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The cohesin and mediator complexes control immunoglobulin class switch recombination

Les complexes cohésine et médiateur contrôlent la commutation isotypique

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Table of contents

List of figures

List of abbreviations

γH2AX:	phosphorylated histone H2AX
3'RR:	3' regulatory region
4C-Seq:	Chromosome conformation capture (3C) with high-throughput sequencing
53BP1:	p53 binding protein 1
Ab:	antibody
Ag:	antigene
AID:	activation-induced cytidine deaminase
aNHEJ:	alternative non-homologous end joining
APE:	apurinic/apyrimidinic (AP) endonuclease
APLF:	aprataxin and PNK-like factor
APOBEC:	apolipoprotein B mRNA-editing catalytic polypeptide
ATM:	ataxia telangiectasia mutated
ATR:	ataxia telangiectasia and Rad3-related protein
BATF:	(Basic leucine zipper transcription factor, ATF-like
BCR:	B cell receptor
BER:	base excision repair
BL:	burkitt's lymphoma
bp:	base pair
C region:	constant region
CDC5L:	cell division cycle 5 like
CdLS:	Cornelia de Lange Syndrome
CFSE:	carboxyfluorescein succinimidyl ester
CHD1:	chromodomain helicase DNA-binding protein 1
ChIP:	chromatin immunoprecipitation
ChIP-Seq:	ChIP followed by deep sequencing
CSR:	class switch recombination
CT:	chromosome territories
CTCF:	CCCTC-binding factor
CTIP:	CtBP-interacting protein
CTNNBL1:	catenin beta like 1
DDR:	DNA damage response
DNA-PK:	DNA-dependent protein kinase
DNA-PKcs:	catalytic subunit of the DNA-PK
DSB:	double stranded break
Eμ:	enhancer μ
ES cells:	embryonic stem cells
Exo1:	exonuclease 1
FACT:	histone chaperone facilitates chromatin transcription

GANP:	GC-associated nuclear protein
GC:	germinal center
GL:	germline
H3K9ac:	histone H3 lysine 9 acetylated
H3K9me:	H3K9 methylated
HIGM:	hyper-Immunoglobulin M
HoxC4:	Homeobox C4
HP1:	heterochromatin protein 1
hs:	DNase hyper sensitive site
Hsp90:	heat shock protein 90
Id2:	inhibitor of DNA binding 2
IFNγ:	interferon γ
Ig:	immunoglobulin
IgH:	immunoglobulin heavy chain
IgL:	immunoglobulin light chain
IL-4:	interleukin 4
iPS cells:	induced pluripotent cells
KAP1:	KRAB domain associated protein 1
Lig4:	DNA Ligase 4
LPS:	lipopolysaccharide
LSR:	locus suicide recombination
Mb:	mega base pair
MDC1:	mediator of DNA damage checkpoint 1
Med1:	mediator complex subunit 1
MH:	microhomology
miR:	micro RNA
MMR:	mismatch repair
MRN complex:	MRE11, RAD50, NBS1 complex
MSH2:	MutS protein homolog 2
NCS:	neocarzinostatin
NES:	nuclear export signal
NF-κB:	nuclear factor kappa-light-chain-enhancer of activated B cells)
NHEJ:	non-homologous end joining
NLS:	nuclear localization signal
PARP:	poly(ADP-ribose) polymerase
PCNA:	prolifzrating cell nuclear antigen
PKC:	protein kinase C
Pol II:	RNA polymerase II
PTBP2:	Polypyrimidine-tract binding protein
PTIP:	Pax transactivation-domain interacting protein

qPCR:	quantitative PCR
RAG1/2:	recombination activating genes 1/2
RPA:	replication protein A
RSS:	recombination signal sequences
S3:	serine 3
Sμ:	switch region μ
SHM:	somatic hypermutation
Smc:	structural maintenance of chromosome
Sp:	specificity protein
ssDNA:	single stranded DNA
STAT6:	Signal transducer and activator of transcription
T1 B cells:	transitional 1 B cells
T27:	threonine 27
TAD:	topologically associating domains
TdT:	terminal deoxynucleotidyl transferase
TCR:	T cell receptor
TF:	transcription factor
TGF-β:	transforming growth factor
UNG:	uracil N-glycosylase
V region:	variable region
V(D)J genes:	Variable, diversity, joining genes
WT:	wild-type
XLF:	XRCC4-like complex
XRCC4:	X-ray repair cross-complementing protein 4
Y184:	tyrosine 184
YY1:	yin-yang 1

Introduction

I. Assembly of the B cell receptor

1. The B cell receptor (BCR)

To efficiently fight infections, B cells bear on their surface highly diversified antigen receptors, called immunoglobulins (Ig), that recognize a wide variety of antigens (Ag) from bacteria, virus and other pathogens. Immunoglobulins (Ig) can either be bound to the membrane to constitute the B cell receptor (BCR) or they can be secreted and are called antibodies (Ab).

Abs are composed of two heavy (H) chains and two light (L) chains (figure 1). The heavy chains form a homodimer linked by disulfide bonds and each heavy chain is bound to a light chain by a disulfide bond. Light and heavy chains are composed of a variable region (V) at the N-terminus, and a constant region (C) at their C-terminus. The variable regions of the light and heavy chains form together the antigen-binding site, which is responsible for the recognition of the antigen and the diversity among the receptors. In the light chain, the constant region is either κ or λ chain whereas in the heavy chain, the constant region can be μ, δ, γ, ε or α and determine the isotype expressed (IgM, IgD, IgG, IgE or IgA) and the effectors' function of the Ig. When attached to the B cell membrane, the two heavy and light chains associate with Igα and Igβ invariant subunits to compose the BCR. These subunits are responsible for initiating signaling after antigen recognition.

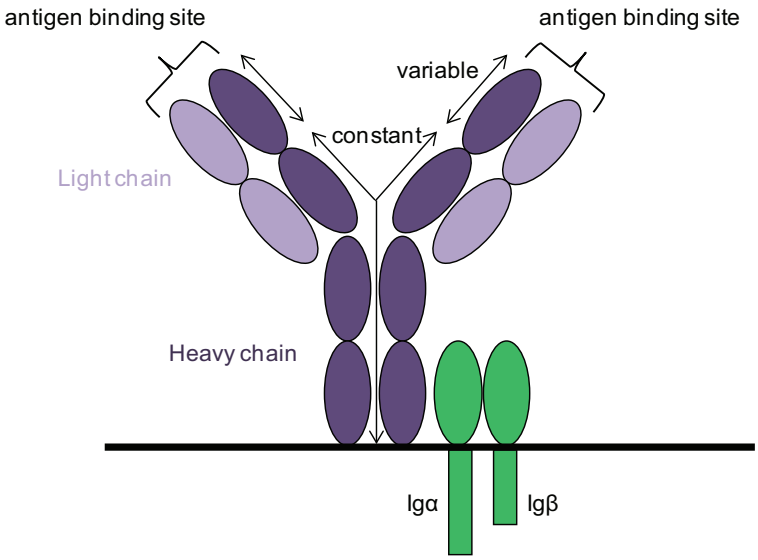

Figure 1: Structure of the B cell receptor (BCR)
The B cell receptor is composed of two heavy (H) chains and two light (L) chains. Each chain harbors a variable (V) region and a constant (C) region. Combined V regions form the antigen binding site, whereas the C region of the H chain is the isotype expressed and confers the function of the BCR. Upon antigen recognition, the Igα and Igβ subunits mediate signal transduction.

The Ig repertoire is generated through genomic rearrangement (recombination and mutation) that occur at the IgH and IgL loci. Four different mechanisms have been described to contribute to Ig diversification: V(D)J recombination, somatic hypermutation (SHM), class switch recombination (CSR) and gene conversion in some species like chicken.

2. The murine immunoglobulin loci

The mouse IgH locus spans around 3 Mb on the long arm of the chromosome 12. It is composed of Variable (V), Diversity (D), Joining (J) gene segments forming the V region of IgH followed by exons coding for the C region of IgH. The IgH locus contains around 150 V_H segments of gene, depending on the mouse strain and are clustered into 16 gene families defined by sequence similarities. Depending on their position relative to D_H-J_H gene segments, V_H genes can be divided into proximal (3' part of the V_H cluster, close to D_H-J_H gene segments, for example V_H7183), intermediate (for example, V_HGam3.8) or distal (5' part of the V_H cluster, for example V_HJ558) families. Downstream of the V_H gene segments lye 10 to 15 D_H elements, followed by 4 J segments. The gene segments coding for the V_H region are then followed by 8 C_H region exons coding for the different isotypes (Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε and Cα). The heavy chain constant region genes are organized as individual transcription units containing a cytokine responsive promoter upstream of an intervening exon (I-exon), an intronic switch region (S), with the exception of Cδ, and a constant exon (C). Switch regions are repetitive but non-homologous regions with GC-rich sequences (figure 2).

The IgH locus also contain 3 main regulatory elements, the PDQ52 germline promoter-enhancer [1], located between D_H and J_H segment genes; the Eμ enhancer [2], located downstream of the J_H4 and at the 5' of C_H genes; and the 3' enhancer, also called 3' regulatory region (3'RR) that lie downstream of Cα and contain 7 DNAse I hypersensitive sites (hs3a, 3b, 1-2, 4, 5, 6 and 7). hs3a, 3b, 1-2 and 4 have been described to play a role as enhancer, whereas hs5,6,7 contain CTCF binding sites and are suggested to be insulators [3], elements creating boundaries in chromatin.

The IgL chain is composed by either κ or λ chains, which by opposite to the IgH chain are only composed of V, J and C genes. The choice of IgL chain is not random and in mice majority of the IgL chains are κ. The κ locus is located on chromosome 6 and span over 3 Mb. It is composed of around 140 $V_κ$ followed by 4 functional $J_κ$ gene segments and a single $C_κ$ exon. Some of the $V_κ$ gene segments are in reverse orientation compared to $J_κ$ segments, therefore recombination has to occur through inversion of the DNA to allow the expression of these gene segments.

The mouse Igλ locus is located on chromosome 16 and span 200 kb. Gene segments are not organized as in the chains previously described, where gene segments are clustered by family (V or J) but instead are present rather as units. Three different cassettes of $V_λ/J_λ$ followed by $C_λ$ exons exist and the rearrangement occur preferentially with $V_{λ1}$ and $V_{λ2}$ which recombine with the most proximal $J_λ$ ($V_{λ1}$ with $J_{λ1}$ or $J_{λ3}$ and $V_{λ2}$ with $J_{λ2}$) [4]. Given the low number of $V_λ$ gene segments, the repertoire of mouse $Ig_λ$ recombination is more restricted than the Igκ repertoire.

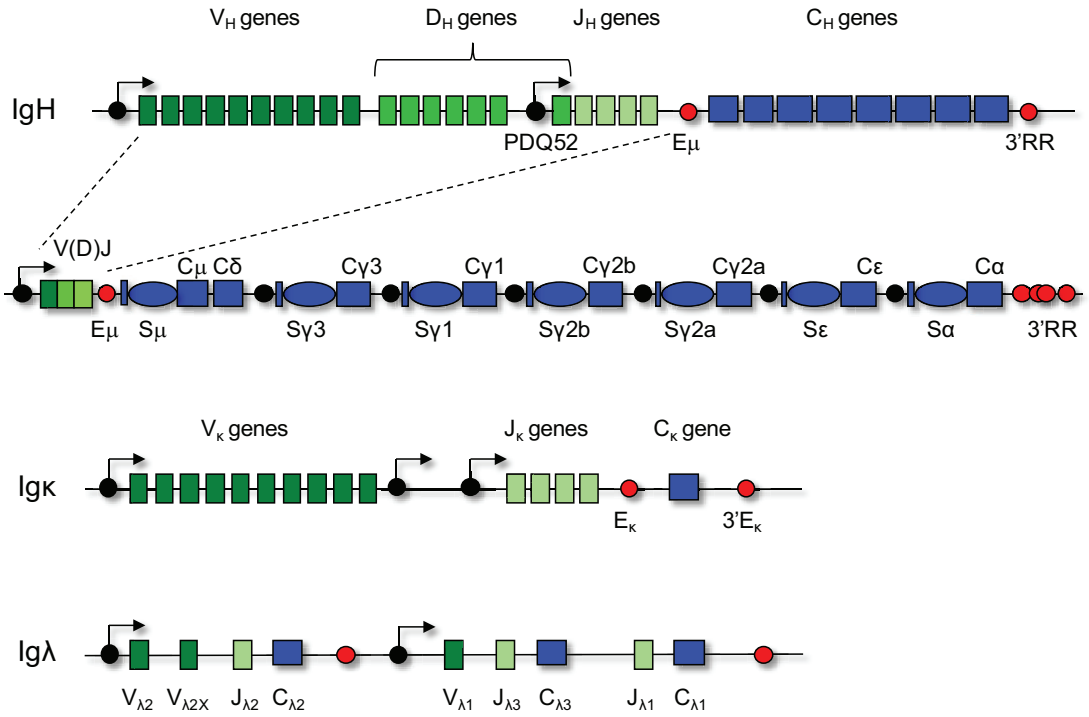

Figure 2: The murine Ig loci
The mouse IgH locus is composed of V, D and J gene segments that code for the variable region of the Ig, followed by transcription units coding for the constant region composed of a promoter, an intervening (I) exon, a switch region and a constant exon. The L chains are either κ or λ and contain V, J and C genes that are organized by family for κ chain and as distinct cassettes for λ chain. Regulatory regions are indicated. Adapted from [4,5].

3. V(D)J recombination

V(D)J recombination is a somatic rearrangement which assembles the variable region of B and T cells antigen receptors from variable gene segments generating a diverse antigen receptor repertoire. During B and T cell development, V(D)J recombination joins discontinuous Variable (V), Diversity (D) and Joining (J) gene segments to generate a functional receptor.

3.1. Mechanism

V(D)J recombination is dependent on the activity of the recombinase complex, composed of recombination activating gene 1 (RAG1) and 2 (RAG2), a protein complex that recognizes the conserved recombination signal sequences (RSS) that flank V, D and J gene segments. RAG1/2 binds to these sequences and introduces DNA breaks between RSS and the gene segments. The DNA ends are then processed and linked by the non-homologous end joining (NHEJ) pathway (figure 3).

All V, D and J segments are surrounded by RSS that consist of a conserved palindromic heptamer and a conserved AT-rich nonamer, both separated by a 12 or 23 bp spacer that is less conserved [6]. The V(D)J recombination occurs preferentially between a 12RSS and a 23RSS providing the order of the assembly [7]. For example, V_H and J_H elements are flanked by 23RSS, whereas D_H segments are

flanked by 12RSS on both sides. In this case, according to the 12/23 rule, this conformation prevents direct recombination between V_H and J_H segments.

The RAG recombinase recognizes RSSs through two domains of RAG1: the nonamer-binding domain, which binds the nonamer region of RSS and its core region that is able to bind the heptamer domain. RAG2 binds RAG1-heptamer domain enhancing the interaction with RSS and is indispensable for DNA cleavage [8]. RAG recombinase binds to one RSS and then captures a second RSS lacking bound proteins to form a paired complex. The RAG complex introduces a single stranded DNA (ssDNA) break between the heptamer and the gene segment. Then the free 3'OH group attacks the opposite strand leading to double stranded break (DSB) generation with blunt RSS ends and the formation of a hairpin at the side of the paired complex, which are then called coding ends as they lack RSS.

Additionally to its role in DNA cleavage, RAG protein is also important for the repair of the DSB by the non-homologous end joining (NHEJ) pathway. Indeed, RAG2 C-terminus has been implicated in the choice of DNA repair pathway used, favoring repair by the classical NHEJ [9,10] and in suppressing genomic instability, as mutation of the C-terminus of RAG2 in p53 knock out mice increase tumorigenesis [11]. The NHEJ pathway mediates the joining of the two coding ends and the processing of the RSS ends. Free ends are recognized by Ku70/80 which recruits the catalytic subunit of DNA-PK (DNA-PKcs), forming the DNA-PK holocomplex, which targets DNA repair proteins like Artemis and the histone variant H2AX. The endonuclease activity of Artemis opens the hairpins at the coding ends and induces additional deletion of nucleotides from the exposed ends [12]. These deletions and the addition of random nucleotides by the terminal deoxynucleotidyl transferase (TdT) favor the generation of further diversity at the coding joints. Finally, recruitment of XRCC4 and DNA ligase 4 (Lig4) leads to the ligation of the coding ends. On the other hand, the blunt signal ends are simply ligated together and usually deleted from the genome as episomal circles (figure 3).

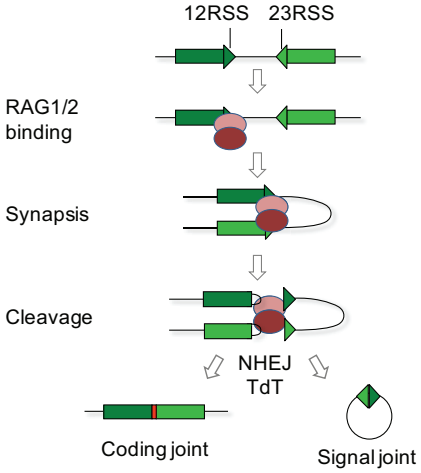

Figure 3: V(D)J recombination steps
Gene segments (green rectangle) are flanked by a 12 or a 23 recombination signal sequence (RSS) (green triangle). RAG1 and RAG2 proteins (pink ovals) bind the 12 and the 23RSS to form a paired complex, within which RAG complex induces double stranded breaks (DSB) between the gene segment and the RSS. RAG complex subsequently cooperates with non-homologous end joining (NHEJ) repair factors to ligate the ends. The coding joint contains additional nucleotides (red rectangle) added by the terminal deoxynucleotidyl transferase (TdT) whereas the signal joint is simply ligated. Adapted from [5].

3.2. V(D)J regulation

V(D)J recombination occurs through the introduction of DNA DSB around the selected gene segments and their subsequent ligation. To ensure that this damage is properly repaired, V(D)J recombination is tightly controlled by different mechanisms.

First of all, RAG proteins being absolutely required for V(D)J recombination, their pattern of expression is regulated. Indeed, RAG proteins are highly expressed only during the early stages of development of B and T cells, so the reaction is lineage-specific. Secondly, antigen receptor loci recombine in a specific order at specific stages (figure 4).

In B cells, the IgH locus recombines before the IgL locus at the pro-B cell stage. RAG complex is expressed and targets the locus for D_H to J_H recombination prior to V_H to D_HJ_H rearrangement. Functional IgH locus recombination leads to the expression of the IgH protein combined to a surrogate IgL that form together the pre-BCR [13]. The pre-BCR inhibits further recombination at the IgH locus to maintain B cell monospecificity by allelic exclusion. Finally, RAG is then reexpressed at the pre-B cell stage to allow V(D)J recombination of the light chain. Association of a functionally rearranged light chain with the heavy chain allows the expression of a complete BCR.

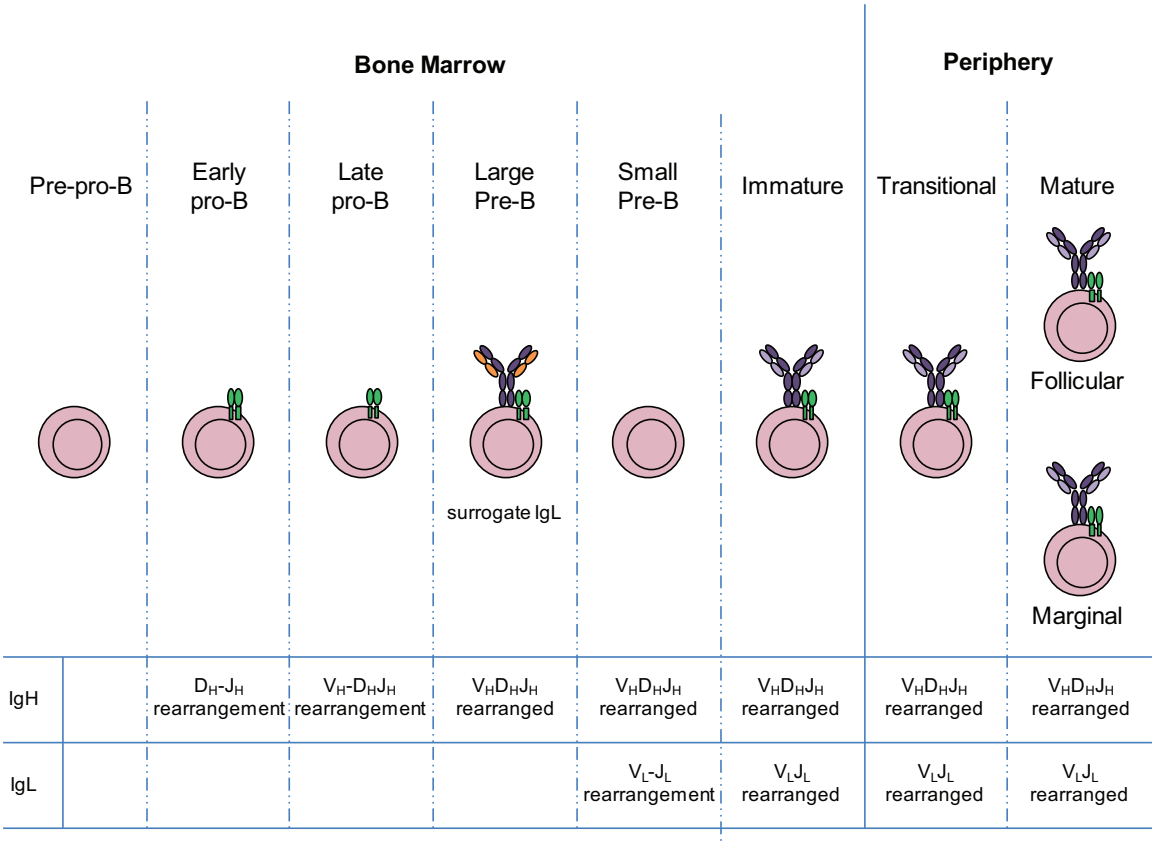

Figure 4: B cell development
The earliest committed B cell precursors are pre-pro-B cells. At the pro-B cell stage, the IgH locus is recombined. D_H-J_H assembling occurs prior to V_H-D_HJ_H. At the pre-B cell stage, the pre-BCR, composed of the rearranged heavy chain and a surrogate light chain, is expressed. Pre-B cells then recombine the light chain locus to express a functional receptor at the immature stage. B cells migrate to the periphery where they further maturate into follicular or marginal B cells. Adapted from [14,15]

The ordered and selective assembly of antigen receptor genes raises questions about the mechanisms by which a common enzyme and well-conserved RSS substrates are tightly regulated. The model that is currently proposed to explain how and when rearrangements occur depicts 3 levels of regulation: the first one being the control of accessibility to RSS, the second one being the nuclear location of the loci and last but not least, the architecture of the loci themselves.

The accessibility model emerged from the finding that V_H gene segments are transcribed prior to their recombination [16]. At the IgH locus, the promoter DQ52 becomes active before D_H to J_H rearrangement to generate the μ0 transcripts [1]. To the same extent, promoters located upstream of each V_H elements initiate V_H germline transcription prior to V_H to D_HJ_H joining [17] which is dependent on IL7 receptor signaling [18].

The model was also supported by observations demonstrating that V(D)J recombination correlates with open chromatin marks like histone acetylation. Acetylated H3K9 and H3K4 are broadly distributed along D_H-J_H gene segments in pro-B cells undergoing D_H to J_H rearrangement [19]. Prior to V_H to D_HJ_H rearrangement, H3 and H4 from V_H to J_H become acetylated [20] and the hyperacetylated profile of V_H gene segments is only present in cells that have already joined D_HJ_H gene segments [21]. H3K4 dimethylation, another open chromatin mark, has also been shown to be present at the D_H and J_H clusters in cells undergoing D_H to J_H rearrangement [19] . Moreover, RAG2 is able to bind di- or trimethylated H3K4 through its PHD domain, which is critical for V(D)J recombination [22-24]. On the other hand, cytosines methylated in CpG dinucleotides are hallmarks of silenced genes that are present at the Ig loci before V(D)J recombination. The methylation is removed before D_H to J_H recombination in one allele, the second one staying in a repressive environment to favor allelic exclusion.

Additionally, the localization of the locus within the nucleus accounts for the recombination efficiency. Before undergoing rearrangement, the IgH locus is relocated from the periphery to the center of the nucleus [25] to allow D_H to J_H recombination.

Finally, loci also undergo conformational changes and the recombination between distal V_H and D_HJ_H gene segments seems to be dependent on long-range interactions and looping of the IgH locus. Pax5, a B cell specific transcription factor, has been implicated in this process as locus compaction and rearrangements to distal V genes are abolished after its depletion [26].

Efficient V(D)J recombination at the IgH locus leads to the expression of the variable region and the Cμ exon located downstream. Association of the rearranged heavy and light chains leads to the exposure of a functional IgM antibody on the cell surface, which constitutes the primary repertoire. However as V(D)J recombination is a random process, it produces also self-reactive antibodies that are negatively selected before leaving the bone marrow [27,28]. Immature B cells migrating to the secondary lymphoid organs are referred to as transitional (T1 and T2) B cells: T1 B cells that have entered the follicles acquire cell surface IgD and the ability to recirculate and are subsequently called T2 B cells. Finally, T2 B cells can further mature into follicular B cells or marginal B cells [29].

Many marginal B cells express BCRs that are less specific than those expressed by follicular B cells due to a poorly diversified V(D)J genes [30]. These BCRs allow the recognition of multiple highly conserved microbial determinants, similarly to toll-like receptors (TLR) [30]. Additionally, marginal B cells express high levels of TLRs which render them able to produce low-affinity antibodies as soon as their BCR and their TLRs recognize microbial molecules such as LPS [31,32]. This rapid response has been suggested to take place to bridge the temporal gap required for the generation of high-affinity antibodies by follicular B cells. However, marginal B cells can also diversify their repertoire after antigen recognition through the same mechanisms used by follicular B cells and detailed below [33].

II. Diversification of the B cell receptor

Naïve B cells that arise from the bone marrow express low affinity IgM receptors that recognize a large panel of antigens. In the periphery, after antigen recognition, B cells migrate to secondary lymphoid organs where they form germinal center. In these structures, B cells further diversify their repertoire according to the specific pathogen and to the stimuli received notably by T cells. Three antigen-dependent mechanisms of BCR diversification exist:

The first one is somatic hypermutation (SHM) which modifies the affinity of the receptor for the antigen by introducing mutations in the V region of the heavy and light chains of Ig. The second mechanism is class switch recombination (CSR), which replaces the isotype expressed by a deletional recombination reaction between switch regions that precede each C_H gene. The last one is gene conversion which uses pseudogenes as template to diversify their Ig. This mechanism occurs only in some species like chicken or lampreys, but since I focused on the Ig diversification in mice during my work, I will not detail this mechanism. All these mechanisms are dependent on the activity of activation-induced cytidine deaminase (AID), an enzyme that deaminates cytosines into uracil in DNA.

1. AID

1.1. Domains and function

AID was identified, using a PCR-based cDNA subtraction screen, as a gene strongly induced when CH12 cells (a mouse B cell line) were stimulated to undergo class switch recombination [34]. The discovery of the essential role of AID in CSR and SHM comes from work done in two different laboratories. In Tasuku Honjo laboratory, they generated AID-deficient mice that showed a striking inability to undergo CSR and SHM [35] and in Anne Durandy laboratory, the characterization of patients affected by the hyper-IgM syndrome 2, due to a loss of AID, showed a block in both processes, thereby confirming also in human the essential role of AID in CSR and SHM [36].

AID is a small protein of 198 amino acids that belongs to the APOBEC family of DNA/RNA cytidine deaminases. AID bears at its N-terminus a nuclear localization signal (NLS) and a nuclear export

signal (NES) at its C-terminus, which determine its subcellular localization [37,38] (figure 5). The N-terminus domain of AID has specifically been implicated in SHM by studies on different N-terminus mutants [39] whereas the C-terminus of AID has been shown to be specifically required for CSR [40-42]. AID cytidine deaminase motif displays the highest homology with APOBEC1 which deaminates a specific cytidine in apolipoprotein B mRNA [43]. On the contrary, other members of the family deaminate cytosines on ssDNA from different substrates. Its homology with APOBEC1 firstly suggested that AID might target mRNA. However, several lines of evidence indicate that AID targets DNA. Through *in vitro* studies, it has been shown that AID is able to mutate *E. Coli* DNA and that these mutations are enhanced by uracil N-glycosylase-deficiency (UNG), which removes uracils from DNA [44]. Other *in vitro* studies further demonstrated that AID is a ssDNA specific cytidine deaminase that converts dC into dU with no effect on RNA, dsDNA or RNA:DNA hybrids [45-47]. Finally, AID and RPA, a protein that binds and stabilizes ssDNA, have been shown to interact [48] and ChIP experiments revealed that AID is associated with switch region in B cells undergoing CSR [49]. These studies revealed that AID converts cytosines into uracils in ssDNA, introducing dU:dG mismatches that are differentially processed to result in SHM and CSR.

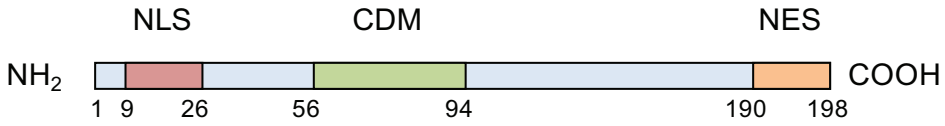

Figure 5: AID domain organization
Schematic representation AID structure. The protein harbors a nuclear localization signal (NLS) at the N-terminus, a cytidine deaminase motif (from amino acid 56 to 94) and a nuclear export signal (NES) at the C-terminus. Adapted from [50].

1.2. AID regulation

AID, due its mutagenic ability, is a potentially dangerous protein that needs to be tightly regulated. Most human lymphomas are of B cell origin and are usually associated with translocations of Ig genes, like the BCL2-Ig translocations in follicular lymphoma or the c-myc-Ig translocations in Burkitt's lymphoma (BL). Most of the Ig breaks are located in V or S regions and have been suggested to be linked to V(D)J recombination, SHM and CSR [51]. Additionally, in the c-myc-IgH translocations, AID-mediated deamination was implicated in the initiation of genome instability [52,53]. Indeed, they have been shown to occur in B cells stimulated to undergo CSR [53] and c-myc has been shown to be relocated closed to Ig regulatory regions leading to its constitutive expression [54]. AID-initiated DSBs during CSR are the likely substrates for translocation, and although DSBs are not intermediate of SHM, they might still occur as translocation between c-myc and IgL locus have been depicted in BL [55]. Concerning the induction of DSBs on partner chromosomes, they might arise from AID off-targeting. Indeed, AID can also target non-Ig genes, which are highly transcribed [56,57] and their close proximity to the IgH locus might increase the chances of translocations and make these non-Ig genes AID "hotspots" [58].

In addition to initiating the translocations mentioned above, AID has been shown to mutate 25% of transcribed genes of germinal center B cells deficient for UNG and MSH2, which respond to DNA damage [59] and can induce transformation by mutating oncogenes [60]. Consistent with this, AID overexpression leads to extensive mutations of non-Ig genes [61] and its constitutive expression leads to T cell lymphomas development and micro-adenomas [62]. Moreover, AID expression has been associated with numerous cancer such as gastric cancer [63] and oral squamous cell carcinoma [64].

Additionally to this "not so" specific targeting, abnormal repair of AID-mediated damages might increase propensity for malignant transformation in B cells. In order to avoid genomic aberrations induced by AID that might lead to cell transformation and cancer, B cells tightly control AID activity at different levels.

1.2.1. Transcriptional regulation

The Aicda locus contains four regions of transcription factors-binding sites: region 1 is located directly upstream of the TSS and contains transcription factors-binding sites for NF-κB, STAT6, HoxC4 and Sp elements [65-68]. STAT6 and NF-κB binding are induced by IL-4 signaling and CD40 ligation and their deletion impairs AID expression [67]. Additionally, estrogen and progesterone can also modulate AID expression: estrogen might activate HoxC4 expression or directly bind AID promoter [69,70], whereas progesterone has been shown to display an inhibitory effect through its binding around the regulatory region 1 [71]. The second region is located between exon 1 and 2 and contains binding sites for inhibiting proteins like c-Myb, Id2, Id3 and E2F proteins but also for activating proteins like Pax5 and E-box-binding proteins [65,72-74]. Id2 and Id3 have been shown to interact with Pax5 and E-proteins to impair their binding to this region [72,73]. On the other hand, Pax5 has been suggested to remove the inhibition induced by c-Myb and E2F [65,74]. The third region lies downstream of the exon 5 and contains a BATF (Basic leucine zipper transcription factor, ATF-like) binding site that is suggested to be an enhancer as deletion of BATF abrogates AID expression [75]. The final regulatory region is located 8 kb upstream of the TSS and contains binding sites for STAT6, SMAD3/4, NF-κB and C/EBP proteins.

The stability of AID transcripts is also regulated: miR-181b and miR155 have been shown to bind to AID 3'UTR and to regulate AID expression: miR-181b is normally expressed in resting cells and has been suggested to impair inappropriate expression of AID as inducing its expression in activated cells led to decreased AID mRNA and protein level [76]. miR-155 is expressed in cells undergoing CSR and its function might be to limit the amount of AID transcripts as deletion of its binding site leads to increased IgH/c-myc translocations [77,78].

1.2.2. Compartmentalization of AID activity

Subcellular localization represents another level to control AID activity (figure 6). As AID exerts its function in the nucleus, its localization is predominantly cytoplasmic limiting its access to the substrate [37,79]. At its C-terminal region, AID owns a NES, which is important for its nuclear export [80] through CRM-1 [81], but also an anchor sequence required for its retention in the cytoplasm [38]. AID has been shown to interact with eEF1A (elongation factor 1 alpha), a protein involved in protein synthesis, through its C-terminal and more specifically through its residue 187 to mediate AID cytoplasmic retention as its mutation induced an increased nuclear localization [82].

Additionally, despite its small size, its nuclear import requires interaction with importin-α3 [38]. CTNNBL1, a nuclear protein, also interacts with AID NLS and has been suggested to be implicated in AID nuclear import as its deletion in DT40 cells (a chicken B cell line) bearing an AID mutant for its NES showed a reduced nuclear accumulation [83] associated with a reduced frequency of SHM and gene conversion [84]. However, its knock down in CH12 cells did not revealed any defect in CSR [85], suggesting that either this factor is specifically required for gene conversion and SHM or that its role is redundant with other proteins. GANP has also been suggested to play a role in AID nuclear import as its overexpression increases AID nuclear fraction [86].

Subcellular localization of AID also affects its stability: to ensure its limited lifespan in the nucleus, AID can be degraded by the proteasome in a ubiquitin-dependent [87] or independent way through its interaction with REGγ [88], as REGγ deficiency displayed an increased accumulation of AID. Nevertheless, AID can also be stabilized in the nucleus, through its interaction with YY1, an ubiquitously expressed zinc finger transcription factor, which has been shown to modulate AID level in the nucleus [89]. On the other hand, AID is stabilized in the cytoplasm by histone chaperones [90,91]. Indeed, AID has been shown to interact with HSP90 [91] and HSP40 DnaJa1 [90] and their deficiencies reduced stability of AID and thus its protein level.

1.2.3. Post-translational modifications

Post-translational modifications are another level to control protein activity and AID function is largely regulated by phosphorylation on several residues. AID can be phosphorylated on its serine 3, threonine 27, serine 38, serine 41, serine 43, threonine 140 and on tyrosine 184. Phosphorylation of S3 by protein kinase C (PKC) has been suggested to inactivate AID activity as a S3A mutation increases CSR efficiency [92]. Residues T27 and S38 are phosphorylated by PKA and regulate AID interaction with RPA [93,94]; mutating these residues impairs CSR and SHM [93-97]. Phosphorylation of S41 and S43 have not yet been shown to be implicated in CSR or SHM [98]. On the other hand, phosphorylated residue T140 seems to be implicated more particularly in SHM, as it replacement by an alanine impairs SHM, while CSR is only slightly affected [99]. Finally, mutation of Y184 by an alanine did not affect CSR, suggesting that this site is not critical for AID regulation [93].

AID is also tightly regulated by protein cofactors that coordinate its recruitment and functions during antibody diversification mechanisms (figure 6). I will detail some of its partners and their role in CSR and SHM in the following chapters.

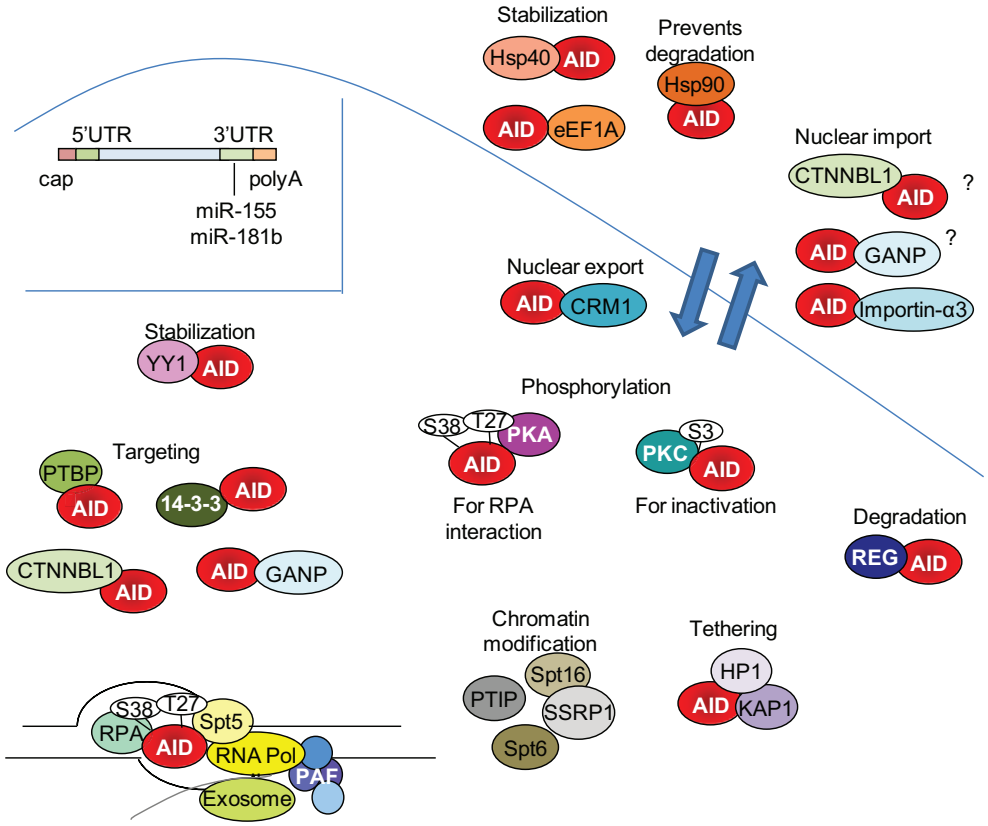

Figure 6: AID regulation
AID is regulated at different levels: Its mRNA stability is regulated by microRNA (miR-155, miR-181b). AID cellular localization is modulated by importin-α3 and CRM1. AID protein stability is regulated in the cytoplasm by Hsp40 Dnaja1, Hsp90 which prevent its degradation through the proteasome. In the nucleus, AID is stabilized by YY1 or destabilized by REGγ. Finally, AID activity is controlled by post translational modifications and by factors implicated in its targeting to DNA.

