

Apurinic/apyrimidinic endonuclease 1 (APE1) is dispensable for activation-induced cytidine deaminase (AID)-dependent somatic hypermutation in the immunoglobulin gene

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Abstract

Activation-induced cytidine deaminase (AID) initiates DNA breakage in the variable (V) and switch (S) regions of the immunoglobulin gene, which results in somatic hypermutation (SHM) and class switch recombination (CSR), respectively. Apurinic/apyrimidinic endonuclease 1 (APE1) has been shown to be important for CSR, and is supposed to cleave at abasic sites when AID-dependently deaminated cytidine is removed by uracil DNA glycosylase. However, APE1 is unexpectedly dispensable for SHM in the S region and translocation between immunoglobulin heavy chain (IgH) and c-myc genes in the mouse B lymphoma cell line, CH12F3-2A. This suggested that APE1 is not involved in AID-dependent DNA breakage, but rather, in DNA repair. In order to investigate detailed molecular mechanisms underlying APE1's involvement in CSR and SHM, we measured apurinic/apyrimidinic (AP) sites via aldehyde reactive probe labeling. Results indicated that the frequencies of AP sites in the S regions were not different between APE1^{-/-}CH12F3-2A and wild-type CH12F3-2A cells. To carry out similar experiments in SHM of the V region, we generated an APE1 knockout (APE1^{-/-}) human Burkitt's lymphoma cell line, and compared SHM between APE1-proficient and -deficient BL2 lymphoma cells. SHM frequencies in the V regions of APE1^{-/-}BL2 and APE1-proficient cells were also similar. Taken together, we showed that AID does not induce AP sites in the S region of the IgH gene, and that APE1 is not necessary for SHM in the V and S regions; however, it is required for DNA repair following DNA breakage in CSR.

Keywords: abasic site, aldehyde reactive probe, DNA deamination, RNA editing

Introduction

Three types of genetic alterations, somatic hypermutation (SHM), class switch recombination (CSR) and gene conversion (GC), can take place in the immunoglobulin heavy chain (IgH) gene when mature B lymphocytes are stimulated upon encountering pathogens at the periphery. While SHM, CSR and GC are distinct genetic alterations of the IgH locus, all events are initiated by activation-induced cytidine deaminase (AID)-dependent DNA cleavage at actively transcribed loci in the variable (V) or switch (S) regions (1–4). SHM takes place during error-prone repair of single-stranded breaks (SSBs) in the V region, while CSR requires joining of two separate double-stranded break (DSB) ends in the S regions. DSBs in the S regions are occasionally translocated to other loci, such as c-myc during the repair phase, which may induce tumorigenesis (5).

The N-terminal domain of AID is required for DNA cleavage; AID N-terminus mutants cannot induce either SHM or CSR (6, 7). On the other hand, the C-terminus of AID is specifically involved in DNA repair during CSR; mutations in the C-terminus of AID result in inefficient CSR and SHM, despite the introduction of equivalent or higher levels of DNA breaks when compared with wild-type AIDs (7–9). Although AID-mediated DNA breaks are initiated as staggered type SSBs in both V and S regions (10), two adjacent SSBs in the S region are simultaneously processed to generate a blunt-end DSB (11), which are repaired by the general non-homologous end-joining pathway. CSR requires multiple steps, including processing of SSBs into DSBs, appropriate pairing of two S-region break ends and synapsis

formation (12). Compared to CSR, SHM is simpler and consists of two steps: AID-dependent SSB generation, followed by error-prone DNA synthesis with translesion polymerases (TLPs) (13, 14). Studies have shown that Pol ζ -deficient mice exhibit a SHM frequency that is 5% that of wild-type mice, which clearly demonstrated that a major step in SHM following AID-dependent DNA breakage is mediated by translesion DNA synthesis via Pol ζ .

There is a long-standing debate over whether AID deaminates cytidine (C) to uridine (U) of RNA (RNA editing model) or of DNA (DNA deamination model) (Supplementary Figure 1). In the DNA deamination model, AID directly deaminates deoxy-cytidine to generate deoxy-uridine (dU)/deoxy-guanine (dG) mismatches, which are either recognized by base excision repair (BER) enzymes, uracil DNA glycosylase (UNG) and apurinic/apyrimidinic endonuclease 1 (APE1), or by the mismatch repair system. However, the DNA deamination model has several flaws that do not necessarily match experimental results. For example, the DNA deamination model cannot explain the specific functions of the N- and C-terminal domains of AID for DNA breakage and repair, respectively (11). Additionally, UNG deficiency greatly suppresses CSR, but has no effect on SHM (15). Surprisingly, catalytically inactive UNG mutants can rescue CSR in UNG knockout mouse B cells. Therefore, UNG appears to serve as a scaffold protein that recruits non-homologous end-joining proteins, which is critical to DNA repair (15). UNG deficiency does not reduce, but rather augments SHM; UNG mutants can suppress SHM, most likely because of their ability to recruit other repair proteins at the DNA break to serve as competitors with the TLP complex (15, 16).

On the other hand, the RNA editing model proposes that the N-terminus-dependent activity of AID edits miRNA precursors, which causes down-regulation of topoisomerase 1 (Top1) mRNA translation. Reduction of the Top1 protein facilitates the formation of non-B DNA structures in actively transcribed V and S regions of the immunoglobulin locus, which can generate irreversible DNA cleavage sites covalently bound to Top1 in the S and V regions (17, 18). AID-dependent DNA breakage is dependent on hnRNP K, whereas hnRNP L, SERBP1, and hnRNP U function as cofactors that are specific to DNA repair during CSR (19, 20). All of these RNA-binding proteins interact with AID in SHM and/or CSR. Although Top1 is ubiquitously expressed, and is also involved in maintenance of the helical DNA structure, Top1 preferentially cuts V and S regions of the immunoglobulin gene DNA, probably because of the particular chromatin environment such as modification by H3K4 trimethylation, accumulation of the FACT complex and the SWI/SNF complex including SMARCA4 (21, 22).

APE1 is a major endo/exonuclease that accounts for >95% of the total apurinic/apyrimidinic (AP)-endonuclease activity in yeast (23), it cleaves the phosphodiester bond at AP sites of damaged genomic DNA caused by oxidization, alkylation or UV. In general, AP sites are spontaneously produced not only in damaged cells, but also in normal mammalian cells at a rate of >12 000 per cell per day (24) by hydrolysis of N-glycosylic bonds. Deaminated cytosine and adenine are converted into uridine and hypoxanthine, respectively, by spontaneous hydrolysis (24, 25). This dU/dG or dT/hypoxanthine pair is processed into the AP site by excision via DNA

N-glycosylases such as UNG. AP site formation is accelerated at single-strand DNA (ssDNA) regions associated with replication or transcription, as cytosine deamination proceeds a hundred times faster at ssDNA as compared with that at double-stranded regions (26). Additionally, CSR-committed B cells generate high levels of mitochondrial reactive oxygen species (27), which can cause oxidative DNA damage that needs to be corrected by BER. These lines of evidence suggested that transcribed I μ /S μ and V regions of the IgH gene in activated B cells are susceptible to AP site formation by spontaneous hydrolysis or oxidation, even without deamination by AID. In the DNA deamination model, APE1 has been proposed to function downstream of UNG, as APE1 deficiency significantly reduces CSR.

However, we demonstrated that APE1 knockout CH12 F3-2A B lymphoma cells have normal DSB levels in the S region to induce IgH/cMyc translocation in an AID-dependent manner (28). These results suggested that APE1 is required not for DSB, but for DNA repair following cleavage.

To directly examine whether AP sites are increased in the immunoglobulin locus following AID activation, we employed aldehyde reactive probe (ARP), [N-(aminooxyacetyl)-N'-biotinylhydrazine], an aminooxyl base fused with a biotin tag that has been shown to be useful for detecting AP sites produced by DNA damage in genomic DNA (29, 30). We applied this probe to detect locus-specific AP site formation in the genome, and evaluated AP sites in the immunoglobulin S region during CSR. Wild-type APE1 overexpression in its knockout cells decreased methyl methane sulfate (MMS)-induced AP sites as compared with that in parental APE1 knockout cells, showing that AP site frequency is quantitatively detected. However, AID activation does not increase AP site frequency in the S μ and S α regions of the IgH locus. Furthermore, we examined whether APE1 is required for SHM in the V region via generation of APE1^{-/-} human Burkitt's lymphoma (BL2) cells. We found that the mutation frequency is not changed by APE1 deficiency in either the V or the S regions. These results clearly demonstrated that APE1 does not contribute to DNA cleavage through processing of AP sites, but rather functions at the repair step following DNA breakage during CSR, as we have previously proposed (28).

