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Combinatorial H3K9acS10ph Histone Modifications in *IgH* Locus S Regions Target 14-3-3 Adaptors and AID to Specify Antibody Class Switch DNA Recombination

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SUMMARY

Class switch DNA recombination (CSR) is central to the antibody response, as it changes the immunoglobulin heavy chain (IgH) constant region, thereby diversifying biological effector functions of antibodies. The AID-centered CSR machinery excises and rejoins DNA between an upstream (donor) and a downstream (acceptor) S region, which precede the respective constant region DNA. AID is stabilized on S regions by 14-3-3 adaptors, which display a high affinity for 5'-AGCT-3' repeats, as recurring in all S regions. However, how 14-3-3, AID and the CSR machinery target exclusively the donor and acceptor S regions is poorly understood. Here we showed that histone methyltransferases and acetyltransferases were induced by CD40- or TLR-signaling and catalyzed H3K4me3 and H3K9ac/K14ac histone modifications, which were enriched in S regions but did not specify the S region target of CSR. By contrast, the combinatorial H3K9acS10ph modification specifically marked the S regions set to recombine and directly recruited 14-3-3 adaptors for AID stabilization there. Inhibition of the enzymatic activity of GCN5 and PCAF histone acetyltransferases reduced H3K9acS10ph in S regions, 14-3-3 and AID stabilization, and CSR. Thus, H3K9acS10ph is a histone code that is specifically "written" in S regions and "read" by 14-3-3 adaptors to target AID for CSR as an important biological outcome.

INTRODUCTION

Immunoglobulin (Ig) class switch DNA recombination (CSR) and somatic hypermutation (SHM) underpin the generation of class-switched high affinity antibodies. These are critical for the effectiveness of vaccines and the neutralization of pathogens, such as bacteria and viruses, and tumor cells, or the response to self-antigens (autoantibodies). SHM inserts point-mutations in antibody V(D)J region(s) at a high rate to provide the structural substrate for positive selection of higher affinity mutants by antigen (Casali, 2013). CSR substitutes

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the Ig heavy chain constant region (C_H), e.g., Cμ, with a downstream Cγ, Cα or Cε, thereby giving rise to IgG, IgA or IgE antibodies with new and diverse biological effector functions, without changing the structure or specificity of the antigen-binding site (Xu et al., 2012). CSR entails introduction of double-strand DNA breaks (DSBs) in the upstream ("donor") switch (S) region (Sμin naïve B cells) and a downstream ("acceptor") S region (an S region lies upstream of each C_H region exon cluster), and proceeds through resolution of such DSB by DNA repair. This leads to the juxtaposition of the originally recombined V_HDJ_H DNA with a downstream C_H exon cluster by looping out the intervening DNA as an "S circle" (Figure S1).

Triggering of CSR requires both "primary" and "secondary" CSR-inducing stimuli (Li et al., 2013; Xu et al., 2012). Primary stimuli comprise a T-dependent stimulus, i.e., CD40 engagement by CD154, and T-independent stimuli, such as dual engagement of a Toll-like receptors (TLR) and the B cell receptors (BCR) by microbe-associated molecular patterns (MAMPs) and antigen epitopes, respectively. This is exemplified by *E. coli* lipopolysaccharides (LPS), which engage TLR4 and BCR through the monophosphoryl lipid A moiety and polysaccharidic moiety, respectively (Pone et al., 2012a; Pone et al., 2012b). Primary stimuli induce B cells to proliferate and express CSR-related genes through activation of a variety of B cell differentiation stage-specific transcription factors, including NF-κB, HOXC4 and E2A (Mai et al., 2010; Mai et al., 2013; Murre, 2005; Park et al., 2009; Sayegh et al., 2003; Tran et al., 2010). Secondary stimuli consist of cytokines, such as interleukin-4 (IL-4), transforming growth factor-β (TGF-β) and interferon-γ (IFN-γ, in mouse, but not human). When enabled by primary stimuli, secondary stimuli direct CSR to specific Ig isotypes: IgG (four subclasses in both human and mouse), IgA and IgE – the only exception being CSR to IgG3 in the mouse, which is induced by LPS alone. They do so by activating transcription factors, such as STAT6 (IL-4), SMAD3/4 and RUNXs (TGF-β) and STAT1/2 (IFN-γ), for induction of germline I_H-S-C_H transcription (Xu et al., 2012). This starts at a specific I_H promoter and elongates through the I_H exon, intronic S region and C_H exon cluster, eventually giving rise to germline Iμ-Cμ, Iγ-Cγ, Iε-Cε or Iα-Cα transcripts after RNA splicing.

In addition to germline I_H-S-C_H transcription, a further reflection of an open chromatin state is provided by the enrichment in activating histone modifications, such as histone 3 lysine 4 trimethylation (H3K4me3) and H3 K9/K14 acetylation (H3K9ac/K14ac), and concomitant decrease in the repressive H3K9me3 in the S regions that are set to undergo recombination (Li et al., 2013). This is suggested by the analysis of Sμ and Sγ3 or Sγ1 chromatin in B cells stimulated by LPS, LPS plus BCR crosslinking or LPS plus IL-4 (Chowdhury et al., 2008; Daniel et al., 2010; Jeevan-Raj et al., 2011; Kuang et al., 2009; Pavri et al., 2010; Stanlie et al., 2010; Wang et al., 2006; Wang et al., 2009; Yamane et al., 2011). Open chromatin would be necessary to allow access of CSR machinery elements to S regions (Xu et al., 2012). Further suggesting an important role of activating histone modifications in CSR are the decreased Sα H3K4me3 enrichment and reduced CSR to IgA in an fashion independent of germline Iα-Sα-Cα transcription in CH12 mouse B cells after abrogation of H3K4 methyltransferases expression (Stanlie et al., 2010).

In species that use CSR to diversify their antibodies, all S regions contain at high density repeats of the 5'-AGCT-3' motif (Xu et al., 2010), an iteration of the 5'-RGYW-3' mutational hotspot (Chang and Casali, 1994). CSR requires activation-induced cytidine deaminase (AID, encoded by *AICDA* in human and *Aicda* in mouse) (Liu and Schatz, 2009; Stavnezer, 2011; Zan and Casali, 2012). In CSR, AID deaminates deoxycytidines (dCs) in S regions, particularly within 5'-AGCT-3' repeats, yielding high densities of deoxyuracils (dUs). dUs are processed by uracil DNA glycosylase (UNG) to generate abasic sites, which are then nicked by an apurinic/apyrimidinic endonuclease (APE), leading to single strand

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DNA breaks (SSBs). Nearby SSBs on opposite strands of S region "core" can readily form staggered DSBs, obligatory intermediates of CSR. Conversion of SSBs to DSBs in the flanking area of S region depends on mismatch repair proteins (MMRs), such as MSH2 and MSH6 and exonuclease 1 (EXO1) (Vallur and Maizels, 2008). In addition to providing substrates for AID DNA deamination, S region 5'-AGCT-3' repeats are bound with high affinity by 14-3-3 adaptors, which function as scaffolds to stabilize AID on S region DNA (Xu et al., 2010) – the REV1 DNA polymerase functions as a scaffold protein to recruit/stabilize UNG (Zan et al., 2012). Thus, 5'-AGCT-3' repeats provide an anchor for the assembly of the CSR machinery on S regions, but not other areas of *IgH* locus or the genome at large. As 5'-AGCT-3' repeats occur at high density in all S regions, how AID and other elements of CSR machinery are selectively recruited/stabilized to/on the donor and acceptor S regions that are set to undergo recombination, but not other S regions, is poorly understood.

A key function of many histone modifications in the regulation of DNA transactions (and, therefore, various biological processes) is to recruit downstream effector proteins (Ruthenburg et al., 2007; Suganuma and Workman, 2011; Taverna et al., 2007; Yun et al., 2011). Thus, histone modifications in S regions may play a critical role in targeting the CSR machinery by directly recruiting CSR factors. Here, we have hypothesized that a specific combinatorial histone modification encrypts a donor and acceptor S region-specific histone code that is to be "read" by the 14-3-3 and AID-centered CSR machinery for specific targeting of CSR. To test our hypothesis, we have used quantitative chromatin immunoprecipitation (qChIP) assays and two-step ChIP assays to identify a combinatorial histone modification (H3K9acS10ph) that exclusively marks the S regions that will undergo recombination, and that is read by 14-3-3 adaptors, which transduce such combinatorial modification through AID stabilization for a biological outcome (CSR). Further, we have performed H3K9acS10ph peptide competition assays to address the role of H3K9acS10ph in recruiting/stabilizing 14-3-3 adaptors to/on S regions. Finally, we have used a specific inhibitor of GCN5/PCAF histone acetyltransferases to reveal the role of H3K9acS10ph in histone writing and CSR targeting.

RESULTS

Primary and secondary stimuli synergize to induce S region histone modifications and CSR

As enabled by primary stimuli, different cytokines direct CSR to different S regions; in a naïve $Ig\mu^+$ B cell to Sγ3, Sγ1, Sα1, Sγ2, Sγ4, Sε or Sα2 (human), and to Sγ3, Sγ1, Sγ2b, Sγ2a, Sε or Sα (mouse). To address the role of histone modifications in selection of S region targets of CSR, we systematically analyzed the induction of activating H3K4me3 and H3K9ac/K14ac histone modifications and germline I_H-S-C_H transcription – both reflections of an open chromatin state – across all S regions by primary CSR-inducing stimuli, secondary CSR-inducing stimuli or both. In resting $Ig\mu^+Ig\delta^+$ B cells and stimulated B cells, the Sμ region was enriched in H3K4me3 and H3K9ac/K14ac and underwent germline Iμ-Sμ-Cμ transcription at a high level (Figure 1); the Iμ promoter and the intronic μ enhancer (iEμ) were also enriched in H3K4me3 and H3K9ac/K14ac, albeit to a lower degree than Sμ.

