Supporting Information

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SI Text

Cell Culture and Stimulation. CH12F3–2 cells were cultured and stimulated for class-switch induction as described in ref. 1. Red blood cell-depleted splenic B cells isolated using a B-cell isolation kit (Myltenyi Biotec) were obtained from 2- to 8-month-old wild-type or AID$^{-/-}$ mice on a C57BL6 background. The cells were cultured with LPS and IL4 as described in ref. 2.

RT-PCR. Total RNA was purified with TRIzol reagent (Invitrogen) and reverse-transcribed by the SuperScriptIII enzyme (Invitrogen) with an oligo(dT) primer. PCR for germline transcripts and AID mRNA detection was performed as described in ref. 3. For the αCTs, we used the reported primer set (4) and LA-Taq (Takara) for PCR amplification, which had an initial denaturing step of 94 °C for 1 min followed by 35 cycles at 94 °C for 30 s, 61 °C for 30 s, and 72 °C for 30 s. GAPDH mRNA was detected as a positive control for RT-PCR. The RNA expression level of Top1, rRNA, endogenous AID, and μ- and α-GLT was quantified by ABI 7900 with each primer set. 18S rRNA TaqMan probe (ABI) or GAPDH primer set was used as internal control. The primer sets for μ- and α-GLT were described in ref. 4, and the others are shown in Table S2.

DC-PCR. Digestion circularization (DC)-PCR was done as described in ref. 5 with some modifications. Briefly, 100 ng of genomic DNA digested with EcoRI was subjected to a self-ligation reaction with T4 DNA ligase at 16 °C overnight. Then, the circularized DNA was amplified for IgH genotyping by using AmpliTaq Gold enzyme with the primers described in Table S2. PCR amplification was done with an initial denaturing step of 94 °C for 9 min followed by 40 cycles at 94 °C for 30 s and 65 °C for 3 min.

Materials and Animals. The antibodies used for flow cytometric analysis by FACS Calibur (BD) were an FITC-conjugated anti-IgM antibody (eBioScience) and PE-conjugated anti-IgA antibody (eBioScience). Camptothecin (CPT) was purchased from Calbiochem and dissolved in dimethyl sulfoxide (DMSO). Anti-human Top1 monoclonal antibodies from Abnova and LifeSpan Biosciences were used to detect Top1 in the nucleoplasmic fraction of AER cells and in splenic B cells, respectively by Western blotting procedure (3). AID knockout mice (3) were maintained at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. Wild-type C57/BL6 mice were purchased from CLEA Japan. Our experimental protocols using mice were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University. AID and its mutant constructs were described in ref. 6. Recombinant retrovirus was prepared and used to infect cells as described in refs. 2 and 7.

Nucleoplasm Fractionation. The nucleoplasm fraction was isolated by the method described by Dieckmann (8) with minor modifications. Briefly, cells were washed with PBS three times. After suspension in hypotonic buffer with 4 mM Mg^{2+}, Triton X-100 was added to the suspension to 0.3%. Cells were homogenized and washed with 0.25 M sucrose. Nuclei were collected, resuspended in 0.34 M sucrose, sonicated, and centrifuged on 0.88 M sucrose cushion to precipitate nucleoli. Supernatants were used as the nucleoplasmic fraction.

CHIP Assay. The CHIP experiment was done with anti-γH2AX antibody (JBW301) as described in ref. 9. Relative amounts of *S*μ, *C*μ, *Gapdh*, and *Icos* regions in the precipitates were measured by real-time PCR with iQ SYBR Green Supermix (Bio-Rad). The values were calculated as a percent of the input. The p value obtained from the one-tailed paired Wilcoxon's t test. Primers used for the real-time PCR are shown in Table S2.

Top1 Protein Decay Analysis. z-VAD-FMK is purchased from Peptide Institute and cycloheximide (CHX) is from Nacalai tesque. The cells were lysed in RIPA buffer. Western blot with anti-Top1 or anti-β-actin antibody was measured by NIH Image software (NIH) and plotted to the semilogarithmic graph.