2. Somatic hypermutation (SHM)

SHM is responsible for the affinity maturation of the primary antibody repertoire. Antigen recognition by B cells expressing low affinity IgM leads these B cells to form germinal center in secondary lymphoid organs in which they diversify their repertoire through SHM. It introduces point mutations and occasionally insertions and deletions in the variable regions of the IgH and IgL loci to generate antibodies with different affinity for the cognate antigen. B cell clones bearing a mutated receptor are selected on the basis of antigen binding affinity to give rise to a higher affinity antibody repertoire [100]. Mutations occur at a frequency of 10^{-5} 10^{-3} per bp per generation across a region that begins around 150 nucleotides downstream of the IgV promoter and extents over 2 kb downstream of the promoter [101]. Most mutations are concentrated within the assembled variable regions that form the antigen binding site [102] (figure 7).

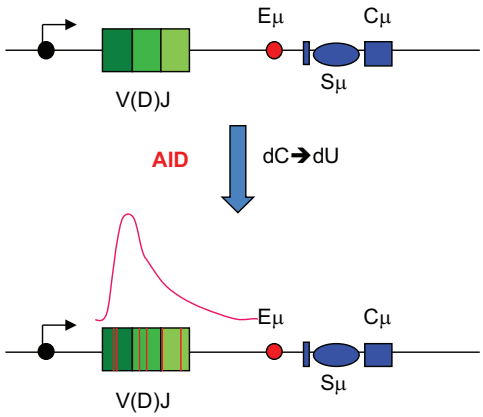

Figure 7: Somatic hypermutation (SHM)
SHM modifies the rearranged V genes in order to express antibodies with higher affinity for the cognate antigen. V genes are modified by the introduction of point mutations, insertions and deletions. The mutation frequency is high close to the promoter and gradually decreases, as shown by the pink peak. Adapted from [103].

Mechanistically, the first step of SHM is the deamination of cytosines to uracils by AID resulting in U:G mismatches. High rate of transcription are required for SHM to occur and both strands are targeted by AID [104]. Moreover, proteins that have been implicated in AID nuclear recruitment, like GANP or CTNNBL1 are also candidate for AID targeting: GANP has been proposed to regulate AID binding to V region [86] and CTNNBL1 ChIP on DT40 cells revealed that it binds to the Igλ locus [84].

U:G mismatches can be processed by several pathways to generate two steps of mutations, the first one targeting G:C bp and the second one the A:T bp leading to around 40% of mutations on G:C bp and 60% of mutations on A:T bp (figure 8). Additionally, many of the mutations are preferentially targeted to the dC within WRCY motifs (W=A/T, R=A/G, Y=C/T) that are hot-spots for SHM [105,106].

Mutations at G:C bp result from the direct activity of AID. In the phase 1a of SHM, replication over U:G mismatches can produce transition events [44]. Uracil residues can also be targeted by the base excision repair (BER) enzyme UNG prior to replication leaving an abasic site that can be replaced by any nucleotide during replication leading to transitions or transversions in the phase 1b.

Phase 2 mutations target A:T bp and are due to further processing of U:G mismatches by the BER and the mismatch repair (MMR) complexes. During processing by the MMR complex, mismatches are recognized by the MSH2/MSH6 complex which recruit additional proteins like EXO1, PCNA or ATR. EXO1 removes the uracil from DNA and proceeds to strand degradation. PCNA has been implicated in the recruitment of error-prone polymerases such as Polη [107] to fill the gap, leading to transition and transversion from A:T bp. Additionally, when dU residues are recognized by the BER complex, UNG removes the uracil residues from the DNA creating an abasic site that is recognized by the apurinic/apyrimidc endocnuclease (APE), which induces a nick and strand degradation as well leading to mutations at A:T bp, as a backup mechanism. Indeed, in the absence of UNG and APE2, global mutation frequency and more specifically mutations at A:T bp are reduced [108]. ATR is part of the DNA damage response pathway and patients bearing mutations in the ATR gene show a normal frequency of SHM with fewer mutations at A and more mutations at T residues (transitions) [109]. Mutations in

MSH2 and MSH6 lead to a decrease in SHM frequency as well as reduced mutations at A:T bp [110,111]. MSH6 deficiency leads also to an increase in G:C mutations (transitions). Polη, an error-prone polymerase recruited by MSH2/MSH6, favors mutations of A:T residues on the non-transcribed strand and patients with *xeroderma pigmentosum* harboring a defect in Polη show a normal frequency of SHM [112]. Furthermore, deletion of Polη in mice revealed a marked decrease in mutations of A and T bases (more pronounced for A bases) and an increase in mutations of C and G (more pronounced for C bases) compared to control [113]. In conclusion, SHM modifies the coding regions located at the antigen binding site. B cells expressing high affinity antibodies but that do not recognize self-antigens are then selected to undergo proliferation and further differentiate into plasma cells or memory B cells [114].

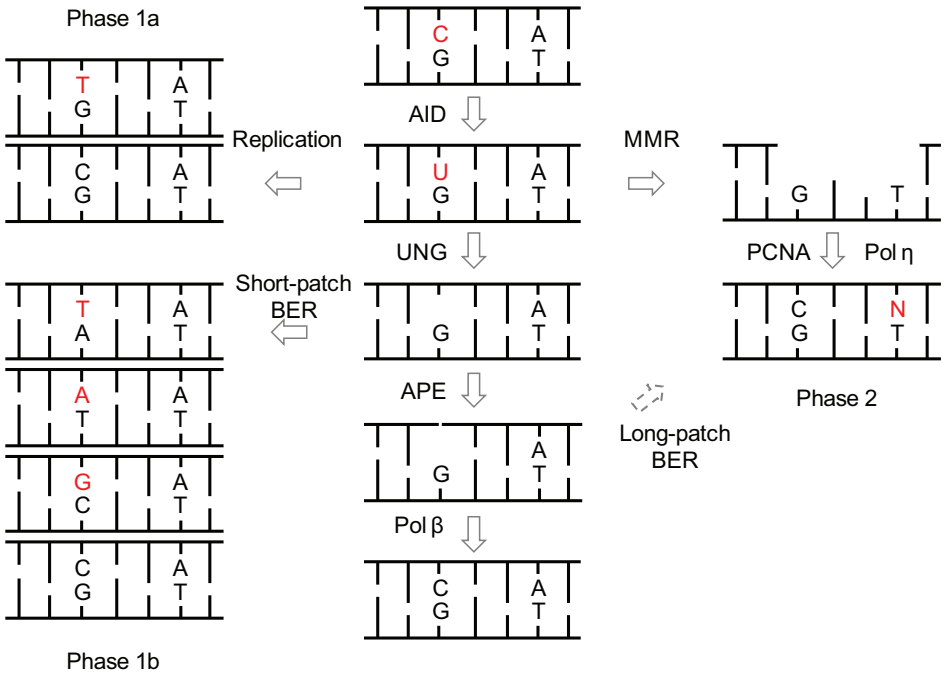

Figure 8: The steps of somatic hypermutation (SHM)
AID deaminates cytosine into uracil at the genes coding for the V region inducing U:G mismatches. In phase 1a: if replication occurs, it can lead to transition from C to T. In phase 1b, the mismatch can be recognized by UNG that removes the uracil leaving an abasic site that can be filled by any of the four bases by error-prone polymerases through short–patch base excision repair (BER). U:G mismatches can also be recognized by the mismatch repair (MMR), which excises the strand bearing the uracil generating a gap that can be filled by error-prone polymerases recruited by PCNA, leading to mutations at A:T bases as well as G:C bases. Long-patch BER can also induce mutations at A:T bases. Adapted from [103].

3. Class switch recombination (CSR)

CSR replaces the antibody isotype expressed from Cμ to one of the downstream constant exon (Cγ, Cε or Cα) to modify its effector functions according to the cytokine environment. CSR is a region specific recombination that occurs between switch (S) regions located upstream of each constant gene (except for Cδ). It is a multistep process that takes place in germinal center after antigen recognition and is initiated by germline transcription of the donor and acceptor S regions (figure 9). AID deaminates cytosines in the ssDNA exposed, leading to U:G mismatches that are processed to

generate DSBs. These breaks are then repaired by the NHEJ pathway. CSR places a new constant gene directly downstream of the variable region exons, modifying the function of the Ig without changing its affinity for the antigen.

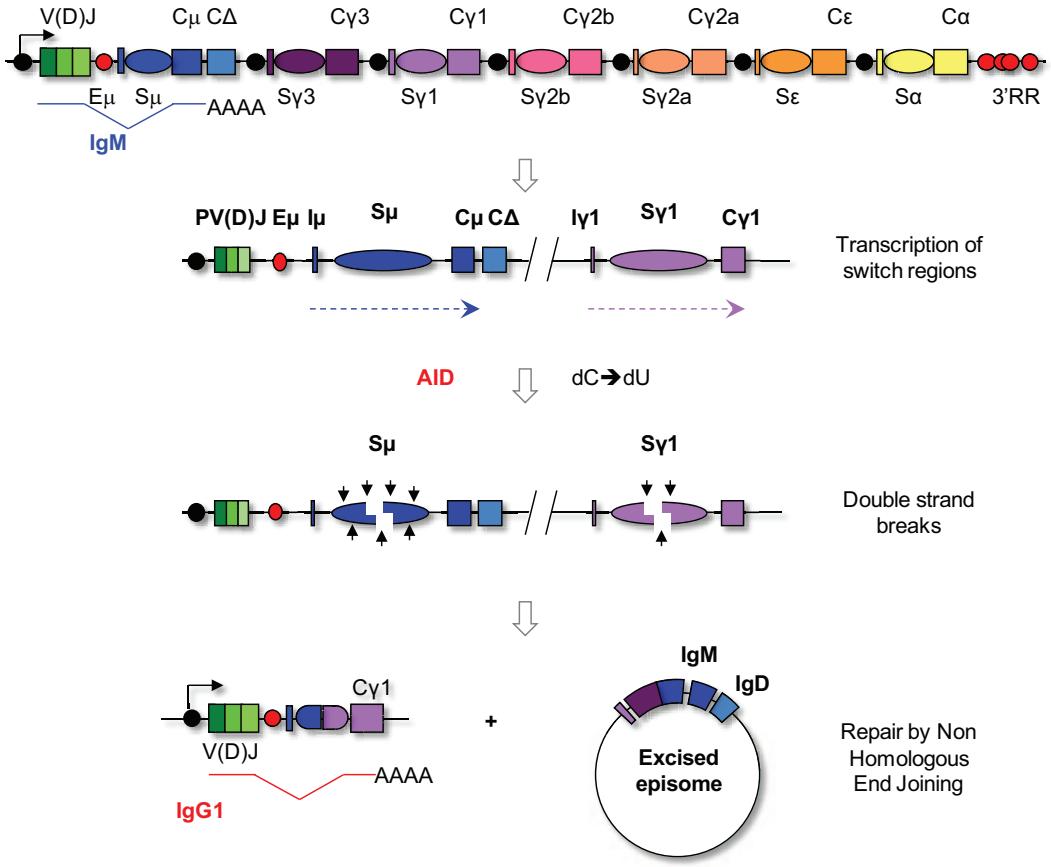

Figure 9: Model of class switch recombination (CSR)
CSR is a multistep process initiated by germline transcription of the donor and the acceptor switch regions. AID is then targeted to the switch regions exposed by transcription and deaminates cytosine into uracil. The mismatches are then processed to generate DSBs that are repaired by the NHEJ pathway.

3.1. Transcription at the IgH locus

Activation of B cells to undergo CSR leads to transcription of the germline (GL) units which are composed of cytokine inducible promoters lying upstream of an intervening exon (I-exon), followed by switch (S) regions and the C_H exons. The primary transcript is spliced to remove the intronic switch region and to join the I-exon to the C_H exon, generating a sterile transcript [115]. The role of transcription seems to direct AID to specific switch regions and to render them accessible for CSR.

In this accessibility model, three roles have been proposed for GL transcription requirement during CSR: First of all, it provides AID substrate for deamination. During elongation, transcription generates R-loop structures in which the template DNA strand forms RNA:DNA hybrid thanks to the G-rich nascent RNA that loops out the non template strand as ssDNA, thus accessible for AID [116,117]. The transcribed strand is hybridized to the nascent RNA and to allow its targeting by AID, the RNA

exosome has been suggested to displace the nascent transcript rendering the transcribed strand accessible [118].

Secondly, GL transcription might also favor CSR by inducing chromatin remodeling to favor AID accessibility and binding. Numerous histone post-translational modifications have been implicated in CSR. In resting cells, the donor switch region bears already activation histone marks, like H3K9ac/K14ac, H3K27ac, H4ac, H3K4me3 and H3K36me3 [49,119-121], which suggests that Sμ is accessible to AID even before the recombination. However, the acceptor switch region is maintained in a repressive state through H3K27me3 until activation, which leads to the removal of this repressive mark [119]. Additionally, active marks such as H3ac and H3K4me3 strongly correlates with transcription at the acceptor switch region [120,122] and regulation of the H3K4me3 by the FACT complex has been shown to be required for CSR [121]. The histone chaperone, Spt6, has also been implicated in CSR, but while its precise role is still not fully understood, it has been suggested to modify the surrounding chromatin [123]. PTIP protein regulates most of the histone modifications at the activated S regions, such as H3K4me3, H3K27ac and H3K36me3 and ChIP-Seq experiments showed that PTIP is able to regulate the association of the RNA Polymerase II (Pol II) to the acceptor switch region [124]. Moreover, H3K9me3 marks present at the donor switch region recruit KAP1 and HP1 proteins to tether AID at Sμ [125].

Finally, GL transcription might recruit AID to the switch region. It has been proposed that AID is recruited to transcribed genes during CSR as AID interacts with the Pol II [49]. Moreover, it has been shown that AID is recruited to switch regions through its interaction with Spt5 and the RNA exosome [118,126], supporting the importance of transcription and transcription-related factors in AID targeting.

3.2. Sequence and structural specificity of the IgH locus

The importance of GL transcription in CSR has been emphasized by mutational studies that showed that deletion of I-exon or promoters affects CSR [127,128]. Transcription of an individual C_H exon induces CSR to that particular isotype and this depends on the promoter located upstream as its replacement induces CSR in a cytokine independent way [129]. In addition, isotype specificity might be depending on the S region itself as deletion of the G-rich region of Sμ or the entire Sγ1 decreases or abrogates CSR, respectively [130,131]. The specificity of S region targeting seems to arise from at least its ability to form R-loops upon transcription and from its sequences. Indeed, the inversion of Sγ1 results in reduced CSR due to the absence of R-loop [131] and mutations of repeat units from Sγ1 to Sγ3 in a plasmid induced switching to IgG1 in the absence of IL-4, revealing the implication of S motifs [132]. Moreover, the 14-3-3 adapter protein has been shown to recruit AID to the 5'AGCT3' repeats located in S regions to enhance AID activity [133].

The IgH locus contains two major regulatory elements, the Eμ enhancer and the 3'RR that have been implicated in CSR. The 3'RR is composed of numerous DNAse I hypersensitive sites (hs) which comprise 4 enhancer elements: hs3a, hs1,2, hs3b and hs4. Single deletion of these elements doesn't

affect CSR efficiency [134,135], suggesting a redundant role for these elements. However, deletion of the entire 3'RR or of both hs3b and hs4 drastically reduces CSR and GL transcription to several isotypes, except Cγ1 which transcription and switching seems to be less dependent on the 3'RR [136,137]. As these elements are located far away from GL promoters, their influence on transcription has been proposed to occur through loop formation between regulatory elements and switch regions (figure 10). Indeed, it has been shown that in resting cells, Eμ and the 3'RR interact together and after B cell activation, the acceptor switch region is additionally recruited within this loop, bringing it in close proximity to the donor switch region [138]. These loops have been shown to be dependent on hs3b,4 as deletion of this region abolishes loop formation concomitantly with CSR and GL transcription defects [138]. On the other hand, deletion of downstream hs sites: hs5, 6 and 7 do not impact CSR efficiency and thereby these sites have been suggested to constitute insulators [139,140].

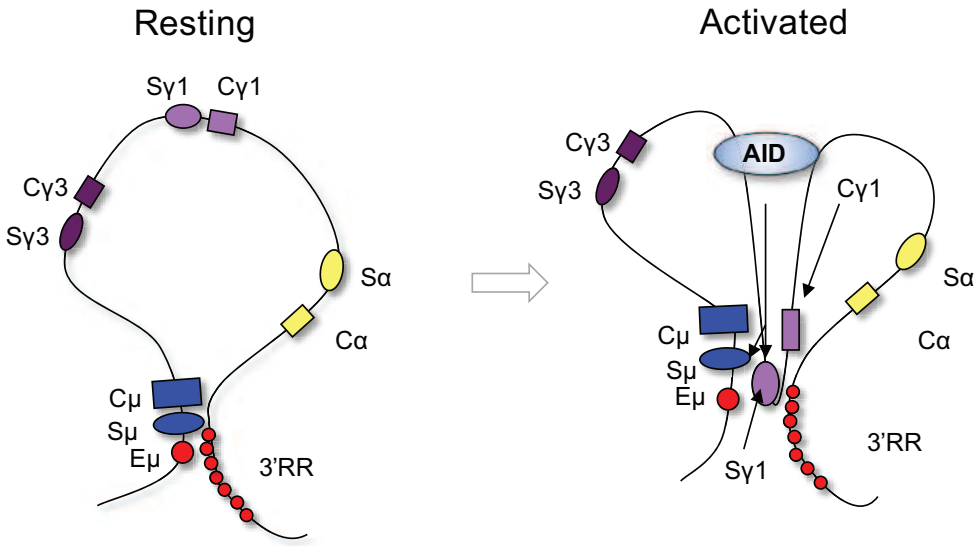

Figure 10: DNA loops at the IgH locus
CSR is additionally dependent on long range interactions: in resting cells, the regulatory elements Eμ and the 3'RR interact and upon activation, the acceptor switch region is brought into close proximity to the donor switch region to allow their recombination.

Upon B cell activation, the 3'RR has been shown to be transcribed and targeted by AID as well, and this might lead to recombination between Sμ and the 3'RR [141]. This process, called locus suicide recombination (LSR), might delete the complete C_H gene cluster leading to B cell apoptosis and has been suggested to regulate B cells homeostasis. LSR has been proposed to induce B cell death to select high-affinity switched clones. Indeed, if the stimulation to switch is not optimal, acceptor switch transcription might not be efficient, leading to LSR [141].

Taken together, these data provide evidences that transcription, DNA sequence and structure are important for AID targeting and efficient CSR.

3.3. AID targeting

GL transcription and the formation of R-loops allow AID to target the non-template strand of the S region. For the targeting of the template strand, different hypothesis have been proposed: a) the formation of negative supercoils at the rear of the elongation Pol II [142]; b) the antisense transcription of switch region [143]; c) the association of AID with RPA, that stabilizes ssDNA exposed during transcription [48]; and d) the RNA exosome complex, which can remove nascent transcripts from transcribed DNA [118]. Additionally, antisense transcripts associated with transcription start sites have been shown to be targeted by the RNA exosome and are strongly expressed at genes that accumulate AID-mutations, suggesting that the RNA exosome recruits AID at sites of antisense transcription, thus gathering the point b) and d) [144].

Numerous proteins have been implicated in AID targeting or tethering to S regions. First of all, AID recruitment has been shown to be dependent on transcription and interacts with Pol II [49] and Spt5 which colocalizes with stalled Pol II [126]. Additionally, the PAF complex, which is part of the Pol II transcription machinery, has been suggested to serve as a platform for AID recruitment on chromatin [145]. However, transcription is not sufficient to explain the pattern of AID recruitment, other cofactors have been involved: RPA has been proposed to stabilize AID on ssDNA [48] and to be specifically recruited to the IgH locus [146]. The interaction between AID and RPA is dependent on AID S38 phosphorylation by PKA [93,94,97] but as AID S38A is still able to bind to S region, the initial recruitment of AID seems independent of RPA [97]. As previously mentioned, 14-3-3 protein interacts with the C-terminus of AID and has been suggested to recruit AID to S regions, nevertheless, AID bearing a mutation at the C-terminus is still able to bind to Sμ, suggesting that 14-3-3 is not implicated in this binding [40]. Polypyrimidine-tract binding protein 2 (PTBP2) knock down has been shown to reduce AID binding at S regions thereby affecting CSR and even if the precise mechanism is not yet known, PTBP2 has been suggested to mediate AID targeting through its interaction with S region transcripts [147]. Furthermore, the RNA exosome complex, which can remove nascent transcripts from transcribed DNA, has been suggested to trigger AID targeting on both strands and to accumulate on S regions in a AID-dependent manner during CSR [118].

The precise mechanism of AID recruitment and regulation is still not fully elucidated leading to the need of further studies.

3.4. Formation of DSBs at the IgH locus

AID deaminates cytosine into uracils on both DNA strands generating mismatches that are processed by the BER and the MMR complexes. Through BER, dU bases are recognized and removed by UNG which generates an abasic site. APE1 then removes the abasic sites thus creating ssDNA breaks. If nicks occur in close proximity on both DNA strands, this might induce DSB formation. The involvement of the BER complex has been highlighted by experiments showing that deletion of UNG or APE1

impairs CSR [148-151]. However, nicks can also be filled by the polymerase β to induce efficient repair as confirmed by the increase CSR efficiency upon pol β deletion [152].

U:G mismatches can also be processed by the MMR complex. The heterodimer MSH2/MSH6 can recognize mismatches and recruit adaptor proteins like MLH1 and PMS2 complex in order to recruit PCNA, RFC and EXO1 proteins to excise the ssDNA that harbors the mismatch. Once again, if the ssDNA breaks occur closely on opposite DNA strands, this could lead to DSB formation. Mutations in genes encoding MMR proteins (MSH2, MSH6, MLH1, PMS2 or EXO1) lead to a reduction in CSR [110,111,153-156]. Additionally, the MMR complex could also be implicated in the generation of DSB from distal single strand breaks (SSB): EXO1 could excise DNA from the closest 5' SSB generated by UNG-APE1 until it reaches a mismatch on the other strand, thus creating a DSB with a 5' overhang [157] (figure 11).

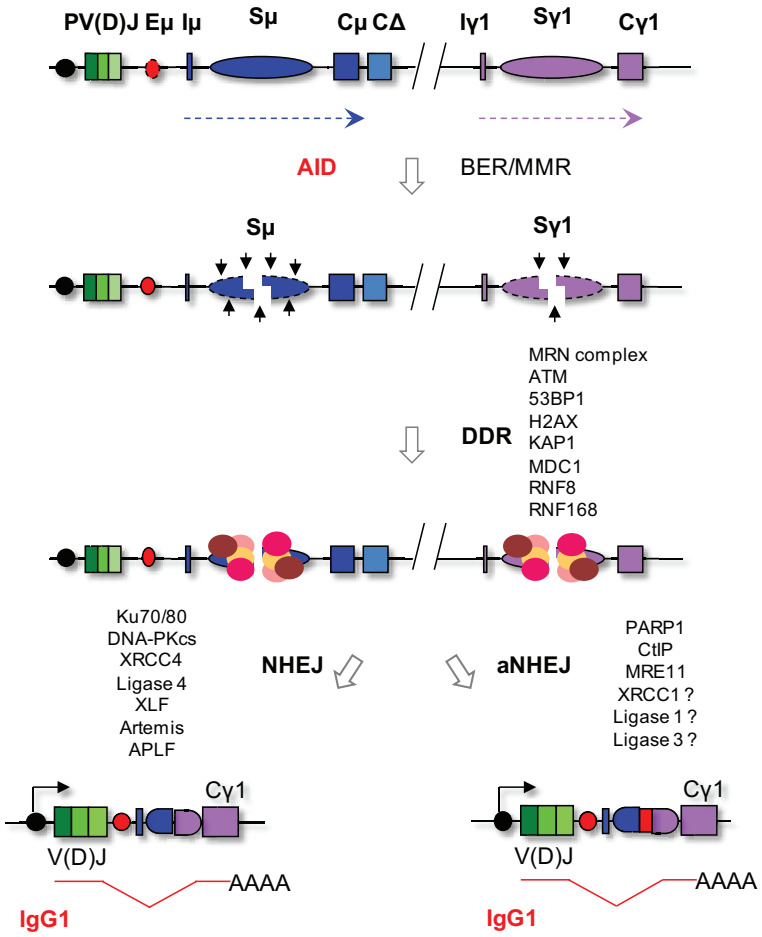

Figure 11: Formation and repair of DSBs during CSR
During CSR, transcription of the donor and the acceptor switch regions lead to the exposure of ssDNA. AID deaminates cytosines into uracils. These mismatches can be processed by the BER and the MMR complexes to generate DSBs. The breaks are recognized and bind by the MRN complex which recruits ATM, 53BP1, MDC1,H2AX. DSBs are then repaired either through the classical NHEJ leading to switch junctions with no or short microhomologies either through the aNHEJ resulting in the presence of junctions displaying longer micrhomologies.

3.5. Double stranded breaks repair

3.5.1. The DNA damage response

Lesions induced by AID are processed to generate DSBs at the donor and the acceptor switch regions that activate the DNA damage response (DDR) [158]. The MRN complex (MRE11, RAD50 and NBS1) is one of the first effectors of the DDR and has been shown to sense DSBs [159]. After its binding, it recruits ATM, a serine/threonine kinase, that undergoes autophosphorylation and also phosphorylates other effector proteins like H2AX [160], NBS1 [161], 53BP1 [162,163], MDC1 [164] and KAP1 [165,166]. Phosphorylation of H2AX on serine 129 forms γH2AX [167] and leads to the recruitment of MDC1 [168,169], 53BP1 [170] and NBS1 [171] which accumulate and spread over the breaks. Additionally, phosphorylated MDC1 recruits RNF8 [172-174] which monoubiquitinates H2A-type histone to recruit RNF168 that will stabilize 53BP1 at DSB sites [175]. Knock out studies confirmed the role of the DDR components in CSR [176-184].

3.5.2. The NHEJ pathway

Mammalian cells possess two major DNA repair pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ). HR requires large regions of homology and is active during S/G2 phases of the cell cycle, whereas NHEJ is active through the entire cell cycle and is the preferential pathway used during G1 phase. This pathway joins DNA ends that have either little or no homology. As AID is active in G1 [158,185] and since the switch junctions do not bear high homology, NHEJ is thought to mediate AID-induced breaks repair. Several proteins compose this pathway: the DNA-PK holoenzyme, which includes Ku70, Ku80 (also called 86) and the catalytic subunit DNA-PKcs, the ligase complex XRCC4/DNA Ligase 4 (Lig4), XRCC4-like complex (XLF also called Cernunnos) and Artemis [186].

During CSR, Ku proteins are believed to bind and protect DSB ends and their deletion induces a severe defect in CSR [187,188]. Ku70 and Ku80 binding leads to the recruitment of the DNA-PKcs, which implication in CSR is controversial as DNA-PKcs deficient B cells undergo variable levels of CSR [189-191]. However, fluorescence in situ hybridization assays showed end-joining defects upon its deletion [192]. The formation of the DNA-PK holocomplex recruits XRCC4 and the Lig4 to mediate the final ligation step. Since XRCC4 or Lig4 deletion leads to embryonic lethality and are essential for V(D)J recombination, their implication in CSR have been shown using transgenic mice bearing an IgH/IgL knock in for XRCC4 [193] and hypomorphic mutation of the Lig4 (Y288C) [194]. In both cases, CSR has been shown to be reduced but not abolished. Additionally, the role of Lig4 in CSR has been confirmed by patients bearing mutations [195]. Artemis is activated by DNA-PKcs and both of them are required for joining a subset of DNA ends that must be further processed before end joining. Similarly to DNA-PKcs, Artemis deletion only moderately affects CSR [196]. XLF-deficient B cells have been shown to only display lower CSR levels [197] and this phenotype might be explain by potential redundant role of XLF with ATM, as combined deletions of these factors drastically affect CSR [198].

B cells deficient for core component of the NHEJ, such as XRCC4 or Lig4, are still able to undergo CSR suggesting that an alternative pathway could be involved in the resolution of AID-induced breaks [193,199].

3.5.3. The alternative NHEJ (aNHEJ)

CSR mediated by the alternative pathway uses a microhomology (MH) biased repair. Indeed, switch junctions bear longer microhomologies and decreased blunt ends when proteins of the classical NHEJ pathway are mutated [200]. A recent study indicated that these two pathways do not compete but seem to be complementary. Indeed, the density of deamination events has been suggested to lead to different types of DSBs which are then repaired either by the classical NHEJ or the aNHEJ [201]. The molecular mechanisms that lead to aNHEJ pathway are not fully understood and only few proteins have been implicated so far: PARP1, CtIP, MRE11, XRCC1, DNA ligase 3 (Lig3) and potentially DNA ligase 1 [186]. PARP1 is known to sense DNA damage and has been shown to play a role in CSR as its deletion leads to decreased MH at the junction and increased insertions [202]. In the aNHEJ, MRE11 seems to be involved in DNA ends processing as its depletion in XRCC4-deficient cells limits end resection [203]. A similar role has been proposed for CtIP, as its knock down in CH12 cells resulted in increased direct joins at the switch junctions [204]. However, its implication in aNHEJ during CSR is not clear as its depletion from B cells didn't reproduce the phenotype previously observed [205]. The Lig3 with XRCC1 or the Lig1 have been suggested to mediate DSB joining during aNHEJ. But their implication is still under debate: Indeed, mice heterozygous for XRCC1 harbor increased blunt ends and reduced MH at the switch junctions [206], whereas its deletion in WT or XRCC4 deficient cells do not impair aNHEJ-mediated switch junction formation [207]. Similarly, Lig3 or Lig1 deletion do not alter MH usage in CSR junctions, suggesting a redundant role for these proteins [207].

3.5.4. Synapsis of the DNA ends

The final phase of CSR requires synapsis of the donor and acceptor switch regions prior to their ligation but the precise mechanism is not fully understood. Different hypotheses have been proposed: a) AID could dimerize and bind the two switch regions bringing them together [38], b) the interactions between promoters and enhancers could promote the formation of synapsis [138,208,209], c) MSH2/MSH6 complex could bind to S regions and favor their synapsis [190,210] and d) DNA damage proteins like 53BP1, H2AX or ATM could participate in S regions synapsis as their absence might lead to increased internal Sμ deletion rather than recombination [179,181,184,211,212]. Additionally, 53BP1 has been shown to bind Rif1 and it has been proposed that their association is required for the protection of DNA end from 5'-3' end resection [213].

CSR replaces the isotype expressed depending on the pathogen. IgG isotype is the most abundant type of Ig in blood and extracellular fluid, whereas the IgA isotype dimerizes and is mostly found in secretions, notably those of the mucus epithelium of the respiratory intestinal tracks. IgE isotype is present at a very low levels in blood and extracellular fluid but binds avidly on receptor on mast cells located in mucosa, along blood vessels in connective tissue and in the skin epithelium. Each Ig isotype has specialized functions: IgG antibodies can neutralize bacteria and viruses by opsonizing them in order to facilitate their phagocytosis and the activation of the complement system, IgA dimer blocks bacteria's entry across the intestinal epithelium and neutralizes viruses, and finally, IgE triggers local defense reactions by activation mast cells that release chemical mediators to provoke coughing or sneezing.

4. Pathologies associated with antibody diversification

Hyper-IgM (HIGM) syndromes, also called immunoglobulin class switch recombination (Ig-CSR) deficiencies, are immunodeficiencies caused by genetic defects that lead to a complete block or impairment of CSR, which can be associated or not with a defect in SHM. Patients suffering from hyper-IgM syndromes have normal or elevated levels of IgM associated with a lower or absent IgG, IgA or IgE. The impaired switching can be due to an impaired cell-cell interaction, a defect in the activation of the intracellular pathway in B cells or to a B cell intrinsic defect [214].

4.1. Ig-CSR deficiencies due to B cell activation defects

The interaction between T and B cell initiates CSR and SHM and occurs through the interaction between CD40 ligand (CD40L), highly expressed on activated T cells, and CD40, expressed on B cells and dendritic cells [215]. Mutations in CD40L gene cause the most frequent Ig-CSR called HIGM type 1 (HIGM1), patients are susceptible to recurrent bacterial infections and they do not generate antibodies against pathogens but anti-polysaccharide IgM can be detected [216]. Their B cells are unable to proliferate and form germinal centers in secondary lymphoid organs, displaying a CSR defect *in vivo*, with impaired IgG and IgA production. However, *in vitro* stimulation induces their proliferation and CSR as B cells are intrinsically normal [217]. Additionally, SHM is also affected by CD40L mutations [218]. Genetic defect in CD40 induces HIGM3 and patients B cells are unable to undergo CSR *in vivo* or *in vitro* by activation via CD40 and cytokines [219]. Patients exhibit similar symptoms than HIGM1 patients with impaired IgG and IgA production. The NF-κB pathway is implicated in B cell activation and defects in NEMO, the essential NF-κB modulator, result also in an Ig-CSR deficiency. Patients are susceptible to mycobaterial infections and have low levels of serum IgG and IgA and due to genetic heterogeneity, *in vitro* CSR and SHM can be either defective or normal [220].

4.2. Ig-CSR deficiencies due to a B cell intrinsic defect

Ig-CSR deficiencies can also be due to intrinsic B cell defects: Indeed, mutations in AID is the most frequent of autosomal recessive Ig-CSR deficiencies and is called HIGM2 syndrome. Patients harbor drastic CSR defects, with low IgG, IgA and IgE serum levels together with SHM defects [36]. However, in some patients bearing mutation in the C-terminus region of AID, CSR defect is associated with a normal frequency and pattern of SHM, suggesting that AID could interact with CSR-specific cofactors through its C-terminus domain [221]. This was confirmed by AID artificial mutant lacking the last 10 residues, which was able to catalyze SHM and GC but not CSR [40].

Mutations in proteins implicated in AID-induced mutation processing have also been implicated in Ig-CSR deficiencies. UNG deficiency lead to a rare Ig-CSR deficiency as only three patients bearing mutations in this gene have been identifieds so far [222]. Patients B cells exhibit a CSR defect *in vivo* and *in vitro* and analysis of SHM profile in mice deficient for UNG revealed a biased profile of mutations [149]. Additionally, PMS2 mutations, a factor which belongs to the MMR pathway, have been shown to lead to the early occurrences of cancers and patients harboring these mutations showed also a specific defect in CSR, therefore PMS2 has been suggested to convert ssDNA breaks in DSBs after UNG-mediated uracil excision [223]. Given their key role in AID induced-breaks repair, it is not surprising that defects in components of DNA repair pathways such as ATM, MRE11, NBS1, XLF, Lig4 or Artemis have also been implicated in Ig-CSR deficiencies [224].

Finally, Ig-CSR deficiencies associated with an unknown molecular defect have also been described. Patients harbor a defective CSR associated with normal SHM. These deficiencies can be classified in two groups: the first one is due to a defect located downstream of GL transcription and upstream of S region DNA cleavage as no DSB were detectable at Sμ, suggesting that AID might not be targeted to S regions [41]. The second group is due to a CSR block downstream of DNA cleavage as DSBs can be detected. However, sequencing and analysis of protein expression of the main components of the BER, MMR and NHEJ pathways have not identified the origin of the Ig-CSR deficiency, making this defect still uncharacterized [225].

III. Long-range interactions

1. Nuclear organization

The eukaryotic genome is highly organized in the nucleus. Based on microscopy approaches, chromosomes have been shown to occupy distinct territories, called chromosome territories (CT), that can overlap to allow interchromosomal interactions [226]. Within these CT, subnuclear domains are formed to separate regions which are transcriptionally permissives with open chromatin marks from regions enriched in heterochromatin marks, favoring DNA interactions within the same domain [227]. Moreover, 3C-based techniques revealed another level of organization called topologically associating domains (TAD) with a size of 1 Mb, in which long-range cis-interactions occur and these TAD appear

to be conserved across species [228,229]. These domains are composed of interactions between enhancers and promoters are found and seem to be restricted by the domains' boundaries, thereby favoring transcriptional activation of specific genes [230]. Chromatin looping is also observed at repressed genes as in the imprinting control of the H19 gene [231,232]. Additionally, interactions between different TAD that are in the same transcriptionally permissive conditions occur.

2. Enhancer / locus control region - promoter interactions

Chromatin looping has been shown to allow interactions between enhancers and developmentally regulated genes at different loci. The first example is the loop formation between hs sites from the β-globin locus control region (LCR) enhancer and the β-globin genes. This loop associates with transcriptional activation during erythroid differentiation [233] and is dependent on erythroid specific activator like KLF1, GATA1 and FOG1: replacement of GATA1 by an oestradiol inducible version of GATA1 showed that interaction of the LCR with the β-globin gene and its transcriptional activation is dependent on oestradiol [234]. Similarly, deletion of KLF1 or the more general transcriptional coactivator Ldb1 has been shown to affect transcription and looping [235,236]. These studies revealed that looping is tightly linked to transcription. However, the maintenance of interactions are not dependent on ongoing transcription as inhibition of the Pol II does not affect them [237]. Also they are most likely occurring prior to transcription as forcing the looping in the absence of GATA1 is sufficient to induce transcription [238].

