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

Mohammed Mansour Abbas Eid, Kazuhiko Maeda, Sarah Ameen Almofty, Shailendra Kumar Singh, Mayuko Shimoda, and Nobuo Sakaguchi

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 GANTP in the repair of AID-initiated DNA damage in chicken DT40 B cells to generate IgV region diversity by gene conversion and somatic hypermutation. GANTP plays a positive role in IgV region diversification of DT40 B cells in a nonhomologous end joining–proficient state. DNA-PKcs physically interacts with GANTP, and this interaction is dissociated by dsDNA breaks induced by a topoisomerase II inhibitor, etoposide, or AID overexpression. GANTP affects the choice of DNA repair mechanism in B cells toward homologous recombination rather than nonhomologous end joining repair. Thus, GANTP 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.

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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, stall factor Sp5, and DNA-PKcs.

DNA repair processes initiated by targeted cutting of DNA by AID/GANP complex in situ at the IgV region loci in germinal 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 diversity sensitization in B cells.

**Materials and Methods**

**Plasmid construction**

For GFP or GANP overexpression (GANP<sup>53E</sup>) transfectants, a GFP or GFP-GANP gene was introduced into pCXN2 (chicken β-actin promoter) vector containing a neomycin-resistant cassette (33). The AID expression vector (pPB-AID-ires-GFP) was a gift from Dr. Tasuku Honjo (Kyoto University, Kyoto, Japan).

**Antibodies**

The following Abs were purchased: histone H4 (L64C1; Cell Signaling Technology), acetyl histone H4 (3H18C4;1C10; Millipore), AID (E7 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 (Thr2609; 10B1; (BioLegend), Ku70 (GANP (A303; Bethyl Laboratories), DNA-PKcs (sc-5282; Santa Cruz Biotechnology), phospho–DNA-PKcs (Thr2609; 10B1; (BioLegend), Ku70

**DT40 cell lines and transfection**

DT40 CL18 (DT40<sup>CL18</sup>) (20), AID<sup>-/-</sup>v<sup>-/-</sup> (27), AID<sup>-/-</sup>UNG<sup>+</sup><sup>-/-</sup> (23), and Rad54<sup>-/-</sup> (35), Ku70<sup>+/+</sup> (36) cells were cultured in IMDM supplemented with 0.1 mM 2-ME, 10% FBS, and 1% chicken serum. Samples were prepared with anti-H4 (Chimp Laboratories), DNA-PKcs (sc-5282; Santa Cruz Biotechnology), and biotinylated mouse anti-chicken IgM (M1) (SouthernBiotech).

**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'-GGCTGCAGATGTTAGGACGCTTGCG-3') and Ex5S (5'-GGCTGCAGTTTGAAGTCGATCTGTTCGA-3') and Ex3P (5'-GGCTGCAGATGTTAGGACGCTTGCG-3') and Ex1S (5'-CAGAGGCGGTTCACGCTCCAACAATAAGTA-3') to detect the targeting vector region, and the primer set of 5'-untranslated region 2 (5'-GGATCAGCACGCTCATGCGG-3') and Puro (5'-CAGCGGGCGAGGCGAgGCGGCGACG-3') were used after 10 d. PCR screening was carried out for the drug-resistant clones using the primer set of Ex5S (5'-GGCTGCAGATGTTAGGACGCTTGCG-3') and Ex3P (5'-GGCTGCAGATGTTAGGACGCTTGCG-3') and ACTIN1 (5'-GGCTCCTAGATGTTAGGACGCTTGCG-3') and ACTIN2 (5'-GGCTCCTAGATGTTAGGACGCTTGCG-3') respectively.

**Chromatin immunoprecipitation–quantitative PCR**

GFP<sup>53E</sup> and GANP<sup>53E</sup> were treated with 1.25, 2.5, and 5 nM of TSA (Wako) for 2 wk in RPMI 1640 culture medium containing 0.1 mM 2-ME, 10% FBS, and 1% chicken serum. Samples were prepared with anti-H4 and anti-actin H4 Abs (Cell Signaling Technology), as previously described (33, 34), and examined using Mouse Blue pPCR MasterMix Plus (Eurogentec) on the Applied Biosystems 7500. Gene-specific primer sets were as follows: chicken AID, sense (5'-TAAGTACAAGGCGCCGATG-3') and antisense (5'-ACCATTGATGCGGTAGACG-3'), and chicken HPRT1, sense (5'-TATGTTTGGAAAC-
TGAGAGGACAAGT-3') and antisense (5'-ACTCACTGCTGTATATCTCATTACAG-3').

