DNA Methylation Dynamics of Germinal Center B Cells Are Mediated by AID

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**DNA Methylation Dynamics of Germinal Center B Cells Are Mediated by AID**

**Highlights**

- B cell transition through the GC is characterized by marked loss of DNA methylation
- B cell transition through the GC is associated with increased methylome diversity
- AID is essential for demethylation and diversification of methylome in GCBs
- AID-dependent epigenetic hotspots are located in genes required for B cell function

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**In Brief**

Dominguez et al. use mouse models to study changes in DNA methylation in B cells during entry into germinal centers in vivo. They determine that site-specific demethylation and diversification of methylome in germinal center B cells are dependent on AID function. AID-dependent epigenetic hotspots affect genes important for lymphocyte development.

**Accession Numbers**

GSE71702

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**Graphical Abstract**

Hypomethylation

Epigenetic heterogeneity

- Aicda⁻/⁻ GCB
- AICDA WT GCB

AID

DNMT1

unmethylated C

methylated C
SUMMARY
Changes in DNA methylation are required for the formation of germinal centers (GCs), but the mechanisms of such changes are poorly understood. Activation-induced cytidine deaminase (AID) has been recently implicated in DNA demethylation through its deaminase activity coupled with DNA repair. We investigated the epigenetic function of AID in vivo in germinal center B cells (GCBs) isolated from wild-type (WT) and AID-deficient (Aicda−/−) mice. We determined that the transit of B cells through the GC is associated with marked locus-specific loss of methylation and increased methylation diversity, both of which are lost in Aicda−/− animals. Differentially methylated cytosines (DMCs) between GCBs and naive B cells (NBs) are enriched in genes that are targeted for somatic hypermutation (SHM) by AID, and these genes form networks required for B cell development and proliferation. Finally, we observed significant conservation of AID-dependent epigenetic reprogramming between mouse and human B cells.

INTRODUCTION
DNA methylation is an epigenetic modification that regulates genomic imprinting, X chromosome inactivation, and cell fates during development (Smith and Meissner, 2013) and chromosomal stability, repression of transposable elements, and gene expression during the lifetime of the organism (Bird, 2002; Jones and Takai, 2001). The majority of the DNA methylation in mammalian genomes takes place within a CpG dinucleotide context, and 5-methyl-C has been called a fifth base in the genome (Novik et al., 2002).

B cell development is associated with significant plasticity and changes of the DNA methylome, occurring from lymphoid commitment of hematopoietic stem cells in the bone marrow to peripheral B cell maturation in the secondary lymphoid organs (Hodges et al., 2011; Ji et al., 2010; Zilbauer et al., 2013). The transition from naive B cells (NBs) to germinal center B cells (GCBs), critical for affinity maturation and generation of an improved, long-lasting immune response, is accompanied by marked demethylation of the genome and more heterogeneous DNA methylation patterning (Lai et al., 2013; Shaknovich et al., 2011). The mechanisms of these modifications of the methylome remain poorly understood and, therefore, we set out to understand how these changes arise in B cells.

In contrast to DNA methylation gain, the identities of the enzymes that catalyze DNA demethylation have largely remained elusive. Activation-induced cytidine deaminase (AID) is highly expressed in GCBs and is necessary for the antigen-dependent activation process by which NBs transition through the germinal center (GC) of the secondary lymphoid organs (Bunting and Melnick, 2013; Klein and Dalla-Favera, 2008). AID converts deoxycytosines (dCs) into deoxyuracils (dUs), producing dU:dG mismatches that are removed by both mismatch repair and base excision repair (Honjo et al., 2005; Zan and Casali, 2013). These repair processes are required for somatic hypermutation (SHM) and class switch recombination (CSR) of immunoglobulin (Ig) genes, necessary steps of affinity maturation within the GC (Conticello, 2008; Muramatsu et al., 2000; Zan and Casali, 2013). Interestingly, AID function is not restricted to Ig loci, and 25% of highly expressed genes in GCBs are targeted by AID (Liu et al., 2008). Moreover, anti-AID chromatin immunoprecipitation (ChIP) experiments showed a genome-wide recruitment of AID in ex vivo activated B cells (Yamane et al., 2011).
Figure 1. Loss of AID Abrogates CpG Methylation Changes during GC Transition
(A) Combined CpG methylation of NBs and GCBs from WT mice (seven replicates; left) and Aicda−/− mice (six replicates; right), determined by ERRBS using a 20% methylation difference threshold and FDR < 0.001, Fisher’s exact test. Hypo-DMCs are indicated in blue, and hyper-DMCs are indicated in yellow. (B) Number of DMCs between GCBs and NBs from WT and Aicda−/− mice determined by ERRBS using a 20% methylation difference threshold and FDR < 0.001, Fisher’s exact test. Hypo-DMCs are indicated in blue, and hyper-DMCs are indicated in yellow. (A) and (B) show that the DNA methylation changes after GC transition in WT mice are abrogated in Aicda−/− mice. (C) Density plot showing delta methylation values (GCB% − NB%) determined by ERRBS in WT and Aicda−/− mice. Aicda−/− delta methylation values are decreased compared to WT, indicating less changes in methylation during GC transition. See also Figure S1.

