

Depletion of recombination-specific cofactors by the C-terminal mutant of the activation-induced cytidine deaminase causes the dominant negative effect on class switch recombination

(AID の C 末端変異体は特異的共役因子を枯渇させるため、クラススイッチ組換えにドミナントネガティブ効果を及ぼす)

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Abstract

Activation-induced cytidine deaminase (AID) is essential for class-switch recombination (CSR) and somatic hypermutation (SHM) of immunoglobulin (Ig) genes. Studies on *in vitro* mutagenized AID as well as its mutations in human patients with Hyper-IgM (HIGM)-syndrome type II revealed that C-terminal AID mutations were defective in CSR whereas their DNA cleavage and SHM activities remained intact. The C-terminal mutants of AID were speculated to exert the dominant negative effect on wild type WT AID whereas its mechanism remains unknown. We generated the JP41 (R190X) mutation in one allele and null mutation on the other allele in mouse B cell line (CH12F3-2A) using CRISPR/Cas9 genome editing tools and studied the effect of JP41 expression on the function of exogenously introduced WT AID fused with estrogen receptor (AIDER) in AID^{JP41/Δ}/AIDER CH12F3-2A cells. We found that JP41 expression strongly suppressed not only CSR but also *Igh/c-Myc* chromosomal translocations by AIDER. We showed the dominant negative effect is not evident at the DNA cleavage step but obvious at both deletional and inversional recombination steps. We also confirmed the dominant negative effect of other C-terminal mutants, JP8Bdel (R183X) and P20 (34-aa insertion at residue 182) in AID deficient spleen B cells. Finally, we showed that the expression of JP41 reduced the binding of AIDER with its cofactors (hnRNP L, SERBP1, and hnRNP U). Together these data indicate that dominant negative effect of JP41 on CSR is likely due to the depletion of the CSR-specific RNA binding proteins from WT AID.

Introduction

Activation-induced cytidine deaminase (AID) is expressed in antigen-stimulated mature B cells and essential to both somatic hypermutation (SHM) and class switch recombination (CSR) (1-3). Mechanistically, AID has dual functions in the immunoglobulin (Ig) locus i.e. DNA cleavage and recombination. DNA cleavage in the switch (S) and variable (V) regions initiates CSR and SHM, respectively (4,5). In SHM, error-prone DNA synthesis introduces point mutations during single-strand break (SSB) repair. During CSR, SSBs located at donor and acceptor S regions are converted to double-strand breaks (DSBs), followed by recombination between the two DSB ends to complete CSR (6-8)

Mutations at the N terminus of AID result in deficiency of both SHM and CSR, indicating the requirement of the N-terminal domain of AID for DNA cleavage in SHM and CSR (9). On the other hand, mutations at the C terminus of AID impair only CSR, indicating a requirement of the C-terminal domain for recombination functions in CSR (9-11). Probably, the AID C terminus is required for efficient synapse formation between two S regions by a mechanism yet unknown (12-14).

Immunodeficiency called Hyper-IgM (HIGM) syndrome consists of heterogeneous genetical disorders characterized by increased serum levels of IgM, and reduced levels of serum IgG, IgA and IgE, due to a defect in CSR (2,15-17). The HIGM syndrome is most frequently caused by either X-linked mutation in CD40 ligand (HIGM1) or autosomal mutation in AID (HIGM2). Intriguingly, a small fraction of the HIGM2 patients harbor heterozygous mutations in the C-terminus of AID, suggesting that the C-terminal mutation in AID may have dominant negative effect on wild-type (WT) AID for CSR (11,18,19). Consistently, in vitro expression of AID C-

terminal mutants in AID-sufficient mouse splenic B cells showed dominant negative effect on CSR (13,14). The best known AID patient showing the dominant negative effect contains JP41 (R190X) and WT AID. JP41 contains C to G mutation at the R190 which results in generation of a premature stop codon and the deletion of 8 amino acids from the AID C-terminus. Human patients heterozygous in this mutation had clear but less severe HIGM2 phenotype whereas patients heterozygous for AID null allele were symptomatically normal (18,19).

Several hypotheses were proposed to explain the dominant negative effect by the C-terminal truncation of AID. Because the loss of the nuclear export signal (NES) located at the C-terminal region of AID results in its nuclear accumulation, it was speculated that C-terminal AID mutants may interfere their trafficking and consequently the function of WT AID (20). Some reports also suggested a requirement of deaminase activity of the C-terminal AID mutant for its dominant negative effect, and proposed that DNA breaks by the C-terminal mutant cannot be properly recombined, and thus result in the destruction of the S region, which renders WT AID non-functional (13,14). Additional mechanisms by which the C-terminal AID mutant could interfere with the normal function of WT AID include the competition of CSR specific recombination factors and the disruption of AID multimeric structure (21). While AID can form monomeric or dimeric structures, C-terminal AID mutants not only failed to form homodimers with itself but also failed to form heterodimers with WT AID, suggesting involvement of the AID's C terminus in stabilizing the dimer structure which appears to be required for CSR.

Several RNA-binding proteins (RBPs) were identified to form ribonucleoproteins (RNP) complexes with AID monomer and dimer which contribute to different functions of AID. For example, the heterogeneous nuclear ribonucleoprotein K (hnRNP K) associates with the AID monomer as a cofactor and contributes to the DNA cleavage (21,22). On the other hand, hnRNP

L, hnRNP U, and Serpine mRNA-binding protein1 (SERBP1) contribute as recombination cofactors and associate with the AID dimer (21,22). Knockdown (KD) of hnRNP L, SERBP1 and hnRNP U reduced CSR in CH12F3-2A cells by causing a defect in the DNA end processing which in turn affected the DNA repair at the recombination phase (21). Based on these findings, we suspected that the dominant negative effect by the JP41 mutant could be due to competition of the cofactors specifically required for the CSR function of AID.

To examine this possibility, we generated CH12F3-2A cell line with the JP41 mutation on one allele by the CRISPR/Cas9 genome editing system. As this CH12F3-2A cell mutant had the null mutation on the other allele, WT AID fused with estrogen receptor (AIDER) was exogenously introduced to generate AID^{JP41/Δ}/AIDER cell line. We found that the expression of JP41 suppressed the AIDER activity for not only AIDER induced CSR but also *Igh/c-Myc* chromosomal translocations. Furthermore, JP41 expression reduced the association of the cofactors hnRNP L, SERBP1, and hnRNP U with WT AID. These observations lend support to the assumption that the dominant negative effect of JP41 is ascribed to depletion of CSR-specific cofactors from WT AID.

Methods

Generation of R190X-HA (JP41-HA) knocked-in B cells by CRISPR/Cas9

Mouse B-cell lymphoma cell line CH12F3-2A harboring Bcl2 was cultured with RPMI medium 1640 containing Penicillin-Streptomycin Mixed Solution (Nacalai Tesque) and 10% (v/v) FCS (Gibco) as previously described (23,24). To generate JP41-HA knock-in CH12F3-2A cell line, guide RNA (gRNA) paired oligonucleotides targeting PAM site (GGT) that is 3 nucleotides before AID exon 5 in noncoding strand of the AID locus were designed using an online software

tool (crispr.genome-engineering.org). The gRNAs were annealed and cloned into linearized GeneArt CRISPR Nuclease CD4 Reporter Vector (Invitrogen) as per manufacturer's instructions. The 200 nucleotide long (Ultramer, Integrated DNA Technologies) single stranded oligonucleotides (ssODN) containing two homology arms flanking predicted CRISPR/Cas9 cleavage site and the desired sequences to generate *R190X-HA* knock in mutation in the AID locus was used as homology directed repair (HDR) template (25-27). In addition, the ssODN template also contained point mutations to abolish the NsiI site in the AID locus. The sequences of ssODN template and guide RNAs are shown in Supplementary Table S1.

Prior to electroporation transfection, CH12F3-2A cells were treated with 10 μ M of SCR7 (Xcessbio) (28,29) overnight (20h), then 1.5x10⁶ of SCR7 treated cells were transfected with ssODN as HDR template and GeneArt CRISPR Nuclease CD4 Reporter Vector which expresses a single guide RNA and Cas9. Following transfection, cells were cultured for another 24h in SCR7 containing media, and Cas9 expressing population was enriched using Dynabeads® CD4 Positive Isolation Kit (Invitrogen). Twenty four hours later, Cas9 expressing cells were subjected to limited dilution to isolate single cell clones, and cultured for another 20 days.

Screening of R190X-HA knock-in clones

Single cell clones were screened for successful knock-in of *R190X-HA* into the AID locus by western blotting using anti-HA (Abcam) and AID antibodies (Cell signaling) as well as sequencing of genomic DNA and mRNA. Genomic DNA was extracted by re-suspending cells in 50 μ l of QuickExtract™ DNA Extraction Kit (Epicenter) as per manufacturer's instructions, and subjected to PCR amplification by KOD-FX-Neo polymerase (Toyobo) with the primers flanking the Exon 5 region of AID locus (Supplementary Table S1). Since ssODN used as HDR

template contain the mutation to abolish the NsiI site in the Exon 5 of the AID locus, above mentioned PCR fragments from the clones with successful incorporation of the sequences from ssODN template are expected to be resistant to NsiI digestion. Therefore, the amplified PCR fragments were subjected to restriction digestion with NsiI (ThermoFisher Scientific) for 3h at 37°C, and run on 2% agarose gel. For genomic DNA sequencing, above mentioned PCR fragments were cloned and sequenced.

To further confirm the knock-in of R190X-HA in to the AID locus, sequencing of AID mRNA was also performed. AID^{JP41/Δ} cells were stimulated with CIT for 48h followed by RNA extraction using TRIzol (Invitrogen). The cDNA was synthesized from 0.5 μg of total RNA using Oligo d(T)-primers and SuperScript III (Invitrogen). The AID mRNA was amplified from the cDNA using Prime STAR GXL enzyme (Takara) and primers flanking AID open reading frame (Supplementary Table S1). The PCR fragments were A-tailed, cloned into the pGEM-T Easy Vector (Promega), and sequenced using universal T7 or Sp6 primers.

Generation of AID^{JP41/Δ}/AIDER cell line

AID^{JP41/Δ} cells (2x10⁶) were transfected with 2μg of SfiI-linearized pEF1α-AIDER-IRES-ZsGreen1 (AIDER) or empty pEF1α-IRES-ZsGreen1 (mock) plasmids, and cultured in RPMI medium for 48h. To enrich pEF1α-IRES-ZsGreen1 transfected population, the cells were subjected to 2 rounds of cell sorting by FACS Aria (BD Biosciences) followed by limited dilution to obtain single cell clones with stable expression of AIDER (AID^{JP41/Δ}/AIDER). The single cell clones were then screened for the protein expression of AIDER and CSR activity.

CSR assay and siRNA transfection

To analyze the CSR activity of endogenous AID, various CH12F3-2A cell lines were stimulated with CIT (CD40-L, IL-4 and TGF- β) for 48h, and surface expression of IgM and IgA were analyzed by staining the cells with FITC-conjugated anti-mouse IgM (eBioscience) and PE-conjugated anti-mouse IgA (Southern Biotechnology Associates) antibodies, respectively (30). Dead cells were excluded by staining with propidium iodide. To analyze dominant negative effect of endogenous JP41-HA on exogenously introduced AIDER, AID^{-/-}/AIDER and AID^{JP41/ Δ} /AIDER cell were stimulated either with CIT or 1 μ M of an estrogen analog 4-hydroxyltamoxifen (OHT) alone or combination of CIT and OHT for 48h, and IgA surface expression was analyzed as described above. To analyze the effect of AID C-terminal mutants, spleen B cells were obtained from 8- to 12-wk old AID^{-/-} mice, were pretreated with LPS and IL-4 then infected by retroviral constructs. Cells were stained with biotinylated anti-mouse IgG1 (Becton Dickinson) followed by allophycocyanin-conjugated streptavidin (eBioscience).

To analyze the effect of the AID cofactor KD on CSR and *Igh/c-Myc* translocations, AID^{JP41/ Δ} /AIDER#12 cells were transfected with chemically modified Stealth siRNAs (ThermoFisher Scientific) against mouse hnRNP L, SERBP1, hnRNP U, or control siRNA (siCONT) with similar GC content using Nucleofector 96-well electroporation system (Lonza) as previously described (21,22). Twenty-four hours post transfection of siRNAs, cells were stimulated with OHT for another 48h, and subjected to CSR assay and genomic DNA isolation to analyze the frequency of *Igh/c-Myc* translocations as described below. Flow cytometry analysis in all CSR experiments was performed using a Becton-Dickinson FACS-Caliber.

Igh/c-Myc chromosomal translocation assay

To analyze *Igh/c-Myc* translocation (derivative chromosome 15), various CH12F3-2A cell lines were stimulated as described above, and genomic DNA was purified using phenol/chloroform extraction. To increase basal frequency of *Igh/c-Myc* translocations, experiments were also performed under the Top1 deficient condition. To KD Top1, cells were transfected with either Top1 or control siRNA (siCONT) with similar GC content as described above (31). The 750 ng genomic DNA that is equivalent to 1.25×10^5 cells was subjected to nested PCR using the Expand Long Template PCR System (32,33). A total of 24-48 aliquots of genomic DNA were analyzed in independent reactions. The conditions for both first and second round of PCR were as follows: 94 °C for 3min, followed by 25 cycles at 94 °C for 15 s; 62 °C for 15 s; 68 °C for 7.5 min and a final extension of 5min at 68 °C. The PCR products were electrophoresed on ethidium bromide-containing 1% agarose gels and subjected to Southern blotting with *Myc* locus specific probe. The primer and probe sequences are shown in Supplementary Table S1.