**DNA repair assay**

A modified pSVlacZ1351+727neo reporter was used for the NHEJ assay. Two HpaI sites of the β-galactosidase (lacZ) gene were mutated individually. Addition of two nucleotides converted the HpaI site to the XhoI site, which induced a frameshift and a nonfunctional lacZ gene. The vector of recombination substrate was constructed by tandem alignment of two differently mutated lacZ' (at the second HpaI site) and lacZ (at the first HpaI 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 5' GFP gene (SceGFP), mutated by insertion of an 18-mer restriction endonuclease site of I-SceI, restriction enzyme I-SceI, was used (39). The 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 concentrations 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 Ambion Nucleofector kit V (program O-06) according to the manufacturer’s protocol. The duplex siRNA against human AID (AICDA; 5'-UUCAGAGUUCUCGAGUUGG-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 two-tailed Student t test. A p value <0.05 was considered statistically significant.

**Results**

GANP augments IgVγ, GCV in DT40 B cells

We attempted to confirm the effect of GANP in an assay at the single cell level to measure IgVγ region diversification using chicken DT40 B cells. We examined GANP⁺/E by WB analysis of both endogenous chicken GANP and exogenous GANP following human GANP transfection of DT40 clone 18 (DT40CL18) cells. DT40CL18GANP⁺/E showed a 2- to 3-fold increase in GANP protein (Supplemental Fig. 1). DT40CL18 carries the rearranged IgVγ 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 DT40CL18GANP⁺/E clone significantly augmented the sIgM gain compared with the GFP-expressing DT40CL18 (DT40CL18GFP) clones (Fig. 1B). In comparison with the parental IgVγ sequence of DT40CL18, the genomic sequence of the IgVγ showed GCV tracts from ψVδ donor segments in all the sIgM⁺ cells (Fig. 1C). A higher rate of nonsense GCV tracts was found in sIgM⁺ DT40CL18GANP⁺/E cells that failed to correct the IgVγ sequence to an in-frame position compared with control DT40CL18GFP cells (Fig. 1D, after sequencing).

Next, we analyzed the usage of ψVγ genes as donor sequences, because a previous study demonstrated the preferential usage of ψV8, which showed the highest similarity to the rearranged IgVγ (43). DT40CL18GANP⁺/E cells used more diverse donor ψVγ genes with decreased usage of the preferential donor ψV8 gene (52%), compared with the donor usage profile of DT40CL18 (ψV8 gene, 68%) (Fig. 1C, 1E). Additionally, the median tract length was also increased to 99 bp in DT40CL18GANP⁺/E cells by 67 bp in DT40CL18GFP cells, representing a significant stabilization of GCV with a donor tract of longer length (Fig. 1F). These results indicate that GANP plays a role in IgVγ region diversification by regulating the GCV/HR mechanism.

GANP is required for IgVγ, 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 DT40CL18 (GANP⁺/+ ) 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 were estimated to be nearly half of parental DT40CL18 (GANP⁺/+ ) cells (Supplemental Fig. 2C, 2D). A subsequent attempt to delete another allele did not provide GANP-homodicient clones even after repeated experiments. This observation was reminiscent of the embryonic lethality of GANP-deficient mice (30), indicating that GANP expression is essential for the maintenance of cells in certain stages of differentiation. Thus, ch-GANP may play an essential role in cellular maintenance for DT40 B cells that are proliferating and undergoing IgVγ region diversification.

We compared the fluctuation of sIgM gain in the sIgM⁺ population from GANP⁺/+ and GANP⁺/- cells during culture. The sIgM gain was significantly decreased during the culture of GANP⁺/- cells compared with GANP⁺/+ cells (Fig. 2A). TSA is a histone deacetylase inhibitor, which increases IgVγ GCV (Fig. 2A), probably by activating the transcription rate through histone acetylation (37). TSA augments GCV significantly in GANP⁺/+ 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⁺/+ versus GANP⁺/- cells) affected IgVγ GCV induced by TSA. This implies that GANP has a unique function in modulating the generation of IgVγ 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 unarranged IgVγ loci using chromatin immunoprecipitation–quantitative PCR after TSA treatment of
GANP<sup>+/+</sup> and GANP<sup>+</sup>/− cells failed to acetylate H4 to the same level as did control GANP<sup>+/+</sup> cells, markedly at 2 wk (Fig. 2B). Reduced H4 acetylation in GANP<sup>+</sup>/− cells suggests that GANP plays a role in augmenting TSA-induced chromatin modification selectively at the rearranged IgV<sub>L</sub> gene. Sequence analysis showed that GCV was significantly reduced in GANP<sup>+</sup>/− cells (2.6%; 1 of 39 clones) compared with GANP<sup>+/+</sup> 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<sub>L</sub> SHM region diversity.

