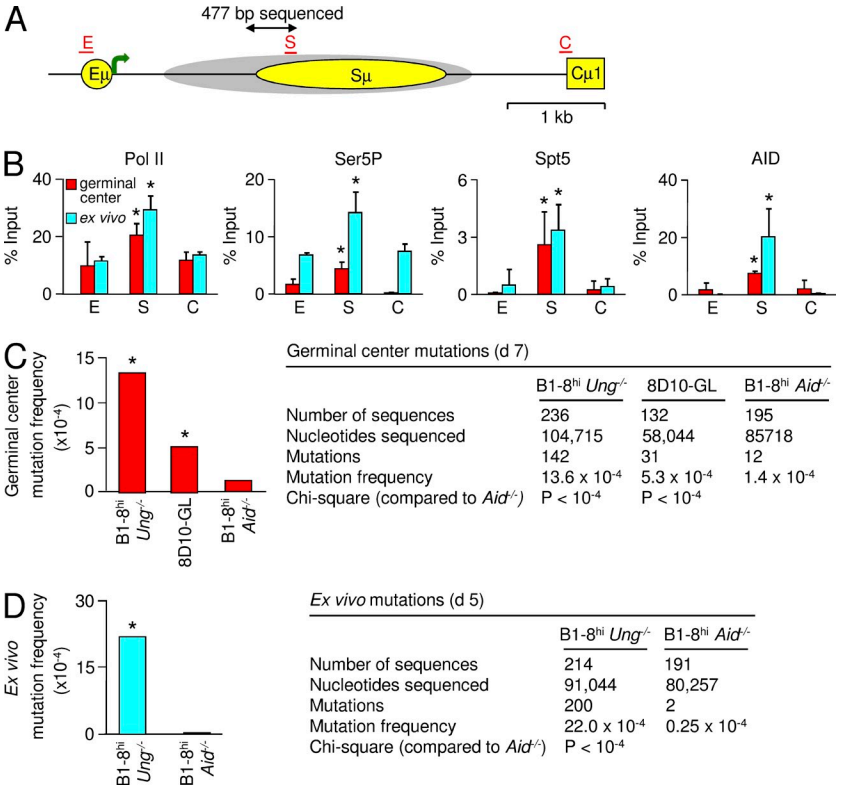


Figure 4. ChIP analyses of proteins bound to V region DNA from activated B cells. (A) Location of ChIP primer sets (red) in *Igh* (V, I, E, and C) and γ-*actin* (*a*, *b*, and *c*) are shown above the loci. Gray oval in the *Igh* locus represents the area of somatic hypermutation. (B–D) Ser5P (B), Spt5 (C), and AID (D) ChIPs from day 7 germinal center (red) and day 2 ex vivo (blue) activation. Error bars represent the SD of values from 9 independent experiments for B1-8^{hi}, and 6 experiments for 8D10-GL, with 1 mouse per experiment. For the *Igh* genes, *, $P < 0.05$ compared with values for C (unpaired two-tailed Student's *t* test). For the γ-*actin* gene, results were averaged between B1-8^{hi} and 8D10-GL cells. *, $P < 0.05$ compared with *c*.

was significantly accumulated at the S primer, and it was the initiating form characterized by Ser5P. Furthermore, Spt5 and AID were significantly associated with the stalled polymerases in both germinal center and ex vivo cells. These results confirm previous reports of ex vivo transcription, which is defined by stalled polymerases (Rajagopal et al., 2009; Wang et al., 2009), Ser5P (Wang et al., 2009), Spt5 (Pavri et al., 2010), and AID (Pavri et al., 2010) and extend the findings to germinal center transcription. Because these proteins are down-regulated in Eμ, the results suggest that they are brought in during de novo transcription from the intronic promoter and become amassed during the stalled migration of pol II through R-loop structures. All these markers of transcription and mutation are made manifest by inducing somatic hypermutation



Germinal center mutations (d 7)

	B1-8^{hi} *Ung*$^{-/-}$	8D10-GL	B1-8^{hi} *Aid*$^{-/-}$
Number of sequences	236	132	195
Nucleotides sequenced	104,715	58,044	85718
Mutations	142	31	12
Mutation frequency	13.6×10^{-4}	5.3×10^{-4}	1.4×10^{-4}
Chi-square (compared to *Aid*$^{-/-}$)	$P < 10^{-4}$	$P < 10^{-4}$	

Ex vivo mutations (d 5)

	B1-8^{hi} *Ung*$^{-/-}$	B1-8^{hi} *Aid*$^{-/-}$
Number of sequences	214	191
Nucleotides sequenced	91,044	80,257
Mutations	200	2
Mutation frequency	22.0×10^{-4}	0.25×10^{-4}
Chi-square (compared to *Aid*$^{-/-}$)	$P < 10^{-4}$	