Methods

Cell culture and reagents

BL2-AID^{-/-} cells expressing AID-ER (estrogen receptor ligand-binding domain) were established previously (31, 32). Cells were maintained at 5% CO₂ and 37°C in RPMI 1640-Glutamax (Thermo Fisher Scientific) medium supplemented with 10% fetal calf serum (Nichirei Biosciences), 100 U ml⁻¹ penicillin, 100 U ml⁻¹ streptomycin (Nacalai), 1× MEM Non-Essential Amino Acids Solution (Thermo Fisher Scientific), 100 mM sodium pyruvate solution (100×) (Thermo Fisher Scientific) and 0.004% (v/v) 2-mercaptoethanol (Nacalai), and were stimulated with 2-4-hydroxytamoxifen (1 μ M, 4-OHT, H7904, Sigma). APE1 knockout (APE1^{-/-}) CH12F3-2A cells were obtained from Kefei Yu (Michigan State University, East Lansing, MI, USA) (33); transfectants with empty and mouse APE1 expression vectors [APE1^{-/-}-CH12-Empty (APE1 deficient) or APE1^{-/-}-CH12-mAPE1 (APE1 proficient)] were

previously established (28). APE1^{-/-}-CH12F3-2A cells were maintained in RPMI supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine (made in our laboratory), 5% (v/v) NCTC-109 (Thermo Fisher Scientific), 20 mM HEPES (made in our laboratory), 0.004% (v/v) 2-mercaptoethanol (Nacalai), 100 U ml⁻¹ penicillin (Nacalai), 100 U ml⁻¹ streptomycin (Nacalai), and were stimulated with a combination of CD40L (made in our laboratory), IL-4 (2.5 µg ml⁻¹, FUJIFILM Wako Pure Chemical) and TGF-β (1 µg ml⁻¹, R&D Systems) (CIT) for AID induction.

In vivo ARP labeling

ARP [*N*-(aminooxyacetyl)-*N'*-biotinylhydrazine] and the DNA Damage Quantification Kit were purchased from Dojindo Laboratories (Kumamoto, Japan) (29). The actual AP site frequency generated by variable concentrations of MMS was measured in APE1-deficient CH12 cells (28) under instructions from the manufacturer. APE1-deficient and -proficient cells were stimulated with CIT for 24 h; non-stimulated cells and MMS-stimulated cells were also cultured in parallel. Cell viability was detected by trypan blue exclusion. Cells (5–10 × 10⁶) were centrifuged and washed twice with 1 ml phosphate-buffered saline (PBS). As a positive control, cells were incubated with 2–20 mM MMS at 37°C for 5 min in 1 ml PBS/5 mM glucose, and were washed twice with 1 ml PBS prior to ARP labeling. Test samples and positive control cells in 1 ml PBS/5 mM glucose were incubated with 3 mM ARP for 60 min at 37°C. Cells were then centrifuged and washed twice with 1 ml PBS. DNA was lysed in buffer [10 mM Tris, pH 8.0, 100 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS)] containing 0.4 µg ml⁻¹ Proteinase K (Wako), and was incubated at 55°C overnight prior to phenol/chloroform extraction. Purified DNA was sonicated by a Covaris S2 Focused-ultrasonicator (Covaris) into 500–700 bp fragments. The sensitivity of this labeling assay in APE1-deficient cells was tested by the DNA Damage Quantification Kit, according to the manufacturer's instruction. APE1^{-/-}BL2 cells are labeled by the same method which is applied to the CH12 cell series.

Dot blot analysis of ARP-labeled DNA

Following DNA purification, 100 ng–2 µg DNA was spotted onto 10 cm × 10 cm Hybond-N+ (GE Healthcare) membranes. Air-dried membranes were cross-linked under 254 nm at 120 mJ cm⁻² with the Stratalinker UV crosslinker 2400 (Stratagene). Non-specific binding was blocked using 4% skimmed milk (30403-55, Nacalai) with 1% bovine serum albumin (BSA; Nacalai); membranes were then incubated with stabilized streptavidin–HRP conjugate (in Chemiluminescent Nucleic Acid Detection Module Kit, 89880D, Thermo Fisher Scientific) at 4°C overnight, and were subsequently treated with the same conjugate for 30 min at room temperature for secondary incubation. The reacting dots were detected by Chemi-lumi One Super and ImageQuant LAS-4000.

Locus-specific AP site labeling assay

Dynabeads MyOne Streptavidin C1 (10 µl for each sample, ThermoScientific) was pre-blocked with 200 µg ml⁻¹

glycogen, 1 mg ml⁻¹ BSA and 500 µg ml⁻¹ yeast tRNA (Thermo Fisher Scientific) in the trapping buffer (5 mM Tris-Cl, pH 8.0, 0.5 mM EDTA, 0.015% Tween20) with 1 M NaCl at 4°C overnight. ARP-labeled DNA (20 µg) was incubated with pre-blocked beads in the trapping buffer with 1 M NaCl for 30 min at room temperature. Beads were washed three times with high stringency RIPA buffer (50 mM Tris-Cl, pH 8.0, 1 M NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate and 0.1% SDS) and three times with lithium chloride buffer (20 mM Tris-Cl, pH 8.0, 250 mM lithium chloride, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate), followed by a single wash with 10 mM Tris-Cl at pH 8.0. After changing the tube, DNA was eluted from beads twice in 100 µl of 98% formamide with 10 mM EDTA beads at 67°C for 15 min. Combined eluates (200 µl) were diluted with 500 µl ultra pure water, and were precipitated with equal volumes of 2-propanol and 200 mM NaCl with glycogen. Purified DNA was quantitated by real-time PCR analysis using PowerUp SYBR (Thermo Fisher Scientific) and the 7900HT Fast Real-Time PCR System (Applied Biosystems) with S_μ, S_α, S_γ1, β-actin (βAct) and β2 microglobulin (β2M) primer sets. All primers used for quantitative PCR (qPCR) are listed in [Supplementary Table 3](#).

Establishment of APE1^{-/-}BL2 cells

We designed two gene-targeting vectors with GFP or blasticidin (BSD)-resistant gene cassettes ([Fig. 3A](#)), and attempted to disrupt the APE1 gene in the human BL2 AID^{-/-}AIDER cell line by the CRISPR/Cas9 technique with the double nicking method (34). PCR fragments used to target the vector were assembled with the Genearr Seamless Cloning and Assembly Kit (ThermoScientific). Oligos encoding guide RNAs were cloned into pX335-U6-Chimeric_BB-CBH-hSpCas9n (D10A) (Addgene). Gene targeting procedures have been previously described (34); oligos and primers used for identifying targeted clones are listed in [Supplementary Table 3](#). Isolated clones were validated by PCR, western blot and genomic quantitative PCR.

Stable transfectants of APE1^{-/-}BL2 cells

JP8Bdel-ER (a C-terminal mutant of AID) (7) was cloned into pEF1α-IRES-DsRed-Express2 (Clontech). Wild-type (mAPE1) and catalytic mutant (mY170F) (35) mouse APE1 with Myc and FLAG in pEF1α-IRES ZsGreen have previously been constructed (28). Constructs were linearized with Sfil (NEB), and were introduced into APE1^{-/-}BL2 cells by electroporation (Lonza). Cells were sorted by FACS Aria II (BD Biosciences) on the basis of fluorescence intensity. The established stable transfectant cell lines were BL2-AID^{-/-}APE1^{-/-}JP8BdelER-Empty (BL2-J-Empty), BL2-AID^{-/-}APE1^{-/-}JP8BdelER-mAPE1 (BL2-J-mAPE1) and BL2-AID^{-/-}APE1^{-/-}JP8BdelER-mY170F (BL2-J-mY170F).

Antibodies

The following antibodies were used for western blot in the present study: anti-APE1 (EPR4023) (Ab133300, Abcam), anti-ER (HC-20) (sc-543, Santa Cruz), anti-GAPDH (MAB374, Millipore) and anti-α-Tubulin Mouse mAb (DM1A)

(CP06, CALBIOCHEM). Anti-mouse IgA antibody labeled with phycoerythrin (Goat Anti-Mouse IgA-PE, #1040-09, SouthernBiotech) was used to detect surface IgA expression of CH12 cells.

Western blot

Cells were lysed with RIPA buffer (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% sodium deoxycholate, 1% TritonX-100 and 1 mM EDTA), and were subjected to western blot following standard protocols. The membranes were incubated with anti-APE1, anti-ER and anti-GAPDH at 4°C overnight for primary incubation. Subsequently, membranes were labeled with rabbit TrueBlot anti-rabbit IgG HRP (18-8816-33) (Rockland), or mouse TrueBlot ULTRA anti-mouse immunoglobulin HRP (18-8817-33) (Rockland) for 30 min at room temperature for secondary incubation. Protein bands were detected by Chemi-lumi One Super (Nacalai) and ImageQuant LAS-4000 (GE Healthcare).

SHM analysis of V and S μ regions

To analyze SHM of the immunoglobulin gene in BL2 cells, genomic DNA was prepared from cells stimulated with or without 4-OHT, and were incubated for 24 h. V α 39-J α 5 gene (31) and 5' regions of core S μ , I μ /S μ regions (36) were amplified from genomic DNA with PrimeSTAR GXL DNA polymerase (TaKaRa). The primer sets were V α 4-n 5'-GAGGCTGCCTCTGATCCCAG-3' and J α 5-n 5'-CCTGGCAAGCTGAGTCTCCC-3' for the V region, and S μ F 5'-GACATGGTAAGAGACAGGCAGCCG-3' and S μ R5'-GAAAGCTGGATGGAGTTGTCATGGCC-3' for the S μ region. The PCR cycling parameters were as follows: 95°C for 3 min, followed by 35 cycles of 98°C for 10 s for denaturation, 60°C for 30 s for annealing, and 68°C for 1 min for extension. PCR products were excised from 1% agarose gels, and were purified with the Wizard SV Gel and PCR Clean-Up system (Promega). Purified products were cloned into pGEM-T Easy Vector (Promega), and were sequenced with T7 primer using an ABI 3130x1 Genetic Analyzer (Applied Biosystems). The 310–416 and 710–830 bp sequences for the V and S μ regions, respectively, were analyzed with Sequencher software v4.8 (GeneCodes).