T-dependent (CD154) and T-independent (LPS, lipid A and BCR crosslinking) primary stimuli but not secondary stimuli induced H3K4me3, H3K9ac/K14ac and germline I_H-S-C_H transcription in Sγ3 and to a lower extent (LPS only) in Sγ2b (Figures 1, S2, S3 and S4). Induction of H3K4me3, H3K9ac/K14ac and transcription in any other downstream S regions required addition of cytokines (IL-4, TGF-β or IFN-γ), which synergized with primary stimuli to induce CSR, as shown by expression of post-recombination Iμ-C_H (Iμ-Cγ3, Iμ-Cγ1, Iμ-Cγ2b, Iμ-Cγ2a, Iμ-Cε and Iμ-Cα) transcripts, which are direct indexes of

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CSR, and class-switched IgH chains (Igγ3, Igγ1, Igγ2b, Igγ2a, Igε and Igα). The S regions marked by H3K4me3 and H3K9ac/K14ac and underwent germline I_H-S-C_H transcription were in general targets of recombination: Sγ1 and Sε, as induced by CD154 plus IL-4 (IL-4 alone induced germline Iγ1-Sγ1-Cγ1 transcription but not histone modifications in any S regions or CSR), Sγ1 by LPS plus IL-4, Sγ3 by LPS or lipid A or lipid A plus BCR crosslinking, Sγ3, Sγ2b and Sα by LPS plus TGF-β and BCR crosslinking, and Sγ2a by LPS plus IFN-γ (Figures 1D, S2D, S3 and S4D). However, little if any CSR to IgG3 was detected in B cells stimulated by LPS plus IL-4, IFN-γ or TGF-β, or CD154 plus IL-4, TGF-β and BCR crosslinking, in spite of Sγ3 enrichment in H3K4me3 and H3K9ac/K14ac, germline Iγ3-Sγ3-Cγ3 transcription and high levels of AID in these B cells (Figures 1, S2 and S3), suggesting that induction of these histone modifications and germline I_H-S-C_H transcription do not precisely predict the subsequent unfolding of CSR. This was also indicated by the extensive Sγ3 H3K4me3 marking but lack of CSR to IgG3 in B cells stimulated by CD154 alone or CD154 plus TGF-β, CD154 plus IFN-γ, or CD154 plus IL-4, TGF-β and BCR crosslinking (Figure S2).

Thus, Sμ, iEμ and Iμ are highly enriched in activating H3K4me3 and H3K9ac/K14ac histone modifications in resting B cells. Primary stimuli enable cytokines to induce H3K4me3, H3K9ac/K14ac and germline I_H-S-C_H transcription, which are reflections of an open chromatin state, in selected S regions. H3K4me3 and H3K9ac/K14ac modifications as well as germline I_H-S-C_H transcription are generally associated with, but they are confined to the S regions that will be targets of CSR.

Combinatorial H3K9acS10ph modification specifies the S regions set to undergo CSR

The failure of a variety of combined primary and secondary stimuli to induce CSR to IgG3 despite Sγ3 enrichment in H3K4me3 and H3K9ac/K14ac and AID expression prompted us to hypothesize that the selection of the S regions to be recombined is determined by a more specific histone modification, which encrypts a S region histone "code" (Jenuwein and Allis, 2001), which is to be read by the AID-centered CSR machinery. In an attempt to identify such specific hallmark histone modification, we first analyzed the induction of H3 serine 10 phosphorylation (H3S10ph), a modification critical for cell division (mitosis) (Wei et al., 1999) – B lymphocyte division is critical for CSR unfolding. In resting B cells, Sμ, but not iEμ, Iμ or Cμ, was constitutively enriched at high levels in H3S10ph. Upon application of primary and secondary stimuli, this histone modification, however, was induced in both recombining and non-recombining acceptor S regions as well as in I_H promoters and C_H exons (Figure 2A). By contrast, the combinatorial H3K9acS10ph mark, as identified by an antibody specific for the epitope as specified by H3S10ph and adjacent H3K9ac on the same H3 amino-terminal tail, was exclusively enriched in the donor Sμ and was induced (in a time-dependent manner) in the cytokine-selected acceptor S regions, at levels much higher than those in I_H promoters or C_H exons (Figure 2A, S5 and S6). Further, K9acS10ph and K4me3 marked the N-terminal tail of H3 of the same/adjacent nucleosome(s) in the donor Sμ and the same acceptor S region DNA, as shown by sequential ChIP (Figure 2B). Finally, the specific H3K9acS10ph combinatorial modification marked only the recombining Sγ3 or Sγ1 region in B cells stimulated with LPS or LPS plus IL-4, respectively. This specificity contrasted with the widespread H3S10ph enrichment and non-discriminating H3K4me3 and H3K9ac/K14ac in Sγ3, as further visualized by gel analysis of precipitated B cell chromatin (Figure 2C).

Thus, the combinatorial H3K9acS10ph histone modification exclusively marks the donor and acceptor S regions that are set to undergo recombination.

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H3K9acS10ph stabilizes 14-3-3 and AID on the S regions that are to undergo CSR

The exclusive enrichment of H3K9acS10ph in both donor Sμ and acceptor S regions that are to undergo recombination prompted us to define the functional meaning of such combinatorial mark. The only known direct “interactors” of H3S10ph are the seven highly homologous 14-3-3 isoform adaptors (Winter et al., 2008). The interaction of H3S10ph with 14-3-3 is enhanced by acetylation of the adjacent H3K9 residue (Winter et al., 2008;Walter et al., 2008). As we have shown, 14-3-3 adaptors can potentially bind Sμ and all downstream S regions owing to their high “affinity” for S region 5′-AGCT-3′ repeats (Xu et al., 2010). We, therefore, hypothesized that H3K9acS10ph targets 14-3-3 adaptors onto S regions (“14-3-3 docking”) for their stabilization (“14-3-3 locking”) by the S region 5’-AGCT-3’ repeats. Indeed, in B cells stimulated by LPS or LPS plus IL-4, 14-3-3 adaptors were recruited only to the recombining Sμ and Sγ3 or Sγ1 regions, the only S regions displaying specific enrichment in H3K9acS10ph, but not H3K9ac/K14ac and H3S10ph (Figures 3A and S7). Consistent with our demonstration that AID stabilization on S regions depends on 14-3-3, particularly 14-3-3γ (Xu et al., 2010), LPS- and LPS plus IL-4-stimulated *14-3-3*$\gamma^{-/-}$ B cells displayed an H3K9acS10ph enrichment in Sγ3 and Sγ1 comparable to their *14-3-3*$\gamma^{+/+}$ B cell counterparts, but reduced AID binding to these S regions (Figure 3B). Importantly, enforced expression of exogenous 14-3-3γ restored colocalization of AID with H3K9acS10ph in the acceptor Sγ3 region in LPS-stimulated *14-3-3*$\gamma^{-/-}$ B cells (Figure S8).

To prove the specificity of the interaction of 14-3-3 adaptors with S region H3K9acS10ph, we performed *in vitro* competition assays involving LPS-induced *14-3-3*$\gamma^{-/-}$ B cells, a free H3K9acS10ph peptide, a free H3K9S10ph peptide, a free H3K9acS10 peptide or a free “negative control” H3K9S10 peptide (Figure 3C). In these induced B cells, Sγ3 (enriched with H3K9acS10ph) was precipitated by purified recombinant GST-14-3-3γ protein but not GST alone (Figures 3D and 3E). GST-14-3-3γ precipitation of H3K9acS10ph-marked Sγ3 chromatin was inhibited by the H3K9acS10ph peptide, which effectively competed with the Sγ3 region H3K9acS10ph for targeting by GST-14-3-3, but not by the H3K9acS10 peptide, the H3K9S10ph peptide or the H3K9S10 peptide (Figure 3F).

Thus, H3K9acS10ph is read by 14-3-3 adaptors and recruits/stabilizes these adaptors and AID to/on the donor and the acceptor S regions that are to undergo recombination.

Primary stimuli induce histone-modifying enzymes through activation of NF-κB

Patterns of histone modifications, i.e., histone “codes”, are “written” (and can be “erased”) by histone-modifying enzymes in a cell- and gene-specific fashion (Campos and Reinberg, 2009). We reasoned that given the induction of S region-specificity of histone modifications in stimulated B cells, H3K9 acetyl and H3K4 methyl “writers” must be induced by the same stimuli that induce CSR. Indeed, primary stimuli (CD154, LPS, lipid A, BCR crosslinking or CpG), but not secondary stimuli (IL-4), induced *Gcn5* and *Pcaf* (encoding the highly conserved catalytic subunit of the H3K9 acetyl writer SAGA and PCAF acetyltransferase complexes, respectively) as well as histone acetyltransferase genes *p300* and *Cbp* (Figures 4A and S9). They also induced genes encoding non-catalytic structural components of SAGA and PCAF acetyltransferase complexes (Lee and Workman, 2007), including *Spt3*, *Ada2a*, *Ada2b*, *Ada3*, *Mbip* and *Trrap*. These genes were induced up to 30-fold by primary stimuli, within 16 hr of stimulation and before completion of the first B cell division (not shown). The same primary stimuli, but not IL-4, induced the genes encoding catalytic subunits MLL1, MLL2, MLL3 and MLL4 of MLL methyltransferases (a family of highly homologous and dominant H3K4 trimethyl writers) and respective non-catalytic structural components, such as the obligatory subunits WDR5 and ASH2L, and other major cofactors of MLL complexes, such as RBBP5 and CXXC1 (Figure 4B).

