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GANP Regulates the Choice of DNA Repair Pathway by DNA-PKcs Interaction in AID-Dependent IgV Region Diversification

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RNA export factor germinal center–associated nuclear protein (GANP) interacts with activation-induced cytidine deaminase (AID) and shepherds it from the cytoplasm to the nucleus and toward the IgV region loci in B cells. In this study, we demonstrate a role for GANP in the repair of AID-initiated DNA damage in chicken DT40 B cells to generate IgV region diversity by gene conversion and somatic hypermutation. GANP plays a positive role in IgV region diversification of DT40 B cells in a nonhomologous end joining–proficient state. DNA-PKcs physically interacts with GANP, and this interaction is dissociated by dsDNA breaks induced by a topoisomerase II inhibitor, etoposide, or AID overexpression. GANP affects the choice of DNA repair mechanism in B cells toward homologous recombination rather than nonhomologous end joining repair. Thus, GANP presumably plays a critical role in protection of the rearranged IgV loci by favoring homologous recombination of the DNA breaks under accelerated AID recruitment. The Journal of Immunology, 2014, 192: 5529–5539.

A nigen binding selectively induces the rapid proliferation and maturation of Ag-specific B cells. Ab maturation occurs with diversification of the rearranged IgV region gene segment by inducing activation-induced cytidine deaminase (AID) in the germinal centers of peripheral lymphoid organs (1–4). In mice and humans, IgV region diversification appears to occur with somatic hypermutation (SHM) accumulated nearby cytidines at WRC (W = A/T, R = A/G, and C) hotspot motifs (5, 6). The initial C deamination causes various DNA injuries, including DNA single-strand breaks and double-strand breaks (DSBs). Repair of these DNA injuries accompanies the high-frequency mutation during the process of mismatch repair (MMR) and base excision repair (BER) (7–13). In mammals, most DSBs occurring in various phases of the cell cycle are repaired by the nonhomologous end joining (NHEJ) repair mechanism, which is mediated by end-binding with Ku70 and Ku80 proteins catalyzed by DNA-PKcs (14–18). The DNA end joining is then catalyzed by DNA ligase IV, Artemis, and XRCC4. Such NHEJ repair potentially accompanies extreme sequence alterations because the DNA joining and extension do not read the template sequence and allow deletion or insertion of the nucleotide sequence during DNA ligation. NHEJ repair at the IgV region gene may predispose to the extensive alteration of the V region sequence, resulting in extinguishment of the Ag-binding capacity (19).

B cells, particularly in birds, diversify their IgV region repertoire through gene conversion (GCV). GCV involves alteration of the rearranged Ig L chain V region (IgVL V) sequence by copying a segment from one of the 25 upstream pseudo-V (ψV) donor gene sequences to the downstream IgVL gene (20). The IgVL GCV is well evidenced in the DT40 chicken B cell line (21–23). In the presence of AID and uracil nucleotide glycosidase (UNG), the IgVL GCV is initiated with DNA injury and processed by a DNA repair mechanism similar to homologous recombination (HR) (23, 24). The HR DNA repair mechanism is employed against a large proportion of DNA damage responses associated with DNA replication at late S and G2 phases of the cell cycle (11, 25). After invasion of the homologous chromosome aided by Rad51 recombination, the HR repair response promotes the precise reading of the template DNA strand sequence using DNA polymerases, usually with error-free activity (DNA polymerase δ), but occasionally with error-prone activities such as in Ag-driven B cells to generate SHM (12, 26). In DT40 B cells, AID can induce both SHM and GCV at the IgVL V region (24, 27). AID induces abundant IgVL GCV in the presence of UNG, but predominantly induces SHM in UNG-deficient DT40 B cells (23). AID-initiated DNA injury is repaired through a DNA repair mechanism associated with GCV/HR and SHM in DT40 B cells, thus providing a suitable system to study the mechanisms of IgV region diversification after AID-induced DNA injuries.

Germlinal center–associated nuclear protein (GANP) is upregulated in proliferating germinal center B cells (28–30). The central
region of GANP is homologous to the Saccharomyces cerevisiae SAC3 protein, a component of the SAC3/THO complex involved in RNA export following transcription in yeast, and therefore GANP is proposed as a component of the mammalian transcription/export-2 complex (31, 32). Recently, we demonstrated that GANP interacts with cytoplasmic AID and assists its nuclear localization to augment its transport to the transcribed IgV region segment in B cells (33). In this process, the histone acetyltransferase region of GANP is involved in the selective recruitment of GANP toward the terminal region of the rearranged IgV region exons and the site-selective acetylation of the linker histone H1 (34). Thus, AID is targeted to the selective DNA region of the rearranged IgV loci with the assistance of GANP. AID converts C to U, creating a mismatch that can be processed by DNA-modifying enzymes such as UNG and APE1, leading to DNA breaks at the transcribed IgV region loci. AID interacts with the ribonucleoprotein complex, including RNA polymerase II, stalling factor Sp5, and DNA-PKcs.

DNA regions with DSBs initiated by intensive targeting of AID must be repaired during the interval phase before the mitotic phase of the cell cycle to rescue and generate IgV region diversity in daughter Ag-reactive B cells. Our proteomics analysis revealed that GANP is associated with DNA-PKcs prominently among the DNA repair molecules (34). Given that GANP interacts with DNA-PKcs, it might be the initial member of the DNA repair pathway interacting with the AID/GANP complex in situ at the IgV region loci in germline-center B cells.

In this study, we demonstrate that GANP probably participates in the regulation of post-AID DNA repair during transcription-coupled IgV region diversification in B cells.

Materials and Methods

Plasmid construction

For GFP or GANP overexpression (GANPΔex) transfectants, a GFP or GFP-GANP gene was introduced into pCK2 (chicken β-actin promoter) vector containing a neomycin-resistant cassette (33). The AID expression vector (pPB-AID-RES-GFP) was a gift from Dr. Takasu Honjo (Kyoto University, Kyoto, Japan).

Antibodies

The following Abs were purchased: histone H4 (L64C1; Cell Signaling Technology), acetyl histone H4 (3H8-4C10; Millipore), AID (L2E7 and 30F12; Cell Signaling Technology), β-actin (AC-15; Sigma-Aldrich), GANP (A303; Bethyl Laboratories), DNA-PKcs (sc-5282; Santa Cruz Biotechnology), phospho–DNA-PKcs (Thr2669; 10B1; BioLegend), Ku70 (sc-9033; Santa Cruz Biotechnology), mouse IgG (sc-2025; Santa Cruz Biotechnology), and biotinylated mouse anti-chicken IgM (M1; SouthernBiotech).