Figure 5. ChIP analysis and mutation frequency in the Sμ region from activated B cells. (A) Location of ChIP primer sets for E, S, and C (red) are shown above the loci. Double-sided arrow represents the region sequenced for mutations. Green arrow, intronic promoter. Gray oval represents the area of somatic hypermutation. (B) ChIP from day 7 germinal center (red) and day 2 ex vivo (blue) activation. Error bars represent the SD of values from 9 independent experiments for B1-8^{hi} with 1 mouse per experiment. *, $P < 0.05$ compared with values for C (unpaired two-tailed *t* test). (C and D) Hypermutation analysis in germinal center and ex vivo activated cells. Germinal center mutation frequencies (red) were measured in B1-8^{hi} *Ung*$^{-/-}$, 8D10-GL, and B1-8^{hi} *Aid*$^{-/-}$ (3–4 independent experiments with 1 mouse per experiment). *, $P < 10^{-4}$ compared with the B1-8^{hi} *Aid*$^{-/-}$ control. Ex vivo mutation frequencies (blue) were measured in B1-8^{hi} *Ung*$^{-/-}$ and B1-8^{hi} *Aid*$^{-/-}$ (3–4 independent experiments with 1 mouse per experiment). *, $P < 10^{-4}$ compared with B1-8^{hi} *Aid*$^{-/-}$.

in the Sμ region from germinal center cells (Fig. 5 C) and ex vivo cells (Fig. 5 D). Therefore, the Sμ region is accessible for mutation under both conditions of cell activation, perhaps because of the constitutively active intronic promoter and unique DNA sequence.

Deletion of the 3′ regulatory region (3′RR) reduces Pol II and ablates Spt5 and AID in J_H4 regions

The 3′RR on the heavy chain locus is implicated in controlling transcription, somatic hypermutation, and class switch recombination in mature B cells (Vincent-Fabert et al., 2010; Rouaud et al., 2013). To determine if it affects the recruitment of Spt5 and AID to the V region in germinal center cells, we examined mice which have a 30-kb deletion of the 3′RR (Vincent-Fabert et al., 2010). Mice were immunized with phycoerythrin for 7 d, and $GL7^+$ B cells were isolated from spleens for ChIP assays. Because the mice contain a heterogeneous repertoire of VDJ rearrangements, we focused on the J_H4 intron, with the caveat that only 25% of the cells may contain rearrangements to J_H4. To see if transcription could be measured in these heterogeneous cells, RNA pol II ChIPs were performed using J_H4 and E primers (Fig. 6 A). The results in Fig. 6 B showed that in WT cells, pol II was reduced fourfold compared with similar regions in the knock-in B1-8 mice (Fig. 2 C), confirming that WT mice had fewer rearrangements to J_H4. In 3′RR cells, pol II molecules were drastically reduced 80% in both J_H4 and E regions, compared with WT cells. Pol II binding in the mutant mice was unaffected in primers located at the TATA box in the *γ-actin* gene, and the *β-globin* gene was a negative control. In accord with the ChIPs from knock-in mice, Spt5 was present in J_H4 from WT cells, albeit fourfold lower than from B1-8^{hi} cells, but not in the E region, suggesting that the initiating form of pol II is retained over J_H4. However, Spt5 was absent over J_H4 in 3′RR cells. As controls, Spt5 was bound to primers located in the promoter of the *γ-actin* gene from both types of mice. In confirmation of a previous study (Rouaud et al., 2013), the AID protein was also missing in the J_H4 region from 3′RR mice. Therefore, in the absence of the 3′RR, transcription was markedly reduced, and both Spt5 and AID proteins were not recruited to the J_H4 region.

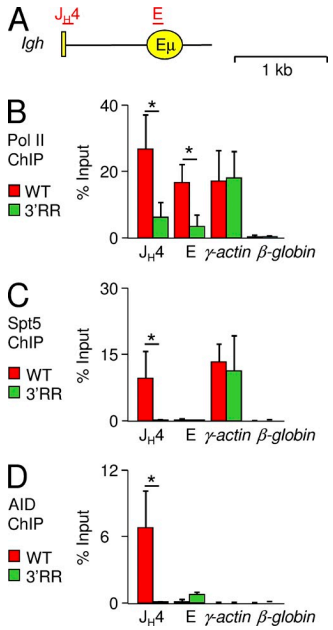


Figure 6. Protein recruitment in the absence of 3′RR. (A) Location of ChIP primer sets (red) in the *Igh* loci (J_H4 and E) are shown above the loci. (B–D) Pol II (B), Spt5 (C), and AID (D) ChIPs from germinal center cells 7 d after immunization. Error bars represent the SD of values from 3 independent experiments for WT (red) and 3 experiments for 3′RR-deficient (green) cells, with 1–2 mice per experiment. *, $P < 0.05$ comparing WT to 3′RR cells (unpaired two-tailed Student's *t* test).

DISCUSSION

V genes have long been enigmatic in efforts to understand how they are substrates for AID activity during transcription. In this first report of AID targeting in germinal center B cells, we examined features of transcription that are unique to V genes, which provide insight into their ability to sustain high levels of somatic hypermutation. Two distinct events occur after transcription.