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Primary CSR-inducing stimuli induce activation of NF-κB, a master B cell transcription factor mediating a variety of functions in immune cells, such as B and T lymphocytes as well as NK cells (Pone et al., 2012b; Vallabhapurapu and Karin, 2009). We analyzed the dependency on NF-κB of the induction of histone-modifying enzymes and germline I_H-S-C_H transcription initiated at Iμ, Iγ3 and Iγ1 promoter, all of which possess at least one consensus NF-κB-binding site (not shown). To circumvent complications arising from potential defects in follicular mature B cells in mice deficient in NF-κB subunits, we blocked IKKβ-mediated phosphorylation of IκB and subsequent activation of p65 (canonical NF-κB pathway) in B cells from C57BL/6 mice using TPCA-1 (Iwasaki et al., 2011). Consistent with the regulation of multiple genes by NF-κB, blocking of activation of this transcription factor by TPCA-1 decreased the expression of components of H3K9 acetyltransferase and H3K4 methyltransferase complexes as well as expression of *14-3-3γ* and *Aicda* in B cells stimulated by LPS, LPS plus IL-4 or CD154 (Figures 5A and S10) – all these genes contain NF-κB binding sites in their promoter (not shown). Indeed, in B cells stimulated with LPS, NF-κB p65 was recruited to the promoters of *Gcn5*, *Pcaf*, *Ada2a*, *Ada2b*, *Mll3*, *Mll4*, *Rbbp5*, *Ash2l* and *Wdr5* as well as those of *14-3-3γ* and *Aicda*. NF-κB p65 recruitment to these promoters was inhibited by TPCA-1 (Figure S11). Blocking NF-κB activation also reduced the enrichment of H3K4me3 and H3K9acS10ph in both Sμ and Sγ3 in B cells stimulated by LPS or CD154, and in Sμ, Sγ3 and/or Sγ1 in B cells stimulated by LPS plus IL-4 (Figures 5B and S10).

Thus, primary but not secondary stimuli induce specific H3K9 acetyltransferases and H3K4 methyltransferases, and they do so through activation of the NF-κB pathway.

Inhibition of GCN5/PCAF histone acetyltransferases reduces S region combinatorial H3K9acS10ph modification, binding of 14-3-3 and AID, and aborts CSR

To further address the role of the S region combinatorial H3K9acS10ph histone modification, which stabilizes 14-3-3 and AID on S regions, in CSR, we blocked the enzymatic function of GCN5 and PCAF using the highly specific inhibitor CPTH2 (Chimenti et al., 2009) - both GCN5 and PCAF catalyze the H3K9ac histone modification (Jin et al., 2011). In B cells stimulated with LPS or LPS plus IL-4, CPTH2 significantly impaired CSR to IgG3 or IgG1 (by as much as 70%), as shown by decreased proportion of switched $Ig\gamma3^+$ or $Ig\gamma1^+$ B lymphocytes, decreased amounts of secreted IgG3 or IgG1 and lower levels of post-recombination Iμ-Cγ3 or Iμ-Cγ1 transcripts, without affecting cell proliferation or viability (Figures 6A, 6B, 6C and 6D). Unlike the global impairment of histone modifications and gene transcription by the IKKβ inhibitor TPCA-1, CPTH2 did not alter overall H3K9ac/K14ac levels, expression of histone-modifying enzymes (*Gcn5*, *Pcaf*, *Wdr5*, *Ash2l* and *Cxxc1*, MLL-associated *Paxip)* or the open chromatin state of Sγ3 or Sγ1 regions (shown by normal enrichment of H3K4me3 and H3S10ph and unaltered levels of germline I_H-S-C_H transcription) in B cells – *Aicda* expression was only marginally reduced (less than 28%) if at all (Figures 6D, 6E and 6F). Rather, CPTH2 reduced the H3K9ac/K14ac and H3K9acS10ph modifications in the S region (Sγ3 or Sγ1 but not Sμ) that was set to undergo recombination, thereby decreasing 14-3-3 and AID binding (recruitment/stabilization) to that S region (Sγ3 or Sγ1) (Figure 6F).

Thus, inhibition of the enzymatic activity of the GCN5 and PCAF histone acetyltransferases, which catalyze the combinatorial H3K9acS10ph histone modification, inhibits recruitment/stabilization of 14-3-3 adaptors and AID to/on S regions and aborts CSR.

DISCUSSION

Here, we identified H3K9acS10ph as a combinatorial histone modification that specifically marks the *IgH* S regions, which are set to undergo S-S recombination. We defined how

H3K9acS10ph is written as a histone code (by histone-modifying enzymes induced by primary stimuli) and is read (by 14-3-3 adaptors) to "transduce" (through 14-3-3 and AID) a specific functional outcome (CSR) (Figure 7). We showed that 14-3-3 adaptors, which possess no enzymatic activity, function as histone code readers to recruit/stabilize downstream effector molecules, which *per se* cannot read histone codes, as postulated by the "histone code hypothesis" (Suganuma and Workman, 2011; Wang et al., 2008). Overall, our findings highlight the central role of epigenetic modifications in the specification of a biological information output in response to environmental stimuli. This is exemplified by *E. coli* LPS, which elicited class-switched antibodies by inducing unique epigenetic changes, including, as shown here, histone modifications.

Resting $Ig\mu^+$ $Ig\delta^+$ B cells consistently displayed H3K9ac/K14ac, H3K4me3 and H3K9acS10ph histone modifications in the Sμ region as well as germline Iμ-Sμ-Cμ transcription, indicating that in these lymphocytes the Sμ region is constitutively in an open chromatin state and poised for switching. Primary stimuli (T-dependent and T-independent) alone, but not secondary stimuli (cytokines), induced H3K9 acetyl writers GCN5/PCAF and H3K4 methyl writers MLLs and likely did so through their characteristic activation of NF-κB. Indeed, blocking of NF-κB activation by TPCA-1 dampened the upregulation of H3K9 acetyltransferases and H3K4 methyltransferases, thereby reducing H3K9acS10ph and H3K4me3 enrichment in upstream Sμ and downstream Sγ3 or Sγ1 regions. It also decreased germline I_H-S-C_H transcription and induction of 14-3-3γ and AID. Highly specific inhibition of the GCN5/PCAF histone acetyltransferases activity by CPTH2 significantly decreased H3K9acS10ph in the downstream S region targets of CSR, such as Sγ3 and Sγ1,without affecting transcription of many CSR-related genes, B cell proliferation or B cell viability. H3K9acS10ph in Sμ was, however, unaffected, suggesting that it is mediated by other acetyltransferases, such as p300 and CBP – p300 and CBP were also induced by primary stimuli and might mediate the normal levels of overall H3K9ac/K14ac and expression of many genes in CPTH2-treated B cells.

Cytokines alone failed to induce histone-modifying enzymes and, therefore, histone modifications in any acceptor S regions. When enabled by primary stimuli, they, however – in addition to activating transcription factors for germline I_H-S-C_H transcription – induced histone code writing in S region(s) to specify S region recombination, e.g., IL-4-specified Sγ1 and Sε (as enabled by CD154), TGF-β specified Sγ2b and Sα (as enabled by LPS and BCR cross-linking) and IFN-γ specified Sγ2a (as enabled by LPS). The Sγ3 region underwent germline Iγ3-Sγ3-Cγ3 transcription and was marked with H3K4me3 and H3K9ac/K14ac in B cells induced by primary stimuli alone, possibly as a result of NF-κB activation and reflecting an open chromatin state. Sγ3 open chromatin state persisted even in the presence of IL-4, TGF-β or IFN-γ, which did not erase the primary stimuli-induced Sγ3 transcription or H3K4me3 and H3K9ac/K14ac in this S region. An exception was IL-4, which, in the presence of CD154, inhibited the induction of those histone modifications in Sγ3, suggesting that this cytokine activated yet to be identified repressive factor(s). This would hamper binding of NF-κB to the Iγ3 promoter and subsequent histone writing in Sγ3. Upon activation by primary stimuli alone or together with cytokines, H3S10ph was enriched in all S and I_H promoter regions as well as C_H exons, possibly as introduced by H3S10ph kinases, such as Aurora kinases, mitogen-and stress-activated kinases (MSKs), ribosomal subunit protein S6 kinases (RSKs) or IκB kinase-α (IKKα).

