Distinct Requirements of CHD4 during B Cell Development and Antibody Response

Highlights

- CHD4 is essential for early B cell development
- Naive, mature B cells can sustain loss of CHD4
- CHD4 represses p53 expression and promotes B cell proliferation
- Loss of CHD4 influences recruitment of AID to the Igh locus and impairs CSR

Authors

Wei-Feng Yen, Rahul Sharma, Montserrat Cols, ..., Alexander Y. Rudensky, Sergei B. Koralov, Jayanta Chaudhuri

Correspondence

chaudhuj@mskcc.org

In Brief

Yen et al. demonstrate that CHD4, a component of the NuRD remodeling complex, is essential for early B cell development, represses p53 expression in mature B cells, and influences the recruitment of AID to DNA during class switch recombination.
Distinct Requirements of CHD4 during B Cell Development and Antibody Response

Wei-Feng Yen,1,4,8 Rahul Sharma,1,8 Montserrat Cols,1 Colleen M. Lau,1 Ashutosh Chaudhry,1 Priyanka Chowdhury,1,3 William T. Yewdell,1 Bharat Vaidyanathan,1,3 Amy Sun,4 Maryaline Coffre,4 Joseph N. Pucella,5 Chun-Chin Chen,2,6 Maria Jasinska,2,5,6 Joseph C. Sun,1,3,5 Alexander Y. Rudensky,1,3,5,7 Sergei B. Koralov,4 and Jayanta Chaudhuri1,3,6,9,*

1Immunology Program, Memorial Sloan Kettering Cancer Center, New York, NY, USA
2Biochemistry, Cellular and Molecular Biology Program, Weill Graduate School of Medical Sciences, New York, NY, USA
3Immunology and Microbial Pathogenesis Program, Weill Cornell Graduate School of Medical Sciences, New York, NY, USA
4Department of Pathology, New York University School of Medicine, New York, NY, USA
5Gerstner Sloan Kettering Graduate School of Biomedical Sciences, New York, NY, USA
6Developmental Biology Program, Memorial Sloan Kettering Cancer Center, New York, NY, USA
7Howard Hughes Medical Institute, Memorial Sloan Kettering Cancer Center, New York, NY, USA
8These authors contributed equally
9Lead Contact
*Correspondence: chaudhuj@mskcc.org
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SUMMARY

The immunoglobulin heavy chain (Igh) locus features a dynamic chromatin landscape to promote class switch recombination (CSR), yet the mechanisms that regulate this landscape remain poorly understood. CHD4, a component of the chromatin remodeling NuRD complex, directly binds H3K9me3, an epigenetic mark present at the Igh locus during CSR. We find that CHD4 is essential for early B cell development but is dispensable for the homeostatic maintenance of mature, naive B cells. However, loss of CHD4 in mature B cells impairs CSR because of suboptimal targeting of AID to the Igh locus. Additionally, we find that CHD4 represses p53 expression to promote B cell proliferation. This work reveals distinct roles for CHD4 in B cell development and CSR and links the H3K9me3 epigenetic mark with AID recruitment to the Igh locus.

INTRODUCTION

Because of the ability to produce and secrete antibodies against an almost infinite array of pathogens, B lymphocytes play a critical role in the adaptive immune response. Antibodies, or immunoglobulins (Igs), are composed of two heavy (Igh) and two light (IgL or Igk) chains covalently linked by disulfide bonds to form a tetrameric complex. The highly diverse N terminus variable regions of Igh and Igk or Igλ contact antigens, while the less divergent C terminus constant region of Igh provides the effector function of the antibody molecule. The variable region exons of Igh and Igk or Igλ are assembled in developing pro- and pre-B cells in the bone marrow from component variable (V), diversity (D), and joining (J) gene segments during the process of V(D)J recombination (Teng and Schatz, 2015). Upon exiting the bone marrow, the naive IgM+ IgD+ B cells migrate to secondary lymphoid organs (spleen, lymph nodes, Peyer’s patches), where they encounter antigens and undergo Igh class switch recombination (CSR) and somatic hypermutation (SHM).

During SHM, which occurs primarily within microanatomical germinal center (GC) structures in lymphoid follicles, genes encoding the variable regions of Igh and Igk or Igλ are mutated at a very high rate ($10^{-3}$ to $10^{-2}$/bp/generation) to ultimately select B cells with higher antigen affinity (Mesin et al., 2016). CSR can occur within GCs or in the extra-follicular regions and exchanges the default Cλ constant region with one of a set of downstream constant region (Cγ, Cε, Cα) (Xu et al., 2012; Yewdell and Chaudhuri, 2017). The B cell thereby changes from expressing IgM to one expressing IgG, IgE, or IgA, with each secondary isotype providing a distinct effector function during an immune response (Xu et al., 2012; Yewdell and Chaudhuri, 2017). Recent evidence strongly suggests that CSR also contributes to the generation of IgD-positive B cells from those expressing IgM (Chen and Cerutti, 2010; Rouaud et al., 2014).

CSR is a deletional-recombination reaction occurring between 1 and 12 kb long transcribed, repetitive switch (S) region DNA elements that precede each Cγ gene segment (Alt et al., 2013; Xu et al., 2012; Yewdell and Chaudhuri, 2017). Prevailing models posit that activation induced cytidine deaminase (AID), a ~24 kDa protein essential for CSR, deaminates cytidines to uridines at transcribed S regions (Xu et al., 2012; Yewdell and Chaudhuri, 2017). Components of the general base-excision and mismatch repair pathways convert the deaminated residues into nicks and single-strand gaps that are ultimately processed into DNA double-strand breaks (DSBs). End-joining of DSBs between donor (usually Sμ) and acceptor S regions (Sγ, Sε, Sα) juxtaposes a new Cγ gene downstream of the V(D)J segment and deletes the intervening DNA sequence as an extra-chromosomal circle to complete the recombination reaction (Xu et al., 2012; Yewdell and Chaudhuri, 2017).

The mechanism by which AID is recruited to the Igh locus during CSR is intricately linked to germline transcription (Pavri,
2017; Yewdell and Chaudhuri, 2017). Each of the Cn genes is configured as individual germline transcription units composed of a cytokine- and activator-inducible promoter, an intervening I exon, an intronic S region, and Cn exons (Pavri, 2017; Yewdell and Chaudhuri, 2017). It is generally believed that transcription generates DNA structures, such as G quadruplexes and R loops, to facilitate AID recruitment and deamination (Chaudhuri et al., 2003; Duquette et al., 2005; Qiao et al., 2017; Yu and Lieber, 2003). Recent reports suggest that transcription also enables recruitment of AID to DNA via interaction with RNA polymerase II (Pol II), Spt5, RNA exosome machinery, PTBP2, and 14-3-3 adaptor proteins (Basu et al., 2011; Nowak et al., 2011; Pavri et al., 2010; Pefanis et al., 2014, 2015; Xu et al., 2010). Additionally, intronic segments of germline transcripts form G quadruplexes, bind AID, and have been implicated in recruiting AID to the IgH locus (Zheng et al., 2015). Thus, there is a generally broad understanding of trans-factors that facilitate recruitment of AID to its DNA substrates. However, what is largely unknown and remains a challenge is an understanding of how the chromatin landscape at the IgH locus interacts with and co-ordinates these various protein-DNA interactions to facilitate efficient AID targeting and CSR.

The N-terminal tails of the four core histones (H2A, H2B, H3, and H4) undergo extensive post-translational modifications (PTMs), including acetylation, phosphorylation, and methylation, to orchestrate the ordered recruitment of proteins that mediate DNA transactions (Tessarz and Kouzarides, 2014). The PTMs also attract factors that modulate chromatin structure via ATP-dependent nucleosome remodeling to alter chromatin accessibility. These combinatorial activities generate a permissive chromatin landscape for factors required for replication, transcription, recombination, and repair (Tessarz and Kouzarides, 2014). The requirement for cell division, germline transcription, recruitment of specific DNA-modifying enzymes (AID), and long-range DSB repair suggests that CSR may use distinct PTMs to co-ordinate these myriad processes in a sequential and highly regulated manner within a clearly demarcated region of the B cell genome (Zan and Casali, 2015).

Consistent with this notion, S regions are hyper-accessible in activated B cells to DNA-modifying enzymes, with the accessibility pattern superimposable with histone PTMs that are generally associated with transcriptionally active chromatin (Wang et al., 2009). In naive unstimulated B cells, Sμ is enriched for activating histone marks including H3K4me3 (histone H3 trimethylated at lysine-4), H3K36me3, and H3K9acS10ph (H3K9ac9 phosphorylated serine 10). Upon B cell activation, these modifications spread to downstream S regions irrespective of their length (Wang et al., 2009). The functional relevance of the histone PTMs in CSR came from studies in which activities of the methyl transferases or acetyltransferases were altered and CSR was found to be perturbed. For example, depletion of Set1 or PTIP, proteins that promote H3K4me3, impaired CSR (Daniel et al., 2010; Stanlie et al., 2010). Likewise, loss of the histone acetyltransferases Pcaf and Gcn5 reduced H3K9AcS10ph at S regions and decreased CSR, mimicking CSR defect in B cells due to 14-3-3 adaptor proteins that interact with H3K9AcS10ph (Li et al., 2013). Because germline transcription is essential for CSR, it is plausible that the PTMs represent transcriptionally active chromatin at Cn genes engaged in CSR.