During erythropoiesis, the α-globin locus is also subjected to loop formation that correlates with transcriptional activation. It has been shown that specific transcription factors (GATA1 for example) are recruited at the enhancer and at the core promoter, independently from each other, and that activating cell differentiation leads to the recruitment of the pre-initiation complex (PIC) to enhancers which are required for PIC's recruitment at the promoter, suggesting that the enhancer favors the recruitment of PIC to the promoter. Interactions between the enhancer and the promoter correlates with transcription activation [239].

Chromatin loops also display inhibitory functions like at the imprinted H19-Igf2 locus which bears differentially methylated regions depending on the parental origin of the alleles. It has been shown that on the maternal chromosome, the Igf2 promoter is located within a loop that prevents its interaction with the enhancer allowing the H19 promoter to interact with it and thereby to be expressed [240].

Long-range interactions have also been described for immune cells: indeed, in T cells, gene clusters activated by distant enhancers have been reported. TH2 cytokine genes locus span a region of 120 kb and comprise IL4, IL5 and IL13 genes and the LCR. Promoters of these genes are located in close proximity through a looping structure that occurs in different cell types (T, B and fibroblast cells) and upon differentiation of T cells to TH2 cells, the LCR has been shown to be recruited to the promoters in a cell type specific way to induce the expression of the cytokines. These interactions are dependent on STAT6, a transcription factor required for IL4 production, as its depletion revealed impaired interactions between the LCR and the rest of the TH2 cytokine locus. Additionally, inducing the

expression of GATA3, another TH2 specific transcription factor, in fibroblasts induces the interaction between one of the hs sites from the LCR with cytokine promoters but is not sufficient to induce their expression [241]. Regulation of major histocompatibility complex class II (MHC-II) expression has also been shown to be dependent on loop structures that bring the proximal promoter closed to the XL9 enhancer, and these interactions are dependent on CIITA, the master regulator of MHC-II, and the transcription factor RFX which bind the proximal promoter, as deletion of these proteins affected the interaction [242]. Long-range interactions might also prevent transcription as suggested at the IFNγ locus which is active in TH1 cells. An initial folding present in neutral TH, TH1 and TH2 cells is changed upon activation by anti-CD3 and in TH1 cells, the locus was less compacted than in TH2 cells [243].

During B and T cell development, V(D)J recombination recombines DJ with V gene segments that spread over two to three Mb. To allow these distal rearrangements, locus compaction has been shown to occur [26,244]. For the IgH locus, locus contraction is known to be dependent on B cell commitment factor Pax5, on Ikaros, which is essential for B cell development and on the ubiquitous transcriptional regulator YY1, which deletion abrogated compaction of the IgH locus and recombination to distal V genes [26,245,246]. Additionally, Ezh2, which belong to the Polycomb group of proteins, has also been shown to impact recombination to distal V genes possibly through chromatin modifications [247].

During CSR, long distance loops are also occurring: it has been shown that the Eμ enhancer interacts with the 3'RR in resting cells and that upon B cell activation, the acceptor switch region is recruited to this loop [138]. However, no protein has been shown to be specifically affecting these long-range interactions so far.

Overall, these studies seem to implicate cell type specific transcription factors in mediating the looping structures. Nevertheless, proteins that regulate higher order chromatin structures have also been involved in this process, like CTCF, a zing-finger protein required for transcription insulation [248], the cohesin and the mediator complexes. As during my PhD, I focused on cohesin and mediator complexes, I am going to describe their functions in details.

IV. Cohesin and Mediator complexes

1. The cohesin complex

1.1. The structure of the cohesin complex

The core cohesin complex is composed of two structural maintenance of chromosomes (Smc) proteins: Smc1 and Smc3, and two non-Smc proteins Rad21 and SA1 or SA2 (figure 12). Smc proteins form an heterodimer and each subunit is composed of a Walker A and a Walker B nucleotide-binding residues at the N and C terminal region, respectively. The central domain is composed of a globular hinge domain surrounded by coiled-coil motifs that associate in an anti-parallel fashion, bringing Walker motifs in close proximity to form an ATP-binding site [249,250]. The two Smc proteins

interact through their hinge domain and the Smc3 and Smc1 ATP binding sites are bound by Rad21 N and C terminal domains, respectively, creating a tripartite ring [249]. Either SA1 or SA2 protein is bound to the central domain of Rad21 and is essential for cohesin loading onto chromatin. The heterodimer Nipbl/Mau2 has been implicated in cohesin loading [251] and Pds5/Wapl is responsible for their unloading [252].

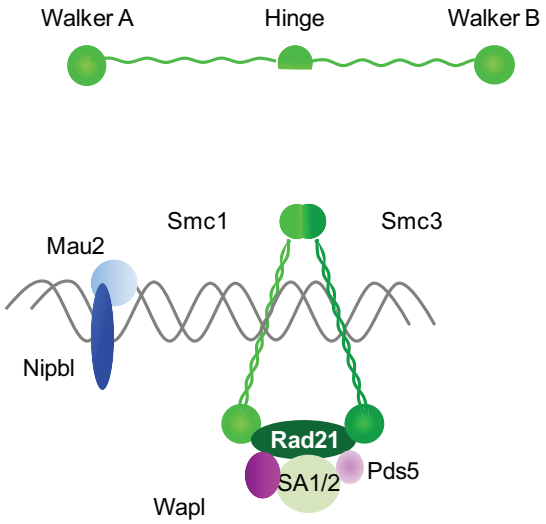

Figure 12: The cohesin complex
Smc protein structure, displaying a Walker A motif at the N-terminus and a Walker B motif at the C-terminus separated by two coiled-coil motifs surrounding a hinge domain. Smc1 and Smc3 interact through their hinge domain and bind Rad21 subunit to form a ring structure. Adapted from [253].

1.2. The function of the cohesin complex

Cohesin function is to hold sister chromatids together from the time they are replicated until they segregate during anaphase to ensure a symmetric distribution of chromosomes to the daughter cells [254]. Mutation of cohesin subunits in yeast has been shown to induce premature separation of sister chromatids [255,256]. On the contrary, maintaining cohesin onto chromosomes has been shown to inhibit their separation and to delay mitosis progression [252].

In vertebrate cells, cohesin loading onto chromatin occurs into early G1 phase and is dependent on ATP hydrolysis [257] and on Nipbl-Mau2 heterodimer [251]. The precise mechanism that allows cohesin loading is not fully elucidated, but it has been shown that Smc1 and Smc3 might retain their binding to Rad21 and that their hinge domain could serve as an entry gate [258]. Cohesin is then established during S phase and this step is dependent on Eco1, which acetylates K112 and K113 residues from Smc3 subunit [259]. This modification counteracts the activity of Pds5/Wapl, which is responsible for cohesin unloading [252,260]. Cohesin is removed during two phases of mitosis: the prophase (the majority of cohesin is removed from chromosomal arms) and the anaphase (removal from centromeres and from the arms). The mechanism occurring during prophase is not elucidated. So far, it has only been shown to be independent of separase cleavage, by opposition to the dissociation occurring during anaphase [261], which requires separase activity. Once all chromosomes are bioriented in metaphase,

the securin protein is degraded through the proteasome and its partner, separase, is then able to cleave the Rad21 subunit leading to unloading of the cohesin complex from centromeres, and thereby allows sister chromatids migration towards opposite poles of the cell in anaphase [262,263].

In addition to its role in sister chromatid cohesion, cohesin is also involved in DNA damage response. Mutation in Scc1 subunit, the Rad21 equivalent subunit in *S. pombe*, has been shown to render cells hypersensitive to UV and IR [264] and Rad21 role has been confirmed in vertebrates [265]. Additionally, mutations in Smc1, Smc3, Scc3 (SA equivalent) and Pds5 in *S. cerevisiae*, was shown to lead to sensitivity to γ-irradiations and to be required for DSB repair [266]. Cohesin has been implicated in HR, probably to favor recombination with sister chromatid, and has been suggested to direct the choice between HR and NHEJ [267]. Cohesin is also implicated in DNA damage checkpoint activation, through phosphorylation of Smc1 and Smc3 by ATM [268-270]. Additionally, cohesin has also been implicated in the G2/M checkpoint since cohesin depletion induced defective recruitment of 53BP1 to DNA damage foci and weaker Chk2 activation [271].

Together, these studies implicated the cohesin complex in cell cycle control and in DNA damage response. Cohesin has also been implicated in gene expression regulation, through its potential ability to control long-range interactions. As cohesin's function in DNA looping is often associated with CTCF or the mediator complex, I will detail it below.

1.3. Cohesin mutations and disease

Mutations in cohesin subunits or regulatory subunits have been shown to cause human syndromes, called cohesinopathies that include Cornelia de Lange syndrome (CdLS) and Roberts Syndrome (RS). CdLS patients present multiple developmental and cognitive abnormalities characterized by growth retardation, malformations of the upper limbs, characteristic facial appearance, gastrointestinal abnormalities and severe mental retardation [253]. More than half of CdLS cases are due to mutations in the Nipbl gene [272,273], whereas Smc1 or Smc3 mutations only account for 5% of CdLS cases [274] and the phenotypes arising from Smc1 or Smc3 mutations are less severe that Nipbl mutations. In addition, cell lines derived from CdLS patients have an increased sensitivity to DNA damage suggested to be due to defective HR-mediated repair [275] and to defective NHEJ [276]. The impaired NHEJ was associated with reduced 53BP1 recruitment to DSB, suggesting that Nipbl might recruit 53BP1 to DSB sites. Rad21 mutations have also been implicated in a cohesinopathy syndrome and patients affected display even milder phenotype compared to Smc mutations with variations in the facial features and mild physical abnormalities [277]. RS on the other hand is caused by mutations in ESCO2 gene and is characterized by upper and lower limb defects, craniofacial abnormalities, mental retardation and growth deficiency [278].

2. The mediator complex

2.1. Structure of the mediator complex

The mediator complex was initially discovered in yeast as a factor required for activator dependent transcription as its absence prevented it *in vitro* [279,280]. The mediator complex was subsequently shown to interact with the C-terminal domain of the Pol II and to be composed of several subunits [281]. Yeast mediator complex is composed of 25 proteins and its structure and function are conserved in higher eukaryote cells, which contain around 30 subunits [282,283] (figure 13). Initial studies revealed that its subunits are organized in different modules called head, middle, tail and kinase, where the head and middle modules are believed to be involved in interactions with the core Pol II machinery, whereas the tail interacts with various activators [284]. The head, middle and tail modules form the core structure, whereas the kinase module associates reversibly with the rest of the mediator complex. Indeed, different laboratories identified two distinct variants of the human mediator complex, the PC2 and the thyroid receptor-associated protein (TRAP), which differed mostly by the absence of the kinase module in the PC2 complex [285,286].

The identification of different types of mediator complexes has been suggested to reflect functional differences: firstly, the presence of the kinase module in the mammalian mediator complex might reflect an inactive form of the mediator complex because it can inhibit basal transcription by phosphorylating the Cyclin H subunit of the transcription initiation factor IIH (TFIIH), thereby repressing its ability to induce transcription [287]. Additionally, the kinase module has been shown to bind in part, to the surface of the core complex that interacts with the Pol II thereby competing with it [288,289]. However, the kinase module has been shown to be also a positive co-regulator dependent on its context. For example, its CDK8, Med12 and Cyclin C subunits are recruited at p21 locus only when the transcription is activated and depletion of CDK8 reduced the level of p21 transcription [290]. Moreover, several single subunit differences have been depicted so far. For instance, Med1 has been shown to exist predominantly in a subpopulation of mediator complex that is enriched for the Pol II [291].

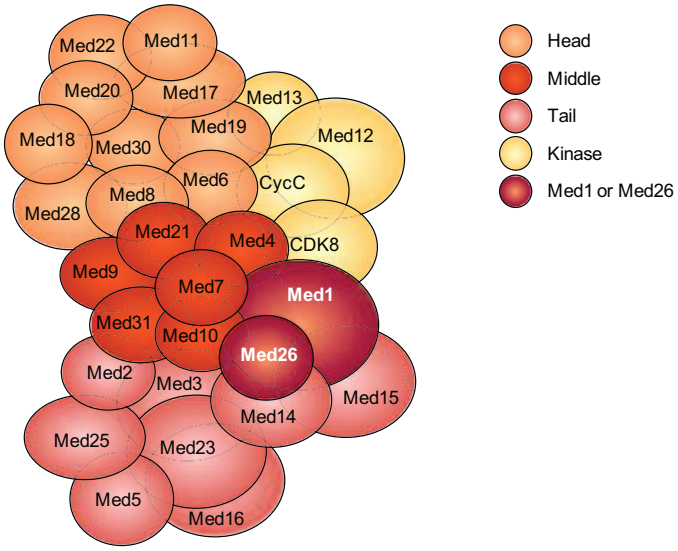

Figure 13: The mediator complex
A schematic representation of the human mediator complex. It is composed of 30 subunits organized in four modules. The relative placement of the subunits is based on published interactions and partial structural data. Med1 and Med26 are not present in all mediator complexes and are most likely located at the junction of the middle and the tail modules. Adapted from [292].

2.2. The mediator complex is implicated in different signaling pathways

The mediator complex has been implicated in different physiological processes and numerous studies revealed that specific activators interact with different subunits suggesting a specificity induced by these interactions. Indeed, Med1 subunit has been shown to be essential for transcriptional activation for several hormone receptors through its interaction with nuclear receptors like the vitamin D receptor [293], estrogen receptor [294] or the PPARγ [295]. Med1 also interacts with GATA family of transcription factors [296] and is required for erythroid development [297], showing that an ubiquitous subunit might regulate specific events. Other subunits have been implicated in specific events, like the Med23 which is required for MAPK signaling as this pathway was defective in embryonic stem (ES) cells deficient for this subunit [298]. Med12 belongs to the kinase module and has been shown to interact with specific transcription factors to mediate activation or repression: Med12 interacts with SOX9 to induce chondrocyte differentiation [299] and with β-catenin to activate transcription in response to Wnt signaling [300,301]. On the other hand, Med12 interaction with Gli3, an effector of the Hedgehog signaling pathway, important for the formation and maintenance of adult stem cells, is required for repression of Gli3 target genes [302].

2.3. The mediator complex and transcriptional regulation

The mediator complex has been implicated in different steps of transcription regulation. The first function of the mediator complex is to recruit the Pol II and to direct the assembly of the pre-initiation complex (PIC). Indeed, the mediator complex was shown to interact and recruit *in vitro* several pre-

initiation factors like TFIID [303] or TFIIB [304]. Mediator also interacts with TFIIH, recruits it to the PIC [305] and might favor phosphorylation of the Pol II CTD by TFIIH [306].

The mediator complex has also been implicated in transcription elongation [307]. At many genes, the Pol II is paused near the proximal promoter and is controlled by transcription elongation factors as DSIF (Spt5 and Spt4) for example. The exact mechanism of the paused Pol II release is not clear, however, it has been shown that the mediator complex interacts with transcription elongation factors, like P-TEFb [308] or DSIF [309] and might facilitate the transition to elongation. It has been proposed that mediator complex recruits P-TEFb which phosphorylates the Pol II CTD [310] and Spt5 to allow elongation [311]. The complex formed by P-TEFb and mediator contains also Brd4, which has recently been implicated in Pol II paused release through activation of P-TEFb [312]. Additionally, the Med23 subunit has been shown to favor the transition to elongation in ES cells, as deletion of this subunit still allows Pol II recruitment but not efficient transcription [313].

Finally, the mediator complex can also favor transcription by regulating the chromatin state. The kinase module can directly phosphorylate H3S10 while GCN5L acetylates the same H3 on K14 and CDK8 depletion induces a depletion of these two histone modifications [314]. Additionally, the mediator complex has been implicated in the recruitment of CHD1 (chromodomain helicase DNA-binding protein 1), a chromatin-remodeling protein that binds H3K4me3, a histone modification present near the beginning of active genes [315]. The mediator complex can also synergize with the histone acetyltransferase p300 to activate transcription at estrogen receptor [316].

Chromatin looping has been implicated in transcriptional activation through activators that can be located at long distances, and the mediator complex has been implicated in the formation and maintenance of these loops. I will detail this function below.

2.4. Mediator mutations and disease

Mutations in mediator subunits have been associated with human diseases comprising malformation, mental retardation and cancer. Med13L subunit (a Med13 paralog) has been shown to be mutated in a patient suffering from mental retardation and malformation of the heart due to transposition of the great arteries [317]. Patients with DiGeorge syndrome, characterized by various cardiovascular abnormalities and facial dysmorphism harbor a chromosomal deletion which include the Med15 gene [318]. Numerous studies of diseases associated with mental retardation have also revealed mutations in several subunits of the mediator complex [319]: Med12 mutations in FG syndrome and Lujan syndrome [320,321]; Med17 mutation in infantile cerebral and cerebellar atrophy [322], Med23 mutation in nonsyndromic intellectual disability [323].

Additionally, mediator subunits have also been implicated in cancer: mutation in Med12 has been reported in more than 50% of uterine leiomyomas which are benign tumors occuring in 60% of women

by the age of 45 years [324]. Finally, Med23 has been shown to be overexpressed in lung cancer samples associated with hyperactive RAS activities [325].

3. Cohesin and mediator as architectural proteins

In addition to specific transcription factors, chromatin loops have also been shown to require ubiquitous proteins like CTCF, cohesin and mediator. These factors have been implicated in gene regulation to maintain embryonic stem (ES) cells state and in the different examples explained above: the β-globin locus in erythroid cells, the expression of T cells specific factors and in V(D)J recombination.

3.1. Implication in the induction or maintenance of pluripotency in ES or iPS cells

In ES cells, a shRNA screen identified subunits of the mediator and cohesin complexes as implicated in the maintenance of ES cells state [326]. These complexes were shown to co-occupy enhancers and promoters from active genes and their binding has been suggested to predict DNA looping. Additionally, it has been reported that their binding profile is cell type specific as their recruitment profile is different in ES cells and in MEF [326].

The implication of cohesin, mediator and CTCF as genome organizer for enhancer–promoter and inter-TAD contacts to maintain or induce pluripotency has been further investigate in recent papers: In a first study, it has been shown that over-expressed pluripotency factors like Nanog or Oct4 bind similarly in induced pluripotent stem (iPS) cells and in unreprogrammed cells, suggesting that their recruitment is not sufficient to induce pluripotency [327]. However, the generation of a loop between the enhancer and the promoter of Oct4 gene was specific to iPS cells and these elements were bound by Smc1 and Med12 and that Smc1 knock down reduced the loop and affected pluripotency [327]. Moreover, Klf4 has been shown to also be bound at Oct4 enhancer and promoter, to interact with cohesin and to be required for cohesin's recruitment at Oct4 locus, suggesting that Klf4 might be responsible for bringing specificity to the long-range interactions mediated by cohesin and mediator [328].

Similarly, focusing on the specific interaction network around Nanog promoter, it has been shown that mediator and cohesin bind to the Nanog locus and that depletion of these proteins leads to disruption of the contact and differentiation of ES cells [329]. Additionally, these long-range interactions were occurring at early reprogramming stages, prior to transcriptional activation [328,329]. Finally, a more global analysis of chromatin conformation in ES cells and neural progenitor cells revealed that CTCF, cohesin and mediator are found at around 80% of chromatin interactions and their differential binding might reflect different types of loops [330]. Indeed, cohesin and CTCF binding reflects long-range

constitutive interactions that can form sub-TAD boundaries, whereas cohesin and mediator were implicated in enhancer-promoter interactions within TAD, but also between TAD in a tissue-specific manner [330] (figure 14). In accordance with this paper, cohesin and CTCF have been shown to be enriched at TAD boundaries [228,230].

Together, these data suggest that architectural proteins mediate inter- and intra-TAD interactions to maintain or favor genome architectural changes during differentiation.

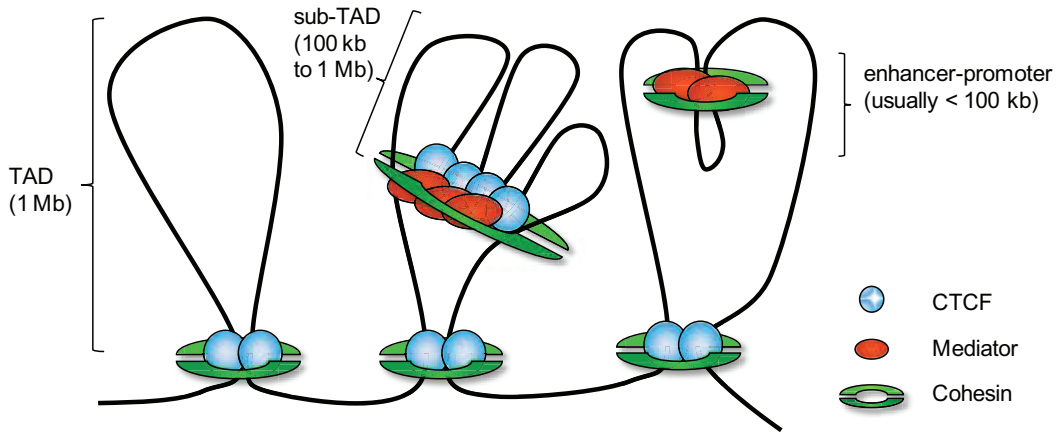

Figure 14: Architectural proteins act combinatorially to organize chromatin at different length-scales
TAD boundaries are enriched for CTCF and cohesin. They can also be recruited in association with mediator to subdivide the TAD in sub-TAD within which mediator and cohesin can mediate enhancer-promoter interactions. Adapted from [331].

3.2. Examples of implication in other cell types

These architectural proteins have also been implicated in the regulation of looping events in other cell types. At the β-globin locus, CTCF binding sites flank the locus and have been shown to participate in the interactions between the LCR and the β-globin genes [332]. CTCF binding sites interact together already in erythroid progenitor cells that do not yet express globin genes [233] and its depletion reduced these interactions and the presence of active chromatin marks around the genes, but did not affected globin transcription [333]. At the α-globin locus, CTCF binding sites were also identified [334] and the mediator complex has been shown to be recruited to the promoter [239], but their implication in DNA looping has not been investigated so far.

The imprinted-control region (ICR) of imprinted H19-Igf2 genes has been shown to be bound by CTCF in the unmethylated allele (maternal) constituting an enhancer-blocking activity that induced Igf2 silencing [335,336]. More recently, cohesin has also been implicated in this transcriptional insulation as it has been shown that cohesin are recruited by CTCF and are required for CTCF insulator function [337].

In T cells, CTCF has been implicated in the looping occurring between XL9 and the promoter of MHC-II to induce its expression [242] and subsequently additional CTCF sites were identified and implicated in differential interactions that were associated or not with gene expression [338]. Additionally, cohesin has also been shown to be recruited to these sites and to be important for DNA interactions with the

promoter [339]. Finally, the regulation of the IFN-γ locus interactions have also been shown to be dependent on cohesin and CTCF binding through knock down experiments that revealed reduced interactions and impaired IFNγ expression when CTCF [340] or cohesin [341] were absent.

During V(D)J recombination, cohesin and CTCF have been implicated in long-range interactions regulation. In pro-B cells, CTCF and cohesin were found to bind through the V_H region and cohesin recruitment was shown to be cell stage specific contrary to CTCF binding [342]. Additionally, CTCF knock down affected the locus compaction [343] and deletion of two CTCF binding sites located upstream of the D_H genes showed decreased rearrangement with distal V_H genes, suggesting that CTCF is implicated in this recombination [344]. However, this effect could be also be due to disruption of PAIR elements and abolished recruitment of other factors like E2A, cohesin and Pax5 that are also bound to these elements, the latter in a cell stage specific fashion [345]. In T cells, cohesin, CTCF and mediator have been shown to bind to promoters and enhancers of TCRα locus and cohesin depletion affected long-range promoter-enhancer interactions, transcription and rearrangement [244].

These different studies implicated the architectural proteins: CTCF, cohesin and mediator complexes in mediating long-range interactions in various cell types and at genes which expression is tightly regulated.

Working hypothesis

AID is a mutagenic protein essential for antibody diversification, however, the precise mechanisms responsible for controlling its activity or its specific recruitment to the Ig genes are not fully elucidated. To gain insight into AID regulation, we performed a proteomic screen to identify AID partners. Among the list of proteins that we identified by mass spectrometry analysis, we found subunits of cohesin and mediator complexes. This was of particular interest because these complexes have been shown to co-occupy active genes and their co-binding can predict genomic long-range interactions [326], characteristics that we find also at the IgH locus in B cells undergoing CSR. Indeed, CSR is a transcription-dependent, long-range recombination event that occurs at the IgH locus and that involves the joining of two S regions, which may be separated by several hundreds of kilobasepairs. Therefore, for CSR to succeed, donor and acceptor S regions must be brought into close proximity and this is believed to occur through three-dimensional conformational changes that involve the generation of transcription-coupled DNA loops [209]. Nevertheless, the precise mechanisms controlling these conformational changes remain to be elucidated.

The fact that AID interacts with cohesin and mediator subunits and that mediator and cohesin complexes are implicated in 3D organization of the genome lead us to hypothesize that cohesin and mediator complexes might be implicated in the generation of DNA loops occurring during CSR. Additionally, AID recruitment to transcribed genes could be dependent on the conformational changes mediated by these complexes (figure 15).

To study the role of cohesin and mediator complexes in CSR, we knocked down subunits of cohesin and mediator complexes in CH12 cells using lentiviruses expressing shRNA targeting Smc1, Smc3, Nipbl, Wapl, Med1, Med12, AID as a positive control and a non specific shRNA. Additionally, we generated Med1$^{f/f}$ Mb1$^{Cre/+}$ mice to study the implication of Med1 in CSR and SHM *in vivo*.

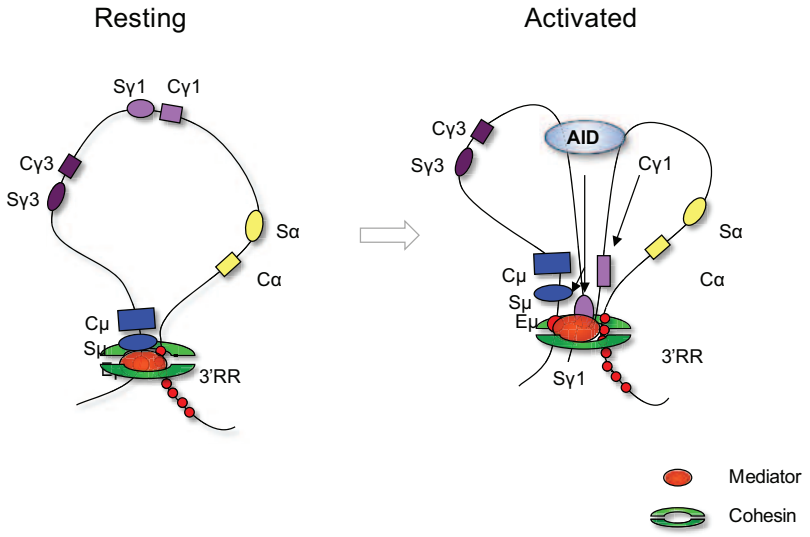

Figure 15: Working hypothesis
DNA loops between Eμ and the 3'RR and Eμ, the 3'RR and the acceptor switch region might be mediated by cohesin and mediator complexes.

Results

The result section is divided in three parts. The first one corresponds to our manuscript published in Journal of Experimental Medicine [346] describing the role of cohesin complex in CSR. The second part corresponds to a first draft of our manuscript in preparation focusing on the role of Med1 in antibody diversification. The third part shows additional results on the characterization of the role of these complexes in cells undergoing CSR, which were either requested by reviewers (and not included in the final manuscript) or preliminary results which need to be confirmed.

I. The cohesin complex regulates class switch recombination

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The cohesin complex regulates immunoglobulin class switch recombination

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Immunoglobulin (Ig) class switch recombination (CSR) is initiated by the transcription-coupled recruitment of activation-induced cytidine deaminase (AID) to switch regions and by the subsequent generation of double-stranded DNA breaks (DSBs). These DNA breaks are ultimately resolved through the nonhomologous end joining (NHEJ) pathway. We show that during CSR, AID associates with subunits of cohesin, a complex previously implicated in sister chromatid cohesion, DNA repair, and the formation of DNA loops between enhancers and promoters. Furthermore, we implicate the cohesin complex in the mechanism of CSR by showing that cohesin is dynamically recruited to the Sμ-Cμ region of the IgH locus during CSR and that knockdown of cohesin or its regulatory subunits results in impaired CSR and increased usage of microhomology-based end joining.

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Abbreviations used: 3′RR, 3′ regulatory region; AID, activation-induced cytidine deaminase; CSR, class switch recombination; DSB, double-stranded DNA break; IgH, Ig heavy chain; MudPIT, Multidimensional Protein Identification Technology; NHEJ, nonhomologous end joining; SHM, somatic hypermutation.

During immune responses, B cells diversify their receptors through somatic hypermutation (SHM) and class switch recombination (CSR). SHM introduces mutations in Ig variable regions that modify the affinity of the receptor for its cognate antigen (Di Noia and Neuberger, 2007). CSR replaces the antibody isotype expressed (from IgM to IgG, IgE, or IgA), providing novel antibody effector functions (Chaudhuri et al., 2007). Mechanistically, SHM and CSR are initiated by activation-induced cytidine deaminase (AID), an enzyme which deaminates cytosines in both strands of transcribed DNA substrates (Petersen-Mahrt et al., 2002; Basu et al., 2011). AID-induced DNA deamination is then processed to trigger mutations in variable regions during SHM or to generate double-stranded DNA break (DSB) intermediates in switch (S) regions during CSR (Chaudhuri et al., 2007; Di Noia and Neuberger, 2007). These breaks activate the DNA damage response (Ramiro et al., 2007) and are resolved through classical and alternative nonhomologous end joining (NHEJ; Stavnezer et al., 2010).

CSR is a transcription-dependent, long-range recombination that occurs at the Ig heavy chain (IgH) locus and that involves the joining of two S regions, which may be separated by several hundreds of kilobase pairs. For CSR to succeed, donor and acceptor S regions must be brought into close proximity. This is believed to occur through three-dimensional conformational changes involving the generation of transcription-coupled DNA loops (Kenter et al., 2012). Nevertheless, the precise mechanisms controlling these conformational changes remain to be elucidated.

The cohesin complex has been described to play a prominent role in sister chromatid cohesion during cell division, in favoring DNA repair by homologous recombination (Nasmyth and Haering, 2009), in modulating gene expression (Dorsett, 2009), and in promoting the transcription-coupled formation of long-range DNA loop structures (Kagey et al., 2010). In addition, cohesin and the transcriptional insulator CTCF (Dorsett, 2009; Nasmyth and Haering, 2009) have been shown to control the RAG1/2-dependent rearrangement of antigen receptor genes during early B and T lymphocyte development by mechanisms involving the regulation of transcription and formation of long-range in

A.-S. Thomas-Claudepierre and E. Schiavo contributed equally to this paper.

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Results part 1

cis DNA interactions (Degner et al., 2011; Guo et al., 2011; Seitan et al., 2011). Here, we have examined the role of cohesin in mature B cells undergoing CSR.

RESULTS AND DISCUSSION

Nuclear and chromatin-bound AID associate with cohesin

We have previously shown that nuclear AID exists in a large molecular weight complex containing proteins that are required for CSR (Jeevan-Raj et al., 2011). To further characterize this complex and investigate the functional role of novel AID partners in CSR, we have performed additional coimmunoprecipitation experiments coupled to identification by mass spectrometry. Nuclear and chromatin extracts prepared from CH12 cells expressing a full-length N-terminally tagged AID protein ($AID^{Flag\text{-}HA}$) or the epitope tags alone (Flag-HA) as negative controls were immunoprecipitated using an anti-Flag antibody. Eluted proteins were submitted for identification by mass spectrometry. Among the proteins identified, we found multiple AID partners previously implicated in CSR and/or SHM (Table S1). In addition, we found several proteins with no known function in CSR (Table S2), including subunits of the cohesin, condensin, Smc5/6 complex and Ino80 complexes. Given the described role for cohesin in mediating long-range recombination during B cell and T cell differentiation, we focused on the potential role of cohesin in CSR. The association between AID and the cohesin complex subunits (Smc1, Smc3, Nipbl, and Wapal) was confirmed by reciprocal coimmunoprecipitations and Western blotting in the nuclear (Fig. 1 A) and chromatin (Fig. 1 B) fractions and was specific, as they did not coprecipitate with an irrelevant tagged protein ($EGFP^{Flag\text{-}HA}$; Fig. 1 C). Importantly, these interactions were not mediated by nonspecific nucleic acid binding, as extracts and immunoprecipitations were done in the presence of the benzonase nuclease. We conclude that endogenous subunits of the cohesin complex associate with a fraction of nuclear and chromatin-bound tagged AID through interactions that do not involve nonspecific nucleic acid binding.

Smc1 and Smc3 are dynamically recruited to the IgH locus during CSR

To determine whether cohesin is recruited to the IgH locus in B cells undergoing CSR, we performed ChIP-Seq experiments on chromatin prepared from resting or activated splenic B cells isolated from wild-type mice and using antibodies specific for Smc1, Smc3, and CTCF (Fig. 2). In resting B cells, we found that Smc1, Smc3, and CTCF are co-recruited to the 3′ regulatory region (3′RR; Fig. 2 A). This is consistent with published ChIP data on CTCF (Chatterjee et al., 2011) in mature B cells and ChIP-Seq results for CTCF and cohesin (Rad21) in Rag1-deficient pro–B cells (Degner et al., 2011). A sharp peak of CTCF, Smc1, and Smc3 binding was observed at Cα. This peak occurred over a region containing a predicted DNaseI hypersensitive site and a CTCF consensus motif (Nakahashi et al., 2013). No significant enrichment was observed at the Eμ enhancer Sμ or Sγ1 (Fig. 2 A). After stimulation, under conditions that induce CSR to IgG1, we found that Smc1 and Smc3 are significantly co-recruited, independently of CTCF, to a region spanning from the 5′ end of the donor switch region (Sμ) to the 3′ end of the Cμ constant region that did not comprise the Eμ enhancer (Fig. 2 B). Surprisingly, we failed to detect a reproducible recruitment of Smc1 or Smc3 over the Sγ1 switch region (Fig. 2 B), suggesting that Smc1 and Smc3 are not recruited to the acceptor switch region upon activation. It is possible, however, that our cell culture conditions (in which ∼15–20% of the cells switch to IgG1) are not robust enough to detect a specific enrichment. Consistent with this, we were unable to reproducibly detect a specific enrichment of AID at Sγ1 by ChIP-qPCR (Fig. 2 E).

The ChIP-Seq signal obtained in resting and activated B cells for Smc1 and Smc3 (Fig. 2, A and B) is consistent with the fact that they are known to exist as a heterodimer and was reproducible and specific, as we did not observe any significant enrichment at the IgH locus when using an IgG antibody as a negative control (Fig. 2, A and B). The recruitment of Smc1 and Smc3 at the IgH locus only partially correlated with that reported for AID (Yamane et al., 2011) and is consistent with the fact that only a fraction of chromatin-bound AID associates with the cohesin complex (Fig. 1 B). This suggests that cohesin is not a targeting factor for AID. The recruitment of Smc1, Smc3, and CTCF in resting and activated B cells observed by ChIP-Seq (Fig. 2, A and B) was confirmed by additional independent analytical-scale ChIP-qPCR experiments, using primer pairs at individual locations across

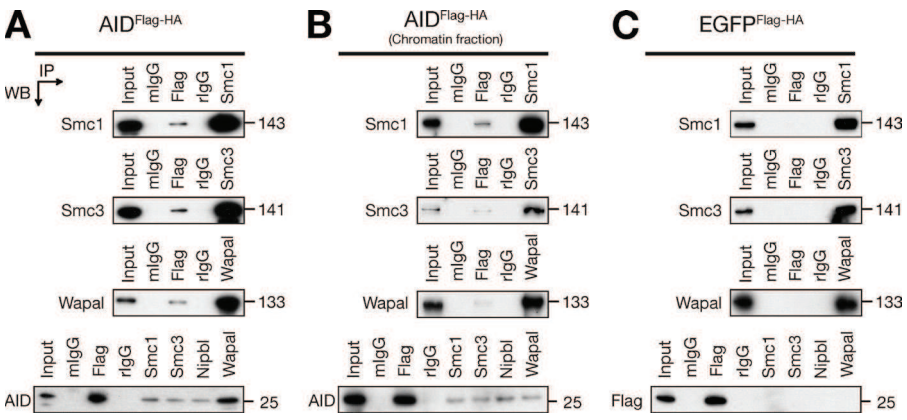

Figure 1. Nuclear AID associates with cohesin subunits. Nuclear extracts (A and C) and chromatin fractions (B) prepared from CH12 cells expressing $AID^{Flag\text{-}HA}$ (A and B) or $EGFP^{Flag\text{-}HA}$ (C) were immunoprecipitated and blotted with antibodies specific for Flag, AID, Smc1, Smc3, Wapal, and Nipbl. Note that the Nipbl antibody works only on immunoprecipitation. Input represents 1% of material used. Theoretical molecular masses in kilodaltons are indicated. Data are representative of three independent experiments.