Analysis of DNA double-strand breaks

Genomic DNAs were prepared from CH12F3-2A/JP41ER cells stimulated with CIT alone or CIT in combination with OHT for 4 or 24 h. DSBs were analyzed by ligation-mediated PCR as previously described (34,35). The primer and probe sequences are shown in Supplementary Table S1.

Retroviral transduction of splenic B cells and IgG1 switch assay:

For retroviral expression of AID in AID^{-/-} spleen B cells, all the constructs shown in Fig.3B were prepared in pFB-IRES-GFP backbone. Retroviral supernatant was prepared by co-transfecting the retroviral plasmid along with the helper plasmid pCL-ECO (2:1 ratio) in 293T cells. Spleen B cells, purified by depleting CD43⁺ cells (MACS, Miltenyi Biotech), were cultured in presence of

LPS and IL4 for 2 days. Viral supernatant containing Polybrene (8 ug/ml, Millipore) was added to the pre-activated B cells (300 ul/1x10⁶ cells), mixed, and subjected to spinfection (3000 rpm, 1h at 300°C). After infection, cells were resuspended in the fresh medium containing LPS and IL4. The retrovirus preparation and the infection of the AID^{-/-} primary B cells were described previously (9,11).

Three days after infection, cells were stained with biotinylated anti-IgG1 (anti-IgG1), followed by APC (allophycocyanin)-conjugated streptavidin for FACS. The dead cells were excluded by PI (Propidium Iodide) staining, live cells positive for GFP were analyzed for surface IgG1 expression.

Co-Immunoprecipitation to analyze the interaction of AID with its cofactors

AID^{JP41/Δ} and AID^{JP41/Δ}/AIDER#12 cells were stimulated by either CIT or OHT alone, or a combination of both, for 24h. A total of 1x10⁷ of cells were washed twice with ice cold PBS, and lysed in 1ml lysis Buffer [30 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 0.05% Na-deoxycholate, and 5 mM EDTA] supplemented with protease inhibitors (Roche) as described previously (21). The clarified lysates were incubated with BSA (1mg/ml) blocked ANTI-FLAG M2 Affinity Agarose Gel (Sigma) with slow end-over-end rotation for 4h at 4°C. The IP complex was washed five times with the lysis buffer containing 1mg/ml BSA. The bound proteins were eluted with wash buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% NP-40) containing 500 μg/ml of 3X-FLAG-peptides (Sigma). Eluted fractions were boiled with SDS-sample buffer, and subjected to western blotting with specific antibodies. The list of antibodies used is shown in Supplementary Table S2.

Western blotting

Cells were washed twice with cold PBS, and suspended in RIPA buffer supplemented with protease inhibitors (Roche), Benzonase® Nuclease (Novagen), and 2 mM MgCl₂. Following incubation on ice for 30 min, lysates were centrifuged at 13000 rpm for 10 min at 4°C. The clarified lysates were electrophoresed on 4–20% SDS–PAGE gradient gel (BIORAD), and subjected to western blotting using specific antibodies. The list of antibodies used is shown in Supplementary Table S2.

Results

Generation of CH12F3-2A cells with HA tagged-JP41 mutation at the AID locus

To establish CH12F3-2A cell line with the JP41 mutation we used clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology to manipulate exon 5 of AID by transfection of guide RNA (gRNA) and Cas9 expressing plasmid, together with 200bp single-stranded oligodeoxynucleotide (ssODN) as a template for homology-directed repair (HDR). The ssODN template contained two homology arms and desired sequences that lead to the insertion of HA tag after residue 189 in the AID protein to generate HA-tagged JP41 mutant of AID (Fig. 1A; Supplementary Table S1). In addition, the ssODN template also contained point mutations equivalent to C568T and A572T in the AID open reading frame. The C568T mutation equivalent to that found in JP41 patients generates a stop codon after the HA tag, leading to R190X-HA knock-in, whereas A572T mutation abolishes the NsiI site in the AID locus. The abolishment of the NsiI site in the AID locus allowed screening of clones with successful homology directed repair with ssODN HDR template, and

the HA-tag was used initially for screening the mutant proteins to distinguish JP41 from WT AID, and later for functional experiments.

The WT Cas9 nuclease generates DSBs which can be repaired by both non-homologous end joining (NHEJ) and HDR pathways. Since NHEJ mediated repair of Cas9 induced DSBs is more efficient than HDR, it inhibits precise genome editing by HDR mediated incorporation of sequences from an exogenous ssODN template containing desired mutations. Therefore, to transiently suppress NHEJ, we treated the cells with a DNA ligase IV inhibitor (SCR7), prior to transfection of gRNA and Cas9 expressing plasmid, and ssODN HDR template (Fig. 1B).

In our system, CD4 co-translated with Cas9 via a self-cleaving 2A peptide, was expressed at the cell surface following the cleavage of the 2A peptide. After transfection, cells with higher expression of Cas9 were enriched by magnetic bead-based enrichment using Dynabeads® CD4 magnetic beads. Subsequently, Cas9 expressing cells were subjected to single cell cloning, and whole cell protein extracts prepared from individual clones were screened for the expression of HA-tagged JP41 by western blotting (Supplementary Fig. S1A and S1B; Fig. 1B and 1C). To confirm the successful repair using ssODN HDR template, the PCR fragments amplified from genomic DNA of a JP41-HA expressing clone as well as WT CH12F3-2A cells with the primers flanking the Exon 5 region of the AID locus were tested for the digestion with NsiI (Fig. 1D). The NsiI restriction site in exon 5 is abolished by the incorporation of sequences from the ssODN template, which serves as a marker of successful repair using the ssODN template. The PCR product from the clone with positive expression of JP41-HA was failed to be cleaved by the restriction digestion with NsiI whereas the PCR fragment from WT CH12F3-2A cell DNA was cleaved by NsiI. To further confirm the successful repair using the ssODN HDR template, we sequenced the genomic DNA of the clone with positive expression of JP41-HA and identified the

JP41-HA sequence on one allele and a 102bp deletion surrounding exon 5 on the other allele (Fig. 1E). We also sequenced AID cDNA of the clone and found that only JP41-HA sequence was present (Supplementary Fig. S2A and S2B). Hence we named the generated cell line as AID^{JP41/Δ}.

JP41 is defective in CSR but active in Igh/c-Myc translocations

We compared CSR efficiency of AID^{JP41/Δ} cell line with those of AID^{WT/WT} and AID^{WT/-} CH12F3-2A cell lines. The CSR activity of AID^{JP41/Δ} was extremely low, while the frequency of the IgA-switching in AID^{WT/-} was decreased to about a half compared to AID^{WT/WT} (Fig. 1F and 1G). Since AID^{WT/-} switching represent the activity of one allele of AID, the C-terminal mutation of AID (JP41) was confirmed to have the drastic loss of the CSR activity in AID^{JP41/Δ} cells in agreement with previous reports (11).

To confirm the DNA cleavage activity of JP41, we tested the frequency of *Igh/c-Myc* chromosomal translocations after CIT stimulation of AID^{JP41/Δ} cells (Fig. 1H-L). The Top1 deficient condition was also employed to increase the frequency of *Igh/c-Myc* translocations (31,33). To detect *Igh/c-Myc* translocations, the PCR amplification products of the translocated genomic DNA using specific primers were subjected to Southern blotting with a *Myc* specific probe (Fig. 1I and 1J). AID^{JP41/Δ} induced *Igh/c-Myc* translocations at a level similar to AID^{WT/-} which represents the activity of one allele of AID regardless of Top1 reduction (Fig. 1J and 1K). The drastic reduction of *Igh/c-Myc* translocations by AID^{WT/-} compared with AID^{WT/WT} was also reported previously (36). The results indicate that the DNA cleavage activities of JP41 and WT alleles are almost identical, in agreement with our previous conclusion that the C terminus of AID is not required for DNA cleavage but only for CSR recombination (9,37).

JP41 showed dominant negative effect on CSR and Igh/c-Myc translocations

In order to test the dominant negative properties of JP41, we introduced WT AID fused with estrogen receptor (ER) into AID^{JP41/Δ} cell line to obtain cells which express both JP41 and AIDER (AID^{JP41/Δ} /AIDER) (Fig. 2A). AIDER can be activated by the addition of OHT to the culture medium. After transfection with linearized plasmid expressing AIDER-IRES-ZsGreen1, the cells were subjected to 2 rounds of cell sorting by FACS Aria followed by limited dilution to obtain single cell clones with stable expression of AIDER (Supplementary Fig. S3A-C). The single cell clones were screened for the AIDER expression and CSR activity. The clones that showed higher CSR activity (AID^{JP41/Δ}/AIDER#12 and AID^{JP41/Δ}/AIDER#45) were selected for further analysis. Similar AIDER transfectants were obtained using AID^{-/-} CH12F3-2A cells which were generated by CRISPR/Cas9 technology (Supplementary Fig. S5). As AIDER expression levels are not identical among different clones, AID^{-/-}/AIDER, AID^{JP41/Δ}/AIDER#12 and AID^{JP41/Δ}/AIDER #45 CH12F3-2A cells showed 5.1%, 9.3% and 11% IgA switching activity by OHT stimulation, respectively (Fig. 2B). It is noteworthy that CIT stimulation increased the AIDER-induced CSR in both AID^{JP41/Δ}/AIDER and AID^{-/-}/AIDER cells. Since all of these cells have no WT AID, this increment is likely due to the CIT-induced increment in the expression of α germ-line transcripts, which is required for efficient CSR (38).

To assess the dominant negative property of JP41 on WT AID, we examined the effect of CIT-induced JP41 on the CSR activity of OHT-activated AIDER in AID^{JP41/Δ}/AIDER (#12 and #45) by comparing with that of AID^{-/-}/AIDER cells. Stimulation of AID^{-/-}/AIDER, AID^{JP41/Δ}/AIDER#12 and AID^{JP41/Δ}/AIDER #45 clones increased IgA switching to 12.2%, 12.9% and 14.8%, respectively (Fig. 2B and 2C). The increment percentage of CIT induced CSR was less in AID^{JP41/Δ}/AIDER than in AID^{-/-}/AIDER cells, indicating a suppression of the WT AID CSR activity by JP41 (Fig. 2C). To confirm the expression of AIDER and JP41, we performed

western blot analysis from the cell extracts, and found that both AIDER and JP41 were efficiently expressed (Fig. 2D). Taken together, these data show that JP41 is dominant negative for CSR induced by WT AID. In addition to AID^{JP41/Δ}, our screening also identified two additional clones (AID^{ΔC5/ΔC11}, AID^{ΔCL/Cin}) of CH12F3-2A expressing novel C-terminal mutants of AID (Supplementary Fig S4A and S4B). As expected, these clones were defective in CSR and exogenously introduced AID barely increased CSR efficiency, indicating a dominant negative effect on wild type AID (Supplementary Fig. S4C-F).

To test whether dominant negative effect of JP41 is also extended to *Igh/c-Myc* translocation, we also measured the JP41 expression effect on AIDER-induced *Igh/c-Myc* translocations in AID^{JP41/Δ}/AIDER#12 cells. To increase the DNA cleavage frequency and thus generation of translocations, the experiment was also performed with the siRNA mediated knockdown of Top1 (Fig. 2E and 2F). Again, the dominant negative effect of JP41 on AIDER was tested by comparing *Igh/c-Myc* translocation frequencies between AID^{JP41/Δ}/AIDER#12 and AID^{-/-}/AIDER cells stimulated with either OHT alone or OHT in combination with CIT. To detect *Igh/c-Myc* translocations, the PCR amplification products of the translocated genomic DNA using specific primers were subjected to Southern blotting with the *Myc* specific probe. We found that CIT-induced expression of JP41 in AID^{JP41/Δ}/AIDER#12 cells reduced the frequency of *Igh/c-Myc* translocations under the Top1 KD condition, whereas AID^{-/-}/AIDER showed no significant reduction under the same condition (Fig. 2G and 2H). The dominant negative effect of JP41 on AIDER was not clearly visible in cells transfected with control siRNA. This is probably because of the low sensitivity of the assay that arises from two factors; (a) the low frequency of DNA cleavage without Top1 KD and (b) depletion of the Sμ break sites by enhanced Sμ-Sα recombination. Since expression of JP41 suppressed AIDER induced translocations, the

dominant negative effect of JP41 extends to recombination in *Igh/c-Myc* chromosomal translocations.

AID C-terminal mutants suppressed WT AID induced IgG1 switching in AID^{-/-} spleen cells

To further confirm dominant negative properties of other well studied AID C-terminal mutants such as JP8Bdel (R183X), P20 (34-aa insertion at residue 182), and JP8B (26-aa frameshift replacement at residue 183), we expressed them in AID-deficient mouse splenic B cells either alone or in combination with WT AID, and assayed the CSR frequency after stimulation with lipopolysaccharide (LPS) and interleukin 4 (IL4) (Fig. 3A). In order to better control the co-expression of both WT and mutant AID, we took advantage of a bicistronic expression vector containing the internal ribosome entry site (IRES), which enables the expression of two genes controlled by one promoter. To monitor the transfection efficiency, either WT or mutant AID was fused to green fluorescence protein (GFP) at its C-terminus. As expected, the transfection of the C-terminal mutants failed to rescue switching to IgG1 in AID^{-/-} spleen B cells, whereas WT AID efficiently rescued IgG1 switching (Fig. 3B and 3C). Furthermore, when co-expressed, the C-terminal mutants of AID suppressed the IgG1 switching activity of WT AID (Fig. 3B and 3C). These data obtained in an independent system further strengthen the dominant negative properties of the AID C-terminal mutants on CSR.