Results

Quantitative measurement of locus-specific AP sites

To investigate the molecular mechanism of APE1's involvement in immunoglobulin gene diversity, AP site formation at AID-induced breakage sites was evaluated by ARP labeling in the presence or absence of APE1 in CH12 F3-2A cells (Fig. 1A) (28). The frequency of AP site induction by the alkylating chemical MMS was compared between APE1^{-/-}-CH12-Empty (APE1-deficient) and APE1^{-/-}-CH12-mAPE1 (APE1-proficient) cells (Fig. 1A–C). Dot blot analysis revealed that the ARP signal is correlated with the amount of DNA and MMS concentration in both cell lines. In particular, it is quite reasonable that APE1-deficient cells showed higher ARP signal as compared with that of APE1-proficient cells, as APE1 endonuclease activity can eliminate AP sites induced by MMS (Fig. 1B).

The densitometry values of ARP signals shown in dot blot analysis were plotted with various amounts of DNA at different MMS concentrations. The ARP signal linearly is increased by DNA amount and by MMS within the range of 2–10 mM (Fig. 1C). DNA (2 μ g) of APE1-deficient cells exposed to 10 mM MMS yielded a densitometry value of 1.5, while DNA (4 μ g) of APE1-proficient cells exposed to 10 mM MMS resulted in a densitometry value of 0.9 (Fig. 1C). This suggested that APE1-deficient cells have approximately three times more AP sites in the genome as compared with APE1-proficient cells.

In order to confirm the sensitivity of this labeling assay, the absolute AP site frequency was evaluated in the positive control; APE-deficient CH12 cells were exposed to 0–20 mM MMS (Fig. 1D). The standard DNA solutions, which were provided in the DNA Damage Quantification Kit, were prepared by heat/acid depurination of calf thymus DNA, and containing 0, 2.5, 5, 10, 20 or 40 AP sites per 10⁵ bp, according to the manufacturer. These standard DNA solutions showed linear correlation between AP site frequency and ARP signal, as expected (Fig. 1D, left). Next, ARP signals in APE1-deficient CH12 cells exposed to 0–20 mM MMS were evaluated, and ARP signal intensity by MMS was revealed (Fig. 1D, middle). By combining with these two results, the association between MMS concentration and AP site frequency was determined (Fig. 1D, right). As shown by the regression line, exposure to 5 mM MMS produced approximately 5 AP sites per 10⁵ bp in APE1-deficient cells. While it is possible that each gene locus exhibits some fluctuations in MMS-mediated AP site frequency, we postulated that 5 mM MMS is sufficient to induce an equivalent or lower level of AP site frequency with SHM in the immunoglobulin S region of non-stimulated CH12 cells.

AP sites in DNA samples exposed to increasing concentrations of MMS were evaluated by trapping biotin-containing ARP using streptavidin beads. AP sites in specific genes were quantified by qPCR (Fig. 1A and E). In accordance with dot blots results, locus-specific AP site frequencies were higher in APE1-deficient cells as compared with those in APE1-proficient cells in all loci examined (S μ , S α , S γ 1, β Act). Locus-specific ARP signals paralleled with MMS concentrations, and the ARP signals in APE1-proficient cells were 5–10 times less as compared with those in APE1-deficient cells (Fig. 1E, upper bar graph). Since both APE1-deficient and -proficient cells showed correlation between ARP signals and DNA content with 2–20 mM MMS treatment, 5 mM MMS treatment was used as the positive control in the following experiments (Fig. 1E, two lower scatter plots).

AP sites in S μ and S α regions did not increase with AID activation

To examine whether the AP site frequency increases with AID expression, APE1-deficient and -proficient CH12 cells were stimulated by CIT, labeled with ARP, and were trapped by streptavidin beads (Fig. 2A). Approximately 10% of APE1-proficient cells switched to IgA following stimulation with CIT, whereas only ~2% of APE1-deficient cells switch to IgA (Fig. 2B), as shown previously (28, 33). Dot blot analysis of AP sites did not detect any signal with CIT stimulation, whereas MMS-treated cells revealed strong ARP signals that were higher than those observed in APE1-deficient CH12 cells (Fig. 2C).