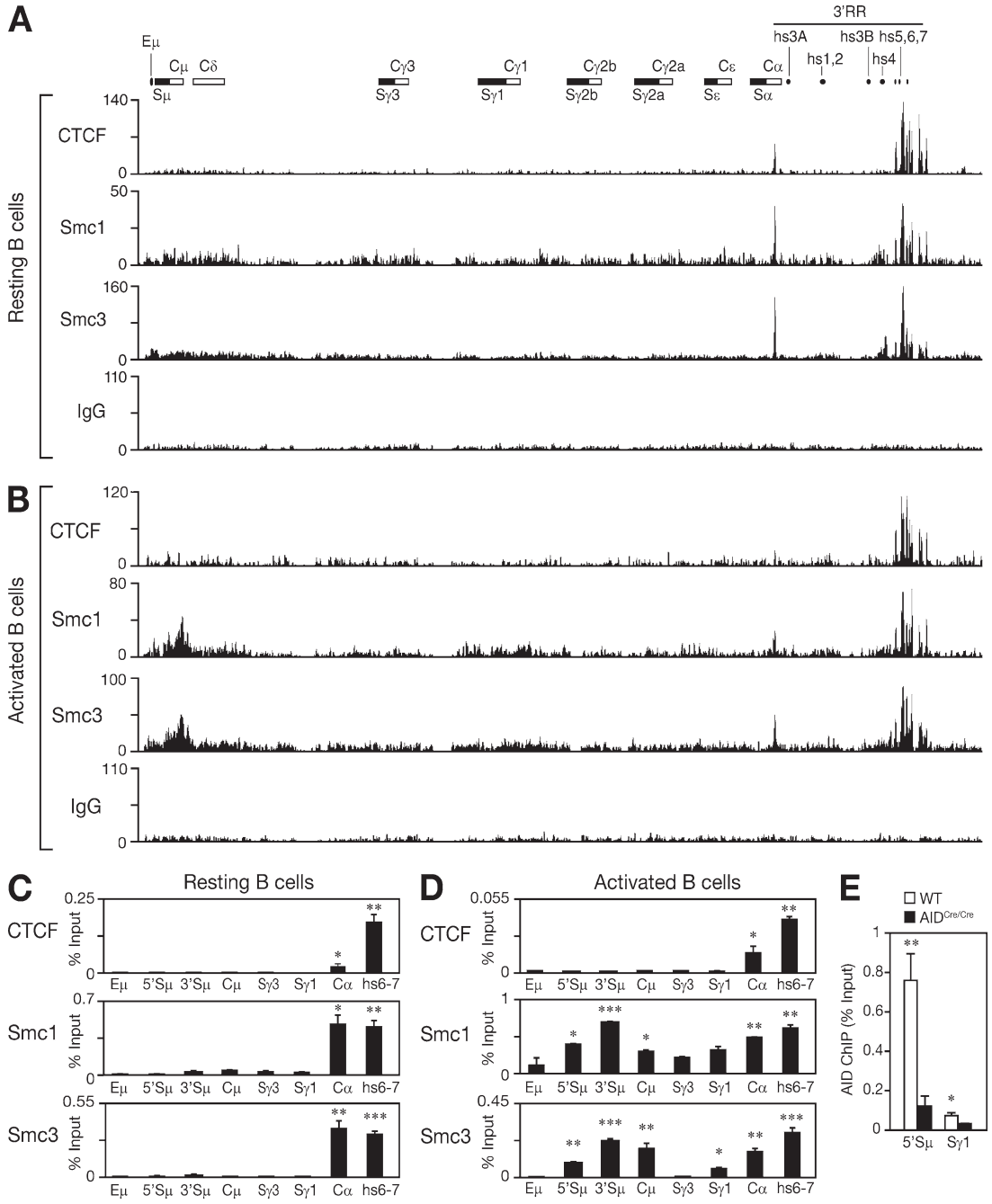

Figure 2. Smc1 and Smc3 are dynamically recruited to the IgH locus during CSR. UCSC genome browser screenshots showing the ChIP-Seq binding profiles of CTCF, Smc1, Smc3, and IgG (negative control) at the IgH locus (chr12:114,438,857–114,669,149) in resting (A) and activated (B; with LPS + IL-4) B cells isolated from wild-type mice. A schematic map of the IgH locus indicates the switch regions (black boxes), the constant region exons (white boxes), the Eμ enhancer, and the DNaseI hypersensitive sites (hs) located in the 3′RR. Similar ChIP-Seq profiles were observed in an additional biological replicate experiment for Smc3 that was conducted in resting and activated B cells (not depicted). Chip-Seq results were verified by analytical-scale ChIP-qPCR experiments performed on chromatin prepared from 10^7 splenic resting (C) and activated (D) B cells. qPCR was performed at several locations across the IgH locus using primers listed in Table S4. Results are expressed as percent input and are representative of two independent biological replicate experiments. Mean of triplicate samples (+SD) is shown. Statistical significance versus Sγ3 (two-tailed Student's *t* test) is indicated: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. Additional statistical analyses across the locus and between resting and activated B cells are shown in Table S5. (E) ChIP analysis for AID occupancy at the Sμ and Sγ1 switch regions in wild-type and *AID*$^{Cre/Cre}$ B cells cultured in vitro with LPS + IL-4 for 60 h. Results are expressed as percent input. Mean of triplicate samples (+SD) is shown. Statistical significance versus *AID*$^{Cre/Cre}$ was determined by a two-tailed Student's *t* test. *, $P \leq 0.05$; **, $P \leq 0.01$. Results are representative of four independent experiments.

Results part 1

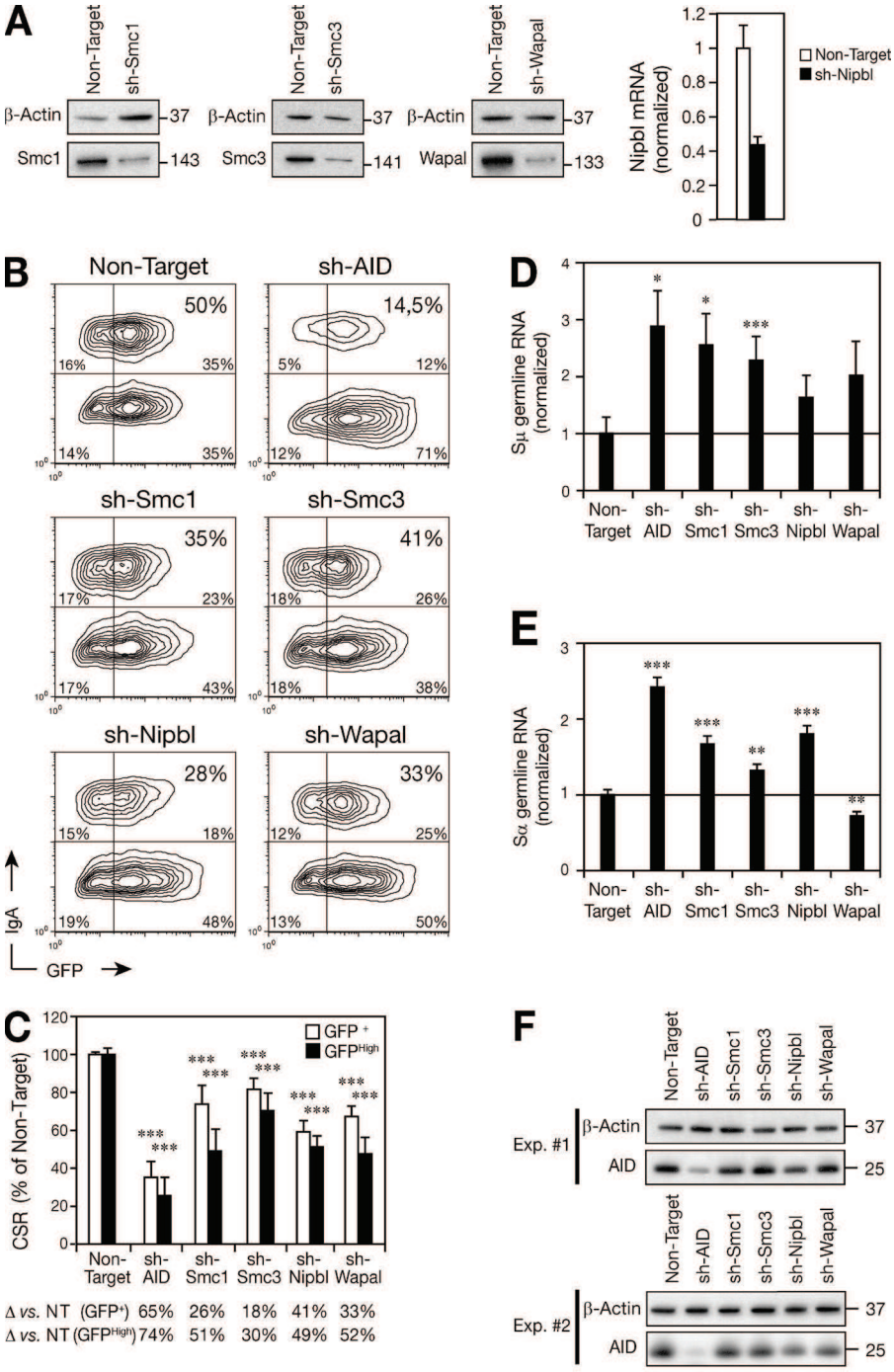

Figure 3. CSR is impaired by the knockdown of cohesin subunits. (A) CH12 cells were transduced with a lentivirus expressing a GFP reporter and shRNAs specific for AID, Smc1, Smc3, Nipbl, Wapal, or a Non-Target control. Transduced cells were stimulated for 48 h and sorted for GFP expression. Protein extracts and cDNAs were prepared and knockdown was determined by Western blotting or qPCR. Western blot for β-actin, Smc1, Smc3, and Wapal and qRT-PCR for *Nipbl* transcripts are shown. Expression was normalized to *Cd79b* and is presented relative to the Non-Target control, set as 1. Mean of triplicate samples (+SD) is shown. Statistical significance versus the Non-Target control (two-tailed Student's *t* test): P = 0.0023. Data are representative of three experiments. (B) CH12 cells treated as in A were analyzed for surface IgA and GFP expression by flow cytometry. Representative plots from four to eight independent experiments are shown. (C) CH12 cells treated as in A were gated on cells expressing GFP (GFP$^+$; white bars) or high levels of GFP (GFP^{High}; black bars). The percentage (+SD) of CSR relative to the Non-Target shRNA control from four to eight independent experiments is shown. CSR in cells expressing the Non-Target shRNA control was set to 100%. The difference in CSR efficiency relative to the Non-Target control (Δ) is indicated below. Statistical significance versus the Non-Target control (two-tailed Student's *t* test) is indicated: ***, P ≤ 0.001. (D and E) cDNA was prepared from CH12 cells treated as in A and qRT-PCR for μ (D) and α (E) germline transcripts was performed. Expression was normalized to *HPRT* mRNA abundance and is presented relative to the Non-Target control, set as 1 (black line). Mean of triplicate samples (+SD) is shown. Statistical significance versus the Non-Target control (two-tailed Student's *t* test) is indicated: *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001. (F) Proteins extracts were prepared from CH12 cells treated as in A. Western blots for β-actin and AID are shown. Data are representative of three independent experiments. Theoretical molecular masses in kilodaltons are indicated.

the IgH locus (Fig. 2, C and D). We conclude that Smc1 and Smc3 are dynamically recruited, independently of CTCF, to the IgH locus (at the Sμ-Cμ region) during CSR. As Eμ is not bound by cohesin in resting B cells, the constitutive long-range interactions between Eμ and the 3′RR that take place in resting B cells (Wuerffel et al., 2007) are most likely cohesin-independent. Nevertheless, given the dynamic recruitment of Smc1 and Smc3 at Sμ-Cμ (and possibly Sγ1) in activated B cells, we speculate that cohesin may play a role in supporting the structural changes occurring at the IgH locus upon B cell activation.

Cohesin is required for efficient CSR

To determine the functional relevance of the cohesin complex in CSR, we undertook knockdown experiments in CH12 cells, a B cell line which can be induced to undergo CSR from IgM to IgA in vitro and which allows the study of the role of specific factors in CSR (Pavri et al., 2010; Willmann et al., 2012). CH12 cells were transduced with lentiviruses expressing a GFP reporter together with shRNAs specific for AID (as a positive control), the core subunits of the cohesin complex (Smc1 and Smc3), the cohesin loader/unloader

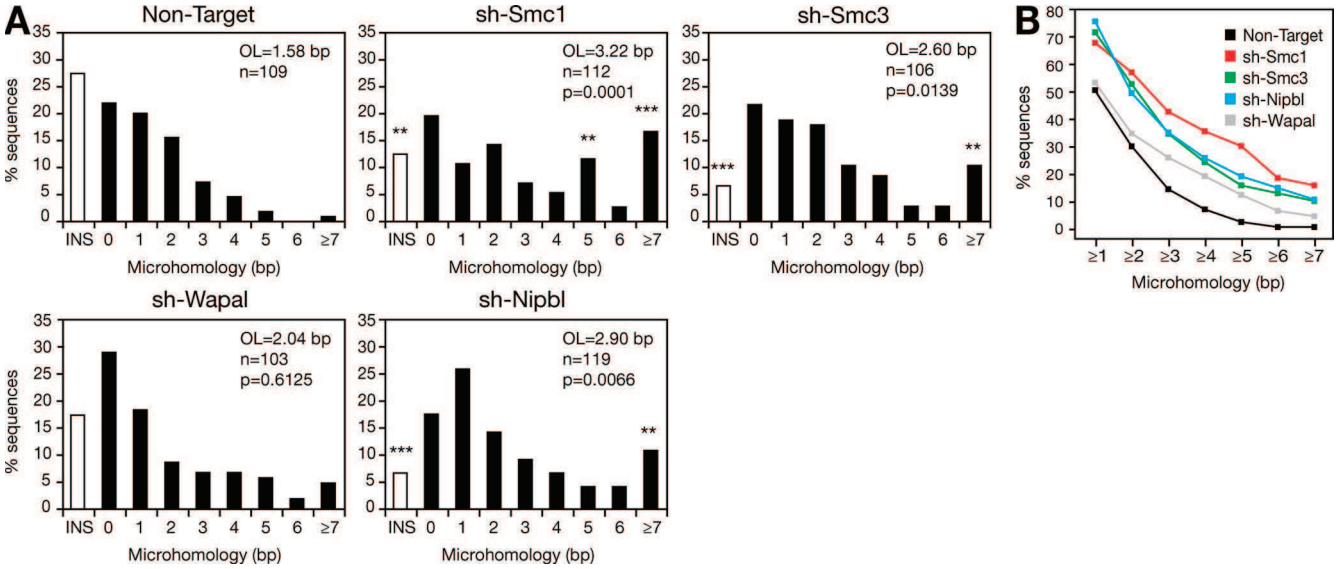

Figure 4. Knockdown of cohesin affects NHEJ. (A) CH12 cells were transduced with a lentivirus expressing a GFP reporter and shRNAs specific for AID, Smc1, Smc3, Nipbl, Wapal, or a Non-Target control. 48 h after stimulation, GFP-expressing cells were sorted. Sμ-Sα switch junctions were amplified by PCR, cloned, and sequenced. Bar graphs show the percentage of switch junction sequences with indicated nucleotide overlap. Number of junctions analyzed (n), mean length of overlap (OL), and p-values relative to the Non-Target control (Mann-Whitney test) are indicated. White bars indicate the percentage of sequences with small (1–4 nucleotides) insertions. Overlap was determined by identifying the longest region of perfect uninterrupted donor/acceptor identity. Sequences with insertions were not included in the calculation of the mean length of overlap. Significant differences relative to the Non-Target control ($\chi 2$ test) are indicated: **, $P \leq 0.01$; ***, $P \leq 0.0001$. Data are from three independent experiments. (B) Cumulative percentage of sequences with a given length of microhomology (bp) and obtained from CH12 cells transduced with lentiviruses expressing shRNAs specific for Smc1 (red squares), Smc3 (green squares), Nipbl (blue squares), Wapal (gray squares), or a Non-Target negative control (black squares) and sorted for GFP expression. Data are from three independent experiments.

subunits (Nipbl and Wapal), and a Non-Target shRNA as a negative control. Knockdown efficiencies were determined by Western blotting or by quantitative RT-PCR (qRT-PCR) on GFP^+ sorted cells (Fig. 3 A). Transduced cells were stimulated for 48 h, and their ability to undergo CSR to IgA was determined by flow cytometry (Fig. 3, B and C). As expected, knockdown of AID resulted in a robust reduction in the efficiency of CSR relative to the Non-Target shRNA control (Fig. 3, B and C). Interestingly, we found that knockdown of Smc1, Smc3, Nipbl, and Wapal resulted in a significant reduction in the efficiency of CSR (18–41%) in GFP^+ cells (Fig. 3, B and C). This reduction was more pronounced (30–52%) when the analysis was performed by gating on cells expressing high levels of GFP (Fig. 3 C). The effect on CSR after cohesin knockdown was not due to decreased survival (Topro-3 staining; unpublished data), strong defects in proliferation (CFSE dilution; unpublished data), significant activation of the DNA damage response and cell cycle checkpoints (Western blot for γ-H2AX and p-Chk1; unpublished data), or defective cell cycle progression (flow cytometry; unpublished data).

To determine whether switch region transcription is affected by the knockdown of cohesin subunits, we measured the level of donor (Sμ) and acceptor (Sα) switch region transcripts by qRT-PCR in activated CH12 cells. We found that the level of Sμ and Sα transcripts was increased after knockdown of AID and cohesin (relative to the Non-Target control), with the exception of Sα transcripts after knockdown of Wapal (Fig. 3, D and E), as expected from cells in which CSR is compromised and that continue to transcribe the switch regions. As no significant reduction in the level of these transcripts after Smc1, Smc3, and Nipbl knockdown was observed, we conclude that switch regions continue to be efficiently transcribed and that they are accessible for DNA deamination by AID. Therefore, cohesin appears not to be involved in the transcriptional regulation of switch regions during CSR. Importantly, we excluded a potential reduction in AID expression levels by Western blot (Fig. 3 F). We conclude that the cohesin complex is required for efficient CSR in CH12 cells. The role of cohesin in CSR appears to be independent of regulating switch region transcription and/or AID accessibility. Concerning a potential more global effect on transcription, we cannot exclude the possibility that the expression of additional genes required for CSR (other than AID) is affected by the knockdown of cohesin.

Knockdown of cohesin affects NHEJ

DSBs triggered by AID in switch regions during CSR are resolved through the NHEJ pathway, and the resulting switch junctions display small insertions and short stretches of microhomology (Stavnezer et al., 2010). In the absence of core NHEJ components, an increase in the usage of microhomology is observed concomitantly with a complete loss of direct joining (Yan et al., 2007). To determine whether cohesin knockdown affects the resolution of DSBs generated during

Results part 1

CSR, we cloned and sequenced Sμ/Sα switch junctions from stimulated CH12 transduced with lentiviruses expressing shRNAs for Smc1, Smc3, Nipbl, Wapal, and a Non-Target negative control (Fig. 4) and sorted for GFP expression. Sequence analysis (Stavnezer et al., 2010) revealed that knockdown of cohesin subunits resulted in a significant increase in the usage of microhomology when compared with the Non-Target control (Fig. 4). Although the mean length of overlap (excluding insertions) was of 1.58 bp for the Non-Target control, it was increased to 3.22 bp for Smc1 ($P = 0.0001$), 2.60 bp for Smc3 ($P = 0.0139$), and 2.90 bp for Nipbl ($P = 0.0066$). The switch junctions obtained after Wapal knockdown displayed an overlap of 2.04 bp that was not statistically different from the Non-Target control ($P = 0.6125$). The increase in microhomology was due to sequences bearing >7 bp of microhomology at the junction and a reduction in those bearing short insertions (Fig. 4), similar to what has been described in human patients with deficiency in DNA ligase IV (Du et al., 2008), Artemis (Du et al., 2008), or ATM (Pan-Hammarström et al., 2006). In contrast to deficiency in core NHEJ components (Stavnezer et al., 2010), we did not find a reduction in the frequency of direct joining events (Fig. 4). We conclude that switch recombination junctions generated after Smc1, Smc3, and Nipbl knockdown (but not Wapal) are biased toward the usage of longer microhomologies. Given the role of Wapal in releasing cohesin from chromatin (Kueng et al., 2006), this suggests that cohesin is recruited but not released from the IgH locus and that NHEJ proceeds unaffected. Therefore, it appears that the loading of cohesin is sufficient to determine the outcome of DSB repair and that cohesin participates in the resolution of AID-induced DNA breaks.

Increased usage of microhomology at the junctions is reminiscent of what is observed in B cells defective for core components of the NHEJ pathway (Yan et al., 2007). Nevertheless, deficiency in XRCC4 or DNA ligase IV also results in a complete loss of sequences repaired through a direct joining (Yan et al., 2007). Therefore, it is unlikely that the cohesin complex is, by itself, part of the NHEJ machinery. As cohesin has been implicated in the recruitment of 53BP1 to γ-irradiation–induced foci (Watrin and Peters, 2009) and 53BP1 deficiency leads to defective CSR, increased DNA end resection, and preferential usage of microhomology (Bothmer et al., 2010), we speculate that cohesin could participate in the recruitment of 53BP1 to AID-induced DSBs and that defective 53BP1 recruitment could account for the increased usage of microhomology observed. Overall, our results implicate the cohesin complex in the mechanism of CSR and provide evidence for its involvement in regulating the repair of programmed DSBs.

MATERIALS AND METHODS

Nuclear extracts and coimmunoprecipitation. Nuclear extracts and chromatin fractions were prepared using standard techniques (in the presence of 100 U/ml Benzonase; Novagen) from CH12F3 cells stably expressing $AID^{FLAG-HA}$, $EGFP^{FLAG-HA}$, or the tags alone (Jeevan-Raj et al., 2011). Coimmunoprecipitations (in the presence of 100 U/ml Benzonase; Novagen) and Western blot analysis were performed as previously described (Jeevan-Raj et al., 2011). See Table S3 for antibodies used.

Mass spectrometry analysis. 20 mg nuclear extract were immunoprecipitated with Flag M2-agarose beads, washed, and eluted with Flag peptide as previously described (Jeevan-Raj et al., 2011). Flag eluates were fractionated by one-dimensional electrophoresis and processed as previously described (Jeevan-Raj et al., 2011) for identification by nano-LC-MS/MS or directly submitted to Multidimensional Protein Identification Technology (MudPIT). MudPIT analyses were performed as previously described (Washburn et al., 2001; Florens et al., 2006). In brief, protein mixtures were TCA-precipitated, urea-denatured, reduced, alkylated, and digested with endoproteinase Lys-C (Roche), followed by modified trypsin digestion (Promega). Peptide mixtures were loaded onto a triphasic 100 μm inner diameter fused silica microcapillary column. Loaded columns were placed in-line with a Dionex Ultimate 3000 nano-LC (Thermo Fisher Scientific) and an LTQ Velos linear ion trap mass spectrometer equipped with a nano-LC electrospray ionization source (Thermo Fisher Scientific). A fully automated 12-step MudPIT run was performed as previously described (Florens et al., 2006), during which each full MS scan (from 300 to 1,700 m/z range) was followed by 20 MS/MS events using data-dependent acquisition. Proteins were identified by database searching using SEQUEST (Thermo Fisher Scientific) with Proteome Discoverer 1.3 software (Thermo Fisher Scientific) against the mouse Swissprot database (2011–02 release). Peptides were filtered with Xcorr versus charge state 1.5–1, 2.5–2, 3–3, 3.2–4, and peptides of at least 7 amino acids in length.

shRNA-mediated knockdown. The lentiviral vectors (pLKO.1 and pLKO.1-puro-CMV-TurboGFP) expressing shRNAs specific for AID (TRCN0000112031), Smc1 (TRCN0000109034), Smc3 (TRCN0000109007), Nipbl (TRCN0000124037), and Wapal (TRCN0000177268) or a Non-Target control (SHC002) were obtained from Sigma-Aldrich. The lentiviral vectors were transiently transfected into Lenti-X 293T cells (Takara Bio Inc.) to produce infectious viral particles as previously described (Willmann et al., 2012). 2 d later, CH12 cells were spin-infected with viral supernatants supplemented with 10 μg/ml polybrene (Sigma-Aldrich). Cells were selected for 5 d with 1 μg/ml puromycin before CSR induction.

Real-time quantitative (q) RT-PCR. RNA and cDNA were prepared using standard techniques. qPCR was performed in triplicates using the Universal Probe Library (UPL) system (Roche) or SyberGreen (QIAGEN) and a LightCycler 480 (Roche). Transcript quantities were calculated relative to standard curves and normalized to β-actin, CD79b, or HPRT mRNA. See Table S4 for primers and probes.

Cell culture and flow cytometry. Lentivirally transduced CH12 cells were cultured with 5 ng/ml IL-4 (Sigma-Aldrich), 1 ng/ml TGF-β (R&D System), 200 ng/ml monoclonal anti-CD40 antibody (eBioscience), and 1 μg/ml puromycin and analyzed after 48–72 h for cell surface expression of IgA by flow cytometry as previously described (Robert et al., 2009). Resting splenic B cells were isolated from 8–12-wk-old C57BL/6 mice using CD43 microbeads (Miltenyi Biotec) and cultured for 60 h with 50 μg/ml LPS (Sigma-Aldrich) and 5 ng/ml IL-4 (Sigma-Aldrich) as previously described (Jeevan-Raj et al., 2011). All animal work was performed under protocols approved by the Direction des Services Vétérinaires du Bas-Rhin, France (Authorization No. 67–343).

Switch junction analysis. Sμ-Sα switch junctions were amplified using previously described primers (Ehrenstein and Neuberger, 1999; Schrader et al., 2002) and conditions (Robert et al., 2009) from genomic DNA prepared from lentivirally transduced CH12 cells stimulated for 72 h and sorted for GFP expression. PCR products were cloned using TOPO-TA cloning kit (Invitrogen) and sequenced using T7 universal primer. Sequence analysis was performed as previously described (Robert et al., 2009).

ChIP-Seq. Resting or activated B cells were cross-linked for 10 min at 37°C with 1% (vol/vol) formaldehyde, followed by quenching with glycine (0.125 M final concentration). Cross-linked samples were then sonicated to obtain DNA fragments 200–500 bp in length using a sonicator (Covaris).

Results part 1

Chromatin (from 10 × 10^7 cells) was precleared with protein A magnetic beads prewashed with PBS 0.05% tween, 5% BSA, and immunoprecipitated in ChIP dilution buffer (0.06% SDS, 20 mM Tris, pH 8.1, 2 mM EDTA, 160 mM NaCl, 1.045% Triton X-100, and 0.05× proteinase inhibitor cocktail) overnight at 4°C with protein A magnetic beads (Invitrogen) coupled to 100 µg Smc1 or Smc3 antibodies and processed according to the Millipore protocol. Cross-links were reversed for 4 h at 65°C in Tris-EDTA buffer with 0.3% (wt/vol) SDS and 1 mg/ml proteinase K. ChIP DNA was extracted with an IPure kit (Diagenode). Libraries were prepared for sequencing according to the manufacturer's protocol (Illumina) and sequenced on the Illumina Genome Analyzer IIx as single-end 50 base reads according to manufacturer instructions. Image analysis and base calling were performed using the Illumina Pipeline and sequence reads were mapped to reference genome mm9/NCBI37 using Bowtie v0.12.7. Peak calling was performed using MACS (Zhang et al., 2008) with default parameters. Global comparison of samples and clustering analysis were performed using seqMINER (Ye et al., 2011).

ChIP-qPCR. Analytical-scale ChIP was performed on chromatin prepared from 10^7 (resting or activated) splenic B cells isolated from a pool of five mice. qPCR was performed at several locations across the IgH locus using primers listed in Table S4. Results are expressed as percent input and represent the mean of three qPCR technical replicates. Error bars represent the corresponding standard deviation.

Accession codes. ChIP-Seq data for CTCF, Smc1, and Smc3 on resting and activated B cells was submitted to GEO (GSE43594).

Online supplemental material. Table S1 lists known AID partner proteins found. Table S2 lists novel AID partner proteins found. Table S3 lists antibodies. Table S4 lists primers. Table S5 shows ChIP-qPCR statistical analysis. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20130166/DC1.

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SUPPLEMENTAL MATERIAL

Thomas-Claudepierre et al., http://www.jem.org/cgi/content/full/jem.20130166/DC1

Table S1. Proteins previously described to associate with AID identified by Flag co-immunoprecipitation coupled to nano-LC-MS/MS and MudPIT analysis

GN	Description	MM	Flag-HA		$AID^{Flag-HA}$		Reference
			Pep	SCs	Pep	SCs	
Aicda	Activation-induced cytidine deaminase	24	0	0	17	981	BAIT
Eef1a1	Elongation factor 1-α	50.1	6	19	18	238	Häsler et al., 2011
Dnaja1	DnaJ homolog subfamily A member 1	44.8	1	1	12	120	Orthwein et al., 2012
Dnaja2	DnaJ homolog subfamily A member 2	45.7	0	0	12	60	Orthwein et al., 2012
Msh2	DNA mismatch repair protein Msh2	104.1	0	0	16	44	Ranjit et al., 2011
Xpo1	Exportin-1	123	0	0	11	20	Jeevan-Raj et al., 2011
Yy1	Transcriptional repressor protein YY1	44.7	2	3	8	15	Zaprazna and Atchison, 2012
Hsp90ab1	Heat shock protein HSP 90-β	83.3	1	5	5	15	Orthwein et al., 2010
Ywhaz	14-3-3 protein ζ/δ	27.8	0	0	4	14	Xu et al., 2010
Ywhae	14-3-3 protein ε	29.2	3	4	4	14	Xu et al., 2010
Ywhaq	14-3-3 protein θ	27.8	0	0	3	13	Xu et al., 2010
Ywhab	14-3-3 protein β/α	28.1	0	0	3	12	Xu et al., 2010
Trim28	Transcription intermediary factor 1-β	88.8	0	0	9	11	Jeevan-Raj et al., 2011
Polr2A	DNA-directed RNA polymerase II subunit RPB1	217	1	3	2	10	Nambu et al., 2003
Prkdc	DNA-dependent protein kinase catalytic subunit	471.1	4	5	8	9	Wu et al., 2005
Psme3	Proteasome activator complex subunit 3 (REG-γ)	29.5	0	0	2	9	Uchimura et al., 2011
Polr2b	DNA-directed RNA polymerase II subunit RPB2	133.8	1	1	1	6	Nambu et al., 2003
Cdc73	Parafibromin	60.5	0	0	4	5	Willmann et al., 2012
Ctr9	RNA polymerase-associated protein CTR9 homolog	133.3	3	3	2	3	Willmann et al., 2012
Supt16h	FACT complex subunit SPT16	119.7	0	0	2	2	Willmann et al., 2012
Ssrp1	FACT complex subunit SSRP1	80.8	1	1	2	2	Willmann et al., 2012
Ctnnbl1	β-catenin–like protein 1	64.9	1	1	2	2	Conticello et al., 2008
Rpa1	Replication protein A 70 kD DNA-binding subunit	69	0	0	1	2	Chaudhuri et al., 2004
Supt5h	Transcription elongation factor SPT5	120.6	0	0	1	1	Pavri et al., 2010

Gene Name (GN), protein description, molecular mass (MM) in kilodaltons, and corresponding number of peptides (Pep) and spectral counts (SCs) found are shown on Flag immunoprecipitations conducted on extracts prepared from CH12 cells expressing $AID^{Flag-HA}$ or the epitope tags alone (Flag-HA) as a negative control.

Table S2. Novel AID partner proteins identified by Flag coimmunoprecipitation coupled to nano-LC-MS/MS and MudPIT analysis

GN	Description	MM	Flag-HA		$AID^{Flag-HA}$	
			Pep	SCs	Pep	SCs
Aicda	Activation-induced cytidine deaminase	24	0	0	17	981
Cohesin complex						
Wapal	Wings apart-like protein homolog	134	3	5	37	254
Nipbl	Nipped-B-like protein	315.3	4	8	11	92
Pds5a	Sister chromatid cohesion protein PDS5 homolog A	150.2	0	0	8	12
Smc1a	Structural maintenance of chromosomes protein 1A	143.1	1	1	9	11
Pds5b	Sister chromatid cohesion protein PDS5 homolog B	164.3	2	2	6	8
Smc3	Structural maintenance of chromosomes protein 3	141.5	1	1	5	6
Stag2	Cohesin subunit SA-2	141.3	2	2	3	6
Stag3	Cohesin subunit SA-3	141.1	1	1	2	4
Condensin complex						
Smc2	Structural maintenance of chromosomes protein 2	134.2	2	2	13	19
Smc4	Structural maintenance of chromosomes protein 4	146.8	2	2	8	14
Ncapd2	Condensin complex subunit 1	155.6	2	2	5	9
Ncapd3	Condensin-2 complex subunit D3	169.3	2	3	5	6
Ncapg2	Condensin-2 complex subunit G2	130.8	0	0	3	4
Ncaph	Condensin complex subunit 2	82.3	1	1	2	2
Smc5/6 complex						
Smc5	Structural maintenance of chromosomes protein 5	128.7	3	3	2	5
Smc6	Structural maintenance of chromosomes protein 6	127.1	1	1	3	3
Ino80 complex						
Ruvbl1	RuvB-like 1	50.2	5	5	14	172
Ruvbl2	RuvB-like 2	51.1	4	9	16	64
Yy1	Transcriptional repressor protein YY1	44.7	2	3	8	15
Ino80	Putative DNA helicase INO80 complex homolog 1	176.4	1	1	4	5
Ino80b	INO80 complex subunit B	40.5	1	1	1	1
Ino80c	INO80 complex subunit C	20.4	0	0	1	1

Gene Name (GN), protein description, molecular mass (MM) in kilodaltons, and corresponding number of peptides (Pep) and spectral counts (SCs) found are shown on Flag immunoprecipitations conducted on extracts prepared from CH12 cells expressing $AID^{Flag-HA}$ or the epitope tags alone (Flag-HA) as a negative control.

Table S3. Antibodies used

Antibody	Clone	Source	Use
AID	Strasbg 9 (AID-2E11)	IGBMC (Jeevan-Raj et al., 2011)	WB, IP
AID	Polyclonal	IGBMC	ChIP
Flag	M2	Sigma-Aldrich	WB, IP
Smc1	A300-055A	Bethyl Laboratories, Inc.	WB, IP, ChIP
Smc3	ab9263	Abcam	WB, IP, ChIP
Nipbl	A301-779A	Bethyl Laboratories, Inc.	IP
Wapal	A300-268A	Bethyl Laboratories, Inc.	WB, IP
CTCF	07-729	Millipore	ChIP
β-Actin	A1978	Sigma-Aldrich	WB
γ-H2AX	JBW301	Millipore	WB
p-Chk1	133D3	Cell Signaling Technology	WB

WB, Western blot; IP, immunoprecipitation; ChIP, chromatin immunoprecipitation.

Results part 1

Table S4. Primers and probes used

Primer	Sequence (5′–3′)	Probe or reference
Germline transcripts		
Iμ-Cμ forward	ACCTGGGAATGTATGGTTGTGGCTT	Jeevan-Raj et al., 2011
Iμ-Cμ reverse	TCTGAACCTTCAAGGATGCTCTTG	Jeevan-Raj et al., 2011
Iα-Cα Fwd*	GGAGACTCCCAGGCTAGACA	UPL probe #27
Iα-Cα Rev*	CGGAAGGGAAGTAATCGTGA	UPL probe #27
Switch junctions		
Sμ-Fwd	AACAAGCTTGGCTTAACCGAGATGAGCC	Schrader et al., 2002
Cα-Rev	CCGGAATTCCTCAGTGCAACTCTATCTAGGTCT	Ehrenstein and Neuberger, 1999
Knockdown		
Nipbl-Fwd*	CCCTTAAGATCTCCTCAACCAG	UPL probe #2
Nipbl-Rev*	TGTAGAATTAAAGGTGGTCTTGAGC	UPL probe #2
CD79b-Fwd*	TGGTGCTGTCTTCCATGC	UPL probe #18
CD79b-Rev*	TTGCTGGTACCGGCTCAC	UPL probe #18
HPRT-Fwd*	GTCAACGGGGGACATAAAAG	UPL probe #22
HPRT-Rev*	CAACAATCAAGACATTCTTTCCA	UPL probe #22
ChIP		
Eμ-Fwd	GGGAGTGAGGCTCTCTCATA	Wang et al., 2009
Eμ-Rev	ACCACAGCTACAAGTTTACCTA	Wang et al., 2009
5′Sμ-Fwd1	TAAAATGCGCTAAACTGAGGTGATTACT	Kuang et al., 2009
5′Sμ-Rev1	CATCTCAGCTCAGAACAGTCCAGTG	Kuang et al., 2009
5′Sμ-Fwd2	TAGTAAGCGAGGCTCTAAAAAGCAT	Pavri et al., 2010
5′Sμ-Rev2	AGAACAGTCCAGTGTAGGCAGTAGA	Pavri et al., 2010
3′Sμ-Fwd	CTGAATGAGTTTCACCAGGCC	Wang et al., 2006
3′Sμ-Rev	GCCTGTCCTGCTTGGCTTC	Wang et al., 2006
Cμ-Fwd*	GTTCTGTGCCTCCGTCTAGC	
Cμ-Rev*	AGCATTTGCATAAGGGTTGG	
Sγ3-Fwd	GCTGAGAGTATGCACAGCCA	Wang et al., 2006
Sγ3-Rev	GGATCATGGAAACTCCTCCG	Wang et al., 2006
Sγ1-Fwd	GGAGGTCCAGTTGAGTGTCTTTAG	Muramatsu et al., 2000
Sγ1-Rev	TTGTTATCCCCCATCCTGTCACCT	Muramatsu et al., 2000
Cα-Fwd*	CTCCTGTCTCACAGGCCTTC	
Cα-Rev*	CATGGGCCTTTACTCCACTC	
Hs6,7-Fwd*	CCCTGGTGACCATGTGTGT	
Hs6,7-Rev*	TCTGGGTCTGTTTTGTTACTGAAA	

Primers designed in this study are marked by *.

Table S5 is provided as an Excel file.

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II. Mediator complex subunit 1 deletion reduces transcription of the acceptor switch region and affects its interaction with Eμ

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Running Title: Med1 is required for CSR

Abstract

Somatic hypermutation (SHM) and immunoglobulin class switch recombination (CSR) are initiated by the transcription-coupled recruitment of activation induced cytidine deaminase (AID) to immunoglobulin loci. During CSR, the IgH locus undergoes dynamic three-dimensional structural changes in which promoters, enhancers and switch regions are brought to close proximity. Nevertheless, little is known about the underlying mechanisms. Here we show that during CSR, conditional inactivation of Med1 in mice results in a B cell intrinsic defect in CSR which is due to decreased acceptor switch region transcription correlating with reduced long range interaction between the acceptor switch region and Eμ at the IgH locus. Furthermore, although mutation frequency during SHM is not affected by Med1 deletion, we show that the pattern of SHM is affected, suggesting an additional role for Med1 in antibody diversification. Our results indicate that Med1 is required for antibody diversification processes.