JP41 interferes with the deletional as well as inversional recombination step of WT AID

In order to further analyze the mechanism of the dominant negative effect of JP41, we generated CH12F3-2A cells stably expressing JP41 fused with ER (JP41ER) (Fig. 4A). Stimulation of these cells with CIT induced switching from IgM to IgA, while the activation of JP41ER by

OHT treatment failed to induce IgA switching as expected. However, when treated with CIT and OHT, IgA switching is strongly suppressed, indicating a dominant negative effect of JP41ER on the CSR activity of WT AID (Fig. 4B). We next performed the DNA double strand break assay by ligation mediated LM-PCR to confirm that JP41ER activation does not affect the formation of DNA double strand breaks by WT AID. As shown in Fig. 4C, JP41ER activation did not drastically affect the formation of DNA double strand breaks. We thus confirmed that the dominant negative effect of JP41 is the post-break phenomenon.

AID-induced DNA double strand breaks at the upstream S μ and the downstream S α regions can be combined in either deletional or inversional orientation with a strong bias towards deletional orientation that successfully completes productive CSR (39). The two types of joining (deletional and inversional) can be detected by digestion-circularization polymerase chain reaction (DC-PCR) as described previously (40). Thus, we next tested if JP41 expression affects the equilibrium between deletional and inversional recombination of two S regions. As expected, OHT-activation of JP41ER failed to induce either deletional or inversional recombination between S μ and S α regions, which is consistent with the incapability of JP41 to induced CSR (Fig. 4D). Interestingly, we found that activation of JP41ER strongly suppressed WT AID induced recombination in both deletional and inversional orientations, suggesting that the dominant negative effect of JP41 is due to the interference with the recombination step of CSR.

JP41 reduced the binding of WT AID with its cofactors

We recently showed that the DNA cleavage and recombination functions of AID depend critically on its RNA-dependent interaction with heterogeneous nuclear ribonucleoprotein (hnRNP) cofactors (21,22). The AID monomer associates with the DNA cleavage cofactor

hnRNP K whereas the AID dimer associates with the recombination cofactors such as hnRNP L, hnRNP U, and SERBP1. These findings prompt us to speculate that the dominant negative effect by JP41 mutant could be due to its inference with the binding of recombination cofactors with WT AID. In order to test this possibility, we examined the effect of JP41 on the association of AIDER with AID cofactors in AID^{JP41/Δ}/AIDER#12 cell line which expresses HA-tagged JP41 and FLAG-tagged AIDER (AID-FLAG-ER) by CIT stimulation and OHT treatment, respectively. Co-immunoprecipitation (co-IP) of FLAG tagged AIDER from the cell extracts prepared from AID^{JP41/Δ}/AIDER#12 cells stimulated with either OHT alone or in its combination with CIT allowed us to examine the interaction of AIDER with JP41 as well as cofactors (Fig. 5A). The co-IP experiments with anti-FLAG antibody did not detect HA-tagged JP41 showing no interaction between JP41 and AIDER. This result is consistent with our previous findings that JP41 as well as other C-terminal mutants of AID are defective for dimer formation (21).

Interestingly, we found that JP41 expression reduced the interaction of the recombination specific cofactors i.e. hnRNP L, SERBP1 and hnRNP U with AIDER whereas the interaction of the cleavage specific cofactor hnRNP K with AIDER remained unaffected (Fig. 5B). It is noteworthy that JP41 expression does not completely dissociate hnRNP L, SERBP1 and hnRNP U from AIDER. This could be due to a relatively lower expression of JP41 as it is expressed from only one allele, while exogenously introduced AIDER is robustly expressed. Taken together, these observations lend support to our speculation that the dominant negative effect of JP41 on WT AID is due to a depletion of recombination hnRNP cofactors from the complex formation with WT AID.

To further strengthen the above conclusion on the mechanism for the dominant negative effect of JP41, we assayed the effect of hnRNP L, SERBP1 or hnRNP U knockdown on the frequency of

AIDER induced CSR and *Igh/c-Myc* chromosomal translocations in AID^{JP41/Δ}/AIDER#12 cells (Fig. 5C). We found that significant depletion of hnRNP L, SERBP1 or hnRNP U protein by siRNA transfection caused 42-70% reduction in the CSR activity (Fig. 5D-5F). We also found that the frequencies of *Igh/c-Myc* translocations were also decreased upon the KD of AID associated RNA binding proteins in AID^{JP41/Δ}/AIDER#12 (Fig. 5G and 5H). SERBP1 KD showed the strongest decrement in *Igh/c-Myc* translocation as well as CSR. These findings indicate that JP41 competes with WT AID or AIDER for the binding of hnRNP L, SERBP1 or hnRNP U required for efficient repair of AID-induced DNA breaks.

Discussion

CSR and SHM require distinct molecular mechanisms of AID, because SHM is mediated by error-prone DNA repair at AID-induced DNA cleavage sites in the V region whereas CSR requires not only DNA cleavage in the S regions but also their efficient recombination of two cleaved ends in donor S_μ and acceptor S regions. A series of genetic studies on natural and artificial AID mutants revealed that AID cleaves DNA in both V and S regions regardless of the C-terminal mutation whereas S region recombination in CSR depends on the intact C-terminus of AID (9-11,37). Analysis of human patients with HIGMII syndrome suggests that C-terminal mutants of AID have the dominant negative effect on CSR by the unknown mechanism.

To study the mechanism of the dominant negative effect of AID C-terminal mutants, we generated CH12F3-2A cell line with the JP41 mutation on one allele and the null mutation on the other by the CRISPR/Cas9 genome editing (Fig. 1). As reported previously, we found that the expression of JP41 in AID^{JP41/Δ} cells fail to induce CSR, whereas AID^{WT/-} cells showed CSR, indicating that the C-terminus of AID is essential for CSR. On the other hand, expression of JP41

induced DNA cleavage as AID^{JP41/Δ} cells induced *Igh/c-Myc* translocations at a frequency similar to AID^{WT/-} cells (Fig. 1). DNA cleavage activity of JP41 was also confirmed by the assay using linker ligation mediated PCR of cleaved DNA end. However, compared to AID^{WT/WT} cells, the frequencies of *Igh/c-Myc* translocations in AID^{JP41/Δ} and AID^{WT/-} cells were greatly reduced, which is in line with the previous reports that the AID haploinsufficiency has more severe effect on the frequency of *Igh/c-Myc* translocations than CSR (36). We found that JP41 expression by CIT stimulation suppressed CSR as well as *Igh/c-Myc* translocation, which is induced by the OHT activation of exogenously introduced AIDER in AID^{JP41/Δ}/AIDER cells (Fig. 2). The dominant negative effect of the C-terminal mutant on WT AID was also confirmed by expression of P20 (or JP8Bdel) and WT AID on single vector in AID deficient spleen B cells (Fig. 3).

Although the AID C terminus is required for efficient S-S synapsis and prevents excessive end-resection of the AID induced DNA double strand breaks, it still remained unclear how AID C-terminal mutants interfere with function of WT AID to induce CSR (12-14). We confirmed that activation of JP41ER in CH12F3-2A strongly suppresses both deletional as well as inversional recombination by WT AID, indicating that JP41 interferes with the recombination step of WT AID induced CSR. Because AID C-terminal mutants affected both CSR and *Igh/c-Myc* translocation by WT AID, the dominant negative effect is unlikely due to a mere shift in the choice of the classical non-homologous end joining (c-NHEJ) to alternative non-homologous end joining (alt-NHEJ) repair pathways. Indeed, we found that expression of JP41 dissociated CSR specific cofactors such as SERBP1, hnRNP L, and hnRNP U from the AID ribonucleoprotein complex (Fig. 5). These findings indicate that JP41 depletes one or more ribonucleoprotein factor(s) and probably associated RNA species from WT AID, and thus prevents the generation of factor(s) required for efficient recombination.

To our surprise, we found a dominant negative effect of JP41 on *Igh/c-Myc* translocations in which the AID C-terminus function is not required (Fig. 5). We also found that siRNA mediated KD of hnRNP L, hnRNP U, or SERBP1 in AID^{JP41/Δ}/AIDER cells decreased the frequencies *Igh/c-Myc* translocations. These findings suggest that JP41 may compete with DNA repair proteins, particularly those implicated in alt-NHEJ for binding to SERBP1, hnRNP L, or hnRNP U. Indeed, these AID co-factors are known to associate with a variety of proteins or noncoding RNAs involved in general DNA damage response (29,41-45). For instance, hnRNP U or hnRNP U like proteins have been shown to interact with NEIL1, DNA glycosylase; MRN (MRE11-RAD50-NBS1), a DSB sensor complex; and DDSR1 (DNA damage-sensitive RNA1), a long non-coding RNA (29,44,45). Similarly, interaction of hnRNP L with repair proteins or long non-coding RNA is also implicated in DNA damage repair (42,43). Recently, we showed a requirement of SERBP1 and hnRNP U in the DNA end processing that is required for DNA repair at the recombination phase of CSR (21). Because JP41 showed a dominant negative effect on *Igh/c-Myc* translocation, it is likely that the human patients with heterozygous C-terminal mutations in AID are less likely to develop AID-associated oncogenic chromosomal translocations than the healthy individuals.

Top1 KD enhances DNA cleavage which augments both CSR and *Igh/c-Myc* translocation. The results indicate that the DNA cleavage efficiency by AID is critical to the efficiency of CSR and *Igh/c-Myc* translocation. In stimulated spleen B cells, cultured *in vitro*, AID induced CSR takes place at a 10⁻¹ level efficiency after a few days while *Igh/c-Myc* translocation occurs at 10⁻⁵ to 10⁻⁶ level. The difference could be due to a lower efficiency at either the *c-Myc* DNA cleavage, its recombination, or both. The mutation frequencies in Ig and *c-Myc* loci are approximately 10⁻³~10⁻⁴ and 0.8~50x10⁻³, respectively, indicating that Ig loci are cleaved at a comparable level

with the *c-Myc* locus (36,46-50). To explain $10^4\sim10^5$ higher efficiency of CSR compared with *Igh/c-Myc* recombination it is reasonable to postulate the recombination efficiency of *Igh/c-Myc* is much lower than that of CSR. Although both *Igh/c-Myc* and CSR require the same hnRNPs, their mechanistic involvements are obviously distinct as the AID C-terminus is required only for CSR. The critical part of the AID function would be to accomplish *cis*-recombination within the Ig locus.

In conclusion, our findings indicate that dominant negative function of AID C-terminal mutant on CSR is due to the dissociation of recombination specific cofactors from WT AID whereas the dominant negative effect on *Igh/c-Myc* translocation is likely due to the competition of JP41 with DNA repair proteins such as NEIL1, MRN, DDSR1, and noncoding RNAs for the binding with SERBP1, hnRNP L and hnRNP U which also function as AID cofactors. However, it remains to be established whether dissociation of recombination specific cofactors by JP41 is due to the titration of recombination specific RNA binding proteins themselves or the associated RNA species which upon editing generate the AID C-terminal-specific product required for CSR recombination.

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References

- 1 Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y., and Honjo, T. 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 102:553.
- 2 Revy, P., Muto, T., Levy, Y., Geissmann, F., Plebani, A., Sanal, O., Catalan, N., Forveille, M., Dufourcq-Labelouse, R., Gennery, A., Tezcan, I., Ersoy, F., Kayserili, H., Ugazio, A. G., Brousse, N., Muramatsu, M., Notarangelo, L. D., Kinoshita, K., Honjo, T., Fischer, A., and Durandy, A. 2000. Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* 102:565.
- 3 Honjo, T., Nagaoka, H., Shinkura, R., and Muramatsu, M. 2005. AID to overcome the limitations of genomic information. *Nat Immunol* 6:655.
- 4 Fails, A., Aoufouchi, S., Flatter, E., Gueranger, Q., Reynaud, C. A., and Weill, J. C. 2002. Induction of somatic hypermutation in immunoglobulin genes is dependent on DNA polymerase δ . *Nature* 419:944.
- 5 Petersen, S., Casellas, R., Reina-San-Martin, B., Chen, H. T., Difilippantonio, M. J., Wilson, P. C., Hanitsch, L., Celeste, A., Muramatsu, M., Pilch, D. R., Redon, C., Ried, T., Bonner, W. M., Honjo, T., Nussenzweig, M. C., and Nussenzweig, A. 2001. AID is required to initiate Nbs1/gamma-H2AX focus formation and mutations at sites of class switching. *Nature* 414:660.
- 6 Kong, Q. and Maizels, N. 2001. DNA breaks in hypermutating immunoglobulin genes: evidence for a break-and-repair pathway of somatic hypermutation. *Genetics* 158:369.
- 7 Wuerffel, R., Wang, L., Grigera, F., Manis, J., Selsing, E., Perlot, T., Alt, F. W., Cogne, M., Pinaud, E., and Kenter, A. L. 2007. S-S synapsis during class switch recombination is promoted by distantly located transcriptional elements and activation-induced deaminase. *Immunity* 27:711.
- 8 Boboila, C., Alt, F. W., and Schwer, B. 2012. Classical and alternative end-joining pathways for repair of lymphocyte-specific and general DNA double-strand breaks. *Advances in immunology* 116:1.
- 9 Shinkura, R., Ito, S., Begum, N. A., Nagaoka, H., Muramatsu, M., Kinoshita, K., Sakakibara, Y., Hijikata, H., and Honjo, T. 2004. Separate domains of AID are required for somatic hypermutation and class-switch recombination. *Nat Immunol* 5:707.
- 10 Barreto, V., Reina-San-Martin, B., Ramiro, A. R., McBride, K. M., and Nussenzweig, M. C. 2003. C-terminal deletion of AID uncouples class switch recombination from somatic hypermutation and gene conversion. *Mol Cell* 12:501.
- 11 Ta, V. T., Nagaoka, H., Catalan, N., Durandy, A., Fischer, A., Imai, K., Nonoyama, S., Tashiro, J., Ikegawa, M., Ito, S., Kinoshita, K., Muramatsu, M., and Honjo, T. 2003. AID mutant analyses indicate requirement for class-switch-specific cofactors. *Nat Immunol* 4:843.
- 12 Sabouri, S., Kobayashi, M., Begum, N. A., Xu, J., Hirota, K., and Honjo, T. 2014. C-terminal region of activation-induced cytidine deaminase (AID) is required for efficient class switch recombination and gene conversion. *Proc Natl Acad Sci U S A* 111:2253.