1. Nakamura M, et al. (1996) High frequency class switching of an IgM+ B lymphoma clone CH12F3 to IgA+ cells. *Int Immunol* 8:193–201.
2. Doi T, Kinoshita K, Ikegawa M, Muramatsu M, Honjo T (2003) De novo protein synthesis is required for the activation-induced cytidine deaminase function in class-switch recombination. *Proc Natl Acad Sci USA* 100:2634–2638.
3. Muramatsu M, et al. (2000) Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 102:553–563.
4. Kinoshita K, Harigai M, Fagarasan S, Muramatsu M, Honjo T (2001) A hallmark of active class switch recombination: Transcripts directed by I promoters on looped-out circular DNAs. *Proc Natl Acad Sci USA* 98:12620–12623.
5. ChuCC, Paul WE, Max EE (1992) Quantitation of immunoglobulin mu-gamma 1 heavy chain switch region recombination by a digestion-circularization polymerase chain reaction method. *Proc Natl Acad Sci USA* 89:6978–6982.
6. Doi T, et al. (2009) The C-terminal region of activation-induced cytidine deaminase is responsible for a recombination function other than DNA cleavage in class switch recombination. *Proc Natl Acad Sci USA* 106:2758–2763.
7. Fagarasan S, Kinoshita K, Muramatsu M, Ikuta K, Honjo T (2001) In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. *Nature* 413:639–643.
8. Dieckmann R, Coute Y, Hochstrasser D, Diaz J-J, Sanchez J-C (2005) in *The Proteomics Protocols Handbook* (Humana Press Inc., Totowa, NJ), pp 79–85.
9. Nagaoka H, Ito S, Muramatsu M, Nakata M, Honjo T (2005) DNA cleavage in immunoglobulin somatic hypermutation depends on de novo protein synthesis but not on uracil DNA glycosylase. *Proc Natl Acad Sci USA* 102:2022–2027.

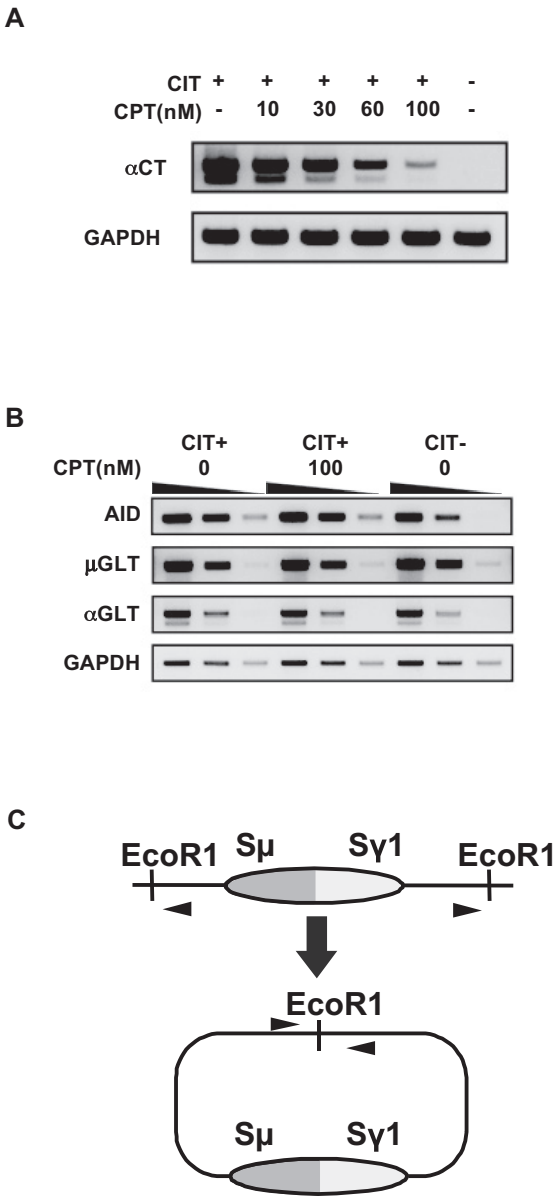


Fig. S1. CPT blocks CSR in CH12F3–2 cells and splenic B cells. (*A*) Reduction of αCT in CPT treated CH12F3–2 cells. Total RNA from cells stimulated with CIT for 24 h in the presence of the indicated concentrations of CPT was subjected to RT-PCR with primers for αCT. (*B*) Total RNA used in panel A was examined for expression of GAPDH, AID, μGLT, and αGLT by PCR with 10-fold serial dilutions. (*C*) Principle of DC-PCR. Upper is the recombined DNA and lower is the self-ligated EcoRI fragment after restriction digestion. Arrows indicate the positions and directions of the primers.