Intriguingly, S regions also accumulate H3K9me3 (histone H3 trimethylated at lysine-9), a mark correlated with inactive chromatin (Chowdhury et al., 2008; Jeewan-Raj et al., 2011; Kuang et al., 2009; Li et al., 2013; Wang et al., 2009). Additionally, over-expression of Suv39h1, the methyltransferase that catalyzes methylation of H3K9, increased CSR while its loss impairs CSR to IgA (Bradley et al., 2006). H3K9me3 was initially linked to heterochromatin formation and gene silencing (Kouzarides, 2007). However, later studies revealed that H3K9me3 interacts with the chromo-domain containing heterochromatin protein 1γ (HP1γ) and that H3K9me3-HP1γ is present at actively transcribing genes and at recombining S regions (Bannister et al., 2001; Chowdhury et al., 2008; Jeewan-Raj et al., 2011; Kuang et al., 2009; Vakoc et al., 2005). Significantly, AID was detected in a complex with HP1γ and KAP1 (KRAB domain associated protein-1), and deletion of KAP1 impaired CSR, leading to the proposal that the H3K9me3 serves to tether AID to S region DNA (Jeewan-Raj et al., 2011).

The chromo-domain-helicase-DNA-binding protein 4, (CHD4, also referred to as Mi-2β) is a ~250 kDa protein that directly binds H3K9me3 through one of its PHD (plant homeodomain) fingers (Musselman et al., 2012). CHD4 also has a C terminus ATPase module, which uses energy from ATP hydrolysis to remodel nucleosomes. In this context, CHD4 is a component of the nucleosome remodeling and histone deacetylase (NuRD) complex, a large ~2,000 kDa complex that also contains histone deacetylases (HDAC1 and HDAC2) and methyl-CpG-binding domain proteins (O’Shaughnessy and Hendrich, 2013). The dual properties of CHD4 that allow it to both recognize H3K9me3 and remodel chromatin via ATP hydrolysis suggest that it might participate in multiple aspects of chromatin accessibility. Indeed, conditional inactivation of CHD4 in different lineages demonstrates its requirement at multiple stages of T cell development, including for normal expression of CD4 (Williams et al., 2004), hematopoietic stem cell self-renewal (Yoshida et al., 2008), striated muscle identity (Gómez-Del Arco et al., 2016), and in the neural differentiation of embryonic stem cells (Hirot a et al., 2019). Furthermore, short hairpin RNA (shRNA)-mediated depletion of CHD4 in a plasmacytoma cell line suggested that CHD4 negatively regulates expression of Cd79a in B cells (Ramirez and Hagma n, 2009).

Because of the highly regulated role of transcription and chromatin remodeling during CSR, as well as the presence of H3K9me3 at the IgH locus, CHD4 has the potential to serve as a critical epigenetic regulator of the process. However, despite the availability of conditionally deficient CHD4 (Chd4fl/fl) mice (Williams et al., 2004), the requirement of CHD4 in B cells has not been investigated, likely because of pleiotropic effects and the impact on cell viability upon CHD4 loss. In this study, we conditionally knocked out Chd4 at different stages of B cell development to demonstrate that CHD4 is essential for early B cell development, with a complete loss of the developing pre-B cell population. Strikingly, mature B cells tolerate the loss of CHD4, allowing us to investigate its role in CSR. Here, we demonstrate that CHD4 promotes proliferation of activated B cells in a p53-dependent manner. Furthermore, we show that
**RESULTS**

**Depletion of CHD4 in Ex Vivo B Cell Cultures Impairs CSR**

We first assessed if CHD4 is expressed in activated mature B cells. Naive splenic B cells were isolated from wild-type mice and stimulated in culture with LPS plus IL-4, a combination that promotes CSR to IgG1. CHD4 was detected in unstimulated B cells by western blotting, and there was no marked change in its expression following stimulation (Figure 1A). Likewise, when naive B cells were activated in culture to simulate a Th2 cytokine (IL-4), or with an alternative Th-dependent stimulation (anti-CD40 plus IL-21), no consistent alteration in CHD4 expression was observed (Figure S1).

To initially assess requirement of CHD4 in CSR, we retrovirally transduced shRNAs into naive splenic B cells stimulated ex vivo with LPS plus IL-4 (Figure 1A). Knockdown of CHD4 led to a significant reduction in CSR to IgG1 compared with scrambled shRNA controls (Figure 1B). CHD4 depletion did not have any discernable effect on AID expression (Figure 1A) or B cell proliferation, as the total B cell numbers were unaltered (Figure 1C). To complement this approach, we retrovirally transduced the GB7 recombinase into naive splenic B cells derived from Chd4fl/fl mice. Depletion of CHD4 protein (Figure 1D) was accompanied by a significant reduction in CSR to IgG1 (Figure 1E). There were no gross changes in AID expression (Figure 1D) or any marked effect on cell survival, as measured by the total number of live B cells at different time points post-stimulation (Figure 1F). Overall, results from these complementary strategies suggested that CHD4 influences CSR and provided the impetus to track B cell development and function in vivo upon stage-specific genetic deletion of CHD4.

**CHD4 Is Essential for Early B Cell Development**

To examine the requirement for CHD4 in vivo, we bred Chd4fl/fl mice to Mb1-cre mice, wherein the Mb1 promoter drives cre expression. The Mb1 gene encodes the Igα subunit of the B cell antigen receptor and is expressed in all B-lineage cells starting from the early pro-B cell stage. Thus, Mb1-cre mice are a convenient tool for B cell developmental studies (Hobeika et al., 2006). Relative to control mice, spleens of Mb1fl/flChd4fl/fl mice were significantly smaller with reduced cellularity and had no

CHD4 associates with the Igh locus, and its loss impairs CSR, potentially because of decreased AID targeting to S region DNA.
detectable B220+ CD19+ mature B cells (Figure 2A). Examination of bone marrow of the Mb1cre/wtChd4fl/fl mice revealed a severe loss of B220+ IgM+ immature B cells and B220+ IgM+ recirculating B cells compared with controls (Figure 2B, top left), with a marked reduction in bone marrow cell numbers (Figure 2B). Further examination showed that Mb1cre/wtChd4fl/fl mice had a near complete loss of pre-B (IgM+ B220+ CD25+ cKit+) cells and a marked increase in the frequency of pro-B (IgM− B220+ CD25− cKit−) cells compared with control mice (Figure 2B). PCR genotyping confirmed that the loss of the floxed CHD4 allele in the bone marrow of the pro-B cells (Figure 2B, right). Thus, CHD4 is required for the development or maintenance of pre-B cells.

To examine if the loss of pre-B cells is due to the activation of a p53-dependent checkpoint associated with V(D)J recombination (Gao et al., 2000; Guidos et al., 1996), we generated Mb1cre/wtChd4fl/fl mice. However, B cell development was not restored to any significant extent even after loss of p53 (Figure 2C). Transgenic expression of the anti-apoptotic Bcl-2 protein has been shown to restore early B cell lymphopoiesis in some instances (McDonnell et al., 1989), yet the Bcl-2 transgene could not restore B cell development in Mb1cre/wtChd4fl/fl mice (Figure 2D). Thus, CHD4 is essential for early B cell development, and its deletion leads to a severe loss of pre-B cells. However, the mechanism by which CHD4 participates in early B cell development is not currently well understood. Notably, this dramatic phenotype indicated that Mb1cre/wtChd4fl/fl mice could not serve as a productive model to study the function of CHD4 in mature B cells.

Deletion of CHD4 in Mature B Cells Impairs CSR

To circumvent the early B cell developmental block, we tried deleting Chd4 at a later stage of B cell development by breeding Chd4floxed mice to Cd19cre-mice. Unlike Mb1-cre, which results in the deletion of floxed alleles at the pro-B cell stage, Cd19cre induces recombination at a later point, primarily during the pre-B stage (Rickert et al., 1997). The spleen of Cd19cre/wtChd4fl/fl mice (hereafter designated CHD4 knockout [KO]) had similar number and frequency of total splenocytes and B220+ IgM+ B cells as control mice, with no marked difference in spleen size (Figures 3A and 2A). Analysis of purified, naive splenic B cells from CHD4 KO mice showed inefficient deletion of Chd4 floxed alleles (Figure 2B) and loss of CHD4 protein (Figures 3B and 2C). Purified naive B cells from CHD4 KO and control mice were cultured ex vivo and stimulated with LPS plus IL-4 (Figure 3C) or anti-CD40 plus IL-4 (Figure 2C) to assess CSR to IgG1. CHD4 deficiency led to a severe defect in CSR, in CHD4 KO cells switching to IgG1 at only 25% of control cells (Figures 3C and S2C). Likewise, when naive splenic B cells were stimulated with LPS to evaluate CSR to IgG3, loss of CHD4 led to a similar reduction in IgG3 CSR as observed for IgG1 (Figure 3C).

In parallel with the above studies, we also generated Cd21cre/wtChd4fl/fl mice wherein the Cd21 promoter in a transgene drives cre expression starting from the IgM+ IgD+ mature B cells (Kraus et al., 2004). The splenic B220+ IgM+ B cell population was similar between Cd21cre/wtChd4fl/fl mice and controls (Figure S2D). Consistent with data generated from Cd19cre/wtCHd4fl/fl mice, B cells from Cd21cre/wtChd4fl/fl also exhibited a severe defect in CSR to both IgG1 and IgG3 (Figure 3D). However, Cd21cre/wtChd4fl/fl mice were born at non-Mendelian ratio (Figure S2E), likely because of non-specific deletion of CHD4 in the germline (Gostissa et al., 2013). We therefore used the Cd19cre/wtCHd4fl/fl mice (CHD4 KO) for further analysis.

