GANP regulates recruitment of AID to immunoglobulin variable regions by modulating transcription and nucleosome occupancy

Shailendra Kumar Singh1,*, Kazuhiko Maeda1,*, Mohammed Mansour Abbas Eid1, Sarah Ameen Almofty1, Masaya Ono2, Phuong Pham3, Myron F. Goodman3 & Nobuo Sakaguchi1

Somatic hypermutation in B cells is initiated by activation-induced cytidine deaminase-catalyzed C→U deamination at immunoglobulin variable regions. Here we investigate the role of the germinal centre-associated nuclear protein (GANP) in enhancing the access of activation-induced cytidine deaminase (AID) to immunoglobulin variable regions. We show that the nuclear export factor GANP is involved in chromatin modification at rearranged immunoglobulin variable loci, and its activity requires a histone acetyltransferase domain. GANP interacts with the transcription stalling protein Spt5 and facilitates RNA Pol-II recruitment to immunoglobulin variable regions. Germinal centre B cells from ganp-transgenic mice showed a higher AID occupancy at the immunoglobulin variable region, whereas B cells from conditional ganp-knockout mice exhibit a lower AID accessibility. These findings suggest that GANP-mediated chromatin modification promotes transcription complex recruitment and positioning at immunoglobulin variable loci to favour AID targeting.
Affinity-maturation of antibodies, which takes place in antigen (Ag)-driven B cells at germinal centres (GCs) of peripheral lymphoid organs is achieved through generation of somatic hypermutation (SHM) at immunoglobulin (Ig) variable (V)-region genes1–4. SHM depends on activation-induced cytidine deaminase (AID), which is specifically upregulated in GC B cells. AID initiates SHM by catalyzing C→U conversion at the rearranged Immunoglobulin variable (IgV)-locus as the initial step of SHM1–4 and class switch recombination5. AID is predominantly localized in the cytoplasm in activated B cells6 but is targeted preferentially to Ig genes in the nucleus through interaction with its recruiting partners. Interacting proteins that have been suggested in various assays to form a complex with AID include single-stranded (ss) DNA binding protein RPA (ref. 7), kinases DNA-PKcs (ref. 8) and Protein Kinase A (refs 9,10), adaptor protein 14-3-3 (ref. 11); binding protein RPA (ref. 7), kinases DNA-PKcs (ref. 8) and others such as EXP1/Crm1 (ref. 20), Hsp90 (ref. 21), Hsp40 DnaJα1 (ref. 22), MDM2 (ref. 23), eEF1A (ref. 24), REG14 (ref. 25), KAP1/Trim28 (refs 17, 26) and HP1 (ref. 26).

Germline centre-associated nuclear protein (GANP) associates with AID in the cytoplasm and facilitates its recruitment to the nucleus, eventually enhancing AID access to actively transcribed IgV-loci27. GANP expression is ubiquitous in mammalian cells but is increased in Ag-driven GC B cells28. B cells from GANP-deficient mice failed to generate high levels SHM at the rearranged IgV-locus and could not generate affinity-maturation of Ag-specific antibodies29. Reciprocally, transgenic overexpression of GANP increased the frequency of high-affinity Ab-producing cells against immunized Ags in vivo30. Thus, GANP is critical in generation of high-affinity antibodies in GC B cells during immune responses31.

GANP is a 210-kDa nuclear protein with multiple domains having different functions. A domain in the middle portion of GANP is homologous to the Saccharomyces Sac3 (refs 32, 33). Sac3 is a component of transcription-export complex 2 (TREX-2), a ribonucleoprotein (RNP) complex involved in mRNA export in yeast34. Besides the Sac3-homology domain, the N-terminal region of GANP is homologous to FG/SP-rich nucleoporin (Nup) proteins32,35. GANP carries a putative RNA recognition motif situated between the Nup domain and Sac-homology domain, and its C-terminus contains a histone-acetyltransferase (HAT) domain that regulates minichromosome maintenance protein 3 (MCM3)36. An alternatively spliced variant of GANP mRNA was reported in humans. A shorter isoform of GANP, GANP/MCM3AP (ref. 37), is likely to encode an 80-kDa protein associated with MCM3 of the DNA helicase MCM-complex composed of MCM2-MCM7 (refs 28, 38) and possesses HAT activity. Based on the Sac3-homology domain, GANP has been shown to play important roles in the mRNA export of mammalian cells35,39. However, the overall function of mammalian GANP has not been determined. In particular, the detailed molecular mechanism describing how GANP regulates SHM at the IgV-locus has largely remained unclear.

Here, we analyse GANP-interacting proteins in B cell nuclei by a proteomics strategy and study the function of GANP in the regulation of chromatin organization and interaction with the RNA Pol-II transcription complex at the IgV-region. Our data show that GANP is involved in histone modification and interaction with transcription elongation factors at the IgV-region, and that this allows for AID recruitment to IgV-loci during SHM in B cells.

