UNIVERSITE DE STRASBOURG
ECOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE

Thèse présentée par:

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pour obtenir le grade de
Docteur de l'Université de Strasbourg

Discipline: Sciences du vivant
Spécialité: Aspects Moléculaires et Cellulaires de la Biologie

Molecular mechanisms underlying AID target specificity during antibody diversification

soutenue publiquement, le 28 Mars 2011

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Memory is a way of holding onto the things you love, the things you are, the things you never want to lose...

- The Wonder Years

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Acknowledgements

Gratitude is a memory of the heart, and there are many people without whom this thesis would have never been possible. So, I'd like to say a few thank you's..

To my mentor – Bernardo, for giving me an opportunity to come to the lab, the freedom to work and most of all for molding me into the "scientist" I probably am. No matter where I go, I'll remember to IMPROVISE, EXPERIMENT, INNOVATE & OVERCOME!!

To Anne, Svend, Françoise, Pierre and Prof Mandel for taking time off to read and access the work I have presented in this dissertation.

To Susan and Philippe, for being the best neighbors ever! All the questions & lab meetings, newspapers & magazines, popcorn & BBQ's! Susan, thanks especially for being my "talking sense" barometer.

To Régine, who I will very fondly remember for her support, without her idea the experiments with the HP1 BOX mice would probably never have happened. Also to Florence, who has always made the time for me – to answer questions and give me loads of polyclonal Ab and plasmids! To all the other members of Régine's lab – Margherita, for her help with my Y2H, Raphael for his expertise on ChIP, Benjamin for the Tif1α mice and Céline for providing an interesting angle to my story because of her's!

To all the girls (and guy) in my lab (past and present) – for good times (and bad)! Sara, with who I will always share a love-hate relationship, for being someone I could always "correct" when it came to English and most importantly for making me go to the Indian restaurant more than I ever wanted to. Ebe for making our lab a very different place.. by being the buffer and of course for baking those awesome muffins! AnneSo's smile has brightened many a gloomy day of mine, and for so bravely taking up the ChIP Seq experiments!!! Vincent, for everything from genotyping mice to MS to teaching me the "grands mots en français" and Mathilde for being a good colleague.
All the other's the lab has seen – Lila, Sonia, Delphine, Barbara, Jean-Pierre, Rania, Fanny, Louis and Niels, for being a part of my life in the lab!

To my dearest Iza, (who I left out on purpose in the last sentence) without whom I would have never come this far so as to write an acknowledgement page to my thesis. Iza, I owe you more than these two lines can hold… and most of all I will miss you!

All the members (past & present) of the SC/PK lab - Mac, Robin, Cai Qi, Attila, Dippy, AnneSo, Eva, Peggy, Ale, Apos and Beate. For all the times I've used their bain marie, stolen glass ware and borrowed the Eppendorf pipette. The coffee & conversations, pot's and everything else! Especially Mac, my first 3 years in the lab would have been very different

without him. C'est Qui for the massages & awesome Chinese and game nights at her apartment, Robin for being the jester around all the time and Attila for being nice to me in his own way and for just being "him"!

To Evi, for always having something encouraging to say to me & Artemis, the only baby who's never cried in my arms. Also the members of Evi's lab – especially Tibor, for letting me use his prized possession "the qPCR machine" as and when I needed it, being the nicest Hungarian (along with Attila) around as well as all the stamps.

To Michael for taking care of my mice, Claudine and Patricia for all the sorts as well as the awesome cakes & cookies, Peggy for always sending my orders out "urgently", Valérie, Maité, Bénedicte and Armelle for all the admin work and Evelyene for helping me send out all those last minute fellowship applications! And of course EDIGE, FRM & ARC for accepting those applications and giving me money!

This would be incomplete if I don't tell all of my friends (both here and back home) how much their support has meant to me. Shwets, Suv, Seemu, Shru, Richa, Marcus, Vadi, Shiv,Pepu, Lavan, Mike .. , you guys are the best-est friends! To Jai, Muru, Maan, Raj & didi, Shankar, Harshal, Amit, Ullas and the rest of the Indian gang, for making this a home away from home. Especially to Shankar for all his support, help and encouragement & also being the person that he is. Finally, Dipp Dipp Dipp, my blue ship – for turning out to be that light at the end of the tunnel by being the girl amidst all the guys ☺, I will be the missing the you ;)

To RK, whom I can never thank enough. You're the best thing that happened to me in Strasbourg.

To Dr.Rahul and Mani Sir who by believing in me have been an inspiration.

To bhejafry.net, raaga.com, FB and Farmville – for all the entertainment during those long (and short) hours in the lab.

And of course to my family: Amma, Appa, Ben, Deeps, Sherry girl, all the cousins, Eby and Woof (and the rest of my family) - thanks for believing in me, without all that love and strength I would have never pulled through. I love you!

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List of abbreviations

5 meC	5-Methylcytosine
γH2AX	Phosphorylated Histone H2AX
53BP1	p53 Binding protein 1
Ab	Antibody
AcH3/H4	Acetylated Histone H3/H4
Ag	Antigen
Aicda	Activation induced cytosine deaminase (gene)
AID	Activation induced cytosine deaminase
APOBEC	Apolipoprotein B mRNA–editing catalytic polypeptide
ATM	Ataxia telangiectasia mutated
BCR	B cell receptor
BER	Base excision repair
BM	Bone marrow
bp	Base pairs
CAF1	Chromatin-assembly factor-1
cd	Chromodomain
CDC5L	Cell division cycle 5 like
CFSE	Carboxyfluorescein succinimidyl ester
ChIP	Chromatin Immunoprecipitation
CK2	Casein kinase 2
Co-IP	Co-immunoprecipitation
CSR	Class switch recombination
CTNNBL1	Catenin beta like 1
DDR	DNA damage response
DNA-PKcs	DNA-dependent protein kinase
Dot1	Disruptor of telomeric silencing-1
DSB	Double stranded DNA break
dsDNA	Double stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced Green Fluorecence Protein
FACS	Fluorescence-activated cell sorting
GCs	Germinal centers
GLP	G9a-like protein
GNAT	Gcn5-related N-acetyltransferase
H3K9	Histone H3 lysine 9
HATs	Histone Acetyl Transferases
HC	Heterochromatin
HIGM	Hyper–immunoglobulin M syndrome
HoxC4	Homeobox C4
HP1	Heterochromatin Protein 1

HS	DNase hyper sensitive site
HSA	Heat stable antigen (CD24)
HSC	Hematopoietic Stem Cell
Id	Inhibitor of DNA binding
IFNγ	Interferon γ
Ig	Immunoglobulin
IGC	Immunoglobulin gene conversion
Igh	Immunoglobulin heavy chain locus
IL-4	Interleukin 4
IRIF	Ionizing radiation induced foci
JmjC	Jumonji C-domain
KAP1	KRAB domain associated protein 1
Kda	Kilodaltons
KRAB	Krüppel-associated box
KRIP-1	KRAB-interacting protein 1
LPS	Lipopolysaccharide
MDC1	Mediator of DNA-damage checkpoint 1
MIN	Minutes
MMR	Mismatch repair
MS	Mass Spectrometry
NCS	Neocarzinostatin
NHEJ	Non-homologous End Joining
PARP	Poly(ADP-ribose) polymerase
PTMs	Post-translational modifications
qPCR	Real-time quantitative PCR
RAG 1/2	Recombination Activating Genes 1/2
RBCC	RING, Box-Coiled-Coil
RSS	Recombination Signal Sequences
Sμ region	Switch region (donor)
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SHM	Somatic Hypermutation
ssDNA	Single stranded DNA
TCR	T cell receptor
TGF-β	Transforming growth factor β
Tif1β	Transcription intermediary factor 1 β
TRIM28	Tripartite motif protein 28
Ung/UNG	Uracil DNA glycosylase
WT	Wildtype
XRCC4	X-ray repair cross-complementing protein 4
ZFPS	Zinc finger proteins

INTRODUCTION

I: Mechanisms of B cell receptor assembly and diversification

1. Structure of the B cell receptor

Antibodies (Ab), or the secreted form of the B cell receptor (BCR) have a common structure that consists of two identical light (L) chains (polypeptides of ~22 kilodaltons [Kda]), and two identical heavy (H) chains (~55 Kda or more) that are bound to each other by a disulfide bond and by non-covalent interactions to form a heterodimer (H-L). Similar non-covalent interactions and disulphide bridges link the two identical heavy and light (H-L) chain combinations to each other to form the basic structure of the BCR (Figure 1).

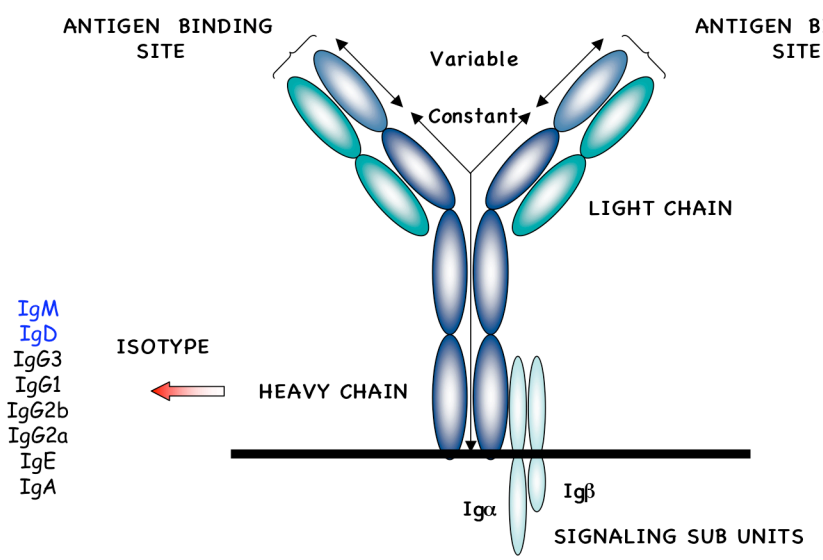

Figure 1: Structure of an antibody: Antibodies are typically made of two heavy chains and two light chains, each consisting of a variable (V) and a constant (C) region. The V regions of the heavy and light chains form the antigen recognition site, while the C regions determine the isotype and thus effector functions of an antibody. The BCR is the membrane-bound form of an immunoglobulin. On binding its cognate antigen, the BCR initiates a signaling cascade through the Igα and Igβ sub-units that results in B cell proliferation.

The first 110 or so amino acids of the amino-terminal region constitute the variable (V) region: V_L in the light and V_H in the heavy chains, respectively. All of the differences in specificity displayed by antibodies can be traced to differences in amino acid sequences of V regions. In fact, most of the variations among antibodies fall within areas of the V regions called complementary-determining regions (CDRs) that form the antigen-binding pocket of an Ab. The regions of constant sequence beyond the variable regions have been dubbed C regions: again C_L and C_H respectively. The light chains can be either kappa (κ) or lambda (λ) and choice of these varies from species to species. While in humans, 60% of the light chains are κ and the rest λ, in mice majority of the light chains are κ. Based on minor differences at the sequence

level, λ light chains are further classified into subtypes: λ1, λ2, λ3, and λ4 (in mice and humans). Similarly, the constant region of heavy chain that determines the class/isotype of an Ab shows five sequence patterns, corresponding to five different C_H regions: μ (IgM), δ (IgD), γ (IgG), ε (IgE) and α (IgA). Again, minor differences in the amino acid sequences of the α and γ heavy chains classify them into sub-isotypes that determines the subclasses of antibody molecules that they constitute. While the V regions determine the specificity of an Ab, the C regions dictate its functions.

Antibody molecules recognize diverse pathogens and help to eliminate them through immune effector mechanisms. Humans and mice are estimated to produce about 10^{11} different antibody specificities and there are four different molecular events that contribute to this (Figure 2): V(D)J recombination, somatic hypermutation (SHM), class switch recombination (CSR) and immunoglobulin gene conversion (IGC) in some species, such as the chicken and sheep.

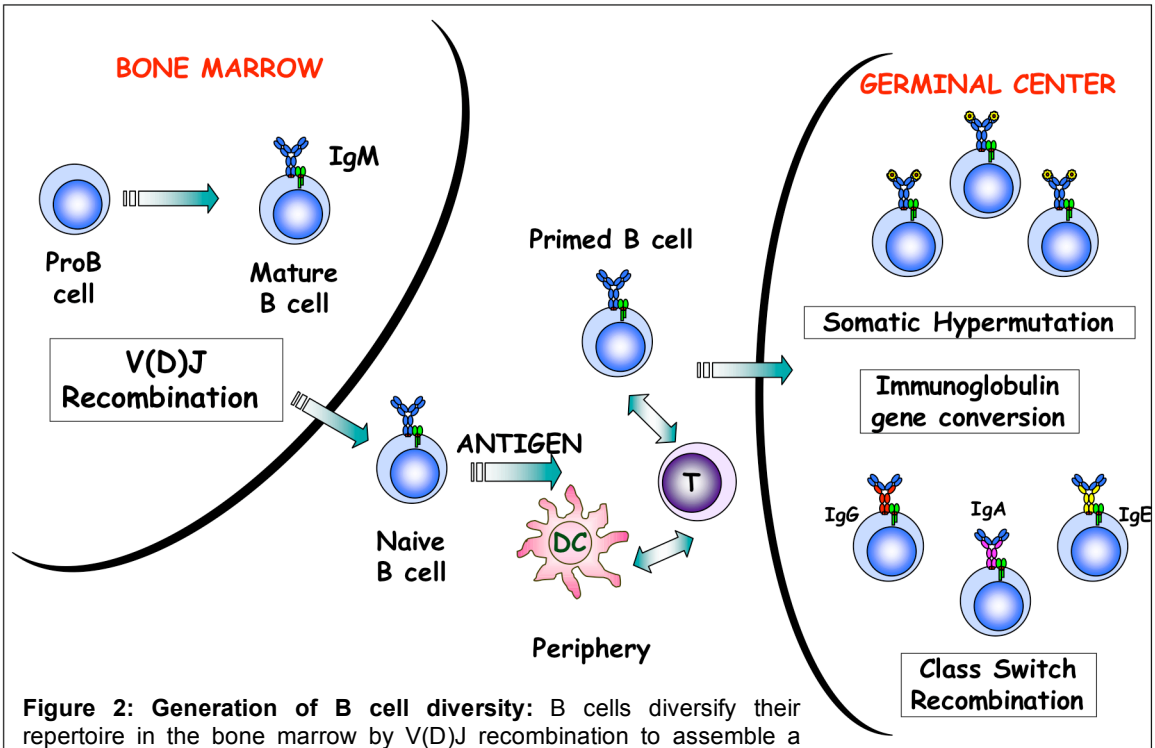

Figure 2: Generation of B cell diversity: B cells diversify their repertoire in the bone marrow by V(D)J recombination to assemble a functional receptor. Naïve B cells exit into the periphery and on encounter with antigen enter the germinal centers where they undergo further diversification by SHM, IGC and CSR.

2. BCR assembly - V(D)J recombination

V(D)J recombination is a genetic recombination reaction used by the cells of the immune system to assemble the BCR and T cell receptor (TCR) genes from preexisting gene segments. V(D)J recombination combines the Variable (V), Diverse (D), and Joining (J) gene segments to assemble a single functional receptor. During lymphocyte development, segments of each type

are thus joined by V(D)J recombination so as to make a V-J or V-D-J array that codes for a variable region, which is later linked by RNA splicing to the constant (C) region.

The mouse Ig*h* locus (Figure 3) contains about 200-1000 variable (V) genes, 12 diversity (D) genes, 4 joining (J) genes and 8 constant (C) region genes within a 3 megabase (MB) sequence. On the other hand, the κ and λ light chain families contain V, J and C segments that rearrange to encode the light chains. The λ locus comprises 3 Vλ segments, 4 Jλ and 4 Cλ segments, of which the Jλ4 is a pseudogene (that do not encode protein), while the κ chain family contains approx 85 Vκ, 5 Jκ (with one pseudogene) and a single Cκ segment. The Ig loci become accessible for recombination and require three major events to occur.

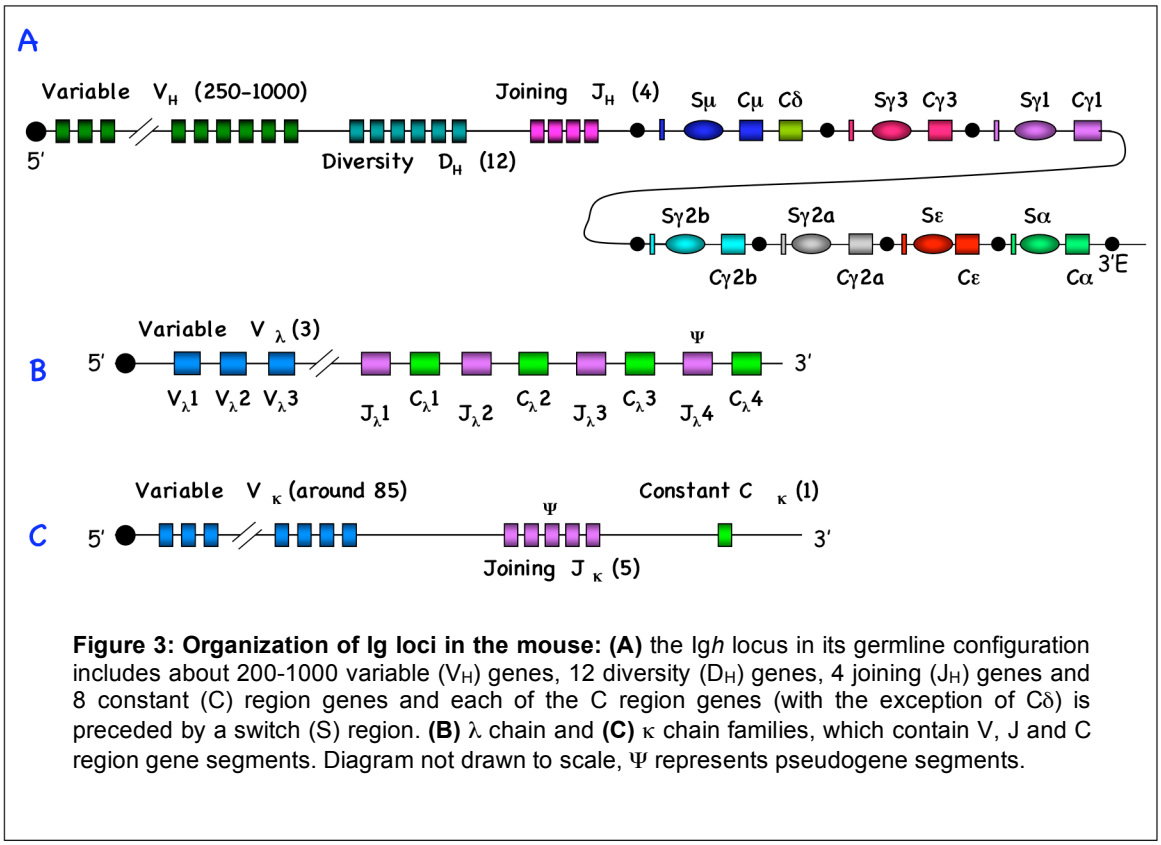

Figure 3: Organization of Ig loci in the mouse: (A) the Ig*h* locus in its germline configuration includes about 200-1000 variable (V_H) genes, 12 diversity (D_H) genes, 4 joining (J_H) genes and 8 constant (C) region genes and each of the C region genes (with the exception of Cδ) is preceded by a switch (S) region. **(B)** λ chain and **(C)** κ chain families, which contain V, J and C region gene segments. Diagram not drawn to scale, Ψ represents pseudogene segments.

First, the recombinase complex, consisting of recombination activating genes RAG1 and RAG2, must be expressed (Oettinger et al., 1990; Schatz et al., 1989). Second, RAG1/2 must be targeted to VDJ segments in order to recognize, bind and induce DNA breaks between the conserved recombination signal sequences (RSS) (Sakano et al., 1979) and its flanking sequences. An RSS contains well conserved heptamer and nonamer sequences separated by 12 or 23 base pairs (bp) of non-conserved DNA called spacers and efficient recombination occurs only between RSSs with 12 and 23 bp spacers (Tonegawa, 1983). Finally, factors that are a part of the general DNA repair and non-homologous end joining (NHEJ) pathway act to process and link the ends into coding and signal joints.

V(D)J recombination (Figure 4) is initiated when a RAG1/2 complex binds an RSS that subsequently binds and captures another RSS in a synapse, to form a paired complex. Within the synaptic complex, the RAG heterodimer cleaves DNA precisely at the junction of the RSSs and the coding segments, yielding two DSBs. This cleavage forms two non-RSS ends and are called coding ends, as they join to encode a new antigen receptor exon, while the other two ends are referred to as signal ends (Gellert, 2002; Roth et al., 1993; Schlissel et al., 1993). By the end of this first phase of V(D)J recombination there is a complex consisting of the RAG1/2 proteins and four DNA ends (Gu and Lieber, 2008). In the second phase of the reaction, the RAG proteins work together with DNA repair proteins to process and ligate the coding and signal ends to form coding and signal joints, respectively. The repair factors involved are those of the NHEJ machinery, which includes the catalytic sub-unit of DNA-dependent protein kinase (DNA-PKcs), Ku70, Ku80, X-ray repair cross-complementing protein 4 (XRCC4), DNA ligase IV, Artemis and XRCC4-like factor (XLF/Cernunnos) (Bassing et al., 2002; Gellert, 2002; Lieber et al., 2003). The processing of coding ends begins with nucleolytic opening of the hairpins by Artemis. These open coding ends undergo loss or addition of small number of nucleotides. Finally, XRCC4-DNA Ligase IV ligate the ends; with the site of rejoining reflecting the presence of short stretches of nucleotide overlap (microhomologies) at both ends (Lieber et al., 2003). On the contrary, signal joint formation is much simpler, requiring only the ligation of two blunt ends. Thus, neither is Artemis nor DNA-PKcs involved, while the other NHEJ proteins are required. Eventually, the signal joints are ligated together, since the RAG proteins are capable of cleaving them to regenerate the two signal ends.

V(D)J recombination is regulated on one hand by the expression pattern of the RAG recombinase, in that rearrangement is lineage restricted – Ig loci undergo rearrangement only in B cells and the TCR genes in T cells. Secondly, rearrangements proceed in a certain order within a lineage. Recombination is initiated in pre-pro-B and pro-B cells at the Ig*h* locus and this always precedes Ig light chain (Ig*l*, κ or λ) rearrangement (occurs in pre-B cells). Additionally, D_H-J_H recombination must take place at the Ig*h* locus before V_H-DJ_H rearrangement can be initiated. Finally, “allelic exclusion” ensures the expression of a receptor from only one allele at both the Ig*h* and Ig*l* segments.

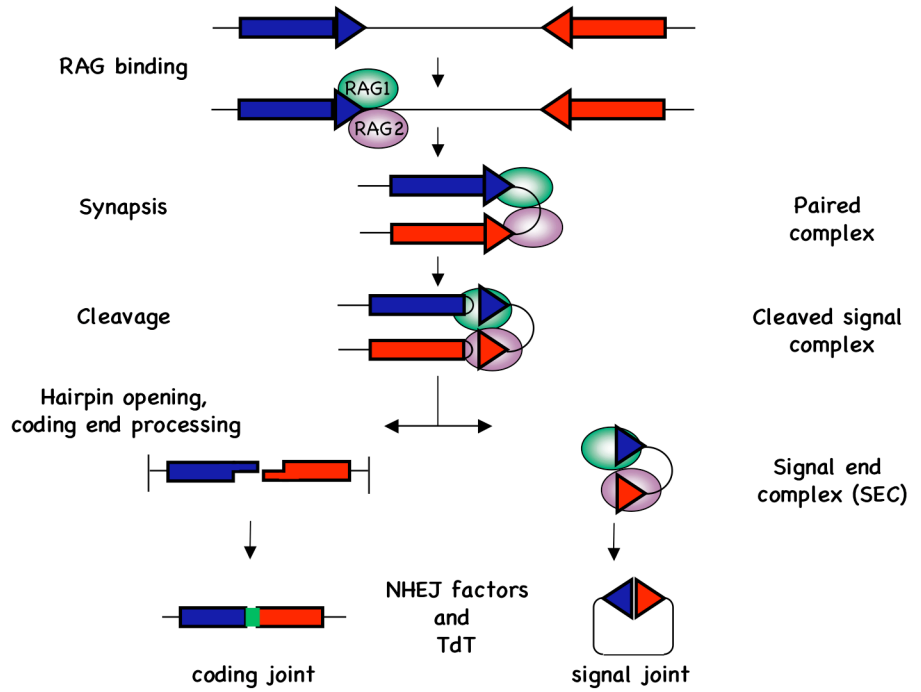

Figure 4: The steps of V(D)J recombination: The substrates (red and blue rectangles) are flanked by a 12-RSS (red triangle) and by a 23-RSS (blue triangle). The RAG proteins (green and pink ovals) are the central players in the reaction leading to the cleaved signal complex. Thereafter, they cooperate with the non-homologous end joining (NHEJ) DNA repair factors in the end processing and joining phase of the reaction. Final coding joints often contain non-templated nucleotides (green rectangle) added by TdT. Adapted from (Schatz, 2004).

As early as 1985, Yancopoulos and Alt proposed that transcription was the key to control when and how rearrangements would occur. More than two decades later, numerous labs have identified several aspects of accessibility control from epigenetic changes, like histone acetylation and methylation (Chowdhury and Sen, 2001; Liu et al., 2007; McBlane and Boyes, 2000) to E2A and EBF mediated, transcription-linked (accompanied by epigenetic modifications) D_H-J_H joining (Goebel et al., 2001; Romanow et al., 2000), IL-7R signaling dependent distal V_H transcription for V_H-DJ_H joining (Bertolino et al., 2005), and Pax5 mediated compaction of the Ig*h* locus for long-range recombination between V_H-DJ_H gene segments (Fuxa et al., 2004). In addition, nuclear organization of the locus either by relocation of the Ig loci to the center of nucleus to initiate recombination, regulation of intra-locus recombination as well as repositioning of one allele into pericentromeric heterochromatin to permit allelic exclusion (Hewitt et al., 2010) dictates efficient recombination.

Productive V(D)J recombination at the Ig*h* locus thus results in the juxtaposition of a variable region upstream of a series of heavy chain (HC) constant region exons. In primary B cells, a mature mRNA that encodes the V(D)J-Cμ protein (μHC) then pairs with either a κ or λ light chain (LC), from a recombined κ or λ locus, to form an IgM molecule, which leads to the production of surface IgM^+ ($sIgM^+$) B cells that constitutes the primary repertoire. As V(D)J recombination is a random process, it could produce self-reactive receptors which are eliminated from the repertoire before B cells can leave the bone marrow and enter the peripheral lymphoid organs (Wardemann et al., 2003).

However, although the antibodies produced by V(D)J recombination are sufficiently diverse to recognize nearly all potential antigens, no species produces an arsenal of antibodies large enough to effectively clear antigens by gene rearrangement alone.

3. Antigen-dependent mechanisms of BCR diversification

The primary antibody repertoire provides a first line of defense by means of polyreactive low affinity IgM antibodies. BCR cross-linking by antigen stimulates rapid proliferation, blast formation and clonal expansion forming the germinal centers (GCs) in the secondary lymphoid organs like the spleen, which is the basis of T-dependent humoral immunity and the ultimate expression of the adaptive immune response. The GC reaction entails three molecular processes that further diversify rearranged Ig loci, namely: SHM and IGC – that underpin affinity maturation, and CSR – that provides means for effective antigen clearance (Figure 5).

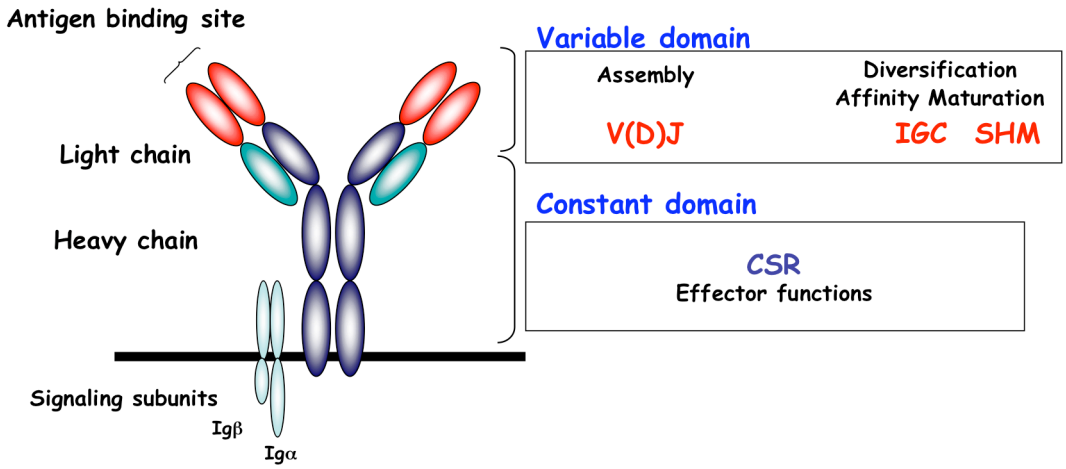

Figure 5: Mechanisms of antibody diversity: B cells assemble a functional receptor in the bone marrow by V(D)J recombination and this constitutes the primary B cell repertoire. Naïve B cells on encounter with antigen enter the germinal centers where they further diversify rearranged Ig loci through SHM, IGC and CSR. SHM introduces point mutations in the heavy and light chain genes and this underpins affinity maturation, while CSR is a recombination reaction that occurs at the heavy chain locus and replaces the isotype of the antibody, thus its effector functions to result in effective antigen clearance.

A. Somatic hypermutation

SHM introduces single nucleotide substitutions in a step-wise manner at a frequency of around 10^{-3}-10^{-4} per base pair (bp) per generation in the variable regions of the Ig*h* and Ig*l* genes. This will generate families of related B cell clones bearing mutated receptors that can then be selected on the basis of antigen binding affinity, known as affinity maturation (Figure 6, Di Noia and Neuberger, 2007). Mutations are introduced across a region that begins about 150

nucleotides downstream of the IgV promoter and extends over about 2 kilobases (kb) (Neuberger and Milstein, 1995; Rajewsky, 1996). Interestingly, most of the mutations are concentrated within the assembled variable regions (or in closely flanking sequences) that form the Ag-binding pocket of an Ab (Papavasiliou and Schatz, 2002). B cells with increased affinity for an antigen are then "clonally expanded" and ultimately intergrate the memory or plasma cell compartments (Meffre et al., 2000; Rajewsky, 1996).

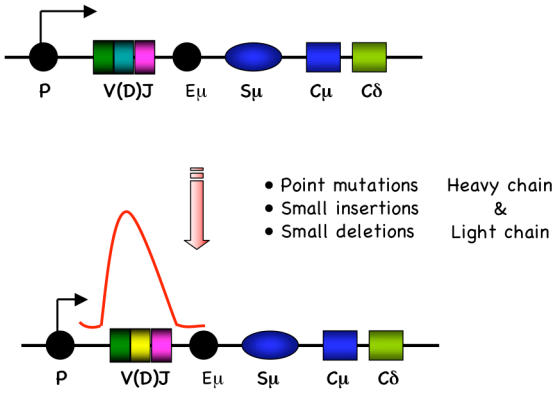

Figure 6: Somatic Hypermutation (SHM) underpins affinity maturation. SHM introduces non-templated single nucleotide mutations and/or rarely small insertions and deletions in the the rearranged heavy and light variable genes. This results in the expression of a mutated receptor with increased affinity for the triggering antigen, and underpins affinity maturation. The red arch represents the frequency of mutations that increases following SHM.