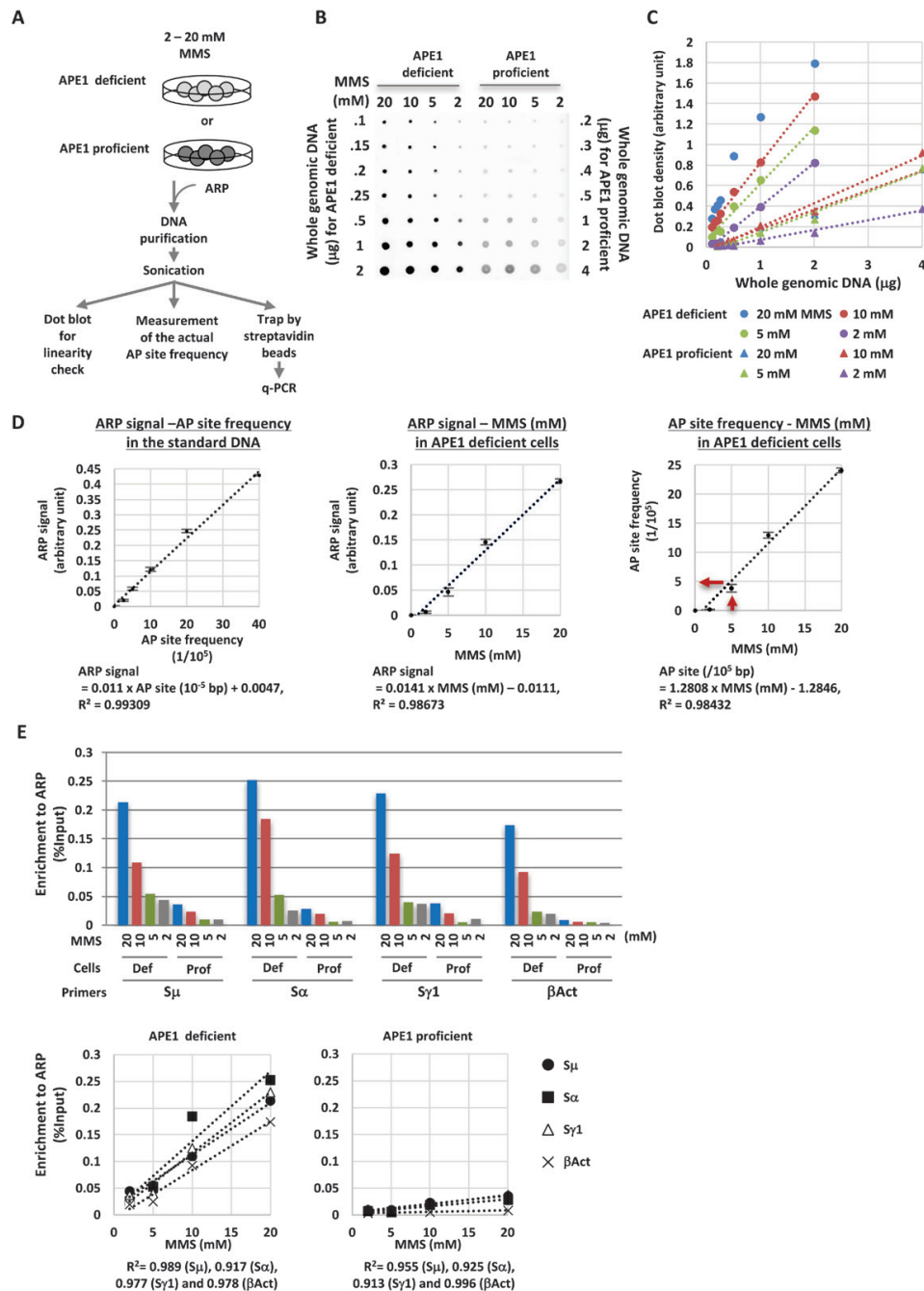


Fig. 1. Detection of general and locus-specific AP site frequency induced by MMS in APE1^{-/-}CH12 cells. (A) Experimental steps in *in vivo* AP site labeling with ARP. (B) Dot blot analysis of APE1-deficient (left) and APE1-proficient (right) cells treated with various concentrations of MMS (mM). The representative of four independent experiments is shown. (C) Association between dot blot density of ARP signal and total DNA amount under various MMS concentrations. Dot blot density of ARP signal from Fig. 1(B) was analyzed by the ImageJ software. R^2 in APE1-deficient cells are 0.997 (10 mM MMS), 0.99 (5 mM MMS) and 0.997 (2 mM MMS) and that in APE1-proficient cells are 0.992 (20 mM MMS), 0.998 (10 mM MMS), 0.984 (5 mM MMS) and 0.985 (2 mM MMS). (D) ARP signal in standard DNA with known AP site frequency, as measured by the DNA Damage Quantification Kit (left). ARP signal of DNA derived from MMS-exposed APE1-deficient cells (middle). AP site frequency generated by MMS (mM) in APE1-deficient cells (right). (E) Locus-specific ARP signal generated by S μ , S α , S γ 1 and β Act primer sets, using genomic DNA exposed to indicated concentrations of MMS. Enrichment to ARP in APE1-deficient (Def) and APE1-proficient (Prof) cell lines (upper). Enrichment signal to ARP (% input) is plotted against MMS (mM) concentration (lower). Regression lines of APE1-deficient cells are $y = 0.0098x + 0.0146$, $R^2 = 0.98921$ (S μ), $y = 0.0131x + 0.0076$, $R^2 = 0.91684$ (S α), $y = 0.0113x + 0.0027$, $R^2 = 0.97707$ (S γ 1), and $y = 0.0091x - 0.0069$, $R^2 = 0.97801$ (β Act) (lower left). Regression lines of APE1-proficient cells are $y = 0.0015x + 0.0054$, $R^2 = 0.95479$ (S μ), $y = 0.0013x + 0.0033$, $R^2 = 0.92548$ (S α), $y = 0.0018x + 0.0024$, $R^2 = 0.91258$ (S γ 1), and $y = 0.0003x + 0.0031$, $R^2 = 0.99617$ (β Act) (lower right).

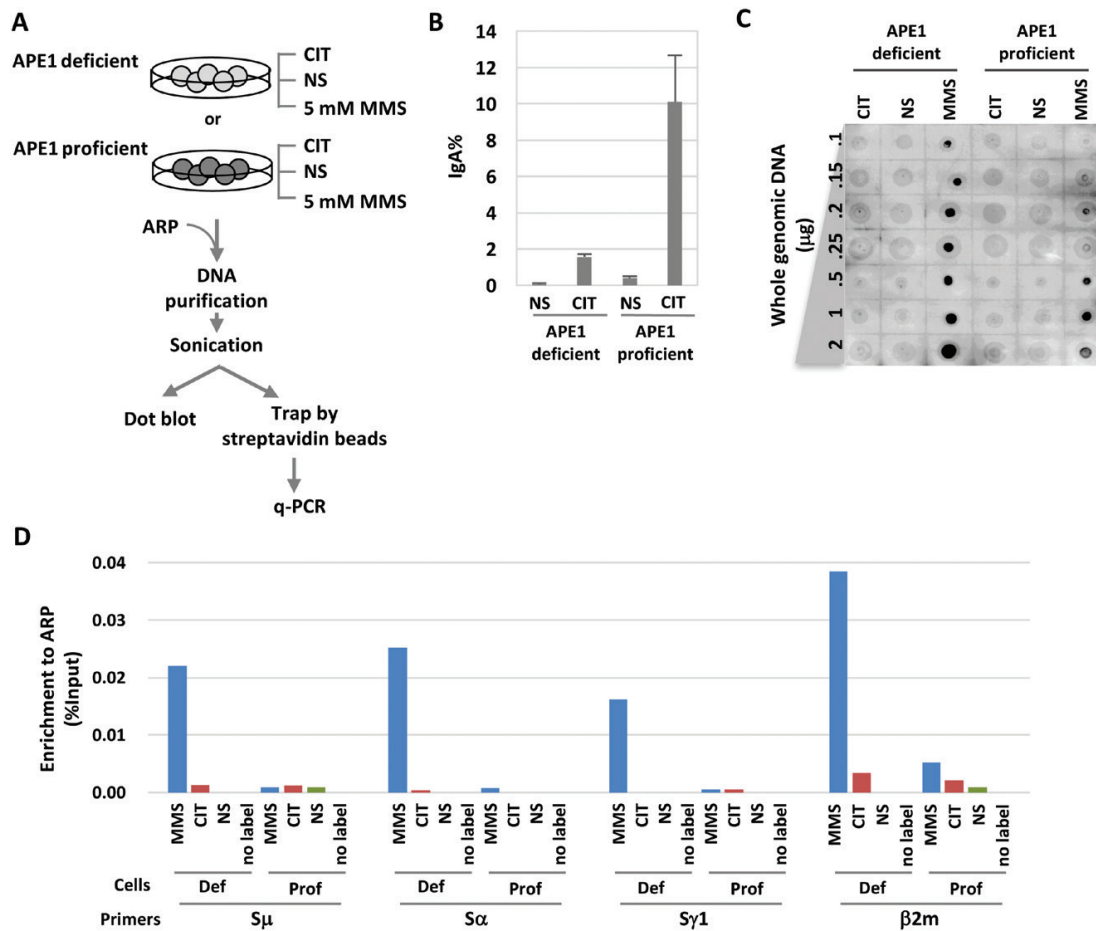


Fig. 2. AP site frequency in the S regions of APE1^{-/-}CH12 cells after AID activation. (A) Schematic of *in vivo* AP site detection with or without AID activation. NS, non-stimulated cells. (B) Cell surface IgA expression (%) following 24-h CIT stimulation. The mean $\pm$ SD from three independent experiments is shown. (C) Dot blot analysis of genomic DNA derived from ARP-labeled APE1-deficient and APE1-proficient cells with or without CIT stimulation. Positive control: 5 mM MMS-treated cells. Representative result of three independent experiments is shown. (D) Locus-specific AP sites were detected by qPCR assay. No label: cells were neither stimulated nor labeled by ARP. Representative result of three independent experiments is shown. Def, APE1-deficient cells, APE1^{-/-}CH12-Empty cells; Prof, APE1-proficient cells, APE1^{-/-}CH12-mAPE1 cells.

Trapping of ARP-labeled DNA from MMS-treated APE1-deficient cells by streptavidin beads and subsequent qPCR analysis with locus-specific primers revealed strong signals in the S μ , S α , S γ 1 and β 2M loci (Fig. 2D, blue bars); these signals were more than 7-fold that of MMS-treated APE1-proficient cells (Fig. 1E). CIT stimulation (dark red bars) induced far less AP sites as compared with that of 5 mM MMS exposure (blue bars) in both S μ and S α regions, where DSB takes place under this condition. As shown in Fig. 1(D), 5 mM MMS yielded 5×10^{-5} AP sites per base pair, while CIT-stimulated DNA resulted in SHM at $\sim 2 \times 10^{-4}$ per base pair in the S μ region; this should introduce similar levels of AP sites according to the DNA deamination model. The S γ 1 and β 2M primer sets were used as the negative control; AP site frequencies of these control regions were similar to those in the S μ and S α regions.

Generation of APE1-deficient BL2 cells for SHM evaluation

To elucidate the function of APE1 in SHM induction in the V region, we generated APE1^{-/-} cells from BL2-AID^{-/-} cells

with two targeting vectors (Fig. 3A). GFP-positive cells were isolated by fluorescence-activated cell sorting (FACS) following first target vector transfections by the GFP-containing vectors. The 13 heterozygous knockout clones were transfected by the second targeting vector and exposed to the BSD for a week. BSD-resistant single clones were picked up and genomic changes were screened by the conventional genomic PCR using the five sets of primers indicated in Fig. 3(B). The clones selected by PCR were all negative for the APE1 protein (Fig. 3C). To exclude aberrant integration of targeting vector fragments by the CRISPR/Cas9 technique, excessiveness of BSD and GFP genes in genomic DNA were assessed by qPCR. Among the 11 APE1^{-/-}BL2 clones (Int3-1, 3, 9, 10, 13, 16, 19, Ext2-2, 11, 12, 14) generated, Int3-3, Int3-19 and Ext2-11 clones were used for further analyses, as they retained the least copy number of BSD and GFP genes (Fig. 3D). Int3-1 and Int3-13 also yielded low copy numbers of BSD and GFP, but were not used due to slow cell proliferation.