CHD4 Promotes Proliferation of B Cells in a p53-Dependent Manner

We sought to investigate potential molecular mechanisms by which CHD4 influences CSR. We observed that CHD4 deletion did not alter AID expression (Figures 3B and S2C) or germline transcription of activated Cγ genes (Figure S3A). Annexin V staining demonstrated that stimulated CHD4 KO B cells did not exhibit increased apoptosis (Figure S3B). However, we observed markedly impaired proliferation of CHD4 KO splenic B cells as assessed by dilution of the tracking dye CFSE (Figure 4A). The defect in cell proliferation observed in CHD4 KO B cells was not observed upon CHD4 deletion by shRNA or cre recombination (Figures 1C and 1F), likely because of residual CHD4 protein, which could not be detected within the sensitivity of the immunoblot.

To gain additional insights into CHD4 deficiency, we dissected its transcriptional network in splenic B cells undergoing CSR. We isolated RNA from purified splenic B cells stimulated with LPS and IL-4 stimulation for 48 h and performed RNA sequencing to identify candidate genes with distinct expression profiles upon CHD4 deletion. After focusing on more highly expressed transcripts (transcripts per million [TPM] > 10), our analysis identified 277 transcripts associated with 257 genes that were differentially expressed (DE; false discovery rate [FDR]-corrected p value < 0.05 and absolute log2 fold change > 0.5) (Figure 4B; Table S1). Among these genes, 133 had gene isoforms that were downregulated upon CHD4 deficiency, 120 had isoforms that were upregulated upon CHD4 deficiency, and 4 genes had isoforms that were either upregulated or downregulated. Gene set analysis among these DE genes showed enrichment of biological processes involved with lymphocyte activation, proliferation, and CSR (Figure 4C). For example, expression of CSR genes Batf and Irf4, two genes that positively regulate AID transcription, was increased, or decreased, respectively in CHD4 KO B cells (Ise et al., 2011; Sciammas et al., 2006). The differential effect of CHD4 loss on the expression of these two factors could explain why AID levels are unaltered in CHD4 KO B cells.

The most relevant data to emerge from this analysis are that several genes associated with proliferation were found to be DE (Figures 4B–4D). These included genes such as Ccd3, Cc15, Fyn, and notably Trp53 (encoding p53 protein) (Figure 4D), with two Trp53 isoforms, among the most significant DE transcripts (Figure 4B). Consistent with these transcript levels, we observed that p53 protein was also elevated in activated CHD4 KO splenic B cells (Figure 4E).

To investigate whether the impaired proliferation of CHD4 KO B cells was p53 dependent, we generated Cd19cre/wtChd4fl/flop53fl/fl mice (henceforth designated double KO [DKO] mice) to delete both p53 and CHD4 in B cells. Purified splenic B cells of all four genotypes (cre control, p53 KO, CHD4 KO, and DKO) were assayed for cell proliferation by CFSE dye
**Figure 2. CHD4 Is Essential for Early B Cell Development**

(A) Mature B cells are absent in spleens of \( \text{Mb}^{-/}\text{Chd4}^{-/-} \) mice. Splenocytes were stained with B220 and CD19 and analyzed using flow cytometry (top panel). Absolute numbers of total splenocytes and CD19\(^+\) B220\(^+\) B cells from each mouse strain are shown (\( n = 3 \); \( **p < 0.005 \)) (bottom left). The spleen from a representative 10-week-old \( \text{Mb}^{-/}\text{Chd4}^{-/-} \) mouse was compared with littermate control (\( \text{Chd4}^{+/+} \)) (bottom right).

(B) CHD4 deletion impairs early B cell development in the bone marrow. Flow cytometric analysis shows expression of IgM and B220 in the bone marrow. Cellularity of bone marrow is shown on the right side of representative flow plots. BM cells were stained with c-Kit and CD25 and analyzed for pre-B (IgM\(^-\)B220\(^+\)CD25\(^-\)c-Kit\(^+\)) and pro-B (IgM\(^-\)B220\(^+\)CD25\(^-\)c-Kit\(^+\)) cells. The red box represents the parent gate for analysis of pre-B and pro-B cells. \( n = 5 \); \( **p < 0.005 \).

Furthermost right panel shows PCR-based genotyping and loss of the floxed \( \text{Chd4} \) allele in total bone marrow cells and sorted pro-B cells. Genotyping PCR from two independent experiments is shown.

(C) Loss of p53 does not restore B cell developmental defects in \( \text{Mb}^{-/}\text{Chd4}^{-/-} \) mice. Absolute numbers of bone marrow from each mouse strain are shown (\( n = 4 \); \( **p < 0.005 \)) (left). Flow cytometric analysis shows expression of IgM and B220 expression or CD25 and c-Kit in bone marrow (BM) (right).

(D) Transgenic expression of Bcl2 does not rescue B cell developmental defects in \( \text{Mb}^{-/}\text{Chd4}^{-/-} \) mice. Absolute numbers of total bone marrow from each mouse strain are shown (left) (\( n = 4 \); \( **p < 0.005 \)). Flow cytometric analysis shows IgM and B220 expression or CD25 and c-Kit expression in total bone marrow (BM) (right).
dilution. Strikingly, p53 deficiency rescued the proliferation defect of CHD4 KO splenic B cells (Figure 4F). However, despite fully restoring the proliferative capacity of DKO B cells, p53 deletion was able to only mildly rescue CSR to IgG1 compared with controls (Figure 4G). On the basis of these results, we conclude that CHD4 influences CSR independent from its role in regulating cell proliferation via p53.

CHD4 Deletion Impairs GC Response and CSR In Vivo

To examine B cell responses in vivo, mice were immunized with sheep red blood cells (SRBC), a complex polyepitope T-dependent model system that induces a robust GC response in the spleen (Muramatsu et al., 2000). We observed that at day 14 (d14) post-immunization, total splenic B cell numbers were similar between control and CHD4 KO immunized mice (Figure 5A). As expected, SRBC immunization of control and AID KO mice led to a marked increase in the frequency of splenic GC (B220+ Fas+ GL7+) B cells over mock PBS immunization (Figure 5B). However, CHD4 KO mice exhibited a severe reduction in the frequency of GC B cells compared with controls at d14 after SRBC immunization (Figure 5B). Additionally, the fraction of IgG1+ B cells among the GC B cell population was significantly reduced in immunized CHD4 KO mice relative to control animals (Figure 5C). Quantification of serum antibodies by ELISA revealed increased IgM levels upon SRBC immunization in both control and CHD4 KO mice (Figure 5D). However, increased IgG1 levels observed for control mice upon immunization was markedly suppressed in CHD4 KO B cells (Figure 5D), consistent with the in vitro data.
with an immunization-dependent defect in CSR. Notably, deletion of p53 (in DKO mice) did not rescue the GC B cell response and in vivo IgG1 CSR defect to control (Figures 5B–5D). Overall, CHD4 deletion led to a severe defect in the GC B cell response to SRBC immunization.

To further characterize the in vivo B cell response, we used NP-CGG immunization, an extensively employed hapten T-dependent model antigen that facilitates identification of NP-specific B cells in the spleens of immunized mice (Jacob et al., 1991). Intracellular staining revealed that at d14 post-immunization, CHD4 protein was present at higher levels in GC B cells relative to naive B cells (Figure S4). In agreement with results obtained with SRBC immunization, CHD4 KO mice had markedly reduced frequency and absolute numbers of GC (B220+ GL7+ Fas+) B cells (Figures 5E–5G and S5). Consistent with a CSR defect in vivo, we observed a marked reduction in the frequency of IgM+ IgD− class-switched cells within the GC B cell population in CHD4 KO mice compared with control animals (Figure 5H). Thus, two different T-dependent immunization models demonstrated that CHD4 plays a critical role in the generation of GC B cells and in CSR in vivo.

The ability to track NP-specific B cells upon NP-CGG immunization allowed us to evaluate if CHD4 influences the emergence of early memory B cells, defined as splenic NP+ cells that are CD38+ GL7+ Fas+ (Figure S5) (Cho et al., 2016; Pucella et al., 2019). We found no significant difference in the frequency of early memory B cells (NP+ of CD38+ GL7− Fas+) between control and CHD4 KO mice (Figure S6A). There was a slight increase in the average absolute number of early memory
B cells; however, this difference was not statistically significant (Figure S6B). In contrast, the frequency of unswitched early memory B cells (IgM+ of NP+ CD38+ GL7/C0 B220+) in CHD4 KO mice was increased (Figure S6C), while the frequency of class-switched early memory B cells (IgM/C0 IgD/C0 of NP+ CD38+ GL7/C0 B220+) was markedly reduced compared with control animals (Figure S6D), consistent with a CSR defect. Thus, it appears that loss of CHD4 does not have a major impact on the generation of antigen-specific early memory B cells, even though the frequency of switched cells among this population is significantly reduced. However, given the complex nature of B cell memory formation and its role in long-lived immunity, more exhaustive studies are required to elucidate the role of CHD4 in this process.

Whereas deletion of CHD4 led to a marked defect in CSR, the reduction in serum IgG1 levels in unimmunized mice was not as striking (Figure 5D). Likewise, at homeostasis, serum IgA levels (Figure S6E) or frequency of IgA+ B cells in the GCs of Peyer’s patches (Figures S6F and S6G) was similar between control and CHD4 KO mice. Thus, even though CHD4 KO B cells were intrinsically deficient for in vitro CSR to IgA (Figure S6H), conditions in vivo likely allow the selection and maintenance of the few cells that have managed to undergo CSR. This lack of correlation between in vitro and in vivo CSR is reminiscent of that observed for other CSR factors such as UNG and MSH2, wherein loss of either factor leads to a severe defect of CSR in vitro but has a much milder effect on homeostatic serum Ig levels in vivo (Rada et al., 2004).