First, RNA polymerases were paused for a long distance downstream of the TATA box. Using both nuclear run-on and ChIP techniques, pausing was detected over 1.2 kb encompassing the VDJ exon and J_H intron in B cells activated either ex vivo or in vivo. In contrast, there was no pausing in the transcribed *γ-actin* or *CD19* genes (unpublished data) from these cells. Prolonged pausing in the V region could be induced during co-transcriptional splicing of the leader and V exons. For example, the transcriptional machinery may be sluggish because of multiple splicing reactions at the leader and rearranged V exons on the heavy and light chain loci, and slow-moving transcription bubbles would increase pol II density. It is notable that CTNNBL1 and PTBP2 factors associated with the spliceosome also bind to AID (Conticello et al., 2008; Nowak et al., 2011). Pol II accumulation could also be due to cis DNA sequences in the J_H introns, which are shared by all V gene rearrangements. Intron sequences have been suggested to form secondary structures during transcription (Golding et al., 1987; Wright et al., 2008), which may impact histone assembly and polymerase migration. Both these predictions can be tested by modifying DNA sequences of rearranged genes in cell lines to see how they affect pol II accumulation. Furthermore, when pol II progression was blocked in DT40 and Ramos cell lines, mutations were increased (Kodgire et al., 2013; Wang et al., 2014). In summary, pausing for more than 1 kb after the promoter is an intrinsic feature of rearranged V genes and can occur regardless of the type of cell stimulation. Paused polymerases then establish the boundary of mutation by exposing single-strand DNA as the substrate for AID in cells from either germinal centers or ex vivo culture. However, although pol II density and single-strand DNA are necessary for somatic hypermutation, they are not sufficient because

the V and I segments from ex vivo activation have virtually no mutations.

Second, although RNA pol II density profiles are similar between germinal center and ex vivo cells, the initiating form of pol II characterized by Ser5 phosphorylation in the C-terminal domain is retained only in germinal center cells. Typically, the conversion to transcriptional elongation occurs within 100 bp after the start of transcription, followed by a rapid loss of Ser5P (Brookes et al., 2012), which we detected in the control *γ-actin* gene. Thus, it is notable that the Ser5P form of pol II was elevated in the V and I primers located 0.6 and 1.2 kb, respectively, downstream from the TATA box preceding the VDJ gene. It is not known what causes the delay from initiation to elongation, but the process catalyzes the recruitment of Spt5 and AID to V genes. Our ChIP data conclusively show that Spt5 and AID are present at the V and I primers in germinal center cells but are totally absent in ex vivo cells. Thus, there is a strong correlation between Ser5P, Spt5, AID, and mutations, as summarized in Fig. 7. The data also indicate that pol II pausing occurs independently of Spt5 recruitment in ex vivo cells and may precede it in germinal center cells. We then examined whether the 3′ RR enhancers affect Spt5 and AID accumulation using mice with a 30-kb deletion of the region. Pol II density in germinal center cells was decreased 80% in the J_H4 and Eμ regions, and Spt5 and AID were nonexistent, explaining the lack of somatic hypermutation in these mice (Rouaud et al., 2013). However, a definitive role for the 3′RR is elusive because transcription was greatly reduced in the knockout mice. Perhaps sub-enhancers—such as hs3a, hs1-2, hs3b, and hs4—within this region may be differentially used by WT cells during activation in vivo compared with ex vivo.

The other targets of somatic hypermutation are the S regions on the *Igh* locus. These regions are a sponge for AID due to the draconian R-loop structures which produce a pile-up of RNA pol II molecules. Wang et al. (2009) have reported that the Ser5P form is also enriched in S regions located 3 kb from transcription start sites in introns. This suggests that retention of the initiating form for long distances in the V and S regions is quite different from the promoter-proximal pausing described in other genes (Kwak and Lis, 2013). Paused initiating polymerases can then more efficiently recruit Spt5, AID, and other factors involved in hypermutation (Willmann et al., 2012; Aida et al., 2013; Singh et al., 2013).

Genome-wide studies of ex vivo–stimulated cells have shown that AID localization mirrors RNA pol II and Spt5 density (Pavri et al., 2010; Yamane et al., 2011). Because RNA pol II and Spt5 are present at most promoters, as many as 6,000 genes could be potential targets of AID (Yamane et al., 2011). We demonstrate here that transcribed V genes are not a target for Spt5 or AID in ex vivo cells and suggest that the number of potential targets may be very different in germinal center B cells. Furthermore, not all genes are mutated equally (Liu et al., 2008; Yamane et al., 2011), which indicates that additional factors regulate AID activity. For example, the mutated *c-myc* gene has a specific RNA pol II pause site in close proximity to the area of mutations (Krumm et al., 1992), and the murine *Bcl6* gene has a significant number of nucleotide repeats near the site of mutations (Liu et al., 2008). Therefore, the sequences of these genes may cause transcriptional perturbation, followed by subsequent pol II accumulation and AID activity. In contrast, we show that the highly transcribed *γ-actin* gene has high levels of the Ser5P form of pol II and Spt5 protein at the promoter region, but no increased accumulation of pol II in the gene body and no detectable AID localization. Thus, a model evolves where AID may be present at open promoters, but the ability to access downstream DNA is regulated by the combined effects of RNA pol II density, DNA sequence, and AID cofactors.

What causes RNA pol II to retain its Ser5P initiating form for a long distance over the V gene and downstream intronic DNA is not known. Conversion to the elongating form in germinal center cells could be blocked by differential usage of transcription factors from either the enhancers or promoter after activation. It has been proposed that the same enhancer may have different factors bound at various times (Pinaud et al., 2011), and the same gene may be controlled by different enhancers during various activation states (Kieffer-Kwon et al., 2013). Another question is what role Spt5 is playing during V gene transcription. The protein is generally associated with the DSIF complex which coordinates pol II promoter release (Kwak and Lis, 2013), or it could potentially be taking a larger role in regulating V region transcription similar to recent results for the A20 gene (Diamant et al., 2012).