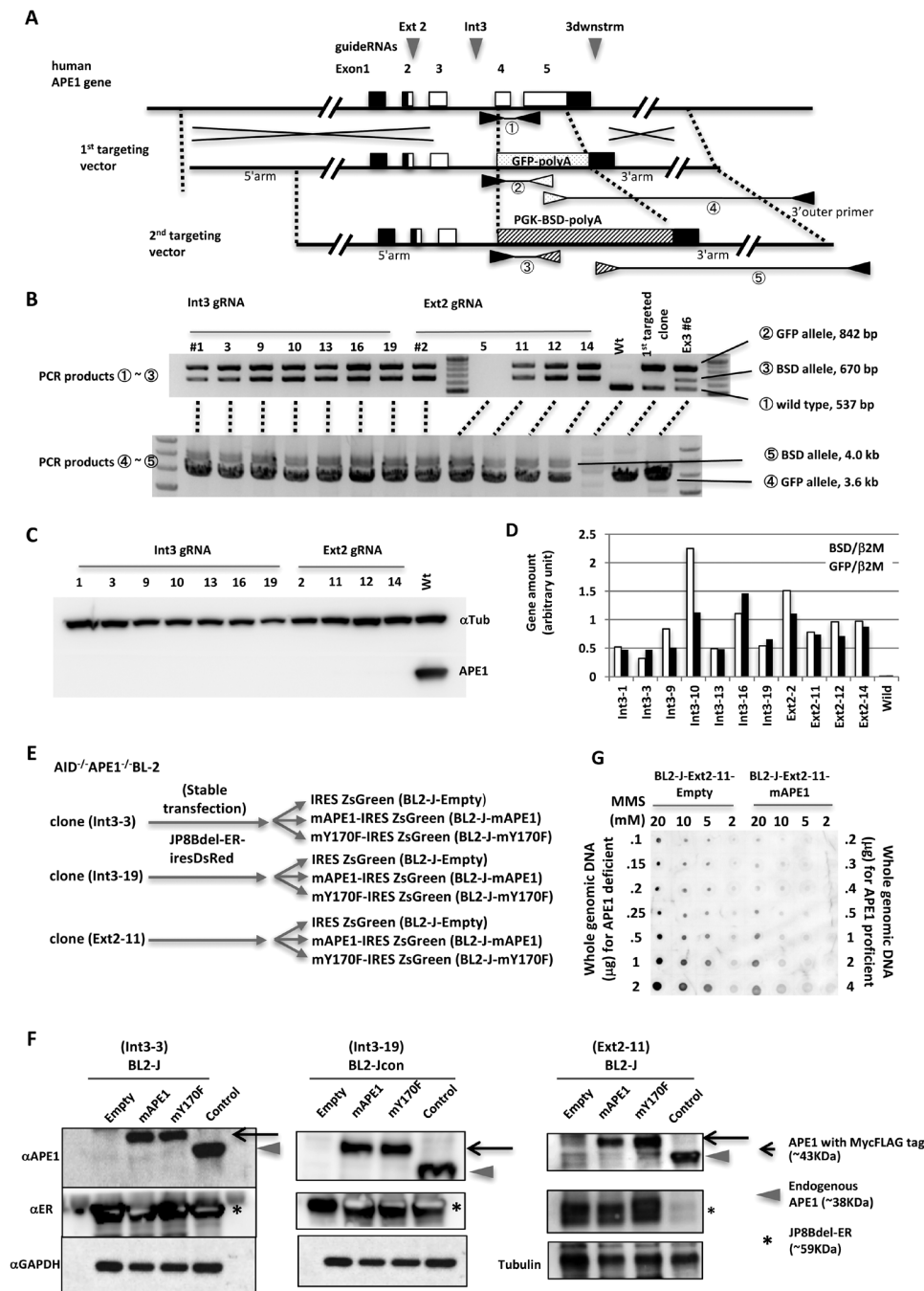


Fig. 3. Gene targeting of APE1 in BL2 cells and introduction of APE1 and the catalytic mutant Y170F. (A) Genomic organization of the wild-type and targeting vectors of the human APE1 gene. Coding regions are indicated by open boxes and were numbered; 5' and 3' UTRs are shown as closed boxes. Guide RNAs are indicated by three gray triangles. The first targeting vector replaces exons 4 and 5 with the GFP-polyA cassette. The second targeting vector replaces exons 4 and 5 with the PGK-BSD-polyA cassette. (B) Genotyping of clones for detection of recombination by first and second targeting vectors. (C) Western blot of the APE1 protein in the clones using anti-APE1 antibodies. Anti-tubulin antibody is used as the loading control. (D) qPCR of genomic DNA for assessing BSD and GFP gene copies. BSD and GFP signals were normalized to $\beta 2M$. (E) Schematic representation of transfection. C-terminus truncated mutant of AID (JP8Bdel) fused with ligand-binding domain of the ER was transfected and sorted by red fluorescence. To the established red cell clusters, wild-type mouse APE1 (mAPE1) or its catalytic mutant (mY170F) was transfected and sorted by green fluorescence to get the stable transfected cells. (F) Protein expression of transfected APE1 and JP8Bdel-ER, as evaluated by western blot in Int3-3 (left), Int3-19 (middle) and Ext2-11 (right) clones. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), GAPDH and anti- α -tubulin antibodies are used as the loading control. (G) Functionality of transfected mAPE1 as evaluated by dot blot analysis of BL2-J-Ext2-11-Empty (APE1-deficient) (left) and BL2-J-Ext2-11-mAPE1 (APE1-proficient) (right) cells treated with various concentrations of MMS (mM).

Since JP8Bdel, a C-terminus truncated form of AID that exhibits four times higher SHM activity as compared with that of wild-type AID (7), JP8Bdel-ER was fused to IRES-DsRed-Express2, and was introduced into Int3-3, Int3-19 and Ext2-11 (Fig. 3E). Subsequently, mAPE1-IRES ZsGreen, a catalytic mutant of mouse APE1 (mY170F)-IRES ZsGreen, or IRES ZsGreen, was introduced to compare their effects on SHM. These cells showed inherent weak green fluorescence due to GFP insertion, and therefore only cells that exhibited both red and strong green fluorescence intensity were isolated by FACS. The nine established cell lines were named as shown in Fig. 3E. Western blot analysis showed that Int3-3, 3-19 and Ext2-11 expressed mAPE1 and mY170F at levels comparable to those of endogenous APE1 of wild-type BL2 cells harboring the JP8Bdel-ER transgene, which was used as controls (Fig. 3F, top panels) (37). The amount of exogenous mAPE1 or mY170F protein in the clones derived from Int3-3 was seemingly lower in the top panel of Fig. 3(F) than the control; however, the loading control GAPDH protein intensity is also lower in mAPE1 and mY170F and correlates with APE1 proteins (Fig. 3F, bottom panel). The amount of mAPE1 and mY170F protein in Ext2-11-derived cells is also relative to the loading control α -tubulin amount. The functionality of the transfected mAPE1 in the Ext2-11 cells was further evaluated by AP site formation under the exposure to MMS with ARP labeling (Fig. 3G). Comparing to Ext2-11-Empty, Ext2-11-mAPE1 obviously has less AP sites, showing that Ext2-11-mAPE1 has more AP site removal activity than their counterpart, 'Empty'. JP8Bdel-ER was almost equally expressed in both Int3-3 and Int3-19 cell lines including the control cells (Fig. 3F, left and middle panels). In all the three derivative cells from Ext2-11, the expression level of JP8Bdel-ER is higher than in the control cells (Fig. 3F, right panel).

APE1 is dispensable for AID-induced mutations in the V and S regions

To evaluate the SHM frequency in the V region, we sequenced the V4-39-J_H5 region from three cell lines (Int3-3, Int3-19 and Ext2-11) carrying Empty, mAPE1 and mY170F vectors. Cells were stimulated with 4-OHT for 24 h, and were further incubated for an additional 48 h (Fig. 4A). In all cell lines, 4-OHT stimulation induced significantly higher SHM (about $5\text{--}15 \times 10^{-4}$) as compared with that in non-stimulated cells (about $0.4\text{--}4.5 \times 10^{-4}$) (Fig. 4B, Table 1). The SHM frequency of the stimulated BL2-J-mAPE1 was comparable with those of BL2-J-Empty and BL2-J mY170F cells (Fig. 4B, Table 1). Some fluctuation of SHM frequency in the stimulated cells was observed; however, there is no statistical significance between them. Furthermore, this fluctuation was not reproducible among these three independent clones (Int3-3, Int3-19 and Ext2-11).

We also analyzed SHM in the I μ /S μ region using BL2 APE1-deficient and -proficient cells (Fig. 4C, Table 2) (28). JP8Bdel-ER activation by 4-OHT resulted in a significantly greater mutation rate ($\sim 4\text{--}15.8 \times 10^{-4}$ versus $0.5\text{--}5.1 \times 10^{-4}$ in non-stimulated cells), with the exception of two cell lines (Fig. 4C, Int3-3 BL2-mAPE1 and Int3-19 BL2-J-Empty). Similar findings to SHM frequency in V4-39-J_H5 region were obtained in all cell lines (Int3-3, Int3-19 and Ext2-11) that expressed their respective APE1 transgenes. Increased or similar levels

of SHM in the S region by mY170F expression in BL2-J cells confirmed that the catalytic activity of APE1 is not required for SHM in the the S region, as shown in the APE1^{-/-}-CH12 F3-2A cell series (28). The mutation base pattern in each cell line showed similar transition/transversion frequencies in both V4-39-J_H5 and I μ /S μ regions (Supplementary Tables 1 and 2). Together, we showed that AID-induced SHM in the IgH gene is not dependent on APE1 in the V4-39-J_H5 and I μ /S μ regions.