In switching B cells, the combinatorial H3K9acS10ph modification was enriched in the nucleosomes (necessarily in the same H3 histone) of donor Sμ and cytokine-selected acceptor S regions (not in the respective I_H or C_H regions) but not in any other (non-recombining) S regions. By contrast, H3S10ph, H3K4me3 and H3K9ac/K14ac marked not only the recombining S regions (Sμ and Sγ1, Sγ2b or Sγ2a) but also the non-recombining

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Sγ3 region in B cells stimulated by LPS plus IL-4, TGF-β or IFN-γ, indicating that the individual H3K9ac/K14ac and H3S10ph modifications mark different nucleosomes or the two different H3 histone molecules within the same nucleosome. The "writing" of the H3K9acS10ph combinatorial modification may depend on both sense and antisense S region transcription. S region sense transcription would allow histone-modifying enzymes to ride on the RNA polymerase (pol) II, on which AID is also hitched through direct interaction with Spt5 and Paf1, to reach S regions during elongation of germline I_H-S-C_H transcription (Figure 7). Upon RNA pol II stalling, likely due to complex secondary DNA structures (e.g., R-loops and cruciform-like structures) or antisense transcription in S regions, the hitched histone-modifying enzymes would catalyze histone modifications in these regions. The S region-specific factor(s) that initiate antisense transcription may actively promote the K9acS10ph marking on the same H3 histone tail around the antisense promoters.

Reading specificity of histone modifications can be increased by combination of different individual histone modifications (Ruthenburg et al., 2007; Suganuma and Workman, 2011; Taverna et al., 2007). For example, binding of TFIID and BPTF to H3K4me3 increased when both H3K9 and H3K14 were acetylated (Vermeulen et al., 2010). Indeed, as we have shown here, H3S10ph synergized with (adjacent) H3K9ac in the same histone tail to encrypt a code for more accurate reading and transduction of the epigenetic information, namely, docking of 14-3-3 adaptors onto recombining S regions and subsequent stabilization of AID. Selection of the S regions to be recombined ("targeting") relies on 14-3-3 docking onto the H3K9acS10ph-modified chromatin of the target S regions, as emphasized by the specific and efficient blocking of 14-3-3 binding by the H3K9acS10ph peptide. After docking on H3K9acS10ph, 14-3-3 proteins would be stabilized (locked) on the S regions by 5′-AGCT-3′ repeats (Figures 7 and S12). The recruitment/stabilization of 14-3-3 to/on S regions through binding to H3K9acS10ph and 5′-AGCT-3′ repeats is reminiscent of the RAG1/RAG2 complex stabilization on H3K4me3 and *V(D)J* DNA recombination signal sequences (RSS) (Schatz and Ji, 2011).

By functioning as scaffolds to assemble macromolecular CSR complexes, which are directly involved in DNA recombination, to S regions, 14-3-3 proteins transduce epigenetic information into specific CSR events, importantly, stabilization of AID on S regions. This is further indicated by our finding that inhibition of the S region combinatorial H3K9acS10ph marking by CPTH2 decreased 14-3-3 recruitment/stabilization and AID targeting to S regions – in B cells stimulated by LPS plus IL-4, the inhibitory effect of CPTH2 might have been compounded by the slight reduction of *Aicda* expression for efficient blocking of CSR to IgG1. Once locked onto S region '5-AGCT-3' repeats, 14-3-3 adaptors would more effectively stabilize AID and PKA to enhance dC deamination (Xu et al., 2010). In Sμ, this process would be enhanced by binding of KAP1-HP-1γ to residual H3K9me3 (Jeevan-Raj et al., 2011), leading to integration of 14-3-3/AID/KAP-1/HP-1γ macromolecular complexes. Colocalization of H3K4me3 and H3K9acS10ph on S region would suggest that these histone modifications may also function together to stabilize the CSR machinery, as H3K4me3 may bind putative plant home domain (PHD)-containing CSR factors through direct interaction with the PHD domain (Suganuma and Workman, 2011). Our findings strengthen the notion that multivalent interactions of effector proteins complexes with modified histones increase overall binding affinity and specificity (Ruthenburg et al., 2007; Yun et al., 2011). The direct interaction between 14-3-3 and UNG (Lam et al., 2013) and those between REV1 and UNG (Zan et al., 2012) would bridge AID and UNG to form an AID/14-3-3/UNG/REV1 complex, therefore enhancing the processing of AID-effected dUs by UNG for generation of DSBs.

Overall, our findings provide a critical evidence for an emerging paradigmatic role of complex histone codes in directing CSR targeting and, likely, the assembly of sequential

macromolecular complexes on S regions for DNA lesion generation and repair. They also provide useful clues to study the role(s) of histone modifications in SHM, as combinatorial histone modifications might play a role in the specific targeting of Ig V(D)J regions by the SHM machinery. Further, our demonstration of histone modifications functioning in combination to provide critical readout specificities in CSR can likely be extended to other biological processes, such as DNA replication and repair. Finally, our studies would have important implications for the development of epigenetic therapeutics that block unwanted CSR, such as those underpinning the generation of IgG autoantibodies or atopic IgE antibodies.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice and *14-3-3*$\gamma^{-/-}$ mice on the C57BL/6 background (Xu et al., 2010) were kept in the pathogen-free barrier vivarium at the University of California, Irvine – all protocols were in accordance to the rules and regulations of the Institutional Animal Care and Use Committee of the University of California, Irvine.

B cells, proliferation and viability

Preparation and purification of spleen and lymph node surface Igμ Igδ^{+} B cells were as we reported (Pone et al., 2012b). For analysis of proliferation, B cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) before culture and then analyzed by flow cytometry for CFSE intensity (CFSE distributes equally into the two daughter cells when a cell divides). B cell divisions were determined using the FlowJo® proliferation platform. For analysis of viability, B cells were stained with 7–AAD and analyzed by flow cytometry.

CSR

B cells were stimulated with mCD154 (3 U/ml), LPS (5 μg/ml, Sigma-Aldrich) or TLR4 ligand monophophoryl lipid A (1 μg/ml, Sigma-Aldrich) plus appropriate cytokine(s) to induce CSR to different Ig isotypes. Anti-δ monoclonal antibody (mAb)-conjugated dextran (anti-δ mAb/dex, Fina Biosolutions) was added where indicated. After 4 days, B cells were analyzed for surface or intracellular expression of Ig (detailed in Extended Experimental Procedures).

Germline I_H-C_H, post-recombination Iμ-C_H and other CSR-related gene transcripts

RNA was extracted from B cells (5×10^6) using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The expression of germline I_H-C_H, post-recombination Iμ-C_H, *14-3-3γ*, *Aicda*, *Paxip*, *Cd79b*, *Gcn5*, *Pcaf, p300, CBP, Spt3, Ada2a*, *Ada2b*, *Ada3*, *Mbip*, *Trrap*, *Mll1*, *Mll2*, *Mll3*, *Mll4*, *Wdr5*, *Ash2l*, *Rbbp5* and*Cxxc1* transcripts were analyzed by real-time quantitative RT-PCR (qRT-PCR) using specific primers (Table S1).

Secreted Ig

Titers of Ig in culture supernatants of *in vitro* cultured B cells were measured using specific ELISAs (detailed in Extended Experimental Procedures).

Inhibition of NF-κB and GCN5/PCAF histone acetyltransferases

For inhibition of NF-κB activation, B cells were pre-treated with FBS-RPMI containing 1 μM of [5-(pfluorophenyl)-2-ureido]-thiophene-3-carboxamide (TPCA-1, EMD Millipore) and then stimulated by LPS, LPS plus IL-4 or CD154, followed by analysis of expressed CSR-related genes and enrichment of histone modifications in *IgH* S regions after 16 hr.

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For inhibition of GCN5/PCAF histone acetyltransferase activity, B cells were pre-treated with FBS-RPMI containing 20 μM of cyclopentylidene-[4-(4′-chlorophenyl)thiazol-2-yl]hydrazine (CPTH2, Sigma-Aldrich) and then stimulated by LPS or LPS plus IL-4, followed by analysis of the expressed CSR-related genes and enrichment of histone modifications in *IgH* S regions after 48 hr, and analysis of CSR after 4 days.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as we described (Mai et al., 2013; Xu et al., 2010) and detailed in Extended Experimental Procedures, using an Ab specific for H3K4me3, H3K9ac/K14ac, H3S10ph, H3K9acS10ph, 14-3-3γ, AID or p65 (Table S2). Precipitated chromatin DNA was analyzed by quantitative qPCR using primers specific for DNA in IgH locus regions or selected genes (Table S3), as detailed in Extended Experimental Procedures.

GST-14-3-3 pull-down and H3K9acS10ph peptide competition assays

LPS-induced *14-3-3γ*$^{-/-}$ B cells (1×10^7) were treated with 0.25% (v/v) formaldehyde for 10 min to crosslink chromatin, which was then sonicated as in the above ChIP protocol. Chromatin was pre-cleared with glutathione-magnetic beads (Pierce) and then incubated with the equal amount of glutathione-magnetic beads-coupled recombinant GST or GST-14-3-3γ proteins at 4°C for 1 hr. Precipitated complexes were then washed and eluted with elution buffer (50 mM Tris-HCl, 0.5% SDS, 200 mM NaCl, 100 μg/ml of proteinase K, pH 8.0), followed by incubation at 65°C for 4 hr to reverse formaldehyde cross-links and digest proteins. DNA was purified and Sγ3 region DNA was specified by PCR. For peptide competition assays, recombinant GST-14-3-3γ proteins were pre-incubated with the H3 N-terminal peptide (NH_2-ARTKQTAR**KS**TGGKAPRKQLA-COOH, H3K9S10 peptide, 2 μg/ml, AnaSpec), the H3 N-terminal peptide containing K9ac (NH_2-ARTKQTAR**K**acSTGGKAPRKQLA-COOH, H3K9acS10 peptide), the H3 N-terminal peptide containing S10ph (NH_2-ARTKQTARK**S**phTGGKA-COOH, H3K9S10ph peptide) or the H3 N-terminal peptide containing both K9ac and S10ph (NH_2-ARTKQTAR**K**ac**S**phTGGKAPRKQLA-COOH, H3K9acS10ph peptide) at 4°C for 2 hr before incubation with chromatin, followed by washing, elution and DNA purification. Sγ3 DNA was specified to assess the specific binding of 14-3-3γ proteins to Sγ3 region H3K9acS10ph. The experimental procedures were adapted from previous reports (England et al., 2010; Meehan et al., 2003).