B. Immunoglobulin gene conversion

During immune responses, some species diversify their immunoglobulin (Ig) light and heavy chain genes by pseudogene templated gene conversion. Although Ig gene conversion (IGC) was initially believed to occur only in birds, it is now clear that most farm animals also use this mechanism to diversify their immunoglobulin gene repertoire. Thus, immunoglobulin diversity in birds (and several other species) is generated by somatic gene conversion events, in which sequences derived from upstream families of pseudogenes replace homologous sequences in unique and functionally rearranged VJ_L or VDJ_H genes. (Figure 7, Arakawa et al., 1996; Arakawa et al., 2002; Butler et al., 1996; Harris et al., 2002; Lanning et al., 2000; McCormack et al., 1991; Meyer et al., 1997; Reynaud et al., 1987).

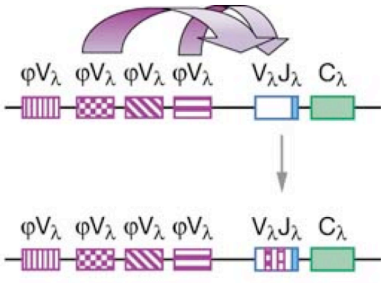

Figure 7: Templated diversification at the chicken λ light chain locus. Chickens have a single functional V and J segment in the light and heavy chain gene loci, and diversity is introduced into the rearranged V(D)J segments by gene conversion using pseudo V genes (represented by Ψ) as "templates". Adapted from (Maizels, 2005).

C. Class switch recombination

In addition to SHM and IGC, B cells also diversify their antibody repertoire through CSR. CSR is a recombination reaction that joins two Ig switch regions by deleting intervening sequences, replacing the heavy chain constant region exons, and thus switching the antibody isotype expressed (from IgM to IgG, IgE or IgA, Figure 8). Therefore, this reaction diversifies the B cell repertoire by producing related B cell clones in which a single variable region is combined with different constant region exons with unique effector functions (Chaudhuri et al., 2007). The heavy chain constant region (C_H) genes are organized as individual transcription units, that are comprised of a cytokine responsive promoter, a switch (S) and a constant (C) region, with the exception of Cδ (Figure 3A, Chaudhuri et al., 2007; Stavnezer et al., 2008). CSR occurs at S regions (repetitive, non-homologous sequences, about 3-12 kb in length) and involves a recombination event between the donor Sμ and an acceptor Sγ, Sε or Sα region. The intervening sequence, including Cμ, being excised results in the juxtaposition of a rearranged VDJ exon with a downstream C_H region (Chaudhuri et al., 2007), producing an antibody with the same antigen specificity but different effector functions.

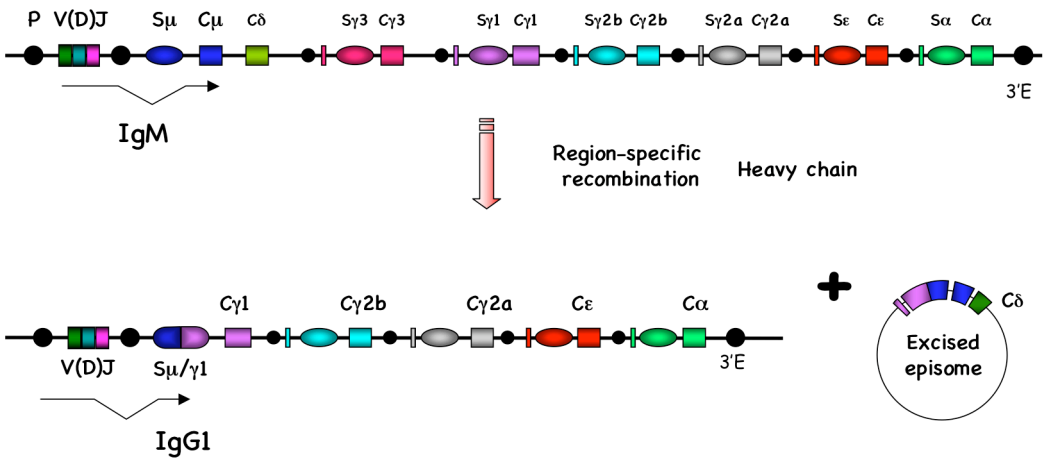

Figure 8: Class Switch Recombination (CSR). CSR is a region-specific recombination that occurs at the heavy chain locus and allows a change of the expressed Ig*h* constant gene. Recombination takes place between 2 highly repetitive "donor" Sμ and "acceptor" (in this case Sγ1) sequences called switch regions. CSR replaces the isotype of the antibody expressed, and thus its effector functions, while preserving its antigen binding specificity.

A major advancement in understanding the mechanisms of SHM, IGC and CSR, came with the identification of activation-induced deaminase (AID), just about a decade ago. Anne Durandy and Tasuku Honjo discovered that AID is required to mediate SHM and CSR in humans and mice (Muramatsu et al., 2000; Revy et al., 2000). Later it was shown that AID was also required for IGC (Arakawa et al., 2002). This outstanding discovery paved way to the understanding of the molecular mechanisms involved in the antigen-dependent diversification of the BCR.

II: Activation induced cytidine deaminase

AID was discovered through a subtractive hybridization screen of cDNAs, which were induced during cytokine-mediated activation of switch recombination, in the mouse B cell line CH12 (Muramatsu et al., 1999). It was identified as the molecule responsible for a subset of human hyper–immunoglobulin M syndromes (HIGM), which result in a complete loss of CSR (characterized by an overproduction of IgM Ab's) and SHM (Revy et al., 2000). At the same time, generation of AID-deficient mice, which exhibit a striking inability to undergo CSR and SHM, confirmed that AID was required for both processes (Muramatsu et al., 2000).

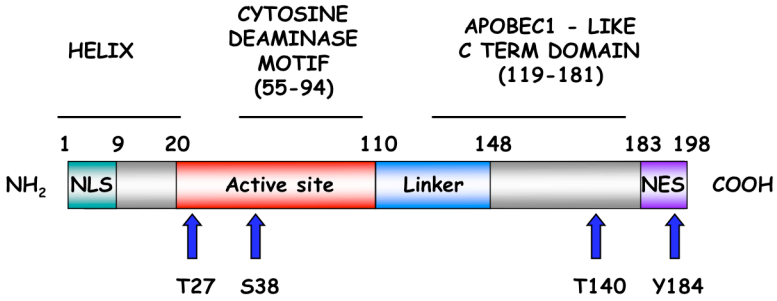

Figure 9: Domain organization of AID: The primary domain structure of AID is shown, depicting the N terminal (NH2) canonical nuclear localization signal (NLS), the C terminal domain (COOH), which houses a nuclear export signal (NES), as well as the active site that contains the cytosine deaminase motif (from amino acid 55-94). The blue arrows indicate the positions of phosphorylation sites threonine 27 (T27), serine 38 (S38), threonine 140 (T140) and tyrosine 184 (Y184).

AID is a small protein of 198 amino acids with a central catalytic domain, an N-terminal domain that appears to be specifically required to initiate SHM (Shinkura et al., 2004) and a C-terminal domain, that in addition to being required to initiate CSR (Barreto et al., 2003; Ta et al., 2003) also contains a nuclear export signal (NES), which controls its subcellular localization (Brar et al., 2004; Geisberger et al., 2009; Ito et al., 2004; McBride et al., 2004) (Figure 9).

Sequence analyses have identified a domain that is common to cytosine deaminases, which includes a His-X-Glu motif separated by a few amino acids from a Pro-Cys-X-X-Cys cluster. This cytidine deaminase domain of AID is most similar to that of the apolipoprotein B mRNA–editing catalytic polypeptide (APOBEC) proteins, with APOBEC1 having the closest homology (Conticello et al., 2005; Muramatsu et al., 1999).

1. RNA-editing model

Owing to its similarity with APOBEC1, AID was hypothesized to act as an RNA editing enzyme. AID was proposed to edit a cellular mRNA to generate a new mRNA that encodes a novel endonuclease required to activate SHM and CSR (Muramatsu et al., 2000). To explain the requirement of AID in both SHM and CSR, it was proposed that AID targets distinct mRNAs - a DNA mutator for SHM and a DNA recombinase for CSR, based on the fact that mutations in AID resulted in either CSR or SHM specific defects (Shinkura et al., 2004; Ta et al., 2003). It was speculated that AID could associate with specific proteins and edit two different mRNAs that encode factors required for SHM and CSR respectively (Honjo et al., 2005). In addition, *de novo* protein synthesis is required post AID expression for DSB induction and for CSR (Begum et al., 2004b; Doi et al., 2003), which could however also be due to the involvement of labile factors that function downstream of AID lesions or in their processing into DSBs. Thus, while data exists which is compatible with the RNA editing model, the ultimate proof, which is the identification of the edited mRNA is still lacking.

2. DNA deamination model

The DNA-editing hypothesis suggests that AID mutates DNA directly, an idea that provides substance to earlier theories proposed by Peters and Storb (Peters and Storb, 1996), Scharff and Edelmann (Wiesendanger et al., 1998) and Neuberger (Neuberger et al., 1998), which suggested the existence of a mutating factor that is directly targeted to Ig loci. Although SHM, CSR, and IGC are distinct processes, the DNA deamination model predicted that they are all initiated by AID-mediated deamination at Ig loci (Figure 10, Petersen-Mahrt et al., 2002). The model made two main predictions – (a) that AID should have the ability to specifically catalyze C-U deamination in DNA, and (b) that a U:G lesion in the Ig locus forms an intermediate in antibody diversification pathways. Thus, modifying or deleting enzymes involved in processing such U:G mismatches would perturb these mechanisms. While DNA synthesis over the AID-generated U:G lesion would result in C-T (or G-A) transition mutations, synthesis over an abasic site that is formed due to excision of the uracils by a uracil-DNA glycosylase (UNG) would lead to both transition and transversion mutations at C:G pairs. In addition, mutations at A:T pairs could result from mutatgenic patch repair, triggered by the recognition of the U:G lesion by the mismatch repair proteins – MSH2/MSH6. In the case of IGC, the U:G mismatch is resolved by recombinational repair using a neighboring IgV pseudogene as template, and would be dependent on the RAD51 paralogues XRCC2, XRCC3 and RAD51B (Sale et al., 2001). Finally, with regard to CSR AID-mediated deamination at proximal dCs on opposite strands could lead

to DNA double stranded breaks at both donor and acceptor switch regions to promote switching.

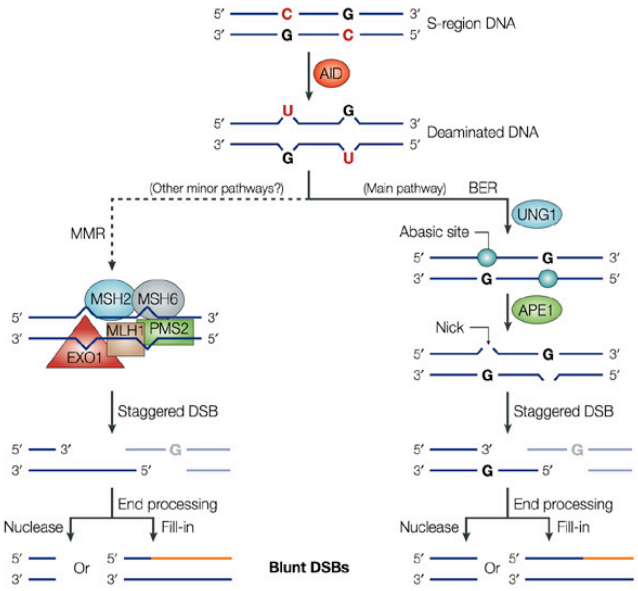

Figure 10: BER and MMR pathways in CSR. AID deaminates dC residues in DNA converting them to dU. This U:G mismatch can be processed by either the base-excision repair (BER) pathway or the mismatch-repair (MMR) machinery to introduce gaps or nicks at S regions. The BER enzyme UNG will excise uracil bases and APE1 will nick the DNA backbone, resulting in a single stranded break. Alternatively, a MMR complex (Msh2/6, Exo1, etc.) can excise multiple base pairs around the mismatch, leaving a single stranded DNA gap. Two single stranded breaks/gaps in close proximity on opposite strands form a DSB, which is processed to yield blunt ends that can then be ligated to similarly created breaks on downstream S-region DNA to complete CSR. Adapted from (Chaudhuri and Alt, 2004).

There are several lines of evidence to support the DNA-editing model: (a) the demonstration that expression of AID cDNA in *E. coli* resulted in an increase in the frequency of mutations to rifampicin resistance, and that these mutations increased drastically in an UNG-deficient background, indicated for the first time that AID could deaminate DNA (Petersen-Mahrt et al., 2002); (b) purified AID proteins are capable of binding and converting deoxycytosines into deoxyuridines on target single-stranded (ss) DNA *in vitro* (Bransteitter et al., 2003; Chaudhuri et al., 2003; Dickerson et al., 2003; Sohail et al., 2003); (c) the interaction between AID and replication protein A (RPA) that binds and stabilizes ssDNA, as well as chromatin immunoprecipitation (ChIP) experiments using exogenous epitope-tagged and endogenous AID showed that AID is associates directly with S regions in B cells undergoing CSR (Chaudhuri et al., 2004; Nambu et al., 2003), strengthening the idea that AID acts on DNA directly; (d) deficiency in the major uracils-excision enzyme UNG perturbs SHM, IGC and CSR (Beale et al., 2004; Di Noia and Neuberger, 2002; Di Noia and Neuberger, 2004; Imai et al., 2003b; Rada et al., 2004; Rada et al., 2002a; Saribasak et al., 2006); (e) the deficiency of the mismatch proteins MSH2 or MSH6 disrupts the generation of A:T mutations (Rada et al., 1998; Wiesendanger et

al., 2000). Consistent with a role for an apurinic/apyrimidinic endonuclease (APE 1/2) (Christmann et al., 2003) in the formation of a ssDNA break, *ape1*$^{+/-}$*ape2*$^{-/-}$ mice show about 40% reduction in CSR (Guikema et al., 2007); (g) and finally, the UNG-MSH2 double knockout mice have a complete block in CSR (Rada et al., 2004) and their B cells accumulate a majority of transversion (C-T, G-A) mutations at S regions.

3. AID and somatic hypermutation

As mentioned above, SHM occurs in germinal centers and such germinal center (B220^{+} Fas/CD95^{+}GL7^{+}) B cells can be purified from the Peyer's patches or lymph nodes of mice that have been immunized, to analyze somatic mutations.

According to the DNA-deamination model, in the first phase of SHM, AID deaminates cytosines to uracils on target DNA to result in U:G mismatches (Figure 11, Petersen-Mahrt et al., 2002).

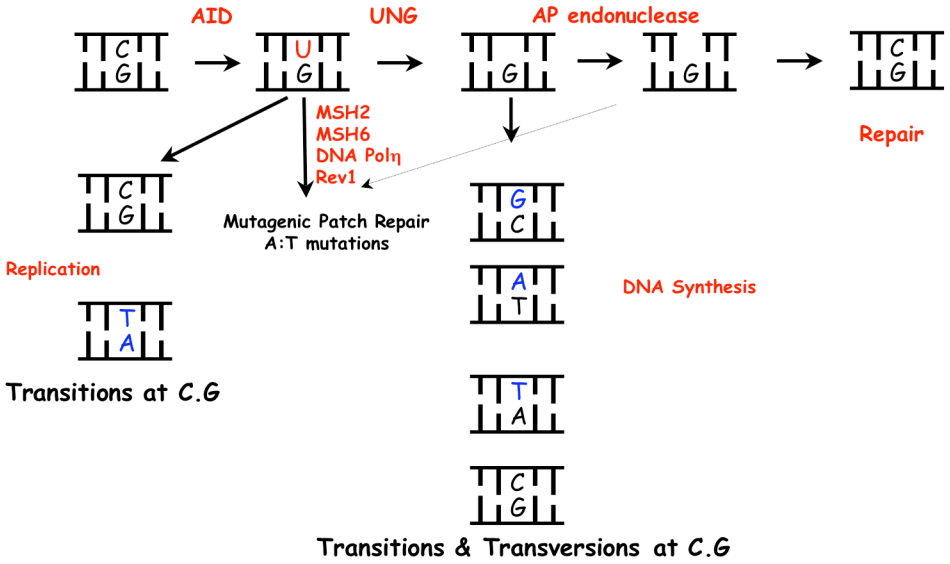

Figure 11: The steps of SHM: The UNG pathway uses UNG to excise the uracils, APE to nick the abasic site and tranlesion polymerases like Rev1 to fill the gap. This results in transition mutations at C:G bps. The MMR pathway involves MSH2-MSH6 heterodimer to bind the U:G mismatches, exonuclease 1 to create a gap and DNA pol η to fill-in. This produces transition and transversion mutations of A:T and could initiate low levels of CSR as well.

All four bp's are targeted for mutation and in mice, C:G and A:T pairs are hit with equal frequencies. However, neither is the choice of individual bases nor the targeted region a chance phenomenon. For example: an A rather than a T on the coding strand is more likely to result in an A:T mutation, although such a strand bias does not exist with respect to C:G pairs (Golding et al., 1987; Milstein et al., 1998). Also, C:G mutations occur within WRCY "hot spots" (where W=A/T, R=A/G and Y=C/T), and such consensus within target loci indicates that the mutations do not occur at random (Betz et al., 1993; Rogozin and Kolchanov, 1992; Sharpe et al., 1991). These U:G mismatches can then be processed by two different pathways to eventually result in

mutations (Figure 11). Replication over the mismatch by high fidelity polymerases can lead to either C-T or G-A transition mutations (that exchanges a pyrimidine for a pyrimidine [C-T] or purine for a purine [G-A]) (Petersen-Mahrt et al., 2002). If the U:G mismatches are removed by UNG to create an abasic site, the abasic site can subsequently be converted to a single-stranded nick by an apurinic/apyrimidinic endonuclease (APE) and then repaired by base excision repair without generating mutations.

On the other hand, mismatches can be recognized by MSH2–MSH6, excised and replaced via resynthesis with error- prone polymerases. This can create additional mutations, including mutations at A-T base pairs. Iterative binding of these proteins, will only result in more mutations downstream of the original C:G pair. Consistent with this, MSH2, MSH6 and exonuclease 1 deficient mice show a drastic drop in A:T mutations (Bardwell et al., 2004; Frey et al., 1998; Martomo et al., 2004; Phung et al., 1998; Wiesendanger et al., 2000). SHM thus makes a dominant contribution to antibody diversity as well as affinity maturation and generation of the secondary repertoire in humans and mice (Milstein and Neuberger, 1996; Rajewsky, 1996).

4. AID and its role in CSR

In the following sections, I will discuss the current model for AID in CSR (Figure 12) and mechanisms underlying each of these steps and regulation of the CSR reaction.

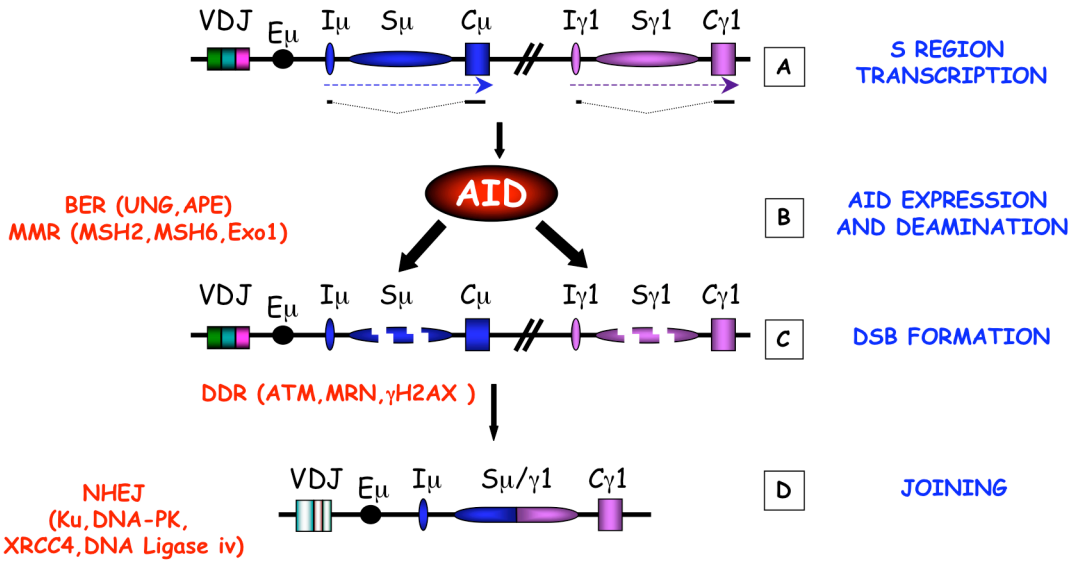

Figure 12: Working model for CSR: (A) CSR is initiated by germline transcription of S regions (blue and purple arrows). **(B)** AID induces DNA lesions by deaminating cytosines; the resulting dU:dG mismatches are processed into DSBs through either the base excision repair pathway or the mismatch repair pathway (MMR). **(C)** DSBs activate the DSB response, bringing about accurate long-range synapsis. **(D)** DSBs in Sμ and a downstream S region are repaired through either NHEJ (major), or alternative NHEJ (minor).

A. The role of transcription in CSR

Transcription through a particular S region is required for successful CSR of that corresponding C_H gene (Manis et al., 2002b). Specific activation of B cells to undergo CSR induces transcription from germline (GL), cytokine responsive promoters that lie upstream of an exon (I-exon) that precede all S regions and the C_H genes, as shown in Figure 3. This primary transcript is spliced to remove the intronic S regions to join the I-exons with the C_H exons, to result in a "sterile" transcript (Manis et al., 2002b), which does not encode any protein. The importance of transcription, and its control elements in CSR has been studied by several groups through gene targeting. Deletion of either an I-exon and/or promoters for a specific C_H gene either completely or partially affected GL transcription and CSR (Jung et al., 1993; Manis et al., 2002b; Stavnezer, 2000).

There are at least three plausible, mutually non-exclusive roles for transcription in CSR. (a) knowing that AID deaminates single stranded DNA (ssDNA) (Bransteitter et al., 2003; Chaudhuri et al., 2003; Dickerson et al., 2003; Pham et al., 2003; Ramiro et al., 2003; Sohail et al., 2003) transcription is thought to provide short stretches of ssDNA in the transcription bubble. In addition, RNA transcribed at S regions is G-rich and can stably associate with the template strand of DNA to form R-loops, in which the template strand is hybridized to the nascent transcript and the non-template strand is displaced and exists as ssDNA (Daniels and Lieber, 1995; Huang et al., 2007; Shinkura et al., 2003; Tian and Alt, 2000; Yu et al., 2003). The role of R-loops in facilitating CSR came from the observations that inversion of the endogenous 12 kb Sγ1 inhibited R-loop formation *in vitro* and resulted in a significant decrease in CSR to IgG1. Furthermore, replacing the Sγ1 sequence with a 1 kb synthetic G-rich sequence, (that contains no S region motifs) permits low levels of CSR, and the ability of this synthetic sequence to mediate CSR was orientation dependent, with CSR observed only in the orientation that favored R-loop formation (G-rich on the non-template strand) (Shinkura et al., 2003). In contrast to mice, switching in *Xenopus* is not very efficient as the Sμ regions are A:T rich and thus do not have G clusters and R-loop formation (Zarrin et al., 2004). R-loops also block transcription as the polymerase could have difficulties in unwinding the RNA-DNA hybrid (Canugovi et al., 2009; Tornaletti et al., 2008). So, another effect of R-loop formation could be to slow down RNA polymerase II (RNA Pol II) as it moves through the S region. (b) To target or recruit AID to the Ig*h* locus. This function of transcription is highlighted by the fact that AID co-immunoprecipitates with RNA Pol II in B cells during CSR (Nambu et al., 2003), and that AID-dependent mutations are concentrated near the 5' end of S regions (Xue et al., 2006). It was thus proposed that AID is recruited to RNA Pol II either during the initiation or elongation phases of transcription (Bransteitter et al., 2006; Longerich et al., 2006; Longerich et al., 2005), and will leave the complex as it moves along the S region. This hypothesis is further substantiated by the fact that AID is targeted to S regions through its interactions with Spt 5, the RNA exosome

and Pol II (Basu et al., 2011; Pavri et al., 2010). (c) Finally, by inducing chromatin remodelling, transcription at the Ig*h* locus can influence CSR in terms of accessibility and AID binding. As this hypothesis forms an integral part of the main questions that I am addressing, it is discussed in detail later.

B. Sequence and structural specificity of the Ig locus in CSR

There are two enhancer regions in the Ig*h* locus (Figure 3): (a) the μ intronic enhancer located downstream of the J_H4 and just 5' of Sμ. It has the Iμ promoter within its core, and deletion of the core reduces CSR on the deleted allele (Sakai et al., 1999). (b) The 3' regulatory regions downstream of Cα, are about 30 kb in length and contain four DNase hypersensitive sites called hs3A, hs1,2, hs3B and hs4. While deletion of hs3A or hs1, 2 has no effect on CSR, combined deletion of hs3B and hs4 reduces GL transcription and CSR to all isotypes except IgG1 (Manis et al., 1998b; Pinaud et al., 2001). As these enhancers are located so far away from the GL promoters, the influence they have on transcription most likely occurs through loop formation between each other, involve the binding of transcription factors and chromatin modifiers that would increase accessibility, and bring the S regions closer to each other. Indeed the 3' hs3B-hs4 enhancers form loops with GL specific promoters in B cells undergoing CSR and are AID-dependent (Wuerffel et al., 2007).

Taken together this provides evidence that S region transcription and transcription-generated DNA structures not only help AID find its substrates, but also functions in the context of sequence motifs and that there could be several mechanisms that target AID to Ig loci.

C. BER and MMR pathways in CSR

In response to activation, transcription, AID expression and deamination, B cells utilize the ubiquitous pathways of base excision and mismatch repair in the next step of the CSR reaction. In canonical BER, UNG binds dU:dG mismatches in DNA, cleaves the uracils and generates an abasic site. This is followed by APE-mediated strand cleavage, and eventually replication over the site by DNA Polβ. During SHM and CSR however, this pathway is altered by resynthesis using translesion polymerases, which introduces mutations and single stranded breaks. Consistent with this, while deletion or inhibition of UNG or APE results in CSR defects (Guikema et al., 2007; Rada et al., 2002b; Schrader et al., 2005), DNA Polβ deficiency increases CSR (Wu and Stavnezer, 2007).

In addition to BER, dU:dG mismatches can be processed through the MMR pathway. This process requires that the MSH2-MSH6 heterodimer binds a U:G mismatch to recruit MLH1 and PMS2 heterodimers (Kunkel and Erie, 2005). This heterotetramer then recruits PCNA, replication factor C (RFC), and Exo 1 to excise the single-stranded segment that contains the

mutated nucleotide (Genschel et al., 2002). The role of the MMR pathway is highlighted by mice deficient for these proteins showing various degrees of reduction in SHM and/or CSR depending on the gene, and are listed in Table 1 (Bardwell et al., 2004; Ehrenstein and Neuberger, 1999; Ehrenstein et al., 2001; Li et al., 2004b; Martin et al., 2003; Martomo et al., 2004; Schrader et al., 1999).

Table 1: Involvement of BER and MMR components in SHM and CSR

Protein	SHM phenotype in absence of activity	CSR phenotype in absence of activity	Role in CSR
BER			
UNG	reduced transversion mutations at G:C base pairs	severely impaired	removes deoxyuridine from S regions to create abasic sites
APE1/APE2		60-70% reduction	creates SSBs by excising abasic sites
DNA Polβ	2-3 fold increase	1.5-2 fold increase	fills single nucleotide gaps
MMR			
MSH2	reduced mutations	2–5-fold reduction	mismatch recognition, end processing
MSH6	reduced mutations	2–5-fold reduction	mismatch recognition, end processing
MSH3	no reduction	no reduction	mismatch recognition, end processing
PMS2	reduced mutations	2–5-fold reduction	mismatch processing, end-region synapsis
MLH1	reduced mutations	2–5-fold reduction	mismatch processing, end-region synapsis
Exo1	reduced mutations	severely impaired	mismatch processing

The prevailing model for the role of this pathway in CSR is to convert single stranded breaks (SSB) on opposite strands, into DSBs (Schrader et al., 2007; Stavnezer and Schrader, 2006). If the SSBs introduced by AID-UNG-APE are in close proximity and on opposite strands they can readily result in DSBs, if not, they are simply repaired. S regions span across kilobases and breaks can appear to occur anywhere within them, and so it is difficult to imagine that SSBs are always juxtaposed so as to form a DSB, MMR could however accomplish this feat (Figure 10). MSH2-MS6 recognize the U:G mismatch caused by AID deamination. MLH1-PMS2 is recruited and Exo1 excises from the nearest 5' SSB created by AID-UNG-APE. Exo1 is thought to proceed through until it finds a SSB on the other strand, thus creating a DSB. This hypothesis is supported by the fact that MMR deficient B cells have lesser DSBs (Schrader et al., 2007) and differ in length and characteristics of Sμ-Sx junctions from those of wild-type B cells (Bardwell et

al., 2004; Ehrenstein et al., 2001; Li et al., 2004b; Schrader et al., 2002). This strongly suggests that the MMR is involved CSR.

D. DNA repair, S region synapsis and joining

DNA lesions induced by AID are processed to result in the formation of double stranded DNA breaks (DSBs) during CSR (Rada et al., 2004; Schrader et al., 2005), and the requirement for AID has been traced to complete block in the formation of DSBs at S regions (Catalan et al., 2003; Petersen et al., 2001; Rush et al., 2004; Schrader et al., 2005), which are necessary intermediates for CSR (Honjo et al., 2002). The two main pathways of DSB repair in higher eukaryotes are, homologous recombination (HR, that requires large regions of homology and provides accurate repair) and non-homologous end joining (NHEJ, which joins ends that either lack or have very short homologies) (Bogue et al., 1998; Bogue et al., 1997; Li et al., 1995; Roth et al., 1985), and extensive work has implicated several proteins of the NHEJ pathway in CSR (Stavnezer et al., 2008).

After formation of the DSBs at donor and acceptor S regions, the S regions are recombined using ubiquitous proteins that perform NHEJ. The NHEJ pathway has seven known components – the DNA-PK holoenzyme, which includes Ku70, Ku80 (or 86) and the catalytic sub-unit of DNA-PK (DNA-PKcs), the ligase complex XRCC4-DNA ligase IV along with the more recently described XRCC4-like factor (XLF)/Cernunnos and Artemis (Table 2). Ku70 and Ku80 bind DSBs and serve as a platform to recruit DNA-PKcs and activate it to form the holoenzyme (Lieber, 2010; Lieber et al., 2003; Rooney et al., 2004). All these three components are essential for NHEJ and are required for CSR at some level. While both Ku70 and Ku80 deficient B cells have severe CSR defects (Casellas et al., 1998; Manis et al., 1998a; Reina-San-Martin et al., 2003), the role of DNA-PKcs is still debated, owing to different results from different mouse models (Bosma et al., 2002; Cook et al., 2003; Manis et al., 2002a; Rooney et al., 2005). However, considering that all these proteins have roles outside NHEJ, some of the effects on CSR (especially the proliferation defects in Ku-deficient B cells) could be due to effects on other cellular processes.

On the other hand, XRCC4 and DNA ligase IV are the only two proteins that have no known role outside of the NHEJ pathway and are absolutely required for V(D)J recombination (Frank et al., 1998; Gao et al., 1998; Li et al., 1995; Taccioli et al., 1998). Although disruption of XRCC4 is embryonic lethal, generation of transgenic or Ig heavy and light chain “knock-in” mice (Soulas-Sprauel et al., 2007; Yan et al., 2007) revealed that XRCC4 is important for, but not required for CSR. Inactivation of DNA ligase IV in mice is also lethal (Barnes et al., 1998; Frank et al., 1998), and defective V(D)J recombination accounts for a complete block in lymphocyte development (Frank et al., 1998). However, CSR is defective as detected in mice expressing a hypomorphic Ligase IV ($LigIV^{Y288C}$) mutation (Nijnik et al., 2009) or peripheral blood cells from

human patients with mutations in DNA ligase IV (Pan-Hammarstrom et al., 2005). More recent additions to this group of proteins are Cernunnos/XLF (Buck et al., 2006; Li et al., 2008) and Aprataxin and PNK-like factor (APLF) (Macrae et al., 2008). Although Cernunnos/XLF is essential for normal NHEJ-mediated repair of DNA DSBs (Zha et al., 2007), XLF-deficient mature B cells are only moderately defective for CSR (Li et al., 2008) and APLF$^{-/-}$ B cells undergo efficient CSR (Rulten et al., 2011).