DT40 cell lines and transfection

DT40 CL18 (DT40Δ115) (20), AID/Δ4V (27), AID/Δ9UNG (23), and Rad54Δ−/− (35), Ku70−/− (36), and CACTIN2−/− (29) cells were cultured in IMDM supplemented with 0.1 mM 2-ME, 10% FBS, and 1% chicken serum (Tissue Culture Biologicals) at 39˚C in a 5% CO2 incubator and maintained by addition of 0.1 mM 2-ME, 10% FBS, and 1% chicken serum (Tissue Culture Biologicals) at 39˚C in a 5% CO2 incubator and maintained by addition of 0.1 mM 2-ME, 10% FBS, and 1% chicken serum (Tissue Culture Biologicals). Dead cells were excluded by staining with 7-aminoactinomycin D (BD Biosciences). Single cells were plated from the GFP+IgM+ population into individual wells of 96-well plates using FlowJo software (Tree Star).

Sequencing of IgV1

DNA samples were purified from wells of DT40 cells by PCR. The targeting vector was constructed by replacing a 4.6-kb fragment encoding the chicken GANP (ch-GANP) open reading frame (from exon 6 to exon 10) with a fused puromycin-resistance gene cassette (38) between the left arm (3.2 kb) and the right arm (2 kb). After the linearized targeting vector was transfected by electroporation, 34 drug-resistant clones were obtained after 10 d. PCR screening was carried out for the drug-resistant clones using the primer set of ExSS (5'-GGCTGCGTTGGAAGTACTGTCGTGTTCA-3') and Ex1S (5'-CAGACGGGGTTCAAGCTCTAAGTA-3') to detect the targeting vector region, and the primer set of 5'-untranslated region 2 (5'-GGATCAGCAGCAGGCGG-3') and Puro (5'-CAGGCAGCCAGGAGGACGGACGAC-3') to detect the targeted integration at the GANP allele. Six ch-GANP gene-haplodeficient DT40 clones (GANPΔ+) were established (targeting ratio, 17.6%; 6 clones from 34 clones).

RT-PCR, Northern blot, and Western blot

For RT-PCR analysis, total RNA samples were purified with a RNeasy Mini Kit (Qiagen) and reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) according to the manufacturers’ protocols. PCR was performed with following primer sets: human GANP, Ex5P (5'-GGCTGCGAGTCTGAATCTGCTCCTGCTCT-3') and Ex3S (5'-GGCTGCTATGATGTTGTGAAGCGCCGTGA-3'), and β-actin, ACTIN1 (5'-CCC-AAGGTACTTCCCCAGGAGGCGATTG-3') and ACTIN2 (5'-GGGTTGGAAGCGCCGTGA-3') to detect the targeting vector region, and the primer set of 5'-untranslated region 2 (5'-GGATCAGCAGCAGGCGG-3') and Puro (5'-CAGGCAGCCAGGAGGACGGACGAC-3') to detect the targeted integration at the GANP allele. Six ch-GANP gene-haplodeficient DT40 clones (GANPΔ+) were established (targeting ratio, 17.6%; 6 clones from 34 clones).

Chromatin immunoprecipitation–quantitative PCR

Chromatin immunoprecipitation (ChIP) was performed using the cell suspension of the corresponding cell line. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). DNA fragments were purified with 0.1 mM 2-ME, 10% FBS, and 1% chicken serum (Tissue Culture Biologicals). Dead cells were excluded by staining with 7-aminoactinomycin D (BD Biosciences). Single cells were plated from the GFP+IgM+ population into individual wells of 96-well plates using FlowJo software (Tree Star).

DNA repair choice by AID-Bound GANP

For KIT-positive cell sorting, a KIT strain was used as a selection marker to purify KIT-positive cells (27). The protocol was similar to the one described above except that DT40 Δ115 AID−cells were used as a target cell.

For TCRαβ/γδ-positive cell sorting, a TCRαβ/γδ strain was used as a selection marker to purify TCRαβ/γδ-positive cells (27). The protocol was similar to the one described above except that DT40 Δ115 AID−cells were used as a target cell.

For TCRβ/γ-positive cell sorting, a TCRβ/γ strain was used as a selection marker to purify TCRβ/γ-positive cells (27). The protocol was similar to the one described above except that DT40 Δ115 AID−cells were used as a target cell.

For TCRδ/γ-positive cell sorting, a TCRδ/γ strain was used as a selection marker to purify TCRδ/γ-positive cells (27). The protocol was similar to the one described above except that DT40 Δ115 AID−cells were used as a target cell.
DNA repair assay
A modified pSVlacZ1351+727neo reporter was used for the NHEJ assay. Two Hpal sites of the β-galactosidase (lacZ) gene were mutated individually. Addition of two nucleotides converted the Hpal site to the Xho1 site, which induced a frameshift and nonfunctional lacZ gene. The vector of recombination substrate was constructed by tandem alignment of two differently mutated lacZ (at the second Hpal site) and lacZ′ (at the first Hpal site) genes. Flow cytometric measurements of β-galactosidase activity were carried out using the FluoroReporter lacZ flow cytometry kit (Molecular Probes). For assessment of HR repair, the recombination reporter system, pDR-GFP, which contains a plasmid with two nonfunctional GFP genes and a recombinant plasmid encoding the restriction enzyme I-SceI, was used (39). The fifth GFP gene (SceGFP), mutated by insertion of an 18-mer restriction endonuclease site of I-SceI, produces a DSB once I-SceI is expressed (40). The truncated 3′ GFP gene (gGFP) serves as a donor to correct the broken SceGFP gene, and the short tract GCV usually restores an intact GFP gene (41). Each clone was transiently transfected with vector for expression of I-SceI enzyme and GANP or empty expression vector.

Colony survival assay
Cells (500–1000 cells/well in a 96-well plate) were cultured in medium with varying concentrations of camptothecin (Cpt; Calbiochem) or etoposide (Etp; Calbiochem). After exposure to the drugs for 48 h, cells were stained with MTT dye (tetrazolium-based colorimetric assay; Roche) and measured at 595 nm according to the manufacturer’s method. Cell survival rates were calculated as the percentage of surviving fraction after exposure to drugs relative to the control.