Several studies in non-lymphoid tissues have demonstrated that AID can also participate in loss of methylation. AID has been implicated in DNA demethylation during zebrafish development (Rai et al., 2008), reprogramming in heterokaryons (Shutani et al., 2010) and pluripotent germ cells (Popp et al., 2010), and late reprogramming of induced pluripotent stem cells in mice (Kumar et al., 2013). The mechanism by which AID demethylates is not completely elucidated, although it is thought to occur via deaminase activity followed by base excision DNA repair and replacement with unmethylated C (Klein and Dalla-Favera, 2008; Küppers, 2005).

No DNA demethylation role for AID has yet been uncovered in vivo in B cells, although several lines of evidence point to such function. We previously demonstrated that hypomethylated regions in human GCBs were enriched for the putative AID binding site RGYW (Shaknovich et al., 2011) and that hypomethylation in GCB-derived lymphomas correlated with AID expression (De et al., 2013). Considering these observations in light of the AID demethylation role in other cell types, we examined the epigenetic function of AID in GCBs. In this study, we establish that AID functions as an epigenetic modifier by promoting loss of DNA methylation and increasing methylation diversity during the GC stage of B cell maturation in vivo in human and murine B cells.

RESULTS

Loss of AID Abrogates CpG Methylation Changes during GC Transition
We previously observed significant loss of DNA methylation in human GCBs (Lai et al., 2013; Shaknovich et al., 2011). We hypothesized that AID would be at least partly responsible for this decrease. To investigate the role of AID in the genome-wide methylation changes occurring during NB to GCB transition, we induced T cell-dependent GC formation with 4-NP-chicken gamma globulin (NP-CGG) in wild-type (WT) (seven replicates) and Aicda−/− (six replicates) mice. Mice were sacrificed at day 10 post-injection, and splenic NBs (B220+GL7+CD95+) and GCBs (B220+GL7+CD95−) were isolated. To profile the methylome of NBs and GCBs, we performed enhanced reduced representation sequencing (ERRBS), an efficient single-nucleotide resolution high-throughput technique that interrogates 2–4 million distinct CpGs (Akalin et al., 2012). Upon rigorous quality control of bisulfite conversion (>99.5% in all samples) and read mapping frequency (>70%), we called differentially methylated CpGs (DMCs) between NBs and GCBs using a combination of statistical difference (false discovery rate [FDR] < 0.001 using Fisher’s exact test) and methylation level difference greater than 20% (see Experimental Procedures). We observed that NB to GCB transition in WT mice was accompanied by significant changes in DNA methylation, including 8,308 hypomethylated DMCs (hypo-DMCs) and 3,390 hypermethylated DMCs (hyper-DMCs) (Figures 1A and 1B). These changes were independent of class-switched B cell receptors, since unswitched (IgM+), GCBs and total GCBs presented similar patterns of methylation (data not shown). This is consistent with our previous results showing a genome-wide loss of methylation in primary human GCB samples compared to NBs (Shaknovich et al., 2011). On the contrary, our profiling of Aicda−/− animals resulted in minimal observed changes in DNA methylation during the transition from NBs to GCBs: only 703 of CpGs revealed hypomethylation and...
also found that during their transit through the GC. For the majority of the methylome changes that B cells undergo (D) CpG methylation variance from MassARRAY data. The histogram shows the difference in variance between WT and (C) Density plot comparing pairwise methylation distance between NBs and GCBs from WT and Aicda-/- mice. NB to GCB transition in Aicda-/- mice is associated with lower pairwise distance than in WT mice, indicating less diversity between Aicda-/- NB and GCB profiles. (D) CpG methylation variance from MassARRAY data. The histogram shows the difference in variance between WT and Aicda-/- mice (varΔWT – varΔAicda-/-), with varΔ being the log-fold change in methylation variance during NB to GCB transition in WT or Aicda-/- cells. Variance in WT animals is statistically greater than in AID-deficient animals (positive values). See also Figures S2 and S3.

172 CpGs revealed hypermethylation (Figures 1A and 1B). We also found that Aicda-/- mice had reduced global methylation differences during the NB to GCB transition, indicating that loss of AID also resulted in less methylene plasticity at non-differentially methylated CpGs (Figure 1C). This occurred despite comparable ERRBS coverage in WT and Aicda-/- cells and similar global methylation levels, as measured using liquid chromatography-mass spectrometry (LC-MS), in NBs from WT and Aicda-/- mice (Figure S1). LC-MS analysis also revealed higher genome-wide levels of 5mC in Aicda-/- GCBs compared to WT GCBs (Figure S1). Our results indicate that AID is responsible for the majority of the methylene changes that B cells undergo during their transit through the GC.