Table 2: Components of the NHEJ pathway in CSR.

Protein	CSR phenotype in absence of activity	Role in CSR
Classical NHEJ		
Ku70, Ku80	severely impaired	end joining?
DNA-PKcs	severely reduced except to IgG1	end joining? synapsis?
DNA-PKcs (SCID)	reduced CSR to all isotypes	
XRCC4 DNA ligase iv	20-50% reduction, mice and human Sμ-Sx junctions have increased microhomology	ligase function
Artemis	does not appear to be affected, but mice and human Sμ-Sx junctions have increased microhomology	end processing
XLF/Cernunnos	20-50% reduction, mild increases in junction microhomology	regulation of ligase function
APLF	does not appear to be affected, but mice have increased Sμ-Sx junction microhomology	end processing
alternative NHEJ		
PARP1	no quantitative effect, Sμ-Sγ3 junctions show increased direct joints	facilitates a-NHEJ ?
CtIP (shRNA knockdown in CH12 cells)	mildly reduced, Sμ-Sα junctions show increased direct joints	end-resection, enhancement of MRN nuclease activity?

Consistent with recombination through NHEJ Sμ-Sx junctions in normal individuals or in B cells from wild type mice are characterized by little or no microhomology between Sμ and Sx at the junction (Dunnick et al., 1993). On the other hand Sμ-Sx junctions from XRCC4$^{-/-}$, Lig IV$^{-/-}$, Artemis$^{-/-}$ or APLF$^{-/-}$ B cells and/or human patients show great increases in microhomology (upto 10 base pairs or more, with absence of direct joints) (Du et al., 2008; Pan-Hammarstrom et al., 2005; Rulten et al., 2011; Yan et al., 2007). This suggests that CSR can also occur via an alternative type of end joining (a-NHEJ) that favors the use of microhomologies. In line with this, we have shown that deletion of the DNA damage sensor Parp1 results in reduced microhomology at Sμ-Sx junctions, favoring repair of switch regions through a microhomology-mediated pathway, underlining the involvement of the a-NHEJ in CSR (Robert et al., 2009). A

comprehensive list of proteins in NHEJ (and a-NHEJ) and their role(s) during CSR is given in Table 2.

One of the earliest effectors of the DDR is the MRN (Mre11, RAD50, Nbs1) complex (Lee and Paull, 2005), which recruits ataxia telangiectasia mutated (ATM) – a serine/threonine kinase. ATM, is a member of a family of kinases which also includes ATR (ATM and RAD3 related) and DNA-PKcs, and is the master regulator of the DDR (Shiloh, 2003). ATM and related kinases can undergo autophosphorylation as well as phosphorylate several other effector proteins including p53, Nbs1 (Falck et al., 2005), H2AX (Burma et al., 2001; Paull et al., 2000; Rogakou et al., 1998), 53BP1 (Anderson et al., 2001; Rappold et al., 2001), MDC1 (Lou et al., 2006) and the more recently described KRAB domain associated protein (KAP1, also called Tif1β, KRIP1 and TRIM 28) (White et al., 2006; Ziv et al., 2006). In response to a DSB, ATM-dependent phosphorylation of H2AX on serine 139 results in the formation of γH2AX (Rogakou et al., 1998). This leads to the recruitment of several other repair factors including MDC1, 53BP1, and Nbs1, which via their ability to bind γH2AX accumulate at the site of damage to form multi-protein "foci" (Kobayashi et al., 2002; Stewart et al., 2003; Stucki et al., 2005; Ward et al., 2003), that can expand upto megabases on either sides of the break (Lou et al., 2006; Stucki and Jackson, 2006). Interestingly, the phosphorylation of MDC1 recruits an E3 ubiquitin ligase, RNF8 (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007) that mediates the monoubiquitination of H2A-type histones and subsequent recruitment of another E3 ubiquitin ligase - RNF168 (Doil et al., 2009; Stewart et al., 2009), which is crucial for the accumulation and stabilization of 53BP1 at DSB sites. These proteins are thought to alter the structure of surrounding chromatin in response to AID-initiated DSBs during CSR, to permit access to other downstream factors and/or hold broken DNA ends together or bring widely separated ends closer together by the formation of large protein complexes. Table 3 includes a list of proteins in the DDR and their role(s) during CSR.

Thus, the initiating lesion by AID during CSR ultimately leads to the formation of DSBs and subsequently employs DSB repair pathways, (NHEJ) for the resolution of these breaks. In addition, this pathway (and possibly an alternative pathway) is also responsible for ligation CSR junctions. Drawing comparisons to V(D)J recombination, although both the reactions generate DSBs as intermediates, V(D)J recombination appears relatively unimpaired in the absence of DDR proteins like H2AX and 53BP1, while CSR is dramatically affected. One possible explanation for this is that these proteins are somehow involved in the long-range synapsis of S regions during CSR by holding the ends together before their joining via NHEJ.

The final phase of CSR requires bringing the DSBs at the donor and acceptor S regions together in a synapsis, which is followed by their ligation. Three hypotheses to explain the synapsis and joining during CSR have been proposed. (a) AID itself participates, by binding the two switch regions and bringing them together by virtue of its dimerization (Wang et al., 2006a).

(b) S regions themselves play a role, either by binding to the MSH2-MSH6 proteins, by binding transcription complexes that could help in juxtaposition, or simply by forming higher order structures (Dempsey et al., 1999; Larson et al., 2005; Manis et al., 2002b). (c) DNA damage proteins like ATM, 53BP1, and H2AX have been proposed to bind and facilitate this step of the CSR reaction, by mediating structural changes in the chromatin as well as anchoring the distal DSBs, as mutations or deletion of these proteins lead to CSR defects without affecting the frequency of AID-initiated DNA damage (Bassing and Alt, 2004; Reina-San-Martin et al., 2004; Reina-San-Martin et al., 2007; Reina-San-Martin et al., 2003).

Table 3: Components of the DNA damage response in CSR.

Protein	CSR phenotype in absence of activity	Role in CSR	Genomic instability
ATM	50–80% reduced, no effect on Sμ mutations and deletions	end processing? synapsis?	+
H2AX	50–80% reduced, no effect on Sμ mutations and deletions	synapsis?	+
53BP1	impaired, intra Sμ deletions	repair, synapsis?	+
(MRN complex) NBS1	2-3 fold reduction	DSB detection and processing. DNA damage sensor	+
(MRN complex) MRE11	CSR defect, milder than that of total MRN loss	DSB detection and processing. DNA damage sensor	+
MDC1	mildly reduced	mediator protein	+
RNF8	2-3 fold reduction	mediator protein for MDC1?	+
RNF168 (shRNA knockdown in CH12 cells)	2 fold reduction	mediator protein for MDC1?	+

E. Internal S region deletions and mutations during CSR

S regions are frequently punctuated by single nucleotide mutations and small deletions (Dunnick et al., 1993) that are AID-dependent (Petersen et al., 2001), and can be detected at Sμ in B cells activated for, but that have not undergone CSR *in vitro*. The analysis of this mutation frequency and pattern is considered a quantitative method to measure AID activity, and I have used this method as a readout of AID action at the Sμ, as presented later. The

recruitment of DNA Pol η results in A:T mutations at the V regions during SHM, and at both unrecombined Sμ and recombined Sμ-Sx junctions during CSR (Delbos et al., 2007; Faili et al., 2004). C:G mutations at the junctions, could either be due to replication probably by DNA Pol θ, or that excision by Exo1 did not lead to the formation of a DSB but was simply repaired. Finally, if DSBs are not synapsed efficiently with a downstream S region it could result in internal Sμ deletions rather that recombination (Dudley et al., 2002; Masuda et al., 2006; Masuda et al., 2005; Zan et al., 2001). Interestingly, mice deficient for ATM, H2AX and 53BP1 accumulate internal Sμ deletions at normal or increased frequencies; suggesting that such factors could somehow be required for long-range S regions synapsis (Reina-San-Martin et al., 2004; Reina-San-Martin et al., 2007; Reina-San-Martin et al., 2003).

5. Pathologies associated with AID

A. Ig class switch recombination deficiencies

X-linked Ig-CSR due to CD40L deficiency

Ig-CSR deficiencies (Ig-CSR-Ds) are immunodeficiencies caused by genetic defects that are characterized by a complete block or impairment in CSR, manifested as either normal or increased levels of IgM with a concomitant decrease or absence of IgG, IgA or IgE. This defect in CSR is either associated, or not, with a defect in SHM. The most frequent and first described syndrome was the X-linked Ig-CSR-D caused by mutations (irregularly distributed in exon 5) in the gene encoding the CD40 ligand (CD40L), a membrane glycoprotein expressed on activated T cells (Allen et al., 1993; Aruffo et al., 1993; DiSanto et al., 1993; Hollenbaugh et al., 1992; Korthauer et al., 1993). CD40L interacts with CD40, a member of the tumor necrosis factor receptor family, which is expressed on B cells and variably on other lymphoid and myeloid cells (Grammer and Lipsky, 2000). CD40L binds to CD40 and induces B cell proliferation (Nishioka and Lipsky, 1994) and AID induction (Muramatsu et al., 1999). *In vitro* stimulated $CD4^+$T cells from these patients exhibit either reduced or no expression on CD40L. Patients are susceptible to recurrent bacterial infections, but their B cells are intrinsically normal i.e. they can be induced to proliferate and undergo CSR *in vitro* in response to stimulation (Durandy et al., 1993). They do not generate antibodies against pathogens or vaccines, but anti-polysaccharide IgMs are usually detected.

Ig-CSR due to defective CD40 signaling

Patients with defects in CD40 are characterized by the lack of surface expression of CD40 on B cells or monocytes, with clinical symptoms similar to the CD40L deficiencies, except that the B cells are unable to undergo *in vitro* CSR (Ferrari et al., 2001). Both the above-mentioned deficiencies are caused by the ablation of upstream signaling pathways leading to CSR and SHM.

X-linked Ig-CSR due to defective NF-κB activation

In addition, the molecular characterization of Ig-CSR-Ds due to defects in NF-κB essential modulator (NEMO), reveals normal to increased levels if IgM, low serum levels of IgG and IgA and either normal or defective *in vitro* CSR and SHM (Kracker et al., 2010). Overall, these deficiencies have provided valuable evidence that the CD40 activation pathway as well as the NF-κB transcription complex play a key role in antibody diversification.

B. B-cell intrinsic Ig CSR deficiencies

Ig-CSR deficiency type 1 caused by AID deficiency

This Ig-CSR deficiency was found to be the consequence of a defect in AID. Patients with AID deficiencies in the form of autosomal recessive mutations in the *AICDA* gene that abrogates the expression or function of AID (characterized by a defect in CSR and SHM) are included in this group. These patients usually have large lymph nodes and correspondingly large GCs, which is presumably the consequence of intense B cell proliferation (Revy et al., 2000). They suffer from a drastically defective CSR, as IgG, IgA and IgE levels are barely detectable in the serum, as compared to elevated levels of IgM in almost all cases. *In vitro*, B cells proliferate normally and produce large amounts of IgM, however they do not undergo CSR to generate other isotypes (Durandy et al., 1997). As the first step of the CSR reaction – production of GLTs was normal, and the rest of the steps were not, this CSR defect was shown to be downstream of transcription and upstream of DNA breaks. Furthermore, SHM was either absent or drastically decreased in these patients. On sequencing *AICDA*, biallelic mutations were found all across the gene – in the NLS, the cytosine deaminase domain and the APOBEC1-like domain. A detailed characterization of these mutations and the description of a defect for both SHM and CSR – that was similar to the phenotype of AID-deficient mice (Muramatsu et al., 2000), led to the determination of the role of AID as a master regulator of CSR and SHM.

AID deficiency with preserved SHM

A selective group of patients with CSR defects caused by AID function, defective CSR *in vivo* and *in vitro*, but a normal frequency and pattern of SHM were described. Strikingly, these patients carried mutations in the C-terminal domain of AID (Ta et al., 2003). The observation of normal SHM and defective CSR caused by these naturally occurring human mutations, was also seen using a C-terminal truncation mutant (deleted of the last ten amino acids) of AID (Barreto et al., 2003). Taken together, these results suggested that AID has a CSR-specific activity most probably by binding to protein cofactor(s) through its C-terminal domain.

Autosomal dominant transmission of AID deficiency

Patients are found to carry heterozygous nonsense mutations located in the NES of AID, and interestingly, although CSR *in vitro* was defective, DSBs were detected at Sμ, tracing the defect

downstream of AID-induced lesions – an observation in line with the hypothesis of a CSR-specific cofactor being a DNA repair protein (Imai et al., 2003a).

Ig-CSR deficiency type 2 caused by UNG deficiency

Ig-CSR type 2 is a consequence of UNG deficiency that leads in humans to a unique CSR deficiency characterized by defective CSR and a skewed SHM pattern. The UNG deficiency is comparatively rare and only three patients have been reported so far (Imai et al., 2003b). Four mutations, all affecting the active site of UNG have been reported. While two patients have small deletions resulting in a stop codon, the other carries a homozygous missense mutation. All patients have CSR defects *in vivo* and *in vitro*, with the *in vitro* deficiency traced upstream of DSB formation (Imai et al., 2003b). Though SHM occurs, majority of the mutations are C:G transitions, similar to mice deficient in UNG that accumulate only C:G mutations in the V regions (Rada et al., 2002b).

A new form of B cell-intrinsic CSR deficiency in three patients with deleterious, homozygous mutations in the gene encoding the PMS2 (a component of the mismatch repair machinery), had also been reported. CSR was found partially defective *in vivo* and markedly impaired *in vitro*, and the CSR specific defect is due to inefficient DSBs at switch regions and abnormal formation of switch junctions. This observation strongly suggested a role for PMS2 in CSR-induced DSB generation (Peron et al., 2008).

Molecularly undefined Ig-CSR deficiency with normal SHM

The CSR defect in these cases is milder than in AID or UNG deficiency, as residual levels of IgG can sometimes be detected. This group of deficiencies is further classified into two categories: (a) Ig-CSR deficiency type 3 – CSR block located upstream of Sμ DNA cleavage. The phenotype of these patients is very similar to one of the patients carrying mutations in the C-terminus of AID. The defect is downstream of GL transcription and upstream of DNA cleavage (as DSBs at Sμ are undetectable) and thus, this defect could be a direct outcome of inefficient AID targeting to the Ig*h* locus (Kracker et al., 2010). (b) Ig-CSR deficiency type 4 – CSR block located downstream of Sμ DNA cleavage. In contrast to most cases of AID deficiency, *in vitro* defective CSR was accompanied by DSBs at Sμ. However, the next step of the reaction was impaired, as post-switch transcripts were not detected, which rules out defective AID targeting. This unique phenotype can therefore be explained either by a defect in the repair of the AID-induced lesions at S regions, a defect in switched B cell differentiation of survival or an inappropriate response to DNA damage (Durandy et al., 2007).

C. The oncogenic potential of AID

Most human lymphomas are derived from mature B cells, and these cancers are usually associated with translocations of Ig genes, for example: BCL2-Ig translocations in follicular lymphoma or the MYC-Ig translocations in Burkitt's lymphoma. Most of the Ig gene breakpoints

are located in the V or S regions, with translocations involving the S regions were thought to be CSR related (Kuppers and Dalla-Favera, 2001). This hypothesis was put to test using mouse plasmocytoma models either by injection of mineral oil or overexpression of IL-6 (Potter and Wiener, 1992; Suematsu et al., 1992). Both studies reported translocations involving the 5' untranslated region (5' UTR) of the *Myc* proto-oncogene to S regions (Potter, 2003) induced by AID catalytic activity (Ramiro et al., 2006; Ramiro et al., 2004; Unniraman et al., 2004).

AID-initiated DSBs during CSR are the likely substrates for translocation, as they are obligatory intermediates. However, the mechanisms that produce DSBs on partner chromosomes could be a result of either random breaks produced during replication, expression of common fragile sites, environmental stress (Arlt et al., 2006; Wyman and Kanaar, 2006), or occasional off-target activity of AID (Gordon et al., 2003; Kuppers, 2005). Although, DSBs are not intermediates during SHM, the activity of AID during SHM can also result in (though not often) translocations, eg: during Burkitt's lymphoma and diffuse large cell lymphoma when the c-myc and bcl-6 genes are translocated to Ig*h* or Ig*l* V regions (Kuppers, 2005). Such Ig*h*/c-myc translocations can be induced in B cells stimulated to undergo CSR and analyzed by long-range PCR (Ramiro et al., 2004) and Southern blotting. I have used the same technique to analyze the frequency of such Ig*h*/c-myc translocations.

In addition to initiating the kind of translocations mentioned above, AID can also induce transformation by mutating oncogenes (Kotani et al., 2005). AID has also been linked to gastric and hepatocaricinogenesis (Endo et al., 2007; Matsumoto et al., 2007) and constitutive and ubiquitous expression of AID in transgenic mice results in both T cell lymphomas and micro-adenomas (Okazaki et al., 2003). Consistent with this, AID overexpression in transgenic mice leads to extensive translocations of non-Ig genes and cancer (Robbiani et al., 2009). Furthermore, even low levels of mutation are sufficient to produce substrates for translocation (Robbiani et al., 2008; Robbiani et al., 2009). AID is also erroneously targeted to non-Ig genes throughout the genome and mutates about 25% of the genes transcribed in germinal center B cells, where it is normally expressed, in the absence of UNG and MSH2 (Liu et al., 2008).

Therefore, although AID-mediated DNA damage is normally restricted to antibody genes, aberrant targeting of AID to non-Ig genes and abnormal repair of AID induced DNA lesions do occur and may account for the increased propensity for malignant transformation in B cells. Thus, the molecular mechanisms that control AID activity and its specific targeting to Ig genes to enforce accurate repair of AID-induced DNA damage need to be in place. I will thus address, in the following section, accessional control of the Ig locus, and how AID is regulated and targeted during antibody diversification (Figure 13).

6. Mechanisms of AID regulation

A. Transcriptional regulation

Transcription of AID mRNA is synergistically induced by IL-4 and CD40 signaling in a STAT6 and NF-κB dependent manner. Pax5 also regulates AID transcription by binding to its promoter in IL-4 treated B cells (Dedeoglu et al., 2004; Gonda et al., 2003). The *aicda* locus contains four regulatory regions in the mouse – the first is located 8 kb upstream of exon 1 (Yadav et al., 2006), and contains potential motifs for binding of transcription factors NF-κB, STAT6, C/EBP and Smad3/4 (Tran et al., 2010; Yadav et al., 2006). The second region is located about 1 kb upstream of exon 1 and contains sites for NF-κB, STAT6, HoxC4 and Pax5 (Dedeoglu et al., 2004; Gonda et al., 2003; Park et al., 2009; Yadav et al., 2006). The third region is between exons 1 and 2 and "houses" binding sites again for NF-κB, E proteins, Pax5 and several others (Gonda et al., 2003; Tran et al., 2010). The last element is about 6 kb downstream of exon 5 and appears to function as an enhancer (Crouch et al., 2007).

The Id1, 2 and 3 proteins that inhibit AID transcription by binding to stimulatory factors E47 and Pax5 (Goldfarb et al., 1996; Gonda et al., 2003; Quong et al., 1999; Sayegh et al., 2003). More recently, the sex hormones estrogen and progesterone have been implicated in upregulating AID in hormone-based cancers and autoimmunity (Petersen-Mahrt et al., 2009), estrogen more specifically binds to, and activates the HoxC4 promoter to mediate AID expression during immune responses (Mai et al., 2010).

Post transcriptionally, the level of AID transcripts is controlled by microRNA (miR) molecules. miR-155 binds to the 3' UTR of AID mRNA to destabilize it. Mutant 3' UTR's of AID, that are unable to bind miR-155 AID expression levels were increased in splenic and Peyer's patch B cells (Dorsett et al., 2008; Teng et al., 2008), accompanied by an increase in c-myc/Ig*h* chromosomal translocations (Dorsett et al., 2008). miR-181b, that binds the 3'-UTR of AID to regulate AID in a similar fashion (de Yebenes et al., 2008). However, as these miRNA's can have an effect on several other genes apart from *aicda,* more work would help better understand their biological role (Figure 13).

B. Sub-cellular compartmentalization

Although the function of AID is strictly nuclear, its localization is primarily cytoplasmic (Rada et al., 2002a; Schrader et al., 2005) and this along with other mechanisms is thought to minimize the off-target activity of AID (Casellas et al., 2009). The localization of AID is governed by four main processes (a) presence of an anchor sequence at the C-terminal (Patenaude et al., 2009) (b) its quick degradation in the nucleus through ubiquitination (Aoufouchi et al., 2008). (c) its inability to passively diffuse into the nucleus, despite its small size and the interaction of its NLS

with importin-α3 that actively transports AID into the nucleus (Patenaude et al., 2009) and (d) its efficient export from the nucleus through a CRM1-dependent mechanism through the C-terminal conserved NES (Brar et al., 2004; Geisberger et al., 2009; Ito et al., 2004; McBride et al., 2004). Thus, the compartmentalization and function of AID is tightly regulated by the competition between active nuclear import mediated by importin-α3 and export by CRM1 (Figure 13).

C. AID and its cofactors

AID is also tightly regulated by protein cofactors that coordinate its recruitment and functions, and extensive studies in several labs attempt to identify AID partner proteins. One of the first advances towards this quest was the identification of replication protein A (RPA) as a protein cofactor for AID. Using an *in vitro* assay that measured AID-mediated deamination of transcribed synthetic SHM substrates enriched in RGYW motifs (Chaudhuri and Alt, 2004), RPA was found to interact with AID purified from B cells through its 32 kDa subunit (RPA has 17, 32 and 70 kDa subunits). Also, while the AID-RPA complex binds transcribed synthetic substrates *in vitro*, the two proteins individually do not. AID houses a cAMP-dependent protein kinase A (PKA) phosphorylation motif at its N terminus, and the serine 38 residue is phosphorylated by PKA (see below), and this modification is required for AID to bind RPA and mediate deamination (Basu et al., 2005; Cheng et al., 2009; McBride et al., 2006; Vuong et al., 2009). Based on ChIP experiments, it has been proposed that CSR requires the assembly of a complex that contains the catalytic and regulatory units of PKA and AID on S regions, leading to the generation of DNA lesions that initiate CSR (Vuong et al., 2009).

Germline (GL) transcription is known to play a key role in targeting the CSR machinery to S regions (Chaudhuri et al., 2007). However, nothing much is known about a role for the components of the splicing machinery or the spliced transcripts themselves (or both) in CSR. As early as 2003, Nambu et al. showed that AID interacts with RNA Pol II (Nambu et al., 2003), although no further analyses were done to map these interactions. More recently, a cytoplasmic yeast two-hybrid screen was used to isolate N-terminal truncations of the nuclear protein catenin beta like 1 (CTNNBL1), which recognizes and binds amino acids 39-42 of AID (Conticello et al., 2008). Previously, CTNNBL1 and cell division cycle 5 like (CDC5L) have been described to be present in a purified splicing complex, and AID could be in this complex as it also associates with endogenous CDC5L. As CTNNBL1 interacts with components of the RNA Pol II spliceosome, it suggests that AID might travel with the transcription complex. And as pieces of the puzzle keep coming together, AID was recently shown to associate with Suppressor of Ty 5 homolog (Spt5). Spt5 is a factor that binds stalled RNA Pol II and ssDNA to strengthen its interaction with RNA Pol II. AID uses Spt5 as a mediator to strengthen its interaction with RNA Pol II to be targeted to Ig (and non-Ig) loci (Pavri et al., 2010).

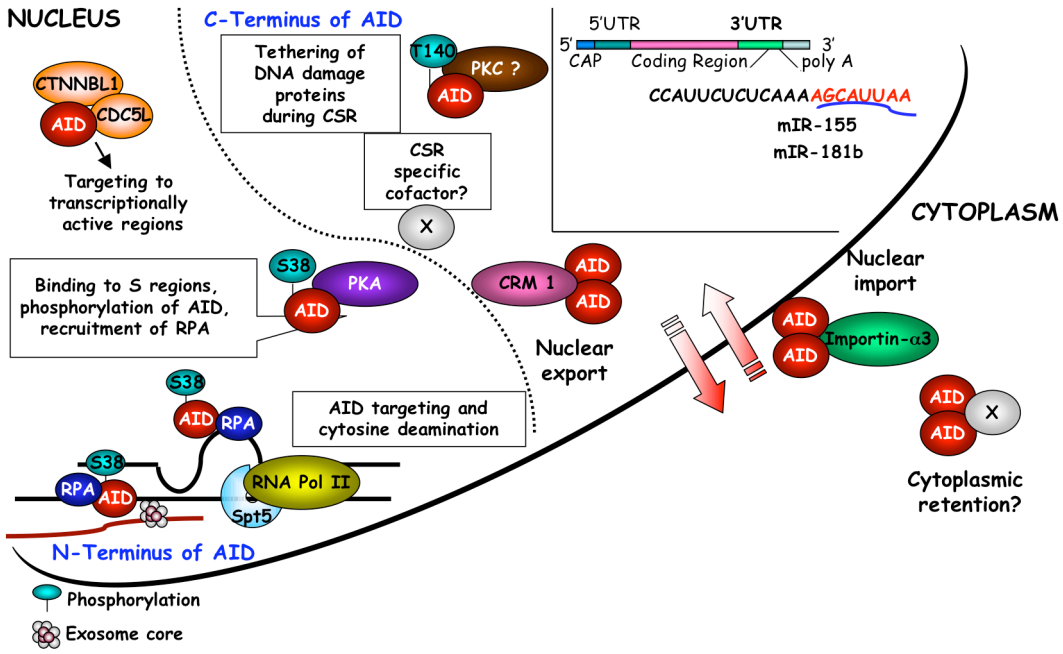

Figure 13: Cellular regulation of AID: AID mRNA stability is regulated by mIR-155 and 181b (inset). The cellular localization and the impact of post-translational modifications also regulate AID function. Every protein, sequence or modification are indicated, the cytoplasm and nuclear functions are divided by the thick black line and the dotted line broadly separates protein interactions and post-translational modifications on the N and C terminus of AID. (The interacting domain(s) for Spt5 and the exosome have not yet been identified).

In *Drosophila*, an evolutionarily conserved RNA processing/degradation complex - the RNA exosome interacts with elongating RNA Pol II via the Spt5/6 transcription factors (Andrulis et al., 2002), and AID was shown to associate with the RNA exosome in B cell lines stimulated to undergo CSR (Basu et al., 2011). The core components of the RNA exosome complex are required for efficient CSR and are thought to target AID activity to both strands of transcribed duplex DNA (Basu et al., 2011). Finally, specificity of AID for binding to S regions during CSR is mediated also by its ability to interact with the polypyrimidine tract-binding protein 2 (PTBP2) (Nowak et al., 2011). Thus, theoretically AID could be targeted to Ig loci by its association with RNA Pol II via CTNNBL1, that is stabilized by Spt5 and the RNA exosome to be loaded onto DNA at donor splice sites so as to interact with RPA and initiate deamination (Figure 13).

AID-initiated DSBs which are obligate intermediates in CSR and recruitment of DDR proteins to the Ig*h* locus during CSR have been proposed to promote changes in chromatin structure that allow for the synapsis of S regions (Manis et al., 2004; Reina-San-Martin et al., 2003) prior to resolution by classical and alternative NHEJ (Bosma et al., 2002; Casellas et al., 1998; Franco et al., 2008; Kotnis et al., 2009; Manis et al., 1998a; Manis et al., 2002b; Reina-San-Martin et al., 2003; Soulas-Sprauel et al., 2007; Yan et al., 2007). Consistent with this, AID is reported to associate with the catalytic subunit of DNA-PK in HeLa cells, as identified by affinity purification and mass spectrometry analysis (Wu et al., 2005). It was proposed that binding of AID to DNA

induced a conformational change in the C-terminus of AID, which in turn recruited DNA-PKcs that disassociates from AID after deamination in order to reconstitute the NHEJ complex.

D. Post translational modifications

While access to genomic DNA and degradation of AID is controlled by its subcellular localization, its function is regulated largely by phosphorylation. Comparing catalytic activity of AID proteins from B cells and the human kidney cell line 293, revealed that deamination activity of AID from 293 cells was reduced than that from B cells (Chaudhuri et al., 2004). Based on mass spectrometry predictions, AID has four known phosphorylation sites: Threonine 27 (T27), Serine 38 (S38), Threonine 140 (T140) and Tyrosine 184 (Y184) (Basu et al., 2005; McBride et al., 2006; McBride et al., 2008; Pasqualucci et al., 2006). T27 and S38 are phosphorylated by PKA and regulate AID's interaction with RPA (Basu et al., 2005; Pasqualucci et al., 2006), and mutation of these residues impairs SHM, CSR and IGC (Basu et al., 2005; Chatterji et al., 2007; McBride et al., 2006; Pasqualucci et al., 2006; Vuong et al., 2009). Of these S38 seems to be the main site, as mice expressing a serine to alanine mutation at this position (AID^{S38A}) have decreased SHM and CSR (Cheng et al., 2009; McBride et al., 2008). On the other hand, T140 is phosphorylated by protein kinase C (PKC), and mutating T140 affects SHM more than CSR, thus suggesting differential phosphorylation at different sites on AID could result in different functions (Figure 13). On the contrary, substituting Y184 by alanine did not have striking effects and thus may not play a role in regulating AID's activity (Basu et al., 2005; McBride et al., 2008).

7. What dictates AID's target specificity?

Extensive sequencing of B cell genes during SHM, showed that although the comparative frequency of mutation at non Ig genes is fairly low, approximately 25% of expressed genes accumulate AID-dependent mutations in GC B cells, indicating that AID activity is genome wide (Liu et al., 2008). While the precise mechanisms that target AID to Ig genes remain unknown, AID-induced mutations are associated with transcription and are most prevalent in a 2 kb region beginning downstream of the promoter (Di Noia and Neuberger, 2007; Peled et al., 2008; Stavnezer et al., 2008; Storb et al., 2007). Examination of the mutational pattern in both SHM and at the S regions during CSR shows that mutations occur in close proximity to the promoters, which has led to the hypothesis that AID is recruited to Ig loci with the transcription machinery. As discussed before, transcription at S regions generates ssDNA in the context of R-loops and such transcribed DNA can serve as targets for AID (Chaudhuri and Alt, 2004; Tian and Alt, 2000; Yu et al., 2003). However, other mechanisms operate to target AID, especially in the context of V region exons, as they do not form R-loops *in vitro,* but are still targeted by AID during SHM (Chaudhuri and Alt, 2004; Papavasiliou and Schatz, 2002).

Knockout mice with partial deletions in Sμ have defective CSR (Luby et al., 2001; Schrader et al., 2007) and complete deletion of the Sμ tandem repeats blocked CSR and SHM (Khamlichi et al., 2004). Thus, based on AID's preference for RGYW sequences *in vitro* (Pham et al., 2003; Yu et al., 2004) and its interaction with RPA (Chaudhuri et al., 2004) it was proposed that the AID-RPA complex binds to and stabilizes ssDNA in the context of transcription bubbles, allowing AID-mediated deamination at the RGYW sequences (Chaudhuri et al., 2004).