Results
GANP-interacted proteins in hypermutating B cell nuclei. We performed a proteomics screen for GANP-interacting proteins in Ramos B cells that constitutively undergo a low level of SHM (ref. 40). Proteins extracted from nuclei were co-immunoprecipitated (IP) with different GANP antibodies and were subjected to analysis by two-dimensional image-converted analyses of liquid chromatography and mass spectrometry (2DICAL)41,42 (see Supplementary Fig. S1a). By comparing about 5,000 peptide peaks in anti-GANP co-IP precipitates to IgG co-IP control (Supplementary Fig. S1b), we identified over 100 peptide peaks present at significantly higher levels (P-value < 0.05) in GANP co-IP precipitates. The largest proportion of the peptides belongs to histones (28%), followed by proteins involved in RNA splicing/processing proteins (22%), ribosomal proteins (16%), nuclear envelope proteins (11%), RNA/DNA helicases (7%), chromatin regulators (4%), and DNA-dependent kinases (3%) (Fig. 1a and Supplementary Table S1 and S2). GANP interactions with several helicases DHX9, DDX21 and DDX39, nuclear envelope proteins Nups, DNA-PKcs, and DNA topoisomerase 2a were confirmed by co-IP assays (Supplementary Fig. S2).

Given the interaction of GANP with histones, we examined whether GANP is associated with chromatin in the nucleus. Nuclear protein extracts were subjected to differential chromatin fractionation using micrococcal nuclease (MNase) (Fig. 1b). Chromatin binding was confirmed for endogenous GANP in Ramos B cells (Fig. 1c). 293 T cells were transfected with GFP-GANP and used for chromatin fractionation. GFP-GANP was observed only in the insoluble (Insol) chromatin fraction but not in the soluble (Sol) chromatin-free fraction. Tight binding to chromatin was confirmed for GANP in 293 T cells (Fig. 1d) and verified by a GANP knockdown experiment (Fig. 1e). GANP knockdown led to a significant decrease in the level of GANP in the chromatin fraction, whereas the level of chromatin-associated RNA Pol-II control protein was not affected. Together, the data suggest that GANP interacts with histones and associates tightly with chromatin.

GANP affects assembly of nucleosomes at the IgV-regions. The C-terminus of GANP contains a domain with a high similarity to HAT (Fig. 2a)36,43. We examined whether the HAT domain of GANP (HATc) possesses histone acetylation activity and plays a role in B cells. First, a recombinant HATc tagged with the glutathione S-transferase at the N-terminus (GST-HATc) was expressed and purified from E. coli and incubated with histone H3 or H1 in the presence of acetyl-coenzyme A (Fig. 2a). Time dependent acetylation of histones H3 and H1 was observed in the presence of GST-HATc, but not GST-control, indicating that the HATc domain has histone acetylation activity (Fig. 2a).

Histone acetylation has been linked to dynamic changes in nucleosome organization that allow gene transcription upon activation44. We examined the effect of GANP on nucleosome assembly in Ramos B cells using a MNase sensitivity assay. Chromatin isolated from Ramos cells transfected with either GFP-GANP, or HAT-domain deleted GFP-GANP (GFP-AHATc) was treated with MNase and the resulting mononuclease and oligonucleosomal DNA was visualized using ethidium bromide (Fig. 2b). GFP-GANP overexpression markedly increased (about 4-fold) the amount of mononucleosomal and oligonucleosomal DNA released by MNase (Fig. 2b, left and right), whereas the amount of released mononucleosomal DNA was not increased in GFP-AHATc transfectants (Fig. 2b, left). Regarding chromatin modification as the whole, GANP overexpression, but not GFP-AHATc overexpression, causes a modest increase of histone H3

REFERENCES
NATURE COMMUNICATIONS | DOI: 10.1038/ncomms2823 | www.nature.com/naturecommunications

© 2013 Macmillan Publishers Limited. All rights reserved.
Acetylation, as a distinct ∼2-fold increase in acetylated H3K9 (Fig. 2b, right). The data indicate that GANP modulates chromatin assembly and its role in nucleosome organization requires the HATG domain with histone acetylation activity.

We investigated the relevance of GANP for nucleosome positioning at the rearranged IgV-coding exons. The effect of GFP-GANP on positioning of nucleosomes at the rearranged VH4(DP63)JH6 locus in Ramos B cells was examined by a MNase assay. Chromatin from GFP-GANP or GFP-alone transfectants was treated with MNase and the 15 overlapping DNA regions (100-bp each) covering the IgV-locus from the leader sequence were analysed by PCR (Fig. 3a). For transfectants with control GFP-alone, MNase resistance was observed at two specific sites of the rearranged IgV-locus: site A at the 5'-side (regions 4 and 5) covering the intron/exon junction and site B at the 3'-side site (regions 13, 14 and 15), situated close to the V-D-J joining site (Fig. 3a). Each site A and site B covers about 160 bp, suggesting that the coding IgV-region supports two stably assembled nucleosomes. GFP-GANP selectively lowered MNase resistance at site B but did not affect site A (Fig. 3a), indicating that GANP specifically disrupts assembly or stability of nucleosomes at the 3'-side of the IgV-locus. The effect of GANP on chromatin at site B depends on the HATG domain, as the MNase-resistant profile of the GFP-GANP-transfectant lacking the HATG domain (GFP-ΔHATG) is similar to the control transfectant (Fig. 3a). In the presence of transcription-elongation inhibitor actinomycin D (ActD)\(^45\), the MNase resistance of site B is significantly decreased, whereas site A is not affected (Fig. 3b), indicating that chromatin organization at the 3'-side of IgV undergoes changes during transcription.