Plasmablast Differentiation Is Not Grossly Affected by CHD4 Deletion

The differential requirement of CHD4 in different stages of B cell differentiation led us to explore if it has a role in plasmablast differentiation. Plasmablasts (B220+ CD138+) represent an intermediate cell type that exhibits a transcriptional program very similar to that observed in plasma cells (Barwick et al., 2016). To assess plasmablast formation ex vivo, we activated naïve B cells with LPS or LPS plus IL-4 for 96 h and assessed emergence of B220+ CD138+ B cells (Barwick et al., 2016). We observed that
CHD4 KO B cells were as proficient in generating plasmablasts as control B cells (Figures 6A and 6B). To assess the impact of CHD4 deletion on plasmablast differentiation in vivo, we used an intravenous immunization model with LPS, a T-independent sub-type 1 (TI-1) antigen that induces a rapid induction of B220<sup>+</sup> CD138<sup>+</sup> plasmablasts in the spleen of immunized animals (Barwick et al., 2016; Pucella et al., 2019). As expected, we observed that relative to PBS-challenged (mock) mice, LPS administration induced robust plasmablast differentiation (Figure 6C). However, there was no difference in the frequency or absolute number of plasmablasts between control and CHD4 KO mice (Figures 6D and 6E). Intracellular staining showed that CHD4 protein expression was markedly diminished in the mice (Figures 6D and 6E). Intracellular staining showed that the frequency of IgG3<sup>+</sup> plasmablasts between control and CHD4 KO administration induced robust plasmablast differentiation (Figure 6F), indicating that the cells have not escaped pre-mediated deletion. Thus, CHD4 is dispensable for formation of plasmablasts ex vivo and in vivo.

LPS administration also induces lower levels of IgG3 CSR as a T-independent extra-follicular humoral response and a small fraction of the plasmablasts express IgG3; however, intracellular staining showed that the frequency of IgG3<sup>+</sup> plasmablasts was similar between control and CHD4 KO mice (Figures 6G and 6H). Additionally, we did not detect any marked difference in the frequency of B220<sup>+</sup> IgG3<sup>+</sup> cells by surface staining between control and CHD4 KO mice (Figures 6J and 6K). However, both the B220<sup>+</sup> IgG3<sup>+</sup> plasmablasts and the B220<sup>+</sup> IgG3<sup>+</sup> B cells still retained significant levels of CHD4 (Figures 6I and 6L). It is possible that LPS challenge induces a strong selection to generate IgG3<sup>+</sup> cells, and because only CHD4 sufficient cells are able to undergo efficient CSR, those cells that have escaped pre-mediated deletion are found in the IgG3<sup>+</sup> population. This could potentially explain the discrepancy in the requirement of CHD4 in inducing CSR in LPS-activated B cells in vivo (Figures 3C and 3D) versus LPS-challenged CSR in vivo.

**CHD4 Promotes AID Recruitment**

Our results have established the role of CHD4 in promoting the intrinsic ability of B cells to undergo CSR. With its well-documented links with chromatin dynamics, it was logical to examine the possibility that CHD4 mediates CSR through a localized interaction with the *Igh* locus. To test this possibility, we first...
Figure 7. CHD4 Mediates Localization of AID to Switch Region DNA
(A) CHD4 binds to Sμ. ChIP analysis was performed on chromatin prepared from LPS plus IL-4-stimulated (48 h) splenic B cells harvested from control (Cd19<sup>cre/wt</sup>), CHD4 KO (Cd19<sup>cre/wtChd4fl/fl</sup>), or Aicda<sup>−/−</sup> mice. Quantification of ChIP signals at Sμ and Cγ1 (negative control) is indicated in each plot as percentage of input (mean ± SD) (n = 6; **p < 0.005).

(B) H3K9me3 epigenetic mark is present at Sμ. Splenic B cells harvested from mice of the indicated genotypes were stimulated with LPS plus IL-4 for 48 h, and ChIP analysis was performed using antibodies specific for H3K9me3. ChIP using histone H3 antibodies and non-specific IgG served as positive and negative controls, respectively. Quantification of ChIP signal is indicated as percent of input (mean ± SD) (n = 6; **p < 0.005).

(C) Loss of CHD4 reduces frequency of 5′-Sμ mutations. Splenic B cells from control (Cd19<sup>cre/wt</sup>), p53 KO (Cd19<sup>cre/wtp53fl/fl</sup>), CHD4 KO (Cd19<sup>cre/wtChd4fl/fl</sup>), DKO (Cd19<sup>cre/wtChd4fl/flp53fl/fl</sup>), and AID KO (Aicda<sup>−/−</sup>) were activated ex vivo for 96 h with LPS plus IL-4, and the 5′-Sμ region was PCR amplified and sequenced. B cells from AID KO mice served as control to estimate the frequency of PCR error. Pie charts illustrate the fraction of sequences with the indicated number of mutations at Sμ.

(D) AID ChIP

(E) Fold change relative to control at Sμ

(F) Day 0 1 2 3 4

(G) % of Max

(H) AID KO

(legend continued on next page)
carried out chromatin immunoprecipitation (ChIP) experiments and observed that CHD4 was significantly enriched at Sµ, but not at the control Cγ1 region. In naive splenic B cells stimulated with LPS plus IL-4 (Figure 7A). The interaction of CHD4 with Sµ was also observed in AID KO B cells (Figure 7A), suggesting that might be recruited upstream of DSBs, potentially through its ability to bind H3K9me3, an epigenetic mark implicated in AID targeting to DNA (Chowdhury et al., 2008; Jeevan-Raj et al., 2011; Kuang et al., 2009; Li et al., 2013; Wang et al., 2009). In agreement with this notion, additional ChIP experiments demonstrated that H3K9me3 marks are enriched at Sµ (Figure 7B). Interestingly, the H3K9me3 was present at Sµ independent of AID or CHD4 (Figure 7B), suggesting that this region is poised to recruit factors that facilitate CSR.

To examine if CHD4 could bridge the interaction between AID and Sµ, we assessed the frequency of mutations at Sµ in LPS plus IL-4-activated splenic B cells. Mutations at Sµ are frequently observed in B cells activated in culture and serve as a surrogate for AID recruitment to DNA (Barreto et al., 2003; Schrader et al., 2003). In control B cells, the mutation frequency at this region was \( \sim 0.6 \times 10^{-5}/\text{bp} \) (Figures 7C and 7D). In contrast, the mutation frequency in CHD4 KO KO B cells was significantly reduced (\( \sim 1.9 \times 10^{-5}/\text{bp} \)) (Figures 7C and 7D). Deletion of p53 in addition to CHD4 did not rescue the mutation frequency to control levels in the DKO cells (Figures 7C and 7D). Although higher than the mutation frequency (\( \sim 0.25 \times 10^{-5}/\text{bp} \)) in AID-deficient B cells, the marked reduction in Sµ mutation frequency in CHD4 deficient B cells compared with controls strongly suggests a defect in AID recruitment to S region DNA.

To further test this hypothesis, we performed additional ChIP experiments to assess AID localization to Sµ. At both 48 and 72 h post-stimulation with LPS plus IL-4, CHD4 KO and DKO B cells had significantly reduced levels of AID bound to Sµ (Figures 7E, S7A, and S7C). Occupancy of AID at Cγ1 as a non-specific control region was at background level because the enrichment was similar to what was observed in AID KO B cells (Figure S7B), and there was no marked difference in the binding of H3 to Sµ in the different genotypes examined (Figure S7C). Importantly, a reduction in both the frequency of Sµ mutations as well as AID occupancy at Sµ provides corroborative evidence that CHD4 influences the interaction of AID with Igh during CSR.

Finally, to test if CHD4 might interact with AID to mediate its recruitment to the Igh locus, we carried out co-immunoprecipitation experiments. We expressed FLAG-HA-tagged AID protein (2T-AID) along with a GFP reporter in AID KO splenic B cells via retroviral transduction (Figure 7F). We found that 2T-AID restored IgG1 switching in AID KO B cells (Figure 7G). When AID was immunoprecipitated from sorted GFP⁺ B cells using HA antibodies, endogenous CHD4 was detected in the immune complex (Figure 7H). Although we could not detect an interaction between endogenous AID and CHD4, this result suggests that AID and CHD4 could form a protein complex in primary B cells, and this interaction could facilitate the optimal recruitment of AID to the Igh locus.

**DISCUSSION**

In this study, we used several genetic models to demonstrate that CHD4 is a versatile effector of B cell development and CSR. Deletion of CHD4 in pro-B cells leads to near-complete loss of the pre-B cell population. The severity of this defect could not be rescued by deletion of p53, suggesting that this phenotype is not likely due to cell cycle checkpoints associated with aberrant V(D)J recombination. Additionally, enforced expression of the anti-apoptotic gene Bcl-2 was unable to rescue pre-B cell development, indicating that CHD4 might not be influencing survival of developing pre-B cells. It is likely that CHD4 regulates expression of genes required for the pro-B to pre-B cell developmental transition, and the identity of such factors will require additional studies.

Remarkably, despite the absolute requirement of CHD4 in early B cell development, it is dispensable for homeostatic proliferation and survival of mature B cells. Loss of CHD4 in pre-B cells or mature B cells does not affect mature B cell numbers or survival. In response to T-dependent antigens, the formation of antigen-specific early memory B cells is not markedly impaired upon loss of CHD4, nor is the generation of plasma blasts in response to T-independent activators such as LPS. It is only under specific conditions of B cell activation such as CSR in vitro with different activators and cytokines, and CSR and GC formation in vivo in response to T-dependent antigens such as SRBC and NP-CGG, that the requirement of CHD4 is fully revealed. This context-dependent requirement of CHD4 at different stages of B cell development and function is reminiscent of its role in distinct stages of T cell development, including its requirement for expression of CD4 but not CD8 (Williams et al., 2004). The mechanism by which CHD4 participates in different cellular processes in a wide variety of cell types will require additional studies.