AID initiated processes are also limited by transcriptional regulators like PTIP, which facilitate RNA Pol II access to specific S regions (Daniel et al., 2010). However, active transcription alone does not allow specific access of AID to DNA and cannot explain why AID primarily targets promoter proximal regions of only some transcribed genes. As stalled RNA Pol II is a characteristic of promoter-proximal regions, the fact that Spt5 (a stalling factor) associates with AID provides an explanation for this. Indeed, Spt5 is required for AID recruitment to S regions as Spt5 and AID accumulation coincide genome-wide and high density of Spt5 predicts AID-dependent mutations. Spt5 is thought to stabilize the interaction between AID and RNA Pol II thereby targeting AID's activity to genomic loci that accumulate paused RNA Pol II. This suggests that AID capitalizes on the phenomenon of Pol II stalling, which is in fact widespread across the B cell genome in order to gain access to its genome-wide targets (Pavri et al., 2010). On a global level, analysis of the enrichment of AID and its cofactor RPA2 indicates that stalled polymerases recruit AID, and can result in off-target mutations across the B cell genome, albeit at low frequencies. On the other hand, efficient hypermutation and CSR require, in addition, the activation of AID (by phosphorylation) and the recruitment of RPA2 (Yamane et al., 2010). However, recruitment of AID is one of the first steps during SHM and CSR and this has to be followed by AID gaining access to its targets. The RNA exosome complex appears to be the factor that can target AID activity to both template and non-template strands, as it has been recently shown to promote AID-mediated deamination of both template and non-template strands of *in vitro* transcribed SHM substrates. Furthermore, the RNA exosome complex associates with AID, and accumulates on S regions in an AID-dependent manner in B cells during CSR, and the core component of the complex is required for efficient CSR (Basu et al., 2011).

Also, as described before, the C-terminus of AID is proposed to associate with CSR-specific factors that could be required for targeting AID to switch regions (Ta et al., 2003), or for recombination downstream of DSB formation either by mediating RNA editing (Doi et al., 2009; Nonaka et al., 2009), or by recruiting factors that promote efficient DNA repair (Barreto et al., 2003; Ito et al., 2004; Kracker et al., 2010; Shinkura et al., 2004; Ta et al., 2003). Interestingly, the recent revelation that Igλ, Ig*h* and *Myc* loci are spatially contained in close proximity within the nucleus, suggests that nuclear organization can also affect both Ig and non-Ig targeting of AID (Wang et al., 2009a). In addition to all this, other transcriptional related events like

epigenetic modifications play a role in coordinating AID activity and targeting the SHM and/or CSR machinery to Ig loci, and are discussed later.

8. An emerging role for AID outside of the immune system

Although AID is primarily an enzyme associated with the immune system, it was found albeit at low levels, in mammalian germ cells and deaminates 5-Methylcytosines (5-meC) resulting in T:G pairing, which could result either in C:T transition mutations in methylated DNA or demethylation if the T:G mismatch is repaired (Morgan et al., 2004). AID also plays a role in the global demethylation of zebra fish embryos by a coupled mechanism that involves the demethylation of 5-meC, followed by thymine base excision by the methyl-domain binding protein 4 (Mbd4), which is promoted by the non enzymatic factor Gadd45 (Rai et al., 2008). The ability to reprogram somatic cells such as fibroblasts into “induced pluripotent stem cells” (iPCs) that are similar to embryonic stem (ES) cells (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007), by expressing of a cocktail of four “factors”, has ushered in a new age in regenerative medicine. It is well established that reprogramming of somatic cells involves extensive demethylation of pluripotency genes. Last year, Bhutani et al. used interspecies heterokaryons (by fusing mouse ES cells to human fibroblasts, which is a model that undergoes cellular reprogramming synchronously, frequently and fast), to demonstrate that AID was required for demethylation of the promoters, and thus induction of pluripotency genes like Oct4 and Nanog (Bhutani et al., 2010). This suggests that the change in methylation status of these genes is essential during reprogramming, and that AID plays a role in active demethylation and initiation of reprogramming of somatic cells towards pluripotency (Bhutani et al., 2010).

On the other hand, by profiling genome-wide DNA methylation in wild type and AID deficient primordial germ cells (PGCs), the Reik lab showed, that AID deficiency interferes with the genome-wide removal of DNA methylation patterns (Popp et al., 2010). As such erasure of DNA methylation in the germ line is a ubiquitous process that limits the potential for epigenetic inheritance, AID seems to also play an important role in epigenetic reprogramming and possibly in restricting the inheritance of epimutations (Popp et al., 2010). Though the actual mechanism by which AID is involved in these processes is still unclear, it is possible that AID works in conjunction with the “DNA methylation” machinery – a system that by itself, is largely unknown.

With this elaborate introduction on B cell diversification mechanisms, AID and its regulation I will now briefly describe chromatin regulation and the role of proteins like HP1 and KAP1 in regulating transcription and DNA repair.

III: Chromatin regulation and epigenetics

1. The structure of chromatin

Epigenetics is the study of factors that modify the packaging of DNA to generate changes in gene expression without altering the underlying sequence. This information can be stored in two ways (a) chemical modifications to cytosine bases and (b) modifications to the histone proteins that package the genome. These chemical changes regulate chromatin structure and DNA accessibility or simply - gene expression. A nucleosome is the basic structural unit of chromatin consisting of ~147 base pairs of DNA and an octamer of histone proteins (two molecules each of H2A, H2B, H3 and H4) with adjacent nucleosomes coupled by a stretch of free "linker DNA" (~10-80 bp in length) (Felsenfeld and Groudine, 2003). Each histone folds to form the histone fold motif, while the N-terminal `tail` extends freely and most of the post-translational modifications (PTMs) are concentrated within the tail extensions. Chromatin is divided between hetero and eu chromatin. The term heterochromatin (HC) refers to chromatin that is different – *"hetero"* from the true *"eu"* chromatin. HC is highly condensed and comprises about 10-25% of total chromatin, while euchromatin is more open and usually associated with active transcription (Yunis and Yasmineh, 1971). There are broadly two types of HC (a) facultative HC that includes genes originally a part of active euchromatin, but which are silenced during development or ageing (b) constitutive HC that refers to transcriptionally inert DNA, which remains silent throughout life, like centromeric satellite DNA and telomeric ends. Heterochromatin is built of basic components that include (i) regulators of histone modifications acetylation and methylation, (ii) DNA sequence-specific transcriptional repressors and co-repressors, (iii) chromodomain containing proteins, (iv) proteins involved in DNA methylation and (v) chromatin remodeling enzymes.

2. Epigenetic modifications and gene expression regulation

Histone modifications modulate the interaction potential of the tail domains and hence influence the folding and functional state of the chromatin (Jenuwein and Allis, 2001). There are at least 8 types of histone modifications (Figure 14), of these, the three most widely studied are acetylation, methylation and phosphorylation. Modifications occur at different sites, and additional complexity is added through the number of modifications on a given residue, e.g: lysine residues can be "*mono, di or tri"* methylated, while arginines are either "*mono or di"* methylated. In this section I will discuss specifically histone phosphorylation, acetylation and methylation, as they are important for the results presented later.

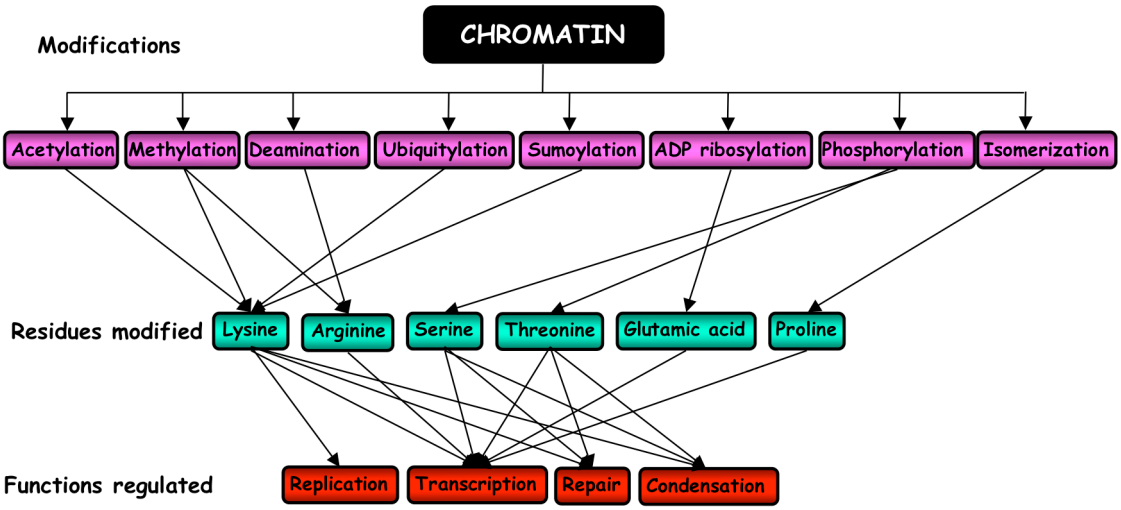

Figure 14: Epigenetic modifications and their functions: Chromatin can be subjected to several posttranslational modifications in order to carry out various physiological processes, represented by the pink and red rectangles. Each of these PTMs can occur on one or more residues (green rectangles), most often on the N-terminal tails of the histones and sometimes within the histone core to regulate several cellular processes (red rectangles).

A. Histone phosphorylation in chromatin remodeling and DNA repair

Histone phosphorylation plays an important role in processes such transcription, DNA repair, chromatin condensation and apoptosis. One of the first events in response to DSBs is the ATM-dependent phosphorylation of the histone variant H2AX on serine 139 (H2AX, Rogakou et al., 1998), that promotes the retention and accumulation of DNA repair proteins at the sites of damage (Figure 15). This phosphorylation event leads to “spreading” of the DNA damage signal across megabases and subsequent recruitment of HATs, ATP-dependent chromatin remodellers, and the cohesin complex to DSBs, serving as a docking site for downstream checkpoint and repair proteins. Phosphorylation of histone H2AX is induced and accumulates at S regions in response to AID-initiated DSBs during CSR and is required for efficient CSR (Petersen et al., 2001). It has also been shown that phosphorylation of linker histones by DNA-PK plays an important role in end-joining during VDJ recombination (Kysela et al., 2005), thus implying an interplay between chromatin modifications and Ag gene receptor diversification (described later).

B. Histone acetylation in transcription and DNA repair

Histone acetylation (Ac) is catalyzed by a group of enzymes known as Histone Acetyl Transferases (HATs), and is thought to weaken histone-DNA interactions or alter protein interactions e.g: histone-histone interactions between adjacent nucleosomes, or between histones and other regulatory proteins (Lee et al., 1993; Luger and Richmond, 1998; Wolffe and Hayes, 1999). Histone acetylation thus promotes transcription by favoring a more open chromatin conformation that is easily accessible for binding of the transcriptional machinery and other factors that promote transcription. (Roth et al., 2001; Shahbazian and Grunstein, 2007). Several HATs have been identified from several organisms ranging from yeast to humans, they include: (a) the Gcn5-related N-acetyltransferase (GNAT) superfamily members Gcn5, PCAF, Elp3, Hpa2, and Hat1, (b) MYST proteins Sas2, Sas3, Esa1, MOF, Tip60, MOZ, MURF, and HBO1, (c) global coactivators p300 and CREB-binding protein, (d) nuclear receptor coactivators SRC-1, ACTR, and TIF2, and (e) TATA-binding protein-associated factor TAF(II)250 and its homologs as well as subunits of RNA polymerase III general factor TFIIIC.

Role of histone acetylation in transcription

Acetylation of histones H3 and H4 at active genes has been associated with the activity of HATs such as Gcn5, which functions as part of the Spt-Ada-Gcn5-acetyltransferase (SAGA) or SLIK (SAGA-like) complexes (Kuo et al., 2000). In addition to transcriptional activators, other factors promote the binding of HAT complexes via a cross-talk between methylation and acetylation. During transcription, histone H3 is methylated mainly at two residues – lysine 4 (K4) at the 5' end of the coding region, and K36 throughout the coding region (Ng et al., 2003). These methylation marks appear to play a role in HAT recruitment, largely through proteins containing chromodomains (de la Cruz et al., 2005). One such family of proteins is the heterochromatin protein (HP) family, which is discussed in the next section.

Histone acetylation in DNA recombination and repair

Acetylation of lysines is most recognized for its ability to regulate gene expression. However, it has also been linked to replication and DNA damage tolerance (Kurdistani and Grunstein, 2003; Kusch et al., 2004; van Attikum and Gasser, 2005). A well studied example of acetylation in response to DNA damage is the histone acetyltransferase Hat1p, specific for acetylation of histone H4 lysine residues 5 and 12 (Qin and Parthun, 2006). Hat1p and nuclear Hat1p-associated histone chaperone (Hif1p) are recruited to sites of DNA damage and are associated with specific changes in histone modifications that occur during DNA repair (Figure 15). More recently, it has been shown that acetylated H3 K56 levels increase following DNA damage and accumulates at DNA repair foci along with γH2AX and phosphorylated ATM (Vempati et al.,

2010). In addition to these PTMs, the acetylation of H4 K5, K8, K12 and K16 are implicated in the DNA damage response especially with respect to antigen gene diversification. Modification of histones H3 and H4 associated with V(D)J recombination (McMurry and Krangel, 2000; Morshead et al., 2003; Osipovich et al., 2004) and with differential targeting of AID to S regions during CSR (Chowdhury et al., 2008; Kuang et al., 2009; Li et al., 2004a; Nambu et al., 2003; Wang et al., 2006b; Wang et al., 2009b) are discussed in detail below.

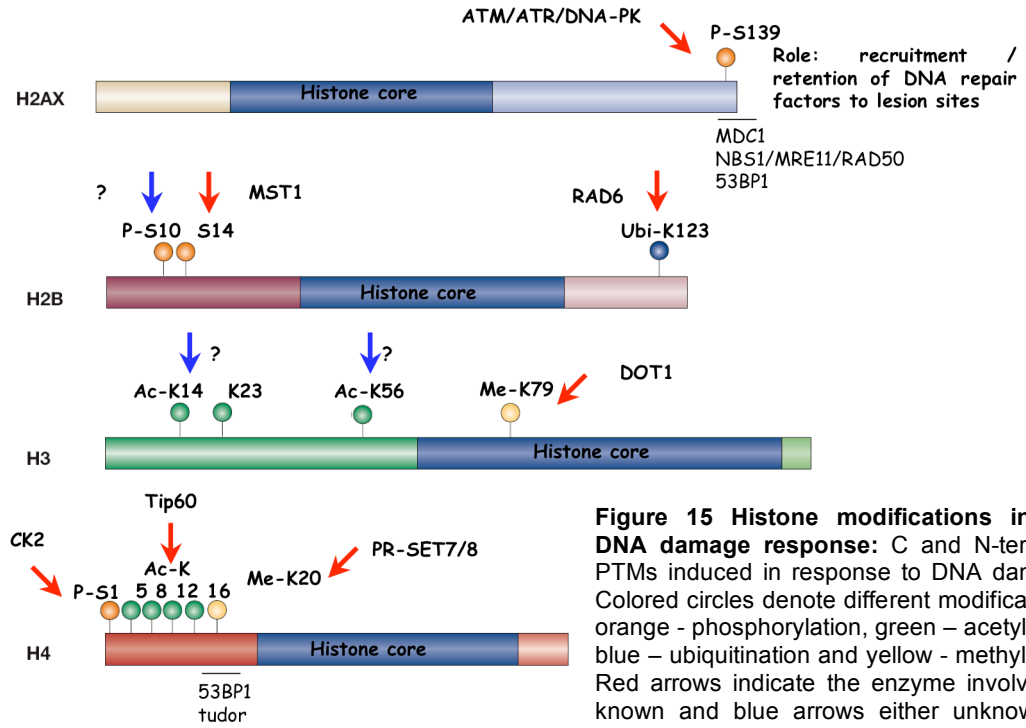

Figure 15 Histone modifications in the DNA damage response: C and N-terminal PTMs induced in response to DNA damage. Colored circles denote different modifications: orange - phosphorylation, green – acetylation, blue – ubiquitination and yellow - methylation. Red arrows indicate the enzyme involved, if known and blue arrows either unknown or possible candidate enzymes. Adapted from (van Attikum and Gasser, 2005).

C. Histone methylation in heterochromatin formation and DNA repair

Histones can also be methylated on arginine and lysine residues, and many sites of histone acetylation are also sites of methylation. The known lysine methylation sites that can be mono, di or tri methylated on histone H3 are K4, K9, K27, K36 and K79, and K20 on histone H4. Lysine residues are methylated by either SET domain or non-SET domain containing lysine methyltransferases, while demethylation is mediated by demethylases, the lysine specific demethylase 1 (LSD1) and the Jumonji C-domain (JmjC) containing proteins (Klose et al., 2006). SUV39 was the first histone methyltransferase to be identified (Rea et al., 2000), with its catalytic activity domain housed within a highly conserved SET domain. The methyltransferase activity of SUV39 is directed against lysine 9 of histone 3 (H3K9).

Methylation and heterochromatin formation

The integration of transgenes into heterochromatic regions leads to silencing phenomena like position effect variegation (PEV), first discovered in drosophila (Wakimoto, 1998). One such PEV suppressor protein is the H3K9 methyltransferase SUV39H1, whose role in heterochromatin function was shown by its association with heterochromatin protein (HP1), as H3K9me provides binding site for chromodomain of HP1 (Bannister et al., 2001). Similarly, the equivalents in mouse – Suv39h1 and h2 are responsible for H3K9 methylation and recruitment of HP1α and HP1β (Bannister et al., 2001; Lachner et al., 2001; Peters et al., 2003; Rice et al., 2003). In addition, G9a and the G9a-like protein (GLP) are two methyltransferases that mediate the bulk of euchromatic H3K9 methylation (Tachibana et al., 2005). Apart from H3K9 methylation, H4K20 methylation by SUV4–20h1 and SUV4–20h2 also marks mammalian heterochromatin (Schotta et al., 2004), and H3K27 methylation is linked to silencing phenomena (Cao and Zhang, 2004).

Interestingly, a more accurate description of the methylation status of histones H3 and H4 could define the chromatin state. In mice, for example: pericenteric HC is enriched in H3K9me3 and H4K20me3, while mono and dimethyl H3K9 and H4K20 mark euchromatin (Peters et al., 2003; Rice et al., 2003; Schotta et al., 2004), defining the recruitment of specific proteins, which in turn are dedicated to unique functions.

Histone methylation and DNA repair

Methylation of histones H3 and H4 play an important role in DNA repair, possibly by serving as a recognition site for DDR proteins. A well-known mark in response to DNA damage is the methylation of histone H3 at lysine 79 by the disruptor of telomeric silencing-1 (Dot1) (Huyen et al., 2004), that is required for the recruitment of mammalian 53BP1 and Rad9 to DNA damage sites *in vivo*. 53BP1 binds H3K79me through its Tudor domain, and Dot1 deletion or mutations in the Tudor domain of 53BP1 abolish the recruitment of 53BP1 to DSBs (Huyen et al., 2004). The complexity underlying interactions between chromatin and repair proteins in response to DSBs is further substantiated by the acetyltransferase Tip60, that is activated by a direct interaction between its chromodomain and H3K9me3 at DSBs, mediated by the MRN complex. Tip60 in turn acetylates histones and ATM, the key kinase in the DDR. Reduced intracellular H3K9me3 blocks activation of the acetyltransferase activity of Tip60, resulting in defective ATM activation and widespread defects in DSB repair. Taken together this implies that changes in histone methylation patterns can alter the efficiency of DSB repair (Sun et al., 2009).

Chromatin structure and integrity is also regulated by the modification of histones and other nuclear proteins by poly(ADP-ribose) polymers, catalyzed by poly(ADP-ribose) polymerases (Parps). Interestingly, physical and functional interactions between Parp-1 and Parp-2 with heterochromatin proteins like HP1 and KAP1 have been suggested to modulate a “histone subcode” that underlies the histone code (Quenet et al., 2008).

3. Epigenetic regulation of antibody gene diversification

A. Chromatin modifications in V(D)J recombination

Extensive studies have been made to understand effects of chromatin modifications including histone modifications, DNase sensitivity, DNA methylation and nucleosome remodeling on V(D)J recombination. Patterns of acetylation at the V regions seems specific to the V_H segment, its promoter and RSSs (Johnson et al., 2003). For example: H3K9Ac, hyperacetylated H4 as well as H3K4me2 mark the D-J_H region around the 5' most D segment and J_H elements in early pro-B cells that are ready to undergo D-J_H rearrangements (Chakraborty et al., 2007). This hyperacetylated status is lost following productive V_H-DJ_H rearrangements thus rendering the locus inaccessible in pre-B cells (Chowdhury and Sen, 2003). However, recently the first direct link between epigenetic control and V(D)J recombination came from the Oettinger and Desiderio labs, when they demonstrated that the PHD finger of RAG2 binds H3K4me3 through a conserved tryptophan residue (W453) (Liu et al., 2007; Matthews et al., 2007). Finally, consistent with the formation of DSBs during V(D)J recombination, γH2AX foci that co-localize with Nbs1 accumulate at breaks associated with rearrangement (Chen et al., 2000). Although γH2AX is not required for coding joint formation, it is known to suppress translocations during V(D)J recombination (Yin et al., 2009).

In addition to histone modifications, the V regions are also sensitized to rearrangement by simple nucleosome packaging. Thus, while the V_H regions becomes nuclease sensitive before V_H-DJ_H rearrangements, they go back to a refractory state after successful recombination has occurred (Chowdhury and Sen, 2003). Furthermore, cytosines in mammalian DNA are methylated in CpG dinucleotides, this can inhibit DNA-protein interactions (Watt and Molloy, 1988) or recruit methyl-CpG binding proteins to silence chromatin (Jaenisch and Bird, 2003). It has been shown, that methylated RSSs can abolish cleavage and rearrangements and that the D_H-J_H cluster is monoallelically demethylated prior to D-J_H recombination at the κ-chain locus (Maes et al., 2001; Mostoslavsky et al., 1998; Storb and Arp, 1983; Whitehurst et al., 2000).

Put together, these studies have helped understand better the aspects of accessibility control within the locus during V(D)J rearrangement.

B. Histone modifications associated with SHM

Epigenetic modifications that initially bring about monoallelic V(D)J rearrangement continue to control antibody diversification in later stages of B cell development, in that the demethylated allele was found to be the "preferred" substrate for SHM (Fraenkel et al., 2007). In addition, SHM can occur only in regions with increased transcription (Fukita et al., 1998; Peters and Storb, 1996), demethylation (Jolly and Neuberger, 2001), and chromatin accessibility (Odegard

et al., 2005; Woo et al., 2003). Interestingly, γH2AX that marks Ig loci during both V(D)J and CSR (Chen et al., 2000; Petersen et al., 2001), both of which involve DSB intermediates, does not seem to have a role during SHM (Bross et al., 2000; Papavasiliou and Schatz, 2000; Zan et al., 2003). Consistent with this, mice deficient for H2AX have defective CSR but SHM is unaffected (Reina-San-Martin et al., 2003).

In 2003, Woo et al. demonstrated that chromatin in the V_H regions were hyperacetylated compared to the C_H regions during SHM, suggesting that histone acetylation was involved in targeting SHM specifically within Ig loci (Woo et al., 2003). A few years later, using ChIP experiments, the Schatz lab found no significant differences in acetylation or methylation states of V regions between naïve and SHM stimulated GC B cells, leading to the conclusion that neither do these modifications accompany SHM nor specifically target SHM (Odegard et al., 2005). However, the phosphorylation of histone 2B at Ser 14 ($H2B^{Ser14P}$) was described as a marker for hypermutating V regions. H2B is known to be phosphorylated on S14 in thymocytes during V(D)J recombination (Fernandez-Capetillo et al., 2004). Unlike γH2AX, $H2B^{Ser14P}$ does not spread from the sites of DNA damage. Detection of $H2B^{Ser14P}$ at Vλ and V_H, as well as Sμ, suggests that in contrast to γH2AX, $H2B^{Ser14P}$ is induced in response to events shared by SHM and CSR. Consistent with this, $H2B^{Ser14P}$ at Vλ and Sμ in GC B cells was dependant on AID and associated with the recruitment of the mammalian sterile kinase (mst1) (Odegard et al., 2005).

C. Histone modifications at the Ig*h locus associated with CSR*

Chromatin modifications also play a critical role in CSR (Figure 16). Histone acetylation at I-exon promoters and switch regions is correlated with the activation of intronic promoters (Li et al., 2004a; Nambu et al., 2003; Wang et al., 2006b; Wang et al., 2009b), indicating that GL transcription is controlled by epigenetic marks. More specifically, histone H3 hyperacetylation at K9 and K14 (H3Ac) correlates with GL transcription at S regions, whereas H4Ac is dependent on AID expression, suggesting that epigenetic modifications could not only label the Ig locus but also serve as markers for AID-initiated DNA damage during CSR (Wang et al., 2006b). In addition, it was suggested that RNA Pol II facilitates recruitment of chromatin modifiers to enable accessibility of the locus. Thus, following GL transcription the I-S regions display increased H3Ac and H3K4me3, rendering them accessible. On the contrary, the C_H regions have been shown to remain hypoacetylated, which probably makes them inaccessible (Wang et al., 2009b). Taken together, this suggests that GL transcription and epigenetic marks are responsible for setting up active and repressed chromatin environments that correlate with S and C regions respectively during CSR. Furthermore, γH2AX foci accumulate at sites of DNA damage during CSR (Petersen et al., 2001) and H2AX is proposed to regulate chromatin remodeling to facilitate switch region synapsis (Reina-San-Martin et al., 2003).

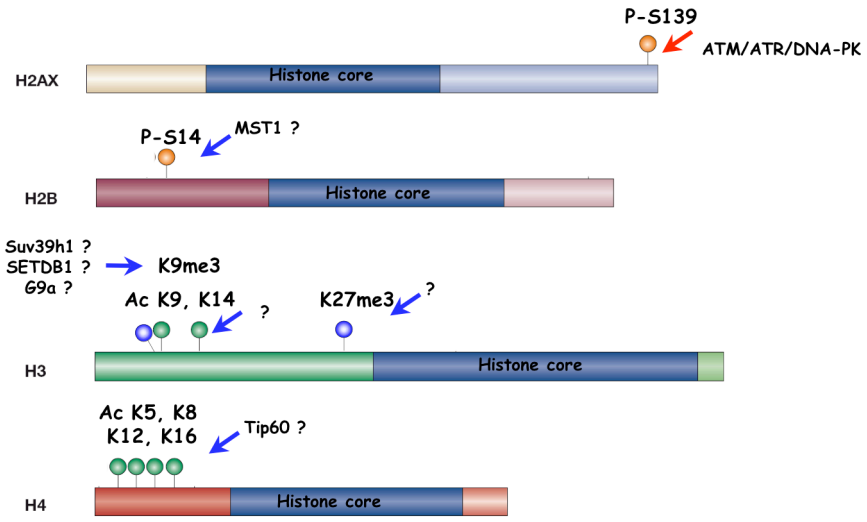

Figure 16: Epigenetic modifications in CSR: Epigenetic modifications induced at the C and N-terminal tails of histones during CSR. Colored circles denote different modifications: orange- phosphorylation, green – acetylation and blue – methylation. Red arrows indicate the enzyme involved, if known and blue arrows either unknown or possible candidate enzymes.

Interestingly, Chowdhury et al. in analyzing the epigenetic landscape of the Ig*h* locus in human B cells found that H3K9me3 was enriched specifically at γ and ε S and C_H regions in response to IL-4 /anti-CD40L stimulation, suggesting a role for this modification during CSR (Chowdhury et al., 2008). Following this, H3K9me3 as well as H3K9Ac marks were found to correlate with the pair of recombining S regions in an AID independent manner. Interestingly, the kinetics of these modifications and the regions of the Ig*h* locus that they associate with led to the hypothesis that these epigenetic marks could play a role in targeting the CSR machinery (Kuang et al., 2009). Although epigenetic modifications have been correlated with targeting of AID to initiate CSR or downstream of AID-dependent DSBs to promote repair and joining, a direct link between such marks and CSR has not been established.

The H3K9me3 mark is known to be in complex with the heterochromatin family of proteins, and is a well-established mark for silenced chromatin (Bannister et al., 2001; Rice et al., 2003), suggesting that such proteins that are involved in the building of heterochromatic structures could also play a role in CSR.

IV: The heterochromatin protein family

1. Structure and functions of the HP1 proteins

Heterochromatin protein 1 (HP1) was initially identified in Drosophila, and the family of HP1 proteins are phylogenetically highly conserved (Singh et al., 1991) from Schizosaccharomyces pombe (*S. pombe*) to mammals.

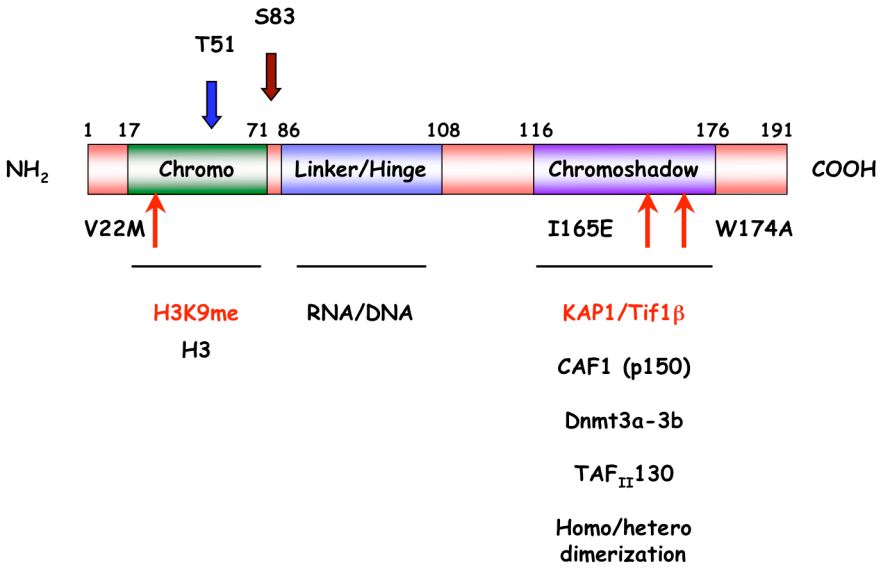

Figure 17: HP1 domain organization: Schematic representation of the HP1 family of proteins illustrating the domain structure and relevant interacting proteins below each domain. Blue and brown arrows indicate phosphorylation sites and the red arrows indicate amino acid substitutions known to affect HP1 function. (V22M: H3K9me binding, I165E: dimerization and W174A: PxVxL interactions).

HP1 is a small 25kDa protein with two conserved domains, an N-terminal 'chromodomain' (CD) and a C-terminal 'chromo shadow domain' (CSD), separated by a poorly conserved flexible 'hinge' or linker region (Figure 17). The CD domain of HP1 interacts with di or tri methylated histone H3 (H3K9me2/3) (Ball et al., 1997; Bannister et al., 2001; Jacobs and Khorasanizadeh, 2002; Lachner et al., 2001; Nielsen et al., 2001) to build and maintain the heterochromatic structure, a function that is conserved from yeast to man. In addition to binding H3K9me, HP1 interacts directly or indirectly with many non-histone proteins for several functions (Figure 18). While the hinge region's flexibility is required for its interaction with RNA and DNA, the CSD is implicated in interactions with several proteins involved in transcriptional regulation, chromatin modification, chromosomal maintenance, replication, and DNA repair, as well as homo and/or heterodimerization (Brasher et al., 2000; Dinant and Luijsterburg, 2009; Kwon and Workman, 2008). Analysis of several HP1 partner proteins identified a pentapeptide motif – P_XV_XL, called

the HP1BOX, which is necessary and sufficient to sustain interactions with the HP1 proteins (Le Douarin et al., 1996; Murzina et al., 1999; Smothers and Henikoff, 2000).