**AID Contributes to Epigenetic Diversity within GCBs**

We hypothesized that AID might also be responsible for the previously described increased methylation diversity in GCBs compared to NBs (De et al., 2013). To that end, we evaluated the epigenetic diversity within the NB group and GCB group compared to NBs (De et al., 2013). To that end, we evaluated the epigenetic diversity within the NB group and GCB group from WT and Aicda-/- mice, calculating all pairwise distances between ERRBS profiles (see Experimental Procedures). We found that WT GCB replicates had greater pairwise methylation distance to each other than WT NB samples had to each other, corresponding to higher average methylation diversity (Figures 2A and 2B; NB WT:GCB WT, Wilcoxon p = 4.5e-09). This is consistent with epigenetic diversification of B cells during their passage through the GC. Importantly, Aicda-/- GCB replicates
displayed significantly lower intra-group methylation distance than WT GCB replicates (Figures 2A and 2B; GCB WT:GCB Aicda−/−, Wilcoxon p = 3.58e-10), indicating that loss of AID results in a more homogeneous GCB methylome, closer to the methylome of Aicda−/− NBs (Figures 2A and 2B; NB Aicda−/−:GCB Aicda−/−, Wilcoxon p = 0.00323). We also found lower pairwise methylation distance during transition from Aicda−/− NBs to GCBs than from WT NBs to GCBs (Figure 2C; Wilcoxon p = 1.76e-20). This decreased diversity is consistent with the abrogation of methylation changes during the NB to GCB transition observed in AID-deficient mice (see Figure 1C).

To further validate these findings, we utilized an orthogonal DNA methylation quantification approach based on Sequenom’s MassARRAY Epityper, which detects the mass difference between methylated and unmethylated CpGs using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. We performed MassARRAY on ten randomly selected genomic loci spanning 30 kb in three replicate pairs of NBs and GCBs from WT and Aicda−/− mice. We then calculated log-fold change in methylation variance to determine whether methylation diversity increased during the NB to GCB transition in WT (varΔWT) and Aicda−/− cells (varΔAicda−/−).

Most studied CpGs had greater methylation variance augmentation in WT as compared to Aicda−/− cells (positive values) during GC transition (Figures 2D and S2). These results altogether strongly suggest that, in addition to its established role in SHM and CSR, AID contributes to the diversification of DNA methylation in the GCB genome.

Previous experiments with AID depletions within an ex vivo system showed no methylation changes (Fritz et al., 2013). In order to reconcile our in vivo AID-dependent methylation changes with these earlier observations, we activated CD43+ splenic WT cells (NB) in the presence of lipopolysaccharide (LPS), interleukin-4 (IL-4), and anti-CD40 and infected them with either empty vector (EV) or a vector expressing the full-length Aicda cDNA (AID). We confirmed by qPCR and western blot that the AID-overexpressing cells expressed higher levels of AID than EV-infected cells (Figure S3A). Moreover, we detected a higher percentage of class-switched splenocytes when AID was overexpressed (Figures S3B and S3C). We performed ERRBS profiling on sorted GFP+IgG1+ cells (class-switched infected cells) and observed few differences in methylation between AID-overexpressing and EV-infected B cells, with less than 1,000 hyper- and hypomethylated CpGs (class-switched infected cells) (Figure S3D). We also calculated the pairwise methylation distance and found a high degree of homogeneity among all sample ERRBS profiles, suggesting that methylation diversity between AID-overexpressing and EV-infected B cells was similar and comparable to NB diversity (Figure S3E). These results support the previous findings obtained by Fritz et al. (Fritz et al., 2013).

The ex vivo stimulated B cells differ from GCBs in that they present significantly reduced levels of SHM—predominantly targeting the Sμ region—compared to GCBs induced in vivo (Liu et al., 2008; McKean et al., 1984; Robbiani et al., 2009). A reduced rate of SHM and the absence of methylyme modifications in ex vivo activated B cells support the hypothesis of convergence of the mechanisms for SHM and methylome editing.

**Methylation Changes in Aicda−/− GCBs Are Not Due to Changes in the Cellular Composition or Clonality within the GC**

In order to rule out the possibility that abrogation of methylation changes in Aicda−/− GCBs arises due to changes in the cellular composition within the GC (content of centroblasts [CBs] versus centrocytes [CCs]) or clonal diversity, we carried out detailed analysis of GCBs from WT and Aicda−/− animals. Flow cytometry analysis of the spleen confirmed hyperplasia of the GC in Aicda−/− mice (Figure S3F), in agreement with previous reports (Muramatsu et al., 2000; Robbiani et al., 2009). Both WT and Aicda−/− animals had the same proportion of CB (CXCR4high) and CC (CXCR4low) within the GC (Figure S3G). To confirm this result, we performed RNA sequencing (RNA-seq) on GCBs isolated from WT and Aicda−/− mice and compared their expression profiles for the genes that constitute the CB (dark zone [DZ]) and CC (light zone [LZ]) signatures, identified by Victora et al. (Victora et al., 2010) (Figure S3H). The expression for these CB- and CC-specific genes was highly correlated between WT and Aicda−/− cells (Pearson correlation coefficient = 0.984 for DZ genes and 0.989 for LZ genes), indicating that both genotypes had comparable gene expression profiles. In addition, we investigated whether there were any differences in clonal complexity in the GC between WT and Aicda−/− mice. For that purpose, we amplified rearranged IgH, Igκ, and Igλ regions using primers capturing the most abundant families of Ig rearrangements (Chang et al., 1992; Cabaleza et al., 2007; Schlissel et al., 1991) and performed high-throughput sequencing using the Illumina MiSeq (PE2x150). Statistical analysis of the Ig rearrangements (see Experimental Procedures) revealed no significant difference in clonal complexity and in composition of VH regions between WT GCBs and Aicda−/− GCBs (p = 0.8571, Wilcoxon rank sum test) (Figure S3I). Altogether, these findings indicate that the composition and the clonality of WT and AID-deficient GCBs are equivalent.