**GANP augments IgV<sub>L</sub> SHM in DT40 B cells**

AID induces IgV<sub>L</sub> 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<sub>L</sub> SHM. In the assay for IgV<sub>L</sub> SHM, we used another DT40 clone that expresses the productive IgV<sub>L</sub> mRNA to express slgM. IgV<sub>L</sub> SHM is represented by the loss of slgM from slgM<sup>+</sup> cells and by sequencing of IgV<sub>L</sub> in slgM<sup>−</sup> cells (27). We measured the effect of GANP in generation of IgV<sub>L</sub> SHM by introducing exogenous AID in DT40 B cells devoid of endogenous AID and ψV genes (AID<sup>−/−</sup>/ψV<sup>−</sup>). This type of cell is called AID<sup>R</sup> in the original report (27) (Fig. 3A). AID<sup>R</sup>/ψV<sup>−</sup> 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<sup>R</sup> augmented the slgM loss in AID<sup>−/−</sup>/ψV<sup>−</sup> cells (Fig. 3B), confirming that AID is a primary requirement for generation of IgV<sub>L</sub> SHM. GANPO/E significantly augmented slgM loss by nearly 2-fold compared with mock-transfected AID<sup>R</sup>/ψV<sup>−</sup> 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<sup>R</sup>/ψV<sup>−</sup> cells (Fig. 3C, 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<sup>−/−</sup>/UNG<sup>−/−</sup>) (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 2. Effect of GANP haplodeficiency in IgV_L, 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 IgV_L after TSA treatment. The acetylation status of histone H4 both active (solid line) and inactive (dashed line) IgV_L loci were measured between GANP+/+ (●) and GANP+/− (∆) cells. IgG was used as the negative control. (C) Analysis of IgV_L sequence between the GANP+/+ (upper) and GANP+/− (lower) clones after TSA treatment for 2 wk. Each horizontal line represents the IgV_L sequence region of CL18. The bold bars show the GCV tracts. The donor ψV genes used for the IgV_L GCV are shown on the left side. The number of clones with each pattern is indicated on the right side.