Cell cycle analysis
DT40 cells were harvested and washed once with PBS, then subcultured in six-well plates at concentration of 0.5 × 10⁶ cells/ml in RPMI 1640 culture medium containing 0.1 mM 2-ME, 10% FBS, and 1% chicken serum for 24 h. Cells were then harvested, washed once with PBS, and stained with propidium iodide for 1 h. Data were collected using a FACSCalibur (Becton Dickinson). Data were analyzed with the FlowJo software (Tree Star).

Drug treatment in Ramos B cells
Ramos B cells were incubated in RPMI 1640 culture medium containing 0.1 mM 2-ME and 10% FBS with 50 μM Etp for 2 h, and then the medium was replaced with fresh culture medium and incubated for additional indicated periods. For kinase inhibition studies, cells were pretreated with 10 μM NU7026 (DNA-PKcs inhibitor; Sigma-Aldrich) for 1 h, and then incubated with 50 μM Etp for another 2 h in the presence of the inhibitor. After Etp treatment, cells were incubated in fresh medium with the inhibitor until cells were harvested.

Nuclease treatment
Cell extracts from Ramos nuclei were preincubated with either 250 μg/ml RNase A (Nippon Gene) or 50 U Turbo DNase I (Ambion) for 10 min before immunoprecipitation (IP).

Small interfering RNA treatment
Downregulation of AID with small interfering RNA (siRNA) in Ramos cells was carried out as described previously (42). Ramos cells (8 × 10⁶ cells) were transfected with 2 μM siRNA using the Amaxa Nucleofector kit V (program O-06) according to the manufacturer’s protocol. The duplex siRNA against human AID (AICDA; 5′-UUCAAAAAUGCCGC-UUGGCGT-3′) and negative control SIC were obtained from Sigma-Aldrich.

Statistical analysis
The statistical significance of differences among the groups was analyzed by an unpaired 2-tailed Student t test. A p value <0.05 was considered statistically significant.

Results
GANP augments IgV L GCV in DT40 B cells
We attempted to confirm the effect of GANP in an assay at the single cell level to measure IgV region diversification using chicken DT40 B cells. We examined GANP(DOE) by WB analysis of both endogenous chicken GANP and exogenous GANP following human GANP transfection of DT40 clone 18 (DT40(CL18)) cells. DT40(CL18)GANP(DOE) showed a 2- to 3-fold increase in GANP protein (Supplemental Fig. 1). DT40(CL18) carries the rearranged IgV L with a frameshift mutation at nucleotide position 129 (by the G insertion) and therefore does not express sIgM. This frameshift is corrected by GCV, thus regaining sIgM expression (sIgM gain assay), which can be used to measure the frequency of GCV (Fig. 1A) (22, 24). The DT40(CL18)GANP(DOE) clone significantly augmented the sIgM gain compared with the GCV-expressing DT40(CL18) (DT40(CL18)GFP) clones (Fig. 1B). In comparison with the parental IgV L sequence of DT40(CL18), the genomic sequence of the IgV L showed GCV tracts from ψV donor segments in all the sIgM+ cells (Fig. 1C). A higher rate of nonsense GCV tracts was found in sIgM+ DT40(CL18)GANP(DOE) cells that failed to correct the IgV L sequence to an in-frame position compared with control DT40(CL18)GFP cells (Fig. 1D, after sequencing).

Next, we analyzed the usage of ψV genes as donor sequences, because a previous study demonstrated the preferential usage of ψV8, which showed the highest similarity to the rearranged IgV L sequence (43). DT40(CL18)GANP(DOE) cells used more diverse donor ψV genes with decreased usage of the preferential donor ψV8 gene (52%), compared with the donor usage profile of DT40(CL18)GFP (ψV8 gene, 68%) (Fig. 1C, 1E). Additionally, the median tract length was also increased to 99 bp in DT40(CL18)GANP(DOE) cells from 67 bp in DT40(CL18)GFP cells, representing a significant stabilization of GCV with a donor tract of longer length (Fig. 1E). These results indicate that GANP plays a role in IgV region diversification by regulating the GCV/HR mechanism.

GANP is required for IgV L GCV in DT40 B cells
We attempted to disrupt the ch-GANP gene by deleting the sequence encoded by the 6th to 10th exons in DT40 B cells (Supplemental Fig. 2A). Heterozygous GANP-deleted clones in DT40(CL18) (GANP(DOE)) were selected using a puromycin-resistant gene, and the targeted regions were confirmed (Supplemental Fig. 2B). The mRNA and protein expression levels of ch-GANP in GANP+/− cells as donor sequences, compared with GANP+/+ cells (Fig. 2A). TSA is a histone deacetylase inhibitor, which increases GCV significantly in DT40 B cells (Fig. 2A), probably by activating the transcription rate through histone acetylation (37). TSA augments GCV significantly in GANP(DOE) DT40 cells, serving as a positive control and validating GCV data in our assay system compared with other laboratories (37, 44–46). The change in the gene dosage for endogenous GANP (GANP(DOE) versus GANP(DOE) cells) affected IgV L GCV induced by TSA. This implies that GANP has a unique function in modulating the generation of IgV L GCV over the enhanced transcription induced by the histone deacetylase inhibitor.

We further examined the effect of GANP in selective acetylation of both rearranged and unrearranged IgV L loci using chromatin immunoprecipitation–quantitative PCR after TSA treatment of
GANP+/- and GANP^-/^- cells failed to acetylate H4 to the same level as did control GANP^+/+ cells, markedly at 2 wk (Fig. 2B). Reduced H4 acetylation in GANP+/+ cells suggests that GANP plays a role in augmenting TSA-induced chromatin modification selectively at the rearranged IgV_L gene. Sequence analysis showed that GCV was significantly reduced in GANP+/-/^- cells (2.6%; 1 of 39 clones) compared with GANP+/+ cells (34.9%; 15 of 43 clones) (Fig. 2C). These results confirmed that GANP is an integral molecule for regulation of the GCV/HR pathway to generate IgV_L region diversity.