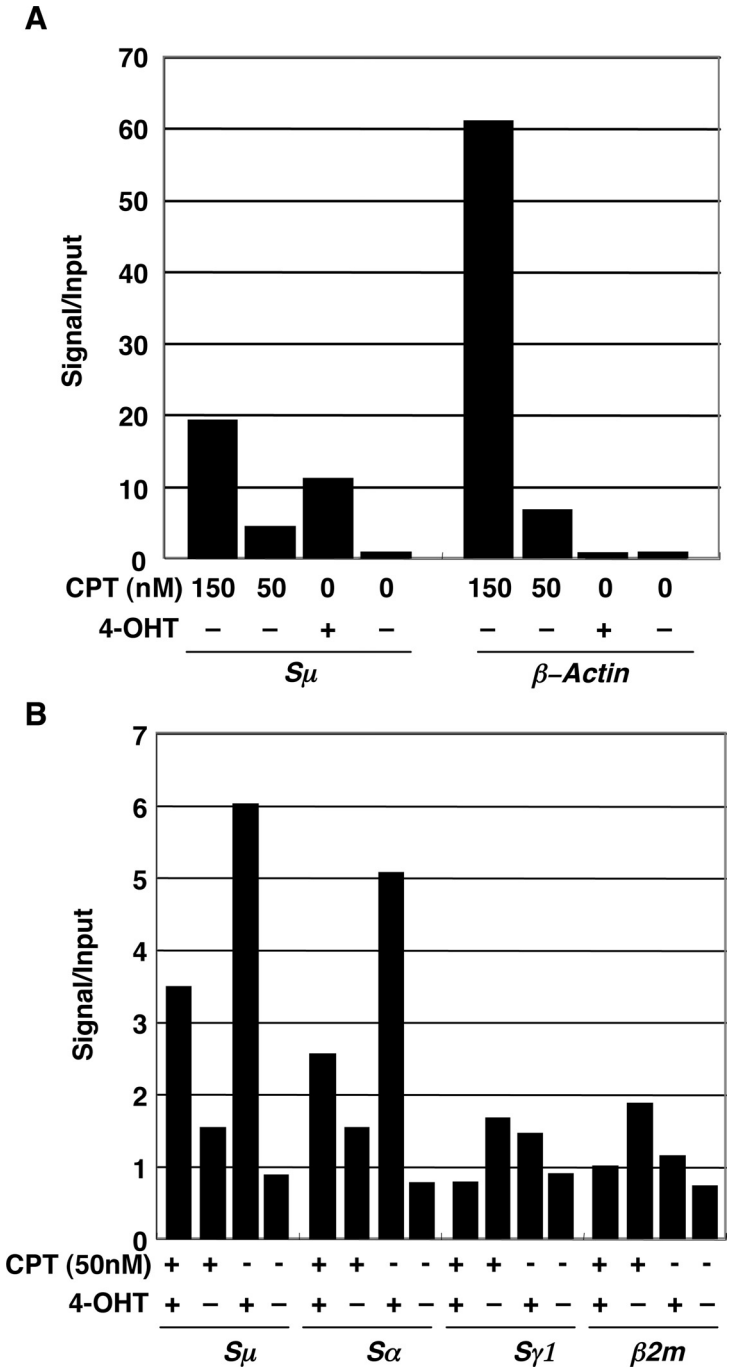


Fig. S2. High concentrations of camptothecin (CPT) cause nonspecific DNA break. (*A*) AER cells were treated with 150 or 50 nM CPT with or without 1 μM 4-OHT, for 16 h. (*B*) The same cells were treated with 50 nM CPT with or without 1 μM 4-OHT for 16 h as well.