- 13 Zahn, A., Eranki, A. K., Patenaude, A. M., Methot, S. P., Fifield, H., Cortizas, E. M., Foster, P., Imai, K., Durandy, A., Larijani, M., Verdun, R. E., and Di Noia, J. M. 2014. Activation induced deaminase C-terminal domain links DNA breaks to end protection and repair during class switch recombination. *Proc Natl Acad Sci U S A* 111:E988.
- 14 Ucher, A. J., Ranjit, S., Kadungure, T., Linehan, E. K., Khair, L., Xie, E., Limauro, J., Rauch, K. S., Schrader, C. E., and Stavnezer, J. 2014. Mismatch repair proteins and AID activity are required for the dominant negative function of C-terminally deleted AID in class switching. *J Immunol* 193:1440.
- 15 Durandy, A. and Kracker, S. 2012. Immunoglobulin class-switch recombination deficiencies. *Arthritis Res Ther* 14:218.
- 16 Durandy, A., Peron, S., Taubenheim, N., and Fischer, A. 2006. Activation-induced cytidine deaminase: structure-function relationship as based on the study of mutants. *Hum Mutat* 27:1185.
- 17 Notarangelo, L. D., Duse, M., and Ugazio, A. G. 1992. Immunodeficiency with hyper-IgM (HIM). *Immunodeficiency reviews* 3:101.
- 18 Imai, K., Zhu, Y., Revy, P., Morio, T., Mizutani, S., Fischer, A., Nonoyama, S., and Durandy, A. 2005. Analysis of class switch recombination and somatic hypermutation in patients affected with autosomal dominant hyper-IgM syndrome type 2. *Clin Immunol* 115:277.
- 19 Kasahara, Y., Kaneko, H., Fukao, T., Terada, T., Asano, T., Kasahara, K., and Kondo, N. 2003. Hyper-IgM syndrome with putative dominant negative mutation in activation-induced cytidine deaminase. *J Allergy Clin Immunol* 112:755.
- 20 Ito, S., Nagaoka, H., Shinkura, R., Begum, N., Muramatsu, M., Nakata, M., and Honjo, T. 2004. Activation-induced cytidine deaminase shuttles between nucleus and cytoplasm like apolipoprotein B mRNA editing catalytic polypeptide 1. *Proc Natl Acad Sci U S A* 101:1975.
- 21 Mondal, S., Begum, N. A., Hu, W., and Honjo, T. 2016. Functional requirements of AID's higher order structures and their interaction with RNA-binding proteins. *Proc Natl Acad Sci U S A* 113:E1545.
- 22 Hu, W., Begum, N. A., Mondal, S., Stanlie, A., and Honjo, T. 2015. Identification of DNA cleavage- and recombination-specific hnRNP cofactors for activation-induced cytidine deaminase. *Proc Natl Acad Sci U S A* 112:5791.
- 23 Nakamura, M., Kondo, S., Sugai, M., Nazarea, M., Imamura, S., and Honjo, T. 1996. High frequency class switching of an IgM+ B lymphoma clone CH12F3 to IgA+ cells. *Int Immunol* 8:193.
- 24 Stanlie, A., Aida, M., Muramatsu, M., Honjo, T., and Begum, N. A. 2010. Histone3 lysine4 trimethylation regulated by the facilitates chromatin transcription complex is critical for DNA cleavage in class switch recombination. *Proc Natl Acad Sci U S A* 107:22190.
- 25 Canver, M. C., Bauer, D. E., Dass, A., Yien, Y. Y., Chung, J., Masuda, T., Maeda, T., Paw, B. H., and Orkin, S. H. 2014. Characterization of genomic deletion efficiency mediated by clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 nuclease system in mammalian cells. *The Journal of biological chemistry* 289:21312.
- 26 Canver, M. C., Bauer, D. E., Dass, A., Yien, Y. Y., Chung, J., Masuda, T., Maeda, T., Paw, B. H., and Orkin, S. H. 2017. Characterization of genomic deletion efficiency mediated by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 nuclease system in mammalian cells. *The Journal of biological chemistry* 292:2556.
- 27 Inui, M., Miyado, M., Igarashi, M., Tamano, M., Kubo, A., Yamashita, S., Asahara, H., Fukami, M., and Takada, S. 2014. Rapid generation of mouse models with defined point mutations by the CRISPR/Cas9 system. *Scientific reports* 4:5396.

- 28 Chu, V. T., Weber, T., Wefers, B., Wurst, W., Sander, S., Rajewsky, K., and Kuhn, R. 2015. Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nature biotechnology* 33:543.
- 29 Srivastava, M., Nambiar, M., Sharma, S., Karki, S. S., Goldsmith, G., Hegde, M., Kumar, S., Pandey, M., Singh, R. K., Ray, P., Natarajan, R., Kelkar, M., De, A., Choudhary, B., and Raghavan, S. C. 2012. An inhibitor of nonhomologous end-joining abrogates double-strand break repair and impedes cancer progression. *Cell* 151:1474.
- 30 Begum, N. A., Stanlie, A., Nakata, M., Akiyama, H., and Honjo, T. 2012. The histone chaperone Spt6 is required for activation-induced cytidine deaminase target determination through H3K4me3 regulation. *The Journal of biological chemistry* 287:32415.
- 31 Kobayashi, M., Aida, M., Nagaoka, H., Begum, N. A., Kitawaki, Y., Nakata, M., Stanlie, A., Doi, T., Kato, L., Okazaki, I. M., Shinkura, R., Muramatsu, M., Kinoshita, K., and Honjo, T. 2009. AID-induced decrease in topoisomerase 1 induces DNA structural alteration and DNA cleavage for class switch recombination. *Proc Natl Acad Sci U S A* 106:22375.
- 32 Boboila, C., Oksenyich, V., Gostissa, M., Wang, J. H., Zha, S., Zhang, Y., Chai, H., Lee, C. S., Jankovic, M., Saez, L. M., Nussenzweig, M. C., McKinnon, P. J., Alt, F. W., and Schwer, B. 2012. Robust chromosomal DNA repair via alternative end-joining in the absence of X-ray repair cross-complementing protein 1 (XRCC1). *Proc Natl Acad Sci U S A* 109:2473.
- 33 Husain, A., Begum, N. A., Taniguchi, T., Taniguchi, H., Kobayashi, M., and Honjo, T. 2016. Chromatin remodeller SMARCA4 recruits topoisomerase 1 and suppresses transcription-associated genomic instability. *Nature communications* 7:10549.
- 34 Schrader, C. E., Linehan, E. K., Mochegova, S. N., Woodland, R. T., and Stavnezer, J. 2005. Inducible DNA breaks in Ig S regions are dependent on AID and UNG. *J Exp Med* 202:561.
- 35 Xu, J., Husain, A., Hu, W., Honjo, T., and Kobayashi, M. 2014. APE1 is dispensable for S-region cleavage but required for its repair in class switch recombination. *Proc Natl Acad Sci U S A* 111:17242.
- 36 Takizawa, M., Tolarova, H., Li, Z., Dubois, W., Lim, S., Callen, E., Franco, S., Mosaico, M., Feigenbaum, L., Alt, F. W., Nussenzweig, A., Potter, M., and Casellas, R. 2008. AID expression levels determine the extent of cMyc oncogenic translocations and the incidence of B cell tumor development. *J Exp Med* 205:1949.
- 37 Doi, T., Kato, L., Ito, S., Shinkura, R., Wei, M., Nagaoka, H., Wang, J., and Honjo, T. 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 U S A* 106:2758.
- 38 Shi, M. J. and Stavnezer, J. 1998. CBF alpha3 (AML2) is induced by TGF-beta1 to bind and activate the mouse germline Ig alpha promoter. *J Immunol* 161:6751.
- 39 Chen, X., Kinoshita, K., and Honjo, T. 2001. Variable deletion and duplication at recombination junction ends: implication for staggered double-strand cleavage in class-switch recombination. *Proc Natl Acad Sci U S A* 98:13860.
- 40 Chu, C. C., Paul, W. E., and Max, E. E. 1992. Quantitation of immunoglobulin mu-gamma 1 heavy chain switch region recombination by a digestion-circularization polymerase chain reaction method. *Proc Natl Acad Sci U S A* 89:6978.
- 41 Dutertre, M., Lambert, S., Carreira, A., Amor-Gueret, M., and Vagner, S. 2014. DNA damage: RNA-binding proteins protect from near and far. *Trends Biochem Sci* 39:141.
- 42 Gaudreau, M. C., Grapton, D., Helness, A., Vadnais, C., Fraszczak, J., Shooshtarizadeh, P., Wilhelm, B., Robert, F., Heyd, F., and Moroy, T. 2016. Heterogeneous Nuclear Ribonucleoprotein L is required for the survival and functional integrity of murine hematopoietic stem cells. *Scientific reports* 6:27379.

- 43 Li, M., Gou, H., Tripathi, B. K., Huang, J., Jiang, S., Dubois, W., Waybright, T., Lei, M., Shi, J., Zhou, M., and Huang, J. 2015. An Apela RNA-Containing Negative Feedback Loop Regulates p53-Mediated Apoptosis in Embryonic Stem Cells. *Cell stem cell* 16:669.
- 44 Polo, S. E., Blackford, A. N., Chapman, J. R., Baskcomb, L., Gravel, S., Rusch, A., Thomas, A., Blundred, R., Smith, P., Kzhyskowska, J., Dobner, T., Taylor, A. M., Turnell, A. S., Stewart, G. S., Grand, R. J., and Jackson, S. P. 2012. Regulation of DNA-end resection by hnRNPU-like proteins promotes DNA double-strand break signaling and repair. *Mol Cell* 45:505.
- 45 Sharma, V., Khurana, S., Kubben, N., Abdelmohsen, K., Oberdoerffer, P., Gorospe, M., and Misteli, T. 2015. A BRCA1-interacting lncRNA regulates homologous recombination. *EMBO reports* 16:1520.
- 46 Liu, M., Duke, J. L., Richter, D. J., Vinuesa, C. G., Goodnow, C. C., Kleinstein, S. H., and Schatz, D. G. 2008. Two levels of protection for the B cell genome during somatic hypermutation. *Nature* 451:841.
- 47 Kato, L., Begum, N. A., Burroughs, A. M., Doi, T., Kawai, J., Daub, C. O., Kawaguchi, T., Matsuda, F., Hayashizaki, Y., and Honjo, T. 2012. Nonimmunoglobulin target loci of activation-induced cytidine deaminase (AID) share unique features with immunoglobulin genes. *Proc Natl Acad Sci U S A* 109:2479.
- 48 Dorsett, Y., McBride, K. M., Jankovic, M., Gazumyan, A., Thai, T. H., Robbani, D. F., Di Virgilio, M., Reina San-Martin, B., Heidkamp, G., Schwickert, T. A., Eisenreich, T., Rajewsky, K., and Nussenzweig, M. C. 2008. MicroRNA-155 suppresses activation-induced cytidine deaminase-mediated Myc-Igh translocation. *Immunity* 28:630.
- 49 Dorsett, Y., Robbani, D. F., Jankovic, M., Reina-San-Martin, B., Eisenreich, T. R., and Nussenzweig, M. C. 2007. A role for AID in chromosome translocations between c-myc and the IgH variable region. *J Exp Med* 204:2225.
- 50 Nagaoka, H., Muramatsu, M., Yamamura, N., Kinoshita, K., and Honjo, T. 2002. Activation-induced deaminase (AID)-directed hypermutation in the immunoglobulin Smu region: implication of AID involvement in a common step of class switch recombination and somatic hypermutation. *J Exp Med* 195:529.

Figures' legends

Figure 1: Generation of CH12F3-2A cell line expressing JP41 equivalent mutant of AID

(AID^{JP41/Δ}). (A) A schematic illustration showing a portion of mouse AID locus (Exon 5) along with guide RNA sequence, PAM site, and NsiI restriction site. (B) Experimental design showing the time-course from pre-transfection to screening of clones with desirable changes in AID locus of CH12F3-2A cells. (C) Western blot screening for the CH12F3-2A clones with repair using ssODN HDR template. Whole cell lysates prepared from the individual clones following CIT stimulation were subjected to western blotting with α -HA, α -AID, and α -Tubulin antibodies. A clone showing HA expression, as a result of successful repair using ssODN HDR template, is highlighted with dotted rectangle. (D) Confirmation of the successful incorporation of the sequences from ssODN HDR template by the NsiI digestion of the genomic DNA fragments obtained by the PCR amplification with the primers flanking the Exon 5 region of AID locus of AID^{JP41/Δ} and AID^{WT/WT} CH12F3-2A cells. (E) A schematic illustration of the sequence of the modified AID locus of the AID^{JP41/Δ} cell line. (F) Representative FACS profiles of the percent IgA-switching in AID^{WT/WT}, AID^{JP41/Δ}, and AID^{WT/-} cells upon CIT stimulation. The numbers in the FACS plots represent the percentage of the cells expressing IgA on their surface. (G) Summary of the IgA switching data derived from three independent experiments. (H) Experimental design for *Igh/c-Myc* chromosomal translocation assay in AID^{WT/WT}, AID^{JP41/Δ} and AID^{WT/-} cells following transfection with Top1 (siTop1) or control siRNA (siCont). At 24 h after electroporation of siRNAs, cells were cultured for another 48 h in the presence (+) or absence (-) of CIT, and genomic DNA were isolated. (I) PCR amplification scheme to detect *Igh/c-Myc* chromosomal translocations (derivatives of chromosome15). Triangles represent the positions of primers used to amplify the rearranged regions. The position of the *Myc* probe used in the

Southern blot hybridization is shown as a horizontal black bar. (J) Southern blot analysis of PCR-amplified fragments with *Myc*-specific probe. (K) Frequency of *Igh/c-Myc* chromosomal translocations from two independent experiments calculated as number of translocations per 1×10^6 cells. (L) Western blotting to confirm siRNA mediated knockdown of Top1 in the indicated cell lines. An * sign indicates the stop codon, whereas Δ indicates deletion of nucleotides. ssODN, single-stranded oligodeoxynucleotides; HA, hemagglutinin; SCR7, an inhibitor of DNA ligase IV. β -actin is used as loading control. CIT, CD40L-IL4-TGF β ; IgA, immunoglobulin A; IgM, immunoglobulin M; Error bars represent the standard deviation (SD).