As a protein that responds to cellular stress including DSBs, p53 induces cell-cycle arrest and apoptosis and enforces genomic integrity in B cells from AID-induced genome-wide collateral damage during CSR (Robbiani et al., 2009; Zilfou and...
Lowe, 2009). However, p53 activity must be downregulated for B cell survival and proliferation in the face of ongoing Igh DSBs during CSR (Phan and Dalla-Favera, 2004). Our results clearly demonstrate that CHD4 represses p53 mRNA expression to promote B cell proliferation. Regulation of p53 expression likely represents the role of CHD4 as an essential component of the NuRD transcriptional repressor complex. The NuRD complex also contains the HDAC1/2 deacetylases, which could also influence p53 stability via regulating its acetylation (Hirota et al., 2019; Polo et al., 2010). Whether both transcriptional and post-transcriptional activities of the NuRD complex contribute to regulate p53 levels in activated B cells is not clear at present, but both appear to be mediated via NuRD-dependent function of CHD4. In this regard, another NuRD complex associated factor ZMNYD8 has recently been shown to influence CSR by regulating the activity of the 3’ Igh super-enhancer (Delgado-Benito et al., 2018). Additionally, on the basis of the ability of the NuRD complex to regulate acetylation of p53 protein, it is possible that it could also impact PTMs of AID and other factors that participate in different stages of B cell development and differentiation.

Our results provide clear evidence that CHD4 influences the recruitment of AID to Igh. One possible mechanism for CHD4-mediated AID targeting is through the ability of CHD4 to directly bind H3K9me3, a histone PTM enriched at S regions (Chowdhury et al., 2008; Jeevan-Raj et al., 2011; Kuang et al., 2009; Li et al., 2013; Wang et al., 2009). Although generally associated with transcriptionally repressed chromatin states, H3K9me3 mark has been found on some transcribed genes (Vakoc et al., 2005) and has also been implicated in establishing an environment permissive to long-range chromosomal interactions (Jeevan-Raj et al., 2011; Jia et al., 2004). In this regard, AID interacts with the KRAB domain-associated protein 1 (KAP1):heterochromatin protein 1 (HP1) complex that is tethered to H3K9me3 at the Igh locus (Jeevan-Raj et al., 2011), and CHD4 has been implicated in the recruitment of KAP1-HP1 to H3K9me3-marked chromatin (Price and D’Andrea, 2013). Therefore, it is quite possible that CHD4 facilitates recruitment of AID to DNA by targeting a KAP1-HP1-AID complex to H3K9me3 at S regions during CSR. Such a mechanism, wherein only a subset of transcriptionally active genes retains the H3K9me3 mark, could potentially explain how AID is targeted to a relatively limited number of transcribed genes in B cells.

CHD4 has been shown to participate in the repair of DNA DSBs (O’Shaughnessy and Hendrich, 2013). It is recruited to sites of DNA damage and, as a chromatin remodeler, could generate an environment permissive to amplification of the DNA-damage response and recruitment of factors required for DNA repair (O’Shaughnessy and Hendrich, 2013). Whether CHD4 influences DSB repair during CSR is yet to be determined. However, the observations that both Kap-1 and CHD4 participate in DNA damage response in an ATM-dependent manner (Urquhart et al., 2011; Ziv et al., 2006) is consistent with a general theme (Vuong et al., 2013) that CSR proceeds in a highly coordinated reaction wherein the factors that promote DSB formation at the Igh locus also promote repair, providing one mechanism by which genomic integrity can be maintained in B cells in the face of deliberate DNA damage.

In summary, we have identified CHD4 as a stage-specific effector of B cell development and differentiation. We have demonstrated that CHD4 influences the formation of GC B cells upon immunization with T-dependent antigens and plays a critical role in B cell proliferation in a p53-dependent fashion. Finally, we have established that CHD4 influences the recruitment of AID to the Igh locus during CSR. These results lead to new outstanding questions regarding the distinct roles of CHD4 across multiple facets of B cell biology and how epigenetic modifications specify AID recruitment in a CHD4-specific manner to the Igh locus, potentially sheltering the rest of the genome from AID-induced destabilization during CSR.

STAR METHODS

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SUPPLEMENTAL INFORMATION

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**AUTHOR CONTRIBUTIONS**

W.-F.Y., R.S., M.Cols, C.M.L., A.C., P.C., W.T.Y., B.V., A.S., M. Coffre, C.C.C., M.J., J.C.S., S.B.K., and J.C. conceived and designed the experiments. W.-F.Y., R.S., M.Cols, A.C., P.C., W.T.Y., B.V., A.S., M. Coffre, and C.-C.C. performed the experiments. W.F.Y., R.S., M.Cols, A.C., P.C., W.T.Y., B.V., A.S., M. Coffre, C.C.C., M.J., J.C.S., S.B.K., A.Y.R., and J.C. analyzed the data. C.M.L. performed bioinformatics analysis. W.F.Y., R.S., M.Cols, W.T.Y., C.M.L., and J.C. wrote the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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### STAR Methods

#### Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse monoclonal anti α-tubulin | Sigma | Cat# T9026; RRID: AB_477593 |
| Rabbit monoclonal anti-CHD4 | Cell Signaling Technology | Cat# 11912; RRID: AB_2751014 |
| Mouse monoclonal anti-CHD4 | Abcam | Cat# ab70469; RRID: AB_2229454 |
| Rabbit polyclonal anti-H3 | Abcam | Cat# ab1791; RRID: AB_302613 |
| Rabbit polyclonal anti-H3K9me3 | Abcam | Cat# ab8898; RRID: AB_306848 |
| Goat polyclonal anti-p53 | Santa Cruz Biotechnology | Cat# sc-6243-G; RRID: AB_653754 |
| Rat monoclonal anti-B220 BV510 | BioLegend | Cat# 103247; RRID: AB_2561394 |
| Rat monoclonal anti-B220 FITC | BD Biosciences | Cat# 553098; RRID: AB_394618 |
| Rat monoclonal anti-B220 PerCP Cyanine 5.5 | Thermo Fisher Scientific | Cat# 45-0452-82; RRID: AB_1107006 |
| Rat monoclonal anti-B220 PE-Cy7 | BD Biosciences | Cat# 552772; RRID: AB_394458 |
| Rat monoclonal anti-B220 AF700 | Thermo Fisher Scientific | Cat# 56-0452-82; RRID: AB_881458 |
| Rat monoclonal anti-B220 APC/eF780 | Thermo Fisher Scientific | Cat# 47-0452-80; RRID: AB_1518811 |
| Rat monoclonal anti-CD3 BU737 | BD Biosciences | Cat# 564380; RRID: AB_2738781 |
| Rat monoclonal anti-CD95 BV510 | BD Biosciences | Cat# 45-0193-82; RRID: AB_1106999 |
| Rat monoclonal anti-CD19 PE | BD Biosciences | Cat# 557399; RRID: AB_396682 |
| Rat monoclonal anti-CD19 PE-Cyanine7 | Thermo Fisher Scientific | Cat# 25-0193-81; RRID: AB_65766 |
| Rat monoclonal anti-CD25 PE-Cyanine7 | BD Biosciences | Cat# 552880; RRID: AB_394509 |
| Rat monoclonal anti-CD25 APC-eF780 | Thermo Fisher Scientific | Cat# 47-0251-82; RRID: AB_1272179 |
| Armenian hamster monoclonal anti-CD95 BV510 | BD Biosciences | Cat# 563646; RRID: AB_2738345 |
| Mouse monoclonal anti-CHD4 PE | Novus | Cat# NBP2-50163PE; N/A |
| Rat monoclonal Anti-Mouse CD117 (c-Kit) APC | Thermo Fisher Scientific | Cat# 17-1171-82; RRID: AB_469430 |
| Rat monoclonal Anti-Mouse CD117 (c-Kit) PE | Thermo Fisher Scientific | Cat# 12-1171-82; RRID: AB_465813 |
| DAPI | Invitrogen | Cat#D1306; RRID: AB_2307445 |
| Rat monoclonal Anti-GL7 FITC | BD Biosciences | Cat# 553666; RRID: AB_394981 |
| Rat monoclonal Anti-GL7 eFluor450 | Thermo Fisher Scientific | Cat# 48-5902-82; RRID: AB_10870775 |
| Rat monoclonal Anti-IgA PE | Thermo Fisher Scientific | Cat# 12-4204-83; RRID: AB_465918 |
| IgA antibody | SouthernBiotech | Cat# 1040-01; RRID: AB_2314669 |
| Mouse monoclonal IgA antibody | BD Biosciences | Cat# 553476; RRID: AB_479590 |
| Goat Anti-Mouse IgA-HRP antibody | SouthernBiotech | Cat# 1040-05; RRID: AB_2741231 |
| Rat monoclonal Anti-IgD AF700 | BioLegend | Cat# 405729; RRID: AB_2563340 |
| Rat monoclonal Anti-IgG1 APC | BD Biosciences | Cat# 550874; RRID: AB_398470 |
| Goat polyclonal Anti-Mouse IgG1 | SouthernBiotech | Cat# 1070-01; RRID: AB_2794408 |
| Mouse monoclonal IgG1 | SouthernBiotech | Cat# 0102-01; RRID: AB_2793845 |
| Goat polyclonal Anti-mouse IgG1 | SouthernBiotech | Cat# 1070-05; RRID: AB_2650509 |
| Rat monoclonal Anti-IgG3 FITC | BD Biosciences | Cat# 553403; RRID: AB_394840 |
| Rat monoclonal Anti-IgM FITC | BD Biosciences | Cat# 553408; RRID: AB_394844 |
| Rat monoclonal Anti-IgM eF450 | Thermo Fisher Scientific | Cat# 48-5790-82; RRID: AB_2574073 |
| Rat monoclonal Anti-IgM PE | Thermo Fisher Scientific | Cat# 12-5790-81; RRID: AB_465939 |
| Rat monoclonal mouse IgM APC-eF780 | Thermo Fisher Scientific | Cat# 47-5790-82; RRID: AB_2573984 |
| Rat monoclonal Anti-IgM BV595 | BD Biosciences | Cat# 743329; RRID: AB_2741430 |
| Goat polyclonal Anti-Mouse IgG1 | SouthernBiotech | Cat# 1070-01; RRID: AB_2794408 |
| Mouse IgM isotype control | Thermo Fisher Scientific | Cat# 14-4752-81; RRID: AB_470122 |
| Goat polyclonal Anti-IgM | SouthernBiotech | Cat# 1020-05; RRID: AB_2794201 |

