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Brief Communication

Topoisomerase I deficiency causes RNA polymerase II accumulation and increases AID abundance in immunoglobulin variable genes

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ABSTRACT

Activation-induced deaminase (AID) is a DNA cytosine deaminase that diversifies immunoglobulin genes in B cells. Recent work has shown that RNA polymerase II (Pol II) accumulation correlates with AID recruitment. However, a direct link between Pol II and AID abundance has not been tested. We used the DT40 B-cell line to manipulate levels of Pol II by decreasing topoisomerase I (Top1), which relaxes DNA supercoiling in front of the transcription complex. Top1 was decreased by stable transfection of a short hairpin RNA against Top1, which produced an accumulation of Pol II in transcribed genes, compared to cells transfected with sh-control RNA. The increased Pol II density enhanced AID recruitment to variable genes in the λ light chain locus, and resulted in higher levels of somatic hypermutation and gene conversion. It has been proposed by another lab that AID itself might directly suppress Top1 to increase somatic hypermutation. However, we found that in both $AID^{+/+}$ and $AID^{-/-}$ B cells from DT40 and mice, Top1 protein levels were identical, indicating that the presence or absence of AID did not decrease Top1 expression. Rather, our results suggest that the mechanism for increased diversity when Top1 is reduced is that Pol II accumulates and recruits AID to variable genes.

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1. Introduction

B cells express activation induced-deaminase (AID) to generate diversity in antibody genes. AID converts cytosine to uracil in single-strand DNA, and the rogue uracil initiates a cascade of error-prone repair pathways to introduce mutations into the immunoglobulin loci (reviewed in [1]). Mutations which alter the variable (V) gene coding sequence on the heavy and light chain loci are then selected for increased affinity to antigen, producing a robust antibody response. Mutations also occur in switch regions preceding each constant (C) gene on the heavy chain locus, which initiate heavy chain class switching to further diversify antibodies. While AID primarily targets immunoglobulin genes, off-target events can occur in several oncogenes including *Bcl6*, *Pax5*, and *Myc* [2–4]. This potential for AID-induced genomic instability warrants continuing investigation into the mechanisms of AID targeting.

One essential component of AID targeting is the need for transcription [5]. In vitro studies have demonstrated that when RNA polymerase II (Pol II) is paused, AID generates multiple mutations [6]. Recently, we reported that AID mutagenesis correlated with Pol II accumulation in the switch and V regions of mice [7,8]. In switch regions, it has been proposed that the DNA sequence forms RNA:DNA hybrids, or R-loops [9], which inhibit transcriptional elongation and increase Pol II accumulation. The paused Pol II complexes then recruit Spt5, a transcription initiation factor, and the RNA exosome, which degrades RNA, to resume elongation [10,11]. Both of these factors have been shown to directly interact with AID, suggesting they play a role in recruiting AID. In V regions, however, there are no R-loops, and it is not known what directs AID to these regions. Nonetheless, Pol II accumulation appears to be involved, as we identified paused Pol II complexes that were associated with Spt5 in germinal center B cells [8]. Furthermore, Pol II-Spt5 complexes correlated with AID accumulation, suggesting similar mechanisms of AID targeting in both switch and V regions. However, the hypothesis that increased Pol II density can actively promote AID mutagenesis has not been directly tested in vivo.

Topoisomerase I (Top1) is an essential enzyme which maintains proper helical tension in DNA. The maintenance of helical tension is especially important during the process of transcription. As Pol II separates the DNA strands, positive supercoils are generated ahead of the transcribing polymerase, followed by negative supercoils behind it (reviewed in [12]). It has been proposed that Top1 nicks positively supercoiled DNA to relieve the tension and enhance

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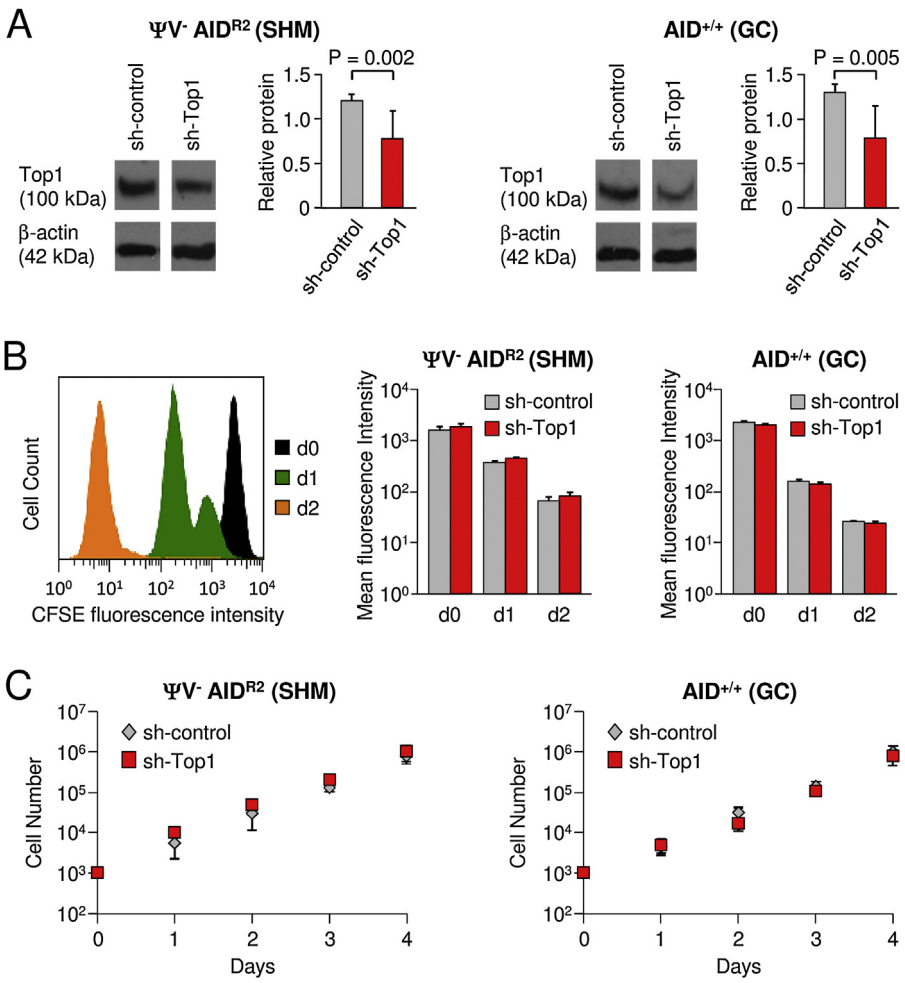