Figure 2: JP41 shows dominant negative effect on WTAID induced CSR and *Igh/c-Myc* chromosomal translocations. (A) *Top*: A schematic representation of the AIDER plasmid construct used to generate AID^{JP41/ Δ} /AIDER cells. *Bottom*: Experimental design to establish AID^{JP41/ Δ} cells stably expressing AIDER (AID^{JP41/ Δ} /AIDER). (B) FACS profiles of IgA switching in two different clones of AID^{JP41/ Δ} /AIDER cells cultured in the absence (none) or the presence of OHT alone or OHT in combination with CIT for 48 h. (C) Summary of the IgA switching data derived from 3 independent experiments. The data is represented as relative change in AIDER-induced CSR upon expression of JP41-HA by CIT stimulation. (D) Western blot analysis of AIDER and JP41-HA in AID^{JP41/ Δ} /AIDER and AID^{-/-}/AIDER cells. β -actin was used as loading control. (E) Experimental design to detect *Igh/c-Myc* translocations in AID^{-/-}/AIDER and AID^{JP41/ Δ} /AIDER#12 cells following transfection with Top1 (siTop1) or control siRNA (siCont). At 24 h after electroporation of siRNAs, cells were cultured for another 48 h in the absence (none) or the presence of OHT alone or OHT in combination with CIT, and genomic DNA were isolated. (F) Western blotting to confirm siRNA mediated knockdown of Top1 in the

indicated cells. Tubulin is used as a loading control. (G) Southern blot analysis of PCR-amplified fragments with *Myc*-specific probe. (H) Frequencies of *Igh/c-Myc* translocations in AID^{-/-}/AIDER and AID^{JP41/Δ}/AIDER#12 cells cultured in the indicated conditions. The data is represented as relative change in frequency of AIDER induced *Igh/c-Myc* translocations upon CIT induced expression of JP41-HA. CIT, CD40L-IL4-TGFβ; OHT, 4-hydroxytamoxifen; AIDER AID fused with the hormone-binding domain of the estrogen receptor (ER). Error bars represent the standard deviation (SD).

Figure 3: AID C-terminal mutants suppressed IgG1 switching induced by WT AID. (A)

The scheme shows the time course of stimulation and retroviral transduction of splenic B cells isolated from AID deficient mice. (B) Representative FACS profiles of IgG1 switching in AID^{-/-} B cells following retroviral expression of AID and the mutants as indicated below each profile. The retroviral constructs used to express the indicated AID species are also depicted above the FACS profiles. Retroviral transduction was monitored by the GFP expression in the infected spleen B cells. Percentage of IgG1 switched population in GFP⁺ gate was shown on the upper right corner of each plot. (C) A summarized view of the result based on three independent experiments is shown. LPS, lipopolysaccharide; IL4, Interleukin 4.

Figure 4: Activation of JP41ER interfered CSR associated recombination but not the formation of DNA double strand breaks. (A)

Generation of a CH12F3-2A line that stably expressed retro virally introduced AID mutant JP41 fused to Estrogen Receptor (ER) at the C-terminus. The expressed JP41ER remains in an inactive state and undergoes functional activation upon 4-hydroxytamoxifen (OHT) treatment. In the absence of CIT, expression of endogenous AID is barely detectable. (B) A representative plot showing the typical IgM to IgA switching profile of JP41ER cell line. Suppression of CIT induced IgA switching is evident when the OHT

was added to activate JP41ER. Immunoblot analysis confirms the expression of both JP41ER and WT AID in the generated cell line. Indicated cell lines were either left untreated or stimulated by OHT and CIT for 24 h. (C) *Left*: A simplified scheme of Ligation mediated PCR (LM-PCR) assay used to detect switch region ($S\mu$) DNA double strand breaks. *Right*: LM-PCR analysis of the AID-induced DNA double-strand breaks in the $S\mu$ region of CH12F3-2A/JP41ER cells. Southern blot analysis using an $S\mu$ probe and the semiquantitative PCR of the GAPDH gene served as an internal control for the DNA samples used. Wedges indicate a threefold increase in DNA amount. The DSBs were only detectable upon the treatment of CH12F3-2A/JP41ER cells with CIT and/or OHT. (D) The scheme illustrates the orientation specific resolution of CSR associated DNA breaks: the productive or deletional recombination that results in CSR and the non-productive or inversional recombination which does not lead CSR. Both the pathways are involved in *trans*-recombination, which is responsible for chromosomal translocations between *Igh* and non-*Igh* loci.

Figure 5: JP41 reduced the binding of WT AID with RNA binding proteins (A) *Top*: Experimental design to analyze the effect of JP41 expression on the binding of AID with RNA binding proteins in AID^{JP41/Δ}/AIDER#12. *Bottom*: The AID^{JP41/Δ}/AIDER#12 cells cultured in the presence of OHT alone or OHT in combination with CIT. CIT stimulated AID^{JP41/Δ} cells were used as a control to confirm the non-specific binding of the FLAG antibodies or the agarose beads. (B) Cell extracts from AID^{JP41/Δ} and AID^{JP41/Δ}/AIDER#12 cells stimulated as shown in panel A (bottom) were immunoprecipitated with FLAG-M2 agarose, and input lysates as well as immunoprecipitated fractions were subjected to immunoblotting with the indicated antibodies. (C) Experimental design to detect *Igh/c-Myc* translocations in AID^{JP41/Δ}/AIDER#12 cell following transfection with control (siCont) or siRNAs against indicated RNA-binding proteins.

At 24 h after electroporation of siRNAs, cells were cultured for another 48 h in the absence (-) or the presence of OHT. (D) Immunoblots with hnRNP L, SERBP1, or hnRNP U antibodies to confirm siRNA mediated knockdown of these factors. β -actin was used as internal loading control. (E) Representative FACS profiles of the percent IgA-switching in AID^{JP41/ Δ} /AIDER#12 cells upon knockdown of the indicated RNA binding proteins. The number in the FACS plot represents the percentage of the cells expressing IgA on their surface. (F) Summary of the IgA switching data derived from two independent experiments. (G) Southern blot analysis of PCR-amplified fragments with *Myc*-specific probe. (H) Frequency of *Igh/c-Myc* chromosomal translocations calculated as number of translocations per 1×10^6 cells. Error bars represent the standard deviation (SD).