(Continued on next page)
| Reagent/Resource | Source | Identifier |
|------------------|--------|------------|
| Hamster monoclonal Anti-CD40 | Thermo Fisher Scientific | Cat# 16-0402-82; RRID: AB_468945 |
| Rabbit polyclonal Anti-AID | Chaudhuri et al., 2003 | N/A |
| Bacterial and Virus Strains | | |
| MSCV-Cre | Addgene | Plasmid# 24064 |
| Biological Samples | | |
| Sheep Red Blood cells | Innovative Research | Cat# IC100-0210 |
| Chemicals, Peptides, and Recombinant Proteins | | |
| NP-PE | Biosearch Technologies | Cat# N-5070-1 |
| NP-CGG | Biosearch Technologies | Cat# N-5055D-5 |
| Zombie Red viability dye | BioLegend | Cat# 423109 |
| Trizol | Invitrogen | Cat# 15596026 |
| LPS | Sigma-Aldrich | Cat#L4130 |
| IL-4 | R&D Systems | Cat#404-ML-100 |
| IL-5 | Peprotech | Cat#215-15 |
| TGF-β | Fischer Scientific | Cat#240-B-010 |
| Retinoic acid | Sigma-Aldrich | Cat#R2625-50-MG |
| BAFF | AdipoGen Life Sciences | Cat#AG-40B-0022-3010 |
| IFN-γ | Gemini Bio-Products | Cat#300-311P |
| IL-21 | R&D Systems | Cat#594-ML-010 |
| Cell Trace CFSE | Thermo Fisher Scientific | Cat# C34554 |
| LPS from *Salmonella minnesota* R595 | Enzo LifeSciences | Cat# ALX-581-008-L002 |
| SYBR Green master mix | Applied Biosystems | Cat#A25741 |
| Critical Commercial Assays | | |
| ChIP assay kit | Millipore Sigma | Cat#17-295 |
| RNeasy mini kit | QiAGEN | Cat#74104 |
| SMART-Seq v4 Ultra Low Input RNA Kit | Clontech | Cat#63488 |
| KAPA Hyper Prep Kit | Kapa Biosystems | Cat#KK8504 |
| Deposited Data | | |
| RNA-seq | This paper | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE128321 |

Experimental Models: Cell Lines

| HEK293T | ATCC | Cat#ATCC® CRL-3216 |

Experimental Models: Organisms/Strains

| C57BL/6 mice | Jackson labs | Cat# JAX: 000664 |
| CHD4 fl/fl | Williams et al., 2004 | N/A |
| Aicda<sup>−/−</sup> | Muramatsu et al., 2000 | N/A |
| Mb<sup>1cre/cre</sup> | Hoboika et al., 2006 | N/A |
| Aicda<sup>cre/cre</sup> | Robbiani et al., 2008 | N/A |
| CD19-Cre | Jackson labs | Cat# JAX: 006785 |
| CD21-Cre | Jackson labs | Cat# JAX: 006368 |
| Vav-2<sup>Bcl2</sup> | Egle et al., 2004 | N/A |
| p53<sup>−/−</sup> | Jackson labs | Cat# JAX: 008462 |

Oligonucleotides

| S 5′-TGGATGACATCAAGAGAAG-3′ Actb | This paper | N/A |
| AS 5′-CGGATGTCAGACATCTTTGAC-3′ Actb | This paper | N/A |
| S 5′-TAGTGAAGGGCTCTAAAAGCA-3′ μ-GLT | This paper | N/A |
| AS 5′-AGAACAGTGCAAGACTGAGCTGA-3′ μ-GLT | This paper | N/A |
| S 5′-GGCCCTTCCAGATCTTTTGAG-3′ γ1-GLT | This paper | N/A |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jayanta Chaudhuri (chaudhuj@mskcc.org).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Aicda−/− (Muramatsu et al., 2000), Mb1cre/cre (Hobeika et al., 2006) and Vav-Bcl2 (Egle et al., 2004) mice were respectively, kindly provided by Tasuku Honjo (University of Kyoto, Japan), Alexander Tarakhovsky (Rockefeller University, USA) and Ari Melnick (Weill Cornell Medical School). Aicdacre/cre mice (Robbiani et al., 2008) were obtained from the Nussenzweig lab (Rockefeller University, USA). Generation of Chd4fl/fl mice was described previously (Williams et al., 2004). Cd19cre/cre, Cd21cre, p53fl/fl and C57BL/6J were purchased from the Jackson Laboratories (Bar Harbor, ME). 8-16 week old mice of both sexes were used and whenever possible littermates were used as controls. Mice used in the experiments were maintained in specific pathogen free conditions in Memorial Sloan Kettering Cancer Center vivarium according to the institutional guidelines.

METHOD DETAILS

Purified B cell cultures

Splenic B cells from naive mouse were purified by anti-CD43 negative selection according to manufacturer’s protocol (Miltenyi Biotec #130-049-801). Purified B cells were cultured in B cell media as described earlier (Pucella et al., 2019). Naive B cells were stimulated with LPS (30 μg/ml, Sigma, #L4130) for IgG3 CSR and LPS plus mouse IL-4 (12.5ng/ml, R&D Systems) or anti-CD40 (10ng/ml, eBioscience clone HM40-3, #16-0402) plus IL-4 (12.5ng/ml) for CSR to IgG1. For IgA CSR, B cells were cultured with LPS (5 μg/mL), BAFF (20ng/mL), IL-4 (10ng/mL), IL-5 (10ng/mL), TGF-β (10ng/mL) and Retinoic acid (10nM). Naive B cells were also cultured in LPS (10 μg/mL) plus mouse IFN-γ (50ng/mL, Gemini Bio-products, #300-311P) or mouse anti-CD40 (10ng/mL) plus IL-21 (50ng/mL, R&D systems). Usually, B cells cultures were initiated at 0.5-1.0x10^6/ml and diluted 1:2 at 48h and 72h and analyzed for CSR at 96h.

Retroviral infection

Short hairpin RNAs against mouse CHD4 (5′-GACTACGACCTGTCAAGCAG-3′) or scrambled (5′-GCGAAAGATGATAAGCTAA-3′) was obtained from Addgene (plasmid 15379). MSCV-cre was obtained from Addgene (plasmid 24064). Retroviral transduction of splenic B cells has been described earlier (Drane et al., 2017). Briefly, retrovirus supernatant was prepared by co-transfecting

Software and Algorithms

R (v.3.3.3) https://cran.r-project.org/ https://cran.r-project.org/
Trimmomatic (v.0.36) Bolger et al., 2014 http://www.usadellab.org/cms/?page=trimmomatic
Salmon (v0.8.2) Patro et al., 2017 https://salmon.readthedocs.io/en/latest/index.html
bximport (v.1.8.0) Soneson et al., 2015 https://bioconductor.org/packages/release/bioc/html/bximport.html
DESeq2 (v.1.14.1) Love et al., 2014 http://bioconductor.org/packages/release/bioc/html/DESeq2.html
GOseq (v.1.26.0) Young et al., 2010 https://bioconductor.org/packages/release/bioc/html/goseq.html
Prism 7 GraphPad software Inc. N/A
FlowJo 9.9 FloJo LLC N/A
Other
CD43 (Ly-48) MicroBeads Miltenyi Biotec Cat# 130-049-801

Software and Algorithms

R (v.3.3.3) https://cran.r-project.org/ https://cran.r-project.org/
Trimmomatic (v.0.36) Bolger et al., 2014 http://www.usadellab.org/cms/?page=trimmomatic
Salmon (v0.8.2) Patro et al., 2017 https://salmon.readthedocs.io/en/latest/index.html
bximport (v.1.8.0) Soneson et al., 2015 https://bioconductor.org/packages/release/bioc/html/bximport.html
DESeq2 (v.1.14.1) Love et al., 2014 http://bioconductor.org/packages/release/bioc/html/DESeq2.html
GOseq (v.1.26.0) Young et al., 2010 https://bioconductor.org/packages/release/bioc/html/goseq.html
Prism 7 GraphPad software Inc. N/A
FlowJo 9.9 FloJo LLC N/A
Other
CD43 (Ly-48) MicroBeads Miltenyi Biotec Cat# 130-049-801
pMIG, 2T-AID, or CHD4 shRNA plasmids with packaging vector pCL-Eco into HEK293T cells using calcium phosphate. Purified splenic B cells (2x10^6) were plated at a density of 1-2 x 10^6 cells per/ml and stimulated with 30 μg/ml LPS plus 12.5 ng/ml IL-4. After stimulation for 24 h and 48 h, media were aspirated, leaving approximately 1 ml of media per well of a six-well dish, and retroviruses were added. Six-well dishes were spun at 2,000 g for 90 min at 32°C, after which viral supernatants were aspirated and fresh B cell media plus LPS and IL-4 was added. Cells were analyzed 48h after the second round of infection.