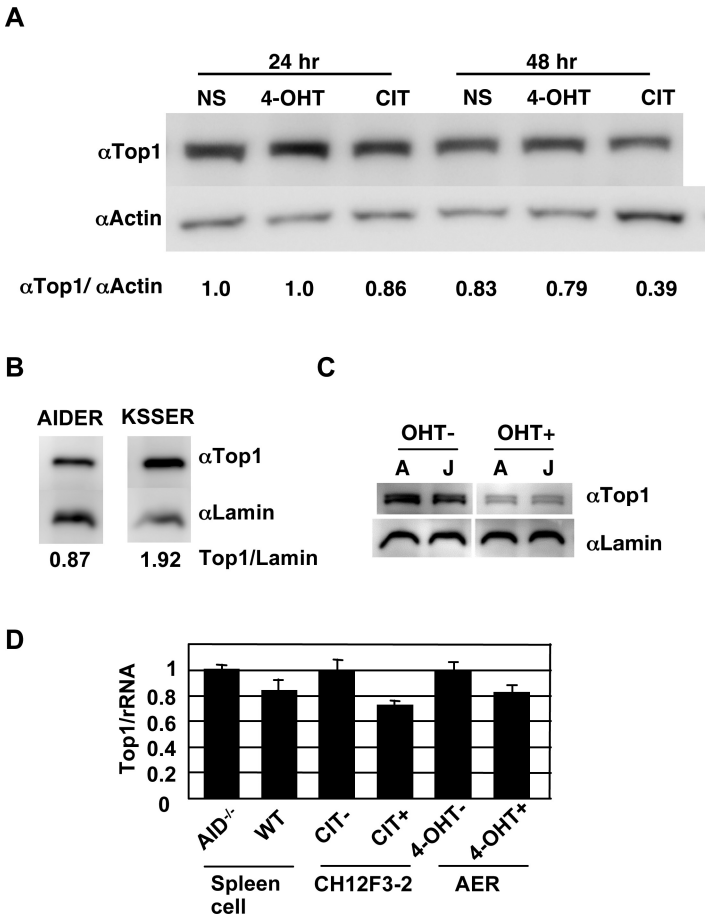


Fig. S3. Top1 protein is decreased by AID expression. (*A*) The decrease in the Top1 protein amount in whole cell extract from AER cells after AID activation. AER cells were stimulated with 1 μM of 4-OHT or CIT. After 24 or 48 h, cells were harvested and whole cell extract was recovered with RIPA buffer with Benzonase nuclease. Western blot signal by each antibody was measured and Top1/Actin ratio was calculated. NS, nonstimulated AER cells. (*B*) NIH 3T3 fibroblast cells were infected by a pMSCV retrovirus harboring AIDER- or KSSER-IRES-GFP. After sorting the GFP positive infected cells, they were incubated with 4-OHT for 3 days. The nucleoplasm fraction was extracted and submitted to Western blot analysis. (*C*) Top1 protein level in the nucleoplasm fraction of AER cells (A) or CH12F3–2 with JP8Bdel-ER (J) 6 h after stimulation with 1 μM 4-OHT. (*D*) Top1 mRNA level in AID expressing cells. AID$^{-/-}$ and wild-type (WT) splenic cells were stimulated by LPS and IL-4 for 48 h. CH12F3–2 and AER cells were stimulated for 48 h by CIT or 1 μM 4-OHT, respectively. Top1 mRNA and rRNA were quantified by qPCR.

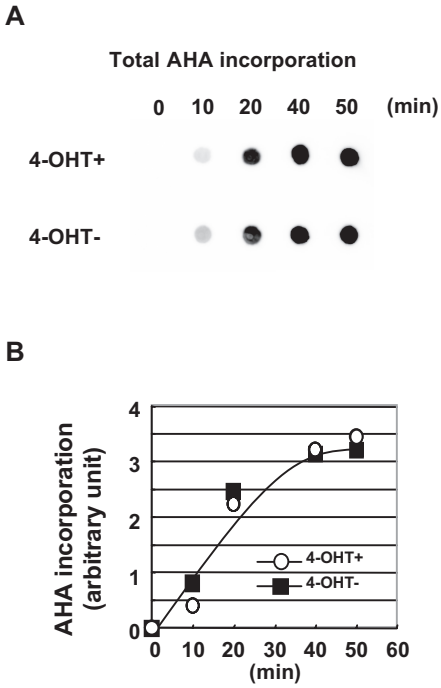
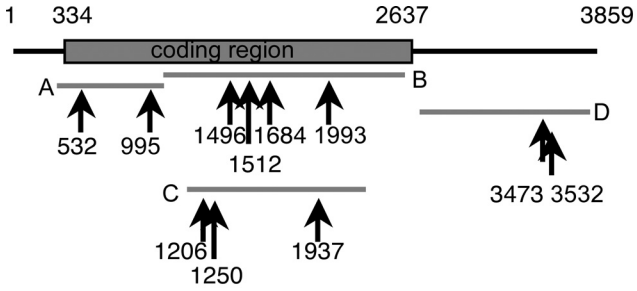


Fig. S4. Total protein synthesis is not affected by AID expression. (*A*) Total AHA incorporation is examined by dot blotting of input samples. Total protein synthesis labeled by biotinylated AHA was detected by streptavidin-conjugated HRP-labeled antibody and chemi-luminescence. (*B*) Signal detected in panel A was measured and plotted.