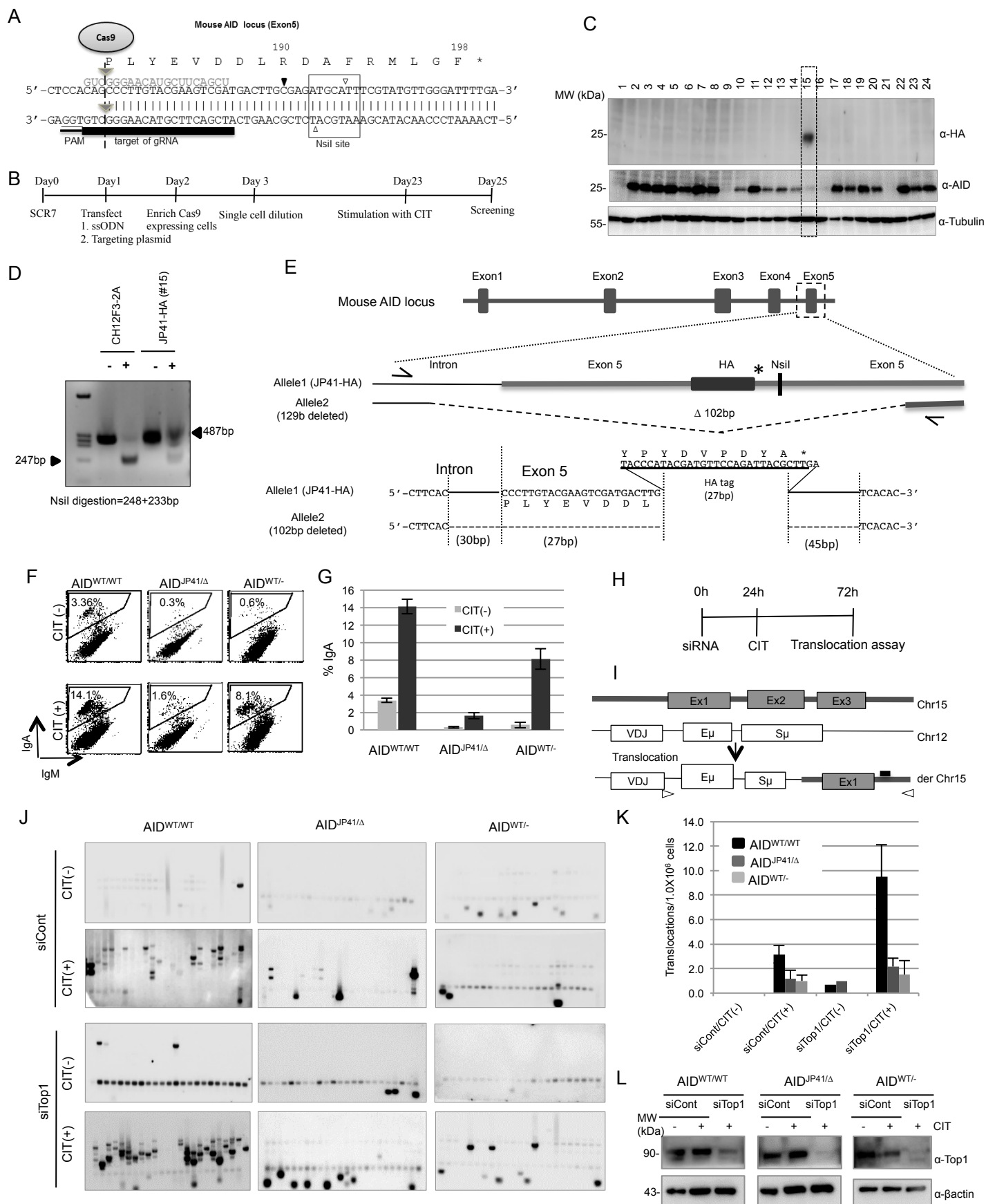


Figure 1

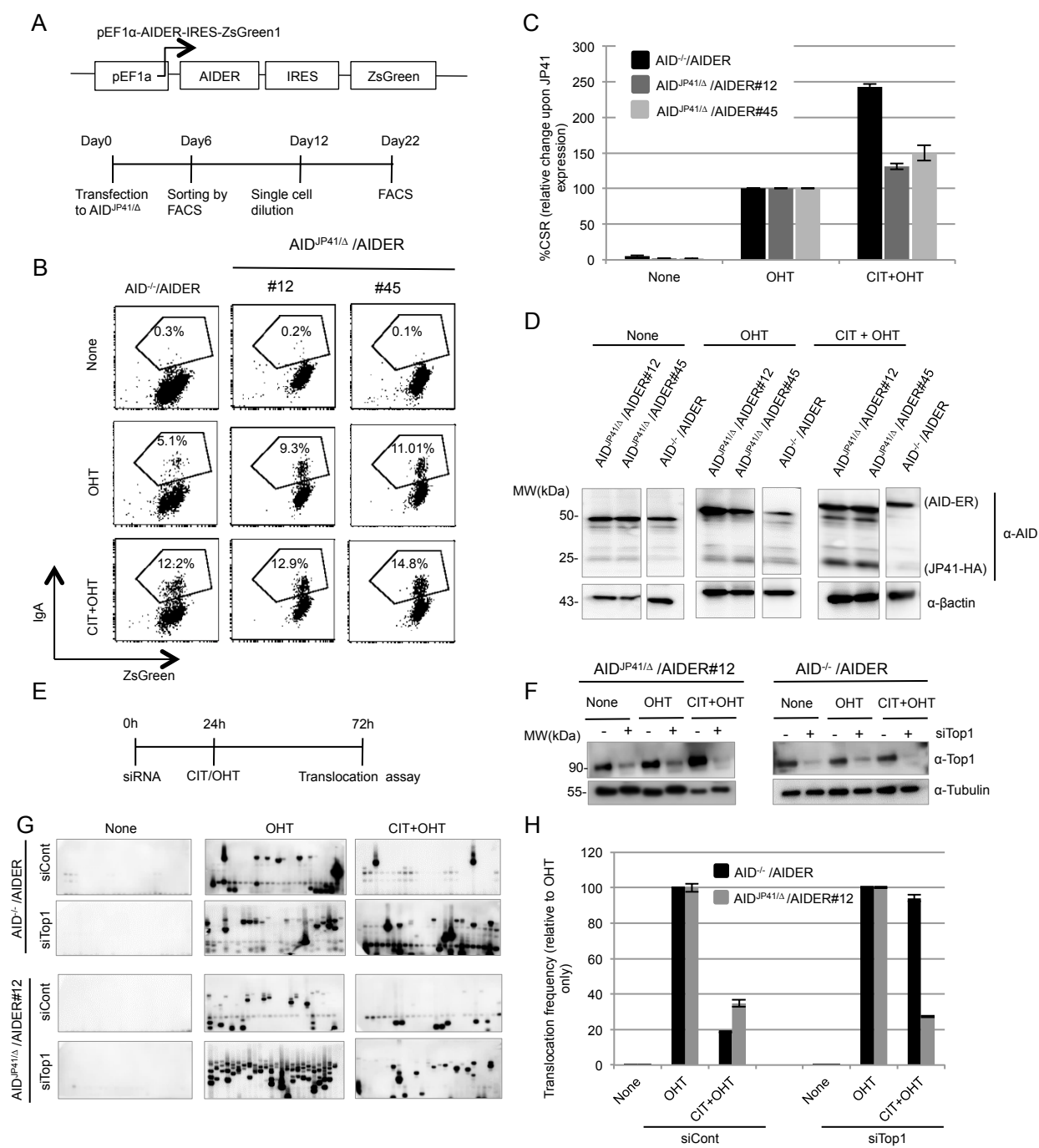


Figure 2

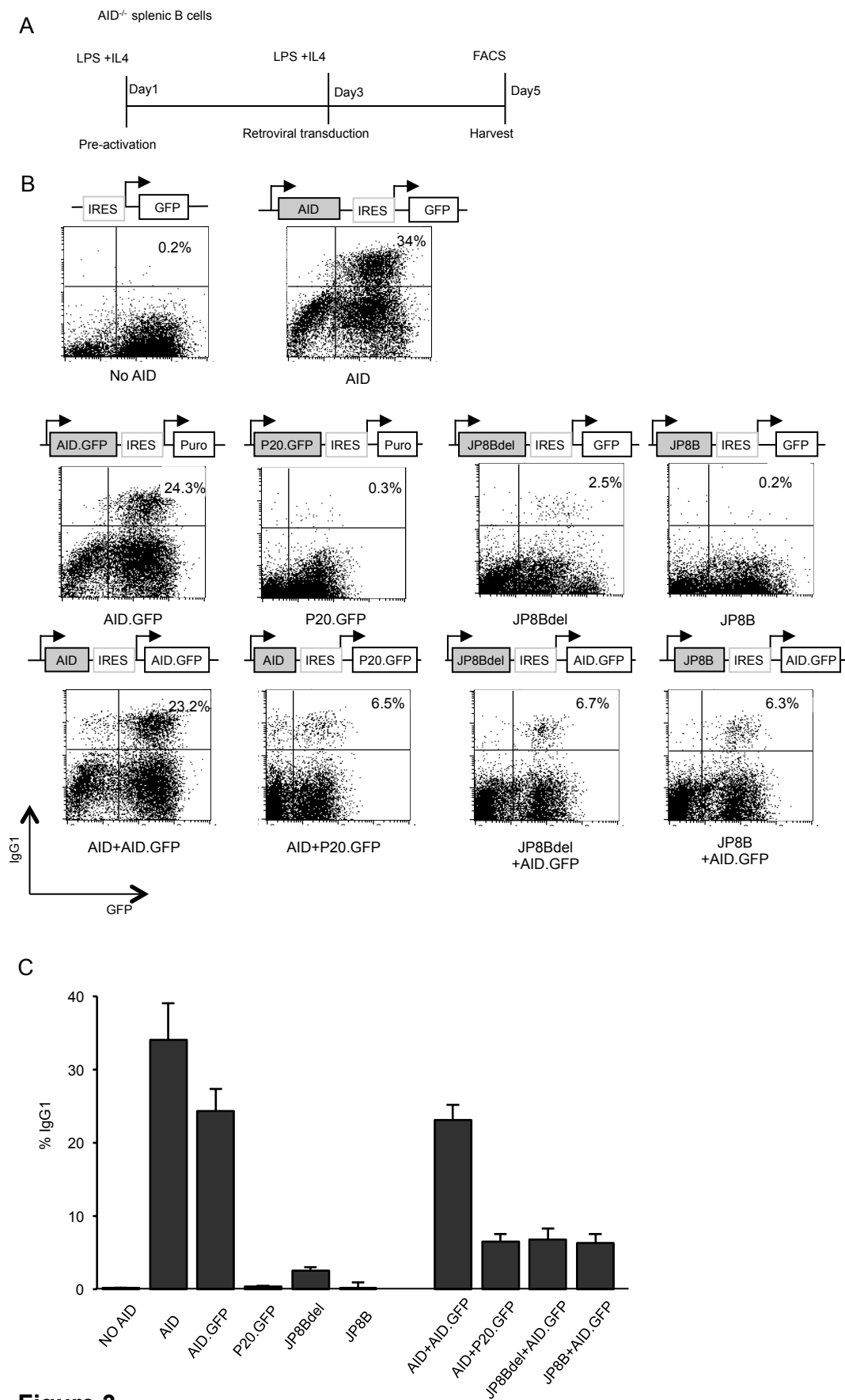


Figure 3

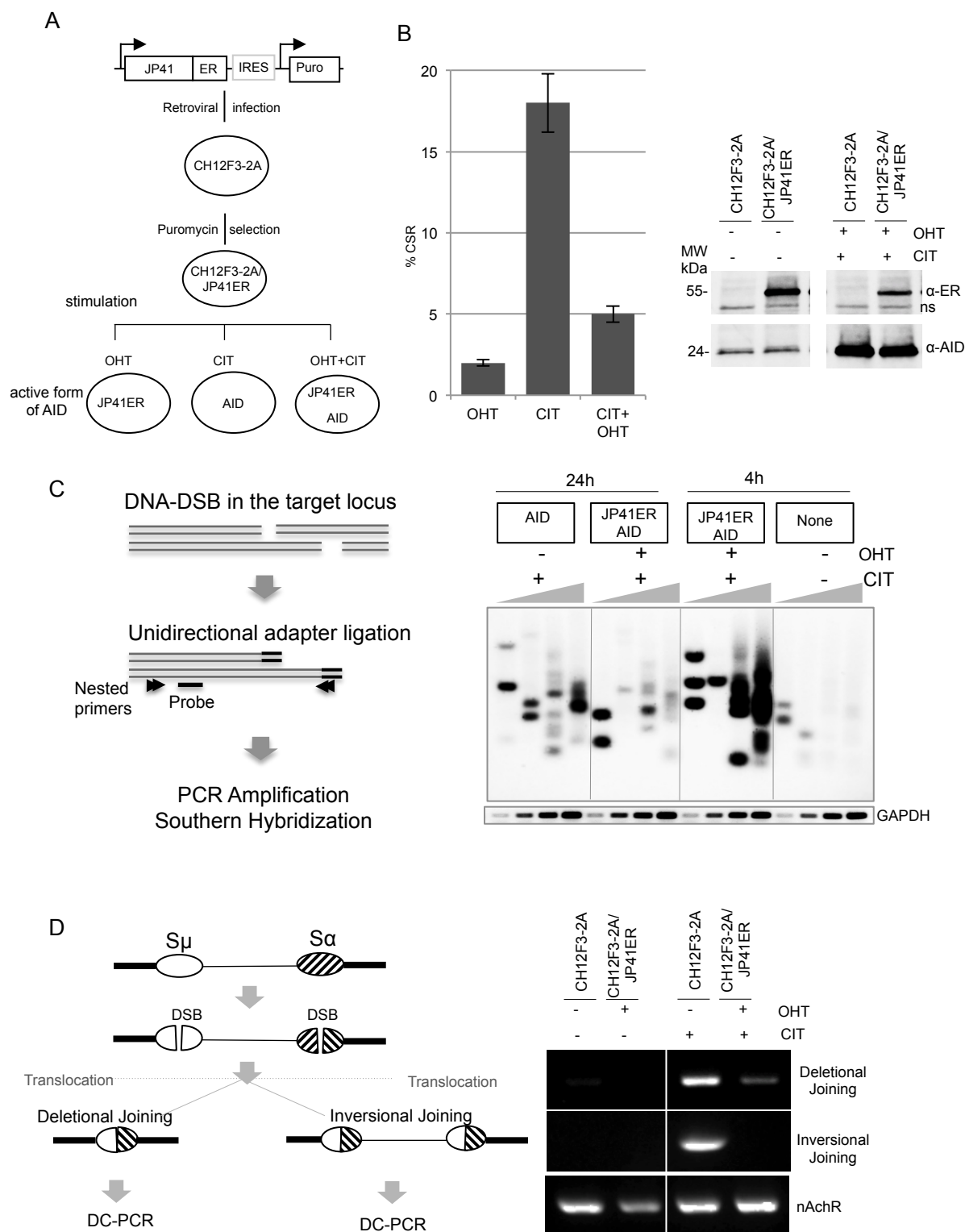


Figure 4

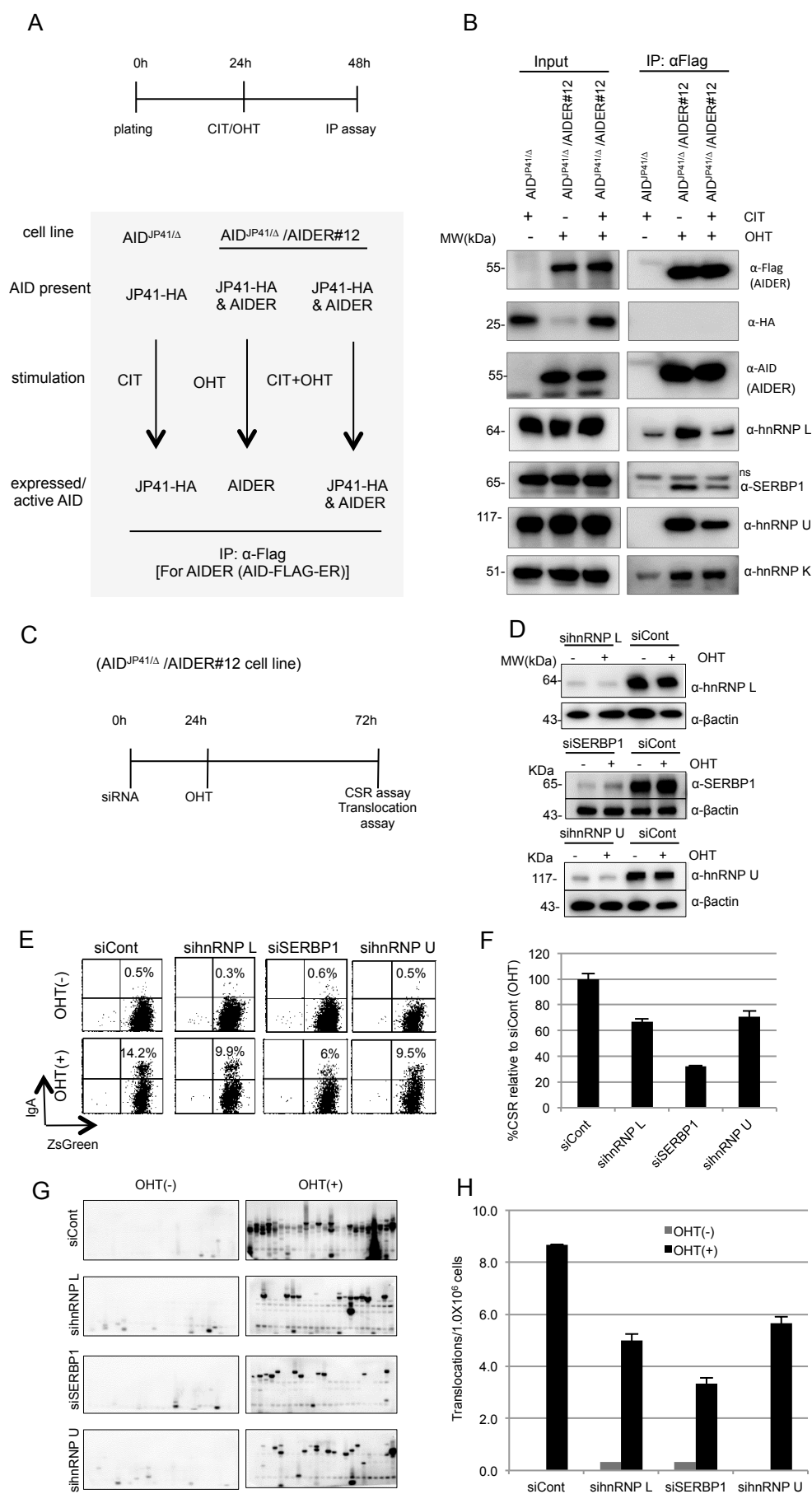
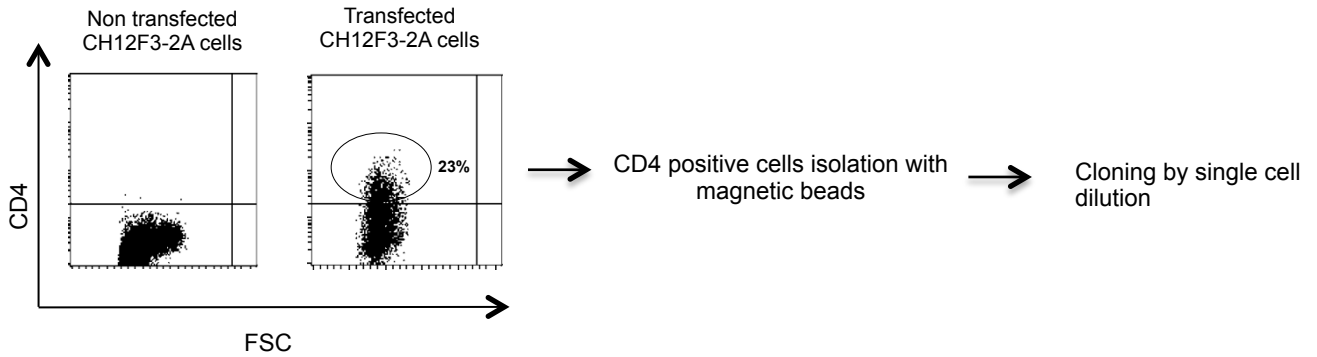


Figure 5

Supplementary data

A



B

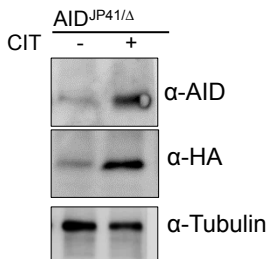


Fig. S1. (A) FACS analysis of CH12F3-2A cells post transfection. Cells highlighted with a circle indicate CD4 expressing cells that have successfully transfected with GeneArt CRISPR Nuclease CD4 Reporter Vector. CD4 expression cells were purified using Dynabeads® CD4 Positive Isolation Kit (Invitrogen). CD4 expressing cells were subjected to limited dilution to isolate single cell clones, and cultured for another 20 days (see Fig1). (B) The clone with modified AID locus was analyzed for AID and HA expression using western blot analysis after 48 h CIT stimulation. Tubulin was used as internal control.

