Supporting Information

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SI Materials and Methods

Expression Vectors and Cell Lines. Mouse activation-induced cytidine deaminase (mAID) cDNA was cloned in-frame to the 5′ end of EGFP-FLAG-HA sequence in pBluescript by a three-fragment ligation, and the resulting cDNA cassette encoding mAID-EGFP-FLAG-HA (mAID-GF) was excised and cloned into a retroviral vector pFB-ires-Puro (pFBP). Expression vectors of human AID (hAID) and AID mutants of human and mouse origins were generated by replacing the mAID fragment in pFBP-mAID-GF with the corresponding AID cDNAs. Retroviruses were produced by transient transfection of each pFBP vector into Plat-E cells using FuGENE 6 transfection reagent (Roche) and used to infect CH12F3-2 cells. Infected cells were selected with puromycin.

Immunopurification and Mass Spectrometry. Cytosolic extracts were prepared as described (1). Briefly, cells were washed once and resuspended in 1 mL/10^8 cells of hypotonic buffer [10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM NaCl, Complete Protease Inhibitor Mixture (Roche)] and incubated on ice for 30 min. Cells were then disrupted by Dounce homogenization (50 strokes with a tight pestle) and centrifuged at 800 × *g* for 15 min at 4 °C. Supernatants were mixed with 0.11 volume of 10× cytosolic extraction buffer [0.3 M Hepes (pH 7.9), 30 mM $MgCl_2$, and 1.4 M NaCl] and ultracentrifuged at 100,000 × *g* for 1 h at 4 °C. Resulting supernatants were supplemented with 10% glycerol and used as cytosolic extracts. Cytosolic extracts were precleared with mouse IgG-agarose (Sigma) for 30 min at 4 °C, then incubated with anti-FLAG M2-agarose (Sigma) for 2 h at 4 °C. After extensive washing with immunoprecipitation (IP) buffer [20 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 150 mM NaCl, 0.2 mM EDTA, 10% glycerol, Complete Protease Inhibitor Mixture (Roche)], coimmunoprecipitated proteins were eluted twice with 100 μg/mL of 3× FLAG peptides in IP buffer. The eluates were separated on 6–9% SDS/PAGE gels and visualized with MS-compatible silver staining. Each band on gels was excised with a scalpel and subjected to MS as described previously (2). Briefly, proteins were in-gel digested with trypsin, and molecular-mass analysis of tryptic peptides was performed by MALDI-TOF/MS with an Ultraflex MALDI-TOF/TOF system (Bruker Daltonics). Mass spectra were subjected to peptide mass fingerprinting using the Mascot (Matrix Science) search engine against the NCBInr database.

IP and Western Blotting. IP with anti-FLAG antibody was performed as described above, except that coimmunoprecipitated proteins were eluted by boiling in Laemmli buffer. For IP with anti-Spt6 antibody, cytosolic extracts were first precleared with Protein G-Sepharose pretreated with 0.1% BSA in IP buffer for 30 min at 4 °C, then incubated with anti-Spt6 mAb 2-1 (kindly provided by Dr. Handa, Tokyo Institute of Technology, Tokyo, Japan) for 1 h at 4 °C, and the immune complexes were captured for 1 h at 4 °C by Protein G-Sepharose pretreated as above. Coimmunoprecipitated proteins were eluted by boiling in Laemmli buffer. Eluted proteins were separated by SDS/PAGE, transferred to PVDF membranes, and probed with the following antibodies: an anti-Spt6 polyclonal antibody S2-B generated in our laboratory, anti-FLAG M2 antibody (Sigma), anti-Nucleolin polyclonal antibody (Novus Biologicals), anti-eEF1α monoclonal antibody (Upstate), and anti-KAP1 polyclonal antibody (Abcam). Signal was visualized using ECL Plus Western Blotting Detection Reagents (GE Healthcare) and detected with LAS4000 (Fujifilm). To analyze the effect of DNase I and RNase A treatment, cytosolic extracts were incubated at room temperature for 10 min either with RNase A (130 μg/mL) or with DNase I (4,000 U/mL) and subjected to IP.