As nucleosomes are assembled at site A and site B of the rearranged IgV-locus (Fig. 3a), we examined GANP association with chromatin at the DNA regions surrounding the promoter and leader sequences of IgV\(_H\) (regions q1, q2 and q3) and the regions at the V(D)J site (q4 and q5) (Fig. 3c). Ramos B cells were transfected with GFP-GANP, GFP-ΔHATG or GFP-alone and subjected to ChIP assay by an anti-GFP Ab. GFP-GANP preferentially interacts with the q5 region of IgV\(_H\) (Fig. 3d). GANP without the HATG domain does not exhibit a preference in binding to the q5 region, indicating that GANP association with the q5 region is dependent on the HATG domain (Fig. 3d).

### IgV-region chromatin modification regulated by GANP

We examined epigenetic chromatin modifications at the VH4(DP63)JH6 locus (Fig. 3c) by comparing the levels of histone H3 acetylation at Lys9 (H3K9ac) and Lys27 (H3K27ac) in each of the q1 to q5 regions (Fig. 3d). Modified histone H3 is present at higher concentrations in regions q2 (H3K9ac) and q3 (H3K9ac and H3K27ac) that span the promoter and the immediate downstream leader sequence. GANP overexpression caused significant increases in H3K9ac and H3K27ac at the q2 region (Fig. 3d, orange columns), H3K27ac at the q3 region, H3K9ac in the q4 region and H3K27ac at the q5 region. GANP appears to have no effect on chromatin modification at the promoter upstream region q1. The increase in histone H3 modifications at q2 to q5 regions is dependent on the HATG domain (Fig. 3d, green columns). Although GANP does not seem to possess histone-methyltransferase activity, GANP also increased the active transcription through chromatin modification with H3K4me3 presumably via regulation of methyltransferase activity (Supplementary Table S1 and Supplementary Fig. S3). The preferential association of GANP with the q5 region and its ability to interact with and acetylate histone H1 and H3 (Supplementary Table S2 and Fig. 2) suggest that GANP might be involved in regulation of acetylated H1K63ac and H3K27ac at q5 region.
Interaction of GANP with the IgV transcription complex. We investigated whether GANP interacts with the proteins involved in the transcription and targeting of AID to the IgV-locus. Co-IP analysis showed that RNA Pol-II and a transcription-stalling factor Spt5 associate with GANP in Ramos B cells (Fig. 4a), RNA Pol-II and Spt5 have been shown to interact with AID at IgV-loci\(^\text{16,18}\). The phosphorylation state of RNA Pol-II C-terminal domain (CTD) at Ser2 (pSer2) and Ser5 (pSer5) is often associated with changes in transcription stages\(^\text{46}\). Ser5 phosphorylation is required for the initiation of transcription at the promoter regions, whereas Ser2 phosphorylation is associated with mRNA elongation\(^\text{46}\). A ChIP assay with antibodies specific to pSer5 and pSer2 showed that GANP overexpression enhanced the binding of both pSer5 and pSer2 RNA Pol-II CTD to the q3–q5 region DNA of the IgV-region (Fig. 4b). GFP-ΔHAT\(_G\) transfectants with a HAT\(_G\) deletion, however, did not enhance RNA Pol-II binding to the IgV-region (q3–q5) (Fig. 4b), suggesting that the HAT\(_G\) domain has an important role in facilitating RNA Pol-II access to the IgV-locus, presumably through histone modification. The concentrations of pSer5 and pSer2 are low and appeared unaffected by GANP at q1 region (Fig. 4b).

Preferential association of GANP with chromatin at the q5 region (Fig. 3d) was further examined for endogenous GANP in Ramos B cells. A ChIP assay using antibodies targeting either the N-terminus (N1) or C-terminus (C1) of GANP confirmed a preferential interaction of GANP to the q5 region of the IgV\(_H\) (Fig. 4c). A ChIP assay was also used to examine the distribution of the histone H1, Spt5 and transcription pausing factor DSIF (Spt4-Spt5 complex) across the IgV-locus. The levels of unmodified histone H1 were similar in all q1 to q5 regions, but a significantly higher level of acetylated H1K63ac was observed at the q5 region (Fig. 4c). Similarly, the highest concentration of Spt5 was observed at the q5 region (Fig. 4c). In contrast to the enrichment of acetylated H1K63ac and Spt5 at the q5 region, the transcription insulator/repressor protein CTCF\(^\text{47}\) exhibited preferential binding at the q4 region and lowest binding to the q5 region (Fig. 4c), suggesting that CTCF accesses the IgV-locus in a different manner. Full-length GANP enhanced the accessibility of DSIF at all regions with DSIF concentration distributed more or less evenly from the q1 to q5, suggesting that GANP overexpression promotes early recruitment of the elongation pausing factor DSIF to the transcription complex (Fig. 4d). GANP-mediated DSIF recruitment to IgV\(_H\) is dependent on the HAT\(_G\) domain (Fig. 4d). The effect of GANP on recruitment of DSIF to the IgV gene has an important implication for a possible mechanism of AID targeting. Perhaps, GANP-facilitated recruitment of the transcription factor DSIF causes stalling of RNA Pol-II at the IgV-region, providing AID access to the ssDNA within the transcription bubbles.