A

	1		80
JP41	Atggacagccttctgatgaagcaaaagaagtttctttaccatttcaaaaatgtccgctgggccaaaggacggcatgagac		
JP41-HA	Atggacagccttctgatgaagcaaaagaagtttctttaccatttcaaaaatgtccgctgggccaaaggacggcatgagac		
WT-AID	Atggacagccttctgatgaagcaaaagaagtttctttaccatttcaaaaatgtccgctgggccaaaggacggcatgagac		
	81		160
JP41	ctacctctgtacgtggtgaagaggagagatagtgccacctctgtcactggacttcggccaccttcgcaacaagtctg		
JP41-HA	ctacctctgtacgtggtgaagaggagagatagtgccacctctgtcactggacttcggccaccttcgcaacaagtctg		
WT-AID	ctacctctgtacgtggtgaagaggagagatagtgccacctctgtcactggacttcggccaccttcgcaacaagtctg		
	161		240
JP41	gctgccacgtggaattgttgttcctacgctacatctcagactgggacctggaccgggcccgggtgttaccgcgtcacctgg		
JP41-HA	gctgccacgtggaattgttgttccctacgctacatctcagactgggacctggaccgggcccgggtgttaccgcgtcacctgg		
WT-AID	gctgccacgtggaattgttgttccctacgctacatctcagactgggacctggaccgggcccgggtgttaccgcgtcacctgg		
	241		320
JP41	tccacctctggagcccgtgctatgactgtgccggcacgtggctgagtttctgagatggaaccctaacctcagcctgag		
JP41-HA	tccacctctggagcccgtgctatgactgtgccggcacgtggctgagtttctgagatggaaccctaacctcagcctgag		
WT-AID	tccacctctggagcccgtgctatgactgtgccggcacgtggctgagtttctgagatggaaccctaacctcagcctgag		
	321		400
JP41	gattttcaccgcgcgcctctacttctgtgaagaccgcaaggctgagcctgaggggctgcgggagactgcaccgcgtgggg		
JP41-HA	gattttcaccgcgcgcctctacttctgtgaagaccgcaaggctgagcctgaggggctgcgggagactgcaccgcgtgggg		
WT-AID	gattttcaccgcgcgcctctacttctgtgaagaccgcaaggctgagcctgaggggctgcgggagactgcaccgcgtgggg		
	401		480
JP41	tccagatcgggatcatgaccttcaaagactatttttactgctggaatacattttagaaaaatcgtgaaagaactttcaaa		
JP41-HA	tccagatcgggatcatgaccttcaaagactatttttactgctggaatacattttagaaaaatcgtgaaagaactttcaaa		
WT-AID	tccagatcgggatcatgaccttcaaagactatttttactgctggaatacattttagaaaaatcgtgaaagaactttcaaa		
	481		560
JP41	gcctgggaagggctacatgaaaattctgtccggctaaccagacaaacttcggcgcatccttttgccttgtacgaagtcca		
JP41-HA	gcctgggaagggctacatgaaaattctgtccggctaaccagacaaacttcggcgcatccttttgccttgtacgaagtcca		
WT-AID	gcctgggaagggctacatgaaaattctgtccggctaaccagacaaacttcggcgcatccttttgccttgtacgaagtcca		
	561	597	
JP41	tgactttgtga		
JP41-HA	tgactttg <u>tacccatacga</u> tgttccagattacgcttga		
WT-AID	tgactttg-----cgagatgcatttcgtatgttgggattttga		

B

JP41	MDSLLMKQKKFLYHFKNVRWAKGRHETYL CYVVKRRDSATSCSLDFGHLRNKSGCHVELL	60
JP41-HA	MDSLLMKQKKFLYHFKNVRWAKGRHETYL CYVVKRRDSATSCSLDFGHLRNKSGCHVELL	60
WT-AID	MDSLLMKQKKFLYHFKNVRWAKGRHETYL CYVVKRRDSATSCSLDFGHLRNKSGCHVELL	60

JP41	FLRYISDWLDPGRCYRVTWFTSWSPCYDCARHVAEFLRWNPNSLRIFTARLYFCEDRK	120
JP41-HA	FLRYISDWLDPGRCYRVTWFTSWSPCYDCARHVAEFLRWNPNSLRIFTARLYFCEDRK	120
WT-AID	FLRYISDWLDPGRCYRVTWFTSWSPCYDCARHVAEFLRWNPNSLRIFTARLYFCEDRK	120

JP41	AEPEGLRRLHRAGVQIGIMTFKDYFCWNTFVENRERTFKAWEGLHENSVRLTRQLRRL	180
JP41-HA	AEPEGLRRLHRAGVQIGIMTFKDYFCWNTFVENRERTFKAWEGLHENSVRLTRQLRRL	180
WT-AID	AEPEGLRRLHRAGVQIGIMTFKDYFCWNTFVENRERTFKAWEGLHENSVRLTRQLRRL	180

JP41	LPLYEVDDL-----	190
JP41-HA	LPLYEVDDL <u>YPYDVPDYA</u>	198
WT-AID	LPLYEVDDL-----RDAFRMLGF	198

Fig. S2. (A) To confirm the knock in of R190X-HA in to the AID locus, AID mRNA was sequenced. RNA extracted from CIT stimulated AID^{JP41/Δ} cells was subjected to cDNA synthesis. The AID was amplified from the cDNA with primers flanking AID open reading frame (Supplementary Table S1). The PCR fragments were cloned into the pGEM-T Easy Vector (Promega), and 12 clones were sequenced. All of the 12 clones contained identical sequence of AID which upon alignment with WT-AID showed the presence of HA-tagged JP41. The underlined sequence is HA tag followed by early stop codon in JP41 compared to WT AID (also see Fig 1. E). (B) The amino acid sequence shown of AID^{JP41/Δ} (JP41-HA) aligned with those of JP41 and WT AID. The underlined sequence is HA tag followed by early stop codon in JP41 compared to WT-AID.

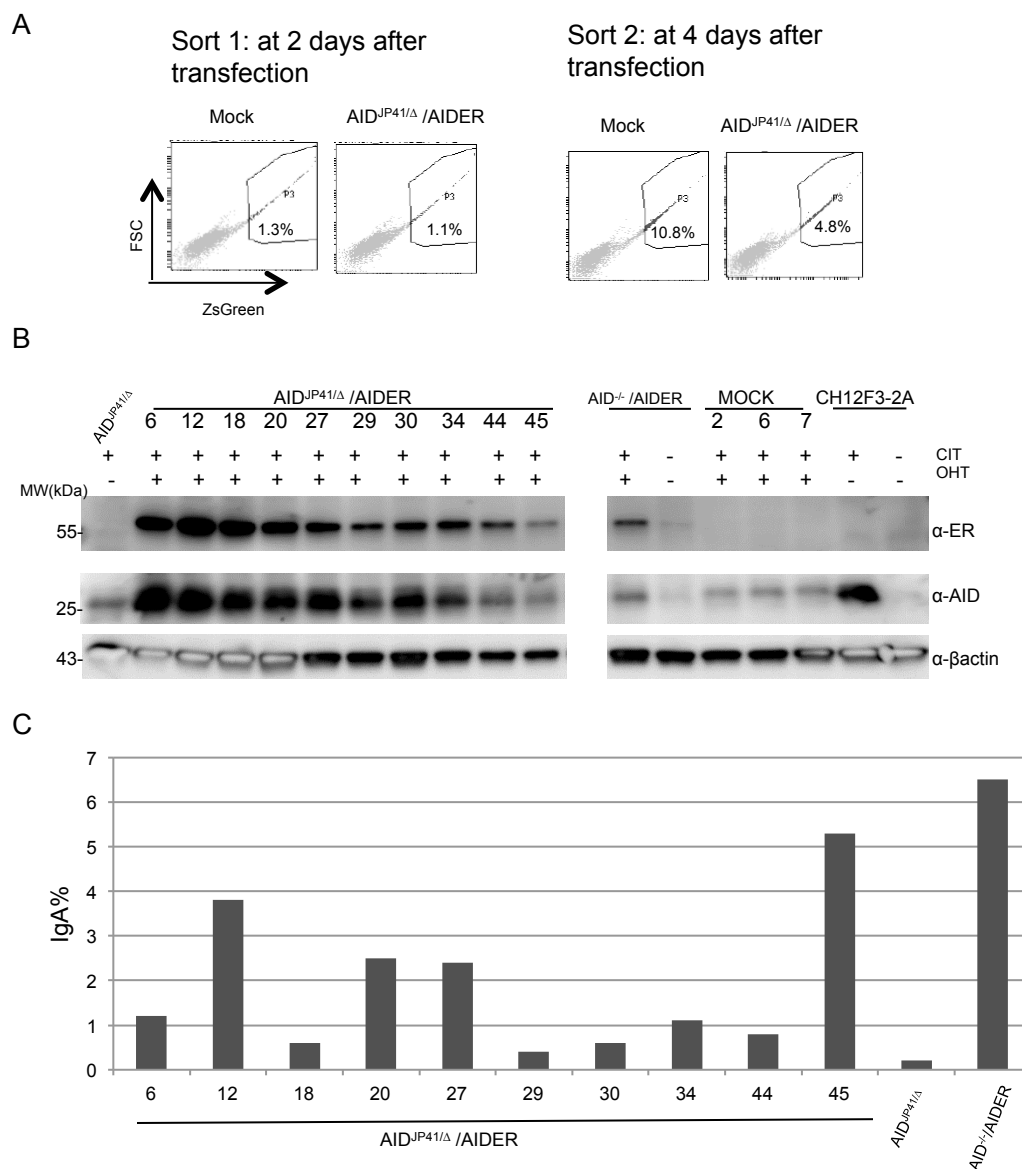


Fig. S3. (A) AID^{JP41/Δ} cells were transfected with AID-ER (see Fig.2) and Mock (vector without AIDER) as negative control. Cells were sorted using FACS-Aria for ZsGreen positive cells and subjected to limited dilution to obtain single cell clones with stable expression of AIDER (AID^{JP41/Δ}/AIDER). (B) Screening of AID-ER expressing clones in CIT+OHT treated CH12F3-2A cells. (C) Plot of % IgA switching after 48h OHT stimulation. Clones that showed higher CSR activity (#12 and #45) were selected for further studies (See Fig. 2).