**AID-Dependent Hypo-DMCs Are Enriched at SHM Hotspot Genes and dsDNA Breaks**

To investigate the genomic distribution of AID-dependent methylaton changes, we defined AID-dependent hypomethylated (blue rectangle, Figure 3A) or hypermethylated (yellow rectangle, Figure 3A) DMCs as CpGs that are hypomethylated (blue rectangle, Figure 3A) or hypermethylated (yellow rectangle, Figure 3A) during NB to GCB transition in WT animals but show no respective differential methylation changes in Aicda−/− animals. We found that these AID-dependent hypo- and hyper-DMCs were significantly depleted in promoters of genes and enriched in introns and intergenic areas (binomial test, p < 0.001) (Figure 3B). AID-dependent hypo- and hyper-DMCs were also depleted in CpG islands, shores, and shelves and enriched in open sea (binomial test, p < 0.001) (Figure 3C). Since AID-dependent DNA demethylation is thought to be carried out via deamination and subsequent DNA repair, similar to SHM, we investigated whether AID-dependent DMCs were enriched in genes reported to be targets of SHM in GCBs (Liu et al., 2008). Interestingly, AID-dependent hypo-DMCs were enriched in SHM hotspot genes (binomial test, p < 0.001) (Figure 3D). As SHM occurs at highly expressed genes, we also tested if AID-dependent DMCs were enriched in genes highly expressed in WT GCBs (fragments per kilobase of
We found no enrichment for hypo-DMCs (data not shown), suggesting that hypo-methylation results from AID targeting specific genomic loci, not simply as a consequence of open chromatin structure or regions with high transcriptional activity. We also found enrichment of AID-dependent hypo-DMCs in AID-associated double-stranded DNA (dsDNA) breaks identified using high-throughput genomic translocation sequencing (Meng et al., 2014) (Fisher’s exact test, p < 0.001) (Figure 3D) and in loci associated with double-strand breaks defined through γ-H2AX occupancy (Barlow et al., 2013) (Fisher’s exact test, p < 0.001) (Figure 3D), suggesting an association between AID-dependent hypomethylation and DNA breaks.

Endogenous retroviruses are present in multiple copies in mammalian genomes and constitute up to a staggering 8% of human and mouse genomic DNA (gDNA) (Ryan, 2004; Stocking and Kozak, 2008). As AID-dependent DMCs are enriched in introns and intergenic regions (Figure 3B), we investigated whether AID targets repetitive elements present in those regions of the genome. We annotated our AID-dependent DMCs according to RepeatMasker (see Experimental Procedures) and identified six intergenic repetitive elements that were significantly enriched for the presence of AID-dependent DMCs, including L1 repeat element, IAPEY3_LTR, and MLT1J1 (Figure S4A).

We also found two intragenic repetitive elements that were significantly enriched to contain AID-dependent DMCs (Figure S4B). In summary, although we do find cases of enrichment at specific repetitive elements, AID acts mostly upon non-repetitive DNA sequences.
Epigenetic Hotspots and Effect on Gene Expression

We next investigated if, similar to SHM targets, there were hotspots of AID epigenetic activity in GCBs. In order to identify such “epigenetic hotspots,” we looked for differentially methylated regions (DMRs) based on the presence of at least five DMCs, a maximum distance of 250 kb between DMCs, and at least 10% difference between average methylation within the region. We identified 119 DMRs between NBs and GCBs from WT mice, distributed throughout all chromosomes and consisting predominantly of hypo-DMRs (Figure 4A, left). We observed that these DMRs were mostly AID dependent, and 88 hypo-DMRs and 16 hyper-DMRs were lost in Aicda−/− animals (Figure 4A, right; Table S1), in agreement with our results from the analysis of DMCs (Figure 1). We validated these findings using the MassARRAY Epityper on several loci and on replicates of WT and AID-deficient NBs and GCBs. Figure S5A shows that the transition from NB to GCB in Aicda−/− mice is accompanied by lower demethylation in AID-dependent DMRs compared to WT mice. As a result, the percentage of methylation in Aicda−/− GCBs is statistically higher than in WT GCBs although still lower than in Aicda−/− NBs due to AID-independent demethylation mechanisms (Figure S5A).

In order to understand the significance of AID-dependent methylation changes, we evaluated the effect on gene expression in NB and GCB using RNA-seq data for all RefSeq genes. We found that although there is a clear difference in expression profiles according to cell type, there is no significant difference in profiles according to AID genotype (Figures S5B and SSC). However, we did identify several genes containing AID-dependent hypo-DMRs, including B cell-relevant genes (e.g., Cdk6, Abcc4, and FancA) and known targets of SHM (e.g., Pax5 and Cd83), that exhibit small gene expression differences between the respective genotypes (Figure 4B). These genes represent epigenetic targets of AID, which may be deregulated not only by SHM or translocations but also via changes in DNA methylation. We also analyzed the expression of genes that have been implicated in DNA methylation (DNMTs, TETs, and IDHs) and found no significant difference between WT and Aicda−/− cells (data not shown). An unsupervised pathway analysis revealed that AID-dependent hypomethylated genes are involved in pathways critical for B lymphocyte development, such as proliferation, B cell commitment, lymphocyte arrest of differentiation, and antibody response (Figures 4C and 4D). To assess whether DMR-associated genes have decreased diversity of expression in Aicda−/− GCBs, we calculated inter-sample pairwise correlation distances based on the expression of genes associated with AID-dependent hypo-DMRs. We observed that AID-dependent hypo-DMR-associated genes show a trend toward decreased heterogeneity of expression in the Aicda−/− GCBs compared to WT GCBs (Figure 4E). Even though this change is not statistically significant, the potential biological consequence of epigenetic diversification may be deregulated gene expression and predisposition toward neoplastic transformation (Hansen et al., 2011).