**Flow cytometry and sorting**

For flow cytometric analysis, cells were washed with FACS buffer (1 x PBS, 2.5% FBS), resuspended in 100 μl FACS buffer, incubated with an Fc blocking reagent (BD PharMingen) and stained at 4°C with antibodies to various cell surface antigens. Viability was assessed with DAPI (Invitrogen) or Zombie red fixable (BioLegend). Cells were washed 1x with FACS buffer, and resuspended in 200 μl FACS buffer. Flow cytometric analysis of CFSE or Annexin V stainings was performed according to manufacturer’s instructions (CellTrace CFSE Cell Proliferation Kit, InVitrogen and FITC Annexin V Apoptosis Detection Kit, BD Biosciences. Briefly, purified naive B cells were stained with 5 μM CFSE and cultured for 96 h in LPS plus IL-4 and CFSE dilution was monitored by flow cytometer. GFP* cells or bone marrow B cell populations were sorted in a FACSria cell sorter (BD Biosciences). Flow cytometry was performed using an LSR-II flow cytometer (BD Biosciences), and data analyzed using FlowJo software (version 9.9).

**Immunization**

Mice were intra-peritoneally injected with 1x10^8 packed sheep red blood cells in 200 μl of PBS (Innovative Research #IC10-0210) on day 0, followed by a boost of the same number of packed cells on day 10. The spleens of immunized or PBS mock-injected control mice were analyzed on day 14. For NP-CGG immunization, NP(31)-CGG (no. N-5055D-5; Biosearch Technologies) was precipitated with Imject alum adjuvant (no. 77161; Thermo Fisher Scientific). Mice were immunized intra-peritoneally in 100 μl of 200 μg NP-CGG in alum on day 0, followed by a boost of 50 μg NP-CGG in alum on day 10. B cells in the spleens of immunized or PBS-injected mice were analyzed d14. For LPS challenge, TRlgrade LPS from *Salmonella minnesota* R595 (no. ALX-581-008- L002) was purchased from Enzo Life Sciences. Mice were immunized intravenously with 50 μg LPS in sterile PBS (100 μl) on d0 and sacrificed on d5 to assess plasmablast formation.

**Sμ mutation analysis**

The 5’ end of Sμ was amplified by PCR using Phusion High-Fidelity DNA Polymerase (NEB) with primers SμF (5’-AATGGATACCT CAGTGGTTTTAATGGTGGGTTTA-3’) and SμR (5’-CGGCCCCGTCATTACCGATTTAAG-3’) as described previously (Barreto et al., 2003). The PCR amplification conditions were as follows: 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C (1 min). PCR products were cloned into TOPO PCR Cloning vector (Invitrogen) and then sequenced (GENEWIZ). Sequences were aligned with Lasergene (DNASTAR) and analyzed for mutational frequencies.

**Protein analysis**

Protein extracts from primary B cell cultures or sorted GFP* splenic B cells were prepared using NP-40 lysis buffer (20 mM Tris-Cl, pH 8.0, 150-650 mM mM NaCl, 1 mM EDTA, 0.5% NP-40) plus protease inhibitors (complete Mini, EDTA-free Roche Cat#11836170001). For immunoprecipitation, lysates were centrifuged at 15,000 x g for 20 min, and the supernatant was collected as cell-free extracts. Extracts (0.5 mg) were precleared with agarose beads and then incubated with anti-HA agarose (Pierce) at 4°C overnight and subsequently with protein A agarose beads for another 90 min. The immunoprecipitate was washed twice with low-salt buffer (150 mM NaCl), once with high-salt buffer (500 mM NaCl), and then with 1 x Tris-buffered saline. The proteins were eluted with 2 x SDS sample loading buffer, and analyzed by immunoblotting. The AID antibody used in this study was described earlier (Chaudhuri et al., 2003).

**Chromatin immunoprecipitation**

Splenic B cells from 8-10 week old mice were isolated and ChIP was performed according to protocol described in the ChIP Assay Kit (Millipore, 17-297). Briefly, splenic B cells were stimulated with LPS (30 μg/ml) plus mouse IL-4 (12.5ng/ml) for 48h or 72h. Approximately 1.0x10^7 activated cells were cross-linked by adding fresh 11% formaldehyde to a final concentration of 1% and incubating at room temperature for 10 minutes. The reaction was neutralized by adding 1:20 volumes of 2.5M glycine (pH 8.0). Cross-linked cells were washed with PBS-based Cell Wash Buffer (PBS, 0.1% FBS, 2mM EDTA) and lysed according to the protocol of truChIP Chromatin Shearing Kit with Formaldehyde (Covaris, 520154). Cell lysates were sonicated in the M220 Focused-ultrasonicator (Covaris, 5002295) to shear the chromatin, and debris was removed by centrifugation. Sonicated lysates were diluted to 1.5 mL in ChIP dilution buffer and pre-cleared according to manufacturer’s protocol. Pre-cleared lysate was divided into three parts of 500 μL each and incubated with 1 μg of anti-H3 antibody (Abcam), 2 μg of anti-AID antibody (Chaudhuri et al., 2003) or control IgG (Jackson Immunoresearch Labs) at 4°C overnight. Immunocomplexes were recovered with salmon sperm DNA/Protein A agarose slurry and washed with the sequence of buffers listed in the protocol (1ml per wash). Immunocomplexes were eluted from the beads using 500 μL of fresh elution buffer (1% SDS and 0.1M NaHCO3) and cross-linking was reversed by incubation with 5M NaCl (final concentration 0.3M) and RNase A (30 μg/ml) at 65°C for 4-6h. The samples were then treated with 20 μL of 1M Tris pH 6.5, 10 μL of 0.5M EDTA and 2 μL proteinase K (10mg/ml) for 1 hour at 45°C. DNA was recovered by phenol-chloroform extraction and ethanol precipitation.
with carrier glycogen. Pellets were resuspended in 35 μL of water for use as ChIP DNA or 50 μL of water for use as input DNA. DNA was analyzed by qPCR using PowerUp SYBR Green Master Mix (Applied Biosystems) for Sμ and negative control locus Cγ1.

ELISA
Coating antibodies for binding IgA, IgM and IgG1 were purchased from Southern Biotechnology Associates (key resources table). Isotype standards used to calculate absolute concentration values are listed in the key resources table. HRP-labeled secondary antibodies for detecting IgA, IgM and IgG1 were purchased are listed in the key resources table. eBioscience ELISA diluent (no. 00-4202-56) was used as blocking buffer, TMB substrate (no. 00-4201-56) was used to develop, and 1 M phosphoric acid was used to stop development. Plates were read at 450 nm on a BioTek Synergy HT detector. Absolute concentrations of serum Abs were determined by interpolation from the standard curve, with attention paid to keeping values within standard and sample linear ranges. All samples were analyzed in duplicate over an eight-step dilution series.

RNA sequencing and analysis
Splenic B cells were isolated and stimulated with LPS plus IL-4 for 48h. RNA was isolated using TRIzol (Invitrogen) and RNeasy Mini Kit (QIAGEN catalog # 74104) according to instructions provided by the manufacturer. After RiboGreen quantification and quality control by Agilent BioAnalyzer, 2 ng total RNA with RNA integrity numbers ranging from 8.1 to 9.6 underwent amplification using the SMART-Seq v4 Ultra Low Input RNA Kit (Clontech catalog # 63488), with 12 cycles of amplification. Subsequently, 10 ng of amplified cDNA was used to prepare libraries with the KAPA Hyper Prep Kit (Kapa Biosystems KK8504) using 8 cycles of PCR. Samples were barcoded and run on a HiSeq 4000 in a 50bp/50bp paired end run, using the HiSeq 3000/4000 SBS Kit (Illumina). An average of 56.8 million paired reads were generated per sample and the percent of mRNA bases per sample ranged from 58% to 75%.

Paired-end reads were trimmed for adaptors and removal of low quality reads using Trimmomatic (v.0.36) (Bolger et al., 2014). Transcript quantification was based on the mm10 UCSC Known Gene models and performed using the quasi-mapping-based mode of Salmon (v.0.8.2) (Patro et al., 2017), correcting for potential GC base pair bias. Differential analyses were performed on the transcript level using tximport (v.1.8.0) (Soneson et al., 2015) and DESeq2 (v1.14.1) (Love et al., 2014). Transcripts were considered differentially expressed (DE) if they showed a false discovery rate (FDR)-adjusted P value < 0.05, an absolute log2 fold change of 0.5, and an average transcripts-per-million (TPM, calculated by Salmon) > 10.

Gene set analysis was performed using GOseq (v.1.26.0) (Young et al., 2010), using gene IDs from DE transcripts, and the “biological process” subgroup of gene sets curated by the Gene Ontology Consortium (Ashburner et al., 2000). Transcripts that were missing gene IDs as designated by UCSC were assigned Entrez IDs based on gene name using the org.Mm.eq.db R package (v.3.4.0) before performing gene set analysis. Transcripts that had no ID assigned were dropped. FDR-corrected P values were calculated from P values calculated by GOseq, and only gene ontologies passing a threshold of p < 0.05 and contained at least 10 DE genes were considered. Top 10 gene sets that contained the highest proportion of DE genes out of the total gene set are depicted. The gene set titled, “adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains” was renamed to “CSR genes.”