GANP augments IgV_L SHM in DT40 B cells

AID induces IgV_L diversification in DT40 B cells with both GCV and SHM through a common intermediate (27). Under specific conditions lacking either the donor ψV segments or UNG, DT40 mutant cells selectively display AID-induced IgV_L SHM. In the assay for IgV_L SHM, we used another DT40 clone that expresses the productive IgV_L mRNA to express slgM. IgV_L SHM is represented by the loss of slgM from slgM^+ cells and by sequencing of IgV_L in slgM^- cells (27). We measured the effect of GANP in generation of IgV_L SHM by introducing exogenous AID in DT40 B cells devoid of endogenous AID and ψV genes (AID^-/-ψV^-). This type of cell is called AID^R in the original report (27) (Fig. 3A). AID^R ψV^- cells are DNA repair-proficient and devoid of any background GCV activity due to deletion of donor ψV segments, and therefore they represent an appropriate system to study the mechanism of AID-initiated SHM in further detail (23). The AID^R augmented the slgM loss in AID^-/-ψV^- cells (Fig. 3B), confirming that AID is a primary requirement for generation of IgV_L SHM. GANPO/E significantly augmented slgM loss by nearly 2-fold compared with mock-transfected AID^R ψV^- cells (Fig. 3B). The mutation analysis showed a pattern of mutations (transition versus transversion and G:C versus A:T) similar to that of AID^R ψV^- cells (Fig. 3B, Supplemental Fig. 3A), indicating that GANP does not affect the mutation pattern itself but rather augments the effect of AID.

To further address whether GANP is associated merely with the initial process of AID-dependent C deamination or also with the later DNA modifying error-prone mutational processes, we evaluated the SHM frequency on the basis of AID and UNG double-deficient DT40 cells (AID^-/-UNG^-/-) (Fig. 3D). The MSH-dependent MMR pathway is not functional in SHM in DT40 (47). AID-initiated lesions (C→U) create mutations after replication at G

**FIGURE 1.** GANP regulates IgV_L GCV in DT40 B cells. (A) IgV_L GCV fluctuation assay. The original DT40^CL18^ carries a frameshift mutation in the rearranged IgV_L gene. The frameshift was eliminated by GCV, resulting in expression of slgM. (B) Clonal analysis of slgM gain. The rate of slgM gain from slgM^- cells was clonally studied after culturing for 3 wk. Solid bars represent the median values. The difference between the rates of slgM gain in DT40^CL18^GFP (n = 52) and DT40^CL18^GAPNO/E (n = 51) clones was statistically significant (*p < 0.01). (C) IgV_L sequence analysis of cells showing sIgM gain between DT40^CL18^GFP (left) and DT40^CL18^GAPNO/E (right) clones. Each horizontal line represents the IgV_L sequence region of parental CL18. The bold bars show the GCV tracts and the dots on the lines indicate point mutations. The donor ψV genes used are shown on the left side. The number of clones with each pattern is indicated on the right side. (D) The gray areas show the rate of IgV_L GCV in the pie graphs. The data were obtained from sIgM+ and sIgM- populations. The number of sequences characterized is shown in the center of each graph. (E) Preferences for ψV gene usage as a donor sequence. The frequencies of the individual putative ψV genes among the total numbers of IgV_L sequences with GCV are shown. The total numbers of sequences analyzed are shown in the central circles. (F) Comparison of the GCV tract lengths. The tract length was analyzed by comparison with the known IgV_L sequences, *p < 0.05 by two-tailed unpaired t test.
AID targets is determined by AID itself. The transcription of chicken coldspot preferences, suggesting that the specificity for hotspot targeting (48). Supplemental Fig. 3H summarizes AID hotspot and GANP+/– used for the eating transitions and transversions. UNG by UNG creates abasic sites followed by the nick and BER, creating transition mutations at G:C. GANPO/E did not cause any change in sIgM loss in AID+/+ cells and the viability of AIDR cells is similar to that of AID+/- cells after GANPO/E, indicating that AID expression is within harmless levels comparable to those of mutating B cells (Supplemental Fig. 3B). Notably, the number of clones with each pattern is indicated on the right side.

**FIGURE 2.** Effect of GANP haplodeficiency in IgVh, GCV in DT40 B cells. (A) The rate of sIgM gain was clonally studied after sorting of sIgM- cells and culturing for 2–3 wk with or without TSA. Each dot represents the percentage of sIgM+ cells. Solid bars represent the median values. The difference between the rates of sIgM gain in GANP+/- (+/+, n = 12) and GANP+/- (-/-; n = 18) clones was statistically significant (*p < 0.00085, **p < 0.005, ***p < 0.002, ****p < 0.002). (B) Chromatin immunoprecipitation–quantitative PCR analysis of histone H4 acetylation at IgVh after TSA treatment. The acetylation status of histone H4 both active (solid line) and inactive (dashed line) IgVh loci were measured between GANP+/- (●) and GANP+/- (△) cells. IgG was used as the negative control. (C) Analysis of IgVh sequence between the GANP+/- (upper) and GANP+/- (lower) clones after TSA treatment for 2 wk. Each horizontal line represents the IgVh sequence region of CL18. The bold bars show the GCV tracts. The donor of genes used for the IgVh GCV are shown on the left side. The mutation profile also showed an increase in mutations at the putative CDRs region loci as GANP-mediated AID augment the SHM frequency (nearly 10-fold) with the C→G transition at sIgM loss variants showed that GANP interacts with DNA-PKcs in Ramos B cell nuclear extracts (34). This suggests a possible role of GANP in the regulation of the DNA repair mechanism required for generation of GCV. To investigate the function of GANP against DNA damage, two different DNA-damaging reagents, Etp and Cpt, were used. Etp is a topoisomerase II inhibitor that causes DNA DSBs that are repaired exclusively by Artemis-independent NHEJ (49), even in the S–G2 phase of the cell cycle (50). NHEJ-deficient DT40 cells are sensitive to Etp (51). GANPO/E rendered DT40 B cells more sensitive to Etp (52) and relaxing chromatin (34).