We further explored the phenotypical consequences of the AID-dependent DNA methylation diversity. We hypothesized that if CpG methylation diversity was functional, it would not be randomly distributed throughout the genome but rather associated with specific gene expression patterns and specific gene function. To address this, we investigated whether the GCB methylome contained any hotspots for CpGs with high methylation variability. We defined “divergent CpG hotspots” as contiguous 1-kb regions enriched for divergent CpGs (methylation interquartile range > 25% among replicates; Kemp et al., 2014) using a hypergeometric mean distribution test. We found 6,952 divergent CpG hotspots in WT GCB replicates and only 788 hotspots in Aicda−/− GCBs (Figure S5D). We also observed that 96.7% (n = 6,725) of these divergent CpG hotspots were AID dependent, as they were lost in the Aicda−/− animals (Figure S5D). Interestingly, AID-dependent divergent CpG hotspots were located mostly in introns, distal regions, and intergenic areas (Figure S5E), similarly to AID-dependent DMCs (Figure 3B).

A gene expression analysis revealed that Aicda−/− GCBs had higher expression in genes overlapping divergent CpG hotspots (n = 3,055) than WT GCBs (gene set enrichment analysis FDR q < 0.001; Figure S5F). Pathway analysis of these genes showed enrichment in pathways regulating caspase activity and innate and adaptive immune responses (Figure S5G).

To further elucidate the biological consequences of the AID-mediated epigenetic changes in the GCB genome, we investigated whether the methylome differences in WT and Aicda−/− GCBs affected the proliferation and/or differentiation capacity of these populations. We observed no differences in the cell-cycle distribution based on bromodeoxyuridine (BrDU) incorporation and 7-AAD staining of GCBs from WT and Aicda−/− mice (Figure S5H). Although there was an increase in the percentage of GCB in the Aicda−/− mice, we observed a reduction in the NP-specific population, indicating a defect in the formation of antigen-specific GCBs in the absence of AID (Figure S5I). We also found that Aicda−/− GCBs presented lower expression of the genes that are normally expressed upon terminal differentiation into plasma and memory cells when compared to WT GCBs (Figure S5J). Even though the cause of such effect on plasmacytic differentiation in Aicda−/− mice is rooted in the absence of AID-dependent affinity maturation, further investigation of the contribution of DNA methylation to this process is warranted.

Conserved Epigenetic Function of AID between Human and Mouse B Cells

To investigate whether the AID epigenetic program in mouse GC was conserved in human GC, we sorted NBs (CD20−IgD−CD77−) and GCBs (CD20−IgD−CD77+) from reactive human tonsils (see Experimental Procedures) and profiled their methylome using ERRBS. Using the same criteria applied to mouse data to call DMCs, we confirmed that human GCBs also underwent extensive hypomethylation compared to NBs and displayed greater epigenetic diversity than NBs. Even though this change is not statistically significant, the potential biological consequence of epigenetic diversification may be deregulated gene expression and predisposition toward neoplastic transformation (Hansen et al., 2011).

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Figure 4. AID Epigenetic Hotspots and Effect on Gene Expression

(A) Ideogram of DMRs in WT (left) and Aicda−/− (right) cells showing depletion of DMRs in Aicda−/− cells. Hypo-DMRs are indicated in blue, and hyper-DMRs are indicated in yellow.

(B) Genes overlapping AID-dependent hypo-DMRs with >1.2-fold GCB/NB expression ratio between WT and Aicda−/− cells. Upregulated genes in Aicda−/− cells are shown in red, and downregulated genes in Aicda−/− cells are shown in blue.

(C) Pathway analysis (IPA) of the genes overlapping AID-dependent hypo-DMRs. The graph shows the name and the functional annotations of the genes with hypo-DMRs in WT cells that are absent in Aicda−/− cells.

(D) Heatmap showing significance of enrichment of the pathways identified by IPA, using hypergeometric p values.

(E) Density plot showing expression correlation distance for genes overlapping AID-dependent hypo-DMRs between WT and Aicda−/− GCB replicates. Variability of gene expression is lower in Aicda−/− GCBs.

See also Figure S5.
Figure 5. Conserved Epigenetic Function of AID between Human and Mouse B Cells

(A) Combined CpG methylation values (top) and DMCs (bottom) between NBs and GCBs from human tonsils (four replicates) determined by ERRBS using a 20% methylation difference threshold and FDR < 0.001 (Fisher’s exact test). Hypomethylated CpGs are indicated in blue, and hypermethylated CpGs are indicated in yellow.

(B) Heatmap showing pairwise methylation distance between ERRBS profiles of NB and GCB replicates from human tonsils with GCBs showing greater intra-sample methylation distance (green versus yellow).