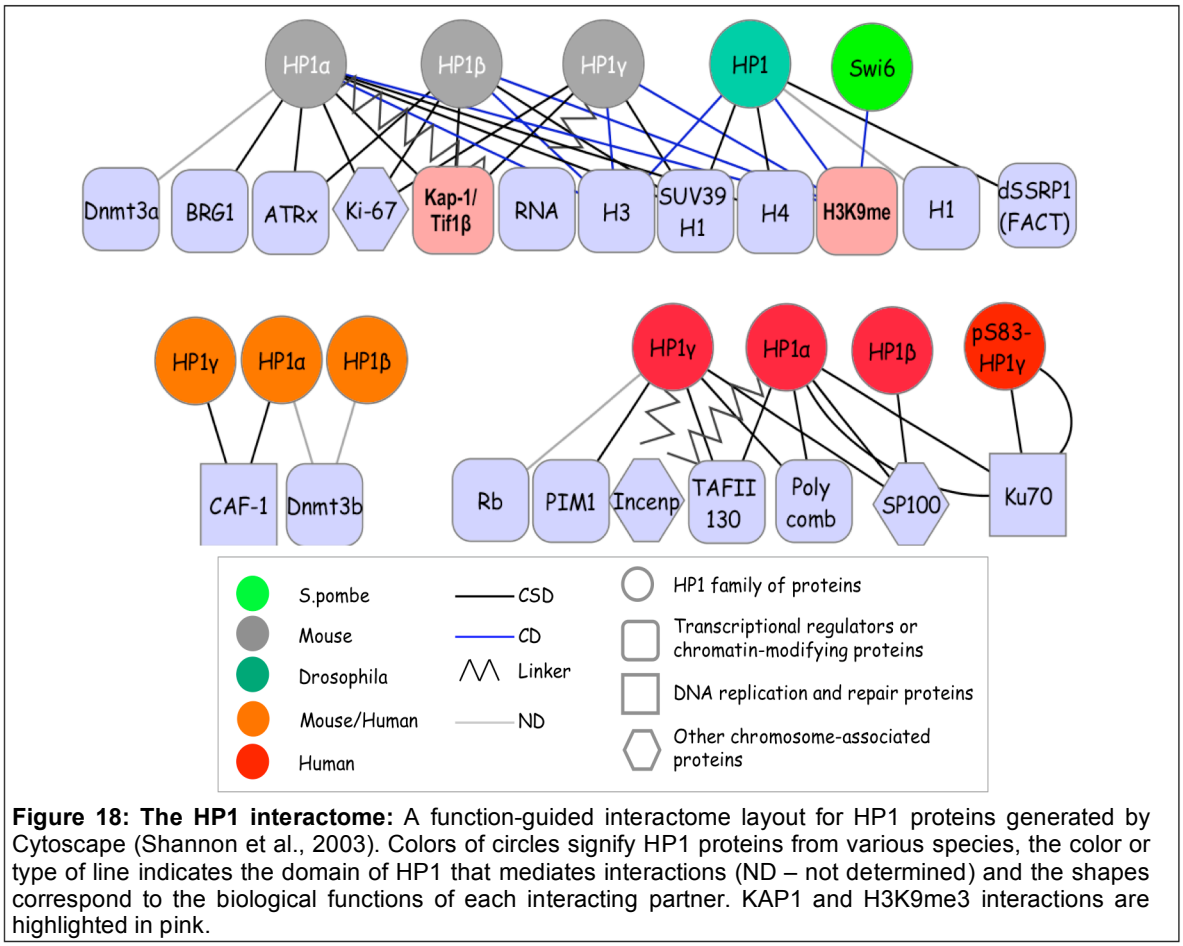

Figure 18: The HP1 interactome: A function-guided interactome layout for HP1 proteins generated by Cytoscape (Shannon et al., 2003). Colors of circles signify HP1 proteins from various species, the color or type of line indicates the domain of HP1 that mediates interactions (ND – not determined) and the shapes correspond to the biological functions of each interacting partner. KAP1 and H3K9me3 interactions are highlighted in pink.

One of the most characterized of HP1 interacting partners is the KRAB-domain associated protein KAP1 (also called Tif1β, TRIM28 or KRIP1, Figure 18), which is required for spermatogenesis and gastrulation (Cammas et al., 2000; Weber et al., 2002). Interestingly, disruption of the HP1BOX has revealed the integrity of the HP1BOX is essential for interaction between HP1 and its partners like KAP1 (Cammas et al., 2004; Cammas et al., 2002; Nielsen et al., 1999) and the histone chaperone CAF1 (Quivy et al., 2008).

The mammalian HP1 family has three members: HP1α, HP1β and HP1γ (Jones et al., 2000), that display discrete sub-nuclear localization patterns and associate with centromeric and other HC regions to varying extents. Although, discovered to be a major constituent of heterochromatin and important for gene silencing, recent studies have implicated HP1 in various other functions, namely: transcriptional regulation, centromeric sister chromatid cohesion, telomere maintenance, replication and DNA repair. In addition, the HP1 proteins are regulated by posttranslational modifications (Figure 17) such as acetylation, phosphorylation, methylation, ubiquitination and SUMOylation (Lomberk et al., 2006). Interestingly, phosphorylation on Ser 83 (S83) of HP1γ, defines a subpopulation of this isoform that is exclusive to euchromatin (Lomberk et al., 2006), suggesting that the sub-nuclear localization of

HP1 proteins is determined by a combination of protein interactions as well as particular posttranslational modifications.

A. HP1 in heterochromatin formation and gene expression regulation

The multi-domain organization allows HP1 proteins to function as structural adaptors, which are vital for the assembly of macromolecular complexes in chromatin. As mentioned earlier, HP1 preferentially binds H3K9me, and Suv39h1 mediates this PTM. Interestingly, it has been shown that Suv39h1 interacts with the HP1 proteins to propagate heterochromatin once an initiating site has been established (Cheutin et al., 2003). A more detailed account of HP1/H3K9me-mediated silencing, with respect to KAP1 is described later. HP1 also plays an important role in maintenance of pericenteric HC. The concentration of HP1 within pericenteric HC, which in turn depends on its interactions with Suv39h1 and H3K9 methylation ensures the proper segregation of chromosomes and gene expression (Malik and Henikoff, 2009). Furthermore, heterochromatin compaction is associated with homodimerization of HP1α or its association with HP1β, mediated by the CSD domain (Smothers and Henikoff, 2000; Thiru et al., 2004). In addition, HP1 also interacts with DNA methyltransferase (cytosine-5)-1 and 3a (Dnmt1, Dnmt3a) that are involved in maintaining heritable methylation patterns during DNA replication and *de novo* DNA methylation, respectively. The HP1 CSD also binds to the large subunit p150 of chromatin-assembly factor-1 (CAF1, see previous page), a factor that is implicated in histone deposition during both replication and repair via its interaction with PCNA. This HP1–CAF-1 complex is thought to function as a built-in replication control for heterochromatin (Murzina et al., 1999; Quivy et al., 2008).

Interestingly, though HP1 protein family members primarily associate with heterochromatin, HP1β and HP1γ also localize to euchromatin sites. In contrast to its "classical" role in HC formation and maintenance, di-and tri-methylation of H3K9 occurs in the transcribed regions of active genes along with HP1γ, in a manner dependent on RNA Pol II elongation, suggesting novel roles for H3K9me and HP1γ in transcription or related processes (Vakoc et al., 2005).

B. HP1 is involved in the DNA damage response

Recently, HP1 has also been shown to play a role in DNA damage response and repair pathways either by (a) dissociating from heterochromatin or (b) by playing a role in the DDR itself (Figure 19). All three HP1 isoforms are recruited to UV-induced DNA lesions in both human and mouse cells. This occurred, either by the binding of P_XV_XL-motif containing proteins to DNA lesions and the subsequent recruitment of HP1, or HP1 could by itself recognize structural alterations in chromatin caused by UV lesions.

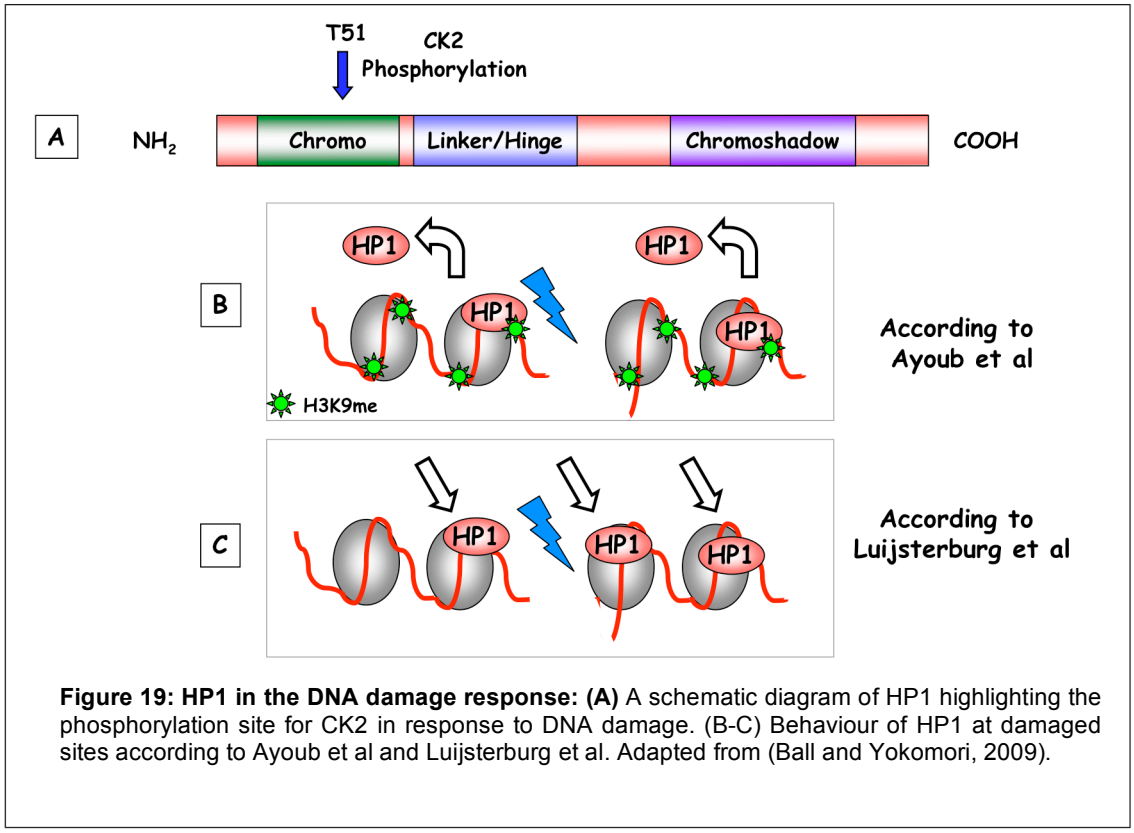

Figure 19: HP1 in the DNA damage response: (A) A schematic diagram of HP1 highlighting the phosphorylation site for CK2 in response to DNA damage. (B-C) Behaviour of HP1 at damaged sites according to Ayoub et al and Luijsterburg et al. Adapted from (Ball and Yokomori, 2009).

ATM was found to facilitate DSB-repair in heterochromatic regions, and chromatin relaxation is mediated by ATM-dependent phosphorylation of KAP1 (Goodarzi et al., 2008; White et al., 2006; Ziv et al., 2006). In line with this, reduced levels of all three HP1 isoforms alleviate the requirement of ATM in HC repair; possibly by loosening the heterochromatin to permit access and efficient processing of the DNA breaks (Ziv et al., 2006).

In support of this, Ayoub et al. showed, that DNA damage-induced casein kinase 2 (CK2) mediated-threonine 51 (T51) phosphorylation of HP1β reduces its affinity for H3K9me, and triggers its dissociation from chromatin to facilitate the DNA damage response (Figure 19, Ayoub et al., 2008). In contrast to these results, Luijsterburg et al. show that HP1 is specifically recruited to both DNA cross-links and DSB sites, suggesting a positive effect of HP1 in the DDR (Figure 19, Luijsterburg et al., 2009). Although these aspects are under debate, reconciliation between these two studies could be that HP1 is phosphorylated and disassociates from H3K9me in response to DSBs, while it is also actively recruited to DSBs by an H3K9me3-independent mechanism.

Though, the HP1 family of proteins has been found to play a role in DNA damage and repair, the exact function of the different HP1 isoforms and mechanisms that recruit them to site of DNA damage are still unanswered questions. However, as mentioned before, HP1's interaction with KAP1 is one of its most well characterized interactions and while on one end the interaction is known to be required for heterochromatin formation and transcriptional repression, it is also proposed to be required for DNA repair.

V: The KRAB-domain associated protein

1. KRAB-containing zinc finger proteins

The largest family of zinc-finger transcription factors is comprised of the Krüppel-associated box (KRAB) domain, present in tetrapod vertebrates. KRAB-containing proteins are characterized by the presence of a DNA-binding domain bearing between 4-30 zinc-fingers and a KRAB domain. The KRAB domain is usually located at the N-terminus and behaves as a transcriptional repressor by binding co-repressor proteins, while the C_2H_2 zinc-finger motifs recognize and bind DNA. The KRAB domain spans approximately 50-75 amino acids and is divided into the KRAB A and KRAB B boxes, and mammalian KRAB-containing zinc-finger proteins can belong to the KRAB-A, KRAB-B or KRAB-A+B sub-families. The members of this family of proteins are associated with transcriptional repression of RNA Pols I, II and III promoters, RNA splicing and control of nucleolus function, and are also involved in cell differentiation and proliferation, apoptosis and neoplastic transformation (Urrutia, 2003).
Several laboratories have identified a core repressor protein for KRAB domains called KRAB-associated protein (KAP1), which binds the KRAB domain via its RING, Box-Coiled-Coil (RBCC) domain to mediate transcriptional repression (Friedman et al., 1996; Kim et al., 1996; Moosmann et al., 1996)

2. Structure and functions of KAP1

KAP1 is a large nuclear phosphoprotein, ~100 Kda in size, and is an essential cofactor of KRAB box zinc finger proteins (KRAB-ZFPs). KAP1 is also known as tripartite motif protein 28 (TRIM28), transcription intermediary factor 1 β (Tif1β) or KRAB-interacting protein 1 (KRIP-1). It was initially identified in a yeast two-hybrid screen by its ability to bind all the isoforms of mammalian HP1 (Le Douarin et al., 1996). KAP1 belongs to a family of proteins that till date have four characterized members in humans and mice – Tif1α (or TRIM 24) (Le Douarin et al., 1995), Tif1β (or TRIM 28, KAP1) (Friedman et al., 1996; Kim et al., 1996; Le Douarin et al., 1996; Moosmann et al., 1996), Tif1γ (or TRIM 33) (Venturini et al., 1999) and Tif1δ (or TRIM 66) (Khetchoumian et al., 2004). All the members have a similar structure that is broadly divided into three domains (a) N-terminal RING finger, B-boxes, coiled-coil (RBCC) domain, collectively referred to as the TRIM domain, (b) C-terminal bromodomain, (c) plant homeo domain (PHD) and a pentapeptide PxVxL or LxxLL motif.
KAP1 is the only member of the Tif1 family that directly binds to the KRAB domain (Peng et al., 2002). KRAB-containing proteins bind to target DNA sequences to recruit KAP1, which subsequently forms a protein scaffold to silence genes by forming a facultative HC environment,

suggesting a KAP1-mediated assembly of a co-repressor complex (Ayyanathan et al., 2003; Riclet et al., 2009; Sripathy et al., 2006; Urrutia, 2003). The primary amino acid sequence of KAP1 reveals a RBCC domain, a PHD finger, and a bromodomain (Friedman et al., 1996; Kim et al., 1996; Le Douarin et al., 1996; Moosmann et al., 1996) (Figure 20), all of which are involved in multiple protein-protein interactions (Figure 21).

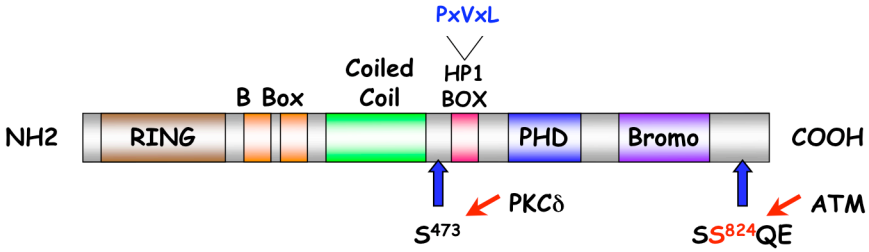

Figure 20: Schematic domain organization of KAP1: KAP1 is a multi-domain protein with a N-terminal RING Box Coiled Coil domain and a C-terminal plant homeo domain (PHD) and bromodomain (Bromo). KAP1 contains a peptapeptide motif – PxVxL or the HP1BOX that is required for its interaction with the HP1 family of proteins. Blue and red arrows indicate phosphorylation sites and the corresponding enzymes respectively.

KAP1 plays an essential role in early embryonic development and terminal cell differentiation, as well as functions that are indispensable for gastrulation and spermatogenesis (Cammas et al., 2004; Cammas et al., 2000; Weber et al., 2002). It interacts directly with the methyltransferase SETDB1 and is also an intrinsic component of two main histone deacetylase complexes – N-CoR1 and NuRD (Schultz et al., 2002; Schultz et al., 2001; Underhill et al., 2000).

KAP1 interacts with the members of the HP1 family through the PxVxL motif or the HP1BOX (Cammas et al., 2007; Le Douarin et al., 1996; Nielsen et al., 1999; Ryan et al., 1999; Thiru et al., 2004) (Figure 21, red circles). Interestingly, several labs have demonstrated that this interaction between KAP1 and HP1 is required for, (a) KAP1-mediated transcriptional regulation, which also requires histone deacetylase activity (Nielsen et al., 1999; Ryan et al., 1999), (b) the relocation of KAP1 from euchromatin to heterochromatin during primitive endodermal differentiation in F9 carcinoma cells (Cammas et al., 2002), and (c) progression through terminal differentiation of F9 cells (Cammas et al., 2004). More recently, the integrity of the HP1BOX was shown to be required for early post-gastrulation development, but dispensable for KAP1 function specifically in Sertoli cells (Herzog et al., 2010).

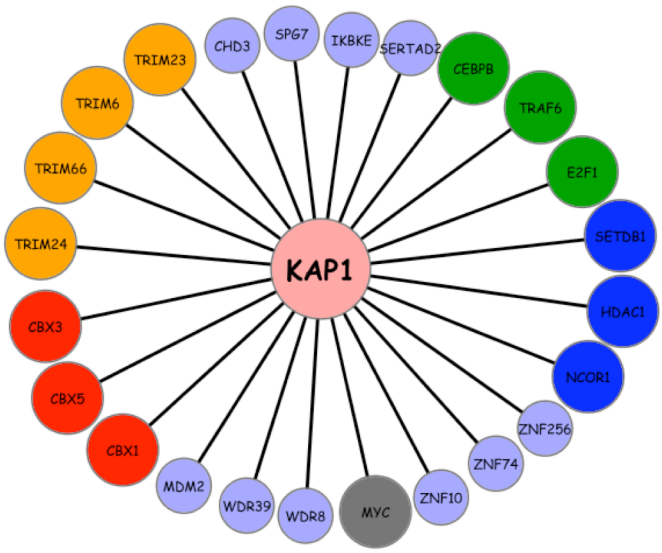

Figure 21: The KAP1 interactome: A function-guided interactome layout for KAP1 and its interacting proteins generated by Cytoscape (Shannon et al., 2003). Orange, red, blue and grey circles are specifically highlighted by color and size to emphasize well-characterized interactions. Orange - other members of the same family, red – the HP1 family members, blue – chromatin modifiers and grey - myc.

A. KAP1 coordinates functions that regulate chromatin structure

KAP1 functions as a molecular scaffold to regulate chromatin structure through its interaction with Mi-2α, a core component of the multi-subunit NuRD histone deacetylase complex (Schultz et al., 2001), the histone H3K9-methyltransferase SETDB1 (Schultz et al., 2002), and the CSD of HP1 family via the HP1BOX *in vitro* and *in vivo* (Lechner et al., 2000; Nielsen et al., 1999; Ryan et al., 1999). This interplay, between KAP1 and its interacting elements results in the formation of a facultative heterochromatin environment during cellular differentiation and development. In addition to recruiting chromatin modifiers, KAP1 also regulates chromatin structure by shuffling within the nucleus. In non-differentiated F9 carcinoma cells, KAP1 interacts with HP1β and HP1γ within euchromatin, while in differentiated cells, KAP1 selectively associates with HP1β in heterochromatin, suggesting that relocation of KAP1 from euchromatin to HC could create compartments in which it may exert differential functions (Cammas et al., 2002). KAP1 also functions to coordinate activities that dynamically regulate changes in histone modifications and deposition of HP1, to establish a *de novo* microenvironment of heterochromatin, which is required for repression of gene transcription by KRAB- ZFPs (Sripathy et al., 2006) (Figure 22).

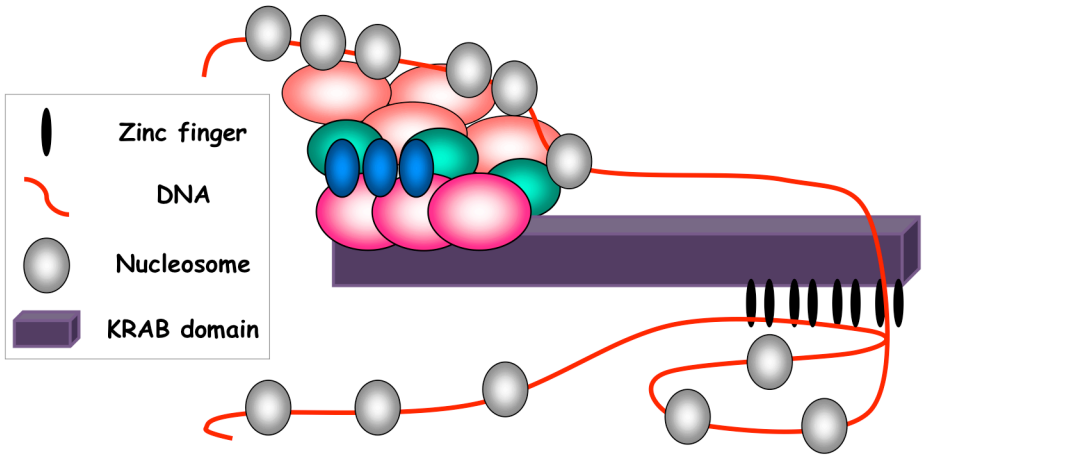

Figure 22: KRAB-domain mediated repression through KAP1: KRAB-domain containing proteins bind target DNA sequences through the zinc fingers. KAP1 is the only known protein that recognizes and binds the KRAB domain to form a scaffold that recruits the HP1 family members, the histone methyltransferase-SETDB1 and nucleosome-remodelling deacetylases in order to form heterochromatin. Adapted from (Urrutia, 2003).

Depletion of endogenous KAP1 by siRNA inhibits KRAB-mediated transcriptional repression of a chromatin template, along with reduction of HP1α/β/γ and SETDB1. Direct tethering of KAP1 to DNA was sufficient to reverse this and induce repression. This activity is dependent on the interaction of KAP1 with HP1 and as well as lysine SUMOylation of KAP1, which in turn is facilitated by its PHD finger and bromodomain (Ivanov et al., 2007; Li et al., 2007; Zeng et al., 2008). On the contrary, the phosphorylation of KAP1 at Ser 473 ($KAP1^{S473}$, located close to the PxVxL motif) by protein kinase C-δ (PKCδ), inhibits the KAP1-HP1 binding (Chang et al., 2008), suggesting that KAP1 functions are regulated largely through its protein partners as well as PTMs.

Thus, the recognition of H3K9me by the CD of HP1 (Ball et al., 1997; Bannister et al., 2001; Jacobs and Khorasanizadeh, 2002; Lachner et al., 2001; Nielsen et al., 2001), that interacts with KAP1, in turn tethered to DNA via its interaction with KRAB-ZFPs is sufficient to repress transcription. KAP1-induced repression involves a decrease in RNA Pol II recruitment, reduced levels of histone H3K9Ac and H3K4me, enrichment of H3K9me3, H3K36me, and HP1 deposition at proximal regulatory sequences of the target genes (Sripathy et al., 2006). HP1 can therefore bridge histones to other chromatin bound proteins to promote HC spreading (Kwon and Workman, 2008). Consistent with this, recent studies have demonstrated that KRAB-ZFPs and KAP1 could mediate long-range transcriptional repression through heterochromatic spreading (Groner et al., 2010).

B. KAP1 is a novel effector of the DNA damage response

ATM activates the DSB-signaling response (Kurz and Lees-Miller, 2004) and also influences DSB repair (Riballo et al., 2004) by modifying the structure of the chromatin in the vicinity of the DSB. Induction of ionizing radiation induced foci (IRIF) is one of the methods that uses 53BP1 or γH2AX as readout for the presence of a DSB and induction of DSBs with neocarzinostatin (NCS), a drug that creates relatively homogenous "complex" DSB termini has been a useful and powerful tool to study the role of several proteins in the DDR. A majority of radiation-induced DSBs (~85%) are repaired with fast kinetics in a predominantly ATM-independent manner. The remaining 15% that are repaired with slower kinetics require ATM and Artemis, as well as mediator proteins like MDC1 and 53BP1 (Darroudi et al., 2007; Iliakis et al., 1991; Lobrich et al., 1995; Riballo et al., 2004). Such DSBs with slow repair kinetics arise in HC, which has led to the hypothesis that chromatin structure rather than damage complexity influences the kinetics of repair.

Apart from their roles in transcriptional repression, KAP1 and HP1 also play a major role in the DDR. Following induction of a DSB, heterochromatin undergoes several changes to facilitate repair triggered by ATM. ATM-dependent KAP1 phosphorylation at serine 824 (γ-KAP1) promotes both DSB repair within HC (Goodarzi et al., 2008), and enhances survival post irradiation (Ziv et al., 2006). Interestingly, while the depletion of KAP1 or expression of a phospho-mimic ($KAP1^{S824D}$) alleviates the requirement of ATM in repair, a phospho-mutant ($KAP1^{S824A}$) results in a HC DSB repair defect even in the presence of ATM (Goodarzi et al., 2008), suggesting that HC is a barrier to DSB repair that is relieved by ATM signaling and that γ-KAP1 is critical to the changes that are necessary for this repair to proceed. γ-KAP1 is usually concentrated where ATM activity is localized (Ziv et al., 2006), and the accumulation of γ-KAP1 at sites of DNA damage is essential for repair within HC (Noon et al., 2010). More specifically, cell lines depleted of 53BP1 fail to form γ-KAP1 IRIF that overlap with HC, due to the inability to activate ATM, and thus phenocopy the HC DSB repair defects of ATM deficient cells. Thus, heterochromatic DSB repair requires specific and high concentrations of ATM that correlates with the induction and localization of γ-KAP1 (Noon et al., 2010).

Although, the mechanistic effects of γ-KAP1 are still not clear, the consequences, other than successful DSB repair, are the transient relaxation of chromatin resulting, in its susceptibility to nuclease digestion *in vitro* (Ziv et al., 2006), and alleviating repression of certain genes (Lee et al., 2007). The phosphorylation of KAP1 at S824 is thought to interfere with its ability to auto-SUMOylate itself (Li et al., 2007), which is turn is required for its functions in repression (Ivanov et al., 2007).

Interestingly, depletion of KAP1 or any of the heterochromatin components (the HP1 isoforms, HDAC1+2 or SUV39H1 and SUV39H2) all bypass the requirement of ATM during DSB repair to

various extents (Goodarzi et al., 2008). Thus, which of these is the actual barrier to DSB repair that is relieved by γ-KAP1 in response to ATM signaling, is still unidentified. What is clear though, is that the slow repair that is mediated by ATM/53BP1 with KAP1 as an effector, correlates with heterochromatic regions, and tampering with KAP1 does not enhance the repair kinetics (Goodarzi et al., 2008). This suggests, that although HC is made open to repair by ATM, the processes that bring about fast repair in euchromatin are in some way retarded within heterochromatin.

VI: Working hypothesis

The working hypothesis of my thesis was built on several observations regarding the C-terminal domain of AID. In addition to its role in nuclear export, the C-terminus of AID is required for CSR but not SHM. Indeed, C-terminal truncations in human AID result in normal SHM but defective CSR (Ta et al., 2003). Furthermore, mouse B cells expressing AID with a C-terminal truncation also display a selective defect in CSR as reconstitution of $AID^{-/-}$ B cells with a C-terminal deletion mutant of AID, restores SHM and IGC but not CSR (Barreto et al., 2003). Taken together, this made the C-terminal of AID a topic of my keen interest.

This CSR-specific defect associated with the C-terminus of AID very closely resembles the hyper IgM syndrome 4 syndrome that is characterized by normal AID expression, efficient induction of DSBs at Sμ, and normal SHM but defective CSR (Imai et al., 2003a). Interestingly, a subset of these patients displays increased radiosensitivity (Peron et al., 2007) and a defect involving the DNA repair machinery was suggested to account for this phenotype. However, the involvement of most DNA repair proteins known to play a role in CSR: H2AX, 53BP1 and MDC1 have been ruled out by gene sequencing (Durandy et al., 2007), and no molecule directly involved in DNA repair in this CSR deficiency has been identified.

Finally, the fact that mice deficient for DNA damage response proteins like ATM and H2AX (Lumsden et al., 2004; Reina-San-Martin et al., 2004; Reina-San-Martin et al., 2003) also display a phenotype similar to the AID C-terminal truncations, of defective CSR and normal SHM, suggests that the C-terminal domain of AID associates with DNA repair proteins that are required exclusively for CSR.

Based on all these observations, We hypothesized that AID associates with a factor(s) having a specific role in CSR, that binds to its C-terminal domain, and that could be involved in DNA repair. We wanted to identify proteins that interact with AID that could have a role in CSR. My thesis involved the identification of one such a factor and to carry out the detailed molecular analysis of its function in SHM and CSR.

RESULTS

I: Overview of thesis work

My main objective was to identify AID cofactors that could be involved in mediating CSR. To do this, we expressed full length AID in the CH12 B cell line, prepared nuclear extracts, performed tandem affinity purification, and identified co-immunoprecipitating proteins by mass spectrometry (MS, Figure 23). From this screen, we identified several proteins with known functions in SHM and CSR: Ikaros (Sellars et al., 2009), MSH2 and MSH6 (Rada et al., 2004) and DNA-PK_{CS} (Wu et al., 2005). We also identified proteins that regulate AID functions, CRM1, which is required to export AID from the nucleus (Brar et al., 2004; Geisberger et al., 2009; Ito et al., 2004; McBride et al., 2004), as well as other proteins with not known function in CSR and/or SHM. The fact that we identified proteins with known functions in CSR, was an indication that our approach was working, and that from the other proteins identified we would find one or more proteins with a role in CSR and/or SHM. However, we identified more than 300 proteins from this screen, and it was difficult to decide which of these proteins to choose and to analyze further. In order to complement our approach with an alternate strategy that was aimed at identifying proteins that bind to the C-terminal domain of AID. To do this, we fused the C-terminus of AID to EGFP ($EGFP^{AID182-198}$) and expressed this in CH12 cells, prepared nuclear extracts, performed tandem affinity purification, and identified and partner proteins by MS (Figure 23). By combining the list of proteins identified in both these screens from two (or more) independent experiments, and looking for common hits, we found the transcriptional repressor KAP1. KAP1 was an interesting candidate, as it had recently been implicated in the DNA damage response as a downstream effector of ATM in response to γ-irradiation (White et al., 2006; Ziv et al., 2006) and as we had identified it in both proteomic screens. We had thus identified KAP1 as a novel protein that associates with AID, possibly via its C-terminal domain and the main focus of my thesis was to delineate the role(s) of KAP1 in antibody diversification mechanisms.

To investigate the role of KAP1 in CSR and SHM we established a collaboration with Régine LOSSON at the IGBMC. We bred mice bearing a conditional KAP1 allele, that they had generated (KAP1F/F, Cammas et al., 2000), with $CD19^{Cre}$ transgenic mice (Rickert et al., 1997) to specifically delete KAP1 in developing B cells. The conditional inactivation of KAP1 allowed us to assess its role in SHM and CSR. We found that while deletion of KAP1 resulted in a 40-50% reduction in CSR to all isotypes tested, while SHM appeared to be unaffected. We concluded, that AID associates with KAP1 and that KAP1 has a CSR-specific function.