Number of point mutation found in each region

	clone mutated/unmutated	sequence mutation/bp
--(A region)--		
4-OHT(+)	2/79	2/55932
4-OHT(-)	4/93	4/65844
--(B region)--		
4-OHT(+)	4/47	4/75012
--(C region)--		
4-OHT(+)	3/77	3/93940
--(D region)--		
4-OHT(+)	2/39	2/45006
4-OHT(-)	3/46	3/52946

Fig. S5. No site specific editing on Top1 mRNA. To examine if AID edits Top1 mRNA, sequencing analysis was performed. CH12F3–2 cells expressing AIDER were treated with 4-OHT for 12 h. Total RNA was extracted by TRIzol (Invitrogen) then Top1 cDNA was amplified by RT-PCR with PrimeStar polymerase (Takara). The fragments were cloned into pBlueScript-SK vector at restriction enzyme sites placed in each amplification primer and sequenced. Shown is the combined result of four sequencing experiments in which four parts, designated as A to D regions, were subjected to each analysis. The diagram shows the map of mouse Top1 cDNA with the position of the A–D regions below. The number represents a nucleotide position according to the c DNA sequence data of NM_009408. One arrow indicates one point mutation found in 4-OHT treated samples. Total mutation numbers were summarized in the table. Sequence data from 4-OHT nontreated samples at regions A and D indicate the background level of this analysis. Primers used for cloning and sequencing were the following: 5′-ATGAATTCGCCCGCAGCGTTCGCACGCCGGCCGAC-3′; 5′-ATGGATCCATCATAGTAAAACTTGACACTCTCTGG-3′; 5′-ATGGATCCTTTTCACAGAACCCCTGCCGAGACTGG-3′; 5′-ATCAGAAGAGGAAGAGGATG-3′; 5′-GCATCAAATGGAAATTCCTAG-3′; 5′-CCAGTTCACGAATCAAGGGT-3′; 5′-TTCATTGATAAGCTTGCTCTG-3′; 5′-ACCATCCAATTCTGGGTGT-3′; 5′-GTGCCAATCGAGCTGTTGCA-3′; 5′-ATGGATCCAGTCTCGGCAGGGGTTCTGTGAAAAGG-3′; 5′-ATAAGCTTCGTTAAGTTGTAGGAGTTTATTTTAAA-3′.

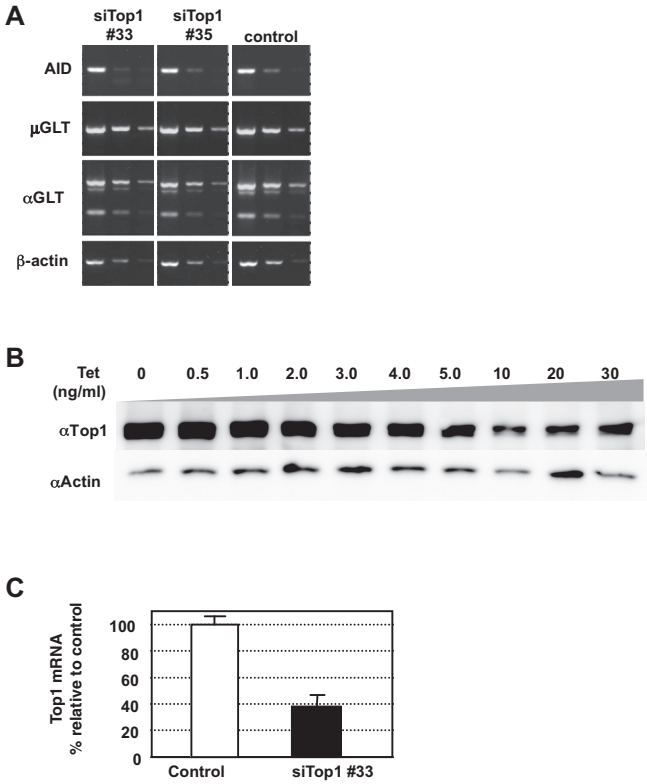


Fig. S6. Top1 knockdown enhances CSR in AER and spleen cells. (*A*) Absence of transcription suppression of GLT by Top1 knockdown. RNAs for endogenous AID, μGLT, and αGLT in Top1 knockdown AER cells were measured by PCR with 4-fold serial dilutions. (*B*) Top1 protein in AER cells expressing miR-Top1 was measured by Western blot 48 h after addition of Tet at indicated concentrations. (*C*) Top1 mRNA was measured for the control and knocked-down spleen cells analyzed in Fig. 5*D* by q-PCR. Top1 mRNA levels were normalized to the GAPDH mRNA and shown as a percentage relative to control Top1 mRNA level. Data are the average with S.D. ($n = 3$).