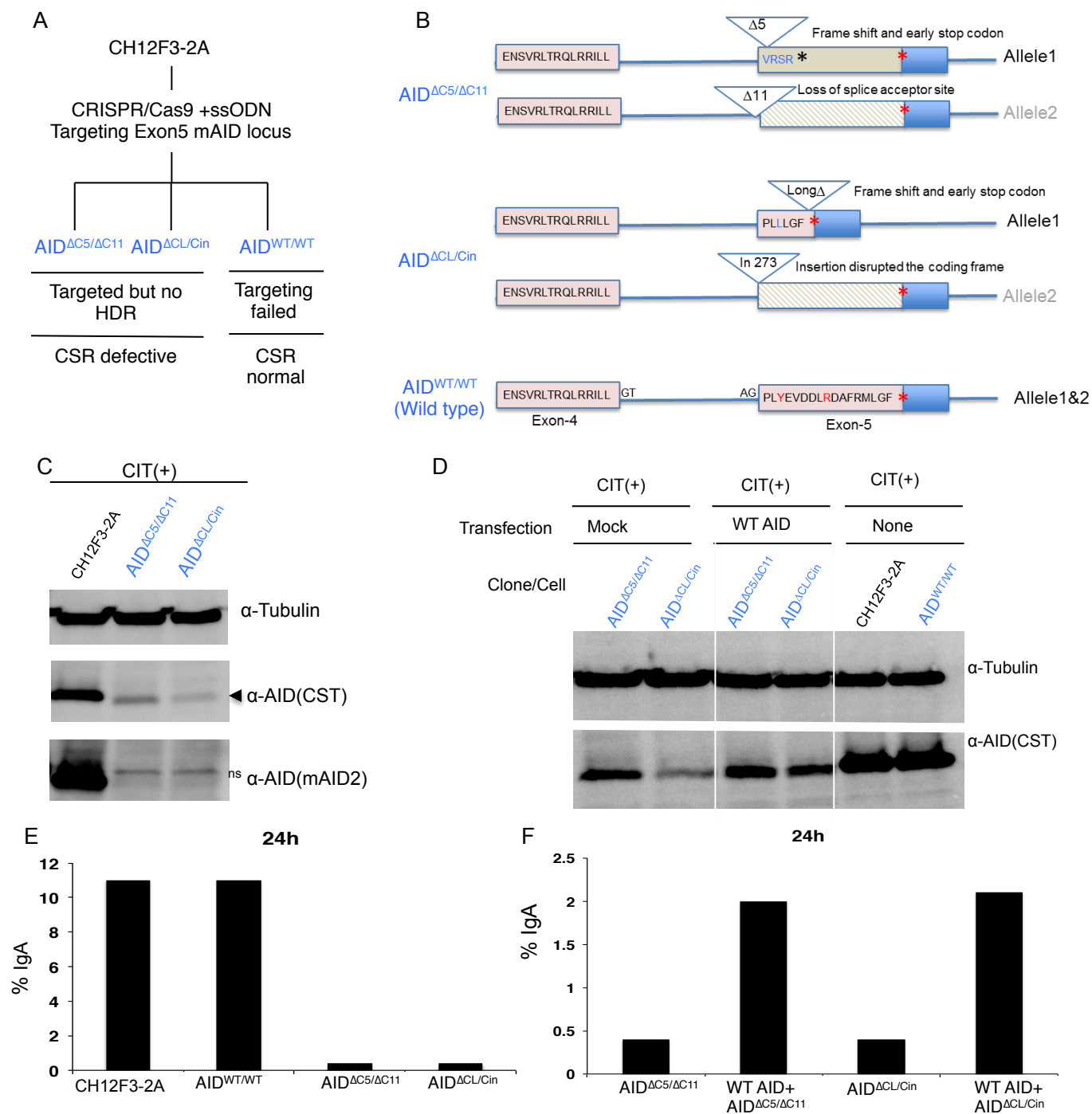


Fig. S4. CRISPR/Cas9 targeting to AID exon-5 yielded two new AID-C terminal mutant clones that blocked CSR. (A) The scheme gives an overview of the three CH12F3-2A clones designated as $AID^{\Delta C5/\Delta C11}$, $AID^{\Delta CL/Cin}$, and $AID^{WT/WT}$, respectively. The former two clones represent two novel C-terminal mutants of AID. (B) Schematic view of mouse AID locus encompassing the exon 4 and 5. Genomic DNA of each clonal line was subjected to PCR amplification of the target locus by appropriately designed primer pair. PCR products were cloned, sequenced and analyzed. The status of the targeted locus in the individual allele is depicted. The red asterisk represents the natural stop codon, whereas the black one was generated during NHEJ repair of the targeted locus. The two amino acids ("Y" and "R") in exon 5 are color coded to indicate the position of stop codons in AID mutant JP8Bdel and JP41, respectively. (C-D) Western blot analysis of parental CH12F3-2A line versus $AID^{\Delta C5/\Delta C11}$, $AID^{\Delta CL/Cin}$ and $AID^{WT/WT}$ clones. α -AID (Cell Signaling Technologies) detects a C-terminal epitope around Leu165, away from the exon 5 encoded region. The mAID2 antibody recognizes the very C-terminus of AID, encoded mainly by exon 5. Cells were stimulated by CIT for 24h to induce AID expression. (E-F) The bar plots show the representative IgM to IgA switching profile of three lines ($AID^{\Delta C5/\Delta C11}$, $AID^{\Delta CL/Cin}$, and $AID^{WT/WT}$) in reference to the parental line. Both $AID^{\Delta C5/\Delta C11}$ and $AID^{\Delta CL/Cin}$, which expressed C-terminally defective AID, were unable to induce CSR. Transient transfection of WT AID partially promoted CSR but could not fully restore CSR in the presence of C-terminally truncated AID.

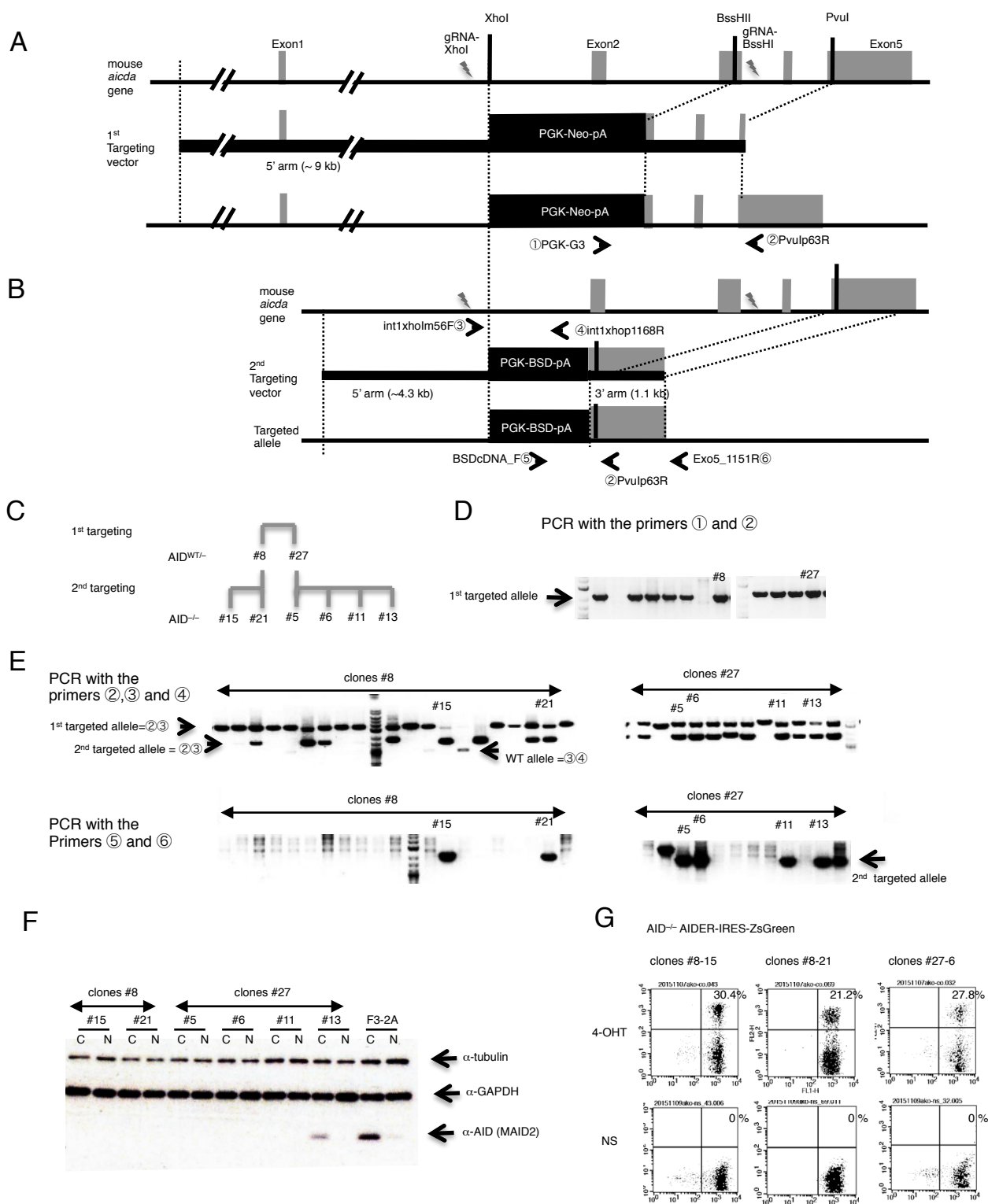


Fig.S5. Generation of AID-knockout CH12F3-2A cells by CRISPR/Cas9 nickase.

(A)(B) Targeting vectors used in 1st (A) and 2nd (B) targeting with double CRISPR/Cas9 nickases encoding two gRNAs based on the vector, pX335-U6-Chimeric_BB-CBh-hSpCas9n(D10A), Addgene. 1st targeting vector is exactly the same with the vector used for AID knockout mouse generation (1). 2nd targeting vector is constructed by PCR amplification of the fragments and assembled to the KpnI-SacI-digested pBlueScript SK(+) by GeneArt Seamless Cloning and Assembly Kit (ThermoFisher). gRNA sequences, primers for constructing the vectors and genotype screening are listed in Table S1. (C) Serial targeting procedure and the clone number information. (D) Genotyping PCR result after 1st targeting. Clones #8 and #27 are used for further targeting step. The targeted allele is shown by the arrow. (E) Genotyping PCR result after 2nd targeting. The targeted alleles and wild type (WT) allele are shown by the arrows. (F) Detection of endogenous AID protein after CIT stimulation with anti-mouse AID antibody 2 (mAID2, eBioscience) and conventional Western blotting method. Cell lysate was prepared by RIPA buffer and 4 mg lysate is loaded to the each lane. Anti-tubulin (Calbiochem) and anti-GAPDH antibodies (Millipore, MAB374) are used for loading control. C, CIT; N, not stimulated. (G) Clones #8-15, #8-21 and #27-6 are stably transfected by pEF1a-AIDER-IRES-ZsGreen1 as AID^{JP41Δ}/AIDER.

Table S1. List of primers

Primers	Sequence
<i>Igh/c-Myc</i> translocation	
Sμ5'1a (first)	ACTATGCTATGGACTACTGGGGTCAAG
c-Myc5'1a (first)	GTGAAAACCGACTGTGGCCCTGGAA
Sμ5'1b (second)	CCTCAGTCACCGTCTCCTCAGGTA
c-Myc5'1b (second)	GTGGAGGTGTATGGGGTGTAGAC
c-Myc probe	DIG-GGACTGCGCAGGGAGACCTACAGGGG
mAID cDNA sequencing	
F	CAAGAAAGTCACGCTGGAGACCGAT
R	CGTGTGACATTCCAGGAGGTTGCTT
CRISPR/Cas9 targeting in CH12F3-2A for JP41 deletion of <i>aicda</i> gene	
gRNA-F	CAGCCCTTGACGAAGTCGAC <u>CGGTG</u>
gRNA-R	TCGACTTCGTACAAGGGCTG <u>GTTTT</u>
JP41-HA-ssODN-RC (200bp)	TTCTATCCAGTCTCTTCAGAGAAATTTATCAGTGTGACATTCCAGGAGGTT GCTTTTCAAAATCTTAACATACGAAATGCAACTCAAGCGTAATCTGGAACATCGT ATGGGTACAAGTCATCGACTTCGTACAAGGGCTGTGGAGAAAGAGAGATGAT ATAAACGGAGTGAAGGGCTTTAAATTTGATCTCTGGAAAGG
Primers for genomic DNA sequencing and screening by NsiI digestion	
F	GCTTGCCTCTTGCCTCCTCTTCGCTCAGTC
R	GTAGATGGCGATGTTGCACAGCAAGCTCAG
CRISPR/Cas9 targeting in CH12F3-2A for <i>aicda</i> gene knockout	
gRNA-XhoI	GTTTGTGACACGGCTCAGC
gRNA-BssHI	TGCGGAGACTGCACCGCGC
2 nd Targeting vector construction	
aidEx1Xho5ar_FW	AGGGCGAATTGGGTACGTACACAACAGCACTGAAGCAGC
aidEx1Xho5ar_RV	GCGGCCGCTCGAGGGATCAAACCTCAAGACATC
PGK-BSDpolyA_FW	TCCCTCGAGCGGCCGCTATCGAATTCCGATCA
PGK-BSDpolyA_RV	TACAAGGGAAATGCTCTAGAACTAGTGAACCT
aidEx5-3arm_FW	GAGCATTTCCCTTGATCGAAGTCGATGACTTG
aidEx5-3arm_RV	AGGGAACAAAAGCTGGGTCTCTGCGCCCTTGACAGAGCTTC
Genotyping PCR primers for <i>aicda</i> gene knockout	
PGK-G3	GGGCCAGCTCATTCCCCACTC
Pvulp63R	CAGAGTGATTTCTTCGTAGTCC
int1xholm56F	CTCAGCAGGTAAAG GCATAAACTG CC
int1xhop1168R	CTCTGTGTACATGCATGCCTGCATGTCAAAGG
BSDcDNA_F	AGAAGAATCCACCCTCATTGAAAGAGC
Exo5_1151R	CCATTACTAGAAGTTTCTAAGTCACAGGC
LM-PCR	
LMPCR.1	GCGGTGACCCGGGAGATCTGAATTC
LMPCR.2	GAATTCAGATC
5'Sμ-F	GCAGAAAATTTAGATAAAATGGATACCTCAGTGG
5'Sμ-probe	DIG-AGGGACCCAGGCTAAGAAGGCAAT

All sequences are written from 5' to 3'. Underlined sequence in the gRNA are overhangs used to anneal with the GeneArt CRISPR Nuclease CD4 Reporter Vector (Invitrogen)

Table S2. List of antibodies

<u>Antibody</u>	<u>Company</u>	<u>Catalog no.</u>
Tubulin	Calbiochem	CP06
hnRNP K(D-6)	Santa Cruz	sc-28380
hnRNP L(D-5)	Santa Cruz	sc-48391
hnRNP U	Bethyl	A300-689A
SERBP1	MBL	RN056PW
HA Tag	abcam	ab18181
Flag	Sigma	F3165
β -actin	Sigma	A1978
ER	Santa Cruz	sc-787
Top1	Abcam	ab109374
AID	Cell signaling	4949S
AID (mAID2)	eBioscience	K10211
IgM-FITC	eBioscience	E00715-1631
IgA-PE	Southern Biotech	C2904-W100
Biotinylated IgG1	Becton Dickinson	553441
APC streptavidin	eBioscience	E07261-1632