Discussion

Reliability of locus-specific AP site detection by ARP

We have applied the ARP modification assay to quantitate AP sites that are possibly generated in the immunoglobulin gene before and after AID activation. First, we considered whether *in vivo* labeling of AP sites by ARP is possible. Studies have shown that ARP (3 mM) in the culture medium can permeate into human lung fibroblasts, and that the intracellular ARP concentration reaches a plateau within 15 min (38). Next, we examined whether the sensitivity of the ARP labeling assay was sufficient for detection of putative AID-induced DNA cleavage sites. APE1-deficient cells showed a higher AP site frequency as compared with that of APE1-proficient cells, as shown in Figs 1–3. Fung and Demple (39) have previously reported that knockdown of APE1 in HCT116 cells and reversion of APE1 in APE1-deficient TK6 cells increased and decreased AP site signals, respectively. Similarly, the APE1 inhibitor increased AP site signal levels (40). Since AP sites are processed by APE1, the AP site frequency was higher in APE1-deficient cells as compared with that in APE1-proficient cells. In addition, we should be able to detect immunoglobulin locus-specific AP sites at a frequency of 10^{-4} per base pair, which matches the level of SHM frequency in the I μ /S μ region if AP sites represent the majority of AID-induced DNA cleavage sites. A previous report (30) demonstrated that ARP labeling of purified DNA *in vitro* resulted in $1.7 \times 10^{-5}\text{--}1.0 \times 10^{-3}$ AP sites with 2–24 mM MMS, which were linearly correlated. We examined ARP sensitivity by determining the correlation between MMS concentration and AP site frequency in APE1-deficient CH12 cells. Results showed that 5 mM MMS produced detectable levels of AP sites that can be used for evaluation and comparison with the AID-induced SHM frequency (Fig. 1D). Furthermore, we examined whether 2–20 mM MMS produced linear locus-specific AP signals; we found positive association between MMS concentration and AP site frequency in every primer set (Fig. 1E). From these results, we concluded that the current ARP labeling and locus-specific AP site detection method is sufficiently sensitive to evaluate the AP site frequency *in vivo*. Since AP sites are immediately processed by APE1, the AP site frequency was labeled more correctly in APE1-deficient cells as compared with APE1-proficient cells. Lastly, we questioned whether amplification of ARP-labeled DNA templates by PCR is possible, since ARP produces a 331.39 Da adduct, which may structurally interfere with DNA polymerase. Pastor *et al.* (41) reported that 5-hydroxymethylcytosine (5hMC)-containing genomic DNA can be labeled by ARP *in vitro*, trapped by streptavidin beads and sequenced by next-generation sequencing

A

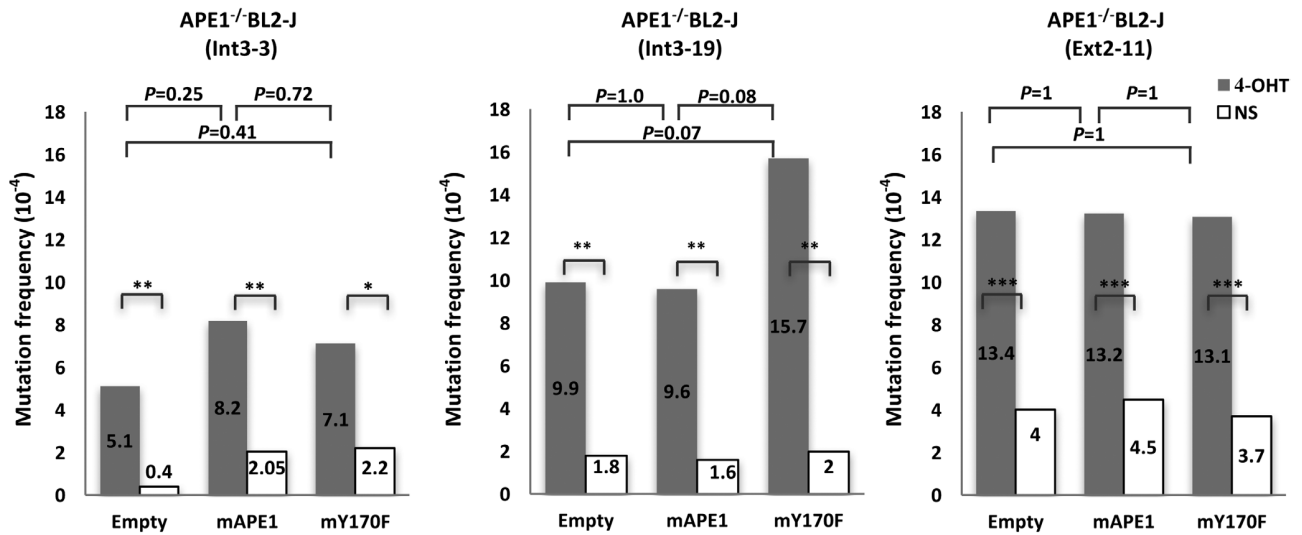
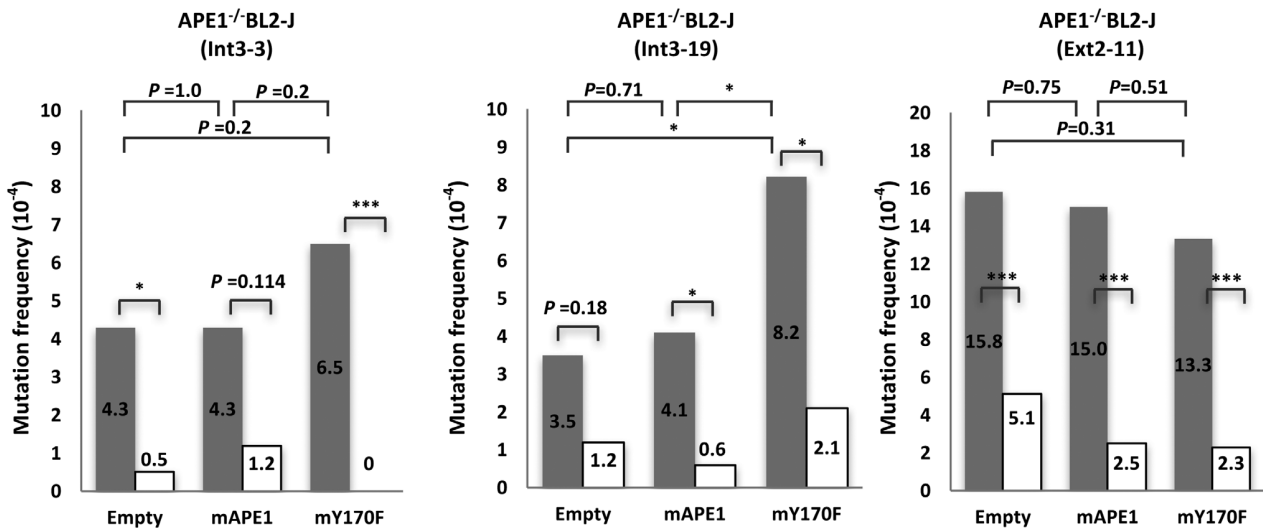
B V4-39-J_H5 regionC I_μ/S_μ region

Fig. 4. SHM analysis of the variable (V) and I_μ/S_μ regions in Empty, mAPE1- or mY170F-introduced AID^{-/-}APE1^{-/-}JP8BdelER BL2 (BL2-J) cells. (A) Time course of the SHM assay procedure. (B, C) Results of SHM analysis in the V4-39-J_H5 (B) or I_μ/S_μ-regions (C) of the Int3-3, Int3-19 and Ext2-11 clones. P values (Fisher's exact test) are shown in graphs. *P value < 0.05, **P value < 0.01, ***P value < 0.001.

to identify 5hMC localization in the whole genome. This suggested that *in vivo* locus-specific AP site detection is possible in the samples which is biotinylated with ARP and trapped by streptavidin.

AID-induced AP site detection in the IgH gene

According to the DNA deamination model (Supplementary Figure 1B), AP sites in the IgH gene are generated by AID and UNG. It was proposed that since SHM is primarily dependent

Table 1. SHM frequency in the V4-39-J_H5 region in BL2-Empty, mAPE1 and mY170F cells

		OHT+				OHT-			
Cell lines		Mutated clone/ total clone	In/del (bp/ clone)	Mutation/ total base	Mutation frequency ($\times 10^{-4}$)	Mutated clone/ total clone	In/del (bp/ clone)	Mutation/ total base	Mutation frequency ($\times 10^{-4}$)
Int3-3	Empty	14/74	0/(1/1)	16/30 784	5.1	1/56	0/0	1/23 296	0.4
	mAPE1	7/21	0/0	14/17 056	8.2	4/47	0/(1/1)	4/19 552	2.1
	mY170F	13/71	0/(1/1)	21/29 536	7.1	5/54	0/(1/1)	5/22 464	2.2
Int3-19	Empty	16/68	0/0	28/28 288	9.9	2/27	0/0	2/11 232	1.8
	mAPE1	13/50	0/0	20/20 800	9.6	2/30	0/0	2/12 480	1.6
	mY170F	22/53	0/(1/1)	35/22 048	15.9	2/24	0/0	2/9984	2.0
Ext2-11	Empty	29/72	0/(16/1)	40/29 952	13.35	11/72	0/0	12/29 952	4
	mAPE1	35/91	0/(1/1)	50/37 856	13.21	16/91	0/(2/2)	17/37 856	4.5
	mY170F	24/81	0/(1/1)	44/33 696	13.06	12/84	0/(1/1)	13/34 944	3.7

Table 2. SHM frequency in the I μ /S μ region in BL2-Empty, mAPE1 and mY170F cells