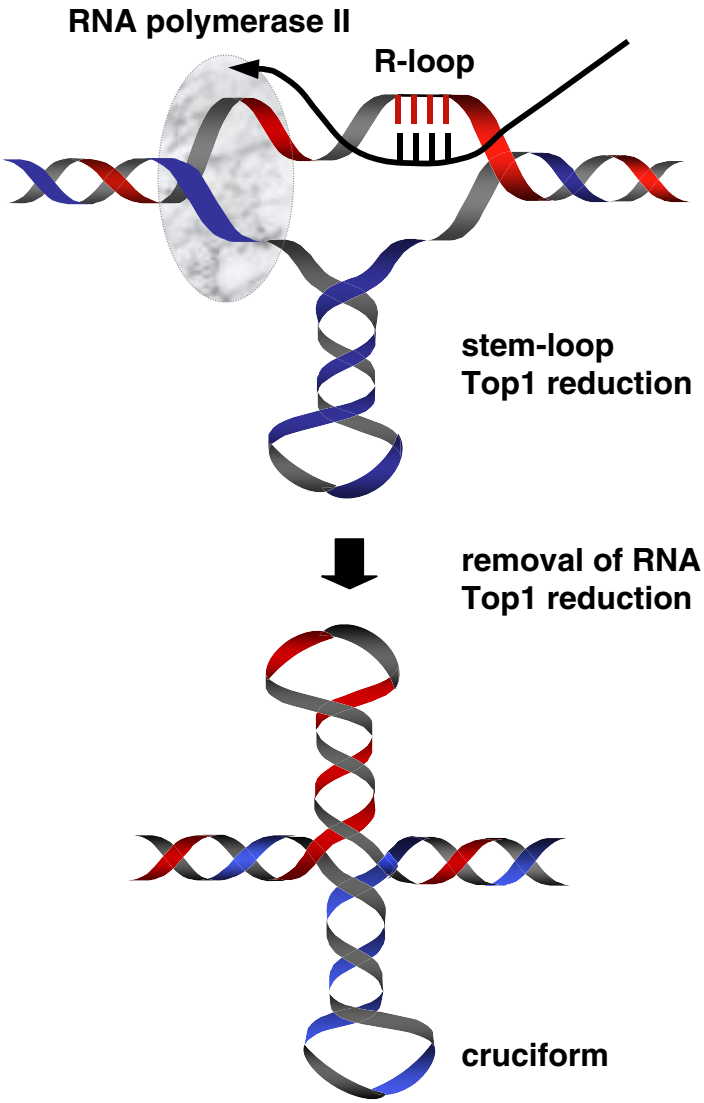


Fig. S7. Scheme for R-loop and non-B DNA structure formation by reduced Top1. RNA polymerase II generates positive and negative supercoil in the front and rear, respectively, of the migrating transcriptional machinery. Negative supercoil in the rear may not be efficiently relaxed when Top1 is reduced. Structural alterations including R-loop and non-B DNA may follow.