(C) Bar plot showing genomic distribution of hypo-DMCs and hyper-DMCs as well as all CpGs represented within ERRBS experiments. DMCs show depletion in promoters and enrichment in introns and distal and intergenic regions.

(legend continued on next page)
in vivo WT and we hypothesized that AID is involved in the active demethylation (Kumar et al., 2013; Popp et al., 2010; Rai et al., 2008), development and epigenetic reprogramming (Bhutani et al., 2008). Since AID is highly expressed in GCBs and completely unknown. Since AID is highly expressed in GCBs (Shaknovich et al., 2011). While the mechanism of the methylome to these processes is not clearly defined.

**DISCUSSION**

In the present study, we have demonstrated using a genome-wide approach that B cell transit through the GC is accompanied by locus-specific hypomethylation and minor gains of methylation, along with a substantial increase in DNA methylation diversity. More importantly, our results indicate that such changes are largely mediated by AID. In the last decade, we have gained in-depth knowledge regarding the function of epigenetic alterations in normal development and cancer biology. DNA methylation is understood to play a key role in gene imprinting, X chromosome inactivation, and regulation of gene expression specific to tissue identity, developmental stage, and cell lineage (Bird, 2002; Jones and Takai, 2001). Changes in the DNA methylome mark specific stages of B cell ontogeny and play an important role in B cell lymphomagenesis (De et al., 2013; Jeong et al., 2014; Kulsh et al., 2012; Lai et al., 2013; Mayle et al., 2014; Shaknovich et al., 2010; Zhang et al., 2014). The GC stage of B cell development is associated with a proliferative burst, affinity maturation of B cells with associated SHM and CSR, all of which contribute to adaptive immune response and determine antibody diversity (Klein and Dalla-Favera, 2008), but the contribution of the methylome to these processes is not clearly defined. Previously, we showed that changes in methylation are required for the successful formation of the GC and that such modifications are dependent on DNMT1, a methyltransferase highly expressed in GCBs (Shaknovich et al., 2011). While the mechanism of DNA methylation gain is well understood, the mechanism of demethylation, the factors responsible for the loss of methylation in GCBs, and its biological significance remain almost completely unknown. Since AID is highly expressed in GCBs and has been implicated in DNA demethylation during embryonic development and epigenetic reprogramming (Bhutani et al., 2010; Kumar et al., 2013; Popp et al., 2010; Rai et al., 2008), we hypothesized that AID is involved in the active demethylation of B cells during GC transition.

To prove this hypothesis, we isolated NBs and GCBs from in vivo WT and *Aicda^−/−* mice and profiled their methylome using ERRBS, a genome-wide approach capable of interrogating three million CpGs. We observed that over 90% of methylome alterations characterizing the transition from NBs to GCBs were lost in *Aicda^−/−* animals, confirming the role of AID in the DNA demethylation of the GCB genome. We also found that AID depletion caused loss of hypermethylation in GCBs. We suspect this to be a result of reduced recruitment of DNMT1 to double-strand breaks (Ha et al., 2011), putatively generated as a consequence of the AID deamination activity (Zan and Casali, 2008).

Several prior attempts to link AID to demethylation in GCBs were made before. Fritz et al. addressed this same question using an ex vivo system, activating primary splenocytes in the presence of anti-CD40, LPS, and IL-4 (Fritz et al., 2013). The authors could not detect AID-induced changes in the B cell methylome, consistent with our results with ex vivo stimulated B cells. This suggests that AID-dependent demethylation is coupled to the rate of SHM, which is much lower in the ex vivo system than in GCBs (McKean et al., 1984; Robbiani et al., 2008). In this regard, it has recently been demonstrated that ex vivo stimulated B cells are defective in SHM because the initiating form of RNA polymerase II is not retained in the variable regions of the Ig genes, hampering the recruitment of the cofactor Spt5 and AID (Maul et al., 2014). Another attempt to delineate the demethylation function of AID in GCBs was made by Hogenbirk et al. using MethylCap sequencing (MethylCap-seq) and failed to find any AID-dependent changes (Hogenbirk et al., 2013). MethylCap-seq is an affinity-purification-based technique, which is likely not to be sufficiently sensitive to detect variable methylation changes in CpGs scattered throughout genome. Here, we have used ERRBS, a genome-wide technique with higher coverage compared to MethylCap-seq and single-nucleotide-level resolution (Rodriguez et al., 2012). We think that the above differences are due to the experimental system and the techniques used in earlier studies. Confirmation of AID-dependent changes that we identified using the MassARRAY Epityper validates ERRBS-based findings.