We then asked what could be the molecular mechanisms underlying the role of KAP1 in CSR? Considering KAP1's role in the DDR and that its deletion phenocopied mice deficient for other DNA damage proteins, ATM and H2AX (Lumsden et al., 2004; Reina-San-Martin et al., 2004; Reina-San-Martin et al., 2003), as well as C-terminal truncations in AID (Barreto et al., 2003;

Imai et al., 2003a; Ta et al., 2003), our hypothesis was that KAP1 could respond to AID-induced DNA lesions during CSR. We performed a detailed analysis of the $CD19^{Cre}$ $KAP1^{F/F}$ B cells to define the role of KAP1 in the various steps of CSR. We found, that the defect in CSR was independent of abnormalities in proliferation, switch region transcription or AID expression, and that CSR junctions were repaired normally through NHEJ. To test if KAP1 responded to programmed DNA breaks induced by AID during CSR, we assayed for the phosphorylation status of KAP1. To our surprise, we found that the role of KAP1 in CSR is independent of its ATM-dependent phosphorylation. Furthermore, unlike B cells deficient for other DNA repair proteins, ATM or XRCC4 (Yan et al., 2007), the deletion of KAP1 did not result in an increase in genomic instability, or in the frequency of Ig*h*/c-myc translocations. Based on these experiments we concluded that KAP1 was required downstream of AID expression and upstream of the DNA lesions that initiate CSR. If this is the case, then we would expect to find reduced levels of DNA damage at S regions in B cells undergoing CSR. To test this, we measured DNA double-stranded breaks (DSBs) by LM-PCR, as well as AID-induced mutations at Sμ. Indeed, we found that KAP1-deficieny results in inefficient DSB induction.

How could we explain the requirement of KAP1 upstream of the DSBs during CSR? At this time, the Scharff lab showed that the H3K9me3 mark was specifically induced at S regions in B cells undergoing CSR, and they suggested that histone modifications could play a role in targeting the CSR machinery to the Ig*h* locus, by providing a binding motif for proteins involved in CSR (Kuang et al., 2009). However, despite such correlations between epigenetic modifications at Ig loci and AID function, a direct link between these has not been shown.

Given that AID associates with KAP1, that KAP1 interacts with HP1 (Le Douarin et al., 2001), and that HP1 can bind H3K9me3, we hypothesized that a KAP1/AID/HP1 complex may be involved in the specific targeting of AID to Ig switch regions bearing H3K9me3.

If so, we needed to show that these proteins exist in a complex that binds H3K9me3, and is recruited *in vivo* to S regions marked by H3K9me3. We performed immunoprecipitation followed by gel filtration experiments from CH12 cells expressing $AID^{Flag\text{-}HA}$ and found that KAP1, HP1 and AID co-elute in the same fractions. To determine if such an AID/KAP1/HP1 complex is able to bind H3K9me3 residues, we performed peptide pulldown experiments and found that KAP1, HP1, and AID exist in a complex that binds an H3K9me3 peptide *in vitro*. In addition, we performed chromatin immunoprecipitation (ChIP) experiments to show that KAP1, HP1 and H3K9me3 are enriched at the Ig*h* locus in B cells before and during CSR. We concluded that KAP1, HP1, and AID exist in a complex that binds H3K9me3 residues *in vitro* and that KAP1 and HP1 are recruited to S regions bearing H3K9me3 *in vivo* during CSR. Therefore, this modification could serve as a binding motif for a complex formed by AID, KAP1 and HP1 during CSR *in vivo*.

Finally, to determine if the interaction between KAP1 and HP1 was required for CSR, we bred mice with a knock-in allele bearing mutations that disrupt the interaction between KAP1 and

HP1 (KAP1$^{V488A\text{-}L490A/F}$, Herzog et al., 2010) with CD19^{Cre} transgenic mice (Rickert et al., 1997) in a KAP1-deficient background. Strikingly, we showed by *ex vivo* stimulation that expression of such a mutant KAP1 protein does not restore CSR. We conclude, that the interaction between KAP1 and HP1 is required for efficient CSR *in vivo*.

Based on these results we have proposed a model in which AID forms a complex with KAP1 and HP1 that is tethered to switch regions bearing the H3K9me3 mark, providing for the first time a direct link between epigenetic modifications and the CSR mechanism.

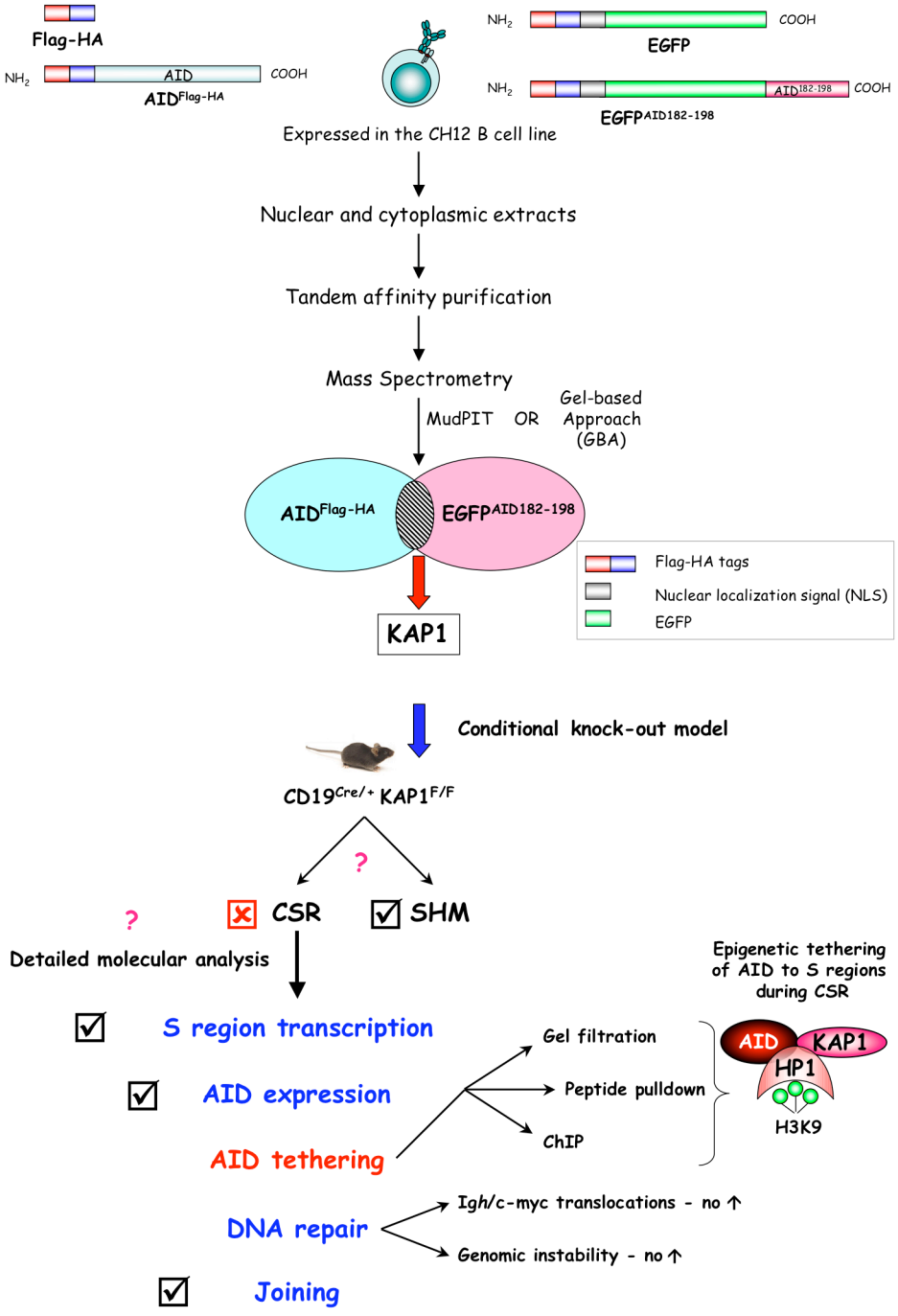

Figure 23: Overview of thesis work. $AID^{Flag-HA}$ and $EGFP^{AID182-198}$ were expressed in CH12 B cells, nuclear extracts were prepared and subjected to affinity purification, followed by mass spectrometry identification of co-immunoprecipitating proteins. KAP1 was identified from the GBA and MudPIT experiments. KAP1 was conditionally inactivated in B cells and the $CD19^{Cre}$ $KAP1^{F/F}$ mice were used to study the role of KAP1 in CSR and SHM. We show that CSR is affected in the absence of KAP1, while SHM is normal and propose a model that links epigenetic modifications to CSR.

II. Manuscript: Epigenetic tethering of AID to switch regions during immunoglobulin class switch recombination

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Running Title: KAP1, HP1 and H3K9me3 tether AID to switch regions.

ABSTRACT

Immunoglobulin class switch recombination (CSR) is initiated by double stranded DNA breaks (DSBs) in switch regions triggered by Activation induced cytidine deaminase (AID). Although CSR correlates with epigenetic modifications at the Ig*h* locus, the relationship between these and AID remains unknown. Here we show that during CSR, AID forms a complex with KAP1 and HP1 that is tethered to the donor switch region (Sμ) bearing H3K9me3 *in vivo*. Furthermore, *in vivo* disruption of this complex results in inefficient DSB-formation at Sμ and a concomitant defect in CSR but not in somatic hypermutation. We propose that KAP1 and HP1 tether AID to H3K9me3 residues at the donor switch region, thus providing a mechanism linking AID to epigenetic modifications during CSR.

INTRODUCTION

During immune responses B cells diversify their immunoglobulin (Ig) genes by somatic hypermutation (SHM) and class switch recombination (CSR). SHM modifies antibody affinities by introducing mutations in the variable region of heavy (IgH) and light chain genes (Di Noia and Neuberger, 2007). CSR modulates antibody effector functions by replacing the antibody isotype expressed through a DNA recombination reaction that joins two switch regions (Chaudhuri et al., 2007). SHM and CSR are initiated by the deamination of cytosines to uracils in single stranded DNA mediated by Activation Induced Cytidine Deaminase (AID, Chaudhuri et al., 2007; Di Noia and Neuberger, 2007; Petersen-Mahrt et al., 2002). The resulting dU:dG mismatches are differentially processed to generate mutations in variable regions during SHM or double stranded DNA breaks (DSBs) in switch regions during CSR (Chaudhuri et al., 2007; Di Noia and Neuberger, 2007). AID appears to find its targets by its binding to Spt5 and RNA polymerase II (Pavri et al., 2010) and distinguishes between variable and switch regions by its association with proteins like RPA, PKA, 14-3-3, or by the formation of higher order DNA structures in switch regions (Chaudhuri et al., 2007; Vuong et al., 2009; Xu et al., 2010; Yamane et al., 2010). AID itself also contributes to this choice, as C-terminal truncations in mouse or human AID results in normal SHM but defective CSR (Chaudhuri et al., 2007; Kracker et al., 2010). This phenotype is also observed in mice deficient for DNA damage response (DDR) components (Ramiro et al., 2007) and in a subset of hyper IgM patients with a CSR-specific defect (Kracker et al., 2010). It has been proposed that this domain associates with CSR-specific factors that could be required for targeting AID to switch regions (Ta et al., 2003), or for recombination downstream of DSB formation either by mediating RNA editing (Doi et al., 2009; Nonaka et al., 2009) or by recruiting factors that promote efficient DNA repair (Barreto et al., 2003; Ito et al., 2004; Kracker et al., 2010; Shinkura et al., 2004; Ta et al., 2003). In addition, epigenetic modifications at the IgH locus, including histone H3 tri-methylation at lysine 9 (H3K9me3), have been suggested to target the CSR machinery to switch regions (Chowdhury et al., 2008; Kuang et al., 2009; Wang et al., 2006b; Wang et al., 2009b). However, a direct causal link between these modifications and AID function in CSR has not been established.

RESULTS

AID associates with KAP1.

To identify nuclear proteins associating with AID, we performed sequential immunoprecipitations followed by mass spectrometry identification using nuclear extracts prepared from CH12 B cells expressing tagged AID ($AID^{Flag\text{-}HA}$). As negative controls we used cell lines expressing the tags alone (Flag-HA) or an irrelevant tagged protein ($EGFP^{Flag\text{-}HA}$). Among the identified proteins specifically co-precipitating with AID but not with the negative controls, we found known AID partners: DNA-PK_{CS} (Wu et al., 2005), CRM1 (Brar et al., 2004;

Geisberger et al., 2009; Ito et al., 2004; McBride et al., 2004), proteins previously implicated in CSR: Ikaros (Sellars et al., 2009), MSH2 (Chaudhuri et al., 2007), MSH6 (Chaudhuri et al., 2007), and proteins with no known function in SHM or CSR, like the KRAB domain associated protein 1 (KAP1; Supplementary Figure 1, [Figure 31]). KAP1 (also known as TRIM28, KRIP1 or Tif1β) was selected for analysis because it has been previously implicated as an effector of the DDR (Ziv et al., 2006). KAP1 is also a transcriptional co-repressor (Cammas et al., 2000) that associates with members of the heterochromatin protein 1 (HP1) family to participate in chromatin packaging and heterochromatin formation (Nielsen et al., 1999). The association between AID and KAP1 was confirmed by reciprocal immunoprecipitations and western blotting (Figure 1A, [Figure 24A]), and was specific, as KAP1 did not co-precipitate with $EGFP^{Flag\text{-}HA}$ (Figure 1B, [Figure 24B]), despite relatively higher expression of $EGFP^{Flag\text{-}HA}$ (Supplementary Figure 1A, [Figure 31A]). To determine whether AID associates with KAP1 through its C-terminus we performed immunoprecipitation and western blot experiments using extracts prepared from CH12 cells expressing an AID C-terminal truncation mutant ($AID^{Flag\text{-}HA\ D182\text{-}198}$). Although the expression level of $AID^{Flag\text{-}HA\ D182\text{-}198}$ is significantly lower than $AID^{Flag\text{-}HA}$ or $EGFP^{Flag\text{-}HA}$ (Supplementary Figure 1A, [Figure 31A]), we found that C-terminal deletion in AID did not disrupt its association with KAP1 (Figure 1C, [Figure 24C]). We conclude that endogenous KAP1 associates with tagged AID through interactions that do not require its C-terminal domain.

KAP1 is required for efficient CSR but is dispensable for SHM.

To determine whether KAP1 plays a role in CSR and SHM, we bred KAP1 floxed mice (KAP1F/F, Cammas et al., 2000) with $CD19^{Cre/+}$ transgenic mice (Rickert et al., 1997) to inactivate KAP1 in developing B cells. Despite efficient Cre-mediated deletion, no differences were observed in cellularity in the bone marrow or the spleen (not shown) and B cell development appeared to be unaffected (Supplementary Figure 2, [Figure 32]). To determine whether CSR is affected by KAP1-deficiency we cultured *in vitro* CFSE-labeled splenic B cells isolated from $CD19^{Cre/+}KAP1^{F/F}$ and control mice, under conditions that induce CSR to various isotypes. We found that KAP1-deficiency had no effect on proliferation (Figure 2A, [Figure 25A]) or survival (not shown). However, KAP1-deficiency resulted in a 40-50% reduction in CSR to all isotypes tested (Figure 2A-C, [Figure 25A-C]). Consistent with this, the level of post-recombination $I\mu\text{-}C_H$ transcripts, which appear only after recombination, was significantly reduced in $CD19^{Cre/+}KAP1^{F/F}$ B cells (Figure 2C [Figure 25C], Supplementary Figure 3, [Figure 33]). We conclude that KAP1-deficiency results in a B cell intrinsic defect in CSR that is independent of abnormalities in survival or proliferation.

To determine whether SHM is also affected by KAP1-deficiency, we immunized $CD19^{Cre/+}KAP1^{F/F}$ and control mice with NP-CGG, sorted germinal centre B cells from the lymph nodes and analyzed J_H4 intron sequences for mutations (Jolly et al., 1997). We did not find

significant differences in mutation frequency (Figure 2D, [Figure 25D]; p=0.12), distribution or pattern (Supplementary Figure 4, [Figure 34]), between sequences obtained from $CD19^{Cre/+}KAP1^{F/F}$ (n=83) and control (n=91) animals. We conclude that KAP1 is involved in mediating CSR but appears to be dispensable for SHM.

The function of KAP1 in CSR is upstream of DSB resolution.

CSR is dependent on transcription (Chaudhuri et al., 2007) and KAP1 is a transcriptional co-repressor (Cammas et al., 2000). To determine whether switch region transcription is affected by KAP1-deficiency we measured by qRT-PCR the level of switch region "germline" transcripts in activated B cells. We found no significant reduction in the level of these transcripts in KAP1-deficient B cells (Figure 3A, [Figure 26A], Supplementary Figure 3, [Figure 33]). However, occasional increases in the levels of γ3, γ1, γ2b and γ2a transcripts were found (Figure 3A, [Figure 26A], Supplementary Figure 3, [Figure 33]). This is similar to what is observed in $ATM^{-/-}$, $H2AX^{-/-}$ or $53BP1^{-/-}$ B cells in which CSR is reduced despite normal or increased levels of switch region transcripts (Reina-San-Martin et al., 2004; Reina-San-Martin et al., 2003; Ward et al., 2004). We conclude that switch regions continue to be efficiently transcribed in KAP1-deficient B cells and that switch regions are accessible for DNA deamination by AID. Therefore, the function of KAP1 is downstream of switch region transcription.

To determine whether KAP1-deficiency affects AID expression we measured the level of AID protein in activated B cells by western blot. For comparison we used AID-deficient ($AID^{Cre/Cre}$) B cells, obtained from mice bearing a targeted insertion of the CRE recombinase cDNA into AID's exon 1 (Robbiani et al., 2008) and which display the same phenotype as $AID^{-/-}$ mice (Muramatsu et al., 2000). Reduced levels in AID protein were found in $AID^{Cre/+}$ B cells (Figure 3B, [Figure 26B]), as has been reported for $AID^{+/-}$ B cells (Sernandez et al., 2008). In contrast, KAP1-deficiency had no deleterious effect on AID protein levels (Figure 3B, [Figure 26B]). Consistent with this, retroviral over-expression of full-length AID in KAP1-deficient B cells did not rescue CSR, whereas it did in AID-deficient B cells (Supplementary Figure 5, [Figure 35]). As expected, over-expression of C-terminal truncation AID mutants had no effect on CSR in either AID- or in KAP1-deficient B cells (Supplementary Figure 5, [Figure 35]). We conclude that defective CSR in KAP1-deficient B cells is not due to reduced levels of AID and that KAP1 functions downstream of AID expression.

KAP1 is phosphorylated at serine 824 (γ-KAP1) by the ATM kinase and accumulates in γ-H2AX-containing foci in response to DNA damage (Ziv et al., 2006). Furthermore, inactivation of DDR components (*i.e.* ATM, H2AX) results in defective CSR (Ramiro et al., 2007). To determine whether KAP1 is phosphorylated during CSR, we assayed for γ-KAP1 by western blot. KAP1-phosphorylation was readily detected in control cells exposed to the DSB-inducing chemical neocarzinostatin (NCS; (Figure 3C, [Figure 26C]). Despite robust levels of AID

expression, γ-KAP1 was not detected in activated B cells (Figure 3C, [Figure 26C]), indicating that KAP1-phosphorylation, if any, is below detection threshold. To determine whether a small fraction of KAP1 is phosphorylated during CSR, we immunoprecipitated γ-KAP1 and blotted for KAP1 (Figure 3D, [Figure 26D]). Although γ-KAP1 was detected under these conditions, KAP1-phosphorylation was not dependent on AID expression (Figure 3D, [Figure 26D]). We conclude that the role of KAP1 in CSR is independent of its phosphorylation status at serine 824.

CSR requires the generation of DSBs in switch regions and their resolution by non-homologous end joining (NHEJ, Yan et al., 2007). To determine whether CSR junctions are affected by KAP1-deficiency we compared IgG3 CSR junctions from $CD19^{Cre/+}KAP1^{F/F}$ (n=52) and control (n=46) B cells. Sequence analysis revealed no significant differences (p=0.9175) in the amount of microhomology at the junctions between groups (Figure 3E, [Figure 26E], Supplementary Figure 6, [Figure 36]). We conclude that CSR junctions are unaffected by KAP1-deficiency and that DNA ends are repaired normally by NHEJ.

Inactivation of core NHEJ or DDR components results in global and Ig*h*-specific genomic instability (Franco et al., 2006; Yan et al., 2007). To determine whether KAP1-deficiency results in the accumulation of unresolved DNA breaks triggered by AID in switch regions we performed two-color Ig*h*-specific fluorescence in situ hybridization (Ig*h*-FISH, Franco et al., 2006; Yan et al., 2007) on metaphase spreads prepared from $CD19^{Cre/+}KAP1^{F/F}$ (n=593) and control (n=780) activated B cells. Contrary to $ATM^{-/-}$ or $XRCC4^{-/-}$ B cells (Yan et al., 2007), we found no significant increase (p=0.9) in the percentage of metaphases with abnormalities in KAP1-deficient B cells (Figure 3F, [Figure 26F] and Table 1, [Table 4]). Consistent with this, we found that KAP1 disruption did not result in increased frequency of AID-dependent Ig*h*/c-myc translocations (Figure 3G, [Figure 26G], Supplementary Figure 7, [Figure 37]). We conclude that in the absence of KAP1 DSBs generated at switch regions are efficiently repaired and do not lead to global or IgH-specific genomic instability. Therefore, KAP1 functions upstream of the AID-induced DNA breaks that initiate CSR.

KAP1-deficiency results in reduced levels of DSBs at Sμ

To detect DSBs in switch regions and determine whether they are formed less efficiently in the absence of KAP1, we performed ligation-mediated PCR experiments on activated B cells (Figure 4A, [Figure 27A]). We found that DSBs at Sμ region were induced at lower frequency in KAP1-deficient B cells (Figure 4A, [Figure 27A], Supplementary Figure 8, [Figure 38]). To assess the level of DNA breaks at switch regions with a more quantitative assay we measured the mutation frequency in the 5' end of Sμ, as mutations in this region are AID-dependent and have been used to assess the level of AID-induced DNA damage (Barreto et al., 2003). We found that the proportion of mutated sequences and the mutation frequency were reduced (p=0.040) in $CD19^{Cre/+}KAP1^{F/F}$ ($F=0.78x10^{-3}$ mutations/bp; n=170) when compared to controls

($F=1.07x10^{-3}$ mutations/bp; n=169; Figure 4B, [Figure 27B]). A significant reduction ($p= 1.5x10^{-19}$) in mutation frequency in the 5' end of Sμ was also found in KAP1-deficient B cells when sequences were analyzed in a larger data set by high-throughput amplicon sequencing (Figure 4C, [Figure 27C]). We conclude that although AID is targeted to Sμ, the generation of DSBs is less efficient in KAP1-deficient cells. This suggests that AID retention at switch regions is sub-optimal in the absence of KAP1.

AID forms a complex with KAP1 and HP1 that binds H3K9me3.

Epigenetic modifications at the Ig*h* locus have been suggested to target the CSR machinery to switch regions by relaxing chromatin or by providing binding motif(s) for factor(s) involved in CSR (Fraenkel et al., 2007; Kuang et al., 2009; Odegard et al., 2005; Wang et al., 2006b; Wang et al., 2009b). Among these, H3K9me3, which is usually associated with silent genes and heterochromatin, was found at transcribed donor and acceptor switch regions (Chowdhury et al., 2008; Kuang et al., 2009). We hypothesized that AID could be tethered to transcribed switch regions bearing this modification through the association between AID, KAP1 and heterochromatin protein 1 (HP1), and the ability of the latter to bind H3K9me3 (Bannister et al., 2001). To determine whether AID also associates with HP1 we performed reciprocal co-immunoprecipitation experiments using nuclear extracts prepared from cells expressing $AID^{Flag-HA}$ and antibodies specific for the different HP1 isoforms. Although all HP1 isoforms co-precipitated with $AID^{Flag-HA}$, only HP1γ reciprocally co-precipitated $AID^{Flag-HA}$ (Figure 5A, [Figure 28A]). To determine whether KAP1, HP1 and AID exist in a complex, we fractionated Flag immunoprecipitates by gel filtration chromatography using a Superose 6 column and assayed the different fractions for AID, KAP1 and HP1 isoforms by western blot (Figure 5B, [Figure 28B]). We found that $AID^{Flag-HA}$, KAP1 and HP1γ co-elute in fractions corresponding to a complex of approximately 670 kDa (Figure 5B, [Figure 28B]). The majority of HP1γ was however eluted in low molecular weight fractions (Figure 5B, [Figure 28B]), indicating that only a small fraction of the complex contains HP1γ or that the *in vitro* association of HP1γ within the complex is weaker. HP1α and HP1β were not detected (Figure 5B, [Figure 28B]). To determine whether the AID/KAP1/HP1 complex is able to bind H3K9me3 residues we performed peptide pulldown experiments using nuclear extracts from cells expressing $AID^{Flag-HA}$, $EGFP^{Flag-HA}$ or $AID^{Flag-HA\ D182-198}$ and biotinylated histone H3 peptides either unmodified or tri-methylated at lysine 9. We found that the H3K9me3 peptide precipitated KAP1, all the HP1 isoforms and $AID^{Flag-HA}$ (Figure 5C, [Figure 28C]). Precipitation of $AID^{Flag-HA}$ was specific, as this peptide did not precipitate $EGFP^{Flag-HA}$ (Figure 5C, [Figure 28C]). The H3K9me3 peptide also precipitated $AID^{Flag-HA\ D182-198}$ (Figure 5C, [Figure 28C]), indicating that the C-terminal domain of AID is not required for the recognition of H3K9me3 residues by the AID/KAP1/HP1 complex. We conclude that KAP1, HP1 and AID exist in a complex that binds H3K9me3 *in vitro*. Therefore, this

modification could serve as a tethering motif for a complex formed by AID, KAP1 and HP1 during CSR *in vivo*.

H3K9me3 and KAP1 mark the donor switch region (Sμ) before and during CSR.

To determine whether KAP1 and HP1 are recruited to switch regions during CSR *in vivo*, and whether this correlates with histone modifications associated with the Ig*h* locus, we performed chromatin immunoprecipitation experiments on resting and activated control and $CD19^{Cre/+}KAP1^{F/F}$ B cells (Figure 6 and Supplementary Figures 9 and 10, [Figures 29, 39 and 40]). Consistent with previous reports (Wang et al., 2009b), we found that histone H3 associated with the pair of recombining switch regions is acetylated at lysine 9 and 14 (H3K9/K14Ac) in B cells undergoing CSR (Figure 6A, [Figure 29A]), and that this correlated with switch region transcription (Figure 3A, [Figure 26A]). Consistent with normal levels of germline transcripts in KAP1-deficient B cells (Figure 3A, [Figure 26A]), we found that H3K9/K14Ac was not affected by KAP1-deficiency (Supplementary Figure 9, [Figure 39]). While H3K9me3 has been reported to be induced at the donor and acceptor switch regions during CSR (Chowdhury et al., 2008; Kuang et al., 2009), we found that H3K9me3 was already present at the Ig*h* locus prior to recombination in resting B cells (Figure 6B, [Figure 29B]). Upon stimulation, the level of H3K9me3 was substantially reduced throughout the locus but was specifically retained (Table 2, [Table 5] for statistics) over the donor switch region (Sμ), when compared to sequences located immediately downstream of the variable region (J_H4), the I exons or the C_H exons (Figure 6B, [Figure 29B]). Surprisingly, H3K9me3 retention was not observed at Sγ3 when cells were stimulated to switch to IgG3 with LPS, nor at Sγ1 when cells were stimulated to switch to IgG1 with LPS +IL-4 (Figure 6B, [Figure 29B]). The H3K9me3 pattern was similar in KAP1-deficient B cells (Supplementary Figure 9, [Figure 39] and Table 3 [Table 6] for statistics), indicating that this modification is not dependent on KAP1. Interestingly, we found that KAP1, together with H3K9me3, are recruited to the Ig*h* locus in resting B cells. KAP1 binding was however predominant over Sμ when compared to downstream regions (Figure 6C, [Figure 29C]). Upon stimulation, KAP1 was released from the locus but was preferentially retained over Sμ (Figure 6C, [Figure 29C]). KAP1 recruitment was specific as no significant ChIP signal above background was found in KAP1-deficient B cells (not shown). HP1γ binding differed from H3K9me3 and KAP1 in that it was distributed throughout the locus in resting and activated B cells (Figure 6D, [Figure 29D]), Supplementary Figure 10, [Figure 40]). Increased HP1γ binding at Cμ in activated B cells was observed (Figure 6D, [Figure 29D]), but was however, not reproducible (Supplementary Figure 10, [Figure 40]). We conclude that H3K9me3, HP1γ and KAP1 are present at the Ig*h* locus as part of a heterochromatic signature in resting B cells and that H3K9me3 and KAP1 specifically mark the donor switch region (Sμ) before and during CSR.

To determine whether the *in vivo* association between KAP1 and HP1 is required to sustain CSR, we bred $CD19^{Cre/+}KAP1^{F/F}$ mice with knock-in mice ($KAP1^{V488A-L490A}$, Herzog et al., 2010) expressing a mutated KAP1 protein that is unable to associate with the HP1 proteins (Cammas et al., 2004), to generate $CD19^{Cre/+}KAP1^{V488A-L490A/F}$ mice. While B cells isolated from $CD19^{Cre/+}KAP1^{V488A-L490A/+}$ mice (expressing a wild-type KAP1 and a mutant protein) underwent CSR at wild-type frequencies (similar to $CD19^{Cre/+}KAP1^{F/+}$), B cells isolated from $CD19^{Cre/+}KAP1^{V488A-L490A/F}$ mice (expressing only the KAP1 mutant protein which cannot associate with HP1, Supplementary Figure 2, [Figure 32]) displayed the same CSR defect observed in $CD19^{Cre/+}KAP1^{F/F}$ B cells (Figure 7, [Figure 30]). We conclude that the *in vivo* association between KAP1 and HP1 is required to mediate efficient CSR and that a complex formed by AID, KAP1 and HP1 is tethered to transcribed immunoglobulin switch regions bearing the H3K9me3 mark *in vivo*.

DISCUSSION

We have found that AID forms a complex with KAP1 and HP1 and that KAP1-inactivation results in a CSR-specific defect, that phenocopies mice deficient for components of the DDR (i.e. defective CSR and normal SHM, Ramiro et al., 2007). Because KAP1 has been reported to be an effector of the DDR downstream of ATM (Ziv et al., 2006) and because KAP1-inactivation results in a CSR-specific defect similar to $ATM^{-/-}$ mice (Lumsden et al., 2004; Reina-San-Martin et al., 2004) we hypothesized that the function of KAP1 in CSR is to respond to AID-induced DNA damage. We found, however that the role of KAP1 in CSR is independent of its phosphorylation status at serine 824 and that its inactivation does not result in defective DNA repair or increased genomic instability. Therefore, KAP1 does not appear to participate in the repair of AID-induced DNA damage during CSR.

Impaired CSR in the absence of KAP1 could be explained by a global re-activation of genes normally silenced by a KAP1-dependent mechanism (Nielsen et al., 1999) or by a substantial deregulation of chromatin structure (Ziv et al., 2006) at the Ig*h* locus. This however, is unlikely as the transcriptional repressor activity of KAP1 requires its association with HP1 (Sripathy et al., 2006) and we show that the expression of a KAP1 mutant protein that cannot associate with HP1 (Cammas et al., 2004) was unable to restore CSR to wild-type levels. Furthermore, no significant alterations in switch region transcription were observed due to KAP1-deficiency. We believe that the long-range interactions between switch region promoters and Ig*h* enhancers, which are controlled by transcription (Wuerffel et al., 2007), are most likely not affected and that the global 3D-structure of the Ig*h* locus is not altered by KAP1-deficiency. The local access of AID to switch region is most likely not affected by KAP1-deficiency since, AID targeting to switch regions appears to be dependent on its association with Spt5 and RNA polymerase II (Pavri et al., 2010), and since KAP1 inactivation reduces (but does not abolish) the formation of DSBs at Sμ.