Effects of GANP knockdown on IgV nucleosome occupancy. We examined the physiological relevance of GANP by siRNA
knockdown in Ramos B cells. Cy3 fluorescent-labelled siRNA (siGANP) targeting endogenous GANP severely reduced its expression in the sorted cells, but did not alter cellular levels of RNA Pol-II pSer2 and pSer5 (Fig. 5a). GANP knockdown caused a higher MNase resistance at both site A and site B of the IgV-locus (Fig. 5b). Consistent with GANP’s role in facilitating RNA Pol-II access to IgV (Fig. 4b), GANP downregulation led to a significant decrease in the levels of both pSer2 and pSer5 associated with each of the q1–q5 regions (Fig. 5c). Spt5 preferentially associates with the q5 region in control Ramos cells (Figs 4c, 5c), and it is noteworthy that there is a significant shift in the Spt5 positioning from the q5 to q3 region in GANP-knockdown cells (Fig. 5c).

The effects of GANP knockdown on the distribution of acetylated histones at the rearranged IgV-locus (Fig. 5c) are consistently opposite to those caused by GANP overexpression (Figs 3d, 4b–d). GANP knockdown and overexpression experiments also showed opposite effects on H3K9ac occupation at the regions q2 and q4 (Figs 3d, 5c). Together, both GANP knockdown (Fig. 5) and GANP overexpression data (Figs 3, 4) suggest that GANP has an important role in epigenetic modification of chromatin at rearranged IgV-loci.

**Role of GANP in the IgV-region SHM.** We examined whether the transient increase of GANP expression can affect SHM at the

![Figure 3](image_url)
IgV-locus. ChIP assay (Fig. 6 and Supplementary Fig. S4) was used to capture IgV-regions that associate with H3K9ac, CTCF, GANP or AID. Mutational analysis of the captured IgV DNA showed that GANP exerted no effect on SHM of IgV associated with H3K9ac and CTCF. These IgV DNA exhibit low SHM with a frequency of 0.3–1.0 mutations per 10$^3$ bp (Supplementary Fig. S3b,c). GANP-bound IgV DNA showed similarly low mutation frequency (<0.3 mutations per 10$^3$ bp)$^{27}$. However, GANP overexpression enhances SHM in AID-bound IgV DNA. The SHM frequency of AID-bound IgV increased 90% in the GFP-GANP transfecants (3.5 mutations per 10$^3$ bp) compared with that of GFP-alone transfecants (1.8 mutations per 10$^3$ bp) (Supplementary Fig. S4a). SHM in the AID-bound IgV DNA are about two-fold higher than IgV SHM in the input DNA, 1.72 mutations per 10$^3$ bp in GFP-GANP transfecants versus 0.85 mutations per 10$^3$ bp in GFP-control transfecants (Supplementary Fig. S4a). SHM in the AID-bound IgV DNA are significantly enhanced in GC B cells with higher GANP expres-

GANP enhances AID-accessibility at IgV-regions in GC B cell. We examined whether GANP affects the chromatin occupancy of AID at the rearranged IgV-locus in B cells during the immune response (Fig. 7). Mice with a conditional Ganp-knockout in CD19 B cells (CD19Cre/Ganp$^{	ext{F/F}}$), the control floxed/floxed C57BL/6 (Ganp$^{	ext{F/F}}$), the Ganp-gene transgenic B cells (Ganp$^{	ext{tg}}$) and the control C57BL/6 (WT) were immunized with nitrophenyl-chicken α-globulin (NP-CGG) in alum conjugates for 14 days and spleen GC B-cells were sorted by using mature GC B cell markers of B220, GL-7 and Fas (Supplementary Fig. S5). The mutant mice showed no measurable difference in Aicda transcription, but the transcription of the IgV$^{	ext{H186.2}}$-region was significantly enhanced in GC B cells with higher GANP expression from ganp$^{	ext{tg}}$ mice (Fig. 7a). In contrast, GC B cells from...
Ganp-deficient (CD19-Cre/Ganp<sup>F/F</sup>) mice showed significantly lower amounts of IgVH186.2-region transcripts (Fig. 7a). The chromatin occupancy was measured by a ChIP assay using a primer combination that amplifies the IgVH186.2-locus encoding the NP-binding IgV heavy chain. GANP overexpression showed a marked increase of AID-binding to the IgVH186.2-region, and particularly to the region that displayed the increased AID binding (Fig. 7b,d). Reciprocally, Ganp deficiency significantly decreased AID-binding to the IgV H186.2-region (Fig. 7c,d). The results indicated that GANP indeed helps AID to access the IgV-region in GC B cells.