Importantly, we have demonstrated that the epigenetic diversification of the B cell methylome during GC transition is dependent on AID activity. It is tempting to speculate that this methylation diversification may contribute, along with SHM, to clonal evolution among normal GCBs. We show here that the genomic distribution of hypo-DMRs in GCBs is similar to the distribution of AID binding sites revealed by Liu et al. (Liu et al., 2008). We also provide circumstantial evidence that the demethylase function of AID may arise from its deaminase activity, showing that AID-dependent hypo-DMCs are enriched within known AID target genes for SHM. Despite expectation that AID-dependent differential methylation would be concentrated around transcription start sites (TSSs) of genes, similar to SHM hotspots, our data reveal that DMCs are enriched in gene introns and intergenic regions. This is consistent with the location of AID-dependent demethylation observed in other systems (Kumar et al., 2013; Popp et al., 2010). We also found enrichment of AID-dependent hypo-DMCs at loci associated with dsDNA breaks. It is possible that, despite the intense focus on SHM target genes, AID may bind genome-wide, with the majority of binding similarly distributed outside of TSS and gene bodies.
This would suggest AID deamination activity to have more far-reaching consequences than we have yet appreciated. It has been proposed that AID-dependent regions of demethylation may extend beyond the deamination sites as a result of the activity of processive DNA repair pathways (mismatch repair or long-patch base excision repair). These pathways can result in the replacement of long stretches of DNA (up to 2 kb) with concomitant possible repair of all somatic mutations (Franchini et al., 2014). Such broad extension of hypomethylation could, in turn, have various consequences, including instability of transposable elements, chromosomal translocations, and gene deregulation, as suggested by the Jaenisch’s group (Gaudet et al., 2003).

In the present study, we were able to detect subtle effects of AID on genes associated with AID-dependent DMRs, including greater variability of gene expression of these epigenetic targets and at the same time consistent changes in a handful of epigenetic hotspots that include genes also targeted by SHM, such as Pax5 and Cd83. The up- and downregulation of gene expression between WT and Aicda−/− cells may be explained by the DMR location in relation to the TSS, since promoter versus gene body methylation may have opposite effects on gene expression (Jaenisch and Bird, 2003), or it may be a secondary effect of a permissive environment for gene regulation through binding of transcription factors or through histone modifications.

There is a significant effect on expression in genes overlapping divergent CpG hotspots—loci with high methylation variability—indicating that AID-dependent methylation diversity is not random and affects specific functions. There is also a detectable statistically significant hypomethylation of repetitive elements, with still uncertain consequences to be investigated in future work.

Although AID loss abrogates the majority of the methylation changes experienced by GCB, we observe residual hypomethylation in Aicda−/− GCBs, suggesting that other demethylation mechanisms are likely to exist in these cells. The most plausible scenario is TET-dependent oxidative demethylation, which has been proposed as an alternative to AID deamination-dependent demethylation (Kohli and Zhang, 2013). Another source of demethylation may be passive loss of methylation in highly proliferative GCBs. The fact that Aicda−/− GCBs are hyperplastic and highly proliferative, and nevertheless have minimal loss of methylation, argues against this theory. Moreover, passive stochastic loss of methylation would likely be randomly distributed throughout the genome, and our results show preferential genomic distribution of the methylation changes. All mechanisms will need more formal examination before the final model of DNA demethylation in GCBs is formulated, but our results indicate for the first time a clear epigenetic role for AID in B cell maturation during GC transition.

**EXPERIMENTAL PROCEDURES**

**Mouse and Human B Cell Isolation**

Aicda−/− mice were a generous gift from T. Honjo. WT (BALB/c) mice were from The Jackson Laboratory. All animals were maintained according to the guidelines of the Research Animal Resource Center of the Weill Cornell Medical College, which approved all mouse procedures. 10- to 12-week-old WT or Aicda−/− mice were immunized intraperitoneally with NP-CGG ratio 20-25 (Biosearch Technologies) in alum (1:1) to induce GC formation. Mice were sacrificed at day 10 after immunization, spleens were dissected, and mononuclear cells were purified using Histopaque (Sigma) gradient centrifugation. Cell suspensions were enriched in B cells by positive selection with anti-B220 magnetic microbeads (Miltenyi Biotech). B cells were separated in NB (B220+GL7+FAS+DAPI−) and GCB (B220+GL7+FAS+DAPI+) using a BD FACSAria II sorter.

Leftover human tonsils were obtained after routine tonsillectomies, performed at New York Presbyterian Hospital. All tissue collection was approved by the Weill Cornell Medical College Institutional Review Board. Tonsils were minced and mononuclear cells were isolated using Histopaque density centrifugation. NBs were separated by positive selection using the AUTOMACS system (Miltenyi Biotech) after incubation with anti-IgD fluorescein isothiocyanate (FITC) (BD Pharmingen) followed by anti-FITC microbeads (Miltenyi Biotec). GCBs were separated by positive selection with anti-CD77 (AbD Serotec) followed by mouse anti-IgM, IgG1 isotype (BD Pharmingen), and anti-mouse-Cy7 microbeads (Miltenyi Biotec).

**Ex Vivo Activated B Cell Cultures**

Activation of B cells and infection with EV or AID-expressing vector was performed as described in Supplementary Experimental Procedures.

**Flow Cytometry Analysis and Antibodies**

Flow cytometry analysis of mouse NBs and GCBs was performed using the following fluorescent-labeled anti-mouse antibodies: APC-conjugated anti-B220 (BD Pharmingen), PE-Cy7-conjugated anti-CD95 (BD Pharmingen), FITC-conjugated anti-GL7 (BD Pharmingen), and PE-conjugated CXCR4 (eBioscience). Cell-cycle analysis was performed using the BruU Flow Kit (BD Pharmingen), and antigen-specific GCBs (NP+GL7+CD95+B220+) were detected using PE-conjugated NP (Biosearch Technologies). Ex vivo stimulated B cells were stained with PE-Cy7-conjugated anti-B220 (eBioscience), PE-conjugated anti-IgD (BD Pharmingen), and APC-conjugated anti-IgG1 (BD Pharmingen). DAPI was used for the exclusion of dead cells. Data were acquired on a MACSQuant analyzer (Miltenyi Biotec) and analyzed using FlowJo 7.8.4 software (Tree Star).