In summary, transcription sets up the V locus as a single-strand substrate by augmenting paused RNA polymerases, perhaps due to the DNA sequence of the region. Proteins associated with hypermutation, such as Spt5 and AID, are recruited to the initiating form of pol II specifically in cells

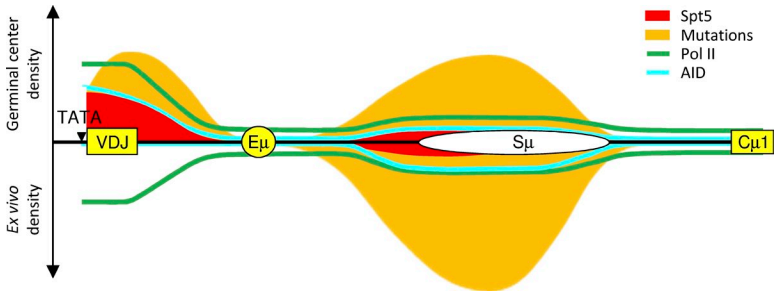


Figure 7. Differential Spt5 and AID accumulation define somatic hypermutation in germinal center– versus ex vivo–activated cells. Representation of protein density and mutations in the *Igh* loci comparing activation in germinal centers (above axis) and ex vivo (below axis). Red-shaded area depicts amassed Spt5, and the orange-shaded area illustrates copious somatic hypermutation. Lines represent the relative density for RNA pol II (green) and AID (blue).

activated in germinal centers. Differential activation programs in B cells induced in germinal centers versus ex vivo may produce an altered landscape of transcription factors that favors initiating polymerases and the cascade of mutation proteins.

MATERIALS AND METHODS

Mice. B1-8^{hi} knock-in mice (Shih et al., 2002) were obtained from Rafael Casellas (National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health). B1-8^{hi} *Ung*$^{-/-}$ and B1-8^{hi} *Aid*$^{-/-}$ mice were generated at the National Institute on Aging. 8D10-GL mice were made at Brandeis and Harvard Universities. 8D10-GL expresses the unmutated version of the mutated heavy chain (VGAM3.8 family VGK7 germline gene rearranged to D and J_H2) from 8D10, a hybridoma derived from the BALB.B memory response to (T,G)-A-L antigen (Press and Giorgetti, 1993). The 8D10 heavy chain rearrangement was cloned; a fragment containing VDJ_H2 but lacking J_H3 and J_H4 was obtained; and V_H codon #92 was changed from TGT to TGC to eliminate a cryptic heptamer site. To construct 8D10-GL, a 123-bp BglII–NdeI fragment (containing the mutated V_H region) was removed from 8D10 VDJ_H2 and replaced with the corresponding piece from the VGK7 germline gene using a genomic phage clone (VFM1.5) from M. Taussig (Babraham Institute, Cambridge, UK; Sims et al., 1992). The pIVHL-2neo^r vector was provided by K. Rajewsky (Harvard University, Cambridge, MA) and contained an HSV-tk cassette (~1.8 kb), a 5′ homology region (~9 kb of BALB/c germline DNA, a BamHI–XhoI fragment 5′ of DQ52), a neomycin resistance gene (neo^r, ~1.3 kb) flanked by two loxP sites, and a 3′ homology region (~0.8 kb of germline DNA from 129/Ola, the NaeI-PvuII fragment upstream of the Eμ enhancer). The 8D10-GL fragment, containing ~1.5 kb of flank, was cloned into this vector after extending the vector's 3′ homology region from 0.8 to 2.5 kb (Sonoda et al., 1997). TC1 embryo stem (ES) cells electroporated with recombinant vector were selected with both G418 and gancyclovir. To screen for recombinant clones, Southern blots of restriction enzyme–digested genomic DNA from ES clones were hybridized with a XbaI-BglII piece from an ~870-bp XbaI–BamHI genomic fragment beginning 5′ of Cμ (Bottaro et al., 1998; Sakai et al., 1999). Recombinant clones, still containing the neo^r cassette, were injected by Laurie Davidson (Harvard University) into C57BL/6 blastocysts to produce chimeric mice. Male chimeras were mated to C57BL/6 or 129SvEv (129S6) females (Taconic), and the F1 progeny were screened by Southern blot analysis of BamHI-digested tail DNA for the presence of the knock-in allele and correct targeting of the locus. Mice containing the knock-in allele were backcrossed onto the 129S6 background and were bred to EIIa-Cre transgenic mice on the 129S6 background (Lakso et al., 1996) to delete the loxP-flanked neo^r cassette in vivo. The 8D10-GL heterozygous strain was backcrossed 9–10 generations onto C57BL/6, and intercrossed to produce 8D10-GL homozygous mice. 3′RR-deficient mice were previously described (Vincent-Fabert et al., 2010). WT C57BL/6 mice were purchased from Charles River.

For immunizations, mice were injected intraperitoneally with 100 μg phycoerythrin (Prozyme) in complete Freund's adjuvant (Sigma-Aldrich) and used 7 d later. All animal procedures were reviewed and approved by the Animal Care and Use Committees of the National Institute on Aging and Brandeis University.