Discussion
GANP has been characterized as a component of the mRNA export TREX-2-complex based on a homology comparison with yeast Sac3 (refs 35, 39). In mammals, the nascent mRNAs need to undergo extensive processing, which includes 5'-capping, splicing and 3'-end polyadenylation to become mature mRNAs. Only properly spliced and mature RNAs can be assembled into a RNP nuclear export complex, which is then exported through the nuclear pores to the cytoplasm (48). 2DICAL analysis revealed GANP interactions with proteins involved in mRNA processing and splicing factors and Nup proteins (Supplementary Table S1 and Supplementary Fig. S2b). These results are consistent with the role of GANP in nuclear export of mRNA (ref. 35). The increase of GANP in GC B cells during the immune response seems to have a distinct role through selective interaction with the rearranged IgV-loci and perhaps in the RNP complex undergoing the unique process of RNA metabolism with nuclear cytidine deaminases in higher eukaryote cells. This may imply a role for GANP distinct from that of the prototype Sac3 (refs 32, 33).

GANP appears to enhance chromatin modifications and it might have a significant role at the initial step of chromatin modification at the rearranged IgV loci. Particularly, the linker histone H1 was found to be associated with GANP by 2DICAL analysis (Supplementary Table S2). Modifications of the linker histone lead to the relaxation of the compact chromatin structure assembled with core histones. ChIP analysis showed that the acetylated histone H1 (H1K63ac) is found preferentially at the q5 region of the rearranged IgV-locus in B cells (Fig. 4c). A similar high concentration of endogenous GANP at the q5 region suggests that HATG domain of GANP is necessary to acetylate histone H1 at this region. The observed increase in acetylated histone H1, which unfolds the chromatin at the q5 region, is
consistent with an increase in mononucleosome release (Fig. 2b) and a higher MNase sensitivity at the 3′-side site B of IgVH in the presence of overexpressed GANP (Fig. 3b). The involvement of GANP in chromatin modification at the IgV-locus is further supported by experiments in which GANP is downregulated by siRNA. GANP knockdown (Fig. 5) and GANP overexpression (Figs 3, 4) lead to opposing effects on the levels of modified histones H1K63ac, H3K9ac and H3K27ac at specific regions within the IgV-locus. Although GANP prefers binding to the 3′-side of the IgV-locus (region q5) and exerts its strongest effects on chromatin modification and nucleosome assembly at site B, it also exerts significant effects on chromatin at other IgV-regions. For example, cells in which GANP is downregulated by siRNA (Fig. 5) exhibit higher resistance to MNase at both sites A and B. Changes in GANP expression also significantly affect the levels of modified histone H3 (H3K9ac and H3K27ac) at the promoter and leader sequences (regions q2 and q3) of the IgV-locus (Figs 3–5).

Transcription at the rearranged IgV-locus is regulated by cooperation of the B cell specific transcription factors operating at the 5′-promoter region and the intronic enhancer region. The chromatin at the Ig-locus inevitably undergoes alterations, transforming from the germ line structure to the functionally rearranged VH-DH-JH region by V(D)J recombination, which randomly joins V, D and J segments and cleaves out the long intervening V, D and J genomic segments. The altered chromatin folding along with extensive histone modification opens up the chromatin structure to allow access by RNA Pol-II and various factors.

Figure 6 | AID targeting and SHM mutation distribution at the rearranged IgV-locus. (a) Mutation spectra of AID-bound IgV-region, H3K9ac-bound IgV-region and CTCF-bound IgV-region in GFP-alone and GFP-GANP Ramos cell transfectants. DNAs isolated from anti-AID, anti-H3K9Ac and anti-CTCF Ab IP samples were subjected to sequencing analysis. SHM mutation profiles of IgV DNA starting from the primer 5 (DP63-05S) were shown. (b) Pie charts showing the distribution of numbers of mutations per a sequenced clone for AID-bound IgV-region from GFP-GANP and GFP-alone transfectants. Data are from one of three independent experiments with similar results.
transcription factors. Indeed, during VH-DH-JH recombination, the RAG-associated complex creates recombination centres containing increased concentration of H3K9ac, H3K4me3 and RNA Pol-II (refs 49, 50). In B cells, GANP is preferentially associated with the 3’-end of the rearranged VH-DH-JH exons (Fig. 4c). In the absence of exogenously expressed GANP, the transcription stall factor DSIF (Spt4–Spt5 complex) is also present at a highest concentration at the same site (Fig. 4c). Importantly, GANP overexpression significantly enhances DSIF association with the 3’-end of RNA Pol-II (refs 49, 50). In B cells, GANP is preferentially associated with the 3’-end of V-D-J junction (Fig. 4d). The uniform distribution of DSIF across these regions (Fig. 4d) suggests that GANP promotes early recruitment of DSIF to the IgV transcription complex. Early recruitment of DSIF at the promoter 5’-site of IgV may ensure the efficient induction of RNA Pol-II stalling throughout the entire IgV-coding region covering complementarity-determining regions 1 and 2. How GANP regulates early recruitment of DSIF to the IgV-locus is not yet clear. GANP interacts with Spt5 (Fig. 4a), and as both proteins associate preferentially with q5 (Figs 4c,5c), we suggest that GANP might be involved in the recruitment of Spt5 to q5. Alternatively, increased transcription of IgV-locus might also be responsible for recruitment of Spt5 or an Spt5-loading factor such as PAF1 (ref. 19). The role of Spt5 in transcription pausing and maintenance of R-loop has recently been proposed at the S-region of the Ig gene during class switch recombination15,16. Similar to the RNA Pol-II stalled model for increased AID access to the S-region, we propose that GANP promotes early recruitment of DSIF, enabling frequent pausing of the transcription complex along the IgV-locus. GANP is associated with the site B nucleosome position at the IgV-locus, the q5 region, and through GANP-AID protein–protein interaction27, recruits AID to the nearby stalled transcription bubbles at IgV (Fig. 8). Within the stalled bubbles, AID can access deoxyctydine on exposed ssDNA of the non-transcribed strand, or through interaction with an exosome complex to access the transcribed strand15.