**Effect of GANP on support of HR in the repair of DNA breaks**

GANP interacts with DNA-PKcs in Ramos B cell nuclear extracts (34). This suggests a possible role of GANP in the regulation of the DNA repair mechanism required for generation of GCV. To investigate the function of GANP against DNA damage, two different DNA-damaging reagents, Etp and Cpt, were used. Etp is a topoisomerase II inhibitor that causes DNA DSBs that are mainly repaired by Artemis-independent NHEJ (49), even in the S–G2 phase of the cell cycle (50). NHEJ-deficient DT40 cells are sensitive to Etp (51). GANPO/E rendered DT40 B cells more sensitive to Etp (Fig. 4A), suggesting a suppressive effect of GANP on NHEJ repair. In contrast, Cpt is a topoisomerase I inhibitor that causes DNA damages that are repaired exclusively by HR (52). DT40 cells lacking NHEJ repair function show resistance to Cpt-mediated cell death (15, 52), which is likely due to the release of the HR pathway from constitutive inhibition by the NHEJ pathway (14, 15, 19). GANP+/- resistant to Etp (Fig. 4A), suggesting a suppressive effect of GANP on NHEJ repair. In contrast, Cpt is a topoisomerase I inhibitor that causes DNA damages that are repaired exclusively by HR (52). DT40 cells lacking NHEJ repair function show resistance to Cpt-mediated cell death (15, 52), which is likely due to the release of the HR pathway from constitutive inhibition by the NHEJ pathway (14, 15, 19). GANP+/- rendered DT40 B cells resistant to Cpt-mediated cell death (Fig. 4B), confirming the enhanced functionality of GCV/HR by GANP expression. GANP+/- DT40 cells displayed the opposite results compared with those of GANP+/- cells in both Etp and Cpt treatments. Next, we examined
DNA REPAIR CHOICE BY AID-BOUND GANP

Regulation of IgV<sub>L</sub> SHM by GANP via an AID- and UNG-dependent mechanism in DT40 B cells. (A) Scheme of the experiment using AID<sup>−/−</sup>ψ<sup>V</sup> cells. AID<sup>ψV</sup> cells were generated by reconstitution of the AID gene in AID<sup>−/−</sup>ψ<sup>V</sup> cells (sIgM<sup>+</sup> background). AID<sup>ψV</sup> cells induced mutations at IgV<sub>L</sub> without GCV. AID<sup>ψV</sup> GANP<sup>OE</sup> cells were generated by introduction of GANP gene into AID<sup>ψV</sup> cells. (B) The rate of sIgM loss from sIgM<sup>+</sup> cells was clonally studied after culturing for 2 wk. Bars represent the median values. The difference between the rates of sIgM loss in AIDRUNG<sup>(ψV)</sup> (n = 16) and AID<sup>ψV</sup> GANP<sup>OE</sup> (n = 16) clones was statistically significant (*p < 0.002). (C) The lacZ<sup>+</sup> frequency significantly reduced the frequency of lacZ<sup>+</sup> cells (Fig. 4D), probably as a consequence of suppressing NHEJ repair. The lacZ<sup>+</sup> frequency was markedly reduced in Ku70<sup>−/−</sup> cells (Supplemental Fig. 4B), confirming that DNA damage is predominantly repaired by NHEJ in this assay. Another reporter, pDR-GFP, can measure the frequency of GCV/HR DNA repair by the expression of GFP signal after induction of DSBs with I-SceI endonuclease (39) (Fig. 4E). In the present study, GANP<sup>OE</sup> cells without GFP tag were used. GANP<sup>OE</sup> increased the frequency of GFP<sup>+</sup> cells, indicating the positive effect of GANP on the DNA repair by GCV/HR (Fig. 4F). The altered expression of GANP either in GANP<sup>−/−</sup> or GANP<sup>OE</sup> does not affect the cell cycle profile (Supplemental Fig. 4C). These data showed that GANP is likely to affect the DNA repair pathways against DSBs, primarily by negatively regulating NHEJ and conversely by promoting HR.

GANP regulates the choice of DNA repair mechanism between the NHEJ and HR pathways

DNA-PKcs is a core molecule in the NHEJ pathway but was recently identified to participate in regulation of the GCV/HR mechanism (53). In fact, DNA-PKcs has a strong suppressive effect on HR (54–56) and IgV<sub>L</sub> GCV (19, 53) in DT40. We examined the effect of DNA-PKcs inhibitor NU7026 on spontaneous IgV<sub>L</sub> GCV in GANP<sup>OE</sup> and GANP<sup>−/−</sup> cells. NU7026 increased GCV rates by sIgM gain ∼2-fold in GANP<sup>OE</sup> cells (Fig. 5A). GANP<sup>−/−</sup> cells recovered the sIgM gain by GCV to levels comparable with wild-type GANP<sup>OE</sup> cells in the presence of DNA-PKcs inhibitor. This supports that IgV<sub>L</sub> GCV is mediated by reducing the influence of DNA-PKcs, presumably through interaction with GANP.

Previous reports demonstrated that the HR-deficient phenotype is rescued by abrogating NHEJ, reflecting that the repair process is dictated by a critical balance between NHEJ and HR (18) and not just by absolute presence or absence of the proteins that are functionally involved in both processes. To further analyze the regulatory role of GANP in the DNA repair mechanism, we examined whether GANP can restore the reduced GCV rate in HR-deficient DT40 cells. IgV<sub>L</sub> GCV was examined in Rad54-deficient DT40<sup>−/−</sup> B cells (Rad54<sup>−/−</sup>). Rad54<sup>−/−</sup> cells showed a decrease in IgV<sub>L</sub> GCV due to their defective HR-mediated DNA repair (Fig. 5B) (35). Overexpression of GANP in Rad54<sup>−/−</sup> cells
significant (**) cells showed the expected increase in GCV/HR compared with
5E), supporting the notion that GANP plays a role in
transfected (n = 9) and GANP-transfected (n = 9) clones was statistically
significant (p < 0.05). (E) Illustration of the pDR-GFP assay system. (F)
Measurement of homology-directed DNA repair using flow cytometric
analysis of lacZ expression in mock-transfected (left) or GANP-transfected
(right) clones. The difference between the rates of lacZ+ cells in mock-
transfected (n = 9) or GANP-transfected (n = 9) clones was statistically
significant (**p < 0.003).

**FIGURE 4.** Effect of GANP on homology-directed DNA repair. (A)
Sensitivity of GANP in DT40 clones to Etp. Cell survival rates from four
different clones were calculated as the percentage of surviving fraction
after exposure (48 h) to drugs relative to untreated cells. (B) Sensitivity of
GANP in DT40 clones to Cpt. Cell survival rates from four different clones
were calculated as the percentage of surviving fraction after exposure
(48 h) to drugs relative to untreated cells. (C) Illustration of pSVlacZ reporter
assay system. (D) Measurement of NHEJ repair using flow cytometric
analysis of lacZ expression in mock-transfected (left) or GANP-transfected
(right) clones. The difference between the rates of lacZ+ cells in mock-
transfected (n = 9) and GANP-transfected (n = 9) clones was statistically
significant (p < 0.05). (E) Illustration of the pDR-GFP assay system. (F)
Measurement of homology-directed DNA repair using flow cytometric
analysis of GFP expression in mock-transfected (left) or GANP-transfected
(right) clones. The difference between the rates of GFP+ cells in mock-
transfected (n = 9) or GANP-transfected (n = 9) clones was statistically
significant (**p < 0.003).