B cell activation. Naive splenic B cells were isolated by negative selection with anti-CD43 and anti-CD11b magnetic beads (Miltenyi Biotec). Cells were plated at a density of 0.5 million cells/ml and stimulated ex vivo with 5 μg/ml *Escherichia coli* LPS serotype 0111:B4 (Sigma-Aldrich) and 5 ng/ml recombinant IL-4 (BioLegend). Germinal center B cells from immunized mice were isolated by staining with FITC-conjugated anti-B220 (Southern-Biotech) and Alexa Fluor 647–labeled anti-GL7 (eBioscience), and B220$^+$ GL7$^+$ cells were isolated by flow cytometry.

AID expression. RNA was extracted from 0.5 million germinal center– or ex vivo–activated B cells using TRIzol reagent (Invitrogen), followed by further purification with RNeasy Mini kit (QIAGEN). cDNA was generated using Superscript III (Invitrogen), followed by qPCR using Power SYBR Green PCR Master Mix (Life Technologies) and the primers listed in Table S1.

Hypermutation analysis. B1-8^{hi} *Ung*$^{-/-}$, B1-8^{hi} *Aid*$^{-/-}$, and 8D10-GL mice were used to detect mutations. Genomic DNA in cells from day 7 immunized germinal centers, day 5 ex vivo activation, and Peyer's patches (4 mo) was isolated and amplified using Herculase II enzyme (Agilent Technologies) with primers listed in Table S1. Ex vivo cells were stimulated in 24 independent wells and analyzed separately to minimize clonal amplification. Frequencies were calculated by dividing the number of mutations by the total number of nucleotides sequenced.

Nuclear run-on. Naive splenic B cells were isolated from B1-8^{hi} *Ung*$^{-/-}$ mice and stimulated with LPS and IL-4. Nuclear run-on experiments were performed on days 0 and 2 as described previously (Rajagopal et al., 2009). Primers for 500-bp probes used in hybridization are listed in Table S1.

μChIP. Experiments on ex vivo– and germinal center–activated B cells were performed using a μChIP protocol (Dahl and Collas, 2008). In brief, 0.25 to 0.5 million cells were cross-linked with 1% formaldehyde for 10 min at room temperature. Cross-linked cells were lysed, and cellular DNA was sheared to a mean size of 500 bp by sonication, followed by incubation with antibody/Dynabead complexes (Invitrogen). Antibodies against pol II (Millipore, clone CTD4H8), pol II CTD-Ser5 (Abcam), SPT5 (clone H-300; Santa Cruz Biotechnology, Inc.), AID (Kohli et al., 2010), and nonspecific rabbit IgG (Millipore) were used at 2.5 μg per reaction. Primers for quantitative PCR are listed in Table S1. The V primer set was positioned at the end of J_H2 to avoid amplification of unrearranged members from the vast J558 V_H family. Calculation of % input was performed by deriving the % input value for the experimental antibody ($2^{Ct(input)-Ct(ChIP)} \times 100$) and subtracting the % input value of the IgG control.

Sodium bisulfite analysis. Sodium bisulfite experiments were performed as previously described (Ronai et al., 2007) on 1–2 × 10^6 naive or germinal center cells from B1-8^{hi} *Aid*$^{-/-}$ mice taken 7 d after immunization with phycoerythrin. DNA was amplified using Taq DNA polymerase (Takara Bio Inc.) with primers listed in Table S1 and sequenced. Analysis of single-strand DNA frequency for individual nucleotides was done by dividing the number of deaminated C:Gs by the total number of C:Gs sequenced.

Online supplemental material. Table S1 lists sequences of primers used for AID expression, DNA sequencing, nuclear run-on, and ChIP assays. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20131512/DC1.

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REFERENCES

Aida, M., N. Hamad, A. Stanlie, N.A. Begum, and T. Honjo. 2013. Accumulation of the FACT complex, as well as histone H3.3, serves as a target marker for somatic hypermutation. *Proc. Natl. Acad. Sci. USA.* 110:7784–7789. http://dx.doi.org/10.1073/pnas.1305859110

Basu, U., F.L. Meng, C. Keim, V. Grinstein, E. Pefanis, J. Eccleston, T. Zhang, D. Myers, C.R. Wasserman, D.R. Wesemann, et al. 2011. The RNA exosome targets the AID cytidine deaminase to both strands of transcribed duplex DNA substrates. *Cell.* 144:353–363. http://dx.doi.org/10.1016/j.cell.2011.01.001

Bottaro, A., F. Young, J. Chen, M. Serwe, F. Sablitzky, and F.W. Alt. 1998. Deletion of the IgH intronic enhancer and associated matrix-attachment regions decreases, but does not abolish, class switching at the mu locus. *Int. Immunol.* 10:799–806. http://dx.doi.org/10.1093/intimm/10.6.799

Brookes, E., I. de Santiago, D. Hebenstreit, K.J. Morris, T. Carroll, S.Q. Xie, J.K. Stock, M. Heidemann, D. Eick, N. Nozaki, et al. 2012. Polycomb associates genome-wide with a specific RNA polymerase II variant, and regulates metabolic genes in ESCs. *Cell Stem Cell.* 10:157–170. http://dx.doi.org/10.1016/j.stem.2011.12.017