Chromatin modification is probably essential for subsequent molecular steps for augmenting SHM in GC B cells. The 3’ nucleosome positioning site (site B) of the rearranged IgV exons may serve as an anchor site for recruitment of GANP, transcription factor Spt5 and other proteins involved in AID targeting. An increase in GANP expression promotes histone H3 modifications at IgV covering all regions from the promoter to the 3’-side (Fig. 3d). GANP-mediated chromatin remodelling at IgV likely provides better access for RNA Pol-II, reflected by

**Figure 7** GANP enhances the accessibility of AID to IgVr-region DNA in mouse GC B cells. (a) RT-qPCR analysis of Ganp, Aicda and IgVrh transcripts in GC B cells from WT, GanpF/F, GanpF/F and CD19-Cre/GanpF/F (n = 3 mice per genotype). (b) ChIP analysis of GC B cells of WT and GanpF/F. Input and ChIP samples were serially diluted 1:3 (wedge) and various genomic DNA sequences (left margin) were amplified with specific primers and were detected by ethidium bromide (Et-Br) staining. Anti-Histone H3 (H3) Ab was used as a positive control. (c) ChIP analysis in GC B cells of the control GanpF/F and Ganp-deficient CD19-Cre/GanpF/F, as in b. (d) ChIP-qPCR analysis of GC B cells from WT, GanpF/F, GanpF/F and CD19-Cre/GanpF/F. ChIP was amplified by qPCR with specific primers. Data (a, d) represent mean ± s.d. calculated from three independent experiments *P < 0.05 and **P < 0.01 (two-tailed unpaired t-test).

**Figure 8** A model of GANP-mediated AID targeting for SHM at the IgV-loci. GANP associates with histones H1 and H3, the IgV transcription-elongation complex, and the transcription stall factor Spt5 in Ramos B cells. The rearranged IgV-locus supports two stably assembled nucleosomes at site A and site B. GANP is involved in chromatin remodelling at the IgV-locus through its HAT domain and causes destabilization of nucleosome assembly at site B (1). Chromatin remodelled IgV provides increased access for both pSer5 and pSer2 forms of RNA Pol-II leading to an increase in IgV transcription (2). GANP facilitates early recruitment of the DSIF transcription stalling complex (Spt4–Spt5) to the IgV-coding region, causing frequent stalling of transcription bubbles, which provide ssDNA substrates for AID-targeting (3).
increases in both phosphorylated pSer5 and pSer2 forms (Fig. 4a). The early recruitment of DSIF to the IgV transcription complex results in an increased concentration of stalled transcription bubbles throughout the IgV-regions to which AID is targeted. Once AID is bound to the ssDNA substrate within the bubbles, once AID is bound to the ssDNA substrate within the bubbles, AID cleaves and deaminates the target DNA, which leads to the formation of IgV region.

B cells were stained with antibodies to GL-7, CD45R/B220 and CD95/Fas (BD (ab5131; Abcam), and pSer2/5 (no. 4735; CST) of RNA Pol-II CTD, DSIF (611106; (no. 4353; CST), CTCF (no. 3418; CST), H1 (ab71594; Abcam), H1K63ac (no. 4620, CST), H3K9ac (Y28, Millipore), H3K4me3 (no. 9751; CST), H3K27ac (no. 9733; Abcam). Cells were maintained in DMEM (for 293 T) and RPMI-1640 (for Ramos) supplemented with 10% heat-inactivated FBS (Invitrogen). Medium (for Ramos) supplemented with 10% heat-inactivated FBS (Invitrogen).

Cell culture

Preparation of native chromatin and nucleosomes. Native chromatin was isolated from Ramos cells by digestion with 10 U of MNase at 37°C for 5 min. The supernatant was collected and ammonium sulphate (0.33 g ml^{-1}) was added to precipitate nuclear proteins. The nuclear proteins were collected by centrifugation at 45,000 r.p.m. (60T1 rotor) for 3 h, the supernatant was collected and ammonium sulphate (0.33 g ml^{-1}) was added to precipitate nuclear proteins. The nuclear proteins were collected by centrifugation at 15,000 r.p.m. for 20 min and resuspended in buffer B (25 mM HEPES pH 7.9, 100 mM KCl, 12 mM MgCl2, 0.5 mM EDTA, 2 mM DTT, 17% (v/v) glycerol) followed by extensive dialysis against Buffer C to remove excess salt. Nuclei were harvested by centrifugation at 4°C. Nuclei were harvested by centrifugation at 4°C. Nuclei were harvested by centrifugation at 4°C. Nuclei were harvested by centrifugation at 4°C.