We pinpoint the role of KAP1 in CSR to a step downstream of AID expression that facilitates DSB-formation at Sμ. We show that AID forms a complex with KAP1 and HP1 that selectively recognizes H3K9me3 *in vitro.* We also show that H3K9me3, KAP1 and HP1 are recruited to the Ig*h* locus in resting B cells and that upon activation H3K9me3 and KAP1 are retained over Sμ. The specific enrichment of KAP1 binding at Sμ suggests that H3K9me3 can serve as a recognition motif for an AID/KAP1/HP1 complex *in vivo* at the donor switch region during CSR. Consequently, KAP1 deficiency may have no impact in the tethering of AID to downstream acceptor switch regions, the efficiency of cytosine deamination and the subsequent generation of DSBs. As DSB formation at acceptor switch regions has been suggested to be a rate-limiting step in CSR (Reina-San-Martin et al., 2003; Schrader et al., 2003), it might explain why the impairment in CSR in the absence of KAP1 is not more profound.

H3K9me3 is usually associated with heterochromatin formation and gene silencing. However, increasing evidence suggests additional roles for heterochromatin (Grewal and Jia, 2007) for example in recruiting factors that facilitate access of RNA polymerase II to heterochromatic regions (Zofall and Grewal, 2006) or by acting as a recruitment platform for factors regulating long-range chromosomal interactions (Jia et al., 2004). The presence of HP1γ throughout the Ig*h* locus before and during CSR is reminiscent of the mating-type switching in the fission yeast *S. pombe*, in which heterochromatin promotes the spreading of the recombination promoting complex across the mating locus and imposes structural constraints that are important for the choice of the recombination donor site (Jia et al., 2004). Indeed, H3K9me3 has been shown to be present, along with HP1γ, at actively transcribed genes in mammalian cells (Vakoc et al., 2005), and at recombining switch regions in mouse and human B cells (Chowdhury et al., 2008; Kuang et al., 2009). Strikingly, we demonstrate that the *in vivo* KAP1-HP1 association is required for efficient CSR by expressing a KAP1 mutant that cannot associate with HP1 (in a KAP1-deficient background).

Our observations are consistent with a model in which AID forms a complex with KAP1 and HP1 that is tethered to transcribed switch regions bearing the H3K9me3 mark. There is precedent for a model based on the recognition of a modified histone, as recognition of hypermethylated histone H3 at lysine 4 by the PHD domain of RAG2 promotes efficient V(D)J recombination (Liu et al., 2007; Matthews et al., 2007). In our model H3K9me3 and associated KAP1 and HP1γ mark the Sμ locus prior to recombination. Upon stimulation, these marks are retained over Sμ and provide a docking motif for AID tethering *in vivo*. AID would then be retained in close proximity to its substrate by KAP1, HP1 and H3K9me3, thus increasing the probability of cytosine deamination, the efficiency of DSB formation and consequently of CSR. Our results thus provide a mechanism linking AID to epigenetic modifications associated with the IgH locus during CSR.

MATERIALS AND METHODS

Mice. KAP1$^{F/F}$ (Cammas et al., 2000), CD19$^{Cre/+}$ (Rickert et al., 1997), KAP1$^{V488A\text{-}L490A/+}$ (Herzog et al., 2010) and AID$^{Cre/Cre}$ (Robbiani et al., 2008) mice were on a C57BL/6 background and were bred and maintained under specific pathogen-free conditions. Age-matched littermates (8–12 week-old) 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).

Splenic B cell purification, CSR assays and two-color IgH-FISH. Resting splenic B cells were isolated, cultured *in vitro* with LPS (CSR to IgG3 and IgG2b), LPS+IL-4 (CSR to IgG1 and IgE), LPS+IFN-γ (CSR to IgG2a) and assayed for CSR as described (Robert et al., 2009). Switch transcripts, switch junctions and Ig*h*/c-myc translocations were analyzed as described (Robert et al., 2009). Metaphase spreads were prepared and subjected to two-color IgH-FISH as described (Franco et al., 2006).

Nuclear extracts and tandem affinity purification. Nuclear extracts were prepared from 12x10^9 cells using standard techniques. 20 mg of clarified extracts were taken into immunoprecipitation buffer (IP-300: 20 mM Tris pH 7.9, 300 mM KCl, 20% Glycerol, 0.25 mM EDTA, 0.125 mM EGTA, 0.5 mM PMSF, 1 mM DTT, 0.025% NP-40, 1X protease inhibitor cocktail (Roche) and Benzonase (100 U/ml; Novagen) and pre-cleared with mouse IgG and protein G-agarose (Amersham) for 1 h at 4 °C. Flag M2 agarose beads (Sigma-Aldrich; 100 ml - 50% slurry) were added and incubated overnight at 4°C. Immune complexes were eluted three times with 100μl of 0.2 mg/ml flag peptide (30 min at 4°C). Elutions were pooled, and subjected to immunoprecipitation with anti-HA agarose beads (Roche; 40 ml - 50% slurry; 4h at 4°C). Immune complexes were eluted twice with 40 ml 0.1 M glycine (pH 2.4; 10 min) or by overnight incubation with 40 ml of 0.6 mg/ml HA peptide. Proteins were fractionated by SDS-PAGE in 4-12% gradient gels (Invitrogen) and stained with Coomassie blue (G250; BioRad) before processing for mass spectrometry.

Mass spectrometry analysis. Whole lanes from one-dimension electrophoresis were systematically excised in ~5 mm bands. Proteins in bands were subjected to in-gel reduction, alkylation and trypsin digestion. Peptides were dried, resuspended in 0.1% formic acid and injected into the nano liquid chromatography (Ultimate 3000, Dionex) coupled with a linear ion trap mass spectrometer equipped with a nanoelectrospray source (LTQ XL ETD, Thermo Fisher Scientific). MS/MS spectra were recorded in the data dependent mode on the five most intense ions observed in MS scan with CID and ETD fragmentation. Peptides selected for MS/MS acquisition were then dynamically excluded for 30 s. Protein ID was done using Proteome Discoverer 1.1 (Thermo Fisher Scientific) and Sequest® searching against the mouse Swissprot database (version 57.9) with the following fixed parameters: precursor mass tolerance of +/- 0.5

Da, product ion mass tolerance +/- 0.8 Da, and 2 missed cleavages, carbamidomethylation of cysteine as fixed modification and methionine oxidation as variable modification. Sequest results were filtered with Xcorr versus charge state 1.5-1, 1.6-2, 3-3, 3.2-4.

Somatic Hypermutation analysis. Germinal centre B cells ($B220^+Fas^+GL\text{-}7^+$) were sorted from the lymph nodes of NP-CGG-immunized mice and analyzed for mutation in J_H4-intron sequences (Jolly et al., 1997) with SHMTool (Maccarthy et al., 2009).

Real time quantitative PCR. RNA and cDNA were prepared using standard techniques. qPCR was performed using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) or QuantiTect SYBR green PCR kit (Qiagen). Approximately 3 ng of cDNA or 3-5x10^6 cell equivalents of de-crosslinked chromatin were run (in triplicate) and analyzed on a LightCycler 480 (Roche). Transcript or DNA quantities were calculated relative to standard curves and normalized to *CD79b* transcripts or input DNA. Gene of interest/ *CD79b* or region of interest/input DNA ratios were averaged across experiments and normalized to the appropriate controls.

LM-PCR. Genomic DNA was prepared by embedding cells in agarose plugs. Linkers were ligated and DSBs in Sμ were detected by PCR and Southern blotting as described (Schrader et al., 2005).

Sμ mutation analysis. The 5' end of Sμ was amplified by PCR using Pfu Turbo as described (Reina-San-Martin et al., 2004) or using Platinum Taq polymerase (Invitrogen) and bar-coded primers (Table 5, [Table 8]) for high throughput amplicon sequencing (HTS). HTS amplification conditions were: 30 cycles - 94°C (15 s), 60°C (15 s), 68°C (1 min). PCR products were purified with AmPure beads (Agencourt), quantified using Quan-iT Pico Green (Invitrogen) and mixed in equimolar ratios prior to sequencing with a 454 GS FLX sequencer (Roche; GATC). Sequences were aligned with Lasergene (DNASTAR) and analyzed with SHMTool (Maccarthy et al., 2009).

Peptide Pulldowns. Avidin-conjugated agarose beads (Pierce) were coupled to biotinylated unmodified or modified H3 peptides (A_1RTKQTAR**K_9(me3)**STGGKAPRKQLATK$_{23}$-Biotin) for 3 h at 25°C with rotation, washed in PBS 1X NP40 0.1% and resuspended as a 50% slurry. Nuclear extracts (5 mg) were pre-cleared with avidin-agarose beads and incubated overnight with peptide-bound resin (40 μl - 50% slurry). Resin was washed and resuspended in 20 μl of Laemmli buffer. Proteins were fractionated by SDS-PAGE and analyzed by western blot.

Chromatin Immunoprecipitation (ChIP). The protocol was adapted from Upstate-Millipore (http://www.millipore.com/userguides/tech1/mcproto407). Briefly, 3x10^7 resting or stimulated B cells were cross-linked at 37°C for 10 min in 5 ml PBS/0.5% BSA with 1% formaldehyde. The reaction was quenched with 0.125 M glycine. Following lysis, chromatin was sonicated to 0.5-1 kb using a Vibracell 75043 (Thermo Fisher Scientific). After 2x dilution in ChIP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl), chromatin was precleared by rotating for 2 h at 4°C with 80 ml 50% protein A/G slurry (0.2 mg/ml sheared

salmon sperm DNA, 0.5 mg/ml BSA, 50% protein A/G; GE Healthcare). 0.3-0.5x10^6 cell equivalents were saved as input and 3-5x10^6 cell equivalents were incubated overnight with specific or control antibodies (Table 9). Immune complexes were precipitated by the addition of protein A/G and processed according to the Millipore protocol.

Gel filtration chromatography. 20 mg of nuclear extract were immunoprecipitated with M2-agarose beads in IP-300 buffer, washed and eluted with Flag peptide (0.2 mg/ml) in 100 ml. Eluted proteins were fractionated using an ÄKTA micro Superose 6 PC 3.2/30 column. Proteins in each fraction (100 ml) were analyzed by western blot.

Online supplemental material. Supplementary Figure 1 (Figure 31) shows western blot analysis of nuclear extracts prepared from CH12 cells expressing $AID^{Flag\text{-}HA}$, $EGFP^{Flag\text{-}HA}$ and $AID^{Flag\text{-}HA\ D182\text{-}198}$, SDS-PAGE (silver stain) analysis of proteins eluted after tandem affinity purification and the KAP1 peptides identified by mass spectrometry. Supplementary Figure 2 (Figure 32) shows that KAP1 deficiency has no major impact on B cell development. Supplementary Figure 3 (Figure 33) displays two additional experiments showing that KAP1-deficiency results in reduced levels of post-recombination transcripts without decreasing germline transcription. Supplementary Figure 4 (Figure 34) shows that KAP1-deficiency has no effect on the frequency of germinal center B cells and the distribution or pattern of somatic mutations. Supplementary Figure 5 (Figure 35) shows that AID overexpression in KAP1-deficient B cells does not rescue CSR. Supplementary Figure 6 (Figure 36) shows alignments of all the Sμ/Sγ3 switch recombination junctions analyzed. Supplementary Figure 7 (Figure 37) shows Southern blot analysis of Ig*h*/c-myc translocations. Supplementary Figure 8 (Figure 38) displays two additional LM-PCR experiments showing that KAP1-deficiency results in inefficient DSB formation at Sμ during CSR. Supplementary Figure 9 (Figure 39) displays two independent experiments showing that KAP1-deficiency does not alter the pattern of H3K9me3 or H3K9/K14Ac marks at the Ig*h* locus. Supplementary Figure 10 (Figure 40) displays an additional ChIP experiment (corresponding to Figure 6, [Figure 29]) showing that H3K9me3 and KAP1 mark the donor switch region (Sμ) before and during CSR. Table 1 (Table 4) shows the two-color Ig*h*-FISH analysis. Table 2, 3 and 4 (Table 5, 6 and 7) show the statistical analysis corresponding to the ChIP experiments on Figure 6 (Figure 29), Supplementary Figure 9 (Figure 39) and Supplementary Figure 10 (Figure 40), respectively. Table 5 (Table 8) shows the primers used. Table 6 (Table 9) shows the antibodies used.

ACKNOWLEDGEMENTS

We thank members of the Losson and Reina-San-Martin laboratory for discussions; Evi Soutoglou and Olivier Pourquié for comments on the manuscript; Klaus Rajewsky for $CD19^{Cre}$ mice; Manuela Argentini and Frank Ruffenach for assistance in mass spectrometry analysis; Isabelle Billas for advice on gel filtration chromatography; Carol Schrader for advice on LM-PCR; Sonia Beroud for assistance with switch junction analysis; Claudine Ebel for assistance with cell sorting; Pascal Eberling for peptide synthesis; Michael Gendron for animal care and immunizations. B.P.J.R. was supported by fellowships from the Association pour la Recherche sur le Cancer (ARC) and the Fondation pour la Recherche Médicale (FRM). F.W.A. is an investigator of the Howard Hughes Medical Institute. This work was supported by grants to B.R.S.M from the Agence Nationale pour la Recherche (ANR), the Association pour la Recherche sur le Cancer (ARC) and the Institut National de la Santé et de la Recherche Médicale (INSERM). B.R.S.M. is an AVENIR-INSERM young investigator.

The authors have no conflicting financial interests.

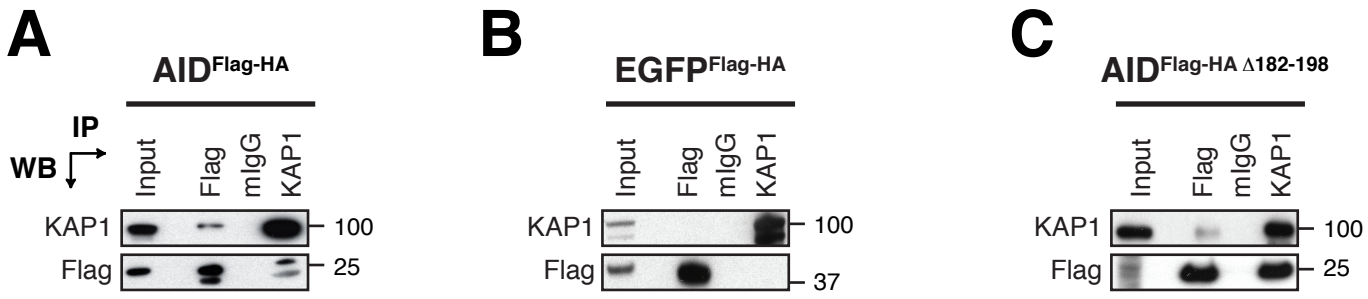


Figure 1 (Figure 24): KAP1 associates with AID. Nuclear extracts prepared from CH12 cells stably expressing (A) AID$^{Flag\text{-}HA}$, (B) EGFP$^{Flag\text{-}HA}$ or (C) AID$^{Flag\text{-}HA\ \Delta 182\text{-}198}$ were immunoprecipitated and blotted with anti-KAP1 and anti-Flag antibodies. Due to lower expression levels of AID$^{Flag\text{-}HA\ \Delta 182\text{-}198}$ when compared to AID$^{Flag\text{-}HA}$ or EGFPFlag-HA (Fig. S1), loading for western blot analysis was adjusted accordingly in (C). Molecular weight markers in kilodaltons (kDa) are indicated.

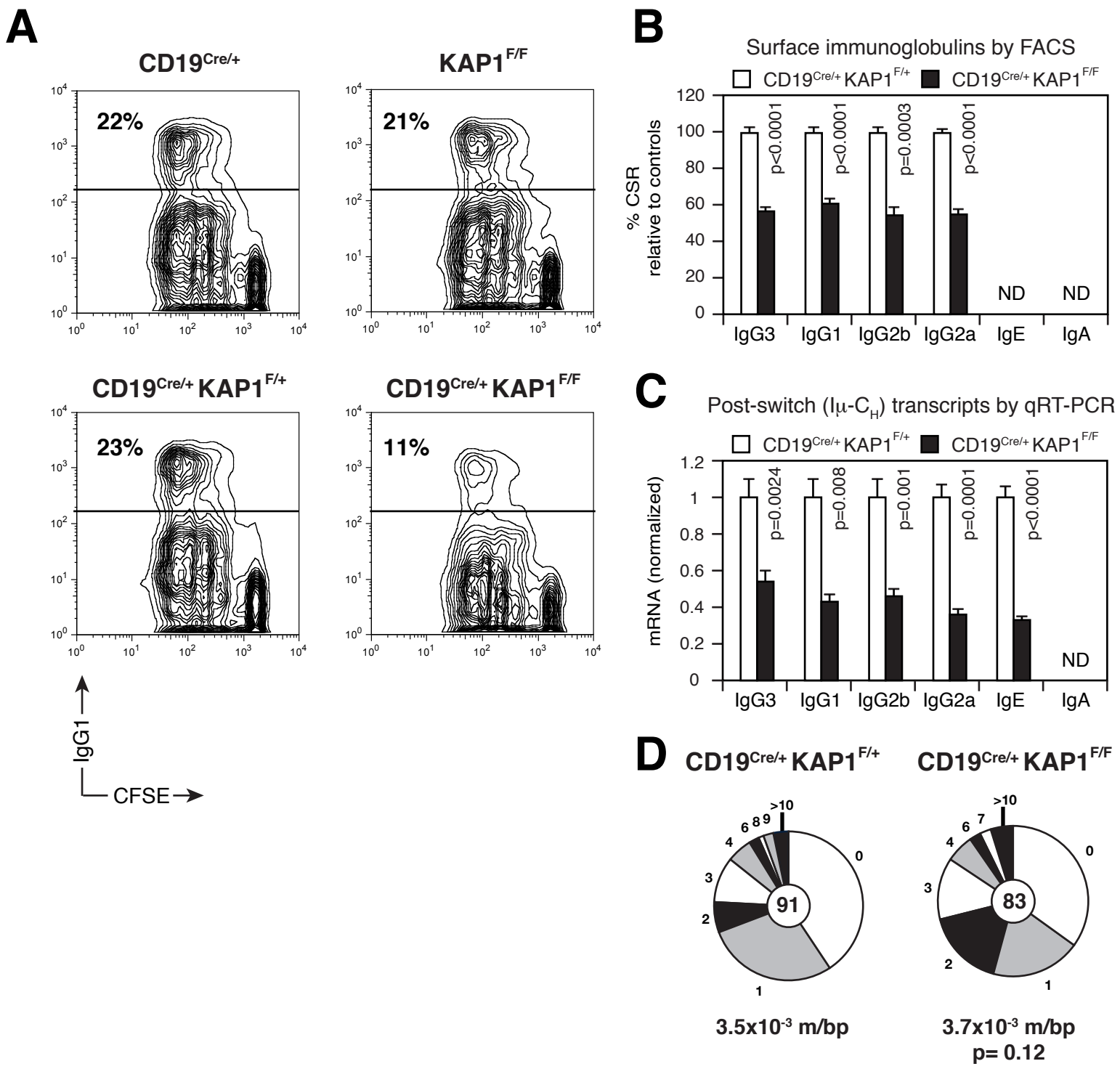


Figure 2 (Figure 25): KAP1 is required for efficient CSR but is dispensable for SHM. (A) IgG1 surface expression and CFSE dilution by flow cytometry in CD$^{19Cre/+}$, KAP1$^{F/F}$, CD19$^{Cre/+}$KAP1$^{F/+}$ and CD19$^{Cre/+}$KAP1$^{F/F}$ B cells stimulated with LPS+IL-4. The percentage of switched cells is indicated in each plot. (B) Percentage (+ s.d.) of CSR in CD19$^{Cre/+}$KAP1$^{F/F}$ (black bars) relative to CD19$^{Cre/+}$KAP1$^{F/+}$ (white bars) for the different isotypes tested (CSR to IgA and IgE could not be determined). CSR in CD19$^{Cre/+}$KAP1$^{F/+}$ B cells was set to 100%. p values, Student's t-test. ND, Not determined. Data are from 5 independent experiments. (C) qRT-PCR for post-switch (Iμ-CH) transcripts in CD19$^{Cre/+}$KAP1$^{F/F}$ (black bars) relative to CD19$^{Cre/+}$KAP1$^{F/+}$ (white bars) B cells. Expression is normalized to *Cd79b* expression and is presented relative to the expression in CD19$^{Cre/+}$KAP1$^{F/+}$ B cells, set as 1. p values, Student's *t*-test. Data are representative of three experiments. (D) J_H4 intron sequences were analyzed for mutations from germinal centre B cells (B220$^+$ Fas$^+$GL-7$^+$) sorted from the lymph nodes of immunized CD$^{19Cre/+}$KAP1$^{F/F}$ and CD19$^{Cre/+}$KAP1$^{F/+}$ mice. Segment sizes are proportional to the number of sequences bearing the indicated mutations. Mutation frequency and number of sequences analyzed are indicated. p value, t-test. Sequences were obtained from 4 independent experiments.

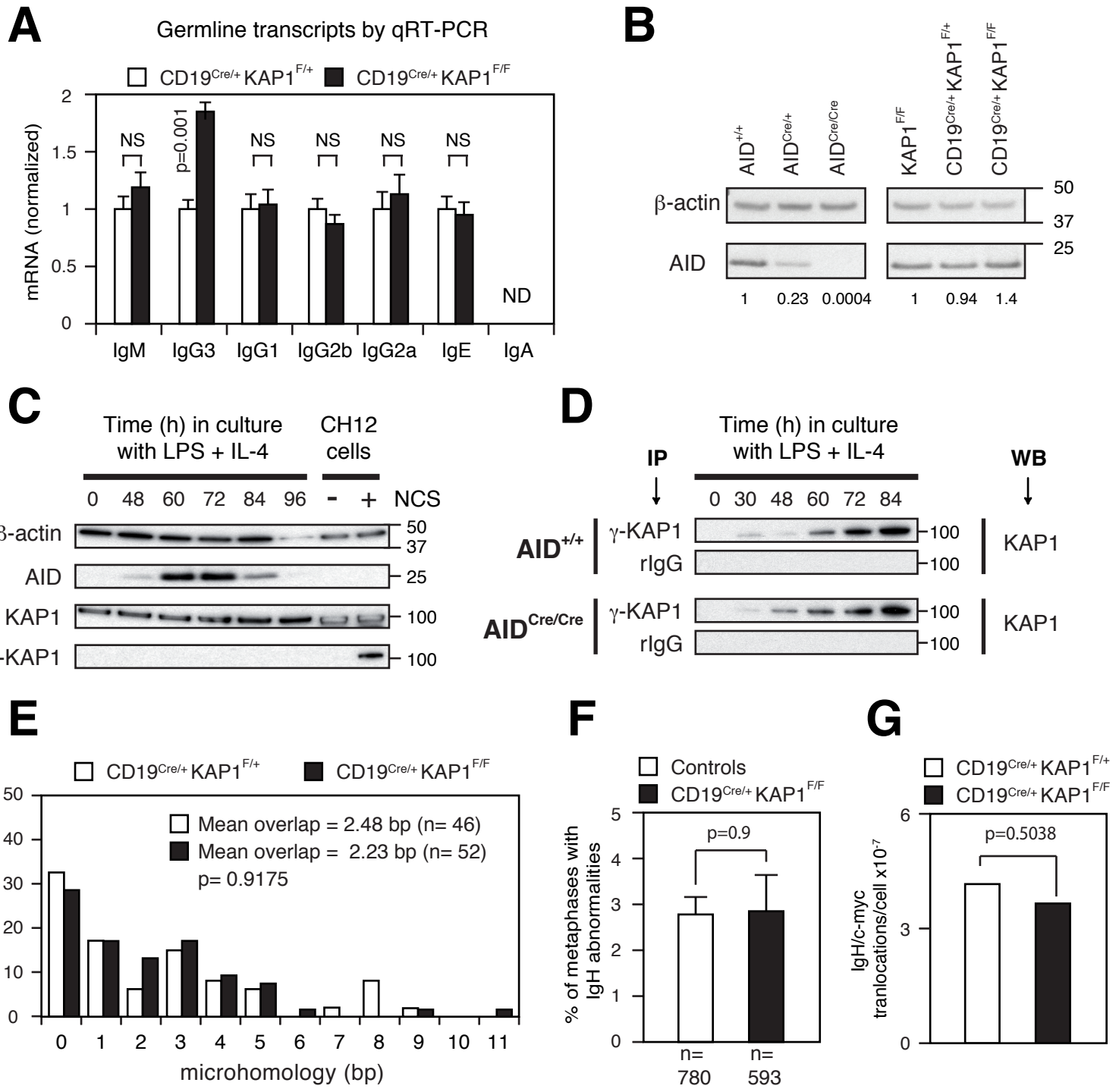


Figure 3 (Figure 26): KAP1 functions downstream of switch region transcription and upstream of DSB formation. (A) qRT-PCR for germline (I_H-C_H) transcripts in CD19$^{Cre/+}$KAP1$^{F/F}$ (black bars) relative to CD19$^{Cre/+}$KAP1$^{F/+}$ (white bars) B cells. Expression is normalized to *Cd79b* and relative to CD19$^{Cre/+}$KAP1$^{F/+}$ B cells, set as 1. p values, Student's *t*-test. NS: Not significant. ND, Not determined. Data are from 3 independent experiments. (B) Western blot for AID, KAP1 and β-actin in KAP1$^{F/F}$, CD19$^{Cre/+}$KAP1$^{F/+}$, CD19$^{Cre/+}$KAP1$^{F/F}$, AID$^{+/+}$, AID$^{Cre/+}$ and AID-deficient (AID$^{Cre/Cre}$) B cells. Numbers below the panel reflect the intensity of bands representing KAP1 and AID protein relative to controls after normalization to β-actin. Molecular weight markers in kilodaltons (kDa) are indicated. (C) KAP1 phosphorylation (γ-KAP1) by western blot in wild-type B cells cultured with LPS + IL-4. Positive control, CH12 cells treated with neocarzinostatin (NCS). (D) γ-KAP1 was immunoprecipitated from activated AID$^{+/+}$ and AID-deficient (AID$^{Cre/Cre}$) B cells and blotted for KAP1. (E) Percentage of Sμ/Sγ3 switch junction sequences with indicated nucleotide overlap from CD19$^{Cre/+}$KAP1$^{F/+}$ (white bars) and CD19$^{Cre/+}$KAP1$^{F/F}$ (black bars) LPS-stimulated B cells from 5 independent experiments. Mean length of overlap in base pairs and the number of sequences analyzed (n) is indicated. p value, Mann-Whitney test. (F) Quantification of Ig*h* locus breaks as determined by Ig*h*-FISH on metaphases prepared from control (white bars; n=780) and CD19$^{Cre/+}$KAP1$^{F/F}$ (black bars; n=593) B cells cultured for three days with anti-CD40- and IL-4. Mean (+s.e.m) is shown. p value, Mann-Whitney test. (G) Frequency of Ig*h*/c-myc translocations in CD19$^{Cre/+}$KAP1$^{F/F}$ and CD19$^{Cre/+}$KAP1$^{F/+}$ B cells determined by long-range PCR and Southern blot in three independent experiments. p value, one-tailed exact Fisher's test.

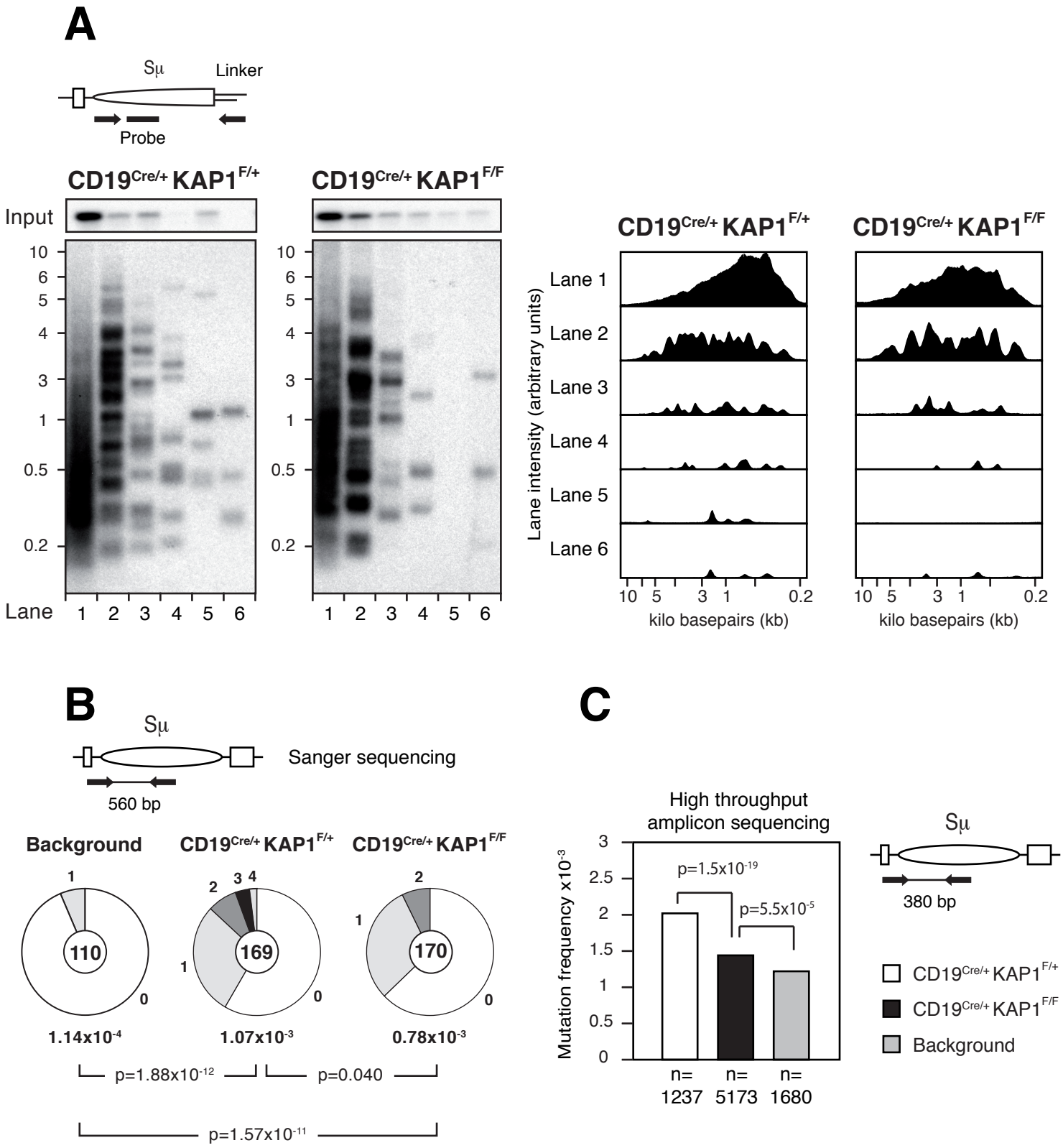


Figure 4 (Figure 27): DSB formation at Sμ is reduced in KAP1-deficient B cells. (A) Ligation-mediated PCR performed on genomic DNA isolated from CD19$^{Cre/+}$KAP1$^{F/F}$ and CD19$^{Cre/+}$KAP1$^{F/+}$ B cells cultured for two days with LPS+IL-4. PCR for the 5' end of Sμ was hybridized with an internal Sμ probe. Histograms corresponding to individual lane intensities (arbitrary units) are shown to the right. Input DNA, Southern blot on aicda PCR. Data are representative of three experiments (Fig. 38). (B-C) Mutation analysis in the 5' end of Sμ in CD19$^{Cre/+}$KAP1$^{F/F}$ and CD19$^{Cre/+}$KAP1$^{F/+}$ B cells as determined by (B) Sanger sequencing or (C) high throughput amplicon sequencing. Pie charts in (B) are as in Fig. 25. Number of sequences analyzed (n) in (C) is indicated below the histogram. p values, two-tailed *t*-test.