QUANTIFICATION AND STATISTICAL ANALYSIS
All statistical analyses were performed using Prism (GraphPad). Graphs represent data from at least 2 independent experiments, each consisting of at least 3 biological replicates. The exact number of biological replicates (n) in the presented dataset is indicated in the figure legends. Results are expressed as mean ± SD. Two-tailed, unpaired Student’s t test was performed on individual biological replicates. Differences were considered significant when P values were < 0.05. P values > 0.05 are either not shown or marked with n.s. (not significant). *p < 0.05, **p < 0.005.

DATA AND SOFTWARE AVAILABILITY
RNA-seq data have been deposited in the Gene Expression Omnibus under accession number GEO: GSE128321.
Supplemental Information

Distinct Requirements of CHD4 during B Cell Development and Antibody Response

Wei-Feng Yen, Rahul Sharma, Montserrat Cols, Colleen M. Lau, Ashutosh Chaudhry, Priyanka Chowdhury, William T. Yewdell, Bharat Vaidyanathan, Amy Sun, Maryaline Coffre, Joseph N. Pucella, Chun-Chin Chen, Maria Jasin, Joseph C. Sun, Alexander Y. Rudensky, Sergei B. Koralov, and Jayanta Chaudhuri
Figure S1. CHD4 expression is not markedly altered upon B cells upon stimulation ex vivo (Related to Figure 1).

Whole cell extracts (~120-150 μg) from naïve or 48h-activated activated splenic B cells from wild type B/6 mice were analyzed by immunoblotting using CHD4 or α-tubulin antibodies. Extracts from LPS plus IL-4 activated CHD4 KO (Cd19<sup>cre</sup>/wtChd4<sup>fl/fl</sup>) B cells were used as controls. The results of two independent experiments are presented.
Figure S2. Deletion of Chd4 in B cells (Related to Figure 3)

(A) Splenocytes were analyzed for the expression of B220 and IgM. Left panel, representative flow cytometry plot. Relative spleen size and total number of B cells were quantified (middle panel); (n=5). Representative spleens from CHD4 KO and control mice are shown (right panel). (B) PCR genotyping of floxed (Flox) and deleted (Del) Chd4 alleles in purified splenic naive B cells derived from CHD4 KO (Cd19cre/wt Chd4fl/fl) and control (Chd4fl/fl) mice. (C) Impaired CSR in CHD4 KO B cells activated with anti-CD40 and IL-4. IgG1 surface expression in CHD4 KO (Cd19cre/wt Chd4fl/fl) and control (Chd4fl/fl) B cells stimulated with anti-CD40 plus IL-4 for 96 h (left panel). CSR was quantified (middle panel) (n=4, **P <0.005). Whole cell extracts from stimulated B cells were analyzed by immunoblotting (right panel). The immunoblot is representative of two independent experiments. (D) Analysis of splenic B cells in Cd21cre Chd4fl/fl mice. Cellularity of spleen and B cell numbers are shown (left panel; n=3). Representative flow cytometry plot of splenic B cells stained with B220 and IgM (right panel). (E) Lower birth rate of Cd21cre Chd4fl/fl mice. Birth rates of mice of the indicated genotypes were calculated from the offspring of crossing Cd21cre Chd4fl/wt males to Chd4fl/fl females or Chd4fl/fl males to Cd21cre Chd4fl/wt females.
Figure S3. Analysis of CHD4 KO B cells (Related to Figure 3).

(A) Germline transcription is not altered in CHD4 KO B cells. Levels of μ and γ1 germline transcripts were determined by qRT-PCR, normalized to β-actin mRNA and control (Chd4^{fl/fl}). Bar graph represents the average of three independent experiments (mean±SD).

(B) Loss of CHD4 does not induce B cell apoptosis. CHD4 KO (Cd19^{cre/wt}Chd4^{fl/fl}) and control (Chd4^{fl/fl}) splenic B cell were stimulated with LPS plus IL-4 and stained with Annexin V. Graph represents the average of three independent experiments (mean±SD).
Figure S4. Germinal center (GC) B cells upregulate CHD4 expression (Related to Figure 5).

(A) Mice were immunized with 50 μg NP-CGG, boosted at d10 and CHD4 expression examined by intracellular staining at d14 post-immunization. Representative flow cytometry histogram of GC+ (Fas+ GL7+ of live B220+) and GC- (Fas- GL7- of live B220+) B cells from the spleens of immunized mice at d14 post-immunization. Non-specific antibody and CHD4 KO B cells served as controls.

(B) Mean fluorescence intensity (MFI) of CHD4 staining in the GC+ versus the GC- B cells of Chd4fl/fl mice was quantified (n=3; *P<0.05).
Figure S5. Gating strategy for quantifying frequency of germinal center (GC) and early memory B cells (related to Figure 5).

Representative gating strategy for identifying GC B cells (Fas$^+$ GL7$^+$ of live B220$^+$) and early memory B cells (NP$^+$ of CD38$^+$ GL7$^-$, gated on live B220$^+$) from NP-CGG immunized mice. Mock represents PBS injected mice. Both populations of cells were further gated for total switched B cells (IgM$^-$ IgD$^-$) or IgM$^+$ B cells.
Fig. S6

A  % NP+ of CD38+ GL7- (gated on B220+ CD19+ live singlets)

B  NP+ CD38+ GL7- cell number (x10^4) (gated on B220+ CD19+ live singlets)

C  % IgM of NP+ (gated on CD38+ GL7+ B220+ live singlets)

D  % IgM of NP- (gated on CD38+ GL7+ B220+ live singlets)

E  IgA concentration (μg/ml)

F  Peyers’s patches

G  Peyers’s patches

H  Experiment #1  Experiment #2

Chd4fl/fl  Cd19cre/wtChd4fl/fl  Aicda^cre/cre

8.79  3.94  0.217

9.61  5.19  0.179
Fig. S6. Early memory B cells and IgA CSR in CHD4 KO mice (Related to Figure 5).

(A-D) Mice were immunized with 50 µg NP-CGG in alum or with PBS (mock), boosted at d10 with 50 µg of NP-CGG in alum and the spleen of immunized mice analyzed at d14 post-immunization. (A) Frequency of NP-specific early memory B cells in the spleen of immunized mice (NP⁺ of CD38⁺ GL7⁻, gated on live B220⁺ CD19⁺). (B) Absolute number of NP-specific early memory B cells in the spleen of immunized mice (NP⁺ CD38⁺ GL7⁻ of B220⁺ CD19⁺ live cells). (C) Frequency of unswitched NP-specific memory B cells (IgM⁺ of NP⁺, gated on CD38⁺ GL7⁻ B220⁺ CD19⁺ live cells). (D) Frequency of switched memory (IgM⁻ IgD⁻ of NP⁺, gated on CD38⁺ GL7⁻ B220⁺ CD19⁺ live cells) B cells. Each symbol represents a different animal, control (Chd4fl/fl, n=3) or CHD4 KO (Cd19cre/wt Chd4fl/fl, n=4). *P<0.05. (E-H) IgA levels at homeostasis and IgA CSR ex vivo. (E) Serum from control (Chd4fl/fl, n=2) or CHD4 KO (Cd19cre/wt Chd4fl/fl, n=3) mice were analyzed by ELISA for IgA levels. (F) Frequency of germinal center (GC) B cells in Peyer’s patches (Fas⁺ GL7⁺ of live B220⁺). (G) Frequency of IgA⁺ B cells amongst the GC B cells in Peyer’s patches (IgA⁺ of Fas⁺ GL7⁺ gated on live B220⁺); (Chd4fl/fl, n=4, CHD4 KO (Cd19cre/wt Chd4fl/fl, n=5)). (H) CSR to IgA ex vivo. Naïve splenic B cells were activated in vitro with a cocktail of LPS, BAFF, IL-4, IL-5, TGF-β and retinoic acid. Frequency of CSR to IgA was determined at 96 h. Splenic B cells from Aicdacre/cre mice in which exon 1 of Aicda is disrupted with cre was used as source of AID deficient B cells.
Figure S7. AID binding to Sμ is impaired in the CHD4 deficient B cells (Related to Figure 7).

(A) ChIP analysis for AID occupancy at the Sμ in control (Chd4<sup>wt/wt</sup>p53<sup>wt/wt</sup>), p53KO (Cd19<sup>cre/wt</sup>p53<sup>fl/fl</sup>), CHD4 KO (Cd19<sup>cre/wt</sup>Chd4<sup>fl/fl</sup>p53<sup>wt/wt</sup>) and DKO (Cd19<sup>cre/wt</sup>Chd4<sup>fl/fl</sup>p53<sup>fl/fl</sup>) activated B cells at 48h after LPS plus IL-4 stimulation. AID-ChIP values were subtracted from IgG control and normalized to control (Cd19<sup>cre/wt</sup>Chd4<sup>wt/wt</sup>p53<sup>wt/wt</sup>). Quantification of AID-ChIP signal is indicated in each plot (mean±SD) (n=3; **P <0.005, *P <0.05).

(B) AID binding to Cγ1 (negative control) in ChIP experiments from 48h activated splenic B cells from the AID ChIP samples in (A) was quantified by real-time qPCR.

(C) AID and H3 occupancy at Sμ in B cells stimulated with LPS+IL-4 for 72h. Results were plotted as percent of input (mean±SD) (n=3; *P <0.05).