Fig. 1. Effects of Top1 knockdown on cell division and proliferation. For ΨV⁻ AID^{R2} cells, 3 sh-control and 9 sh-Top1 clones were tested, and for AID$^{+/+}$ cells, 2 sh-control and 4 sh-Top1 clones were tested. (A) Western blot analysis comparing Top1 levels in sh-control and sh-Top1 clones. Representative blots are shown. Bar graphs represent the average Top1/β-actin ratio; error bars depict standard deviation (SD). *P* value, unpaired two-tailed Student's *t* test. (B) Cell division experiments tracking the dilution of CFSE dye over time. Representative flow cytometry analysis of fluorescence intensity in a sh-control clone on days 0 (black), 1 (green), and 2 (orange). Bar graphs represent the average mean intensity; error bars indicate SD. Value for d 1 includes cells in both peaks. (C) Cell proliferation. Cells were counted on days 0–4.

transcription elongation. In fact, it is noteworthy that Top1 cleavage sites are found throughout transcriptionally active genes but not in silent genes [13,14]. Furthermore, inhibition of Top1 by camptothecin decreased Pol II elongation [15,16], and increased Pol II density in actively transcribed genes [17]. Thus, Top1 is an intricate regulator of Pol II function. To test the role of Pol II density in targeting AID to V genes, we artificially increased Pol II abundance by decreasing Top1 levels in the chicken DT40 B-cell line. We found that increased Pol II density elevated AID mutagenesis.

2. Materials and methods

2.1. DT40 cell lines and mice

Four engineered cell lines were used. For somatic hypermutation (SHM), a cell line was used that was surface IgM$^+$ and lacked Vλ pseudogenes. We generated the ΨV$^-$ AID^{R2} cell line using a DT40cre1 ΨV$^-$ AID$^{-/-}$ IgM$^+$ progenitor cell line [18] that had AID reconstituted (AID^{R2}) using the vector pAidGpt. In this vector, an AID cDNA expression cassette was cloned downstream of the chicken β-actin promoter and upstream of an IRES-GPT sequence. For gene conversion (GC), a cell line was used that was surface IgM$^-$ and had Vλ pseudogenes. The DT40cre1 (called AID$^{+/+}$ hereafter) cell line had a frameshift mutation in the rearranged V-joining (J) gene [19]. For western blots of Top1 and AID, two additional cell lines were used: DT40cre1 AID$^{-/-}$ (called AID$^{-/-}$), and DT40cre1 AID^R with AID reconstituted (called AID^R). For mouse studies, wild type C57BL/6 mice and *Aid*$^{-/-}$ mice [20] on a C57BL/6 background were bred in our mouse colony. All animal procedures were reviewed and approved by the Animal Care and Use Committee of the National Institute on Aging.

2.2. Top1 shRNA cloning, cell culture, transfection, and western blotting

Short hairpin (sh) RNA constructs were cloned into the pLKO.1 vector (Addgene) which contained a puromycin resistant selectable marker. Two shRNAs against Top1 were tried, using oligonucleotides confirmed by Sigma. The one listed in Supplementary Table S1 was selected, along with a control sequence. Cells were cultured in chicken medium (RPMI-1640 supplemented with 10% fetal bovine serum, 1% chicken serum, 2 mM L-glutamine, 0.1 μM β-mercaptoethanol, 100 I.U./ml penicillin, and 100 μg/ml streptomycin) at 41 °C with 5% CO_2. ΨV$^-$ AID^{R2} and AID$^{+/+}$ cell lines were stably transfected with 20–40 μg of linearized sh-control or sh-Top1 plasmids at 25 μF and 700 V with gene pulser Xcell (Bio-Rad). After electroporation, cells were incubated in chicken medium overnight, and the next day, puromycin (Sigma–Aldrich) was added at a final concentration of 0.5 μg/ml. The cells were then incubated

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