Introduction

The B cell receptor diversity is generated by V(D)J recombination, which assembles the variable region of immunoglobulin gene by random combination of variable (V), diversity (D) and joining (J) genes segments during B cell development. The IgH locus is composed of 195 VH genes spanning over 2.44 Mb, 16 DH and 4JH genes (Johnston et al., 2006). To allow distal VH genes to recombine with D and J genes, the IgH locus contracts by DNA looping (Degner et al., 2011).

During immune responses, B cell further diversify their receptors through somatic hypermutation (SHM) (Di Noia and Neuberger, 2007) and class switch recombination (CSR) (Chaudhuri et al., 2007). SHM introduces point mutations, short insertions and deletions to the variable region of the immunoglobulin, modifying its affinity for the antigen. CSR replaces the isotype expressed (from IgM to IgG, IgE or IgA), providing new functions for efficient antigen clearance. Both of these mechanisms are dependent on the activity of activation-induced cytidine deaminase (AID), an enzyme that deaminates cytosine into uracil in the single strand DNA exposed by transcription (Petersen-Mahrt et al., 2002). During SHM, deamination is processed to generate mutations in variable regions. A general model to explain the pattern of SHM involves 2 mutational phases (Peled et al., 2008). Mutations at G:C bp result from phase 1 mutations that can be split in two. Phase 1a mutations result from replication over dU/dG mismatches and phase 1b lead to the removal of the uracil residues leaving an abasic site that can be replaced by any nucleotide during replication. Phase 2 mutations target A:T bp and are due to processing of dU/dG mismatches by the BER and the MMR complexes. Mismatches are recognized by the Msh2/Msh6 complex which recruits Exo1 to remove the uracil from DNA and proceeds to strand degradation, the repair will then be done by error-prone polymerases to fill the gap, leading to transition and transversion from A:T. Additionally, dU residues might be removed by the BER complex which induces strand degradation as well, leading to mutations at A/T bp, as a backup mechanism. Alternatively, during CSR, deamination leads to the generation of double strand breaks (DSB) in switch (S) regions that will be repaired by classical and alternative non-homologous end joining (NHEJ) (Stavnezer et al., 2010). As the donor (Sμ) and the acceptor (Sγ1, Sγ3, Sγ2b, Sγ2a, Sα or Sε) switch regions involved in the recombination can be separated by 200kbp, a DNA looping model has been proposed to explain how they are brought into close proximity (Wuerffel et al., 2007). Nevertheless, the precise mechanisms controlling these conformational changes remain to be elucidated.

Mammalian mediator complex is a multiprotein complex composed of 30 subunits organized in four modules according to published interactions (Conaway et al., 2005b). Depending on its composition, mediator complex has been shown to play different role (Malik and Roeder, 2010). It has been shown to work as a transcriptional coactivator (Carlsten et al., 2013; Conaway et al., 2005a) and binds to transcription factors recognizing enhancer, to those bound to core promoters and to the C-terminal tail of RNA Pol II through different subunits (Malik and Roeder, 2005). This complex is essential for activator-dependant transcription but also for the formation of the transcription initiation complex. Mediator complex has also been implicated in promoting the transcription-coupled formation of long-

range DNA loop structures with cohesin complex by studying Med1 and Med12 subunits which are present in different modules (Kagey et al., 2010). In a previous study, we showed that cohesin is implicated in CSR and affects the AID-induced breaks repair.

In order to investigate the role of the mediator complex in CSR, we generated Med1 deficient mice. Med1 was shown to exist in a mediator subpopulation enriched in RNA pol II (Zhang et al., 2005). We show that deletion of Med1 results in defective CSR due to decreased acceptor switch region transcription that correlates with reduced long range interaction within the IgH locus. Knock down of Med1 subunit in CH12 cells confirmed the reduction in CSR efficiency.

Results and discussion

Med1 is dispensable for B cell development and SHM

To investigate the role of mediator complex in CSR and SHM, we bred Med1$^{f/f}$ mice with Mb1$^{Cre/+}$ mice to inactivate Med1 in developing B cells. Despite efficient Cre-mediated deletion (figure S1A), the proportions of developing B cells in the bone marrow and in the spleen fractions were unchanged in the Med1$^{f/f}$ Mb1$^{Cre/+}$ mice relative to the control mice (Med1$^{f/+}$ or Med1$^{f/+}$ Mb1$^{Cre/+}$) (figure S1B, top and middle). However, within the mature B cells population from the spleen, we observed an increased percentage of marginal B cells and a decreased percentage of follicular B cells in the Med1-depleted mice (figure S1B bottom). We also ruled out an effect of Med1 deletion on long range interactions occurring during V(D)J recombination (figure S1C). We conclude that deletion of Med1 in the early B cell development didn't affect B cells ability to further differentiate and undergo V(D)J recombination in the bone marrow, however a biased differentiation to marginal B cells is observed in the spleen (5 times more marginal B cells than in control).

To determine whether Med1 deletion affects SHM, we immunized Med1$^{f/f}$ Mb1$^{Cre/+}$ and control mice with NP-CGG in the footpad and ten days later, we sorted germinal center B cells from the lymph nodes of individual mice (figure 1A). We analyzed mutation profile in JH4 intron and didn't find significant differences in mutation frequency ($p=0.48$) between Med1$^{f/f}$ Mb1$^{Cre/+}$ and control mice (figure 1B). The analysis of the pattern of mutation revealed that deletion of Med1 induces a biased mutation profile with decreased mutation from A bases and increased mutation from C and T bases (the latter to a lesser extent) (figure S2). Mutation frequency is comparable to control mice, suggesting that Med1 deletion doesn't affect the first steps of SHM and that proteins like AID, UNG or MSH2/6 which initiate mutations are unaffected. The biased profile is similar to those observed when downstream compounds of phase 2 mutation are defective, like ATR (Pan-Hammarstrom et al., 2006) or Polη (Martomo et al., 2005), suggesting that Med1 deletion might affect their expression or their recruitment. The biased differentiation into marginal B cells is likely unrelated to the biased SHM profile, as marginal B cells are able to undergo SHM. It has been shown that the mutation rate of marginal and follicular B cells was equivalent on day 7 after the challenge with the antigen and both populations contain cells that acquired multiple mutations (Phan et al., 2005).

Med1 is required for efficient CSR

To determine whether Med1 plays a role in CSR, we cultured *in vitro* splenic resting B cells from Med1$^{f/f}$ Mb1$^{Cre/+}$ mice and control mice under conditions that induce CSR to different isotypes. B cells were also stained with CFSE in order to track their proliferation. After 72h of stimulation, cell surface expression of the different immunoglobulin and CFSE dilution were monitored by flow cytometry (figure 2A). We found that Med1 deficiency resulted in a 30 to 60% reduction in CSR to all isotypes

tested (figure 2B). Analysis of CFSE dilution revealed that B cells from Med1$^{f/f}$ Mb1$^{Cre/+}$ mice proliferated less than B cells from control mice. However, analysis of switching efficiency per cell division showed that in cells proliferating equally, the defect in CSR is still present when stimulated to switch as shown for IgG1 and IgG3 switching (figure 2C), indicating that Med1 deficiency results in a B cell-intrinsic defect in CSR. The defect of CSR was not due to a block in cell cycle progression visualized by PI staining (figure S3A and S3B), neither to biased balance between follicular and marginal B cells as marginal B cells proliferate and switch more efficiently than follicular B cells, independently of the origin of the cells (WT Fo vs WT Mz and KO Fo vs KO Mz) (figure S3C and S3D). We conclude that Med1 deletion impairs CSR independently of its role in B cell proliferation or in marginal B cell differentiation.

As a subpopulation of mediator complex is naturally depleted from Med1 subunit (Zhang et al., 2005) and as the cellular composition is slightly different after Med1 deletion in mice, we undertook knock down experiments in CH12 cells, a B cell line that can be stimulated to switch from IgM to IgA *in vitro*. CH12 cells were infected by lentiviruses expressing shRNA targeting AID (positive control), Med1 and Med12 (lying in different modules from mediator complex) and a non target shRNA (negative control). The lentiviruses also expressed a GFP reporter, allowing us to determine the infection efficiency. The knock down efficiency was tested by western blot (figure 2D) and the ability of transduced cells to undergo CSR to IgA was checked by flow cytometry after 72h of stimulation (figure 2E and 2F). Knock down of AID resulted in a robust reduction in the efficiency of CSR relative to the non-target shRNA control (65%, Figure 2E and 2F). Similarly, knock down of Med1 and Med12 subunits resulted in a significant reduction in the efficiency of CSR (37%) relative to the non-target shRNA (figure 2E and 2F). We conclude that knock down of Med1 subunit recapitulates the defect in CSR observed in Med1-depleted B cells and that knock down of another mediator subunit also leads to a decreased CSR efficiency, implicating the mediator complex in CSR.

Med1 is required for transcription of the acceptor switch region

As CSR is dependent on transcription (Pavri and Nussenzweig, 2011) and as the mediator complex is a transcriptional cofactor (Carlsten et al., 2013), deletion of Med1 could affect switch region transcription or AID expression. To address the role of Med1 in switch region transcription, we measured the level of donor and acceptor switch transcripts by RT-qPCR in B cells stimulated to switch for 72h. We found that the level of Sμ transcripts is unaffected by Med1 deletion, contrary to the level of acceptor switch transcripts, which is reduced compared to control mice for all isotypes tested (figure 3A). Transcription of acceptor switch region depends on the 3'RR and deletion of part of it, like hs3b, hs4 leads to defect in transcription (Pinaud et al., 2001). This defect in GLT correlates with a defect in the 3D organization of the IgH locus (Wuerffel et al., 2007), suggesting a role for mediator in the looping process occurring during CSR. To determine whether Med1 deficiency affects AID expression, we measured AID protein level in activated B cells by western blot (figure 3B) and didn't observe any significant reduction in AID expression level in Med1$^{f/f}$ Mb1$^{Cre/+}$ mice when compared to

control mice. We conclude that Med1 is required for proper transcription of the acceptor switch region. However, as mediator complex is implicated in basal and activator-dependent transcription, we cannot rule out the hypothesis that Med1 deletion might affect the expression of additional genes required for CSR.

Because our hypothesis is that cohesin and mediator complex favor conformational changes of the IgH locus during CSR, and because cohesin knock down leads defective CSR and AID-induced breaks repair through usage of microhomologies (Thomas-Claudepierre et al., 2013), we decided to investigate the implication of Med1 deletion in the resolution of DSB generated during CSR. We cloned Sμ/Sγ3 switch junctions from LPS-stimulated B cells and analyzed their sequences (figure 3C). The average length of microhomologies was 1.75 bp for junctions from $Med1^{f/f}$ $Mb1^{Cre/+}$ mice which was not statistically different from the overlap of 2.02 bp obtained from controle mice. We found that Med1 deletion doesn't affect the resolution of AID-induced DNA breaks.

Reduced interaction between Eμ and γ1 after Med1 deletion

Mediator complex is an essential compound of the RNA pol II transcription machinery and plays a role in favoring long range interactions. DNA looping and transcription being closely related (Fanucchi et al., 2013), we asked whether the acceptor switch region transcription was affected by a defect in DNA looping. To do so, we performed 4C-Seq experiments on cells from $Med1^{f/f}$ $Mb1^{Cre/+}$ and control mice stimulated or not to switch to IgG1 for 48h. A bait located near the Eμ region was used to visualize the different interactions occurring at the IgH locus. In control cells, Eμ interacts with 3'RR in resting and stimulated cells (figure 4A). After stimulation, Eμ is additionally interacting with the region surrounding Sγ1 and this is significantly different compared to resting cells. In cells from $Med1^{f/f}$ $Mb1^{Cre/+}$ mice, a similar profile is observed: In resting and stimulated cells, Eμ interact with the 3'RR (figure 4B). In stimulated cells, Sγ1 additionally interacts with Eμ. However, when comparing the stimulated profiles obtained from Med1 deficient cells with control cells, we observed that Sγ1 interacts slightly less in Med1 deficient cells than in control cells, despite the fact that the interaction is still more important than in resting control cells (figure 4C), resulting in an intermediate phenotype. Focused analysis on interactions changing upon B cell activation confirmed the differential recruitment observed between resting and stimulated cells. Moreover, this centric analysis revealed that interactions with Sγ1 is significantly reduced in Med1 deficient cells compared to control cells upon activation, suggesting that the interaction with the acceptor switch region is reduced after Med1 deletion. Nevertheless, it is difficult to conclude if Med1 affects the 3D structure of the IgH locus upon B cell activation, leading to defective acceptor switch transcription or if the defect in CSR due to reduced acceptor switch region leads to reduced interaction with Sγ1. This intermediate phenotype could also be due to heterogeneity in cells used for the experiment. First of all, after 72h of stimulation, control cells switch only to 20-25% and Med1 deficient cells to 10-15%, reflecting that not all the cells undergo CSR. Secondly, after 48h of stimulation, among the cells that will switch, all of them are not at the same step of CSR, some of them just start the GLT, some are at the DSB steps and some will have succeed their switching.

Finally, Med1 deletion leads to only 45% reduction of CSR efficiency, indicating that some cells manage to undergo CSR, and to perform DNA looping in the absence of Med1. This suggests that Med1 alone is not essential for CSR, it could be due to the fact that mediator complex can exist without Med1 subunit.

Overall, our results implicate Med1 in CSR and in SHM. Med1 deletion leads to a defect in long-range interactions during CSR associated to a defect in the transcription of acceptor switch regions. Additionally, Med1 seems to play a role in the generation of mutation during SHM.

Materiels and Methods

Lentiviral infection

The lentiviral vectors (pLKO.1 and pLKO.1-puro-CMV-TurboGFP) expressing shRNAs specific for AID (TRCN0000112031), Med1 (TRCN0000099576), Med12 (TRCN0000096466) or a non-target control (SHC002) were obtained from Sigma-Aldrich. CH12 cells were infected with viral particles produced in Lenti-X 293T cells as previously described (Thomas-Claudepierre et al., 2013).

Mice

Med1$^{f/f}$ mice (provided by T. Borggrefe) were crossed with Mb1-Cre mice (provided by P. Kastner). Mice were on a B6/129 mixed background and bred and maintained under specific pathogen-free conditions. 8-12 week old mice were used in all experiments. All animal work was performed under protocols approved by the Direction des Services Vétérinaires du Bas-Rhin, France (Authorization N° 67-343).

Cell culture and flow cytometry

CH12 cells transduced with lentival vectors expressing shRNA were cultured with IL4, TGFB and CD40 to switch to IgGA and selected with puromycin. After 72h, the IgA surface expression was analyzed by flow cytometry.

B cell development: Cells from total BM and spleen were analyzed (LSRII; BD) or purified (FACSAria SORP; BD) as pro/pre-B (B220^{lo}, IgM^{-}), immature (B220^{lo}, IgM^{+}), and mature recirculating (B220^{hi}, IgM^{+}) B cells in the bone marrow fraction; and as immature (B220^{+}, CD93^{-}) or mature (B220^{+}, CD93^{+}) B cells. Within the mature B cell population, marginal (CD21^{+}, CD23^{low}) and follicular (CD21^{low}, CD23^{+}) were distinguished.

Resting splenic B cells were isolated using CD43-microbeads (Miltenyi Biotec), stained with 5µM CFSE (Invitrogen) and cultured for 72h with LPS (50 µg/ml; Sigma-Aldrich) to switch to IgG3 and IgG2b, LPS and IL-4 (5 ng/ml; Sigma-Aldrich) to switch to IgG1 and LPS and IFNg to switch to IgG2a. CSR was assayed by flow cytometry as described (Jeevan-Raj et al., 2011).

For cell cycle analysis, B cells unstimulated or stimulated for 72h have been fixed in 70% ethanol o/n and stained with PI 25 µg/ml (Sigma), RNase A 50 µg/ml (Sigma) in PBS for 30 min at 37°C, and cells have been analyzed for DNA content.

Real time quantitative RT-PCR

RNA and cDNA were prepared using standard techniques. qPCR was performed in triplicates using the Universal Probe Library (UPL) system (Roche) or SyberGreen (Qiagen) and a LightCycler 480 (Roche). Transcript quantities were calculated relative to standard curves and normalized to β-Actin, CD79b or HPRT mRNA. See Table S2 for primers and probes.

Somatic hypermutation analysis

Germinal center B cells were sorted from the lymph nodes from mice immunized with NP-CGG (75 µg/mouse, Biosearch Technologies Inc.). 10 days later, the region downstream of JH4 exon was cloned and analyzed for mutation from each animal individually with SHMTool (Maccarthy et al., 2009).

Switch junction analysis

Sµ Sγ3 junctions were amplified from genomic DNA prepared from B cells stimulated for 72h using Q5 High-Fidelity DNA Polymerase (New England BioLabs) and primers listed in table S2. A first PCR is done to generate megaprimers, amplification conditions were 98°C (30s); 35 cycles at 98°C (10 s), 72°C (1 min); and 72°C (2 min). PCR products were cloned by megawhoop in pUC57, in which BsaI sites were destroyed, amplification conditions were 98°C (30s); 35 cycles at 98°C (10 s), 65°C (10 s), 72°C (40 s); and 72°C (2 min). Megawhoop products are then used to transform competent bacteria and junctions are sequenced using M13F primer. Sequence analysis was performed as previously described (Robert et al., 2009).

V(D)J recombination analysis

PCR analyses of immunoglobulin genes were performed on serial dilution of genomic DNA isolated from B cells stimulated for 72h with published primers (listed table S2; Fuxa et al., 2004). Amplification conditions were 98°C (30s); 35 cycles at 98°C (10 s), 72°C (1 min 40 s); and 72°C (2 min).

4C-Seq experiments

4C-seq was preformed as previously described (Rocha et al., 2012). The primary restriction enzyme used was DpnII and secondary restriction enzyme Csp6I. Primers for the Eµ bait were : 5' TCTGTCCTAAAGGCTCTGAGA 3' and 5' GAACACAGAAGTATGTGTATGGA 3' . The 12 samples (3 replicates, 2 days, 2 genotypes) were sequenced in one lane of a HiSeq2500 rapid run using 50

cycles. Mapping was done using bowtie allowing for zero mismatches against a reduced genome containing all unique 24bps surrounding every DpnII site in the genome. Between 2 to 4 million mapped reads were obtained for all samples and at least half of the reads were mapped to chromosome 12. DESeq 1.6.0 was used to normalize raw read count in 10kb windows centered on each DpnII site in the constant region of IgH (Chr12: 113175000-113475000, mm10) (Anders and Huber, 2010). The following parameters were used with the estimateDispersion function: method=pooled, sharingMode=maximum. The nbinonTest function was used to determine which windows have a significantly different 4C-seq signal. An adjusted p-value of 0.1 following Benjamini-Hochberg FDR correction was used as a cut-off.

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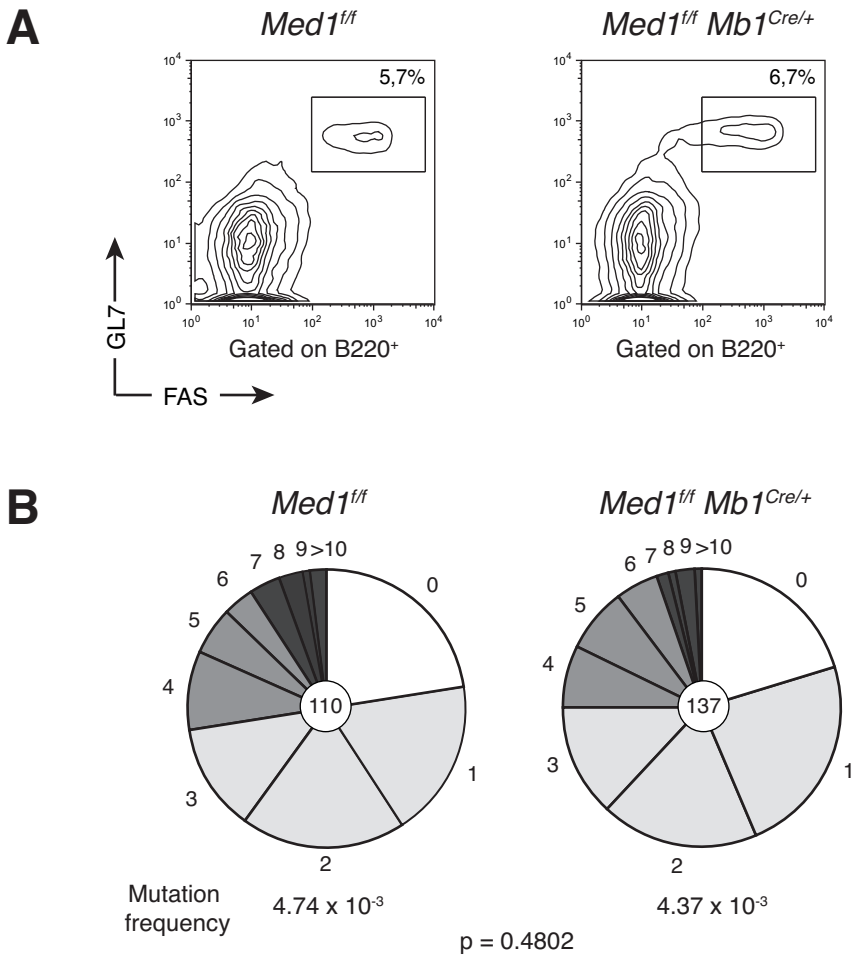


Figure 1: Med1 deletion does not affect SHM frequency
(A) Flow cytometry analysis of Med1$^{f/f}$ and Med1$^{f/f}$ Mb1$^{Cre/+}$ germinal center B cells in lymph nodes of NP-CGG immunized animals at day 10. Plots are gated on B220^{+} cells. The percentage of germinal centre B cells (B220^{+} Fas^{+} GL7^{+}) is indicated above each gate. SHM was analyzed with the SHMtool web application. (B) Mutation analysis in the J_H4 intron sequences comparing control (left panel) and Med1$^{f/f}$ Mb1$^{Cre/+}$ (right panel) sequences. Segment sizes in the pie charts are proportional to the frequency of sequences carrying the number of the mutations indicated in the periphery of the charts. Mutation frequency per base pair is shown below pie charts, number of sequences analyzed is indicated in the center of the pie charts. White portion of the pie charts indicate the proportion of sequences without mutation; light grey, grey and dark grey colors indicate sequences bearing 1 to 3 mutations, 4 to 6 mutations and 7 to >10 mutations, respectively. p-value was determined using two-tailed Student's t-test. Sequences were obtained from two independent experiments with four Med1$^{f/f}$ Mb1$^{Cre/+}$ mice and three control mice each.

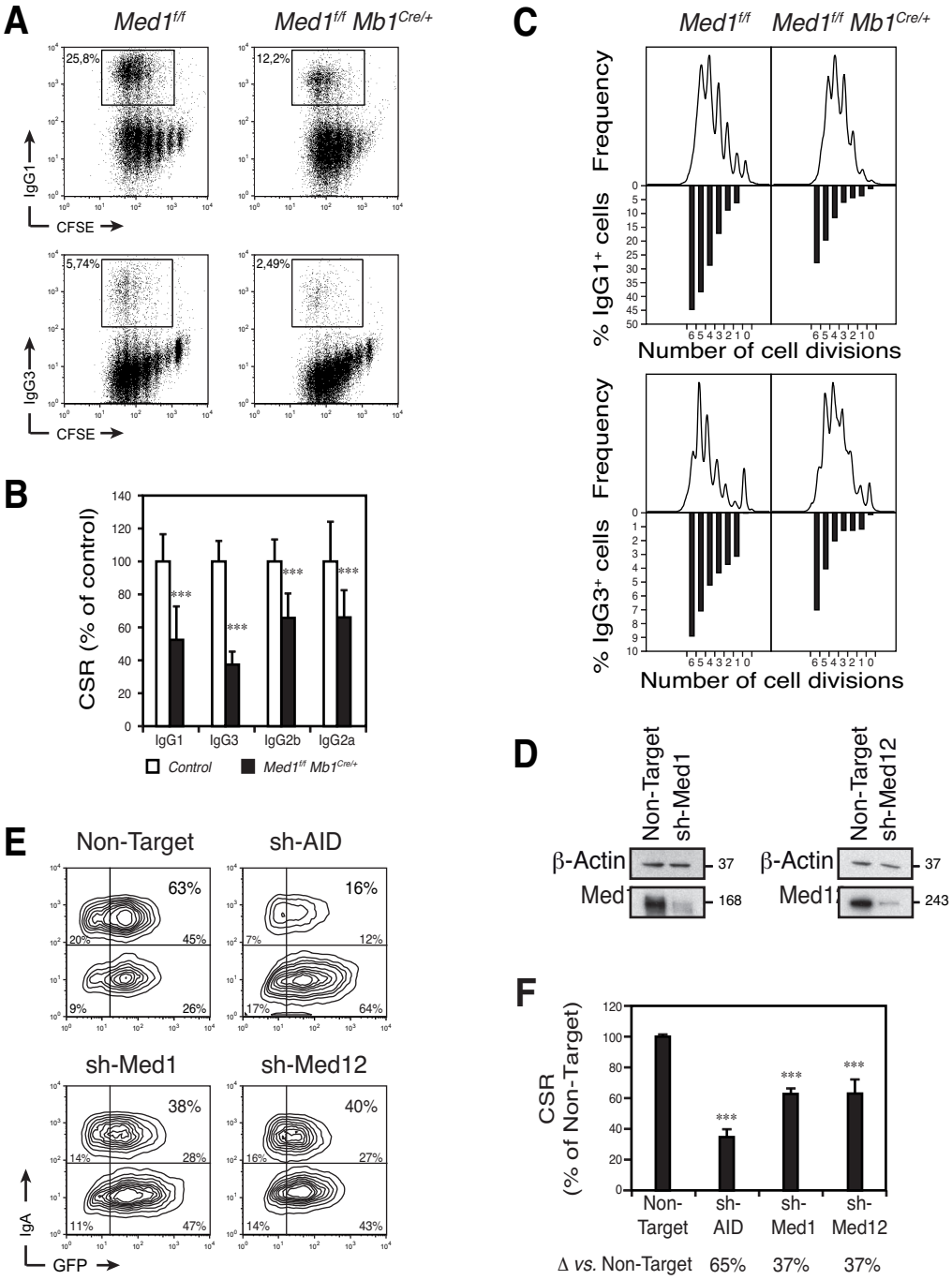


Figure 2: CSR is impaired by Mediator deletion

(A to C) Cells were stimulated to switch to IgG1 with LPS + IL4, IgG3 and IgG2b with LPS and IgG2a with LPS + IFNγ. (A) Surface expression of IgG1, IgG3 and CFSE dilution as determined by flow cytometry in Med1$^{f/f}$ and Med1$^{f/f}$ Mb1$^{Cre/+}$ B cells stimulated for 72h. Percentage of switch cells is indicated. (B) Percentage (+ s.d.) of CSR relative to control cells from three to twenty independent experiments. CSR in control cells was set to 100%. (C) Analysis of CSR per cell division in control and Med1$^{f/f}$ Mb1$^{Cre/+}$ B cells stimulated for 72h. Cell division measured by CFSE dilution is shown as histogram in the upper panel and the percentage of cells expressing switched isotype is indicated in the lower panel. Data are representative of four independent experiments. (D) Knock down efficiencies were determined by western blot on transduced cells stimulated for 48h and sorted for GFP expression. Western blot for β-Actin, Med1 and Med12 are shown. Data are representative from three experiments. (E) IgA surface expression as determined by flow cytometry in stimulated CH12 cells transduced with a lentivirus expressing a GFP reporter and shRNAs specific for AID, Med1, Med12 and a non-target shRNA negative control. Representative plots (gated on live cells) are shown. Percentage of IgA positive cells is indicated. (F) Percentage (+ s.d.) of CSR relative to the non-target shRNA control from four independent experiments by gating on cells expressing GFP. CSR in cells expressing the non-target shRNA control was set to 100%. The difference in CSR efficiency between non-target and shRNA knock down (Δ) is indicated below. Statistical significance vs. the non-target shRNA control (two-tailed Student's t-test) is indicated: ***: p≤0.001.

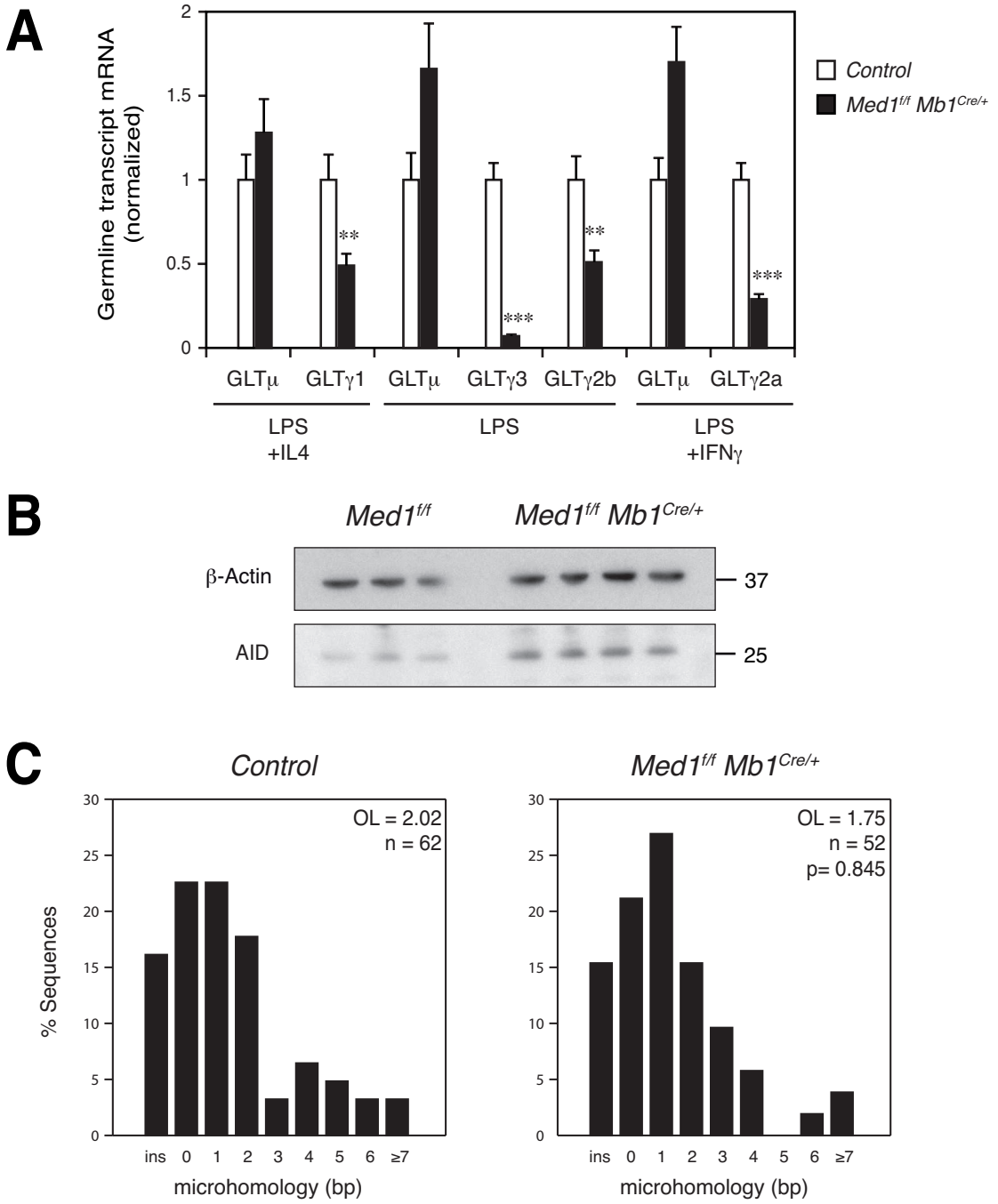


Figure 3: Med1 deletion affects acceptor switch region transcription
(A) RT-qPCR analysis for germline transcripts (GLT) at donor and acceptor switch regions in control and Med1$^{f/f}$ Mb1$^{Cre/+}$ B cells cultured for 72 h with LPS alone or with LPS + IL4 or LPS + IFNγ. Expression is normalized to Igβ and is presented relative to expression in control B cells, set as 1. Mean and sd of triplicate samples are shown. Statistical analysis was performed using Student's t test: *: p≤0.05; **: p≤0.01; ***: p≤0.001. Data are representative of three experiments with two mice per genotype. (B) Western blot for β-Actin and AID on whole-cell extracts from splenic B cells from Med1$^{f/f}$ and Med1$^{f/f}$ Mb1$^{Cre/+}$ mice cultured for 72h with LPS and IL4. Data are representative of three independent experiments. (C) Histograms showing the percentage of switch junction sequences with indicated nucleotide overlap obtained from control and Med1$^{f/f}$ Mb1$^{Cre/+}$ B cells. Number of sequences analyzed (n), average length of overlap in base pairs (OL) and p values relative to the control (Mann-Whitney test) are indicated. Overlap was determined by identifying the longest region of perfect uninterrupted donor/acceptor identity. Sequences with insertions were scored as having no microhomology. Differences relative to the control have been tested by a χ2 test.

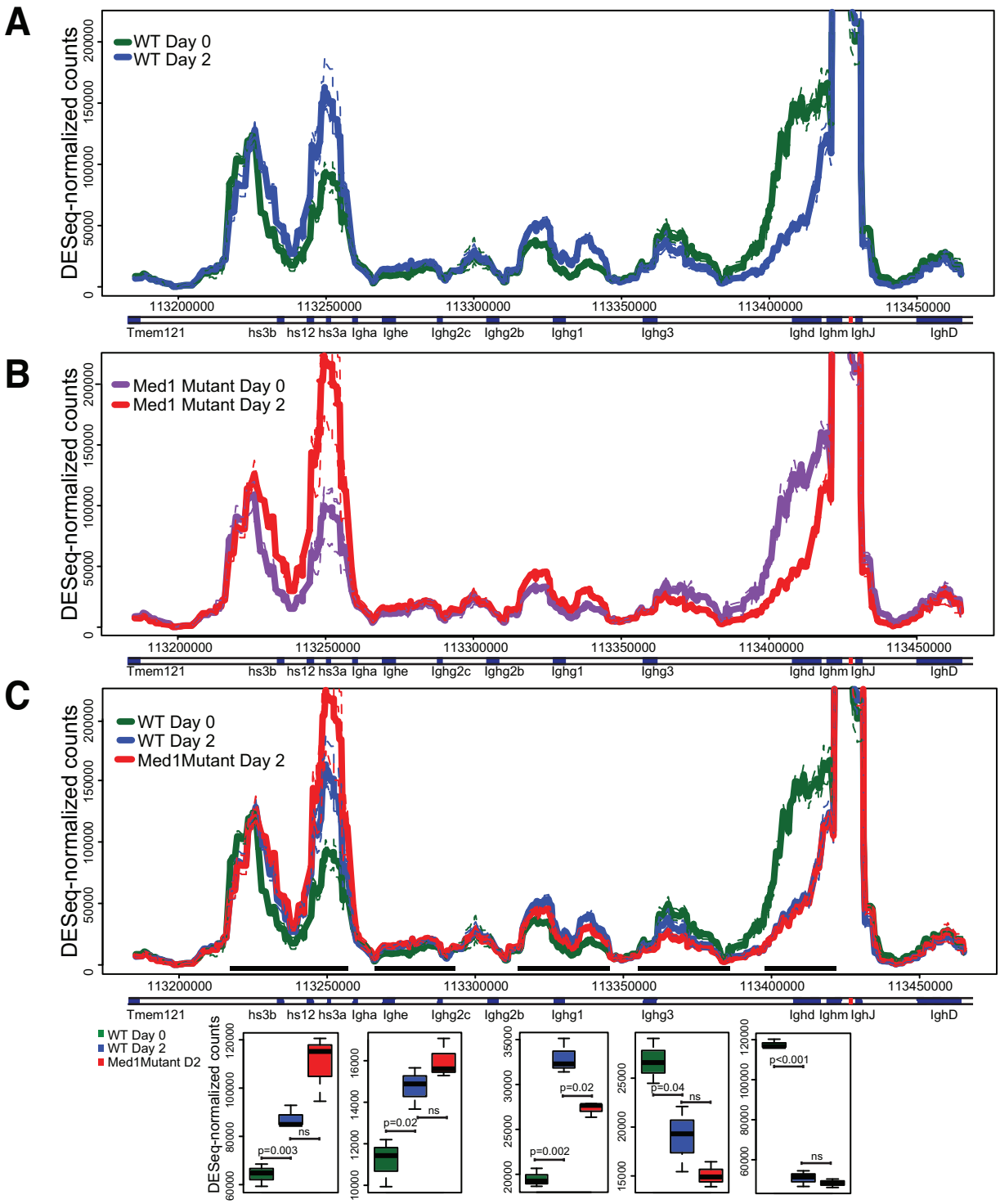


Figure 4: Med1 absence appears to affect Igh chromatin dynamics during CSR
High resolution 4C-seq was performed using a bait on the Eμ enhancer (red box in gene annotation). 4C-seq signal was calculated using 10kb windows centered on DpnII sites located in the constant region of IgH. The DESeq method of analysis was used to determine which windows have a significantly (FDR 0.1) different 4C-seq signal across samples. These significantly different windows are represented as dots. Full lines represent the average of three replicates and dashed lines the signal for each replicate. (A) The interactions between the Eμ enhancer and the region surrounding Cγ1 (Ighg1) are increased upon CSR induction. (B) This increase is also observed in mice mutant for Med1. (C) The comparison of wild-type and Med1 mutants on day 2 of CSR induction did not result in any significantly different windows when the entire IgH constant region was analyzed. However, a comparison centered only on the regions (black bars) where significant changes occur from day 0 to day 2 revealed that in the absence of Med1, interactions with Cγ1 (Ighg1) are decreased (center boxplot). In this focused analysis, boxplots represent the average score for all windows centered within the chosen regions and a Welch's t test (two-sample, two-sided) was used to assess significant differences taking into account the three replicates for each sample.