Preparation of recombinant proteins. Recombinant GST, and GST-HATC; proteins were purified from a BL21(DE3) E. coli strain transformed with pET41 vector. GST-fusion proteins were dialyzed in 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM DTT, 10% (v/v) glycerol and stored at −80°C.

Purification of crude histones. Core histones were purified from Ramos cells by using histone purification kit (Active Motif) according to the manufacturer’s protocol.

Nucleosome positioning assay. Crude nuclear extracts were treated with or without 10 U of MNase to digest the nucleosomes. After incubation at 37°C for 30 min, the genomic DNA was purified. The nucleosome positions recognized by the antibodies were determined by the specific amplification of the DNA region with 15 primer sets (Supplementary Table S3) by qPCR and shown as the ratio of the amount of digested/undigested DNAs. Ramos cells were treated with 0.1 μg of Aced (Nalacui Tesqce). The nuclei were obtained with gentle stirring on ice. After the pellet was washed twice with MNase digestion buffer (0.32 M sucrose, 50 mM Tris-HCl pH 7.5, 5 mM MgCl2, 1 mM CaCl2, 0.1 mM PMSF and 5 mM sodium butyrate), mononucleosomes were prepared by digestion with 10 U of MNase at 37°C for 5 min.

ChIP and qPCR. ChIP assay was performed as previously[27]. Knockdown experiment was carried as follows: 293 T plated in a 10-cm dish with 1 × 10^6 cells was transfected in OPTI-MEM GlutaMAX medium with 2 μM small interfering RNA (siRNA, Sigma, Supplementary Table S3) using lipofectamine RNAiMAX (Invitrogen) transfection reagent. For B cell knockdown experiment, cells were transfected with 2 μM of fluorescent Cy3-labelled control or GFP-targeted siRNA using the Amaxa Nucleofection Kit V (program O-06) and Cy3-positive cells were sorted.

Preparation of recombinant proteins. Recombinant GST, and GST-HATC; proteins were purified from a BL21(DE3) E. coli strain transformed with pET41 vector. GST-fusion proteins were dialyzed in 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM DTT, 10% (v/v) glycerol and stored at −80°C.

Purification of recombinant proteins. Recombinant GST, and GST-HATC; proteins were purified from a BL21(DE3) E. coli strain transformed with pET41 vector. GST-fusion proteins were dialyzed in 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM DTT, 10% (v/v) glycerol and stored at −80°C.

Purification of recombinant proteins. Recombinant GST, and GST-HATC; proteins were purified from a BL21(DE3) E. coli strain transformed with pET41 vector. GST-fusion proteins were dialyzed in 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM DTT, 10% (v/v) glycerol and stored at −80°C.

Purification of recombinant proteins. Recombinant GST, and GST-HATC; proteins were purified from a BL21(DE3) E. coli strain transformed with pET41 vector. GST-fusion proteins were dialyzed in 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM DTT, 10% (v/v) glycerol and stored at −80°C.

Purification of recombinant proteins. Recombinant GST, and GST-HATC; proteins were purified from a BL21(DE3) E. coli strain transformed with pET41 vector. GST-fusion proteins were dialyzed in 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM DTT, 10% (v/v) glycerol and stored at −80°C.

Purification of recombinant proteins. Recombinant GST, and GST-HATC; proteins were purified from a BL21(DE3) E. coli strain transformed with pET41 vector. GST-fusion proteins were dialyzed in 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM DTT, 10% (v/v) glycerol and stored at −80°C.

Purification of recombinant proteins. Recombinant GST, and GST-HATC; proteins were purified from a BL21(DE3) E. coli strain transformed with pET41 vector. GST-fusion proteins were dialyzed in 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM DTT, 10% (v/v) glycerol and stored at −80°C.

Purification of recombinant proteins. Recombinant GST, and GST-HATC; proteins were purified from a BL21(DE3) E. coli strain transformed with pET41 vector. GST-fusion proteins were dialyzed in 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM DTT, 10% (v/v) glycerol and stored at −80°C.
Mice. Gamp^{+/-} (C57BL/6)-Tg(NGANP) meg), WT. Gamp^{+/+} (Mcm3ap{+/-}Imku/ Mcm3ap{+/-}/Imku) and CD19-Cre/Gamp^{+/+} (Cd19^{+/-}Cre/Gamp^{+/+}) X Mcm3ap{+/-}Imku/ Mcm3ap{+/-}Imku/ mice were used. Isolation of GC B cells was described previously.67 All mice were maintained in the Centre for Animal Resources and Development, Kumamoto University. All studies and procedures were approved by the Kumamoto University Animal Care and Use Committee.

Statistical analysis. The statistical significance of differences between two groups was examined by an unpaired two-tailed Student’s t-test. P-values of less than 0.05 or 0.01 were considered significant and very significant, respectively.