Statistical analysis

Statistical analysis was performed using the Excel® software to determine *P* values by paired student t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- ▶ Primary, but not secondary, CSR-inducing stimuli induce histone-modifying enzymes.
- ▶ Primary and secondary stimuli synergize to induce histone modifications in *IgH* S regions.
- ▶ H3K9acS10ph marks exclusively the donor and cytokine-specified acceptor S regions.
- ▶ H3K9acS10ph targets14-3-3 and then AID to the donor and acceptor S regions for CSR.

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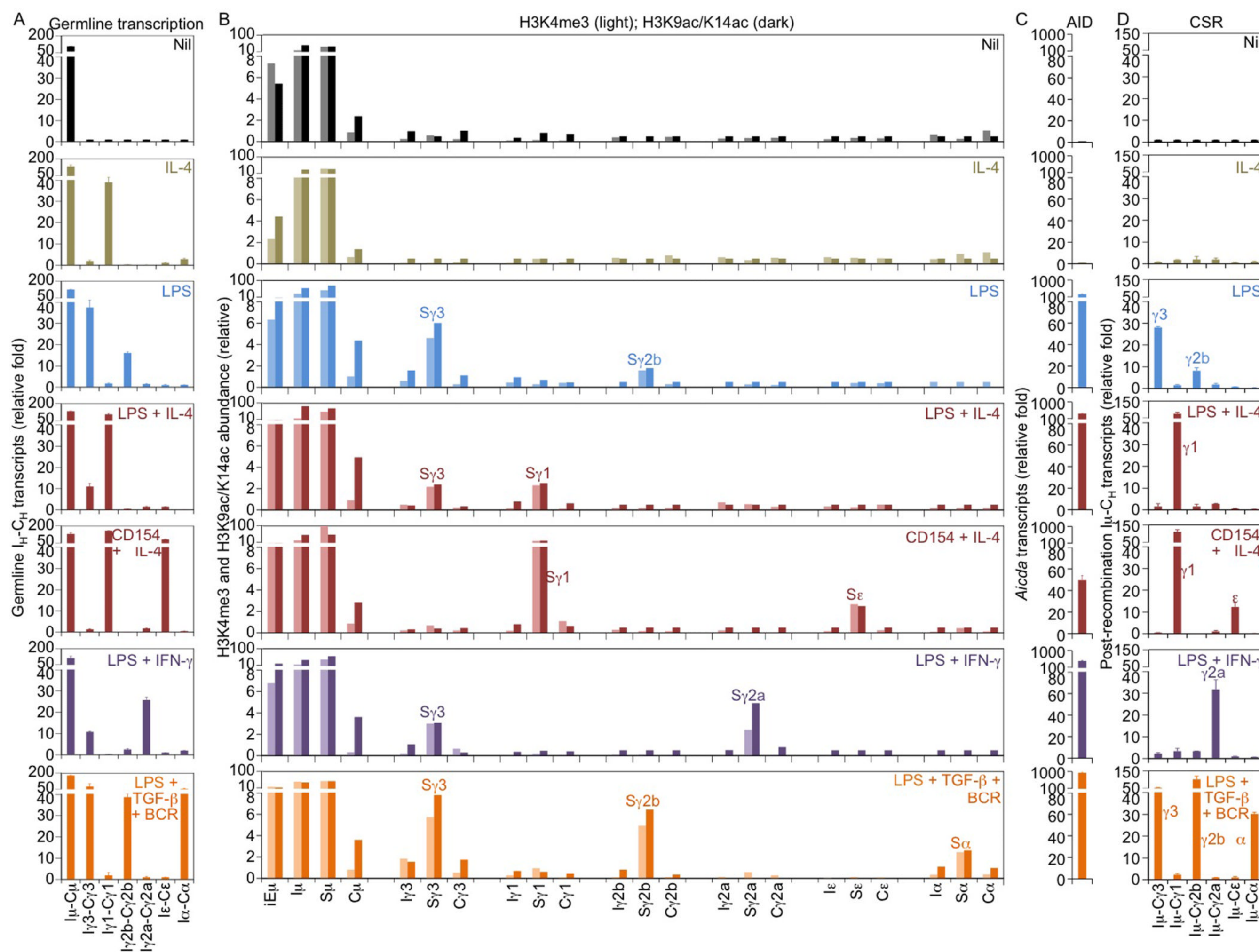

Figure 1. Primary and secondary stimuli synergize to induce S region histone modifications and CSR

(A) Levels of germline I_H-S-C_H transcripts in freshly isolated B cells (nil, black), B cells stimulated by IL-4 alone (yellow), LPS (blue) alone, LPS plus IL-4 (red), CD154 plus IL-4 (red), LPS plus TGF-β (green), LPS plus IFN-γ (purple) or LPS plus TGF-β and BCR crosslinking (anti-δ mAb/dex, orange) for 48 hr (as enabled by primary stimuli, different cytokines direct CSR to different S regions). Data were normalized to the expression of *Cd79b* and are depicted as ratios of values in stimulated B cells to those in resting B cells (mean and s.d. of triplicate samples). Data are representative of those from three independent experiments. The Sμ region underwent a high levels of germline Iμ-Sμ-Cμ transcription in (freshly isolated) resting B cells (the value was thus set as 100). IL-4 alone induced germline Iγ1-Sγ1-Cγ1 but not Iε-Sε-Cε transcription. (B) ChIP assays involving chromatin from resting or stimulated B cells, as in (A), and an Ab specific for H3K4me3 or H3K9ac/K14ac. Depicted is the abundance of iEμ DNA and I_H, S and C_H region DNA in the upstream Igμ and downstream Igγ3, Igγ1, Igγ2b, Igγ2a, Igε and Igα subloci in immunoprecipitated chromatin. Like IL-4 alone (shown), IFN-γ or TGF-β alone did not induce H3K4me3 and H3K9ac/K14ac modifications in any downstream S regions (not shown). IL-4 induces these histone modifications in Sγ1 (as enabled by either CD154 or LPS); it also induces histone modifications in Sε (as enabled by CD154). Data are representative of those from three independent experiments.

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(C and D) Levels of *Aicda* and post-recombination Iμ-C_H transcripts in resting or stimulated B cells, as in (A). Data were normalized to the expression of *Cd79b* and are depicted as ratios of values in stimulated B cells to those in resting B cells (mean and s.d. of triplicate samples). Data are representative of those from three independent experiments.
See also Figures S1, S2, S3 and S4.

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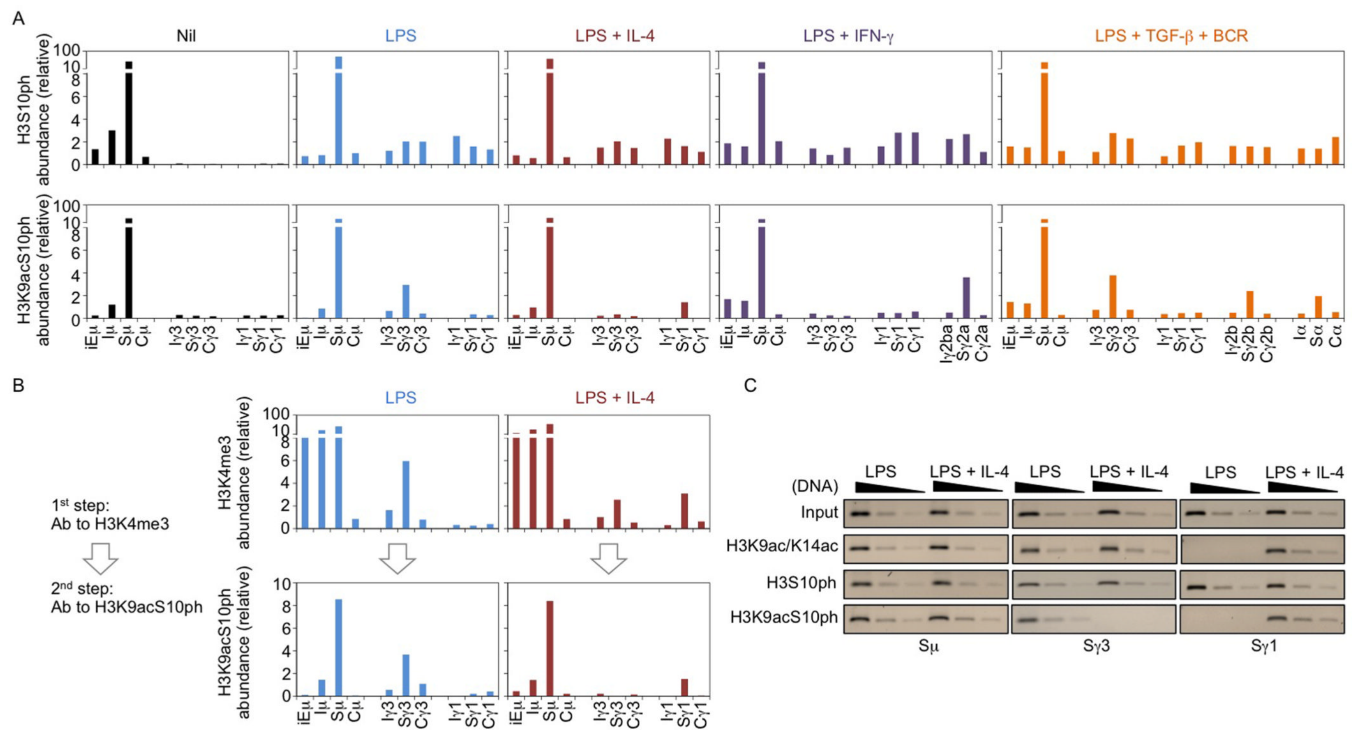