(G→A) and C (C→T) bases (22). Alternatively, the removal of U by UNG creates abasic sites followed by the nick and BER, creating transitions and transversions. UNG−/− cells predominantly create transition mutations at G:C. GANPΔOE did not cause any change in sIgM loss in AID−/− UNG−/− cells (Fig. 3E), confirming that GANP contributes to diversify IgV_L sequences in an AID-dependent manner. The viability of AIDΔ cells is similar to that of AID+/− cells even after GANPΔOE, indicating that AID expression is within harmless levels comparable to those of mutating B cells (Supplemental Fig. 3B). Notably, sequence analysis from slgM loss variants showed that GANP augmented the SHM frequency (nearly 10-fold) with the C→G mutation (55.3%; 779 of 1409) at the predicted sense-strand DNA of the rearranged IgV_L gene (Fig. 3F, Supplemental Fig. 3C–E), which might agree with the observation that UNG favors IgV_L GCV but decreases SHM as reported (23). The mutation profile after GANPΔOE showed predominance at G:C bases and mostly transitions similar to those of UNG−/− cells (23). The mutation profile also showed an increase in mutations at the putative CDRs (Supplemental Fig. 3F, 3G), which occurs selectively at the V region loci but not at the C region loci as GANP-mediated AID targeting (48). Supplemental Fig. 3H summarizes AID hotspot and coldspot preferences, suggesting that the specificity for hotspot targets is determined by AID itself. The transcription of chicken AID does not alter in GANP+/+ and GANP+/− DT40 cells (Supplemental Fig. 3I). These results emphasize the conclusion that GANP augments the frequency of AID-initiated IgV region SHM in B cells, presumably through facilitating AID recruitment (33) and relaxing chromatin (34).
the effect of GANP more directly on DNA repair mechanisms with two kinds of reporter construct. First, we used stable transfectants with pSVlacZ reporter cassette for measuring the NHEJ frequency by lacZ color changes after spontaneous DNA breaks (Fig. 4C). The pSVlacZ vector contains two tandemly aligned and mutated lacZ genes, lacZ′ and lacZ′′, with stop codons at the second HpaI and the first HpaI sites, respectively, precluding expression of productive lacZ. Deletion of the intervening segment led to reconstitution of the first HpaI sites, respectively, precluding expression of productive lacZ. Deletion of the intervening segment led to reconstitution of the functional lacZ gene (Supplemental Fig. 4A). GANPO/E cells were generated by introduction of GANP cDNA into AIDR V cells to rescue the lacZ color variants (Fig. 4D), probably as a consequence of suppressing NHEJ repair. The lacZ′′ frequency was markedly reduced in Ku70−/− 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 DNA-PKcs/GCV-mediated DNA repair by the expression of GFP signal after induction of DSBs with I-SceI endonuclease (39) (Fig. 4E). In the present study, GANPO/E cells without GFP tag were used. GANPO/E cells increased the frequency of GFP+ cells, indicating the positive effect of GANP on the DNA repair by GCV/Hr (Fig. 4F). The altered expression of GANP either in GANP/D transfectants or GANPO/E cells 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 IgVH GCV (19, 53) in DT40. We examined the effect of DNA-PKcs inhibitor NU7026 on spontaneous IgVH GCV in GANP+/+ and GANP+/− cells. NU7026 increased GCV rates by 2-fold in GANP+/+ cells (Fig. 5A). GANP+/− cells recovered the IgVH GCV to levels comparable with wild-type GANP+/+ cells in the presence of DNA-PKcs inhibitor. This supports that IgVH 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. IgVH GCV was examined in Rad54-deficient DT40CL18 B cells (Rad54−/−). Rad54−/− cells showed a decrease in IgVH GCV due to their defective HR-mediated DNA repair (Fig. 5B) (35). Overexpression of GANP in Rad54−/− cells

FIGURE 3. Regulation of IgVH SHM by GANP via an AID- and UNG-dependent mechanism in DT40 B cells. (A) Scheme of the experiment using AID−/ψV− cells. AID−ψV− cells were generated by reconstitution of the AID gene in AID−/ψV− cells (sIgM− background). AID−ψV− cells induced mutations at IgVH without GCV. AID−ψV− GANPO/E cells were generated by introduction of GANP gene into AID−ψV− cells. (B) The rate of sIgM loss from sIgM+ cells was clonally studied after culturing for 2 wk. Bars represent the median values. The difference between the rates of sIgM loss in AID−ψV− (n = 16) and AID−ψV− GANPO/E (n = 16) clones was statistically significant (p < 0.002). (C) Patterns of nucleotide substitutions within IgVH sequences from AID−ψV− (left) and AID−ψV− GANPO/E (right) clones. (D) Scheme of the experiment using AID−/ψ/−− cells. AID−/ψ/−− GANPO/E cells were generated by introduction of GANP cDNA into AID−/ψ/−− (sIgM+ background). AID−ψ/−− cells were generated by reconstitution of AID cDNA into AID−/ψ/−− cells. (E) The rate of sIgM loss variants was clonally studied after 1 wk. Each dot represents the percentage of sIgM− clones. Bars represent the median values. The difference between the rates of sIgM loss in AID−ψ/−− (n = 17) and AID−ψ/−− GANPO/E (n = 17) clones was statistically significant (p < 0.001). (F) Patterns of nucleotide substitutions within IgVH sequences from AID−ψ/−− (left) and AID−ψ/−− GANPO/E (right) clones. The sIgM loss variants were sorted.
significant (*Rad54\(^{5E}\)), supporting the notion that GANP plays a role in transfected (analysis of GFP expression in mock-transfected (Measurement of homology-directed DNA repair using flow cytometric transfected (48 h) to drugs relative to untreated cells. (\(k_B\)) clones. The GCV tract of Rad54\(^{5C}\)). The ability of GANP to rescue the GCV/HR-deficient cells is compatible with the proposed role of GANP in abrogation of the GCV/HR rate compared with the control Ku70\(^{-/-}\) cells (Fig. 5B). The GCV tract of Rad54\(^{5C}\) and DNA-PKcs might be prerequisite components assembled for DNA-PKcs is thought to be a molecular sensor for DNA damage repair capacity.