1. Dignam JD, et al. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 11:1475–1489.

2. Takamatsu H, et al. (2009) Anti-moesin antibodies in the serum of patients with aplastic anemia stimulate peripheral blood mononuclear cells to secrete TNF-alpha and IFN-γ. *J Immunol* 182:703–710.

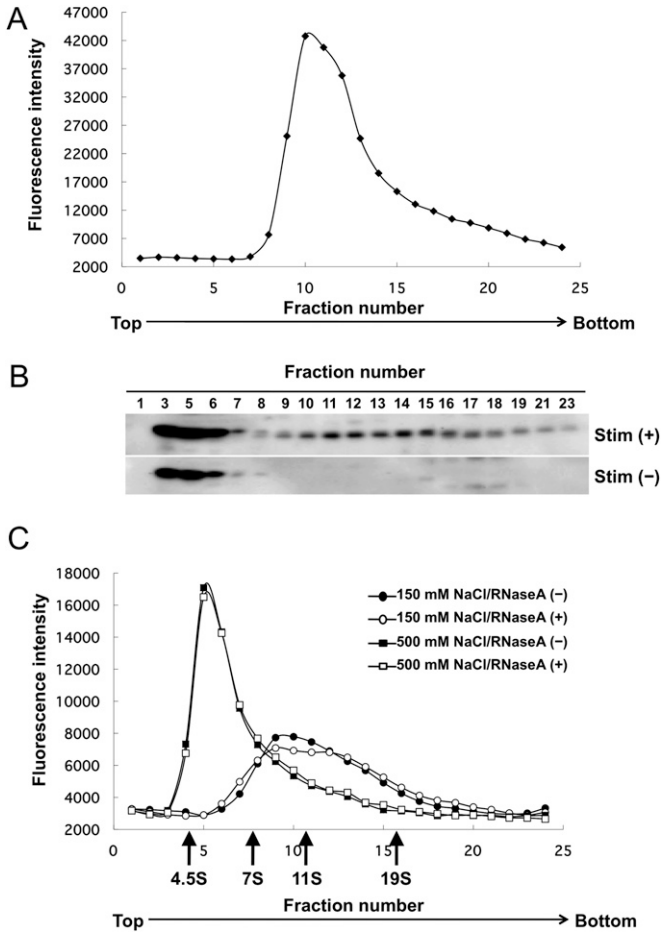

Fig. S1. (*A*) Glycerol gradient sedimentation analysis of AID-GF protein in CH12F3-2A cells. Cytoplasmic extracts of CH12F3-2A cells expressing AID-GF (GFP-FLAG) were fractionated by sedimentation in 10–50% (vol/vol) glycerol gradient at 284,400 x *g* for 17 h. The presence of AID-GF was monitored by GFP fluorescence. (*B*) Glycerol gradient sedimentation analysis of the endogenous AID protein in CH12F3-2A cells. Cytoplasmic extracts of unstimulated and stimulated CH12F3-2A cells were fractionated by sedimentation in 10–50% (vol/vol) glycerol gradient at 284,400 x *g* for 17 h. The presence of AID protein was monitored by Western blotting using anti-AID antibody (KKM-1). Strong signals observed in fractions 3–7 seemed to be nonspecific because they were also observed in unstimulated cells. (*C*) Distribution of AID-GF protein was analyzed by sedimentation in 10–50% (vol/vol) glycerol gradient at 284,400 x *g* for 17 h in the presence of either 150 mM NaCl or 500 mM NaCl in both cytosolic extracts and glycerol gradient. Cytosolic extracts were treated with RNase A before sedimentation. The positions of marker proteins are shown: ovalbumin (4.5S), γ-globulin (7S), catalase (11S), and thyroglobulin (19S).