Buratowski, S. 2009. Progression through the RNA polymerase II CTD cycle. *Mol. Cell.* 36:541–546. http://dx.doi.org/10.1016/j.molcel.2009.10.019

Conticello, S.G., K. Ganesh, K. Xue, M. Lu, C. Rada, and M.S. Neuberger. 2008. Interaction between antibody-diversification enzyme AID and spliceosome-associated factor CTNNBL1. *Mol. Cell.* 31:474–484. http://dx.doi.org/10.1016/j.molcel.2008.07.009

Dahl, J.A., and P. Collas. 2008. A rapid micro chromatin immunoprecipitation assay (ChIP). *Nat. Protoc.* 3:1032–1045. http://dx.doi.org/10.1038/nprot.2008.68

Decker, D.J., P.J. Linton, S. Zaharevitz, M. Biery, T.R. Gingeras, and N.R. Klinman. 1995. Defining subsets of naive and memory B cells based on the ability of their progeny to somatically mutate in vitro. *Immunity.* 2:195–203. http://dx.doi.org/10.1016/S1074-7613(95)80092-1

Diamant, G., L. Amir-Zilberstein, Y. Yamaguchi, H. Handa, and R. Dikstein. 2012. DSIF restricts NF-κB signaling by coordinating elongation with mRNA processing of negative feedback genes. *Cell Reports.* 2:722–731. http://dx.doi.org/10.1016/j.celrep.2012.08.041

Golding, G.B., P.J. Gearhart, and B.W. Glickman. 1987. Patterns of somatic mutations in immunoglobulin variable genes. *Genetics.* 115:169–176.

Huang, F.T., K. Yu, B.B. Balter, E. Selsing, Z. Oruc, A.A. Khamlichi, C.L. Hsieh, and M.R. Lieber. 2007. Sequence dependence of chromosomal R-loops at the immunoglobulin heavy-chain Sμ class switch region. *Mol. Cell. Biol.* 27:5921–5932. http://dx.doi.org/10.1128/MCB.00702-07

Källberg, E., S. Jainandunsing, D. Gray, and T. Leanderson. 1996. Somatic mutation of immunoglobulin V genes in vitro. *Science.* 271:1285–1289. http://dx.doi.org/10.1126/science.271.5253.1285

Kieffer-Kwon, K.R., Z. Tang, E. Mathe, J. Qian, M.H. Sung, G. Li, W. Resch, S. Baek, N. Pruett, L. Grøntved, et al. 2013. Interactome maps of mouse gene regulatory domains reveal basic principles of transcriptional regulation. *Cell.* 155:1507–1520. http://dx.doi.org/10.1016/j.cell.2013.11.039

Kodgire, P., P. Mukkawar, S. Ratnam, T.E. Martin, and U. Storb. 2013. Changes in RNA polymerase II progression influence somatic hypermutation of Ig-related genes by AID. *J. Exp. Med.* 210:1481–1492. http://dx.doi.org/10.1084/jem.20121523

Kohli, R.M., S.R. Abrams, K.S. Gajula, R.W. Maul, P.J. Gearhart, and J.T. Stivers. 2009. A portable hot spot recognition loop transfers sequence preferences from APOBEC family members to activation-induced cytidine deaminase. *J. Biol. Chem.* 284:22898–22904. http://dx.doi.org/10.1074/jbc.M109.025536

Kohli, R.M., R.W. Maul, A.F. Guminski, R.L. McClure, K.S. Gajula, H. Saribasak, M.A. McMahon, R.F. Siliciano, P.J. Gearhart, and J.T. Stivers. 2010. Local sequence targeting in the AID/APOBEC family differentially impacts retroviral restriction and antibody diversification. *J. Biol. Chem.* 285:40956–40964. http://dx.doi.org/10.1074/jbc.M110.177402

Krumm, A., T. Meulia, M. Brunvand, and M. Groudine. 1992. The block to transcriptional elongation within the human c-myc gene is determined in the promoter-proximal region. *Genes Dev.* 6:2201–2213. http://dx.doi.org/10.1101/gad.6.11.2201

Kwak, H., and J.T. Lis. 2013. Control of transcriptional elongation. *Annu. Rev. Genet.* 47:483–508. http://dx.doi.org/10.1146/annurev-genet-110711-155440

Lakso, M., J.G. Pichel, J.R. Gorman, B. Sauer, Y. Okamoto, E. Lee, F.W. Alt, and H. Westphal. 1996. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. *Proc. Natl. Acad. Sci. USA.* 93:5860–5865. http://dx.doi.org/10.1073/pnas.93.12.5860

Lebecque, S.G., and P.J. Gearhart. 1990. Boundaries of somatic mutation in rearranged immunoglobulin genes: 5′ boundary is near the promoter, and 3′ boundary is approximately 1 kb from V(D)J gene. *J. Exp. Med.* 172:1717–1727. http://dx.doi.org/10.1084/jem.172.6.1717

Lennon, G.G., and R.P. Perry. 1985. C $_{\mu}$-containing transcripts initiate heterogeneously within the IgH enhancer region and contain a novel 5′-nontranslatable exon. *Nature.* 318:475–478. http://dx.doi.org/10.1038/318475a0

Liu, M., J.L. Duke, D.J. Richter, C.G. Vinuesa, C.C. Goodnow, S.H. Kleinstein, and D.G. Schatz. 2008. Two levels of protection for the B cell genome during somatic hypermutation. *Nature.* 451:841–845. http://dx.doi.org/10.1038/nature06547

Manser, T. 1987. Mitogen-driven B cell proliferation and differentiation are not accompanied by hypermutation of immunoglobulin variable region genes. *J. Immunol.* 139:234–238.