**ERRBS**

50 ng gDNA was bisulfite converted using the EZ DNA Methylation kit (Zymo Research). Base-pair-resolution DNA methylation analysis was performed in WT mice (n = 7, 3 males 4 females) and Aicda−/− mice (n = 6, 3 males and 3 females) following the ERRBS protocol previously described (Akalin et al., 2012). DMGs were identified by using a 20% methylation difference threshold and a Benjamini-Hochberg-adjusted Fisher’s exact test p value < 0.001 on sum of all methylated and unmethylated CpGs among replicate samples. Delta methylation was calculated by subtracting the combined NB methylation percent from the combined GCB methylation percent. DMGs were detected using RBBSeqeer with parameters of 250 bp maximum distance between DMGs, minimum of five DMGs per region, and 10% total methylation difference for region. Ideogram was generated using the ggbio package (Yin et al., 2012) and UCSC GRCm38 CytoBand data from the Rcircos package (Zhang et al., 2013). Epigenetic diversity and diverse CpG hotspots were calculated as described in Supplementary Experimental Procedures.

**Single-Locus DNA Methylation Assays**

EpiTYPEPER assays were performed on bisulfite-converted gDNA. For the biologic validation of the AID-dependent hypo-DMRs in mouse B cells, primers were designed to cover CpG islands associated with the respective DMGs. All primers were designed using Sequenom EpiDesigner BETA software (http://www.epidesigner.com/). Primer sequences are shown in Supplementary Experimental Procedures.

**Mass-Array-Based Variance Comparisons**

We performed three pairwise comparisons of methylation variances: (1) AID_KO_NB versus AID_KO_GCB, (2) AID_WT_GCB versus AID_KO_GCB, and (3) AID_WT_NB versus AID_WT_GCB. For each pairwise comparison, we calculated the sample variances in each of the two groups across all
Cytosine Methylation Mass Spectrometry
1 μg genomic DNA was denatured by heating at 100°C. 5 U Nuclease P1 (Sigma) was added, and the mixture was incubated at 37°C for 2 hr. A 1/10 volume of 1 M ammonium bicarbonate and 0.002 U venom phosphodiesterase 1 (Sigma) was added to the mixture and the incubation continued for 2 hr at 37°C. Then, 0.5 U alkaline phosphatase (Roche) was added and the mixture was incubated for 1 hr at 37°C. Quantification was done using an LC-ESI-MS/MS system (Agilent 1200 Series liquid chromatography machine in tandem with the Agilent 6410 Triple Quad Mass Spectrometer) in multiple reaction monitoring (MRM) mode as described previously (Song et al., 2005). Chromatographic separation was performed at a flow rate of 220 l/min.

VDJ Rearrangement Analysis
Ig rearrangement analysis was performed on gDNA of mouse WT GCB (n = 3) and Aicda<sup>−/−</sup> mice GCB (n = 4), amplifying IgH, Igκ, and Igλ regions by PCR. Primers annealed to the framework region of the most abundant families of Ig rearrangements, as described previously (Chang et al., 1992; Cobaleda et al., 2007; Schissel et al., 1991). Primer sequences have been described previously (Wanna et al., 2008). Ig rearrangement analysis was performed as described in Supplemental Experimental Procedures.

RNA-Seq
RNA-seq was carried out as described in Supplemental Experimental Procedures.

Pathway Enrichment Analysis
Ingenuity Pathway Analysis (IPA; QIAGEN) was used for the functional analysis of the genes containing AID-dependent hypo-DMRs. For each pathway, we determined the extent to which the pathway was over-represented in the target gene group, using the hypergeometric distribution test. Pathway enrichment for AID-dependent divergent CpG hotspot genes was performed using the iPAGE program (Goodarzi et al., 2009) and gene sets from the Gene Ontology (electronic annotations were not included).

Real-Time qPCR
cDNA synthesis from RNA was performed using the Verso cDNA Synthesis kit (Thermo Scientific). The expression was detected using the Green FastMix kit (Applied Biosystems) on a 7900HT Fast RT-PCR System (Applied Biosystems). Gene expression was normalized to RPL13 using the ΔΔCt method, and results were represented as fold expression compared to NBs. Primer sequences are shown in Supplemental Experimental Procedures.

Immunoblotting
Total cell extracts were prepared after treatment with lysis buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 1% Triton X-100, 0.1% SDS, and 1mM EDTA) supplemented with PMSF (Sigma) and a protease inhibitor cocktail (Roche). Lysates were subjected to SDS-PAGE, transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories), and blotted with anti-AID (LE7, Cell Signaling Technology) or anti-actin (A5441, Sigma). Signals were detected with horseradish-peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) using the ECL system (Thermo Scientific).

ACCESSION NUMBERS
The data discussed in this article have been deposited in NCBI’s Gene Expression Omnibus and are accessible through accession number GEO: GSE71702.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at  http://dx.doi.org/10.1016/j.celrep.2015.08.036.