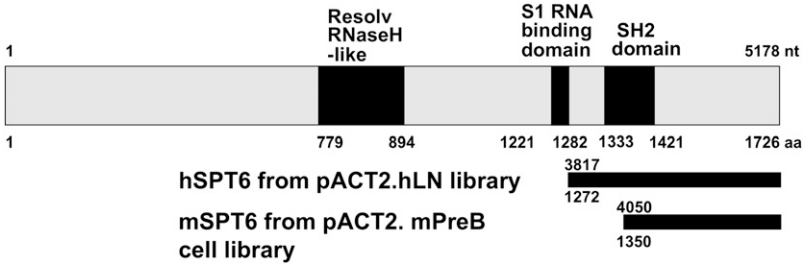

Fig. S2. SPT6 fragments isolated from yeast two-hybrid screening. pACT2 human lymph node cDNA library (Clontech) and pACT2 mouse pre-B-cell cDNA library (gift from Dr. Watanabe, Kyushu University, Fukuoka, Japan) were screened with pGBKT7-hAID and pGBKT7-mAID as bait, respectively. The C-terminal fragments of hSPT6 (3817–5178 nt) and mSPT6 (4050–5178 nt), which contain Src homology 2 domain, were isolated from the human and mouse libraries, respectively.

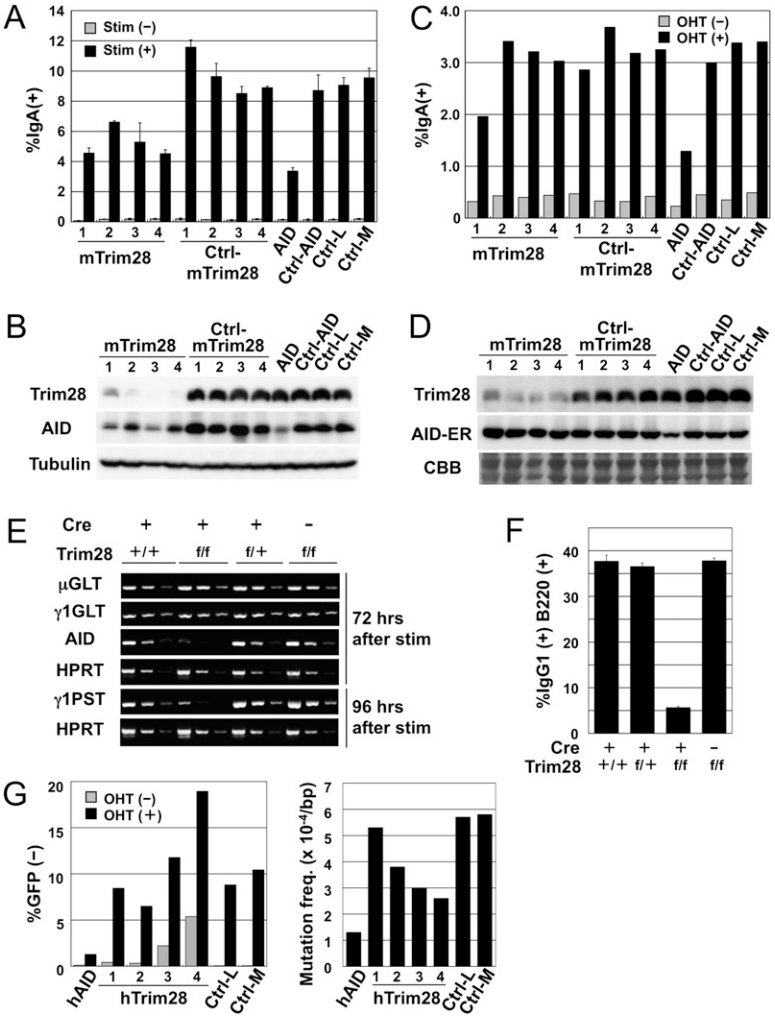

Fig. S3. (*A*) Trim28 knockdown severely reduced class switch recombination efficiency. CH12F3-2 cells (1.5×10^6) were introduced with 1.5 μg of siRNAs against mTrim28, scrambled siRNA for them, an siRNA against mAID, a scrambled siRNA for it, or negative control siRNAs with a low (36%) and medium (48%) GC contents (Ctrl-L and Ctrl-M, respectively). The GC contents of oligos mTrim28-1, -2, -3, and -4 are medium (45–55%), and that of oligo AID is low (35–45%). Twenty-four hours after siRNA introduction, cells were stimulated with CD40L, IL-4, and TGF-β for 24 h. The percentages of IgA^+ cells in the live population are indicated. Mean ± SD values were obtained from triplicate experiments. (*B*) siRNAs against Trim28 efficiently reduced the amount of Trim28 protein but also reduced the amount of AID protein. (*C*) CH12F3-2 cells expressing mAID-ER (1.5×10^6) were introduced with 1.5 μg of siRNAs against mTrim28, scrambled siRNA for them, an siRNA against mAID, a scrambled siRNA for it, or negative control siRNAs with low (36%) and medium (48%) GC contents (Ctrl-L and Ctrl-M, respectively). Twenty-four hours after siRNA introduction, cells were stimulated with OHT and TGF-β for 24 h. Percentages of IgA^+ cells in the live population are indicated. (*D*) siRNAs against Trim28 efficiently reduced the amount of Trim28 protein but did not affect the amount of AID-ER protein. (*E* and *F*) Splenocytes from $Trim28^{f/f}$ × mb1-hCre and control mice were stimulated with LPS and IL-4 in vitro for 3 or 4 d. The amounts of μGLT, γ1GLT, AID, γ1PST, and HPRT were