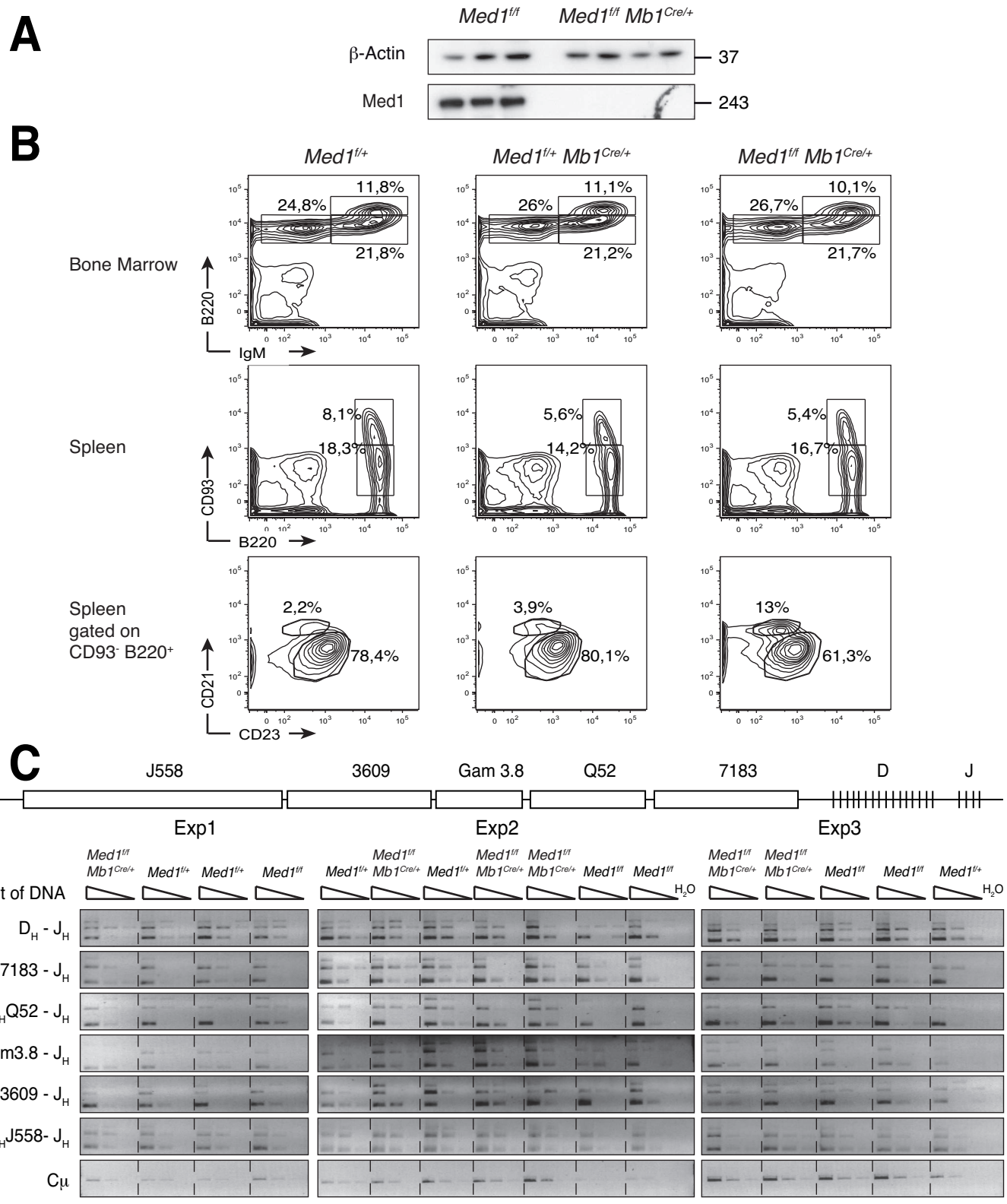


Figure S1: Med1 deficiency does not affect B cell development
(A) Western blot analysis for β-Actin and Med1 on whole-cell extracts from splenic B cells from Med1$^{f/f}$ and Med1$^{f/f}$ Mb1$^{Cre/+}$ mice cultured for 72h with LPS and IL4. (B) Flow cytometry analysis of cells from the bone marrow and the spleen from Med1$^{f/+}$, Med1$^{f/+}$ Mb1$^{Cre/+}$ and Med1$^{f/f}$ Mb1$^{Cre/+}$ mice. Antibodies used are indicated. Percentage of cells within the gates are indicated, further gating is indicated on the left of the plot. In the bone marrow, plots show pro/pre-B (B220^{lo}, IgM^{-}), immature (B220^{lo}, IgM^{+}), and mature recirculating (B220^{hi}, IgM^{+}) B cells. In the spleen, middle plots show immature (B220^{+}, CD93^{-}) and mature (B220^{+}, CD93^{+}) B cells and bottom plots show marginal (CD21^{+}, CD23^{lo}) and follicular (CD21^{lo}, CD23^{+}) B cells among the mature B cell population. Data are representative of three independent experiments. (C) Schematic diagram of the V_H gene cluster of the IgH locus. Only the V_H gene families analyzed and their distal or proximal position within the V_H gene cluster are shown. PCR detection of D_H–J_H and different V_H–DJ_H rearrangements in B cells stimulated to switch to IgG1 for 72h from control and Med1$^{f/f}$ Mb1$^{Cre/+}$ mice. Threefold DNA dilutions were analyzed by PCR. Input DNA was normalized by amplification of a PCR fragment from the IgH Cμ region.

MUTATIONS	*Med1*$^{f/f}$	*Med1*$^{f/f}$ *Mb1*$^{Cre/+}$	Total # sites	# Sites sequenced in *Med1*$^{f/f}$	# Sites sequenced in *Med1*$^{f/f}$ *Mb1*$^{Cre/+}$	Frequency *Med1*$^{f/f}$	Frequency *Med1*$^{f/f}$ *Mb1*$^{Cre/+}$	p value
G->C	16	11	154	16940	21098	0,0009445	0,0005214	0,1781917
G->A	31	46	154	16940	21098	0,0018300	0,0021803	0,5217008
G->T	8	9	154	16940	21098	0,0004723	0,0004266	1,0000000
sum:G	55	66	154	16940	21098	0,0032468	0,0031283	0,9105290
C->G	2	17	83	9130	11371	0,0002191	0,0014950	0,0059032
C->A	4	6	83	9130	11371	0,0004381	0,0005277	NA
C->T	17	26	83	9130	11371	0,0018620	0,0022865	0,6123311
sum:C	23	49	83	9130	11371	0,0025192	0,0043092	0,0419029
A->G	65	54	146	16060	20002	0,0040473	0,0026997	0,0335555
A->C	31	23	146	16060	20002	0,0019303	0,0011499	0,0771006
A->T	45	31	146	16060	20002	0,0028020	0,0015498	0,0138361
sum:A	141	108	146	16060	20002	0,0087796	0,0053995	0,0001515
T->G	4	15	167	18370	22879	0,0002177	0,0006556	0,0673909
T->C	59	61	167	18370	22879	0,0032118	0,0026662	0,3521064
T->A	5	28	167	18370	22879	0,0002722	0,0012238	0,0012714
sum:T	68	104	167	18370	22879	0,0037017	0,0045457	0,2130730
sum:GC	78	115	237	26070	32469	0,0029919	0,0035418	0,2797060
sum:AT	209	212	313	34430	42881	0,0060703	0,0049439	0,0388360
sum:ALL	287	327	550	60500	75350	0,0047438	0,0043397	0,2878856
Tv:GC	30	43	237	26070	32469	0,0011507	0,0013243	0,6357381
Tr:GC	48	72	237	26070	32469	0,0018412	0,0022175	0,3636017
Tv:AT	85	97	313	34430	42881	0,0024688	0,0022621	0,6067213
Tr:AT	124	115	313	34430	42881	0,0036015	0,0026818	0,0261388
Tv:ALL	115	140	550	60500	75350	0,0019008	0,0018580	0,9059060
Tr:ALL	172	187	550	60500	75350	0,0028430	0,0024818	0,2165540

Med1$^{f/f}$

from/to	G	C	A	T	Sum
G	0	5,6	10,8	2,8	19,2
C	0,7	0	1,4	5,9	8
A	22,7	10,8	0	15,7	49,1
T	1,4	20,6	1,7	0	23,7

Med1$^{f/f}$ *Mb1*$^{Cre/+}$

from/to	G	C	A	T	Sum
G	0	3,4	14,1	3,9	20,2
C	5,2	0	1,8	8	15
A	16,5	7	0	9,5	33
T	4,6	18,7	13,5	0	36,5

Figure S2: SHM pattern is affected by Med1 deletion
Table showing mutation type, mutation frequency and deletions/insertions in control and Med1$^{f/f}$ Mb1$^{Cre/+}$ sequences, and the corresponding statistical analysis. Statistical analysis for mutation analysis was performed using $\chi 2$ test. Statistical test for deletion/insertion frequency was performed using a two-tailed Fisher test. Tr, transitions; Tv, transversions. Bottom: Tables showing the percentage of mutations base to base in control and Med1$^{f/f}$ Mb1$^{Cre/+}$ sequences.

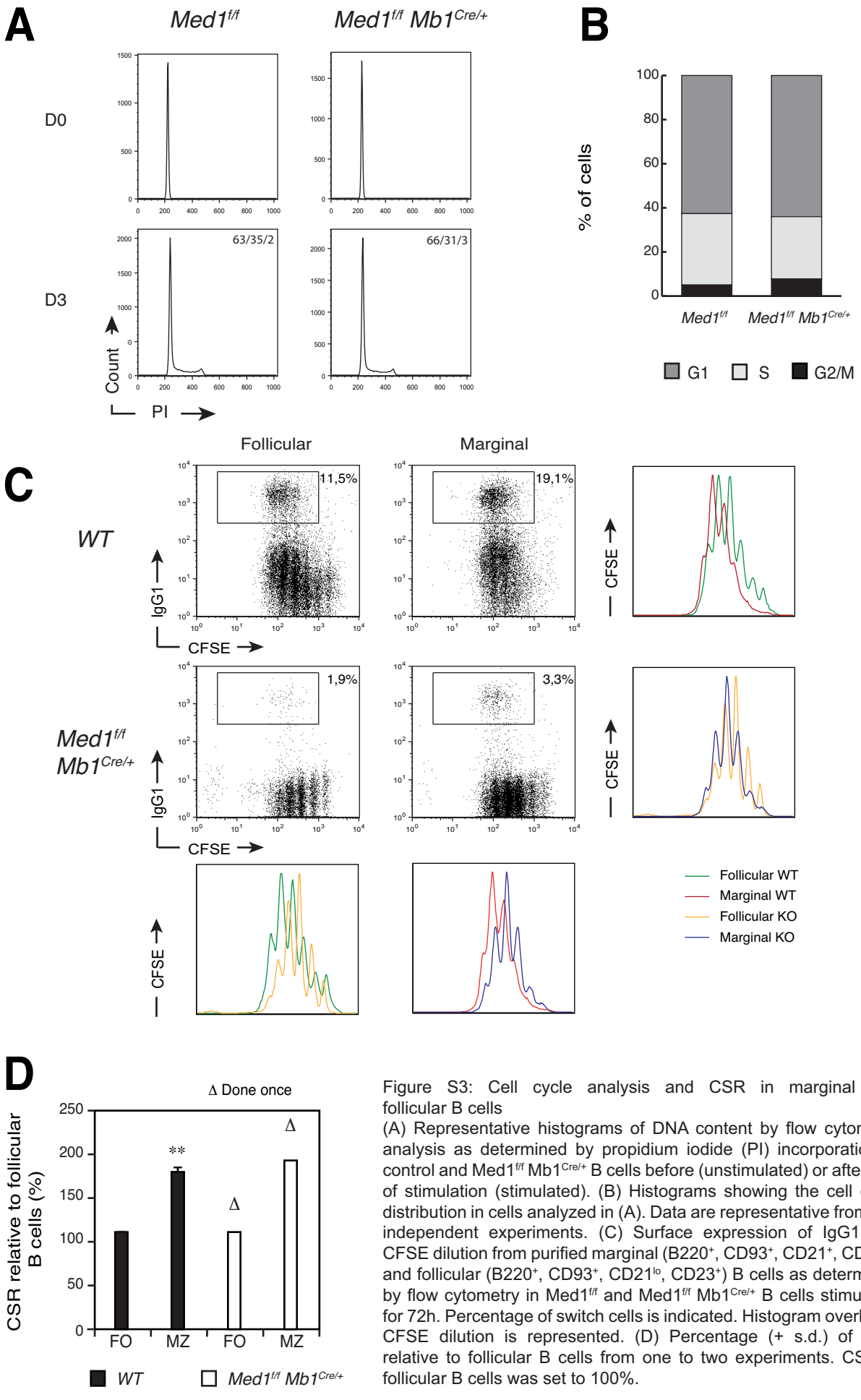


Figure S3: Cell cycle analysis and CSR in marginal and follicular B cells

(A) Representative histograms of DNA content by flow cytometry analysis as determined by propidium iodide (PI) incorporation in control and Med1$^{f/f}$ Mb1$^{Cre/+}$ B cells before (unstimulated) or after 72h of stimulation (stimulated). (B) Histograms showing the cell cycle distribution in cells analyzed in (A). Data are representative from two independent experiments. (C) Surface expression of IgG1 and CFSE dilution from purified marginal (B220^{+}, CD93^{+}, CD21^{+}, CD23^{lo}) and follicular (B220^{+}, CD93^{+}, CD21^{lo}, CD23^{+}) B cells as determined by flow cytometry in Med1$^{f/f}$ and Med1$^{f/f}$ Mb1$^{Cre/+}$ B cells stimulated for 72h. Percentage of switch cells is indicated. Histogram overlay of CFSE dilution is represented. (D) Percentage (+ s.d.) of CSR relative to follicular B cells from one to two experiments. CSR in follicular B cells was set to 100%.

Table S1. Antibodies used.			
Antibody	Clone	Source	Use*
AID	Strasbg 9 (AID-2E11)	IGBMC (Jeevan-Raj et al., 2011)	WB, IP
Flag	M2	Sigma	WB, IP
Med1	n.a.	Abcam	WB, IP
Med12	n.a.	Bethyl	WB, IP
B220-PECy7	RA3-6B2	Biolegend	FC
IgM-Cy5	n.a.	Jackson	FC
CD93-APC	AA4.1	e-biosciences	FC
CD21-FITC	7G6	BDPharmingen	FC
CD23-biotin	B3B4	BDPharmingen	FC
Streptavidin-PE	n.a.	Jackson	FC
GL7-FITC	GL7	BD	FC
CD95-PE	Jo2	BD	FC
IgG1-biotin	A 85-1	BD	FC
IgG3-biotin	R 40-82	BD	FC
IgG2b-biotin	RMG2B1	Biolegend	FC
IgG2a-biotin	n.a.	Biolegend	FC

n.a.: not applicable

* WB: Western blot; IP: Immunoprecipitation; ChIP: Chromatin immunoprecipitation; FC:Flow cytometry

Table S2. Primers and probes used.		
Germline transcripts		
Primer	Sequence (5'-3')	Probe or Reference
Igβ Fwd	TGGTGCTGTCTTCCATGC	UPL probe #18
Igβ Rev	TTGCTGGTACCGGCTCAC	UPL probe #18
CH12		
Iμ-Cμ-Fwd	ACCTGGGAATGTATGGTTGTGGCTT	(Jeevan-Raj et al., 2011)
Iμ-Cμ Rev	TCTGAACCTTCAAGGATGCTCTTG	(Jeevan-Raj et al., 2011)
Iα-Cα Fwd*	GGAGACTCCCAGGCTAGACA	UPL probe #27
Iα-Cα Rev*	CGGAAGGGAAGTAATCGTGA	UPL probe #27
Primary B cells		
Iμ-Cμ2 Fwd	CCCAGACCTGGGAATGTATG	UPL probe #29
Iμ-Cμ2 Rev	GGAAGACATTTGGGAAGGACT	UPL probe #29
Iγ3-Cγ3 Fwd	GCAGAAATCTGCAGGACTAACA	UPL probe #71
Iγ3-Cγ3 Rev	ACCGAGGATCCAGATGTGTC	UPL probe #71
Iγ2b-Cγ2b Fwd	TGGGCCTTTCCAGACCTAAT	UPL probe #88
Iγ2b-Cγ2b Rev	GGGCTGATCTGTCAACTCCT	UPL probe #88
Iγ2a-Cγ2a Fwd	CAGCCTGGGATCAAGCAG	UPL probe #109
Iγ2a-Cγ2a Rev	TGGGGCTGTTGTTTTGGT	UPL probe #109
Iγ1-Cγ1 Fwd	GGCCCTTCCAGATCTTTGAG	
Iγ1-Cγ1 Rev	ATGGAGTTAGTTTGGGCAGCA	
Switch junctions		
Primer	Sequence (5'-3')	Probe or Reference
Sμ-Fwd	CGAATGCATCTAGATATCGGATCCCGGCTTAACCG AGATGAGCC	(Schrader et al., 2002)
Cγ3-Rev	GCAGGCCTCTGCAGTCGACGGGCCCACCCTGACC CAGGAGCTGCATAAC	(Schrader et al., 2002)
V(D)J		
Primer	Sequence (5'-3')	Probe or Reference
D_H Fwd	TTCAAAGCACAATGCCTGGCT	(Fuxa et al., 2004)
V_HJ558 Fwd	CGAGCTCTCCARCACAGCCTWCATGCARCTCARC	(Fuxa et al., 2004)
V_H7183 Fwd	CGGTACCAAGAASAMCCTGTWCCTGCAAATGASC	(Fuxa et al., 2004)
V_HQ52 Fwd	CGGTACCAGACTGARCATCASCAAGGACAAYTCC	(Fuxa et al., 2004)
V_HGam3.8 Fwd	CAAGGGACGGTTTGCCTTCTCTTTGGAA	(Fuxa et al., 2004)
V_H3609 Fwd	KCYYTGAAGAGCCRRCTCACAATCTCC	(Fuxa et al., 2004)
J_H3 Rev	GTCTAGATTCTCACAAGAGTCCGATAGACCCTGG	(Fuxa et al., 2004)
Cμ Fwd	TGGCCATGGGCTGCCTAGCCCGGGACTT	(Fuxa et al., 2004)

III. Additional results

1. Role of cohesin in cell proliferation and cell cycle checkpoint

The cohesin complex has been described to play a prominent role in sister chromatid cohesion during cell division and in favoring DNA repair by homologous recombination [347]. To rule out potential effects of abnormal cell proliferation and survival on CSR after knock down, we assessed IgA surface expression by flow cytometry on cells transduced with lentiviruses expressing specific shRNAs (but lacking the GFP reporter) and that were labeled with CFSE (Figure 16). By CFSE dilution analysis, we found that knock down of Smc1 and Smc3, and to a lesser extent Wapl and Nipbl, resulted in the appearance of a small population of cells that did not dilute the CFSE dye as efficiently as the controls (Figure 16A). Nevertheless, this was independent from the effect on CSR, as revealed by gating on cells having proliferated equally (Figure 16A – Gate 2). Therefore the slight proliferation defects observed after knock down of Smc1, Smc3, Nipbl and Wapl do not contribute significantly to the reduction in CSR observed (figure 16B).

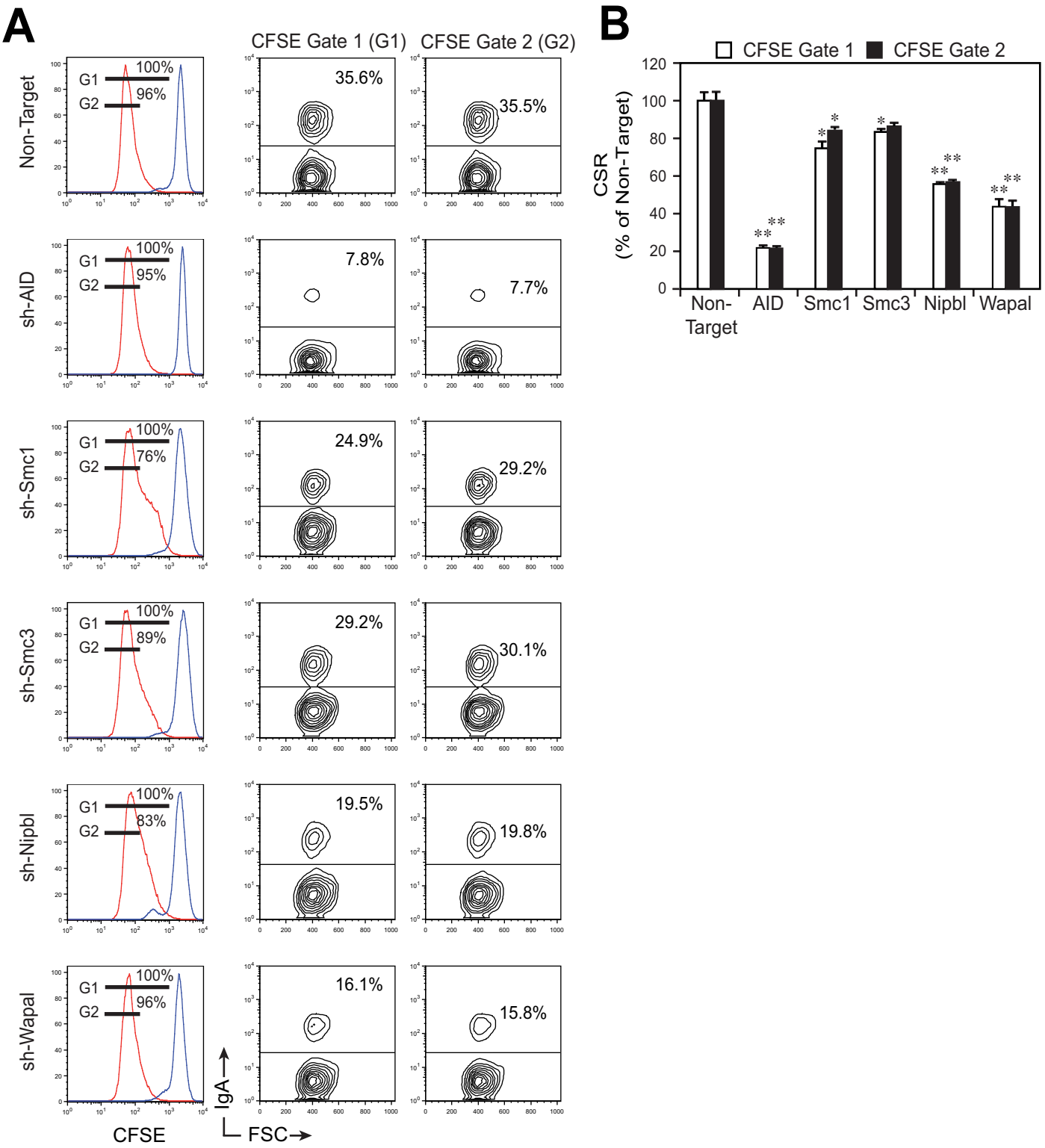


Figure 16: Proliferation and CSR after knock down of cohesin
(A) IgA surface expression determined by flow cytometry in stimulated CH12 cells labeled with CFSE and transduced with a lentivirus (lacking a GFP reporter) and expressing shRNAs specific for AID, Smc1, Smc3, Nipbl and Wapl or a non-target shRNA negative control. Histograms depicting CFSE intensity (Left panel) at day 0 (shown in blue) and after 48h (shown in red) are shown. Gates and percentage of cells gated are indicated. Gate 1 comprises all dividing cells whereas Gate 2 includes only cells having proliferated equally. IgA surface expression analysis on gate 1 (Middle panel) and gate 2 (right panel) is shown. Numbers within the plots indicate the percentage of IgA positive cells. Representative histograms and plots from two independent experiments are shown. (B) Percentage (+ s.d.) of CSR relative to the non-target shRNA control from two independent experiments analyzed on cells gated on gate 1 (white bars) or on gate 2 (black bars). CSR in cells expressing the non-target shRNA control was set to 100%. Statistical significance vs. the non-target control was determined by a two-tailed Student's t-test. *: p≤0.05; **: p≤0.01. Analysis of CSR in the population of equally dividing cells (gate 2) still shows a defect in CSR efficiency upon cohesin knock down.

To determine whether cohesin knock down (before or after stimulation) results in the spontaneous activation of the DNA damage response and in the enforcement of cell cycle checkpoints, we assessed the phosphorylation status of the Chk1 kinase and the histone variant H2AX in transduced cells sorted for GFP expression, both of which are phosphorylated in response to replication defects and sustained DNA damage. While treatment of cells with neocarzinostatin (a radio-mimetic drug) induced the phosphorylation of Chk1 and H2AX and treatment with hydroxyurea (inducing replication arrest and activation of the G2/M checkpoint) resulted in the phosphorylation of Chk1, as expected, no significant phosphorylation of Chk1 and H2AX was observed after cohesin knock down (figure 17A). These results indicate that the knock down of cohesin is not sufficient to induce replication defects and activate the DNA damage response and cell cycle checkpoints.

These results were confirmed by flow cytometry cell cycle analysis after cohesin knock down before and after stimulation. When compared to the positive controls (cells treated with neocarzinostatin and hydroxyurea) and to the negative controls (untreated and untransduced CH12 cells) we did not observe significant differences in the cell cycle progression (figure 17B and 17C). Thus our results suggest that the knock down of cohesin is not sufficient to significantly affect cell cycle progression.

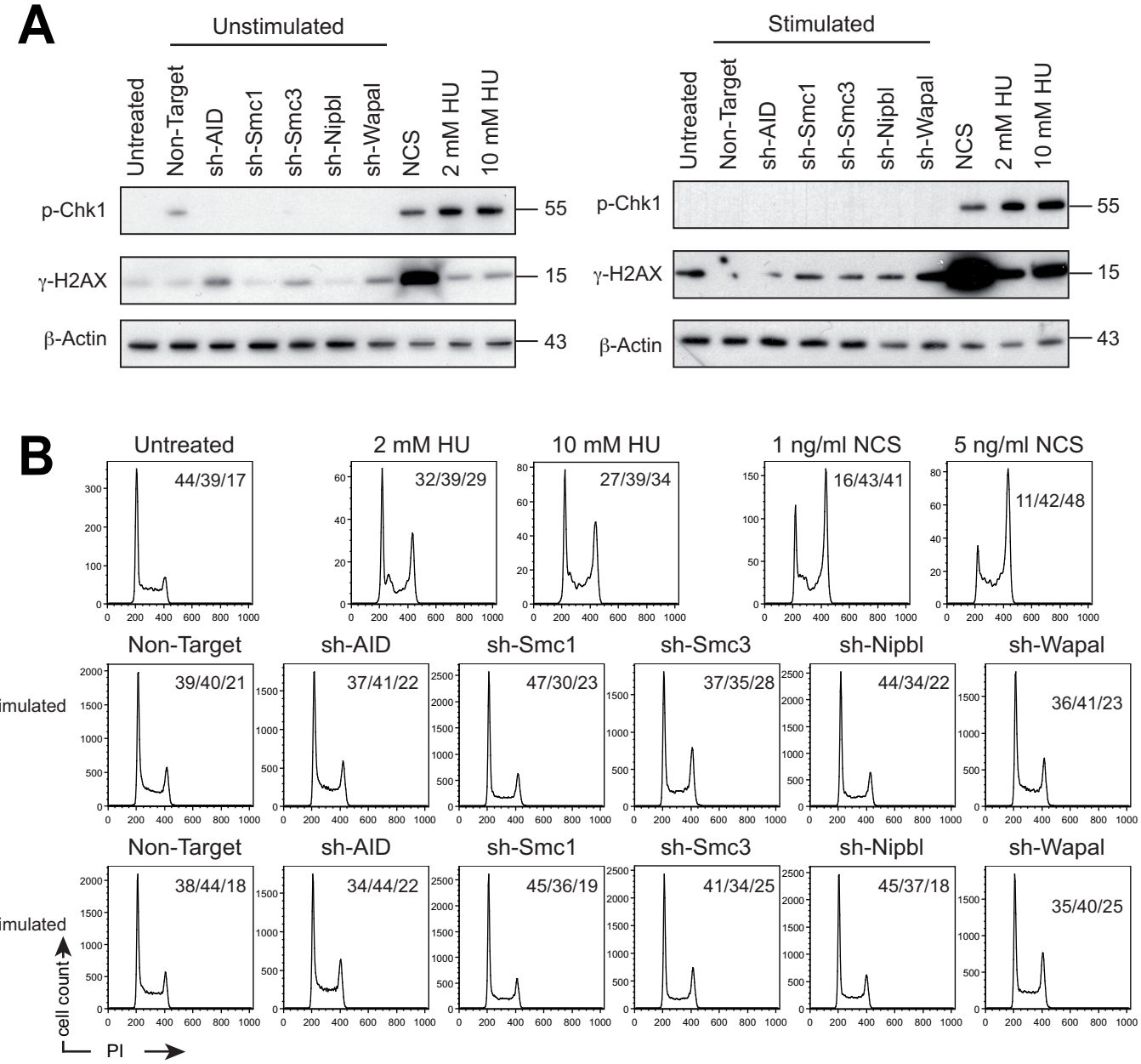


Figure 17: Cell cycle analysis and checkpoint activation after knock down of cohesin
(A) Western blot analysis using antibodies specific for the phosphorylated form of the Chk1 kinase (p-Chk1) and the histone variant H2AX (g-H2AX) and β-actin performed in CH12 cells treated or not with hydroxyurea (HU; 2 or 10 mM) or neocarzinostatin (NCS; 200 ng/ml) and CH12 transduced with lentiviruses expressing shRNAs specific for AID, Smc1, Smc3, Nipbl and Wapl or a non-target shRNA negative control and sorted for GFP expression. (B) Representative histograms of DNA content flow cytometry analysis as determined by propidium iodide (PI) incorporation in cells described in (A) before (unstimulated) or after 48h of stimulation (stimulated). (C) Histograms showing the cell cycle distribution in cells analyzed in (B). Data are representative from two to three independent experiments.

In conclusion, the effect on CSR after cohesin knock down was not due to strong defects in proliferation, significant activation of the DNA damage response and cell cycle checkpoints, nor to defective cell cycle progression.

2. AID recruitment upon cohesin knock down

CSR is dependent on AID recruitment at the IgH locus. To determine whether cohesin is also implicated in this process, we performed AID ChIP experiments on CH12 cells transduced with lentiviruses expressing shRNAs specific for AID, Smc1, Smc3, Nipbl, Wapl and a non-target control, followed by qPCR at Sμ (figure 18). ChIP on CH12 cells depleted from AID showed a decreased binding of AID at Sμ as expected. After cohesin depletion, analysis of the enrichment profile of AID revealed a similar binding profile than the non-target control, suggesting that cohesin is not implicated in AID targeting or tethering.

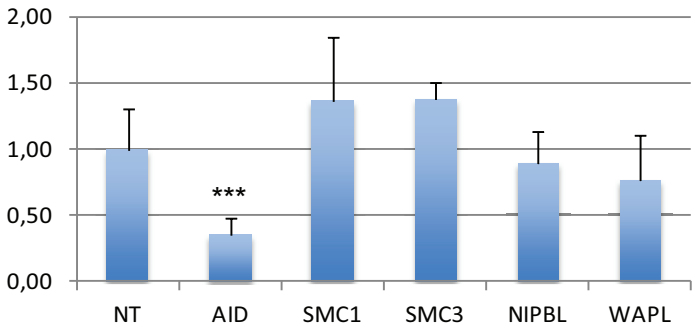


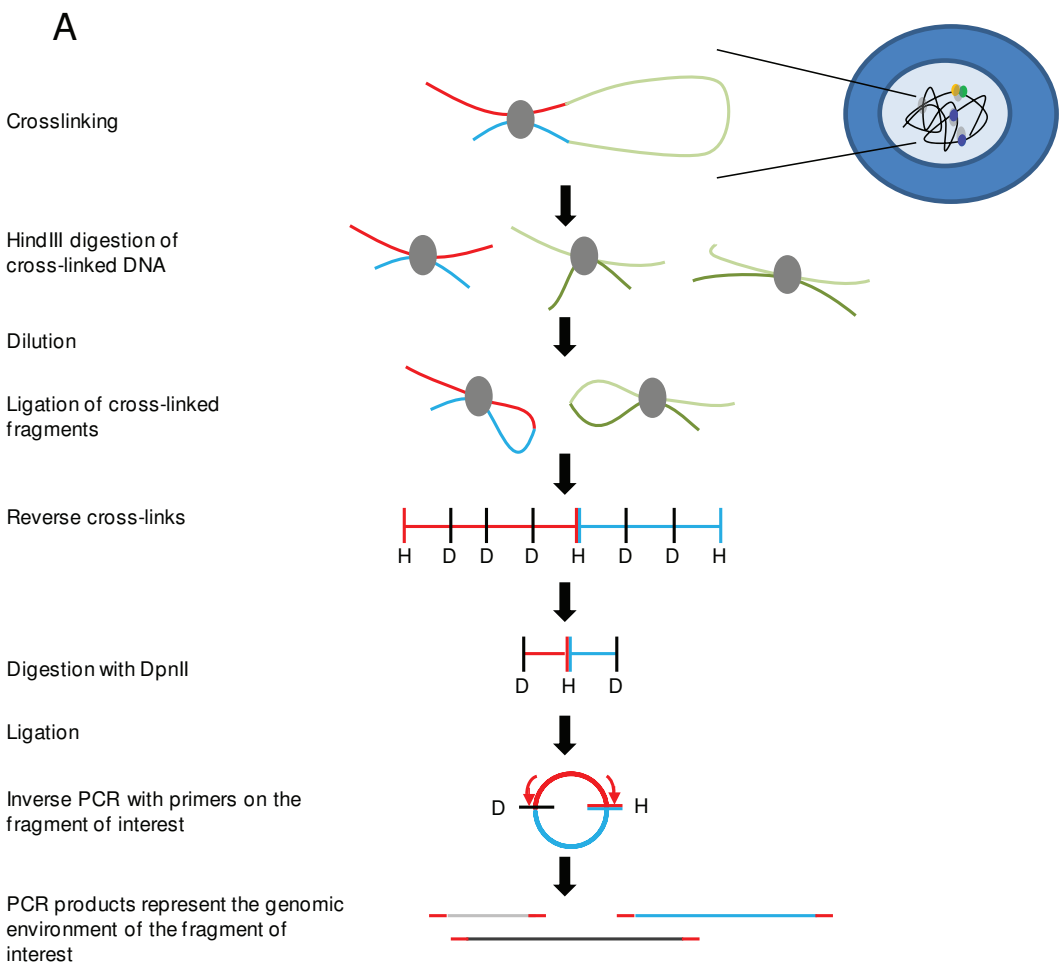
Figure 18: AID recruitment at Sμ upon cohesin depletion
ChIP-qPCR performed on CH12 cells transduced with lentiviral vectors expressing shRNA targeting Smc1, Smc3, Nipbl, Wapl or a non-target control stimulated to switch for 48h and sorted for GFP expression. For each sample, AID-ChIP values (mean ± SD) were normalized to the input control. AID-ChIP signal in non-target control cells was set to 1. Error bars are indicative of the variation between the different PCRs. Statistical significance versus the non-target was determined by a two-tailed Student's t-test. ***, p≤ 0.001. Data are from two independent experiments.

3. Study of the structural conformation of the IgH locus upon cohesin knock down

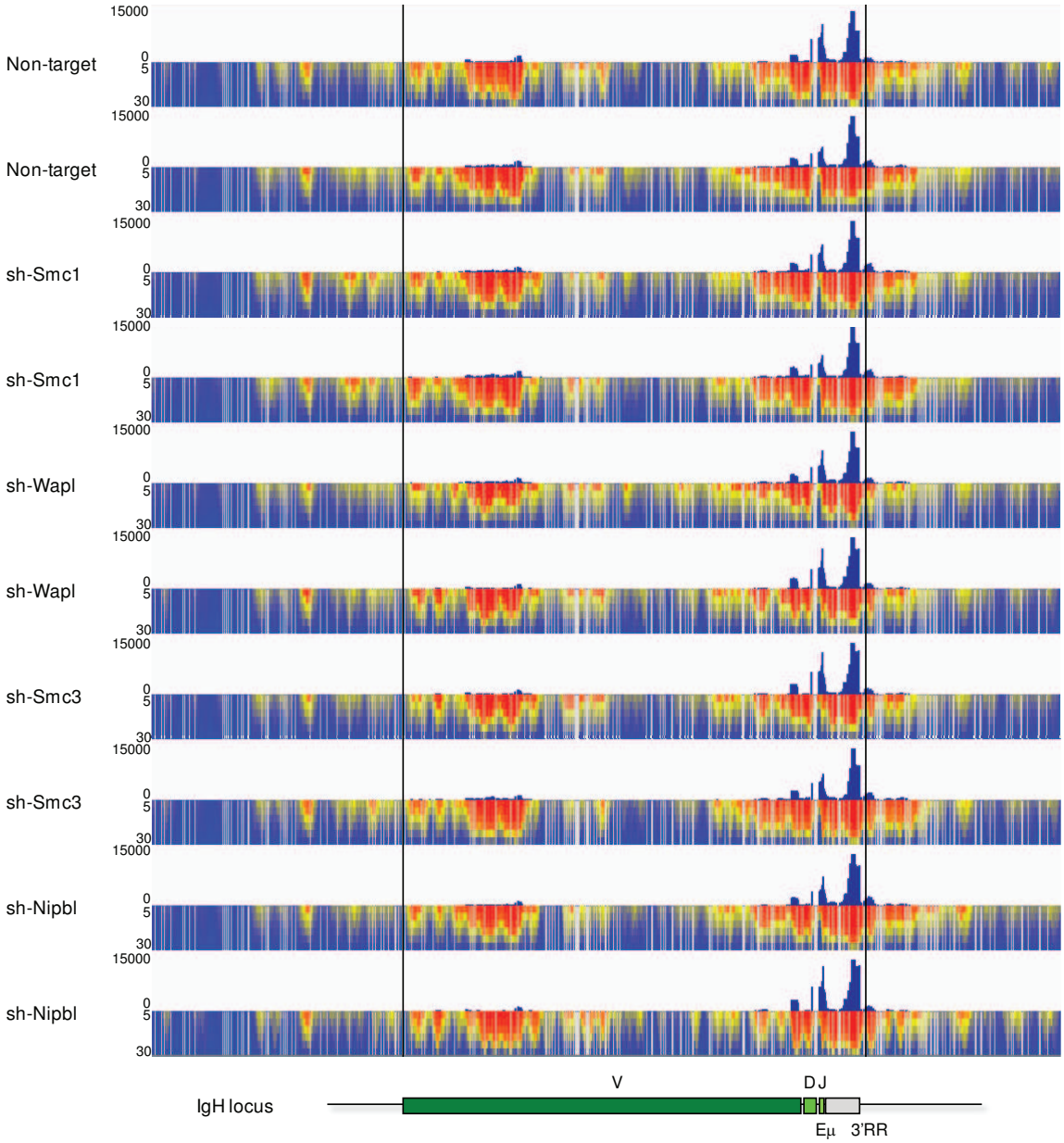
The cohesin complex has been described to play a role in promoting the transcription-coupled formation of long-range DNA loop structures [326]. In addition, cohesin and the transcriptional insulator CTCF [347,348], have been shown to control the RAG1/2-dependent rearrangement of antigen receptor genes during B and T cells development, by mechanisms involving the regulation of transcription and the formation of long-range *in cis* DNA interactions [244,343,344]. We showed that cohesin subunits, Smc1 and Smc3, are dynamically recruited to the IgH locus in B cells undergoing CSR suggesting a role for cohesin in supporting the structural changes occurring at the IgH locus upon B cell activation [346].