Matthews, A.J., S. Husain, and J. Chaudhuri. 2014. Binding of AID to DNA does not correlate with mutator activity. *J. Immunol.* 193:252–257. http://dx.doi.org/10.4049/jimmunol.1400433

Maul, R.W., and P.J. Gearhart. 2010. AID and somatic hypermutation. *Adv. Immunol.* 105:159–191. http://dx.doi.org/10.1016/S0065-2776(10)05006-6

Maul, R.W., H. Saribasak, S.A. Martomo, R.L. McClure, W. Yang, A. Vaisman, H.S. Gramlich, D.G. Schatz, R. Woodgate, D.M. Wilson III, and P.J. Gearhart. 2011. Uracil residues dependent on the deaminase AID in immunoglobulin gene variable and switch regions. *Nat. Immunol.* 12:70–76. http://dx.doi.org/10.1038/ni.1970

McHeyzer-Williams, M.G., G.J. Nossal, and P.A. Lalor. 1991. Molecular characterization of single memory B cells. *Nature.* 350:502–505. http://dx.doi.org/10.1038/350502a0

Milstein, C., M.S. Neuberger, and R. Staden. 1998. Both DNA strands of antibody genes are hypermutation targets. *Proc. Natl. Acad. Sci. USA.* 95:8791–8794. http://dx.doi.org/10.1073/pnas.95.15.8791

Nojima, T., K. Haniuda, T. Moutai, M. Matsudaira, S. Mizokawa, I. Shiratori, T. Azuma, and D. Kitamura. 2011. In-vitro derived germinal centre B cells differentially generate memory B or plasma cells in vivo. *Nat. Commun.* 2:465. http://dx.doi.org/10.1038/ncomms1475

Nowak, U., A.J. Matthews, S. Zheng, and J. Chaudhuri. 2011. The splicing regulator PTBP2 interacts with the cytidine deaminase AID and promotes binding of AID to switch-region DNA. *Nat. Immunol.* 12:160–166. http://dx.doi.org/10.1038/ni.1977

Pape, K.A., J.J. Taylor, R.W. Maul, P.J. Gearhart, and M.K. Jenkins. 2011. Different B cell populations mediate early and late memory during an endogenous immune response. *Science.* 331:1203–1207. http://dx.doi.org/10.1126/science.1201730

Parsa, J.Y., S. Ramachandran, A. Zaheen, R.M. Nepal, A. Kapelnikov, A. Belcheva, M. Berru, D. Ronai, and A. Martin. 2012. Negative supercoiling creates single-stranded patches of DNA that are substrates for AID-mediated mutagenesis. *PLoS Genet.* 8:e1002518. http://dx.doi.org/10.1371/journal.pgen.1002518

Pavri, R., A. Gazumyan, M. Jankovic, M. Di Virgilio, I. Klein, C. Ansarah-Sobrinho, W. Resch, A. Yamane, B. Reina San-Martin, V. Barreto, et al. 2010. Activation-induced cytidine deaminase targets DNA at sites of RNA polymerase II stalling by interaction with Spt5. *Cell.* 143:122–133. http://dx.doi.org/10.1016/j.cell.2010.09.017

Peters, A., and U. Storb. 1996. Somatic hypermutation of immunoglobulin genes is linked to transcription initiation. *Immunity.* 4:57–65. http://dx.doi.org/10.1016/S1074-7613(00)80298-8

Pinaud, E., M. Marquet, R. Fiancette, S. Péron, C. Vincent-Fabert, Y. Denizot, and M. Cogné. 2011. The IgH locus 3′ regulatory region: pulling the strings from behind. *Adv. Immunol.* 110:27–70. http://dx.doi.org/10.1016/B978-0-12-387663-8.00002-8

Press, J.L., and C.A. Giorgetti. 1993. Molecular and kinetic analysis of an epitope-specific shift in the B cell memory response to a multideterminant antigen. *J. Immunol.* 151:1998–2013.

Rada, C., J.M. Di Noia, and M.S. Neuberger. 2004. Mismatch recognition and uracil excision provide complementary paths to both Ig switching and the A/T-focused phase of somatic mutation. *Mol. Cell.* 16:163–171. http://dx.doi.org/10.1016/j.molcel.2004.10.011

Rajagopal, D., R.W. Maul, A. Ghosh, T. Chakraborty, A.A. Khamlichi, R. Sen, and P.J. Gearhart. 2009. Immunoglobulin switch mu sequence causes RNA polymerase II accumulation and reduces dA hypermutation. *J. Exp. Med.* 206:1237–1244. http://dx.doi.org/10.1084/jem.20082514

Reina-San-Martin, B., S. Difilippantonio, L. Hanitsch, R.F. Masilamani, A. Nussenzweig, and M.C. Nussenzweig. 2003. H2AX is required for

recombination between immunoglobulin switch regions but not for intra-switch region recombination or somatic hypermutation. *J. Exp. Med.* 197:1767–1778. http://dx.doi.org/10.1084/jem.20030569