analyzed by semiquantitative PCR using a threefold serial dilution series (*E*). Percentages of $IgG1^+$ cells are indicated (*F*). Mean ± SD values were obtained from triplicate experiments. Data are representative of two mice for each genotype. Note that splenocytes from $Trim28^{f/f}$ × mb1-hCre mice formed blasts, as well as those from control mice upon stimulation with LPS and IL-4, whereas the percentage and absolute number of $B220^+$ B cells, IgA^+ B cells, PNA^{hi} B cells in mesenteric lymph nodes, and Peyer's patches from $Trim28^{f/f}$ × mb1-hCre mice were reduced to approximately 10–25% of those from control mice. (*G*) Trim28 knockdown reduced SHM efficiency in the artificial substrate independently of AID function, presumably by reducing the transcription of the substrate. BL2 cells expressing JP8Bdel-ER and the artificial SHM assay substrate (1.5×10^6) were introduced with 3.0 μg of siRNAs against hTRIM28, siRNAs against hAID, or negative control siRNAs with low (36%) and medium (48%) GC contents (Ctrl-L and Ctrl-M, respectively). The GC contents of oligos mTrim28-1, -2, -3, -4, and AID are medium (45–55%). Twenty-four hours after siRNA introduction, cells were stimulated with OHT for 24 h and incubated for an additional 48 h in the absence of OHT. Percentages of GFP^- cells and mutation frequencies in the GFP sequence are shown. Data are representative of three independent experiments.

Table S1. Mutants of AID used in this study

AID mutants	CSR activity	SHM activity	Binding with Spt6	Reference
WT	100	100	+	
P20 (34-aa insertion at residue 182)	3	71	+	1
P13 (M139V)	5	<1	–	1
P7 (R24W)	1	<1	+	1
M6A	4	1	–	
N7A	90	47	+	
R8A	107	139	+	
R9A	89	93	+	
K10A	59	38	+	
K10R	80	54	+	
L172A	65	28	+	2
JP8Bdel	14	548	ND	2, 3
mG23S	97	11	+	4
W20K	103	17	+	4
W20K-ΔC	–	±	+	
V18S-R19V	53	5	+	4
V18S-R19V-ΔC	–	–	+	
Δ2-26	–	–	–	
Δ2-26-ΔC	–	–	–	
Δ2-5	+	+	+	
Δ2-10	0	0	–	3, 4
Δ2-10-ΔC	–	–	–	

All constructs except for mG23S are derived from human AID. Expected activities for CSR and SHM are indicated as +, –, or ±. CSR, class switch recombination; ΔC, deletion of residues 183–198, ND, not done.