		OHT+				OHT-			
Cell lines		Mutated clone/ total clone	In/del (bp/ clone)	Mutation/ total base	Mutation frequency ($\times 10^{-4}$)	Mutated clone/ total clone	In/del (bp/ clone)	Mutation/ total base	Mutation frequency ($\times 10^{-4}$)
Int3-3	Emp	13/48	0/1	15/35 040	4.3	1/27	0/0	1/19 710	0.5
	APE1	14/48	0/2	15/35 040	4.3	2/22	0/2	2/16 060	1.2
	Y170F	14/42	0/0	20/30 660	6.5	0/23	0/0	0/16 790	0
Int3-19	Emp	8/55	0/3	14/40 190	3.5	2/23	0/0	2/16 790	1.2
	APE1	15/50	0/2	15/36 500	4.2	1/22	0/0	1/16 060	0.6
	Y170F	21/45	0/2	27/32 850	8.2	3/20	0/0	3/14 600	2
Ext2-11	Emp	43/61	0/(7/6)	80/50 630	15.8	31/68	0/(12/5)	29/56 440	5.1
	APE1	39/69	0/(3/3)	86/57 270	15.01	14/72	0/0	15/59 760	2.5
	Y170F	39/64	0/(15/2)	64/48 140	13.3	10/74	0/(2/2)	14/61 420	2.3

on TLPs (13, 14), the number of AP sites should be comparable to that of SHM. SHM in the I μ /S μ region of APE1-deficient cells under CIT stimulation was shown to be $\sim 2.5 \times 10^{-4}$ (28), which may correspond to the AP site frequency in the I μ /S μ region. As we showed that 5×10^{-5} AP sites are produced by 5 mM MMS in APE1-deficient CH12 cells (Fig. 1D, right), we postulated that CIT-treated cells should generate ARP signals that are at least higher than 5 mM MMS-treated cells in the APE1-deficient cell series.

On the basis of this speculation, the measured AID-induced ARP signals in S μ and S α regions under CIT stimulation in CH12 cells (Fig. 2D) were much lower than the expected AP site number from the standpoint of the DNA deamination model. In addition, the lack of difference in S region AP site frequency between APE1-deficient and -proficient CIT-stimulated cells suggested that AP sites were not generated by AID induction.

Molecular function of APE1 in AID-induced IgH diversification

In the present study, we showed that V region SHM in the IgH gene is not dependent on APE1 (Fig. 4); previous research has shown that APE1 is not necessary for AID-induced S region SHM (28). According to the DNA deamination model (Supplementary Figure 1B), APE1 is necessary for processing AP sites formed by AID and UNG activities. If this is the case, SHM is expected to be significantly reduced in the absence of

APE1 (13, 14), and only replication-dependent SHM remains. However, the present study demonstrated that V region SHM was not impaired at all in APE1-deficient BL2-J cells.

In the RNA editing model, APE1 was proposed to be necessary for processing of a staggered-end DSB into a blunt-end DSB via its exonuclease activity, and is only necessary for CSR (Supplementary Figure 1A). We did not detect an S μ - or an S α -specific ARP signal, which is consistent with the idea that knockout of APE1 does not enhance the abasic site signal, and that UNG does not generate abasic sites in the S μ or S α regions following AID activation. APE1's role in CSR is not the processing of AP sites by its endonuclease activity, but the trimming of staggered ends by its exonuclease activity (28, 42) although the 3' to 5' exonuclease activity of APE1 is generally considered to be minor when compared with endonuclease activity *in vitro* (43). APE1 is reported to process the 3'-end of DNA with the adducts such as covalent bonds with tyrosine, which is also a remnant of the Top1-DNA cleavage complex as the previous report showed (42). The role of APE1 in V region SHM and CSR described in previous and present reports is consistent with the RNA editing model rather than the DNA deamination model.

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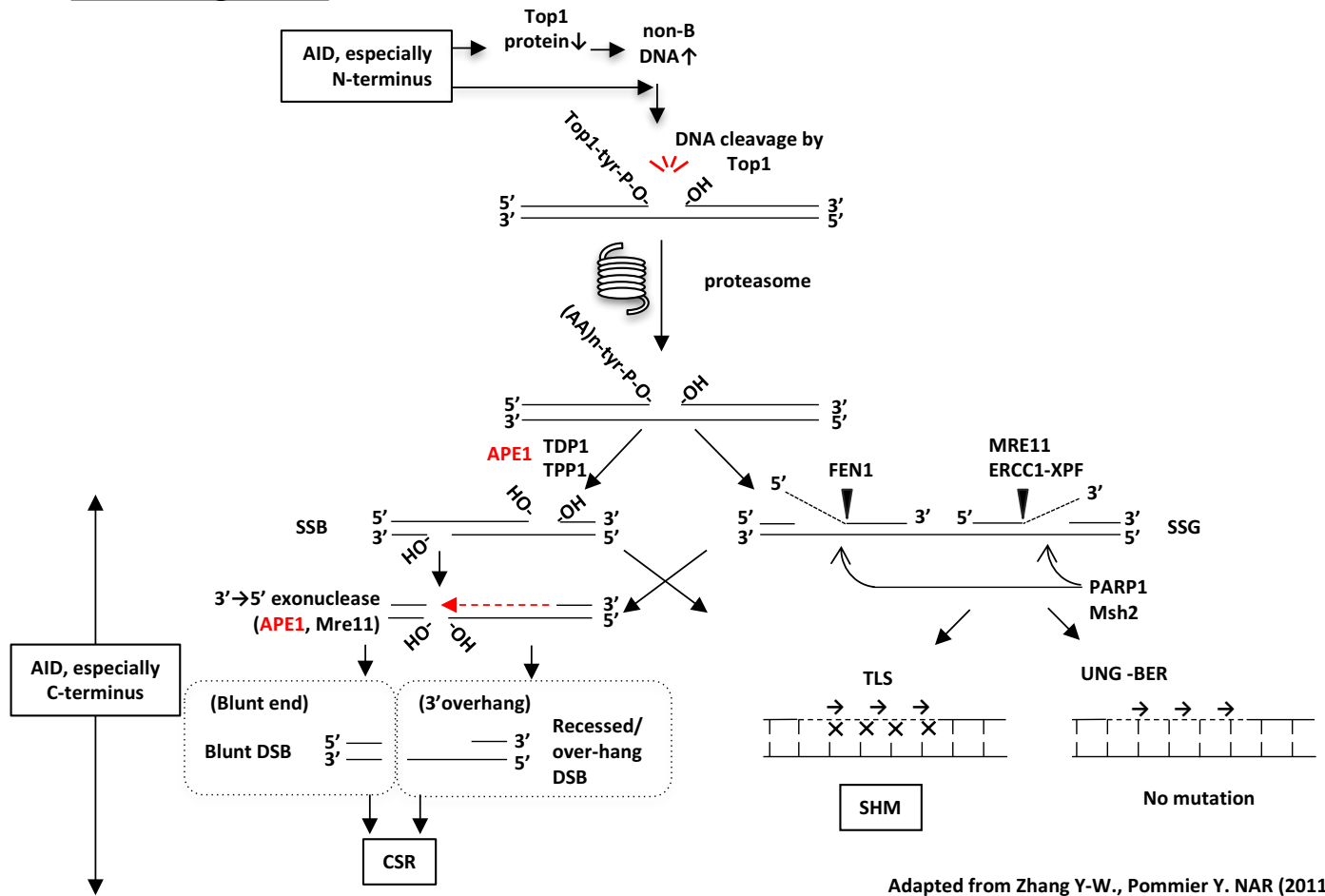
Conflicts of interest statement: the authors declared no conflicts of interest.

References

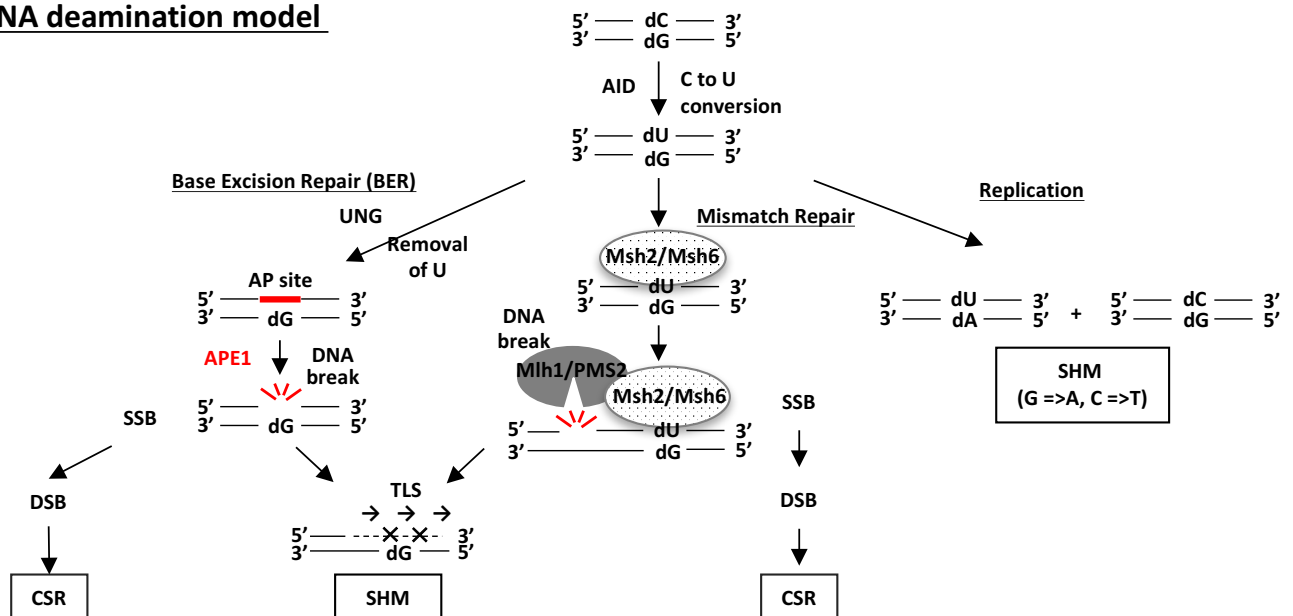
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A