DNA-PKcs is thought to be a molecular sensor for DNA damage and the inducer of various DNA damage responses through phosphorylation of target molecules. A specific inhibitor of DNA-PKcs, NU7026, did not cause any change in the association of DNA-PKcs with DNase I or RNase A (Fig. 6A), indicating that their association is mediated through protein–protein interactions. DNA-PKcs is a core molecule in DNA break repair by NHEJ. We investigated 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 knockdown 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 dissociated during the DNA damage response, particularly at 4 h after DNA breaks. AID-initiated DNA damage shows implicit dependence 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 Thr\(^{2609}\) 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 Thr\(^{2609}\) (54), 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 Thr\(^{2609}\) 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 Thr\(^{2609}\), 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 phosphorylation 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 interaction is not dependent on the PI3K activity of DNA-PKcs itself. GANP and DNA-PKcs might be prerequisite components assembled for IgV region transcription-induced breaks involved in generation of IgV region diversity.

**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\(_C\) locus. Moreover, GANP and DNA-PKcs association suggests an important role for GANP in regulating DNA repair pathways against AID-initiated DSBs. The results demonstrate 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), and

FIGURE 5. Effect of GANP on IgVL GCV in mutant DT40 B cells with DNA repair deficiency. (A) The rate of sIgM gain was clonally studied after sorting of sIgM$^{-}$ cells and culturing for 2 wk. Each dot represents the percentage of sIgM$^{+}$ 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$^{+/+}$ 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$^{+/+}$ clones was statistically significant (**$p < 0.01$). The difference between the rates of sIgM gain in NU7026 treatment of GANP$^{+/+}$ ($n = 29$) and GANP$^{-/-}$ ($n = 20$) clones was statistically significant (**$p < 0.02$). (B) The rate of sIgM gain was clonally studied after sorting of sIgM$^{-}$ cells and culturing for 3 wk. Each dot represents the percentage of sIgM$^{+}$ cells. Solid bars represent the median values. The difference between the rates of sIgM gain in DT40CL18GFP ($n = 8$) and DT40CL18GANPO/E ($n = 8$) clones was statistically significant (*$p < 0.03$). The difference between the rates of sIgM gain in Rad54$^{-/-}$ GFP ($n = 9$) and Rad54$^{+/+}$ GANPO/E ($n = 9$) clones was statistically significant (**$p < 0.006$). (C) Summary of IgVL sequences analysis of sIgM gain variants in Rad54$^{-/-}$ GFP (left) and Rad54$^{-/-}$ GANPO/E clones (right). Each horizontal line represents the IgVL sequence region of parental CL18. The bold bars show the GCV tracts, and dots on the lines indicate point mutations. The $\psi V$ genes used as donors are shown on the left side. 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$^{-}$ cells and culturing for 3 wk. Each dot represents the percentage of sIgM$^{+}$ cells. Solid bars represent the median values. The difference between the rates of sIgM gain in DT40CL18GFP ($n = 9$) and DT40CL18GANPO/E ($n = 9$) clones was statistically significant (*$p < 0.03$). The difference between the rates of sIgM gain in Ku70$^{-/-}$ GFP ($n = 11$) and Ku70$^{-/-}$ GANPO/E ($n = 17$) clones was statistically significant (**$p < 0.001$). The difference between the rates of sIgM gain in DT40CL18GFP ($n = 9$) and DT40CL18GANPO/E ($n = 11$) clones was statistically significant (**$p < 0.02$). (E) Summary of IgVL sequence analyses of sIgM gain variants in Ku70$^{-/-}$ GFP (left) and Ku70$^{-/-}$ GANPO/E clones (right). Each horizontal line represents the IgVL sequence region of parental CL18. The bold bars show the GCV tracts and dots on the lines indicate point mutations. The $\psi V$ 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 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).

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 Ct. GANP′ 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 reduce 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, MMR, and unexpectedly also through HR (12, 26), suggesting that the generation of SHM might be generated on the balance of these DNA repair pathways and affected by the genomic gene configurations. GANP promotes IgV SHM in AID<sup>i</sup>Q<sup>V</sup>- and AID<sup>ΔUN</sup>G<sup>V</sup>-/− 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 aborting 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, κ 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 AIDRUNG. SHM has been characterized through BER, MMR, and unexpectedly also through HR to deal with AID-initiated injuries: BER, MMR, HR, and NHEJ. Annu. Rev. Immunol. 20: 173–181.


Disclosures
The authors have no financial conflicts of interest.

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