Figure 2. Combinatorial H3K9acS10ph modification specifies the S regions set to undergo CSR

(A) ChIP assays using and an Ab specific for H3S10ph or H3K9acS10ph and involving chromatin from B cells stimulated by nil (grey), LPS (blue) or LPS plus IL-4 (red), LPS plus IFN-γ (purple) or LPS, TGF-β plus BCR crosslinking (orange) for 48 hr. Depicted is the abundance of iEμ DNA and I_H, S and C_H region DNA in the upstream Igμ sublocus and downstream Igγ3, Igγ1, Igγ2b, Igγ2a, Igε and Igα subloci (values in ChIP assays involving anti-H3K9acS10ph Ab were ×10) in immunoprecipitated chromatin. Data are representative of those from three independent experiments.

(B) Two-step ChIP assay involving chromatin from B cells stimulated by LPS (blue) or LPS plus IL-4 (red) for 48 hr, followed by sequential precipitation by Ab for H3K4me3 and then Ab for H3K9acS10ph. Depicted is the abundance of iEμ DNA and I_H, S and C_H region DNA in the upstream Igμ sublocus and downstream Igγ3 and Igγ1 subloci (values in ChIP assays involving anti-H3K9acS10ph Ab were ×40) in immunoprecipitated chromatin. Data are representative of those from two independent experiments.

(C) Visualization in electrophoretic gels of upstream Sμ and downstream Sγ3 and Sγ1 region DNA precipitated by Abs for H3S10ph, H3K9ac/K14ac or H3K9acS10ph from B cells stimulated by LPS or LPS plus IL-4. A 4- fold serial dilution of input and immunoprecipitated DNA was amplified with primers specific for the Sμ, Sγ3 and Sγ1 region DNA. Data are representative of those from three independent experiments.

See also Figures S5 and S6.

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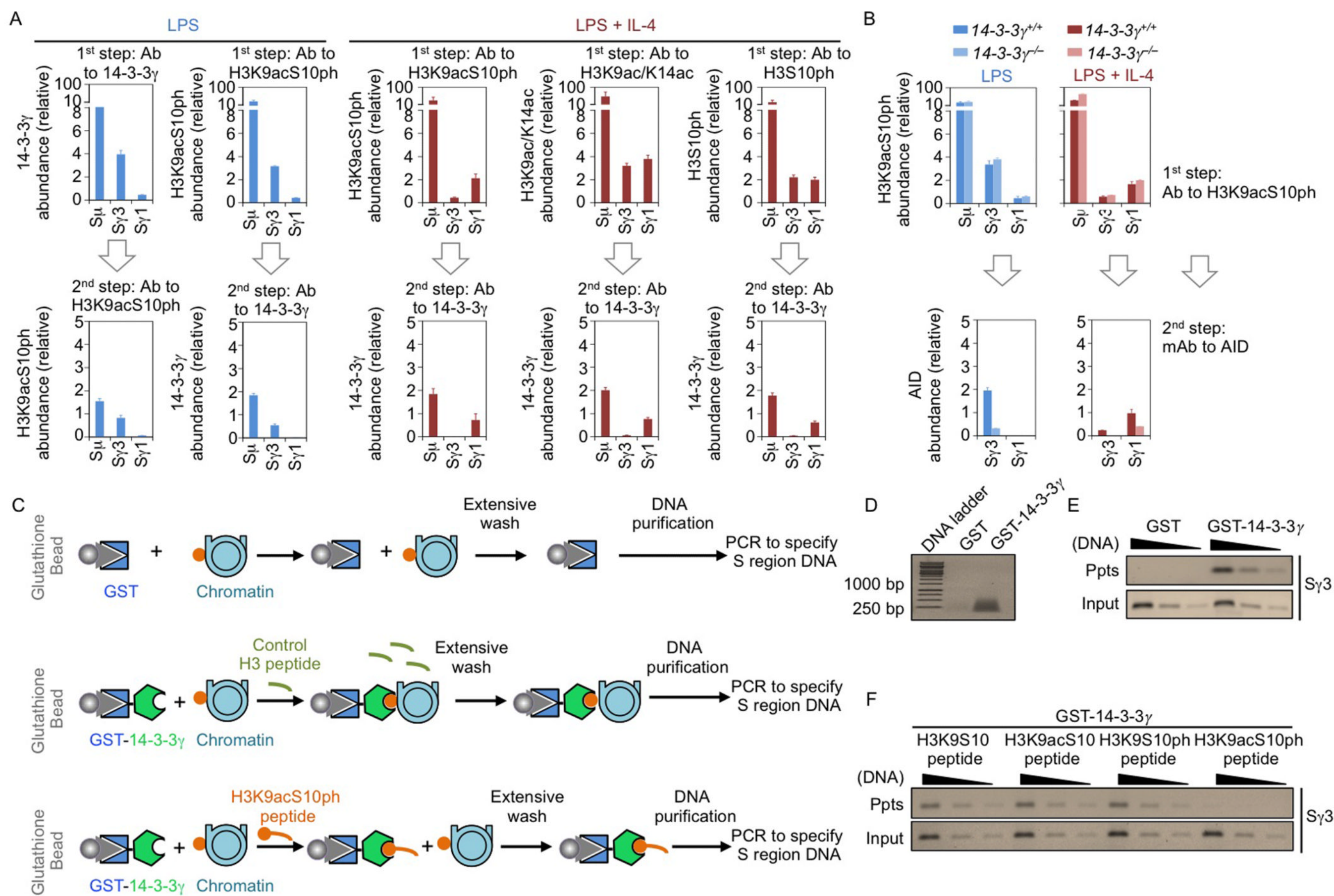

Figure 3. H3K9acS10ph stabilizes 14-3-3 and AID on the S regions that are to undergo CSR

(A) Two-step ChIP assays involving chromatin from B cells stimulated by LPS (blue) or LPS plus IL-4 (red) for 48 hr, followed by sequential precipitation by two antibodies, as indicated. Depicted is the abundance of upstream Sμ region DNA and downstream Sγ3 and Sγ1 region DNA (values after the first-step precipitation using anti-H3K9acS10ph Ab or anti-14-3-3γ Ab were x10; values after the second-step precipitation were ×100) in immunoprecipitated chromatin (mean and s.d. of triplicate samples). Data are representative of those from three independent experiments.

(B) Two-step ChIP assay involving chromatin from *14-3-3γ*$^{+/+}$ and *14-3-3γ*$^{-/-}$ B cells stimulated by LPS (blue) or LPS plus IL-4 (red) for 48 hr, followed by sequential precipitation by Ab for H3K9acS10ph and mAb to AID. Depicted is the abundance of upstream Sμ region DNA and downstream Sγ3 and Sγ1 region DNA (values in ChIP assays involving anti-H3K9acS10ph Ab and anti-AID mAb were ×10 or ×100, respectively) in immunoprecipitated chromatin (mean and s.d. of triplicate samples). Data are representative of those from three independent experiments.

(C) Schematic representation of pull-down and peptide competition assays.

(D and E) *In vitro* pull-down assays involving chromatin from *14-3-3γ*$^{-/-}$ B cells stimulated by LPS for 48 hr, precipitated by purified recombinant GST or GST-14-3-3γ proteins. Total bound DNA was visualized in (D) and a 4-fold serial dilution of precipitated (“Ppts”) and input DNA was specified as Sγ3 in (E). Data are representative of those from three independent experiments.

(F) *In vitro* peptide competition assay involving chromatin from *14-3-3γ*$^{-/-}$ B cells stimulated by LPS for 48 hr, purified recombinant 14-3-3γ proteins and H3K9S10 peptides,

H3K9acS10 peptides, H3K9S10ph peptides or H3K9acS10ph peptides. A 4-fold serial dilution of precipitated (“Ppts”) and input DNA was specified as Sγ3. Data are representative of those from three independent experiments.

See also Figures S7 and S8.