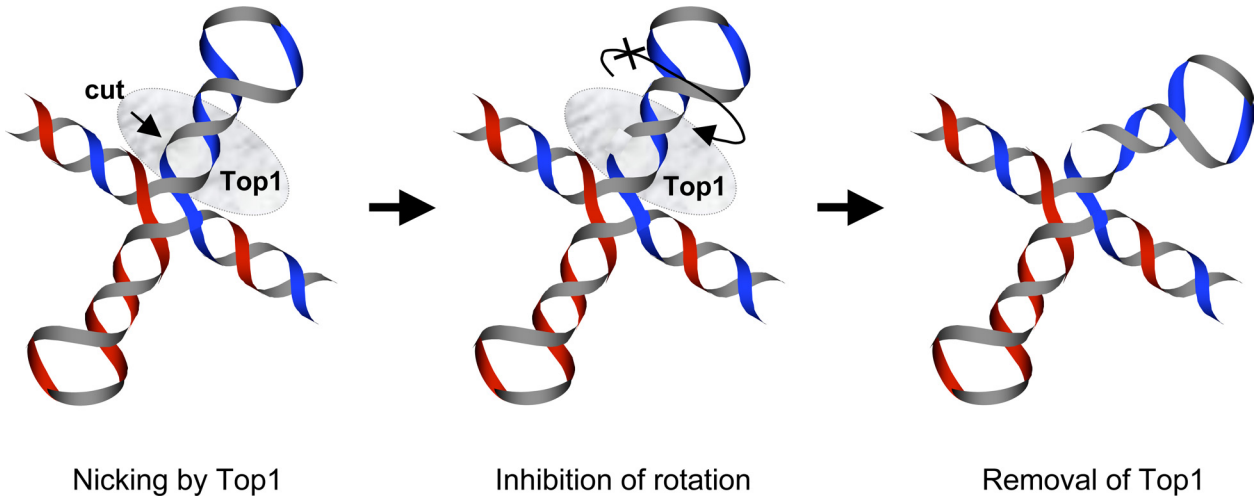


Fig. S8. Non-B structure is prone to nicking by Top1. Red (sense) and blue (anti-sense) strands forms cruciform structure. When Top1 encounters to the non-B structure it nicks DNA, however, cannot rotate DNA around helix and therefore fails to religate. Top1-DNA cleavage complex were cut out by the repair-related enzymes such as tyrosyl DNA phosphodiesterase/polynucletide kinase phosphatase. Thus, irreversible cleavage is created by Top1.

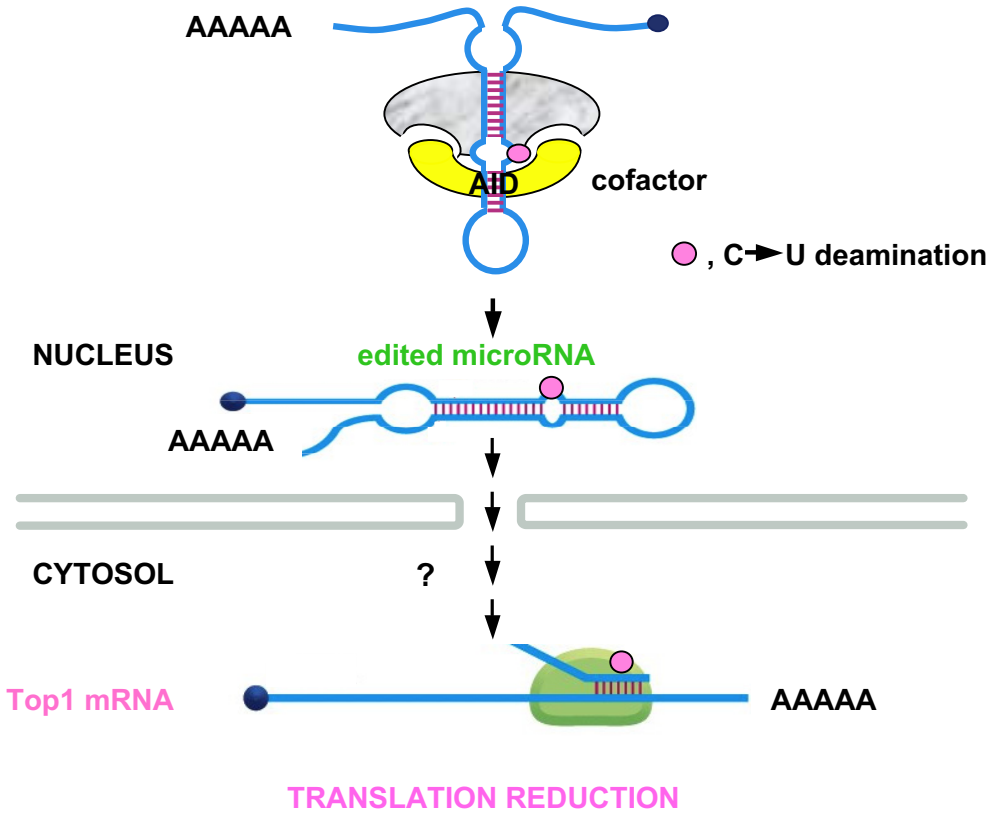


Fig. S9. Model for Top1 mRNA translation inhibition by an AID-edited microRNA. Cytosine (C) of a primitive-microRNA (miR) may be edited into uracil (U) by a complex of AID and its cofactors. Conversion from C to U will increase the interaction of the miR to Top1 mRNA. Therefore, the processed and matured miR will suppress the translation of Top1 mRNA.