To investigate the implication of cohesin subunits in the 3D organization of the IgH locus, we performed 4C-Seq experiments that allow the identification of all the DNA fragments that interact with one fragment of interest. The workflow of the experiment is the following: firstly, the chromatin is fixed using a fixative agent like formaldehyde to maintain all protein-protein and protein-DNA interactions. The fixed chromatin is then digested using a 6 bp cutter enzyme and fragments are religated under diluted conditions to promote intra-molecular ligations. DNA fragments that are far from each other in a linear template but that do interacts can be ligated to each other. A second round of digestion and ligation is done to create small DNA circles. Primers designed to target the fragment of interest and going towards the restriction sites allow us to amplify all sequences contacting this viewpoint, also called the bait. The primers also contain adaptor sequences that allow their identification by sequencing (Figure 19A).

We performed 4C-Seq experiments on CH12 cells transduced with lentiviruses expressing shRNAs specific for Smc1, Smc3, Nipbl, Wapl and a non-target shRNA as a negative control. Transduced cells were stimulated to switch for 60h and chromatin was subsequently digested with HindIII and DpnII. 4C librairies were amplified using a bait located in the 3'RR and were then sequenced and analyzed by our collaborators as previously described [58]. Results are presented as the running mean of the reads obtained for 20 HindIII restriction sites, helping to smooth the data (blue line). Domainograms, in the bottom part, show the frequency of mapped reads in windows of increasing size and are used to identify statistically significant interactions. Running mean for 20 HindIII restriction sites are shown for two biological replicates in figure 19B and 19C. First of all, we see that in CH12 transduced with non-target shRNA the 3'RR interacts with Eμ (figure 19B and 19C) as expected from results obtained on primary B cells [138]. Additionally, the 3' enhancer interacts also slightly with distal V_H genes (figure 19B) and with the region containing the different constant exons (figure 19C). Unfortunately, the analysis of the pattern of interactions occurring in knock down cell lines shows the same profile than the non-target cell line, suggesting that no defect in the 3D organization of the IgH locus is detected upon cohesin deletion.


A
Crosslinking
HindIII digestion of cross-linked DNA
Dilution
Ligation of cross-linked fragments
Reverse cross-links
H D D D H D D H
Digestion with DpnII
D H D
Ligation
Inverse PCR with primers on the fragment of interest
D H
PCR products represent the genomic environment of the fragment of interest

B


Non-target
Non-target
sh-Smc1
sh-Smc1
sh-Wapl
sh-Wapl
sh-Smc3
sh-Smc3
sh-Nipbl
sh-Nipbl
15000
0
5
30
IgH locus
V
D J
Eμ
3'RR

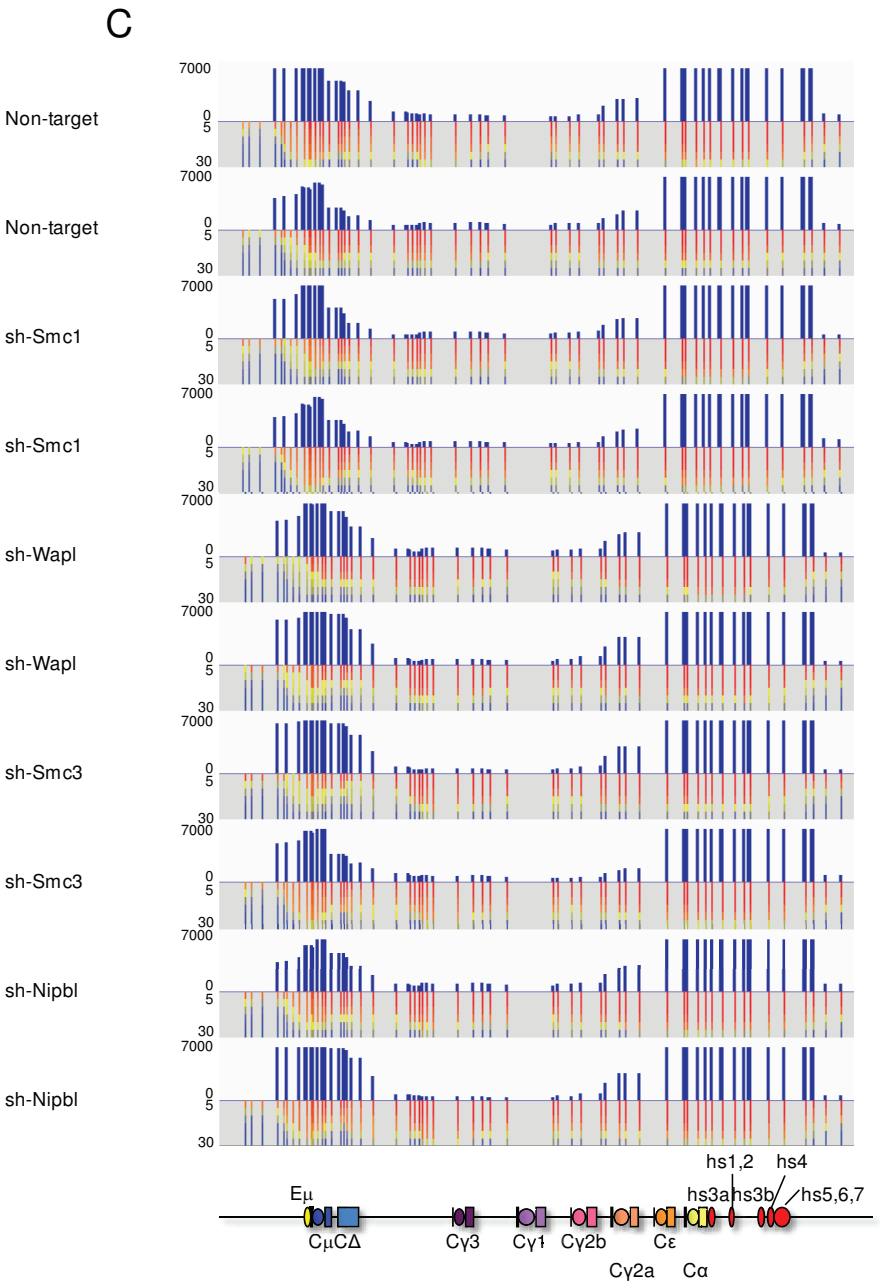


Figure 19: Chromosomal interactions within the IgH locus using a bait located at the 3'RR in CH12 cells
(A) Workflow of 4C-Seq experiments. Cells transduced with lentiviral vectors expressing shRNA targeting cohesin subunits and stimulated to switch for 60h with LPS + antiCD40 + TGFβ. Chromatin was digested with HindIII and DpnII to generate the 4C librairies. Running mean reads for 20 HindIII restriction sites and domainograms are shown. Domainograms were generated by determining the quantile score for each genomic location at increasing length scales (5 to 30 HindIII sites). Quantile score is coded by color with the most frequent interactions represented in red. (B) View of the entire IgH locus. 4C-Seq in a window of 0 to 15000 reads. (C) View of the constant region of the IgH locus. 4C-Seq in a window of 0 to 7000 reads.

4. AID recruitment upon mediator complex subunits depletion

AID recruitment has been shown to be dependent on transcription [45] and Med1 deletion affects acceptor switch region transcription (see part II of the results section). To determine whether mediator complex is implicated in AID recruitment, we performed AID ChIP experiments on CH12 cells

transduced with lentiviruses expressing shRNAs specific for AID, Med1 and Med12 and a non-target control, followed by qPCR at Sμ (figure 20). ChIP on CH12 cells depleted from AID showed a decreased binding of AID at Sμ as expected, and upon depletion of mediator subunits, AID recruitment was also reduced, suggesting that mediator complex is implicated in AID targeting.

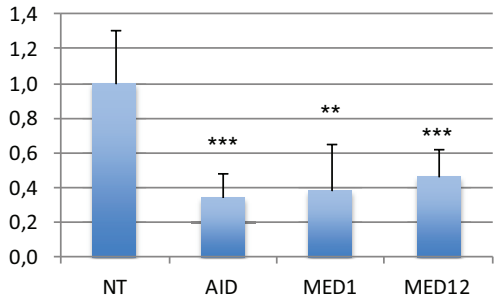


Figure 20: AID recruitment at Sμ upon mediator depletion
ChIP-qPCR performed on CH12 cells transduced with lentiviral vectors expressing shRNA targeting AID, Med1, Med12 and a non-target control stimulated to switch for 48h and sorted for GFP expression. For each sample, AID-ChIP values (mean ± SD) were normalized to the input control. AID-ChIP signal in non-target control cells was set to 1. Error bars are indicative of the variation between the different PCRs. Statistical significance versus the non-target was determined by a two-tailed Student's t-test. **, p≤ 0.01 ; ***, p≤ 0.001. Data are from two independent experiments.

5. Mediator recruitment at the IgH locus

To determine whether mediator complex is recruited to the IgH locus in B cells, we undertook ChIP-qPCR and ChIP-Seq experiments. We performed ChIP-qPCR for Med1 on chromatin purified from resting or activated splenic B cells isolated from WT and Med1-deficient mice using primer pairs located across the IgH locus. Comparing the amplification profile from ChIP done on WT cells to ChIP done on Med1 KO cells allowed us to validate our ChIP experiment and to identify regions where Med1 was recruited. qPCR done on Sγ3 revealed a similar enrichment between WT and Med1-deficient cells, therefore we decided to use this region as a negative control to calculate the Med1 enrichment at other locations. In resting cells, Med1 binds at the Eμ and at Sμ and this profile is unchanged upon B cell activation (figure 21A). Surprisingly, we also detected Med1 recruitment at Sγ1 in resting cells. Nevertheless, validation of the ChIP led us to perform ChIP-Seq experiments on WT and Med1-deficient cells either stimulated or not to switch to IgG1. ChIP efficiency was confirmed by qPCR (figure 21B), which showed again recruitment of Med1 at Eμ and Sμ in resting and stimulated cells, despite an increased enrichment of Med1 at Sγ3 in WT cells. However, sequencing results didn't confirm this recruitment profile. Indeed, ChIP-Seq profile did not show any recruitment of Med1, the profile being similar to an IgG ChIP-Seq, suggesting that the ChIP didn't work (figure 21C).

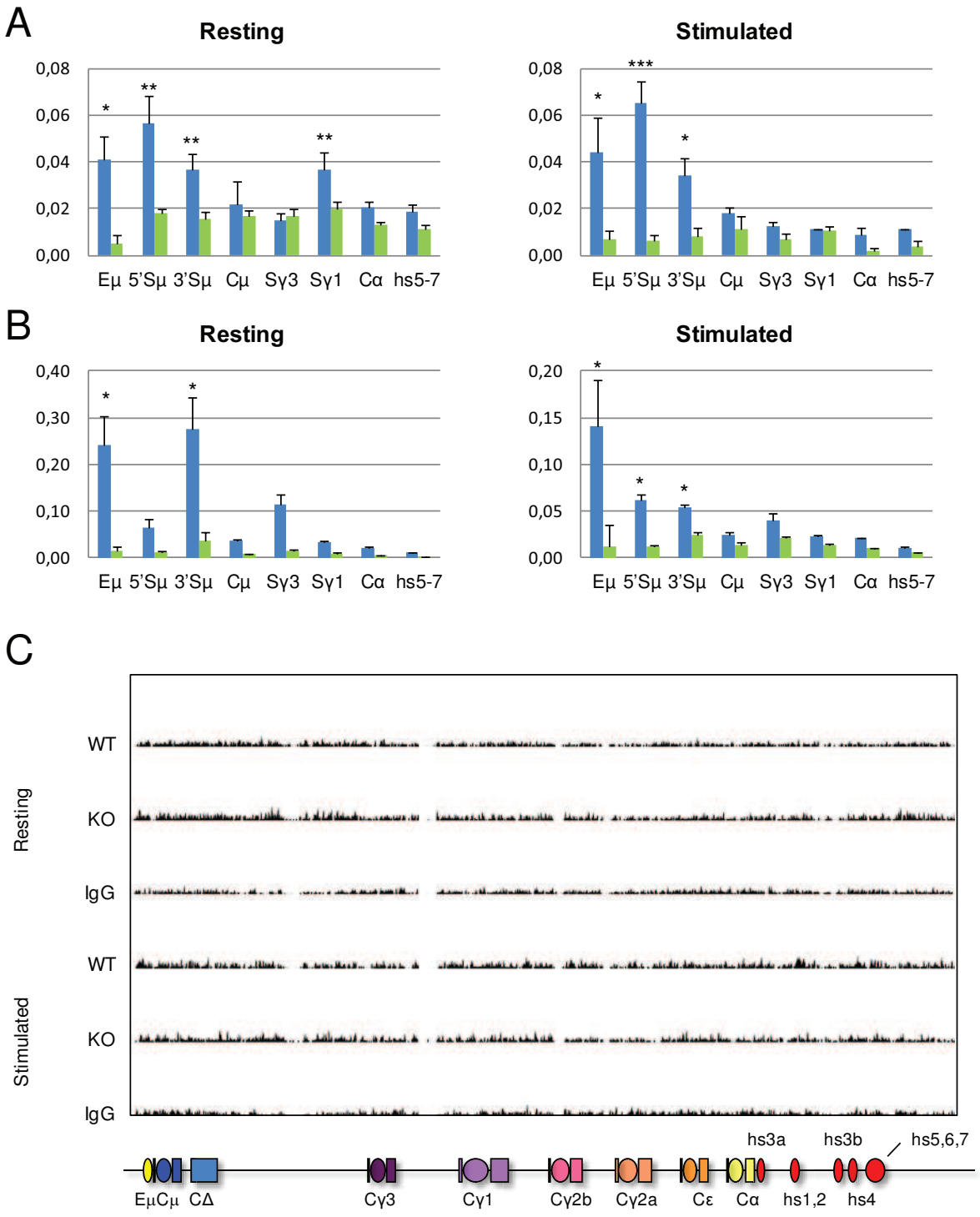


Figure 21: Med1 recruitment at the IgH locus in primary B cells
B cells from WT or Med1$^{f/f}$ Mb1$^{Cre/+}$ mice were stimulated or not to switch for 60h with LPS + IL-4 and chromatin was immunoprecipitated with Med1 antibody. qPCR experiments in resting and activated B cells are shown. For each sample, ChIP values (mean ± sd) were normalized to the input control. Results are expressed as % input. Blue: ChIP on WT cells; green: ChIP on Med1$^{f/f}$ Mb1$^{Cre/+}$ cells. (A) First ChIP experiment. (B) Validation of the ChIP-Seq experiment. Statistical significance versus Sγ3 was determined by a two-tailed Student's t-test. *, p ≤ 0.05; **, p≤ 0.01; ***, p≤ 0.001 . Results are representative of two independent experiments. (C) Genome browser screenshots showing the ChIP-Seq binding profiles of Med1 at the IgH locus (chr12:114,438,857-114,669,149) in resting and activated (with LPS + IL-4) B cells isolated from WT and Med1-deficient mice.

Mediator complex being composed of 30 subunits[283], we decided to undertake ChIP experiments targeting another mediator subunit. We undertook ChIP experiments for Med12 subunit which is located in a different submodules [292]. We performed ChIP on chromatin purified from resting and activated B cells isolated from WT mice and confirmed the enrichment by WB (figure 22A). ChIP samples were then submitted to sequencing but again the profile obtained was similar to IgG ChIP-Seq, with no peaks detectable (figure 22B).

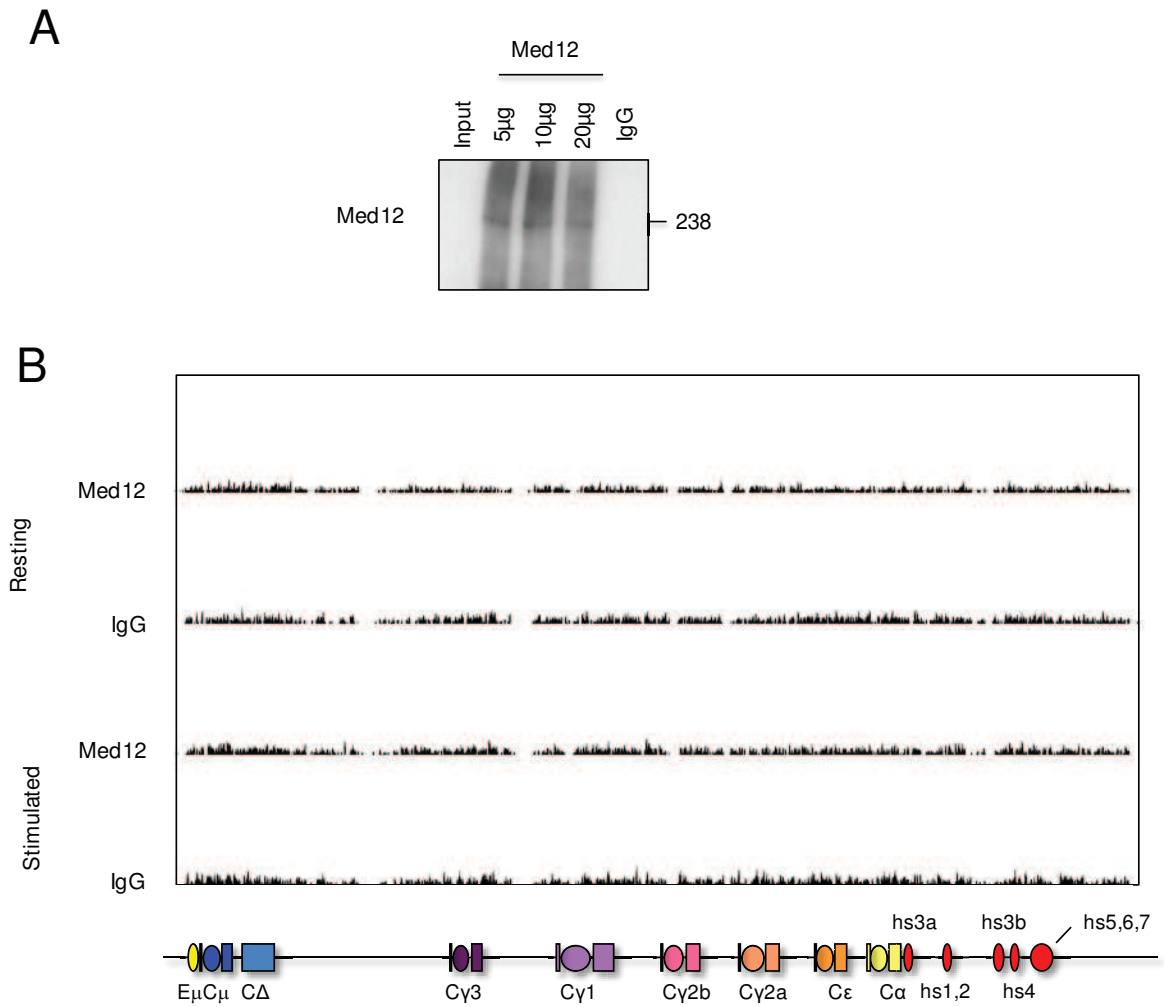


Figure 22: Med12 recruitment at the IgH locus from primary B cells
B cells from WT mice were stimulated or not to switch for 60h with LPS + IL-4 and chromatin was immunoprecipitated using Med12 antibody. (A) WB for Med12 was performed after immunoprecipitation of the equivalent of 10M cells with 5, 10 and 20µg of Med12 antibody. 20µg of IgG antibody was used as control. (B) Genome browser screenshots showing the ChIP-Seq binding profiles of Med12 and IgG at the IgH locus (chr12:114,438,857-114,669,149) in resting and activated (with LPS + IL-4) B cells isolated from WT mice.

6. Study of the structural conformation of the IgH locus upon Med1 deletion

To study the implication of mediator complex in DNA looping upon B cell activation, we performed a first 4C-Seq experiments on stimulated B cells deficient for Med1 in parallel of the experiment done on cohesin KD cell lines. WT and Med1-deficient cells were stimulated to switch to IgG1 for 60h and 4C-Seq experiments were done using the same protocol than the one used for the cohesin KD cell lines. Running mean for 20 HindIII restriction sites are shown for three biological replicates for the WT cells and two replicates for the Med1-deficient cells in figure 23. In WT cells, the 3'RR interacts strongly with Eμ and deletion of Med1 doesn't affect this interaction, suggesting that Med1 is not implicated in the generation of this loop (figure 23A and 23B). Additionally, we detected also peaks distributed along the variable (figure 23A) and the constant regions (figure 23B) in WT cells and the interactions with the variable region occurred more frequently with distal VH genes, especially for the WT samples number 1 and 3 (figure 23A). Surprisingly, the interaction between the 3'RR and distal V genes in Med1-deficient cells seems to be reduced compared to WT cells, suggesting either a defect of interaction during CSR, or a defect of recombination with distal V genes during V(D)J recombination.

Concerning interactions within the constant region, cells being stimulated to switch to IgG1, we expected to see a strong interaction with this region in WT cells. However, interaction between 3'RR and Sγ1 region is not obvious. This result is probably due to the fact that there are not so many HindIII restriction sites around this location. Given the poor resolution quality observed in WT cells, we cannot conclude about the implication of Med1 in this interaction. As this experiment was not conclusive, we performed a second 4C-Seq experiment with higher resolution using DpnII and Csp6I enzymes to investigate the effect of Med1 deletion on the 3D organization of the IgH locus and this result is presented in the second part of the results (manuscript). Also, this second experiment allowed us to study the potential effect of Med1 in the interaction with distal V genes.

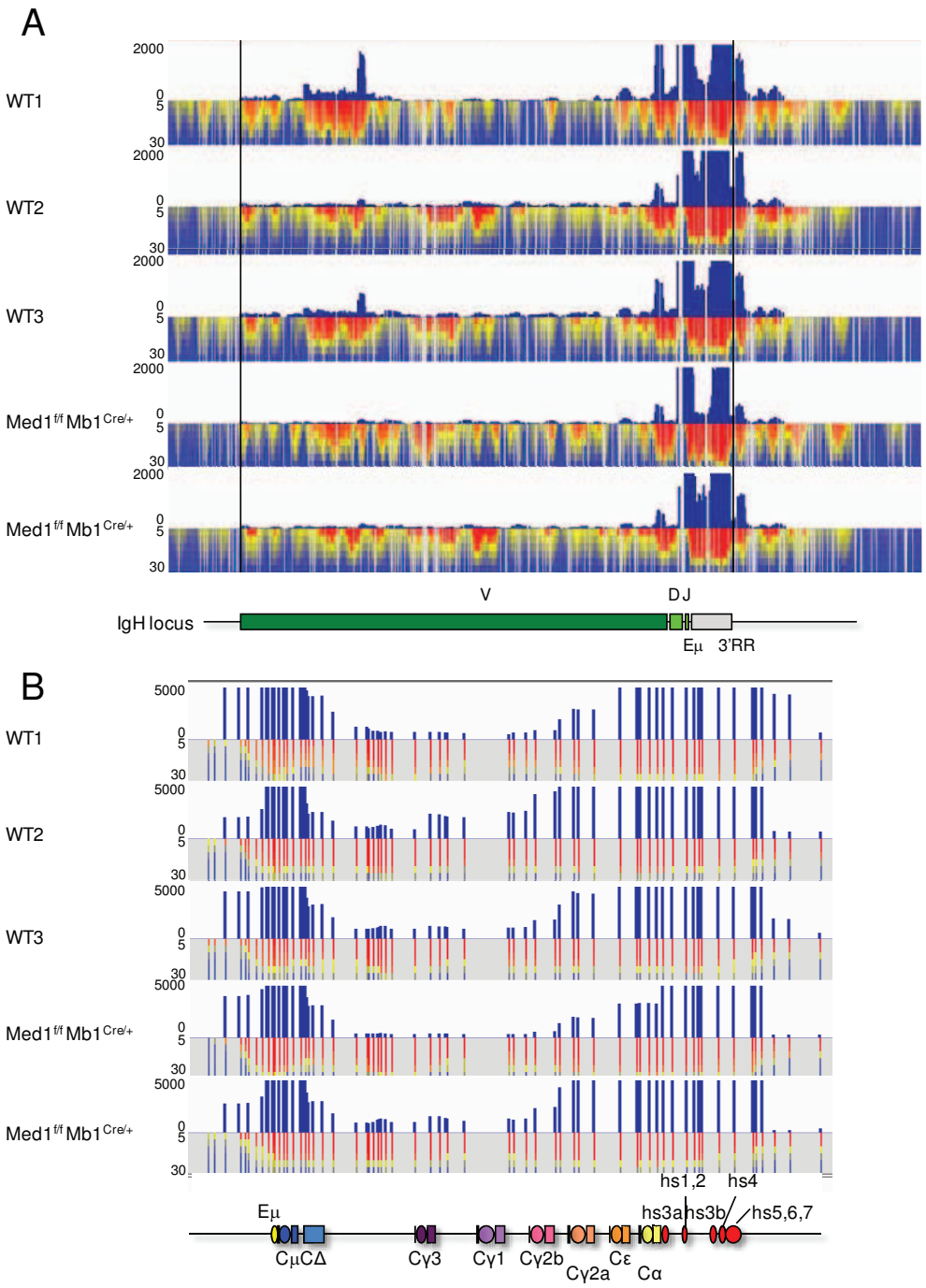


Figure 23: Chromosomal interactions within the IgH locus using a bait located at the 3'RR in primary B cells
B cells were stimulated to switch for 60h with LPS + IL-4 and chromatin was digested with HindIII and DpnII to generate the 4C librairies. Running mean reads for 20 HindIII restriction sites and domainograms are shown. Domainograms were generated by determining the quantile score for each genomic location at increasing length scales (5 to 30 HindIII sites). Quantile score is coded by color with the most frequent interactions represented in red. (A) View of the entire IgH locus. 4C-Seq in a window of 0 to 2000 reads. (B) View of the constant region of the IgH locus. 4C-Seq in a window of 0 to 5000 reads.

7. Cohesin recruitment upon Med1 deletion

To determine whether cohesin binding was affected by Med1 deletion, we performed a preliminary ChIP-qPCR experiment on chromatin from resting and stimulated B cells isolated from WT and Med1-deficient mice using an Smc3 antibody. In resting WT cells, as previously shown [346], Smc3 was recruited to the 3'RR and at Cα and the deletion of Med1 didn't affect Smc3 recruitment at the IgH locus (figure 24). After stimulation, Smc3 is additionally recruited at Sμ and Cμ in WT cells. However, Med1 deletion induced a drastic reduction of Smc3 binding along the IgH regions tested by qPCR, suggesting that Med1 is required for Smc3 recruitment at the IgH locus upon B cell activation. This result needs to be taken with caution as it arises from a unique experiment that needs to be repeated.

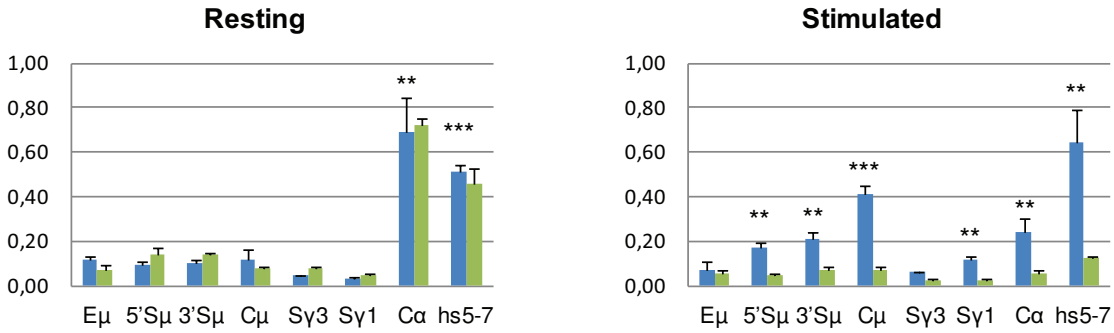


Figure 24: Smc3 recruitment at the IgH locus in WT or Med1$^{f/f}$ Mb1$^{Cre/+}$ B cells
B cells from WT or Med1$^{f/f}$ Mb1$^{Cre/+}$ mice were stimulated or not to switch for 60h with LPS + IL-4 and chromatin was immunoprecipitated with Smc3 antibody. qPCR experiments in resting and activated B cells are shown. For each sample, ChIP values (mean ± SD) were normalized to an IgG ChIP and to the input control. Results are expressed as % input. Blue: ChIP on WT cells; green: ChIP on Med1$^{f/f}$ Mb1$^{Cre/+}$ cells. Statistical significance versus Sγ3 was determined by a two-tailed Student's t test. **, $p \leq 0.01$; ***, $p \leq 0.001$.

Materiels and methods

This section contains the materiels and methods used for the additional results.

Lentiviral infection

The lentiviral vectors (pLKO.1 and pLKO.1-puro-CMV-TurboGFP) expressing shRNAs specific for AID (TRCN0000112031), Smc1 (TRCN0000109034), Smc3 (TRCN0000109007), Nipbl (TRCN0000124037), Wapal (TRCN0000177268) Med1 (TRCN0000099576), Med12 (TRCN0000096466) or a non-target control (SHC002) were obtained from Sigma-Aldrich. The lentiviral vectors were transiently transfected into Lenti-X 293T cells (Clontech) to produce infectious viral particles as described [145]. Two days later CH12 cells were spin-infected with viral supernatants supplemented with polybrene (10 µg/ml; Sigma-Aldrich). Cells were selected for 5 days with puromycin (1 µg/ml) before CSR induction.

Mice

Med1$^{f/f}$ mice (provided by T. Borggrefe) were crossed with Mb1-Cre mice (provided by P. Kastner). Mice were on a B6/129 mixed background and bred and maintained under specific pathogen-free conditions. 8-12 week old mice were used in experiments. All animal work was performed under protocols approved by the Direction des Services Vétérinaires du Bas-Rhin, France (Authorization N° 67-343).

Cell culture and flow cytometry

CH12 cells transduced with lentival vectors expressing shRNA were cultured with IL4, TGFB and CD40 to switch to IgGA and selected with puromycin. After 72h, the IgA surface expression was analyzed by flow cytometry. For proliferation analysis, CH12 cells transduced with lentiviral shRNAs (lacking a GFP reporter) were labeled with 5 µM CFSE (Invitrogen) for 10 min at 37°C prior stimulation. For cell cycle analysis, transduced CH12 knock down lines unstimulated or stimulated for 48h have been sorted for GFP expression and fixed in 70% ethanol o/n. After staining with PI 25 µg/ml (Sigma), RNase A 50 µg/ml (Sigma) in PBS for 30 min at 37°C, cells have been analyzed for DNA content.

Resting splenic B cells were isolated using CD43-microbeads (Miltenyi Biotec) and cultured for 72h with LPS and IL-4 (5 ng/ml; Sigma-Aldrich) to switch to IgG1. CSR was assayed by flow cytometry as described [349].

ChIP-Seq

Resting or activated B cells were crosslinked for 10 min at 37 °C with 1% (vol/vol) formaldehyde, followed by quenching with glycine (0.125 M final concentration). Crosslinked samples were then sonicated to obtain DNA fragments 200–500 bp in length using a Covaris sonicator (Covaris). Chromatin (from 10 × 10^7 cells) was precleared with protein A magnetic beads pre-washed with PBS 0,05% tween, 5% BSA and immunoprecipitated in ChIP dilution buffer (0.06% SDS, 20mM Tris (pH 8.1), 2mM EDTA, 160 mM NaCl, 1.045% Triton X-100, 0.05 X proteinase inhibitor cocktail) overnight at 4 °C with protein A magnetic beads (Invitrogen) coupled to 100 µg of Smc1 or Smc3 antibodies and processed according to the Millipore protocol. Crosslinks were reversed for 4h at 65°C in Tris-EDTA buffer with 0.3% (wt/vol) SDS and proteinase K (1 mg/ml). ChIP DNA was extracted with IPure Kit (Diagenode). Libraries were prepared for sequencing following the manufacturer's protocol (Illumina) and sequenced on the Illumina Genome Analyzer IIx as single-end 50 base reads following Illumina's instructions. Image analysis and base calling were performed using the Illumina Pipeline and sequence reads were mapped to reference genome mm9/NCBI37 using Bowtie v0.12.7. Peak calling was performed using MACS [350] with default parameters. Global comparison of samples and clustering analysis were performed using seqMINER [351].

ChIP-qPCR

Analytical-scale ChIP was performed on chromatin prepared from 10^7 (resting or activated) splenic B cells isolated from a pool of 5 mice. qPCR was performed at several locations across the IgH locus using primers listed in Table S4. Results are expressed as percent input and represent the mean of three qPCR technical replicates. Error bars represent the corresponding standard deviation.

4C-Seq experiments

4C-seq was preformed as previously described [352]. The primary restriction enzyme used was HindIII and secondary restriction enzyme DpnII. Primers for the 3'RR bait were : 5' CTACCCACCTAACTCCAAGC 3' and 5' CCAGACATGTGGGCTGAGAT 3' . Mapping was done using bowtie allowing for zero mismatches against a reduced genome containing all unique 24bps surrounding every HindIII site in the genome. Between 2 to 4 million mapped reads were obtained for all samples and at least half of the reads were mapped to chromosome 12. DESeq 1.6.0 was used to normalize raw read count in 10kb windows centered on each HindIII site in the constant region of IgH (Chr12: 113175000-113475000, mm10) [353]. The following parameters were used with the estimateDispersion function: method=pooled, sharingMode=maximum. The nbinonTest function was used to determine which windows have a significantly different 4C-seq signal. An adjusted p-value of 0.1 following Benjamini-Hochberg FDR correction was used as a cut-off. Domainograms analysis were

were generated by determining the quantile score for each genomic location at increasing length scales (5 to 30 HindIII sites) as performed as in [58].

Table S1. Antibodies used.

Antibody	Clone	Source	Use*
AID		Gift from Michel Nussenzweig	ChIP
Smc3	ab9263	Abcam	ChIP
β-Actin	A1978	Sigma	WB
Med1		Abcam	ChIP
Med12		Euromedex	ChIP, WB
phospho-H2AX	JBW301	Millipore	WB
phospho-Chk1	133D3	Cell Signaling	WB

* WB: Western blot; ChIP: Chromatin immunoprecipitation.

Table S2. Primers and probes used.

ChIP		
Primer	**Sequence (5'-3')**	**Reference**
Eμ-Rev	ACCACAGCTACAAGTTTACCTA	[120]
5'Sμ-Fwd	TAAAATGCGCTAAACTGAGGTGATTACT	[349]
5'Sμ-Rev	CATCTCAGCTCAGAACAGTCCAGTG	[349]
3'Sμ-Fwd	CTGAATGAGTTTCACCAGGCC	[122]
3'Sμ-Rev	GCCTGTCCTGCTTGGCTTC	[122]
Cμ-Fwd	GTTCTGTGCCTCCGTCTAGC	[346]
Cμ-Rev	AGCATTTGCATAAGGGTTGG	[346]
Sγ3-Fwd	GCTGAGAGTATGCACAGCCA	[122]
Sγ3-Rev	GGATCATGGAAACTCCTCCG	[122]
Sγ1-Fwd	GGAGGTCCAGTTGAGTGTCTTTAG	[35]
Sγ1-Rev	TTGTTATCCCCCATCCTGTCACCT	[35]
Cα-Fwd	CTCCTGTCTCACAGGCCTTC	[346]
Cα-Rev	CATGGGCCTTTACTCCACTC	[346]
Hs6,7-Fwd	CCCTGGTGACCATGTGTGT	[346]
Hs6,7-Rev	TCTGGGTCTGTTTTGTTACTGAAA	[346]

Discussion

The recruitment of AID is transcription-coupled [354] and occurs at sites of Pol II stalling [120,126], through interactions with the transcription elongation factor SPT5 [126] and additional transcription-related protein complexes, like the PAF complex [145], RPA [48] or 14-3-3 [133] for example. We have found that nuclear and chromatin-bound AID associates with subunits of the cohesin and mediator complex. Because recruitment of cohesin together with the mediator complex [326] has been reported to be indicative of long-range DNA interactions between enhancers and promoters, and since CSR has been proposed to occur through the formation of a long-range DNA loop involving the intronic promoters, the switch regions, the Eμ enhancer and the 3' regulatory region (3'RR) [209], we hypothesized that the function of cohesin and mediator complexes is to regulate three-dimensional, transcription-coupled, structural changes taking place at the IgH locus during CSR.

In a first part, I will discuss the results that we obtained from the study of cohesin's role in CSR. In a second part, I will focus on the role of mediator and more particularly Med1 subunit in antibody diversification. Finally I will try to match results obtained in the two studies to integrate them in the current knowledge of CSR mechanisms.

I. The cohesin complex in CSR

We have found that nuclear and chromatin-bound AID associates with subunits of the cohesin complex, however, we do not know whether any of the interactions we have described are direct or indirect. Nevertheless, the functional relevance of the association between AID and cohesin is highlighted by defective CSR and abnormal DSB resolution after knock down of individual cohesin subunits.