(Rad54−/−GANPO/E) efficiently restored compromised GCV in
Rad54−/− cells to levels almost comparable with those observed in
DT40CL11GANPO/E cells (Fig. 5B). The GCV tract of Rad54−/−
GANPO/E cells was similar to that of DT40CL11GANPO/E and
Rad54−/−GFP cells, as measured by sequence analysis (Figs. 1C, 5C). The ability of GANP to rescue the GCV/HR-deficient cells is
compatible with the proposed role of GANP in abrogation of the
NHEJ pathway. To confirm that GANP is targeting NHEJ, we
transfected GANP in Ku70−/− cells lacking the NHEJ function
to examine whether it still augments GCV/HR. Ku70−/−GFP cells
showed the expected increase in GCV/HR compared with
DT40CL11GFP cells owing to abolishment of NHEJ. Surprisingly,
Ku70−/−GANPO/E cells led to marked suppression of the GCV/HR
rate compared with the control Ku70−/−GFP cells (Fig. 5D, 5E), supporting the notion that GANP plays a role in IgV region
diversification through interacting with the functional NHEJ complex (54). Ku is thought to play a dominant negative effect in the
absence of the DNA-PK–dependent NHEJ pathway that will limit the
engagement of the other components (57, 58). The results
might suggest that GANP influences the stability of the NHEJ
complex. One possible explanation for the observed phenotype is
that, in the absence of the physiological target of GANP in the
functional NHEJ complex, GANP might stabilize their engage-
ments upon NHEJ repair.

Alteration of GANP interaction with DNA-PKcs during DNA
damage responses

GANP clearly interacted with DNA-PKcs even after treatment
with DNase I or RNase A (Fig. 6A), indicating that their asso-
ciation is mediated through protein–protein interactions. DNA-
PKcs is a core molecule in DNA break repair by NHEJ. We inves-
tigated whether GANP/DNA-PKcs association is DNA damage-
dependent. First, the association of GANP and DNA-PKcs was
examined after induction of DNA damage by AID. Remarkably,
AID overexpression in Ramos B cells decreased DNA-PKcs
coprecipitation with GANP (Fig. 6B); alternatively, AID knock-
down does not alter their association. Etp-induced DNA damage
decreased the GANP/DNA-PKcs association in a time-dependent
manner in bidirectional IP/WB analyses (Fig. 6C). These results
suggest that the interaction of GANP and DNA-PKcs is dissoci-
ated during the DNA damage response, particularly at 4 h after
DNA breaks. AID-initiated DNA damage shows implicit depen-
dence on DNA-PKcs for its repair (19, 59), and it is accompanied
by DSBs that potentially augment the generation of IgV gene
diversity in DT40 B cells (11, 27, 60). Etp-induced DNA breaks
are dependent on NHEJ even in the S–G2 phase of the cell cycle
(50), and they require DNA-PKcs (61). The dissociation of DNA-
PKcs from the GANP complex may affect the choice of DNA
repair pathways at the selective site of DNA breaks targeted by
GANP and its attendants.

DNA-PKcs is activated by phosphorylation at Thr2609 for NHEJ
repair (54, 62). No association was detected between GANP and
phospho–DNA-PKcs in IP/WB analysis (Fig. 6C). Once DNA-
PKcs is phosphorylated at Thr2609, it becomes fully activated
and committed to NHEJ repair. This will cause the dissociation
of phospho–DNA-PKcs from the GANP complex. Again, this finding
consolidates the assumption that GANP binding to DNA-PKcs
hinders its NHEJ repair capacity.

DNA-PKcs is thought to be a molecular sensor for DNA damage
and the inducer of various DNA damage responses through phos-
phylation of target molecules. A specific inhibitor of DNA-
PKcs, NU7026, did not cause any change in the association of
DNA-PKcs and GANP (Fig. 6D). Moreover, NU7026 could not
inhibit the dissociation of DNA-PKcs from the GANP complex
after treatment with Etp (Fig. 6E), indicating that their as-
cociation is mediated through protein–protein interactions. DNA-
P-Kcs is thought to be a molecular sensor for DNA damage
pathway at the

Discussion

The effect of GANP on IgV diversification was confirmed in a
well-characterized system using chicken DT40 B cells in vitro.
GANP highly augments the effect of AID on both GCV and SHM
at the rearranged IgV locus. Moreover, GANP and DNA-PKcs
association suggests an important role for GANP in regulating
DNA repair pathways against AID-initiated DSBs. The results

deemonstrate that GANP favors the HR-mediated DNA repair
pathway at the IgV region loci.
AID-initiated DNA injuries can be repaired by various pathways (12, 63–66). AID attacks and creates DNA single-strand breaks, and DSBs result when these occur on the opposite strand within a localized region. This is further confirmed by the fact that AID attacks C on both strands simultaneously (67). AID-initiated DSBs are the intermediates in IgVL SHM and GCV (12, 19, 26, 53).