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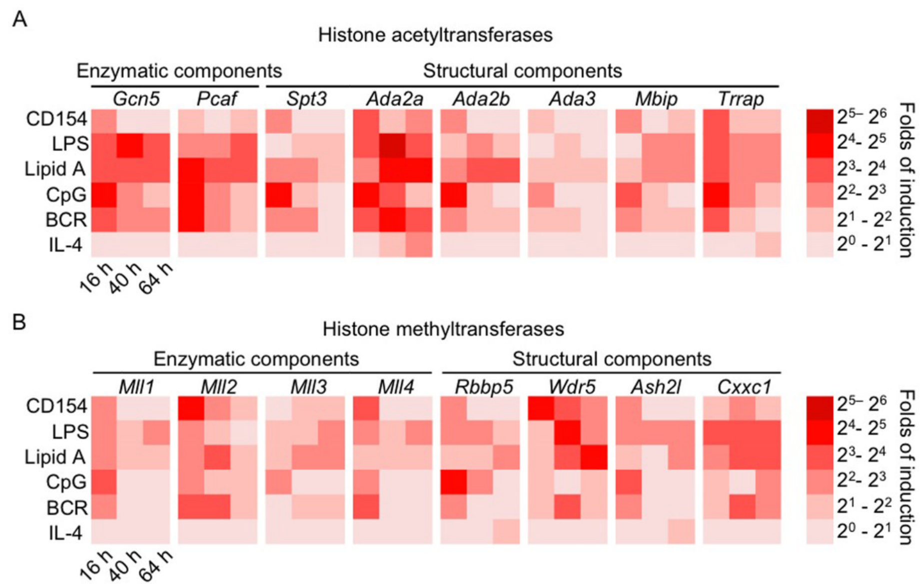

Figure 4. Primary CSR-inducing stimuli induce histone methyltransferases and acetyltransferases

(A, B) Levels of transcripts encoding enzymatic and structural components of histone acetyltransferases (*Gcn5, Pcaf, Spt3, Ada2a, Ada2b, Ada3, Mbip* and *Trrap*, A) or histone methyltransferases (*Mll1, Mll2, Mll3, Mll4, Wdr5, Ash2l, Rbbp5* and *Cxxc1*, B) in freshly isolated B cells or B cells stimulated by CD154, LPS, lipid A, CpG, BCR crosslinking or IL-4 for 16 hr, 40 hr or 64 hr. Data were normalized to the expression of *Cd79b* and are expressed as ratios of values in stimulated B cells at different time-points to those in freshly isolated B cells. Data are representative of those from three independent experiments.
See also Figure S9.

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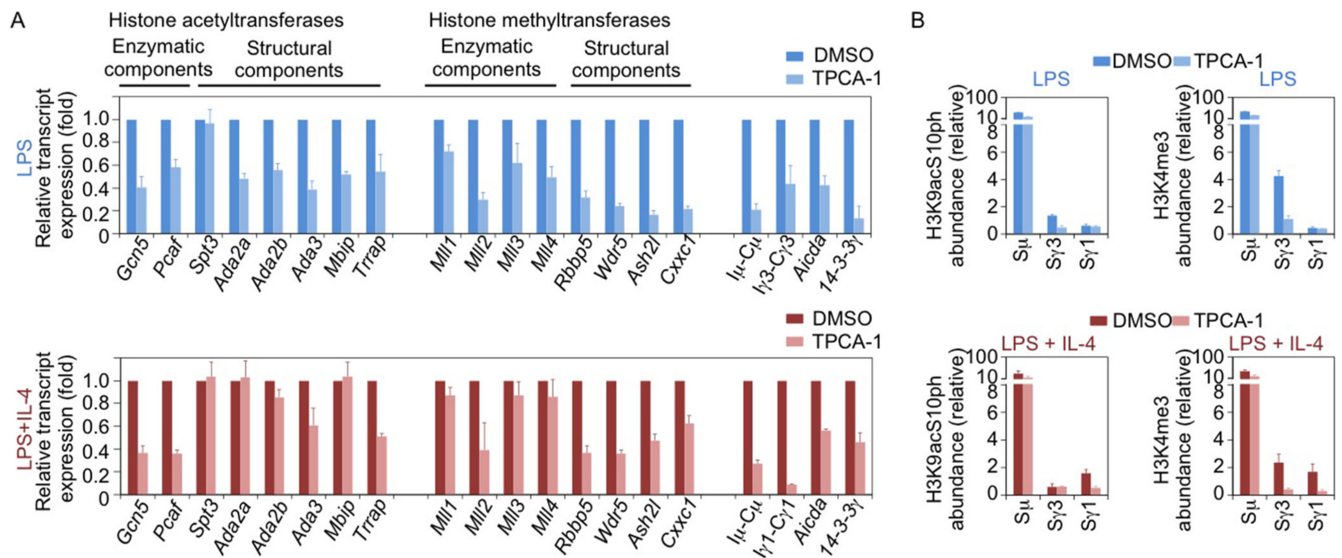

Figure 5. Blocking NF-κB activation by TPCA-1 reduces the expression of histone-modifying enzymes

(A) Levels of histone acetyltransferase and methyltransferase, germline I_H-C_H, *14-3-3γ* and *Aicda* transcripts in B cells treated with DMSO (dark) or TPCA-1 (light) and stimulated by LPS (blue) or LPS plus IL-4 (red) for 16 hr. Data were normalized to the expression of *Cd79b* and are expressed as ratios of values in B cells stimulated in the presence of TPCA-1 to those in B cells stimulated in the presence of DMSO (mean and s.d. of triplicate samples). Data are representative of those from three independent experiments.

(B) ChIP assays involving chromatin from B cells treated with DMSO (dark) or TPCA-1 (light) and stimulated by LPS (blue) or LPS plus IL-4 (red) for 24 hr and Abs specific for H3K4me3 or H3K9acS10ph, as indicated. Depicted is the abundance of upstream Sμ region DNA and downstream Sγ3 and Sγ1 region DNA (values in ChIP assays involving anti-H3K9acS10ph Ab were ×10) in immunoprecipitated chromatin (mean and s.d. of triplicate samples). Data are representative of those from three independent experiments.

See also Figures S10 and S11.

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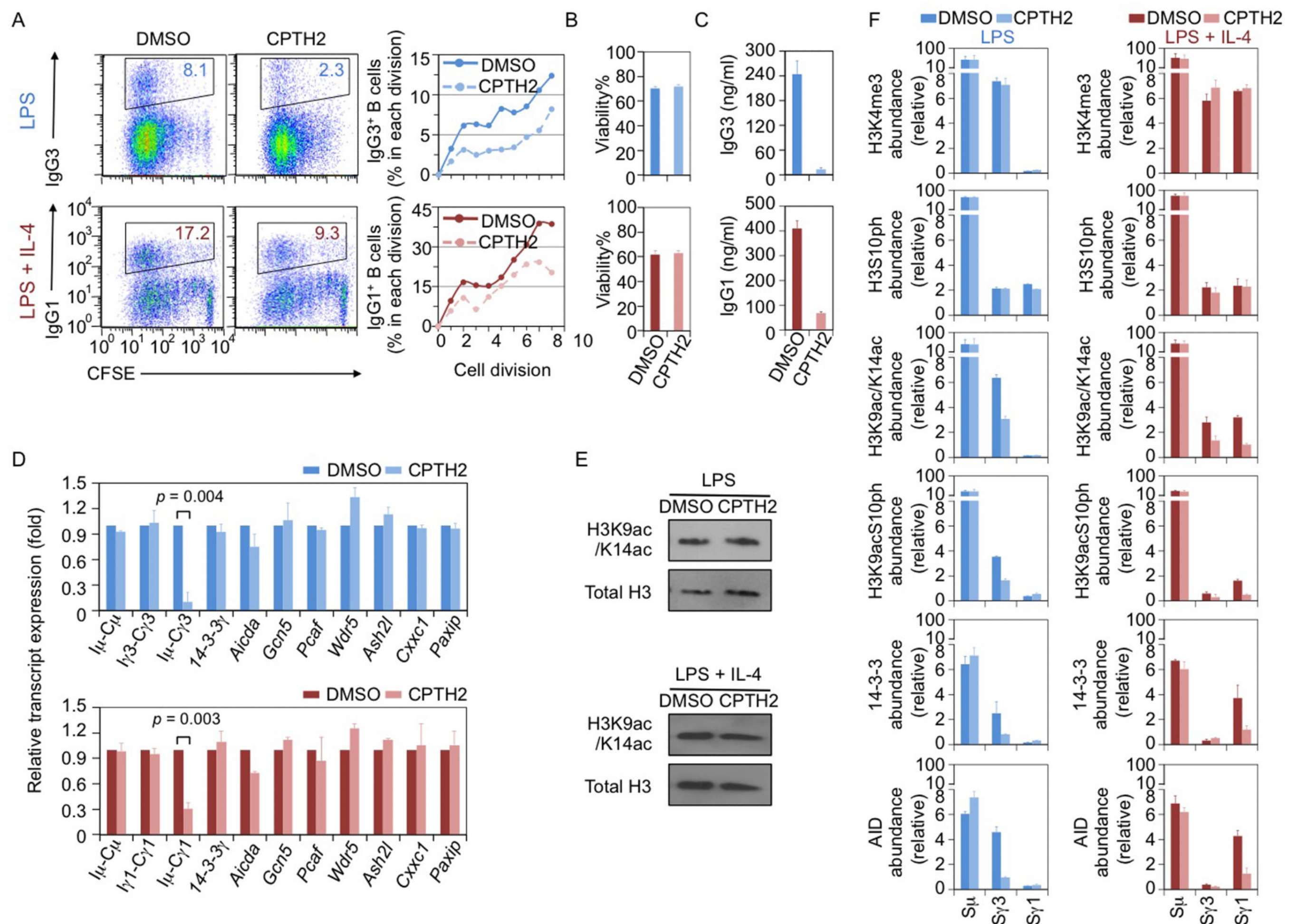

Figure 6. Inhibition of GCN5 and PCAF acetyltransferases decreases H3K9acS10ph, binding of 14-3-3 and AID to S regions and CSR

(A) Proportions of sIgG3+ or sIgG1+ B cells after stimulation of CFSE-labeled B cells with LPS (blue) or LPS plus IL-4 (red), in the absence (dark) or presence (light) of CPTH2 (left panel). Depicted are also proportions of B cells at each cell division that were IgG3+ or IgG1+(right panel). Data are representative of those from three independent experiments.