1. Ta VT, et al. (2003) AID mutant analyses indicate requirement for class-switch-specific cofactors. *Nat Immunol* 4:843–848.
2. 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.
3. Ito S, et al. (2004) Activation-induced cytidine deaminase shuttles between nucleus and cytoplasm like apolipoprotein B mRNA editing catalytic polypeptide 1. *Proc Natl Acad Sci USA* 101:1975–1980.
4. Shinkura R, et al. (2004) Separate domains of AID are required for somatic hypermutation and class-switch recombination. *Nat Immunol* 5:707–712.

Table S2. Proteins interacting with AID in 293T

Symbol	Description	CSR by knockdown*	Knockdown efficiency*
A.	Protein with known direct or indirect interaction with DNA or RNA		
DDX17	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	0.79 ± 0.034	0.32 ± 0.025
DDX20	DEAD (Asp-Glu-Ala-Asp) box polypeptide 20	1.0 ± 0.046	0.12 ± 0.011
hnRNPA1	heterogeneous nuclear ribonucleoprotein A1	ND	ND
IGF2BP1	insulin-like growth factor 2 mRNA binding protein 1	0.93 ± 0.05	0.36 ± 0.13
PABPC1	poly(A) binding protein, cytoplasmic 1	1.4 ± 0.02	0.46 ± 0.14
B.	Proteins involved in degradation of proteins		
PSME3	proteasome (prosome, macropain) activator subunit 3	1.1 ± 0.034	0.55 ± 0.084
C.	Proteins involved in cell cycle regulation		
AFF4	AF4/FMR2 family, member 4	0.94 ± 0.034	0.51 ± 0.27

CSR, class switch recombination; ND, not done.
*Ratio to the control siRNA.

Table S3. Mutation frequency in the GFP sequence in siRNA-treated BL2 cells expressing JP8Bdel-ER

Oligo	Clone (mutated/total)	Mutated (deletion or insertion)	Total bases	Mutation frequency ($\times 10^{-4}$)
OHT+				
AID-1 (M)	5/24	6 (0)	39,792	1.5
AID-2 (M)	0/24	0 (0)	39,792	<0.3
AID-3 (M)	1/24	1 (0)	39,792	0.3
SPT6-1 (L)	17/25	34 (0)	41,450	8.2
SPT6-2 (L)	15/24	26 (1)	39,792	6.5
SPT6-3 (M)	17/24	24 (0)	39,792	6.0
Ctrl-L	12/24	18 (0)	39,792	4.5
Ctrl-M	14/25	21 (0)	41,450	5.1
OHT−				
AID-1 (M)	2/24	2 (0)	39,792	0.5
AID-2 (M)	2/24	2 (0)	39,792	0.5
AID-3 (M)	0/24	0 (0)	39,792	<0.3
SPT6-1 (L)	3/24	3 (0)	39,792	0.8
SPT6-2 (L)	1/24	1 (0)	39,792	0.3
SPT6-3 (M)	0/24	0 (0)	39,792	<0.3
Ctrl-L	0/24	0 (0)	39,792	<0.3
Ctrl-M	0/24	0 (0)	39,792	<0.3

The control siRNA oligo corresponding to each test siRNA is indicated as L (Ctrl-L) or M (Ctrl-M).

Table S4. Mutation frequency in the GFP sequence in NIH 3T3 cells expressing mutant hAID-GFs

hAID-GF constructs	Clone (mutated/total)	Mutated (deletion or insertion)	Total bases	Mutation frequency ($\times 10^{-4}$)
WT	26/43	75 (1)	41,495	18.1
M6A	1/45	1 (0)	43,425	0.2
N7A	20/46	38 (1)	44,390	8.6
R8A	25/35	85 (5)	33,775	25.2
R9A	25/45	73 (2)	43,425	16.8
K10A	18/47	31 (2)	45,355	6.8
K10R	21/46	43 (5)	44,390	9.7
Mock	3/46	3 (0)	44,390	0.7