FIGURE 5. Effect of GANP on IgV\textsubscript{L} GCV in mutant DT40 B cells with DNA repair deficiency. (A) The rate of sIgM gain was clonally studied after sorting of sIgM\textsuperscript{−} cells and culturing for 2 wk. Each dot represents the percentage of sIgM\textsuperscript{+} cells. Solid bars represent the median values. The difference between the rates of sIgM gain in DMSO-treated (n = 28) and in NU7026-treated (n = 29) GANP\textsuperscript{+/+} clones was statistically significant (*p < 0.04). The difference between the rates of sIgM gain in DMSO-treated (n = 20) and in NU7026-treated (n = 20) GANP\textsuperscript{+/−} clones was statistically significant (**p < 0.01). The difference between the rates of sIgM gain in NU7026 treatment of GANP\textsuperscript{+/+} (n = 29) and GANP\textsuperscript{−/−} (n = 20) clones was statistically significant (***p < 0.02). (B) The rate of sIgM gain was clonally studied after sorting of sIgM\textsuperscript{−} cells and culturing for 3 wk. Each dot represents the percentage of sIgM\textsuperscript{+} cells. Solid bars represent the median values. The difference between the rates of sIgM gain in DT40\textsubscript{CL18}\textsuperscript{GFP} (n = 8) and DT40\textsubscript{CL18}\textsuperscript{GANP\textsuperscript{OE}} (n = 8) clones was statistically significant (*p < 0.03). The difference between the rates of sIgM gain in Rad54\textsuperscript{−/−} GFP (n = 9) and Rad54\textsuperscript{−/−} GANP\textsuperscript{OE} (n = 9) clones was statistically significant (**p < 0.006). (C) Summary of IgV\textsubscript{L} sequences analysis of sIgM gain variants in Rad54\textsuperscript{−/−} GFP (left) and Rad54\textsuperscript{−/−} GANP\textsuperscript{OE} clones (right). Each horizontal line represents the IgV\textsubscript{L} sequence region of parental CL18. The bold bars show the GCV tracts, and dots on the lines indicate point mutations. The \(\psi\) genes used as donors are shown on the left. The number of clones with each pattern is indicated on the right side. (D) The rate of sIgM gain was clonally studied after sorting sIgM\textsuperscript{−} cells and culturing for 3 wk. Each dot represents the percentage of sIgM\textsuperscript{+} cells. Solid bars represent the median values. The difference between the rates of sIgM gain in DT40\textsubscript{CL18}\textsuperscript{GFP} (n = 9) and DT40\textsubscript{CL18}\textsuperscript{GANP\textsuperscript{OE}} (n = 9) clones was statistically significant (*p < 0.03). The difference between the rates of sIgM gain in Ku70\textsuperscript{−/−} GFP (n = 11) and Ku70\textsuperscript{−/−} GANP\textsuperscript{OE} (n = 17) clones was statistically significant (**p < 0.001). The difference between the rates of sIgM gain in DT40\textsubscript{CL18}\textsuperscript{GFP} (n = 9) and Ku70\textsuperscript{−/−} GFP (n = 11) clones was statistically significant (***p < 0.02). (E) Summary of IgV\textsubscript{L} sequence analyses of sIgM gain variants in Ku70\textsuperscript{−/−} GFP (left) and Ku70\textsuperscript{−/−} GANP\textsuperscript{OE} clones (right). Each horizontal line represents the IgV\textsubscript{L} sequence region of parental CL18. The bold bars show the GCV tracts and dots on the lines indicate point mutations. The \(\psi\) genes used as donors are shown on the left side. The number of clones with each pattern is indicated on the right side.
HR and NHEJ are the two conserved main pathways evolved to deal with DNA breaks, from yeast to vertebrates. HR plays a dominant role in DSB repair in yeast, whereas NHEJ is the predominant repair mechanism in vertebrates, including birds. The high activity and predominance of NHEJ necessitates the evolution of regulatory mechanisms to choose between HR or NHEJ in vertebrate cells (15). Although DT40 cells are commonly used for their very high rate of gene targeting through HR, NHEJ remains a predominant repair mechanism in DT40 cells. NHEJ repair poses significant competition or hindrance for GCV/HR, both at the level of GCV and IgV region diversification (19, 21, 53), as well as at the level of general HR in DSB repair (52). From our data, abrogating NHEJ in Ku70−/−, a core NHEJ molecule, greatly augments GCV/HR compared with DT40CL18GFP, a predominant repair mechanism in DT40 cells. NHEJ repair locally at the rearranged IgV region, rather than the defective transcription. These results signify that GANP supports GCV/HR through suppressing the NHEJ pathway.

FIGURE 6. DNA-PKcs dissociates from the GANP complex following induction of DNA DSBs. (A) Ramos B cell nuclear extracts were either untreated (−), treated with DNase I (DN), or treated with RNase A (RN) before IP. IgG was used as a negative control. (B) AID overexpression (AID+Etp) affects the interaction between GANP and DNA-PKcs. IP analysis of Ramos B cells after mock or AID transfection and small interfering (si)Control or siAICDA knockdown is shown. IP was performed with anti-GANP Ab and blotted with anti–DNA-PKcs and anti-GANP Abs. (C) DNA-PKcs dissociates from GANP after Etp treatment. Ramos B cells were incubated with 50 μM Etp for 2 h. After Etp treatment, cells were harvested at indicated time points (0, 4, and 8 h). Untreated whole-cell lysates (WCLs) (−) were used as a loading control. The relative GANP binding ratio with DNA-PKcs after Etp treatment was measured by densitometric analysis. (D) NU7026 does not affect the GANP/DNA-PKcs complex. Ramos B cells were either untreated (−) or Etp- (50 μM) or NU7026 (NU; 10 μM) treated before IP with anti-GANP Ab. IgG was used as a negative control for WB. (E) AID overexpression (AID+Etp) affects the interaction between GANP and DNA-PKcs. Ramos B cells were either untreated (−) or treated with Etp and NU before IP with anti-GANP Ab. Untreated WCLs (−) were used as loading controls. Data are from one of three independent experiments with similar results.

Many factors, including DNA-PKcs, can regulate the mechanistic overlap or competition between NHEJ and HR (16, 53–55, 68). DNA-PKcs suppresses both spontaneous and DSB-induced HR at the initial step of DNA repair (56). This was clearly demonstrated by its suppression of GCV in DT40 cells (19, 53). The promotion of GCV/HR by GANP (Fig. 1), together with its binding to DNA-PKcs (Fig. 6A) (34), supports a model in which the regulation of DNA-PKcs could promote GCV/HR through abrogating the competing NHEJ pathway (Fig. 7). GANP clearly suppresses NHEJ, as demonstrated using a LacZ construct (Fig. 4D), and promotes HR, as shown using a pDR-GFP construct (Fig. 4F). These results confirmed that GANP possesses a regulatory function in DNA repair in DT40 cells and mammalian cells. This is further solidified using two different DNA DSB inducers, Etp and Cpt. GANP+Eve rescued Cpt-induced cell death but markedly reduced the survival of Etp-treated cells (Fig. 4A). Thus, GANP could participate in the choice of DNA repair pathways at the actively transcribed genes in situ to use the adequate DNA repair response. Reduction of GCV/HR in GANP−/− cells may be partly due to defective transcription (Fig. 2B); nevertheless, NU7026 DNA-PKcs kinase inhibitor could markedly recover the GCV/HR rate in GANP−/− cells (Fig. 5A), indicating that diminished GCV/HR in these cells is largely due to the enhanced NHEJ function rather than the defective transcription. These results signify that GANP supports GCV/HR through suppressing the NHEJ pathway.