(B) The viability of B cells stimulated by LPS (blue) or LPS plus IL-4 (red), in the absence (dark) or presence (light) of CPTH2. Data are representative of those from three independent experiments.

(C) Titers of IgG3 and IgG1 in supernatants of B cells stimulated by LPS (blue) or LPS plus IL-4 (red), in the absence (dark) or presence (light) of CPTH2. Data are representative of those from three independent experiments.

(D) Levels of germline I_H-C_H, post-recombination $I\mu$-C_H, histone methyltransferases and acetyltransferases, *14-3-3γ*, *Aicda* and *Paxip* transcripts in B cells stimulated by LPS (blue) or LPS plus IL-4 (red) for 48 hr, in the absence (dark) or presence (light) of CPTH2. Data were normalized to *Cd79b* expression and expressed as ratios of values in B cells stimulated in the presence of CPTH2 to those in B cells stimulated in the presence of DMSO (mean and s.d. of triplicate samples). Data are representative of those from three independent experiments.

(E) Immunoblotting of overall H3K9ac/K14ac in B cells stimulated by LPS or LPS plus IL-4 for 48 hr in the absence or presence of CPTH2. Data are representative of three independent experiments.
(F) ChIP assays using Abs or mAbs specific for H3K4me3, H3S10ph, H3K9ac/K14ac, H3K9asS10ph, 14-3-3γ or AID (as indicated) and involving chromatin from B cells treated with DMSO (dark) or CPTH2 (light) and stimulated by LPS (blue) or LPS plus IL-4 (red) for 48 hr. Depicted is the abundance of upstream Sμ region DNA and downstream Sγ3 and Sγ1 region DNA (values in ChIP assays involving anti-H3K9acS10ph Ab, anti-14-3-3γ Ab and anti-AID mAb were x10) in immunoprecipitated chromatin (mean and s.d. of triplicate samples). Data are representative of those from three independent experiments.

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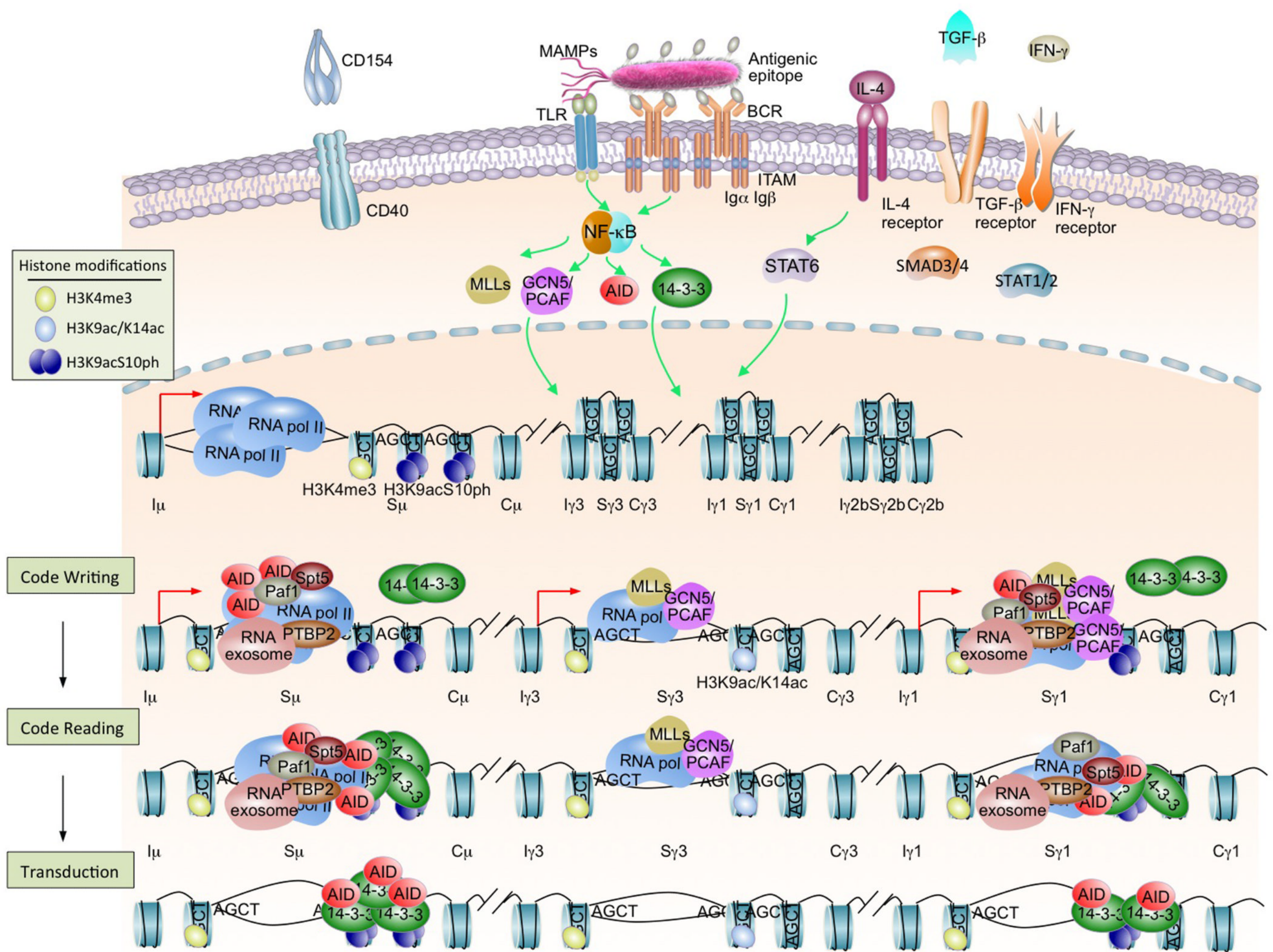

Figure 7. Schematics of the induction of H3K9acS10ph in the donor and acceptor S regions and its role in recruiting 14-3-3 adaptors and subsequently stabilizing the AID-centered CSR machinery

Targeting of the CSR machinery entails "writing" (by induced histone-modifying enzymes), "reading" (by 14-3-3 adaptors) and "transduction" (from 14-3-3 adaptors to AID) of the combinatorial H3K9acS10ph modification that specifically marks the S regions set to undergo recombination (depicted is CSR to γ1). The Sμ region, but not any downstream S region of the *IgH* locus, is constitutively transcribed and marked by H3K4me3, H3K9ac/K14ac and H3K9acS10ph, even in resting $Ig\mu^{+}Ig\delta^{+}$ B cells. Primary stimuli (dual TLR/BCR engagement by MAMPs and antigenic epitopes, respectively, such as on bacteria, depicted here) induce H3K9 acetyl writers GCN5/PCAF and H3K4 methyl writers MLLs as well as expression of 14-3-3 adaptors and AID. As enabled by primary stimuli, secondary stimuli (depicted is IL-4) activate transcription factors that specifically bind *IgH* intervening γ1 promoter to initiate germline Iγ1-Sγ1-Cγ1 transcription in the Sγ1 region. These would allow histone-modifying enzymes (GCN5/PCAF and MLLs) and RNA pol II-associated factors, such as Spt5, PTBP2, RNA exosome and the Paf complex (Basu et al., 2011; Nowak et al., 2011; Pavri et al., 2010; Willmann et al., 2012), to ride on RNA pol II, on which AID is also hitched through interaction with Spt5 and Paf1, to reach S regions during elongation of germline I_H-S-C_H transcription. RNA pol II stalling, likely due to complex secondary DNA structures (e.g., R-loop and cruciform-like structures) or antisense transcription in S

regions, results in deposition of H3K9acS10ph, H3K4me3 on the Sγ1 region. In contrast to widely spread H3KS10ph (not depicted) in all acceptor S and I_H promoter regions as well as C_H exons and non-discriminately marking of H3K4me3 and H3K9ac/K14ac in both recombining and non-recombining S regions, the combinatorial H3K9acS10ph modification exclusively marks the donor Sμ and the acceptor Sγ1 regions at much higher levels than in the respective Iγ1 and Cγ1 regions or any other S, I_H or C_H regions. H3K9acS10ph directly interacts with 14-3-3 proteins, recruits these adaptors ("docking") and, therefore, stabilizing AID on the H3K9acS10ph-marked donor Sμ and acceptor Sγ1 regions for CSR to unfold. The high affinity of 14-3-3 proteins for S region 5′-AGCT-3′ repeats would ensure firm stabilization ("locking") of these adaptors on S regions DNA.

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