1. Cohesin is recruited at the IgH locus

Recruitment of cohesin together with the mediator complex [326] has been reported to be indicative of long-range DNA interactions between enhancers and promoters. Additionally, cohesin mediates transcriptional insulation with CTCF [337] and together they have been shown to control the RAG1/2-dependent rearrangement of antigen receptor genes during early B and T lymphocyte development, by mechanisms involving the regulation of transcription and formation of long-range *in cis* DNA interactions [244,343,344]. Cohesin can also play a role in transcription independently of CTCF via long-range chromosomal interaction [355]. CSR has been proposed to occur through the formation of a long-range DNA loop involving the intronic promoters, the switch regions, the Eμ enhancer and the 3' regulatory region (3'RR) [209], leading us to hypothesize that the function of cohesin is to regulate three-dimensional, transcription-coupled, structural changes taking place at the IgH locus during CSR. Consistent with this, we found that Smc1, Smc3 and CTCF are co-recruited to the 3'RR in resting B

cells and that upon B cell activation, both Smc1 and Smc3 are additionally recruited to Sμ and Cμ, independently of CTCF.

According to the model proposed by Wuerffel and Kenter [138], Eμ and the 3'RR interact in resting cells. The fact that cohesin is absent of Eμ region in resting cells, suggests that cohesin is not implicated in this first loop. Because cohesin and CTCF have been shown to mediate transcriptional insulation in HeLa cells at the H19/IGF2 locus by their binding at the H19 imprinting control region (ICR) or at the β-globin locus through binding at the DNAse hypersensitive site 4 located in the β-globin locus control region (LCR) [337] and because the hs5,6 and 7 from the 3' RR of the IgH locus contain insulator activity [139], it is possible that cohesin and CTCF might be recruited at the 3'end of the IgH locus to prevent communication between IgH enhancers and other genes farther downstream, suggesting that the recruitment of cohesin at the 3'RR is independent of CSR. It would be interesting to assess the transcription level of downstream genes in control and cohesin deficient cells to investigate the insulator role of cohesin at the 3'RR.

Alternatively, considering the ability of cohesin to control long-range DNA interactions, cohesin could also create DNA loops between enhancers and promoters to mediate transcriptional activation. Consistent with this, in stimulated B cells, Smc1 and Smc3 are dynamically recruited to the IgH locus, at a region spanning from the 3' end of the donor switch region (Sμ) to the 3' end of the Cμ constant region and did not comprise the Eμ enhancer. Binding of cohesin at the donor switch region and at the 3'RR might suggest that cohesin is implicated in maintaining interaction between these elements upon B cell activation.

Wuerffel and Kenter also proposed that upon B cell activation, the acceptor switch region is brought into close proximity to Eμ and the 3'RR to favor transcription and recombination. Unfortunately, in stimulated cells, no reproducible recruitment of Smc1 or Smc3 over the Sγ1 switch region could be detected leading to two major hypothesis:

a) cohesin is recruited at Sγ1 region but we are unable to detect it. Indeed, perhaps our cell culture conditions (in which approximately 15% of the cells switch to IgG1) are not robust enough to detect a specific enrichment. Additionally, during stimulation, length of CSR is quite heterogeneous: after 60h of stimulation (crosslinking time point), some cells undergoing CSR might already be $IgG1^+$ (CSR is over), whereas some might still be recombining (beginning of germline transcription or AID targeting or DSB processing or DNA repair...). In this sense, if the recruitment of cohesin at Sγ1 region were transient and only occurring at one specific time during CSR, the heterogeneity of the cellular population might prevent us from detecting cohesin recruitment efficiently. In that case, cohesin might be recruited at the 3'RR and at the donor and acceptor switch regions to create and maintain the loop occurring in cells undergoing CSR.

b) Smc1 and Smc3 are neither recruited to the acceptor switch region nor required for the interaction between acceptor switch region and enhancers upon B cells activation. This suggests that looping structures occurring in stimulated cells are independent of cohesin and thus additional unknown proteins or complexes might be required. Analysis of the proteomic screen showed that mediator [326]

and condensin [356] complexes, which have been involved in chromosome dynamics, interact with AID and thereby could constitute interesting candidates to study within the context of DNA looping of the IgH locus.

Another point that has to be taken into account is the peculiar status of Cγ1 region. Indeed, it has previously been shown that for all isotypes, deletion of hs3b.4 of the IgH locus leads to both strong decreased transcription of the acceptor switch region [136] and decreased DNA looping [138]. However, Cγ1 region seems less affected by this deletion, as only minor effect on transcription and looping can be detected. This leads to two alternatives: either looping and transcription of Sγ1 is less dependent on hs3b,4 than other acceptor switch regions (potentially other enhancers are sufficient like hs1.2 and Eμ), or the IL4 dependent promoter is strong enough to overcome the reduction in looping and transcription. It would be interesting to perform ChIP-Seq experiments on cells stimulated to switch to other isotypes to determine if cohesin is recruited to other acceptor regions and if this recruitment is more pronounced. Given the poor percentage of cells that switch after in vitro stimulation to other isotypes (max 5%) together with the population's heterogeneity, we might not be able to detect clear cohesin recruitment on other acceptor regions neither.

2. Knock down experiments

To assess whether cohesin play a role in CSR, we performed cohesin knock down experiments on CH12 cells that lead to impaired CSR for all the subunits of the cohesin complex. While optimizing cohesin knock down experiments, we observed a direct relationship between knock down efficiency and a defect in cell proliferation. Indeed, increasing the efficiency of the knock down for the cohesin subunits (Smc1, Smc3 and Nipbl) led to a decreased cell proliferation. Deletion of Wapl subunit did not mimic this phenotype, reflecting the requirement of cohesin loading on DNA for proper cell division. To rule out a drastic effect of cohesin knock down on cell division, we performed cell cycle analysis and verified cell cycle checkpoint activation. We found that cohesin knock down doesn't induce significant activation of the DNA damage response or cell cycle checkpoints, nor defective cell cycle progression. This result suggests that residual levels of cohesin are sufficient to ensure the basal cell functions.

The defect in CSR efficiency was not due to reduced AID expression or germline transcription for Smc1, Smc3 and Nipbl knock down. On the contrary, Wapl knock down led to a reduced transcription at the acceptor switch region Sα, suggesting that the CSR impairment observed upon Wapl knock down is due to a reduction in GLT. This result might suggest that retention of cohesin at the IgH locus affects gene expression. However, it is possible that the effect observed upon Wapl depletion can be due to some off-target effects exerted by the shRNA. Concerning a potential more global effect on transcription, we cannot exclude the possibility that the expression of additional genes required for CSR (other than AID) is affected by the knock down of cohesin.

Knock down of Smc1, Smc3 or Nipbl does not have a deleterious effect on IgH locus transcription or AID expression. Additonally, cohesin does not influence the efficiency of AID recruitment to the IgH

locus, as tested by ChIP-qPCR experiments on Sμ, suggesting that cohesin is not implicated in AID targeting. Therefore, the role of cohesin in CSR appears to be independent of regulating switch region transcription and/or AID recruitment at the IgH locus and may be exclusively related to providing a scaffold for the efficient formation of long-range three-dimensional structural changes.

We have also found that knock down of the core cohesin subunits (Smc1, Smc3) and the cohesin loader subunit (Nipbl) has a significant effect on the resolution of AID-induced double stranded DNA breaks, which translates into an increased usage of microhomology at the switch junctions. As the knock down of Wapl, the subunit that is required to remove cohesin from chromatin [252] does not appear to be significantly changed when compared to the non-target control, it appears that the loading of cohesin is sufficient to determine the outcome of DSB. As the initial steps of AID-mediated DSB formation depend on UNG and Msh2/6, it would be interesting to assess their level of expression upon cohesin knock down. Moreover, given that cohesin has been implicated in the recruitment of 53BP1 to γ-irradiation-induced foci [271], and that 53BP1-deficiency leads to defective CSR, increased DNA end resection and preferential usage of microhomology [357], we speculate that cohesin could participate in the recruitment of 53BP1 to AID-induced DSBs and that defective 53BP1 recruitment could account for the increased usage of microhomology observed. Additionally, 53BP1 deficiency favors short-range over long-range interactions, suggesting that 53BP1 could play a role in maintaining synapsis of distal switch regions [211]. It seems that cohesin could be implicated in looping either directly or by 53BP1 recruitment to favor synapsis in a transcription independent way.

3. 3D structure of the IgH locus

To solve the implication of cohesin complex in IgH looping structure, we undertook 4C-Seq experiments on control and cohesin-deficient CH12 cells that were stimulated to switch to IgA. Using a bait in the 3'RR, we were able to detect specific interactions between Eμ and the 3'RR in stimulated CH12 cells transduced with a non-target shRNA, reproducing data obtained in primary B cells [138]. Interestingly, knock down of cohesin subunits did not show any differential interaction between the 3'RR and Eμ compared to the non-target control, which could indicate that this interaction is independent of cohesin complex. However, another explanation could be that knock down of cohesin subunits is not sufficient to detect variation in the 3D structure of the IgH locus reflecting experiments done on ES cells that showed that partial loss of Nanog by knock down (78%) has no effect on contact profiles contrary to full knock out, indicating that complete deletion of protein is required to affect chromosome topology [358]. To solve the question of the implication of cohesin in DNA looping at the IgH locus, 4C-Seq experiments should be done on knock out cells. For this purpose, we are currently generating conditional Smc3 knock out mice that will allow us to investigate this point.

Our observations are consistent with a model in which cohesin binds at the 3'RR to act as a insulator to physically separate IgH locus from downstream genes and that is additionally recruited at Sμ-Cμ

and potentially to acceptor Sγ1 region to favor synapsis between the DSB occurring at the donor and acceptor switch region.

II. The mediator complex in antibody diversification

Because the mediator complex has been implicated in the formation of long-range DNA loop between enhancers and promoters in ES cells (with cohesin complex) [326] and because cohesin complex is implicated in CSR possibly through a role in long-range interaction, we hypothesized that mediator complex could participate along with cohesin complex to the regulation of structural changes taking place at the IgH locus during CSR. We have found that nuclear and chromatin-bound AID associate with subunits of the mediator complex. We have found that conditional deletion of Med1 in developing B cells affects CSR efficiency through reduction of the acceptor switch region transcription that correlates with a reduced interaction between Eμ and the acceptor switch region.

1. Med1 deletion

Med1 subunit of the mediator complex has previously been implicated in the transcriptional regulation of different genes, mostly via its interaction with nuclear receptors [359], or with the GATA family of transcription factors [297]. Interestingly, Med1 only exists in a subpopulation of the Mediator complex that is tightly associated with Pol II and is able to mediate transcription *in vitro* [291]. Knowing that CSR is transcription-dependent, Med1 was thereby a good candidate to mediate the recombination.

As Med1 deletion is known to be embryonic lethal [296], we generated Med1 conditional knock out in B cells and analyzed their development. Although we didn't detect any difference in the cellular composition of the bone marrow, we noticed an increase proportion of marginal B cells in the spleen and a reduction of the follicular population. This could be explained by the fact that Med1-containing mediator complex could modulate the expression of one or several key factors implicated in the development of follicular and marginal B cells. For instance, one interesting candidate could be Notch2, as the Mediator complex has previously been shown to regulate the Notch signaling pathway in Drosophila [360], and as this specific protein is required for proper differentiation in marginal B cells [361]. It would thereby be interesting to assess the expression of Notch2 and other known key factors involved in B cell development, like BTK [362] in Med1-deficient cells.

2. Role of Med1 in SHM

When we analyzed the mutation profile of the J_{H4} intron 10 days after immunization, we found that despite a normal mutation frequency during SHM, Med1 deletion leads to decreased mutation from A bases and increased mutation from C and T bases. Mutations at G:C bp are known to result from the

direct action of AID, whereas mutations at A:T bp derives from the processing of the lesion by the MMR and to a lesser extent by the BER complex. In Med1-deficient cells decreased mutations from A bases suggest that Med1 is implicated in the phase 2 of mutations. Indeed, the mutational profile shares some characteristics with those observed when compounds of phase 2 are defective: for example, patients bearing mutation in ATR, which is recruited by MSH2/6, have a normal frequency of SHM with fewer mutations at A and more mutation at T residues (transitions) [109]. Another example is Polη, an error-prone polymerase recruited by MSH2/MSH6 or PCNA, which favors mutations of A:T residues on the non-transcribed strand as deletion of Polη in mice revealed a marked decrease in mutation of A and T bases (more pronounced for A bases) and an increased mutations of C and G (more pronounced for C bases) compared to control [113] and patients with *xeroderma pigmentosum* harboring a defect in Polη, show a normal frequency of SHM [112]. The fact that mutation frequency is unchanged after Med1 deletion indicates that the first steps of SHM are unaffected and that proteins like AID, UNG or MSH2/6 which are known to initiate mutations are probably unaffected. Our results suggest that Med1 might be implicated either in the expression of downstream factors or in their recruitment to the lesions to favor mutations at A:T bases. Assessing their level of expression or their recruitment profiles by ChIP would allow us to decipher the precise role of Med1 in SHM.

3. Role in CSR

In order to address our hypothesis that mediator complex is implicated in the 3D structure of the IgH locus upon B cells activation, we analyzed the ability of B cells from Med1-deficient mice to undergo CSR *in vitro*. We detected a CSR defect to all isotypes tested (IgG1, IgG3, IgG2b and IgG2a) and observed a reduction in the proliferation of B cells. However, among the cells that proliferate equally, we still detected a decreased CSR efficiency, especially for cells stimulated to switch to IgG1 and IgG3, indicating that reduced CSR is due to a B cell intrinsic defect. Reduction in CSR efficiency per cell division was less obvious for cells stimulated to switch to IgG2b and IgG2a, probably because the CFSE profile did not allow us to identify and separate each cell division properly. The decreased proliferation induced a slight difference in the proportion of cells in each cell cycle phase, however no block in cell cycle progression is occurring upon Med1 deletion.

The biased differentiation towards marginal B cells could also account for the defect in CSR and SHM from $Med1^{f/f}$ $Mb1^{Cre/+}$ mice. Marginal B cells being even more efficient at proliferating and switching than follicular B cells ruled out the impact of this imbalance on CSR. Using transgenic mice, Phang et al. [363], showed that SHM is delayed in marginal B cells compared to follicular B cells 6 days after the challenge with the antigen. However, by day 7, the mutation rate of marginal and follicular B cells was equivalent and both populations contain cells that acquired multiple mutations. As analysis of SHM in Med1-deficient B cells was done after 10 days of immunization, we can expect that at that time point, cells have overcome their delayed SHM process, which is confirmed by the equivalent frequency of mutations. The biased differentiation into marginal B cells is likely unrelated to antigen-dependent antibody diversification.

Additionally, it is important to keep in mind that the mediator complex can actually exist *in vivo* in the absence of its Med1 subunit [291,364]. It is thereby very hard to determine whether Med1-depleted mediator complex could still be able to interact with specific cofactors required for CSR through other subunits. To solve this issue, we knocked down Med1 and Med12, which belong to distinct mediator modules in CH12 cells. *In vitro* CSR assays confirmed the reduction in CSR after Med1 depletion and additionally showed that Med12 also plays a role in the mechanism, pointing towards the role of not only the Med1 subunit, but also the whole mediator complex in CSR recombination. However, confirmation with other core subunits of mediator may be required to completely ensure its essential role in CSR.

As mediator complex is implicated in basal and activator-dependent transcription, deletion of Med1 could affect switch region transcription or AID expression. In Med1-deficient cells, impairment in CSR was independent of AID expression and transcription of the donor switch region. However, acceptor switch region transcription was reduced after Med1 deletion, leading to defect in CSR. Moreover, Med1 deletion might affect the expression of additional genes required for CSR. To investigate this, performing RNA-Seq on WT and Med1-deficient cells would give us good hints concerning the expression profile of key genes involved in this process.

AID recruitment is dependent on GLT, the donor switch region being unaffected by Med1deletion, we decided to investigate the role of mediator complex in AID recruitment at Sμ. We performed AID ChIP on CH12 cells expressing shRNA targeting Med1, Med12, AID and a non-target control. Interestingly, qPCR done at Sμ revealed that AID recruitment was reduced upon mediator depletion, suggesting that additionally to its role in transcription, the mediator complex might be implicated in AID recruitment at the IgH locus.

4. Recruitment of mediator subunits at the locus

To determine whether mediator complex is bound to the IgH locus during CSR, we performed ChIP experiments targeting Med1 subunit. As the protocol used for cohesin ChIP has been shown to be suitable also for mediator ChIP experiments [326], we decided to use the same sample preparation procedure than previously described (see results part I).

Med1 ChIP-qPCR performed on WT and Med1-deficient cells indicated that Med1 is recruited to Eμ and to Sμ region in resting and stimulated cells, suggesting that Med1 recruitment is unchanged during CSR. It also points to the fact that Med1 recruitment at Eμ and Sμ is not important for Cμ expression, as Med1 deletion doesn't affect Cμ transcription. To comfort this data and potentially discover novel binding sites, we performed ChIP-Seq experiments but couldn't detect any peak of enrichment, the obtained profile being almost identical to an IgG ChIP-Seq, despite confirmation by qPCR. One possible explanation for the fact that we could detect specific recruitment in ChIP-qPCR that was not visible in ChIP-Seq might be inherent to the type of method used. Indeed, in ChIP-qPCR, we directly compared the enrichment to the input, whereas in ChIP-Seq, some of the recruitment might

be hidden by the background signal. In this sense, ChIP-qPCR might be more sensitive than ChIP-Seq, making it thereby more likely to reflect what is really happening within the cell. It is also possible, that the efficiency of ChIP is not great and perhaps that's why even in qPCR we might not detect Med1 at other location, like the acceptor switch region for example.

Because our Med1 ChIP-Seq experiment didn't reveal Med1 binding profile, we decided to perform the same type of experiment using another subunit of the mediator complex: Med12. After validation of the ChIP by western blot, we performed ChIP-Seq experiment which unfortunately, and similarly to the one of Med1, didn't give any significant result. As both Med1 and Med12 ChIPs didn't provide great data sets, one possible explanation could be that, considering the size of the mediator complex, the crosslinking we performed in both cases might not be strong enough to maintain interactions between mediator complex and DNA or other proteins. To address this issue, one solution could be to perform a double crosslinking since enhancing strength of interactions might result in an enhanced signal over noise ration during sequencing.

5. Role of Med1 in DNA looping

Transcription of acceptor switch regions depends on the 3'RR [136,137] and correlates with a defect in the 3D organization of the IgH locus [138], indicating that transcription is linked to the looping process occurring during CSR. To solve the implication of Med1 in the looping process during CSR, we proceeded to 4C-Seq experiments on control and Med1-deficient cells. In a first experiment, we digested chromatin from both cell types stimulated to switch to IgG1 with HindIII and DpnII and used a bait located in the 3'RR (in parallel from the experiment done on cohesin knock down cell lines). No strong interaction was detectable with γ1 region, which can be explained by the lack of numerous HindIII restriction sites suitable for 4C experiments in this region, making the analysis of this γ1-3'RR loop difficult to interpret when using HindIII digestion.

Nevertheless, in WT cells, we detected interactions between Eμ and the 3'RR. However, comparing this interaction between control and Med1-deficient cells showed that Med1 deletion didn't affect this loop. Additionally, in WT cells, interactions with different V genes, and especially with distal V genes for two WT out of three were detectable, whereas these interactions were weaker in Med1-depleted cells. This suggests that either Med1 deletion affects these long-range interactions in resting and/or in stimulated cells, or that these V genes are not used in the V(D)J recombination in the conditional knock out cells and thus don't interact with the constant region and the enhancers, suggesting a defect in V(D)J recombination to distal V genes after Med1 deletion. Since similarly to CSR, V(D)J recombination is dependent on both transcription [16] and locus contraction [26], Med1 could play a role in this mechanism. Interestingly, analysis of V(D)J recombination revealed that Med1 deletion was not affecting recombination to distal V genes, thereby indicating that Med1 is probably not implicated in this mechanism.

To parry the poor quality of resolution from the first 4C-Seq experiment and more specifically the lack of HindIII sites within our region of interest, we repeated the experiment using two 4bp cutter enzymes (DpnII and Csp6I), which should increasing the pool of fragment ends available for the analysis [365]. Using Eμ as a bait, in resting WT cells and in accordance with our previously obtained data with HindIII and DpnII digestion, Eμ interacts strongly with the 3'RR (one peak on hs3a-hs1.2 and one centered on hs4). This new type of digestion revealed also novel potential sites of interaction with the constant region Cμ and Cδ as well as with regions surrounding constant exons of the IgH locus (Cγ3 for example). Interestingly after stimulation, Eμ interacts with the region surrounding Cγ1 and Cε. which is expected after stimulation with LPS and IL4 and thereby constitute a nice positive control. We could also observe an enhanced recruitment of the hs3a-1.2 region suggesting that these enhancers are critical for the conformational change of the IgH locus. Replacing each one of these elements by a neomycin cassette affects CSR, but, the effect is most probably due to a false positive since the replacement by LoxP sites is known to restore it [135]. Nevertheless, deletion of only these two elements has not yet been realized and thus the impact on CSR has never been clearly investigated.

Additionally, after stimulation, Eμ interaction with Cδ is significantly reduced. It is possible that this region is looped out to allow the acceptor switch region to come closer to the donor Sμ. Cγ3 interaction is also reduced, but this interaction was initially not very strong in resting condition and might be due to the ability of the IgH locus to interact with constant regions in a non-specific way. In this sense, stimulation could lead to block non-specific interactions with Cγ3 and promote specific interactions with Cγ1 and Cε.

Comparing resting and stimulated cells from Med1-deficient mice revealed the same pattern of interactions than in WT cells: strong interaction with the 3'RR in resting and stimulated cells; increased interactions with the region surrounding Sγ1 and hs3a-1,2 region; slight increased interaction with Cε; lower interactions with Cδ and the region preceding Cγ3 upon stimulation. All of all, this firstly suggests that Med1 deletion doesn't affect the conformation changes of the IgH locus upon B cell activation. Additionally, plotting results from unstimulated WT and Med1-deficient cells indicates that the two profiles are similar, suggesting that the loop between Eμ and the 3'RR is stable even in the absence of Med1 and thereby most likely independent of Med1 subunit. However, plotting profiles from WT resting and stimulated cells with stimulated cells from Med1-deficient cells showed that upon Med1 deletion, the interaction with the region surrounding Sγ1 in stimulated cells is less important in Med1-deficient cells. Analyzing specific regions displaying differences from day 0 to day 2 revealed significant changes in Eμ-Sγ1 interaction between both stimulated WT and Med1-deficient cells. This difference, despite being slight and not being confirmed by an unbiased analysis of the locus, indicates that in the absence of Med1, interactions between the Eμ and Sγ1 might be diminished, suggesting that Med1 deletion might affect the 3D conformation of the IgH locus upon stimulation. Two different hypothesis could possibly account for this intermediate phenotype:

a) Interactions between Eμ and Sγ1 are reduced after Med1 deletion, but again heterogeneity in cells status, as developed previously for cohesin ChIP-Seq and 4C-Seq experiments, could account for the unsettled results. Additionally, Med1 deletion leads to only 45% reduction of CSR efficiency, indicating

that some cells manage to undergo CSR and to perform DNA looping in the absence of Med1, suggesting that Med1 alone is not absolutely required for CSR. It is possible that mediator complex is required for the looping, but that Med1-depleted mediator complex can interact with the IgH locus through other subunits (not as efficiently as Med1 though, as CSR is still affected). To test this hypothesis, deletion of other Mediator complex subunits could allow us to confirm the implication of mediator complex in CSR.

b) Defect in acceptor switch region transcription reduces CSR efficiency leading to reduced interaction between Eμ and Sγ1. In the absence of Med1, acceptor switch region transcription and interactions between Eμ and Sγ1 are affected upon B cell stimulation. CSR efficiency being reduced, we might expect to see diminished interaction between Eμ and Sγ1 region but this doesn't inform us on what causes the defect in CSR. Indeed, germline transcription correlates with the recruitment of the acceptor switch region to regulatory elements. However, we do not know which step comes first. Does the reduction in transcription affect the looping? Or does the reduction in looping affects the transcription? So far, no study has been clearly discriminating between the transcription step and the looping step during CSR.

Analysis of this second experiment (DpnII and Csp6I) revealed that there is no defect of interactions with distal genes in Med1 depleted cells either stimulated or not, suggesting that the reduced interactions with distal V genes observed in the first experiment (HindIII and DpnII) were not due to Med1 deletion and that most likely this first observation was due to heterogeneity inherent to sample preparation.

6. GLT and loops: Which came first the chicken or the egg?

A strong relationship exists between chromatin looping and gene expression. It has been shown that DNA looping can occur prior to gene activation and that transcription is associated with additional looped interactions. Indeed, promoters for genes coding for TH2 cytokines are located in close proximity in different cell types and in TH2 cells, in which they should be expressed, the locus control region is additionally implicated in this structure [241]. Another example is the spatial clustering of hypersensitive sites from the LCR of the β-globin, which change upon cell differentiation [233]. Finally, this link is also present at the IgH locus, as deletion of hs3b.4 affects transcription and IgH looping [138]. However, it has been shown that ongoing transcription or the presence of pol II at regulatory sites is not required to maintain loops, suggesting that the maintenance of long-range interactions is rather dependent on trans-acting factors more than transcription itself [237]. Further insights into the molecular mechanisms that establish and maintain chromatin loops derives from studies of the interactions between LCR and promoters of the β-globin locus. GATA1 and its cofactors FOG1, KLF1 and Ldb1 have been implicated in these LCR - β-globin interactions and depletion of any of these factors leads to both reduced interactions and reduced transcription. It has been shown that forcing juxtaposition of LCR and promoter in cells deficient for GATA1 allows the recruitment of the Pol II, its activatory

phosphorylation and thereby promotes β-globin expression, indicating that looping is a prerequisite for transcription activation at the β-globin locus [238]. It would be interesting to perform this type of experiments to answer if the looping occurs prior to the transcription at the IgH locus during CSR.

Overall, our results describe the role of Med1 in CSR and in SHM. Med1 deletion leads to diminished long-range interactions correlating with defective transcription of acceptor switch regions during CSR. Mediator complex being implicated in the transcription machinery, looping and transcription defects in Med1-deficient cells are likely concomitant. Additionally, Med1 seems to play a role in the generation of mutations during SHM.

III. Cohesin and mediator: synergic effect?

1. DNA repair

Cohesin has been implicated in recruitment of 53BP1 to γ-irradiation-induced foci [271]. Additionally, knock down of cohesin [346] or knock out of 53BP1 [212] led to defective CSR and AID-induced breaks repair through usage of microhomology, suggesting that cohesin could recruit 53BP1 to AID-induced breaks. Additionally, 53BP1 deficiency favors short-range over long-range interactions, suggesting that 53BP1 could play a role in maintaining synapsis of distal switch regions [211]. To address whether mediator could be implicated in 53BP1 recruitment with cohesin and in the repair of AID-induced breaks, we analyzed switch junctions of Med1-deficient cells, but no difference was observed after Med1 deletion. This indicates that Med1 is most likely not implicated in the repair of AID-induced DNA breaks.

2. Recruitment to the IgH locus

Performing Smc3 ChIP-qPCR on Med1-deficient cells stimulated or not to switch to IgG1 could allow us to determine the implication of Med1 in cohesin recruitment to the IgH locus. Indeed, we showed that this recruitment is not affected by Med1 deletion in resting cells. However, upon B cell activation, Smc3 recruitment seems to be abrogated at the Sμ-Cμ region and interestingly also at the 3'RR. It is thereby possible that Med1 is implicated in cohesin recruitment occurring during CSR. However, considering that cohesin recruitment at the 3'RR and at Cα is concomitant to CTCF recruitment and most likely independent of Med1 in resting cells, it is possible that the ChIP experiment didn't work properly. This experiment needs to be reproduced several times to solve this issue. Moreover, if Med1 was responsible for cohesin recruitment at the IgH locus upon B cell activation, we would expect a similar effect than cohesin deletion, switch junctions bearing microhomologies.

3. Cohesin, Mediator and CTCF as architectural proteins

Analysis of long-range interaction in ES cells revealed that distinct combinations of CTCF, mediator and cohesin regulate chromatin organization at different size scales. Indeed, CTCF and cohesin binding reflects more constitutive interactions probably implicated in the formation of invariant subdomains, whereas mediator and cohesin recruitment is implicated in enhancer-promoter interactions and their deletion results in disruption of the chromatin architecture and downregulation of genes [330]. This profile of recruitment of CTCF, cohesin and mediator observed in ES cells correlates with the data that we have in B cells, where binding of CTCF and cohesin at the 3'RR might be implicated in the separation of the IgH locus and downstream genes, whereas deletion of Med1 leads to an impairment in transcription that might be due to loop disruption.

Working model for CSR

We showed that cohesin is recruited with CTCF at the 3'RR of the IgH locus in resting and stimulated B cells. Upon stimulation, cohesin is additionally recruited to a region spanning from Sμ to Cμ and potentially at the acceptor Sγ1 region, indicating that cohesin is dynamically recruited during CSR and might play a role in the 3D structure of the IgH locus. Depletion of Smc1, Smc3 and Nipbl impaired CSR, independently of AID expression or switch region transcription. Analysis of switch junctions revealed that their depletion leads to a microhomology-based repair, involving cohesin in the AID-induced breaks repair. Cohesin being implicated in 53BP1 recruitment to γ-irradiation-induced foci, it is possible that during CSR, cohesin recruits 53BP1 to favor synapsis between switch regions.

On the other hand, we showed that Med1 deletion reduces CSR efficiency. Despite normal donor switch region transcription, acceptor switch region transcription is affected by Med1 deletion and this correlates with reduced interactions between Eμ and Cγ1. Mediator complex has been implicated in basal and activator-dependent transcription, our results suggest that during CSR, Med1 favors acceptor switch transcription through interactions between promoters and regulatory elements. Moreover, we showed that AID recruitment at Sμ is dependent on mediator complex, suggesting that in addition to its role in transcription and looping, the mediator complex might be required for proper AID targeting.

We propose a working model where the mediator complex could be recruited at the Eμ enhancer in a steady-state. Upon stimulation, the mediator complex could be recruited at the acceptor switch region creating a loop between the regulatory elements and the acceptor switch region as suggested by 4C-Seq results upon Med1 deletion. This loop might favor acceptor switch transcription through the recruitment of the PIC and the Pol II. Additionally, the mediator complex could be implicated in AID targeting to the IgH locus, as knock down of Med1 and Med12 subunits affect AID recruitment at Sμ. As mediator complex has been shown to cobind with the cohesin complex at enhancers and promoters in ES and iPS cells [326,327,329,330], the mediator complex could recruit the cohesin complex to maintain this interaction in order to allow the donor and the acceptor switch regions to recombine. Indeed, cohesin ChIPs revealed that Smc1 and Smc3 are recruited to Sμ and potentially to the acceptor switch region upon B cell activation. Cohesin would then favor switch region synapsis and repair through the NHEJ pathway, as cohesin depletion by shRNA leads to a microhomology-based repair of the switch junctions. To do so, cohesin could recruit 53BP1 to favor synapsis between distal switch regions and to protect DNA ends from resection. Indeed, 53BP1 has been shown to be recruited by cohesin at γ-irradiation-induced foci [271] and has been suggested to have two roles in CSR: favoring the synapsis between the switch regions [211,212] and blocking DNA end resection [357], which is required for the production of ssDNA, mandatory intermediates in the repair by aNHEJ. Cohesin depletion might then prevent 53BP1 recruitment, thereby disfavoring long-range recombination and enhancing resection-dependent aNHEJ (figure 25).

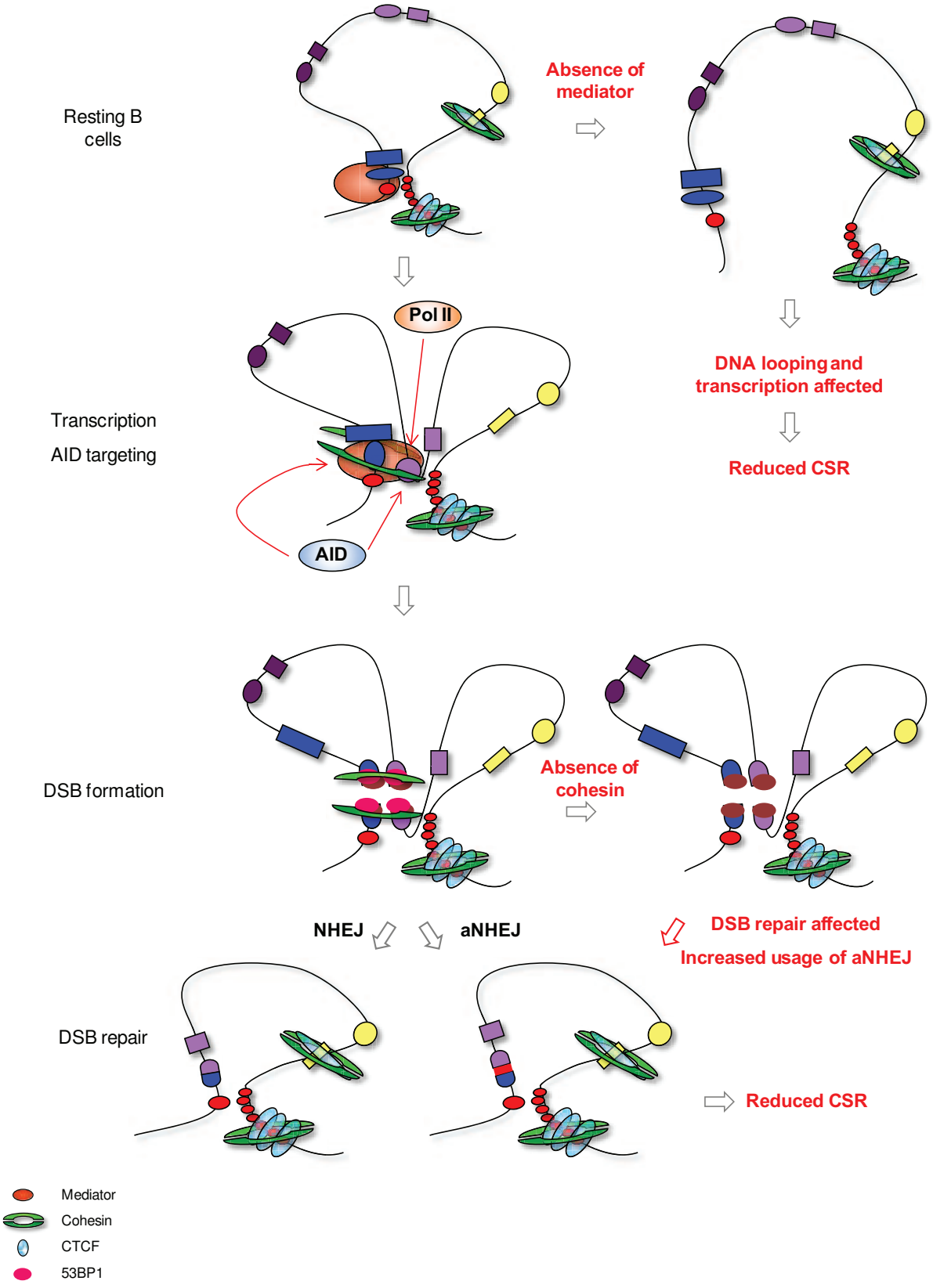


Figure 25: A model for CSR
In our model, the mediator complex would be recruited to Eμ in resting cells. Upon B cell activation, the mediator complex would bind to the acceptor switch region and promote its interaction with Eμ and the 3'RR and induce its transcription through the recruitment of the RNA Pol II machinery. Additionally, the mediator complex could recruit the cohesin complex at Sμ and at Sγ1 to maintain their interaction. The cohesin complex would then reruit 53BP1 to prevent end resection and favor the AID-induced breaks repair through the classical NHEJ pathway.

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The cohesin and mediator complexes control immunoglobulin class switch recombination

Résumé

Lors des réponses immunitaires, les lymphocytes B diversifient leur répertoire par l'hypermutation somatique (HMS) et la commutation isotypique (CI). Ces deux mécanismes sont dépendant de l'activité de « activation-induced cytidine deaminase » (AID), une enzyme qui déamine les cytosines de l'ADN en uraciles générant des mésappariements qui sont processés différemment dans le cas de l'HMS et de la CI. Au cours de la CI, le locus de la chaîne lourde des immunoglobulines subit un changement de conformation qui rapproche les promoteurs, les enhancers et les régions de switch afin de permettre la recombinaison des régions de switch. Cependant, les mécanismes moléculaires sous-jacents n'ont pas encore été identifié. Dans le but de comprednre les mécanismes de régulation d'AID, nous avons réalisé un criblage protéomique et identifié CTCF ainsi que les complexes médiateur et cohésine qui constituent des facteurs préalablement impliqués dans les interactions longues distances. Au cours de ce travail de thèse, nous avons montré que le complexe médiateur est requis pour la transcription de la région de switch acceptrice, pour l'interaction de cette dernière avec l'enhancer Eμ et pour le recrutement d'AID au locus des IgH. D'un autre côté, nous avons montré que le complexe cohésin est impliqué dans la réparation des cassures induites par AID et qu'il pourrait être impliqué dans la recombinaison des régions de switch.

Mots clés : commutation isotypique, complexe médiateur, complexe cohésine, interactions longues distances

Summary

During immune responses, B cells diversify their repertoire through somatic hypermutation (SHM) and class switch recombination (CSR). Both of these mechanisms are dependent on the activity of activation-induced cytidine deaminase (AID), an enzyme that deaminates cytosines into uracils generating mismatches that are differentially processed to result in SHM and CSR. During CSR, the Ig heavy chain (IgH) locus undergoes dynamic three-dimensional structural changes in which promoters, enhancers and switch regions are brought into close proximity. Nevertheless, little is known about the underlying mechanisms. To gain insight into the molecular mechanism responsible for AID regulation during CSR, we performed a proteomic screen for AID partners and identified CTCF, cohesin and mediator complexes, which are factors previously implicated in long-range interactions. We showed that during CSR, the mediator complex is required for acceptor switch region transcription, long-range interaction between the enhancer and the acceptor switch region and AID recruitment to the IgH locus whereas the cohesin complex is required for proper AID-induced breaks repair and might favor switch regions synapsis.

Keywords: class switch recombination, mediator complex, cohesin complex, long-range interactions