Ronai, D., M.D. Iglesias-Ussel, M. Fan, Z. Li, A. Martin, and M.D. Scharff. 2007. Detection of chromatin-associated single-stranded DNA in regions targeted for somatic hypermutation. *J. Exp. Med.* 204:181–190. http://dx.doi.org/10.1084/jem.20062032

Rouaud, P., C. Vincent-Fabert, A. Saintamand, R. Fiancette, M. Marquet, I. Robert, B. Reina-San-Martin, E. Pinaud, M. Cogné, and Y. Denizot. 2013. The IgH 3′ regulatory region controls somatic hypermutation in germinal center B cells. *J. Exp. Med.* 210:1501–1507. http://dx.doi.org/10.1084/jem.20130072

Sakai, E., A. Bottaro, and F.W. Alt. 1999. The Ig heavy chain intronic enhancer core region is necessary and sufficient to promote efficient class switch recombination. *Int. Immunol.* 11:1709–1713. http://dx.doi.org/10.1093/intimm/11.10.1709

Saribasak, H., R.W. Maul, Z. Cao, W.W. Yang, D. Schenten, S. Kracker, and P.J. Gearhart. 2012. DNA polymerase ζ generates tandem mutations in immunoglobulin variable regions. *J. Exp. Med.* 209:1075–1081. http://dx.doi.org/10.1084/jem.20112234

Shih, T.A., M. Roederer, and M.C. Nussenzweig. 2002. Role of antigen receptor affinity in T cell-independent antibody responses in vivo. *Nat. Immunol.* 3:399–406. http://dx.doi.org/10.1038/ni776

Sims, M.J., U. Krawinkel, and M.J. Taussig. 1992. Characterization of germline genes of the VGAM3.8 VH gene family from BALB/c mice. *J. Immunol.* 149:1642–1648.

Singh, S.K., K. Maeda, M.M. Eid, S.A. Almofty, M. Ono, P. Pham, M.F. Goodman, and N. Sakaguchi. 2013. GANP regulates recruitment of AID to immunoglobulin variable regions by modulating transcription and nucleosome occupancy. *Nat. Commun.* 4:1830. http://dx.doi.org/10.1038/ncomms2823

Sonoda, E., Y. Pewzner-Jung, S. Schwers, S. Taki, S. Jung, D. Eilat, and K. Rajewsky. 1997. B cell development under the condition of allelic inclusion. *Immunity.* 6:225–233. http://dx.doi.org/10.1016/S1074-7613(00)80325-8

Vincent-Fabert, C., R. Fiancette, E. Pinaud, V. Truffinet, N. Cogné, M. Cogné, and Y. Denizot. 2010. Genomic deletion of the whole IgH 3′ regulatory region (hs3a, hs1,2, hs3b, and hs4) dramatically affects class switch recombination and Ig secretion to all isotypes. *Blood.* 116:1895–1898. http://dx.doi.org/10.1182/blood-2010-01-264689

Wang, L., R. Wuerffel, S. Feldman, A.A. Khamlichi, and A.L. Kenter. 2009. S region sequence, RNA polymerase II, and histone modifications create chromatin accessibility during class switch recombination. *J. Exp. Med.* 206:1817–1830. http://dx.doi.org/10.1084/jem.20081678

Wang, M., C. Rada, and M.S. Neuberger. 2010. Altering the spectrum of immunoglobulin V gene somatic hypermutation by modifying the active site of AID. *J. Exp. Med.* 207:141–153. http://dx.doi.org/10.1084/jem.20092238

Wang, X., M. Fan, S. Kalis, L. Wei, and M.D. Scharff. 2014. A source of the single-stranded DNA substrate for activation-induced deaminase during somatic hypermutation. *Nat. Commun.* 5:4137. http://dx.doi.org/10.1038/ncomms5137

Willmann, K.L., S. Milosevic, S. Pauklin, K.M. Schmitz, G. Rangam, M.T. Simon, S. Maslen, M. Skehel, I. Robert, V. Heyer, et al. 2012. A role for the RNA pol II-associated PAF complex in AID-induced immune diversification. *J. Exp. Med.* 209:2099–2111. http://dx.doi.org/10.1084/jem.20112145

Wright, B.E., K.H. Schmidt, N. Davis, A.T. Hunt, and M.F. Minnick. 2008. II. Correlations between secondary structure stability and mutation frequency during somatic hypermutation. *Mol. Immunol.* 45:3600–3608. http://dx.doi.org/10.1016/j.molimm.2008.05.012

Xue, K., C. Rada, and M.S. Neuberger. 2006. The in vivo pattern of AID targeting to immunoglobulin switch regions deduced from mutation spectra in *msh2*$^{-/-}$ *ung*$^{-/-}$ mice. *J. Exp. Med.* 203:2085–2094. http://dx.doi.org/10.1084/jem.20061067

Yamane, A., W. Resch, N. Kuo, S. Kuchen, Z. Li, H.W. Sun, D.F. Robbiani, K. McBride, M.C. Nussenzweig, and R. Casellas. 2011. Deep-sequencing identification of the genomic targets of the cytidine deaminase AID and its cofactor RPA in B lymphocytes. *Nat. Immunol.* 12:62–69. http://dx.doi.org/10.1038/ni.1964