References
1. Peled, J. U. et al. The biochemistry of somatic hypermutation. Annu. Rev. Immunol. 26, 481–511 (2008).
2. Odegard, V. H. & Schatz, D. G. Targeting of somatic hypermutation. Annu. Rev. Immunol. 6, 573–583 (2006).
3. Di Noia, J. M. & Neuberger, M. S. Molecular mechanisms of antibody somatic hypermutation. Annu. Rev. Biochem. 76, 1–22 (2007).
4. Maul, R. W. & Gearhart, P. J. AID and somatic hypermutation. Adv. Immunol. 105, 159–191 (2010).
5. Stavnezer, J., Guikema, J. E. & Schrader, C. E. Mechanism and regulation of AID and somatic hypermutation. Annu. Rev. Immunol. 18, 7920–7925 (2011).
6. Pavri, R. et al. Deoxyadenosine 5’-triphosphate complex to dock at the nucleoplasmic entrance of the nuclear pores. EMBO J. 21, 5843–5852 (2002).
7. Gallardo, M., Luna, R., Erdjument-Bromage, H., Tempst, P. & Aguilera, A. Nab2p and the Thp1p-Sac3p complex functionally interact at the interface between transcription and mRNA metabolism. J. Biol. Chem. 278, 24225–24232 (2003).
8. Takei, Y. & Tsujimoto, G. Identification of a novel MCM3-associated protein that facilitates MCM3 nuclear localization. J. Biol. Chem. 273, 22177–22180 (1998).
9. Jani, D. et al. Functional and structural characterization of the mammalian TREX-2 complex that links transcription with nuclear messenger RNA export. Nucleic Acids Res. 40, 4562–4573 (2012).
10. Sale, J. E. & Neuberger, M. S. Tdt-accessible breaks are scattered over the immunoglobulin V domain in a constitutively hypermutating B cell line. Immunology 99, 859–869 (1999).
11. Nakamura, Y. et al. Label-free quantitative proteomics using large peptide data sets generated by nanoflow liquid chromatography and mass spectrometry. Mol. Cell. Proteomics 5, 1338–1347 (2006).
12. Ono, M. et al. Prolly 4-hydroxylation of alpha-fibrinogen: a novel protein modification revealed by plasma proteomics. J. Biol. Chem. 284, 29041–29049 (2009).
13. Yang, X. J. The diverse superfamily of lysine acetyltransferases and their roles in leukemia and other diseases. Nucleic Acids Res. 32, 959–976 (2004).
14. Clayton, A. L., Hazzalin, C. A. & Mahadevan, L. C. Enhanced histone acetylation and transcription: a dynamic perspective. Mol. Cell. 23, 289–296 (2006).
15. Solch, H. M. & Actinomycin, D. N. A. transcription. Proc. Natl Acad. Sci. USA 82, 5328–5331 (1985).
16. Hocine, S., Singer, R. H. & Grunwald, D. RNA processing and export. Cold Spring Harb. Perspect. Biol. 2, a007520 (2010).
17. Filippova, G. N. et al. An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-myc oncogenes. Mol. Cell. Biol. 16, 2802–2813 (1996).
18. Blobel, G. Gene gating: a hypothesis. Proc. Natl Acad. Sci. USA 82, 8527–8529 (1985).
19. Schatz, D. G. & Ji, Y. Recombination centres and the orchestration of V(D)J recombination. Nat. Rev. Immunol. 11, 251–263 (2011).
20. Schatz, D. G. & Swanson, P. C. V(D)J recombination: mechanisms of initiation. Annu. Rev. Genet. 45, 167–202 (2011).
21. Pham, P., Calabrese, P., Park, S. J. & Goodman, M. F. Analysis of a single-stranded DNA-scanning process in which activation-induced deoxycytidine deaminase (AID) deaminates C to U haphazardly and inefficiently to ensure mutational diversity. J. Biol. Chem. 286, 24931–24942 (2011).
22. Kuninger, D., Lundblad, J., Semirale, A. & Rotwein, P. A non-isotopic in vitro assay for histone acetylation. J. Biotechnol. 131, 253–260 (2007).

Acknowledgements
We thank Y. Fukushima for assistance and the supports by the Ministry of Education, Culture, Sports, Science and Technology of Japan (22001036 to N.S.), the Japan Society for the Promotion of Science (23390122, 24659224 to N.S.; 22590436 to K.M.), a Global COE program (Global Education and Research Centre Aiming at the control of AIDS, Kumamoto University) (to K.M. and N.S.). M.O. is supported by the program for Promotion of Fundamental Studies in Health Sciences conducted by the National Institute of Biomedical Innovation of Japan, the Third-TERM Comprehensive Control Research for Cancer and Research on Biological Markers for New Drug Development.
conducted by the Ministry of Health, Labour and Welfare of Japan. P.P. and M.F.G. are supported by US National Institutes of Health grants ES13192 and GM21422.

Author contributions
K.M. and N.S. conceived and designed the study; S.K.S., K.M., S.A.A. and M.M.A.E. did experiments; K.M., M.O., P.P., M.F.G. and N.S. analysed the data; M.O. contributed reagents/materials/analysis tools; K.M., P.P., M.F.G., and N.S. wrote the manuscript.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Singh, S. K. et al. GANP regulates recruitment of AID to immunoglobulin variable regions by modulating transcription and nucleosome occupancy. Nat. Commun. 4:1830 doi: 10.1038/ncomms2823 (2013).

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/3.0/