RNA editing model

B

DNA deamination model

Supplementary Figure Legends

Suppl. Fig. 1 Schema of the (A) RNA editing and (B) DNA deamination models

C, cytidine; U, uridine; TDP1, tyrosil-DNA phosphodiesterase 1; TPP1, previously PTOP/PIP1/TINT1; FEN1, flap endonuclease1; MRE11, meiotic recombination 11; ERCC1-XPF, a nuclease complex in nucleotide excision repair; PARP1, Poly [ADP-ribose] polymerase 1 ; Msh2, MutS protein homolog 2; Msh6, MutS protein homolog 6; Mlh1, MutL homolog 1; PMS2, post meiotic segregation increased 2; TLS, translesion synthesis ;UNG, uracil DNA glycosylase; CSR, class switch recombination; SHM, somatic hypermutation; dC, deoxy-cytidine; dU, deoxy-uridine; dA, deoxy-adenine; dG, deoxy-guanine; SSB, single strand break; DSB, double strand break; SSG, single strand gap

Suppl. Table 1. Base changes of SHM in V4-39-J_H5 region in BL2-Empty, mAPE1 and mY170F cells

Int3-3, VH4-39-JH5											
APE1 ^{-/-} BL2		4-OHT					NS				
		A	G	C	T	Total	A	G	C	T	Total
Empty	A			1		1					
	G	7		2	1	10				1	1
	C				4	4					
	T			1		1					
	Total	16					1				
WT	A										
	G	7		1	2	10			1	1	2
	C	1			1	2				2	2
	T		1	1		2					
	Total	14					4				
Y170F	A										
	G	9		2	1	12	1		1	1	3
	C	1	1		7	9				2	2
	T										
	Total	21					5				

Int3-19, VH4-39-JH5											
APE1 ^{-/-} BL2		4-OHT					NS				
		A	G	C	T	Total	A	G	C	T	Total
Empty	A										
	G	8		3		11					
	C	2			15	17				2	2
	T										
	Total	28					2				
WT	A		1			1					
	G	8		1		9			1		1
	C		1		9	10	1				1
	T										
	Total	20					2				
Y170F	A		1			2					
	G	13			1	14	1				1
	C	1	4		15	20				1	1
	T										
	Total	35					2				

Ext2-11, VH4-39-JH5											
APE1 ^{-/-} BL2		4-OHT				NS					
		A	G	C	T	Total	A	G	C	T	Total
Empty	A										
	G	9		8		17	3		2	1	6
	C	1	4		17	22	1	1		3	5
	T		1			1			1		1
	Total					40					12
WT	A		1		1	2					
	G	20		2	1	23	12				12
	C		2		23	25				5	5
	T										
	Total					50					17
Y170F	A							1		1	2
	G	17		6	2	25	2		3	5	10
	C		1		17	18					
	T			1		1			1		1
	Total					44					13

Suppl. Table 2. Base changes of SHM in I μ /S μ region in BL2-Empty, mAPE1 and mY170F cells

Int3-13, Im/Sm											
APE1 ^{-/-} BL2		4-OHT					NS				
		A	G	C	T	Total	A	G	C	T	Total
Empty	A										
	G	4		3	1	8					
	C	2	2		3	7				1	1
	T										
	Total					15	1				
WT	A		1			1					
	G	5		1	1	7			1		1
	C		2		5	7				1	1
	T										
	Total					15	2				
Y170F	A										
	G	9				9					
	C	1	2		8	11					
	T										
	Total					20	0				

Int3-19, Im/Sm											
APE1 ^{-/-} BL2		4-OHT					NS				
		A	G	C	T	Total	A	G	C	T	Total
Empty	A		1			1				1	1
	G	5		3		8					
	C		1		4	5					
	T								1		1
	Total					14	2				
WT	A										
	G	3			1	4					
	C		1		8	9				1	1
	T		2			2					
	Total					15	1				
Y170F	A							2			2
	G	12		1	1	14					
	C		2		8	10				1	1
	T			3		3					
	Total					27	3				

Ext2-11, Im/Sm											
APE1 ^{-/-} BL2		4-OHT					NS				
		A	G	C	T	Total	A	G	C	T	Total
Empty	A										
	G	29		5	4	38	8		8	2	18
	C	4	13		25	42	1	3		5	9
	T								2		2
	Total					80	29				
WT	A		2		1	3		1			1
	G	33		7	1	41	5		1	1	7
	C	2	5		32	39	1			5	6
	T			3		3	1				1
	Total					86	15				
Y170F	A		1			1					
	G	25		7	2	34	4			2	6
	C	2	10		13	25	2	2		4	8
	T	2	1	1		4					
	Total					64	14				

Suppl. Table 3. Primers and Oligos used

Genotyping primer sets	
① for wild type allele	
hAPEx1_5arm_exon4_m128	gcttgactcgaactccttaggc
hAPEx1_missedExon5p86_R	TGCATTAGGTACATATGCTGTTACC
② for GFP allele	
hAPEx1_5arm_exon4_m128	gcttgactcgaactccttaggc
GFP717R	cttgtagcagctcgtccatgc
③ for GFP allele	
GFPpAp746_F	gccgcgactctagatcataatcagc
3downstrmp3153_R	ccactgatgctccacggtggta
④ for BSD allele	
hAPEx1_5arm_exon4_m128	gcttgactcgaactccttaggc
BSD_32R	gcctttgtctcaagaagaatccac
⑤ for BSD allele	
BSD_233F	cgatcggaatgagaacagg
3downstrmp3153_R	ccactgatgctccacggtggta
Oligos for guideRNAs	
Int3 forward	CACCGgctaagtcatagatacgca
Int3 reverse	AAACTgcgtatctatgacttagcC
3downstrm forward	CACCGttcgtgtcttacccttcg
3downstrm reverse	AAACcgaaggggtaagacacgaaC
Ex2 forward	CACCGGACGCGGTACAGCTGCCCA
Ex2 reverse	AAACTgggcagctgtaccgcgtcC
Primers for targeting vector construction in pBluescript	
APE1_new5'arm_F	AGGGCGAATTGGGTACAACAATAGTGGGGGACATAAGGGA
APE1_new5'arm_R	TTGCTCACCTGTAAGTGAAAAGAAAGGTGGAA
APE1_newEGFP-polyA_F	ACTTACAGGTGAGCAAGGGCGAGGAGCTGTTC
APE1_newEGFP-polyA_R	GGGTGGTGATACATTGATGAGTTTGGACAAAC
APE1_new3'arm_F	CAATGTATCACCACCCCTAAATCACTTTGAGC
APE1_new3'arm_R	AGGGAACAAAAGCTGGCGGACAGTGGGAGTGCCCCCTATGA
5'arm_FW_short	AGGGCGAATTGGGTACccaagtgccagaggagtgaatgc
new5armR	ttcagaccttgctaagtcatagatacgcacggg
newPGK-BSD-pA-F	gacttagcaaggtctgaagaggagtttacgtcc
3'arm PGL-BSD-pA_RV	GGGTGGTGAAATGCTCTAGAACTAGTGAACCT
hAPE1-3arm_FW	GAGCATTTTACCACCCCTAAATCACTTTGAGC
3'arm_RVshort	AGGGAACAAAAGCTGGAggctttgaagcatgtgcctcagg
Primer sets for detecting GFP, BSD and human β2 microglobulin (b2MG)	
BSD233F	cgatcggaatgagaacagg
BSD376R	aattcacgaatcccaactgc
EGFP487F	AAGGTGAACTTCAAGATCCGCCAC
EGFP717R	CTtgtagcagctcgtccatgc
b2MGF	cggctctgcttcccttagac
b2MGR	cgaaaccgctttgtatcaca
Primer set for cloning JP8Bdel-ER into pEF1a-IRES-ZsGreen1 vector	
hAID_F_NheI	agagagctagcatggacagcctcttgatgaacc
hAID_R_EcoRI	agagagaattctcaagctgtggcagggaaaccc

Primer set for qPCR in ARP trapping assay	
SμF	TGGCCAGAACCAGAATCAATT
SμR	GCCTCACATAATCCATGTCAGCTA
SαF	TGAAAAGACTTTGGATGAAATGTGAACCAA
SαR	GATACTAGGTTGCATGGCTCCATTACACA
Sγ1F	GCAATGCTTCTGTCTGTAGACAAGA
Sγ1R	CCCGGATTTCGCTGAGGACAGAGGCT
β2M F	CTGGCTGGCTCTCATTTCAG
β2M R	GGTCAGTGAGACAAGCACCA
βActF	CAGCTTCTTTGCAGCTCCTT
βActR	CTAGCCACGAGAGAGCGAAG