The DT40 cell is unique in the genomic IgV, region configuration with a single productive V segment and the ψV segments...
located in close proximity, which predominantly leads to GCV rather than SHM. In mammals, four types of molecular mechanisms deal with AID-initiated injuries: BER, MMR, HR, and NHEJ. With the exception of NHEJ, SHM was shown to be mediated through BER and MMR, and unexpectedly also through HR (12, 26), suggesting that the generation of SHM might be mediated on the balance of these DNA repair pathways and affected by the genomic gene configurations. GANP promotes IgV SHM in AIDΔNH2V and AIDΔUNG−/− cells, supporting that GANP is not acting as a negative regulator of SHM. Similar to BER and MMR, the HR repair is an error-free repair pathway by default; however, in particular conditions, these pathways are chosen by B cells and subverted to be mutagenic rather than faithful. During generation of IgV SHM (69, 70), B cells might often sustain extensive genomic alterations such as translocations, deletions, and duplications. GANP is not only involved in selective AID targeting to the rearranged IgV loci (33, 34), but also in the process of post-AID-mediated DNA breaks through promoting HR via abrogating NHEJ (Figs. 4, 5). This will favor the maintenance of the IgV region structure and its functional integrity by preventing translocations, truncations, and deletions of the rearranged Ig genes that inevitably cause the extinguishment of Ab maturation (19).

The effect of inactive DNA-PKcs on IgV SHM has been characterized in a system using a special mouse carrying the rearranged IgM-IgD, κ gene transgenes for hen egg lysozyme on the SCID background (71), because DNA-PKcs-null or inactive mice could not generate B cells. The mutant mice did not exhibit apparent changes in the total SHM frequency of germinal center B cells after immunization but demonstrated a change in SHM pattern with increased G·C mutations and decreased C·T mutations. In another study using a similar mutant mouse model, B cells from SCID mice similarly showed skewing of the mutation pattern, affecting C residues on the sense strand (72). These studies imply that total absence of DNA-PKcs may not influence the frequency of IgV SHM; nevertheless, kinase deficiency and probably functionally disturbed DNA-PKcs impact the mutation pattern.

To generate Ag-specific and high-affinity B cell clones, regulation of the repair of post-AID–initiated DNA damage is inevitable. Our data suggest that GANP is associated with AID and reduces the level of functionality of NHEJ at the sites of AID-mediated DNA breaks in situations where HR is required to induce repair, such as replication and/or transcription block, SHM, and GCV. DNA-PKcs might initiate immediate DNA repair at the damaged DNA region through activation of the NHEJ pathway when the DNA damage is minimal. However, the binding of GANP with DNA-PKcs, or its interaction with the NHEJ-dependent pathway, directs the repair toward the HR pathway at the transcriptionally active IgV region loci (Fig. 7). This would ensure, according to the local context, the choice of the proper repair pathway required for preserving viability of B cells and functional integrity of IgV region loci.

In summary, GANP plays multifunctional roles in regulation of IgV region diversification, as follows: 1) change of chromatin conformation, 2) recruitment and selective targeting of AID to the rearranged IgV region loci, and 3) choice of DNA repair pathway against post-AID–initiated DNA breaks. The latter would be beneficial to induce IgV region diversification for affinity maturation of germinal center B cells.

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Disclosures

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Supplemental Figure 1. Generation of GANP stable clones in DT40^{CL18} B cells. (A) Northern blot analysis of GANP gene transcripts. Total RNA (20 µg) purified from DT40^{CL18}GFP (left) and DT40^{CL18}GANP^{O/E} (right) clones was electrophoresed, and hybridized with the h-GANP- (upper) or ch-GANP- (middle) specific probe. Loading control is shown by ribosomal RNA (bottom). (B) Western blot analysis of overexpressed hGANP protein (upper), endogenous ch-GANP (middle), and β-actin (bottom).
Supplemental Figure 2. Generation of GANP-haplodeficient DT40^{CL18} clones. (A) Ch-GANP gene locus in the genome, the targeting construct, and the targeted locus are shown. The solid arrows indicate the locations and orientations of the primers for screening clones. (B) Screening of targeted clones of ch-GANP locus. (C) RT-PCR analysis for the expression of ch-GANP gene. Total RNA from GANP^{+/+} and GANP^{+-} cells was extracted, reverse-transcribed, and then amplified by PCR for ch-GANP (upper panel) or \(\beta\)-actin (bottom panel). (D) Western blot analysis of endogenous ch-GANP protein. Whole cell lysates from GANP^{+/+} and GANP^{+-} cells were electrophoresed, blotted and detected with anti-ch-GANP (upper) or anti-\(\beta\)-actin (bottom) Ab. The density value calculated by densitometry is shown based on the control signal set as 1.0.
Supplemental Figure 3. Mutations in the \(IgV_L\) sequence of GCV-deficient DT40 B cell lines by GANP. (A) Summary of \(IgV_L\) sequence analyses of AID\(^{R\psi V}\) and AID\(^{R\psi V}\)\_GANP\(^{O/E}\). (B) Cell viability assay among DT40 mutants clones. (C) Post culture of AID\(^{R\psi UNG}\)\_-/- cells. FACS profile of GFP\(^+\)\_sIgM\(^+\) positive population for 4 wk after sorting. (D) Reconstitution of AID in AID\^-/- cells. (E) Summary of \(IgV_L\) sequence analyses of of AID\(^{R\psi UNG}\)-/- and AID\(^{R\psi UNG}\)\_GANP\(^{O/E}\). (F) Distribution of mutations in \(IgV_L\). (G) Positions of mutations in \(IgV_L\) from AID\(^{R\psi UNG}\)-/-\_GANP\(^{O/E}\). Data show five independent clones. (H) Summary of AID hotspot and coldspot mutations. (I) qPCR analysis of chicken AID expression among GANP mutants DT40 cells.
Supplemental Figure 4. Effect of GANP on NHEJ-repair in Ku70⁻/⁻ mutant DT40 cells using lacZ DNA-repair construct. (A) Possible DNA repair patterns after DSB in pSVlacZ1351+727 reporter. (B) The rate of lacZ expression was measured after stably transfecting the “lacZ” repair construct in Ku70⁻/⁻ and DT40CL18 cells. (C) Cell cycle analysis of DT40CL18, GANP⁺/⁻ and GANP₀/E cells.