Table S1. Inhibitors tested for CSR

Inhibitors	Targets	Effects on αCT
Etoposide VP16 (2 mM)	Top2a	−
Merbarone (20 mM)	Top2a	−
Doxorubicin (100 nM)	Top2a	−
KU7026 (10 mM)	DNA-PK	+

AER cells, CH12F3–2 cell expressing AIDER were stimulated in the presence or absence of indicated inhibitors and alpha circle transcripts were assayed by RT-PCR as described in Fig. S1B.

Table S2. Nucleotide sequences of primers, oligo RNA, and linker

Top1 knockdown RNA duplex		
#33 (Top1-MSS212033)		UAU CAA UGA AGU ACA GUG CUA CAG C
#35 (Top1-MSS212033)		CUU AGU AGU AUA UUC GUG GUC AAG C
miR		
Top1 knockdown miR		AA TTG CAA CAG CGA TTG GC
DNA break assay		
Biotinylated linker	top strand	biotin-ACA GGT TCA GAG TTC TAC AGT CCG AC
	bottom strand	phospho-GTC GGA CTG TAG AAC TCT GAA C
Second linker	top strand	phospho-TCG TAT GCC GTC TTC TGC TTG
	bottom strand	CAA GCA GAA GAC GGC ATA CGA
Amplification primers	forward	GTT CAG AGT TCT ACA GTC CGA
	reverse	CAA GCA GAA GAC GGC ATA CGA
Gene-locus specific PCR primer sets	*Sμ*	Probe; FAM-CTA GTA AGC GAG GCT CT-MGB (ABI) Primer; TGA TCA AAA TTA AGG GAA CAA GGT and TCA GAG AAG CCC ACC CAT CT
	Sα	Probe; FAM-GTG TCG TCT GAG CTG CAG AG-TAMRA (SIGMA) Primer; ACA TGA TCAA CAG GCA CAA GG and GTC TCC TGT TGC TGC TTT CC
	Sγ 1	Primer; CCTGAGCCCCGAGGATATC and AGTCCATGCCAAACA CATTCC
	β 2-microglobulin (β 2m)	Probe; FAM- CTC GGT GAC CCT GGT CTT T-TAMRA (SIGMA) Primer; CTG GCT GGC TCT CAT TTC AG and GGT CAG TGA GAC AAG CAC CA
	β -actin	Probe; FAM-CCA CCA GGT AAG CAG GGA C -TAMRA (SIGMA) Primer; CAG CTT CTT TGC AGC TCC TT and CTA GCC ACG AGA GAG CGA AG
RT-PCR primer set		
Top1	forward	TGC CTC CAT CAC ACT ACA GC
	reverse	CCC TTC GAG CAT CTG CTA AC
Aicda 3′UTR	forward	GTG GCA TTC ACC TAT AGT TCC
	reverse	AGA ACC CAA TTC TGG CTG TG
β -actin	forward	ACTGGGACGACATGGAGAAG
	reverse	GGGGTGTTGAAGGTCTCAAA
GAPDH	forward	AAAATGGTGAAGGTCGGTGT
	reverse	TGCCGTGAGTGGAGTCATAC
DC-PCR primer set		
recombined	Iγ1 forward	GAGAGCAGGGTCTCCTGGGTAGG
	Iμ reverse	TGGAGACCAATAATCAGAGGGAAGA
Control	nAChRe1	GGCCGGTCGACAGGCGCGCACTGACACCACTAAG
	nAChRe2	GCGCCATCGATGGACTGCTGTGGGTTTCACCCAG
Primers used for ChIP assay		
Sμ	forward	CCCAGCTTTGTGTGCTGATA
	reverse	GCTTCCCTCTGAGACACTGG
Cμ	forward	CTGTCGCAGAGATGAACCCCAATG
	reverse	ATCCTTTGTTCTCGATGGTCACCGG
GAPDH	forward	ATCCTGTAGGCCAGGTGATG
	reverse	GCTCAAGGGCTTTTAAGGCT
Icos	forward	CTGGGCCAGATGTGTTTTCT
	reverse	CCTGCCATGGTTTTTCTTGT