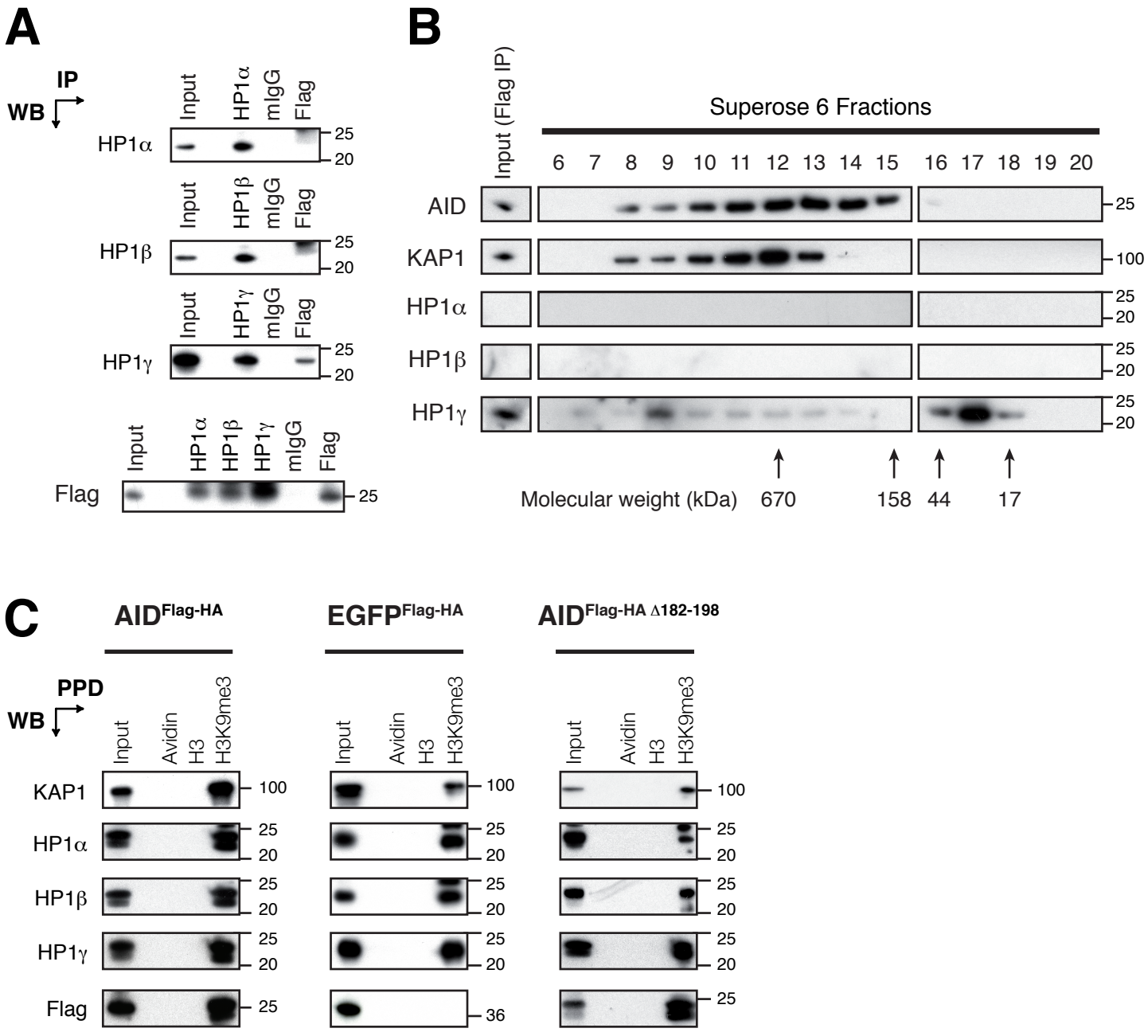


Figure 5 (Figure 28): AID forms a complex with KAP1 and HP1 that binds H3K9me3. (A) Nuclear extracts prepared from CH12 cells expressing AID$^{Flag\text{-}HA}$ were immunoprecipitated and blotted with anti-HP1α, HP1β, HP1 γ and anti-Flag antibodies. Molecular weight markers in kilodaltons (kDa) are indicated. (B) Flag immunoprecipitates eluted with the Flag peptide were fractionated with a Superose 6 gel filtration column. The indicated fractions were analyzed by western blot using antibodies specific for KAP1, HP1α, HP1β, HP1γ and AID. Arrows indicate the elution position of calibration proteins of known molecular weight. (C) Peptide pull downs using biotinylated unmodified (H3) or modified H3 peptides (H3K9me3) and avidin-agarose beads (Avidin) on nuclear extracts prepared from CH12 cells stably expressing AID$^{Flag\text{-}HA}$, EGFP$^{Flag\text{-}HA}$ or AID$^{Flag\text{-}HA\ \Delta 182\text{-}198}$. Precipitated proteins were separated by SDS-PAGE and blotted with antibodies specific for KAP1, HP1α, HP1β, HP1γ and Flag. Molecular weight markers in kilodaltons (kDa) are indicated.

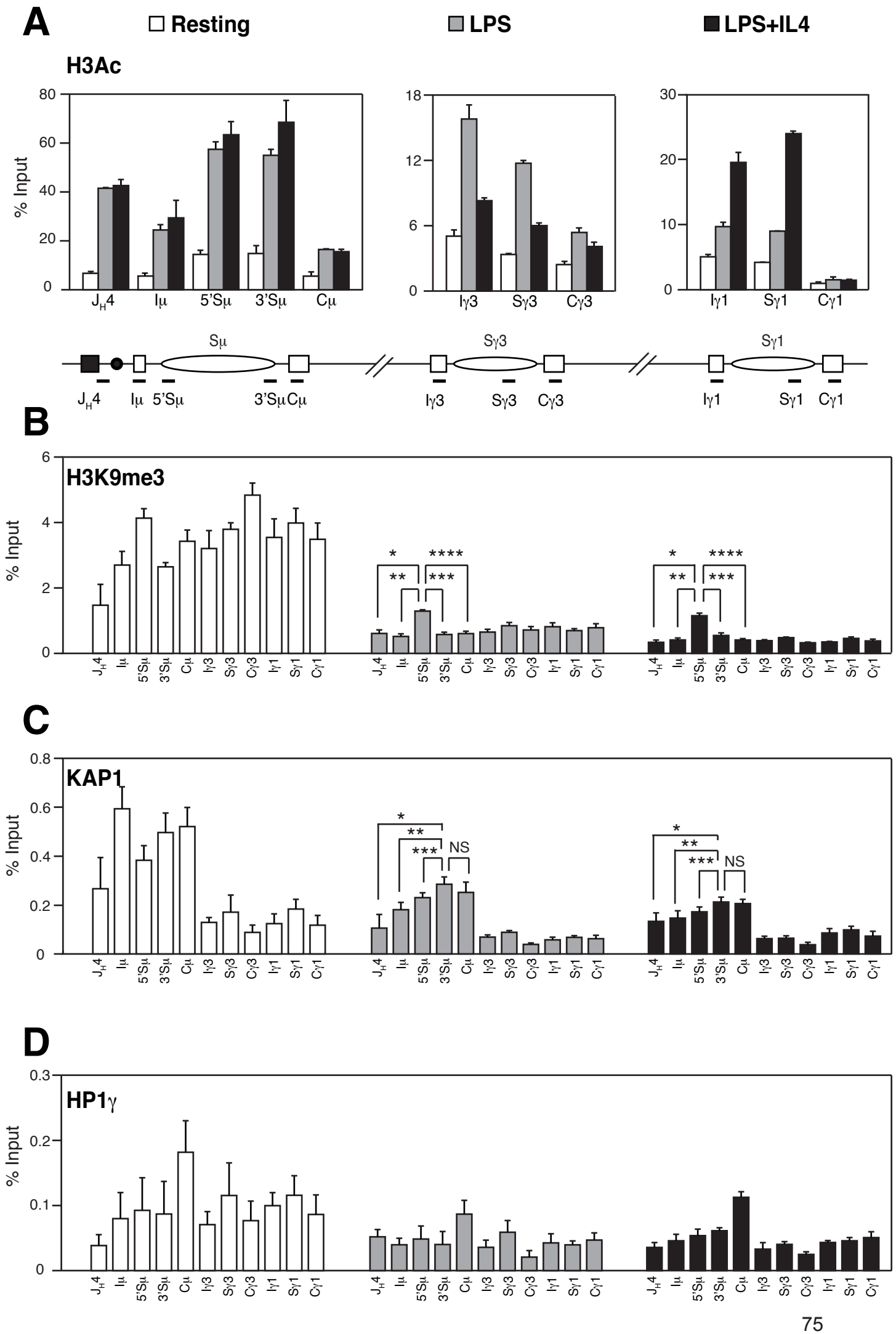

A
Resting
LPS
LPS+IL4
H3Ac
% Input
80
60
40
20
0
18
12
6
0
30
20
10
0
JH4
Iμ
5'Sμ
3'Sμ
Cμ
Iγ3
Sγ3
Cγ3
Iγ1
Sγ1
Cγ1
Sμ
Sγ3
Sγ1
B
H3K9me3
6
4
2
0
C
KAP1
0.8
0.6
0.4
0.2
0
NS
D
HP1γ
0.3
0.2
0.1
0

Figure 6 (Figure 29): H3K9me3 and KAP1 mark the donor switch region (Sμ) before and during CSR. Chromatin immunoprecipitations performed on chromatin prepared from resting (white bars) or LPS-stimulated (grey bars) and LPS+IL-4-stimulated (black bars) B cells obtained from $CD19^{Cre/+}KAP1^{F/+}$ mice using antibodies specific for (A) H3K9/K14Ac (H3Ac), (B) H3K9me3, (C) KAP1 and (D) HP1γ. Real time quantitative PCR was performed by using primer pairs specific for J_H4, Iμ, Sμ, Cμ, Iγ3, Sγ3, Cγ3, Iγ1, Sγ1 and Cγ1. Fold change over control IgG are expressed as percent of input. Mean (+ s.d) of triplicate samples is shown. p values, One-tailed Student's *t*-test. H3K9me3, LPS: * p=0.0003, ** p <0.0001, *** p <0.0001, **** p <0.0001; H3K9me3, LPS+IL-4: * p <0.0001, ** p=0.0001, *** p=0.0004, **** p <0.0001; KAP1, LPS: * p=0.0040, ** p=0.0065, *** p=0.0289, NS: Not significant; KAP1, LPS+IL-4: * p=0.0134, ** p=0.0170, *** p=0.0346. See Supplementary Tables 5 and 6 for detailed statistical analysis and primer sequences respectively. Data are representative of four independent experiments (See Figure S10 for an additional experiment).

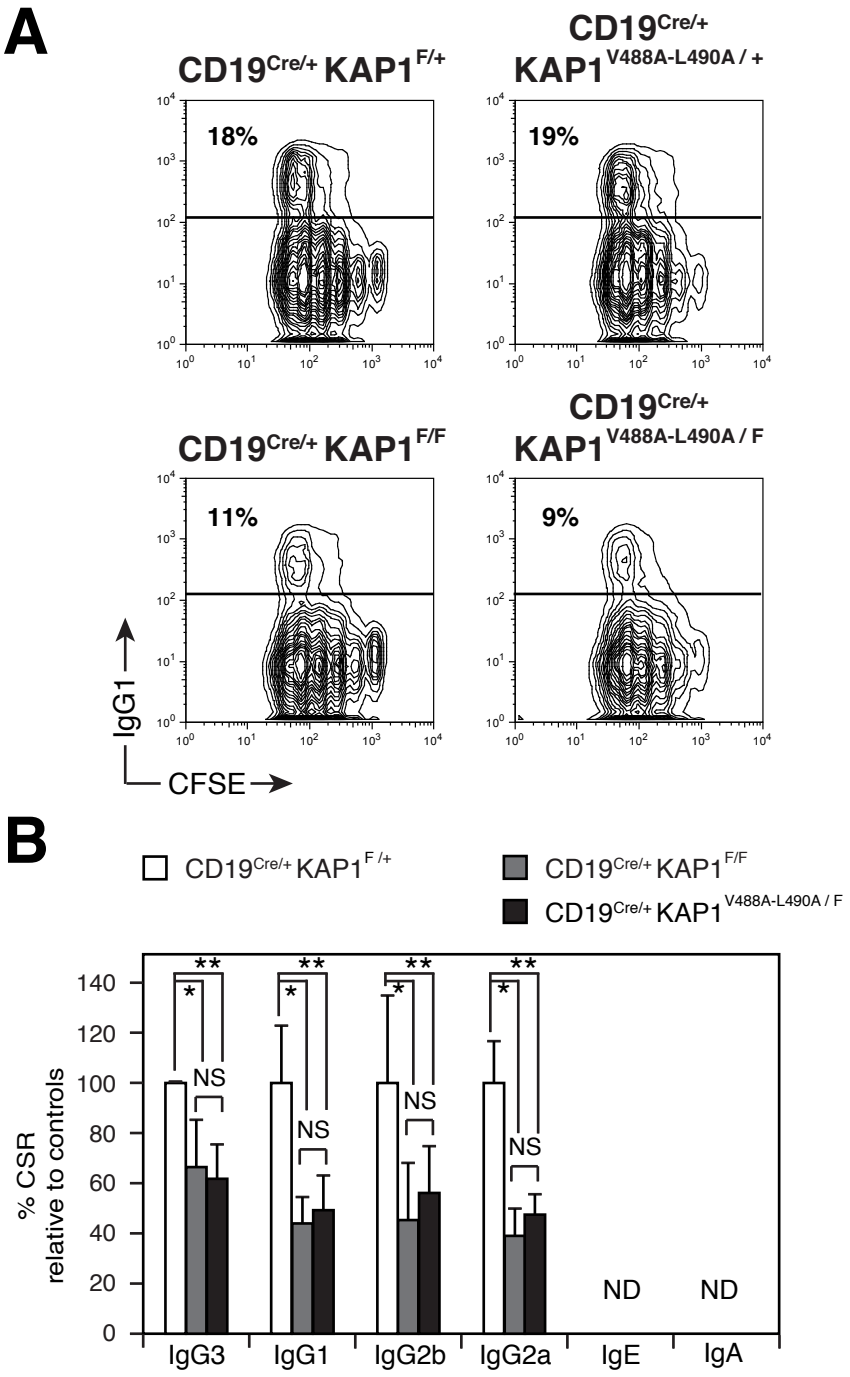
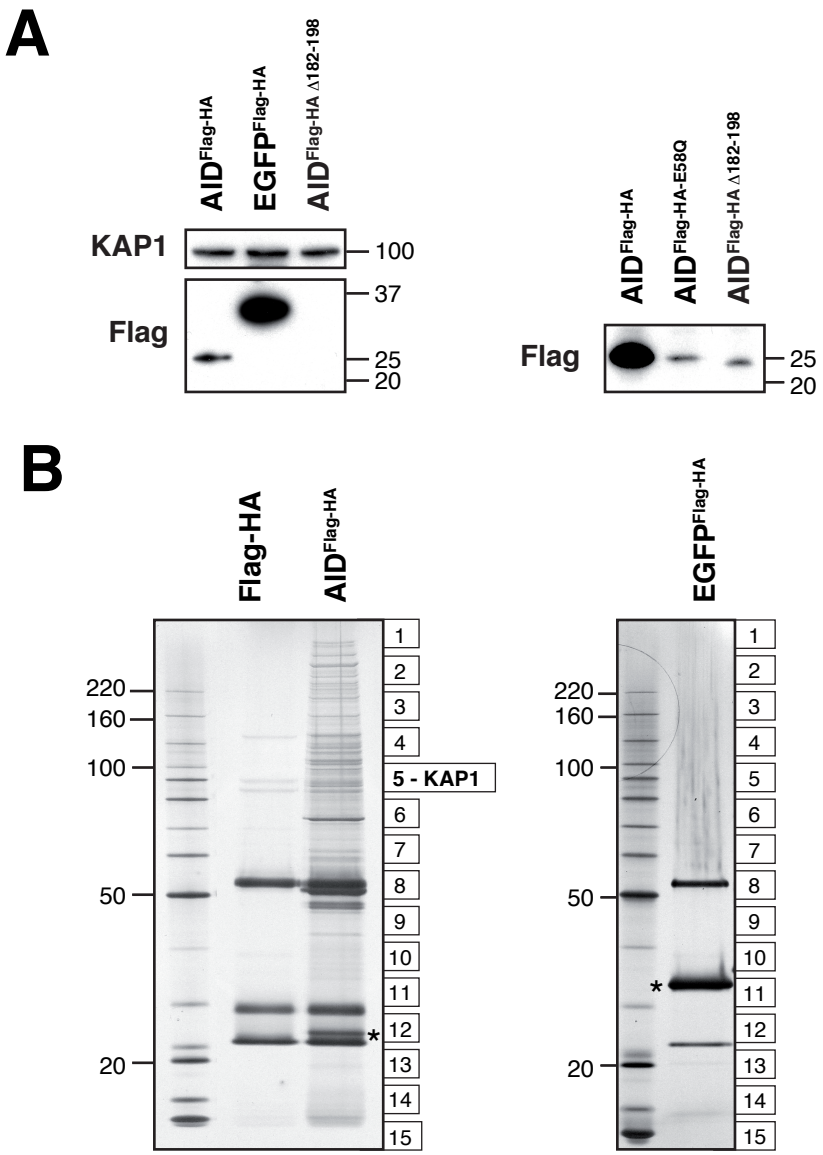


Figure 7 (Figure 30): The in vivo association between KAP1 and HP1 is required for efficient CSR. (A) IgG1 cell surface expression in $CD19^{Cre/+}KAP1^{F/+}$, $CD19^{Cre/+}KAP1^{V488A-L490A/+}$, $CD19^{Cre/+}KAP1^{F/F}$ and $CD19^{Cre/+}KAP1^{V488A-L490A/F}$ CFSE-labeled B cells stimulated with LPS+IL-4 for three days. The percentage of switched cells is indicated in each plot. (B) Percentage (+ s.d.) of CSR in $CD19^{Cre/+}KAP1^{F/+}$, (white bars), $CD19^{Cre/+}KAP1^{F/F}$, (grey bars) and $CD19^{Cre/+}KAP1^{V488A-L490A/F}$ (black bars) relative to $CD19^{Cre/+}KAP1^{F/+}$ (white bars). CSR in $CD19^{Cre/+}KAP1^{F/+}$ B cells was set to 100%. Data are from five independent experiments. p values, one-tailed Student's t-test. *, $CD19^{Cre/+}KAP1^{F/+}$ vs. $CD19^{Cre/+}KAP1^{F/F}$ (IgG3 p=0.0154, IgG1 p=0.0035, IgG2b p=0.0264, IgG2a p=0.001). **, $CD19^{Cre/+}KAP1^{F/+}$vs. $CD19^{Cre/+}KAP1^{V488A-L490A/F}$ (IgG3 p=0.0017, IgG1 p=0.0036, IgG2b p=0.0276, IgG2a p=0.0004). ND, Not determined. NS, not significant (p>0,05).



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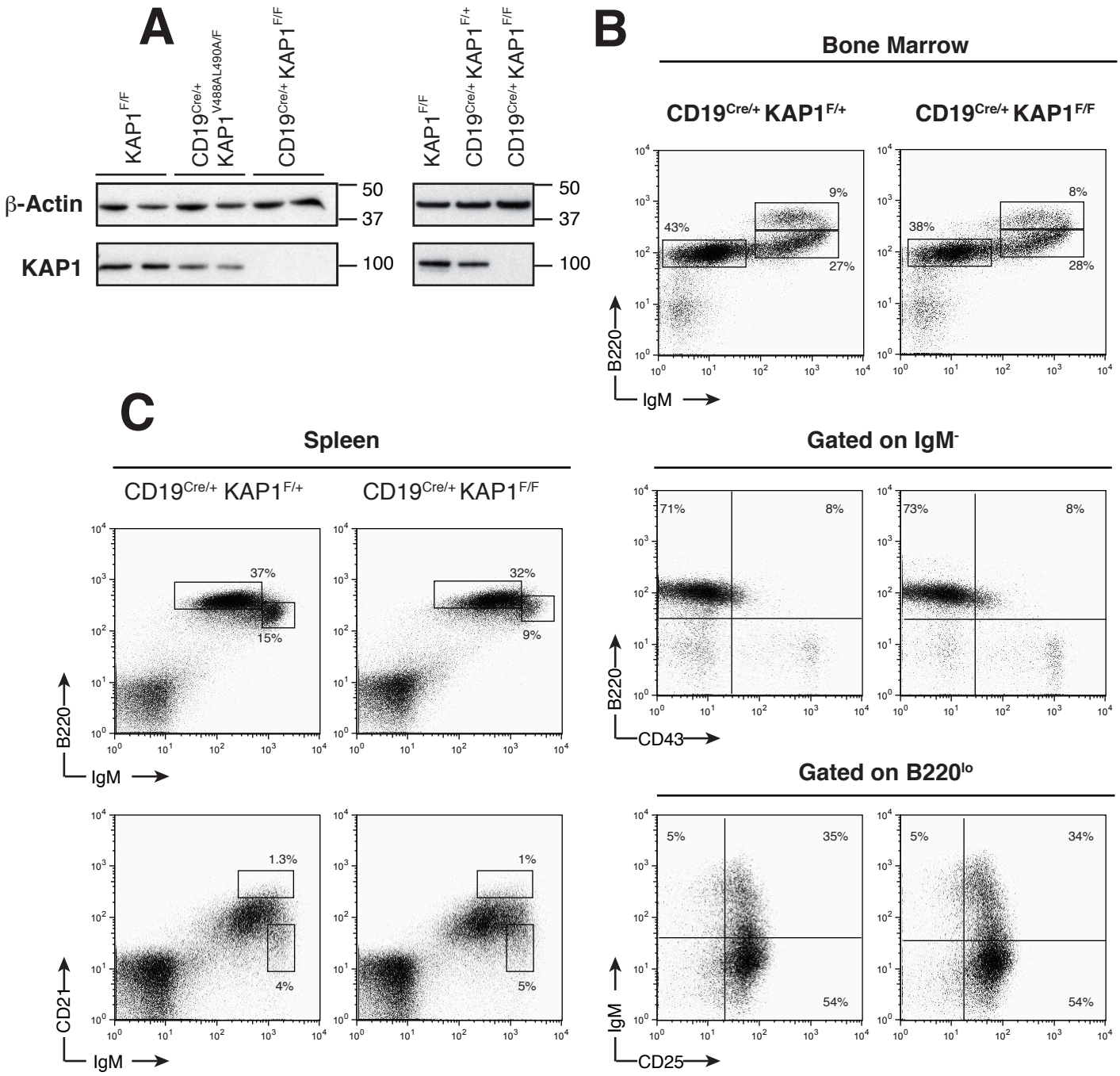
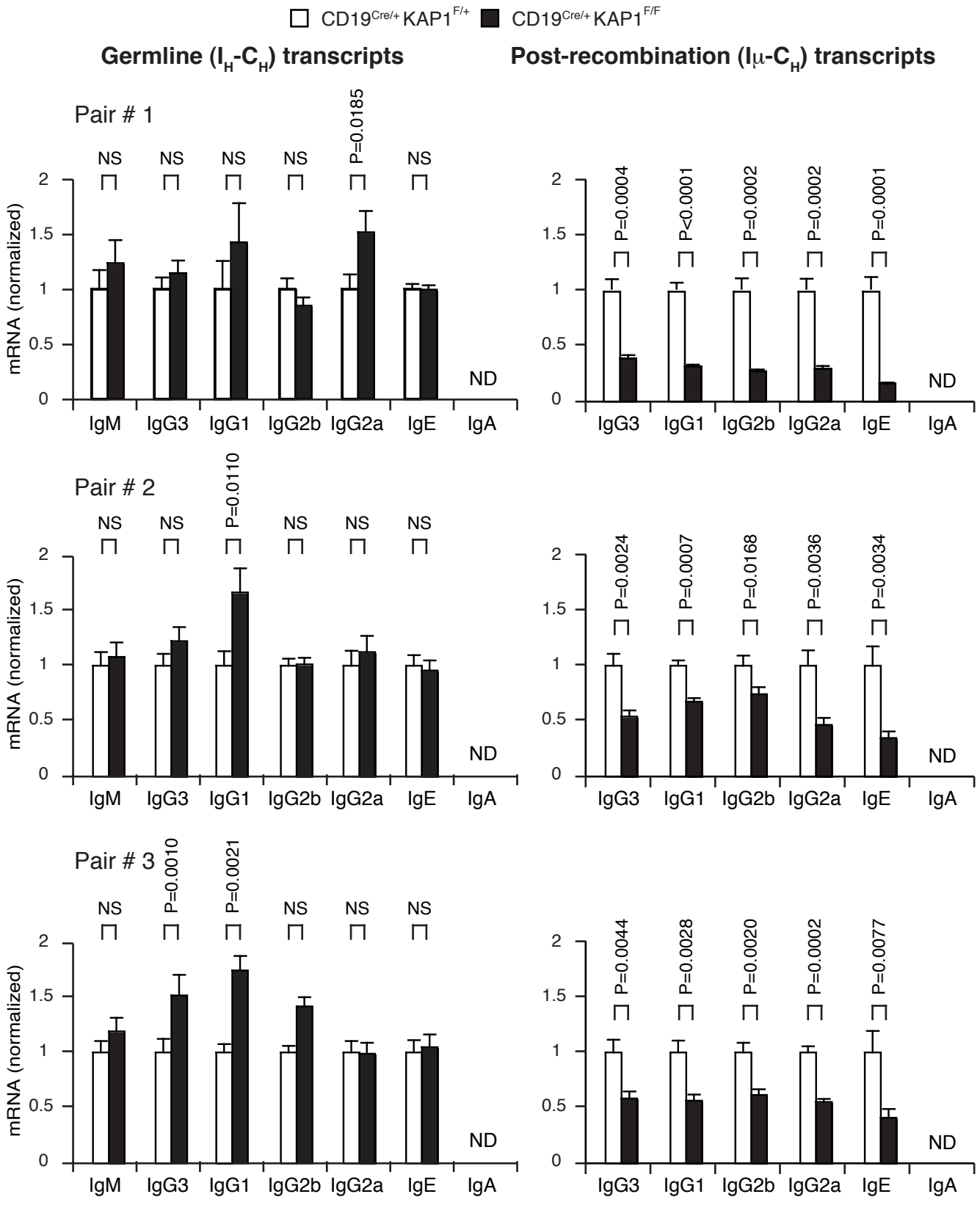
KAP1
Transcription intermediary factor 1beta OS=Mus musculus GN=Trim28 PE=1 SV=3 - [TIF1B_MOUSE] Q62318)

Position	Peptide Sequence	Activation type	Xcorr	z	MH^+(Da)
129-137	DIVENYFmR	CID	2.74	2	1202.29
409-428	IVAERPGTNSTGPGPmAPPR	CID	5.16	3	2020.89
436-469	QGSGSSQPMEVQEGYGFGSDDPYSSAEPHVSGMK	CID	5.92	3	3532.48
473-483	SGEGEVSGLLR	CID	3.04	2	1103.56
751-767	LSPPYSSPQEFAQDVGR	CID	4.58	2	1877.77
780-790	ADVQSIIGLQR	CID	3.09	2	1199.56

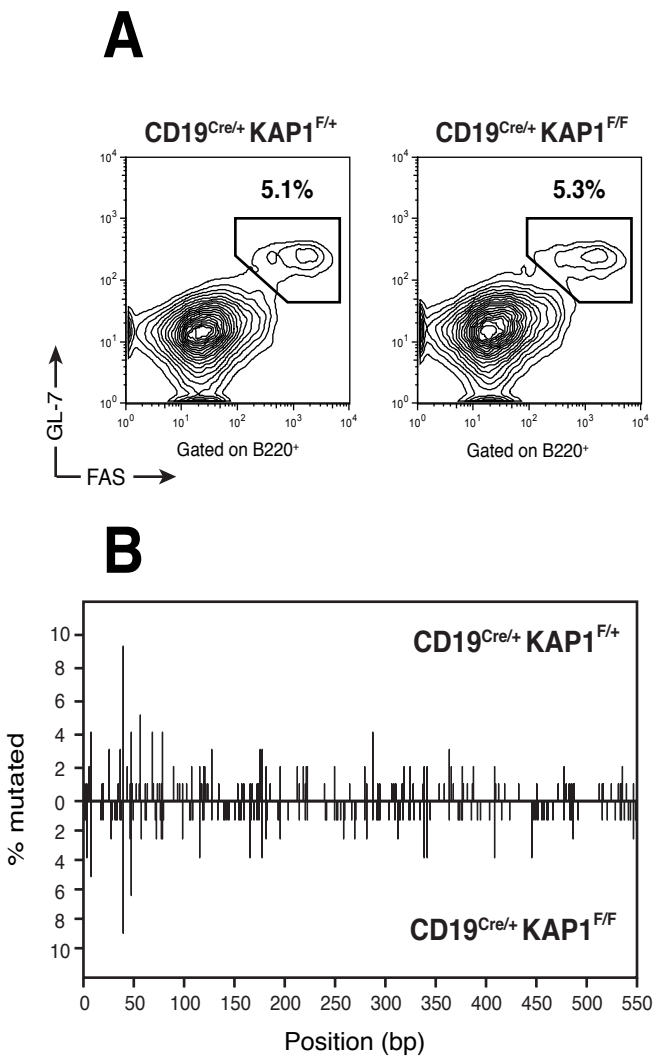
Supplementary Figure 1 (Figure 31): AID associates with KAP1. (A) Western blot analysis showing the relative expression level between $AID^{Flag\text{-}HA}$, $EGFP^{Flag\text{-}HA}$ and $AID^{Flag\text{-}HA\ \Delta 182\text{-}198}$. Comparison only between $AID^{Flag\text{-}HA}$ and $AID^{Flag\text{-}HA\ \Delta 182\text{-}198}$ is shown on the right and includes extracts expressing a catalytic mutant of AID ($AID^{Flag\text{-}HA\text{-}E58Q}$) that was generated but not used in this study. (B) Silver stain of proteins eluted after Flag and HA sequential immunoprecipitations performed on nuclear extracts prepared from CH12 cells expressing tagged AID ($AID^{Flag\text{-}HA}$), the tags alone (Flag-HA) or tagged EGFP ($EGFP^{Flag\text{-}HA}$). Approximate location of gel slices submitted to mass spectrometry analysis are indicated. KAP1 was identified in bands 4 and 5 depending on the experiment. *, positions of the baits. (C) The peptides identified by mass spectrometry in tandem affinity purification experiments performed using nuclear extracts prepared from CH12 cells expressing $AID^{Flag\text{-}HA}$ are indicated. The corresponding peptide position, sequence, fragmentation method (CID or ETD), cross correlation score of the identification by Sequest (Xcorr), charge state of the precursor (z) and corresponding monocharged (MH+) ion are listed.



Supplementary Figure 2 (Figure 32): KAP1 deficiency does not affect B cell development. (A) Western blot analysis for β-actin and KAP1 on whole-cell extracts from splenic B cells cultured in vitro for 72 hrs with LPS from KAP1$^{F/F}$, CD19$^{Cre/+}$KAP1$^{V488A\ L490A/F}$ and CD19$^{Cre/+}$KAP1$^{F/F}$ mice (left panel) as well as LPS + IL-4 activated B cells from KAP1$^{F/F}$, CD19$^{Cre/+}$KAP1$^{F/+}$ and CD19$^{Cre/+}$KAP1$^{F/F}$ (right panel). Molecular weight markers (kDa) are indicated. Flow cytometry analysis of cells from the (B) bone marrow and (C) spleen from CD19$^{Cre/+}$KAP1$^{F/F}$ and control CD19$^{Cre/+}$KAP1$^{F/+}$ mice. Staining antibodies are indicated. The numbers show relative percentages of cells within indicated gates and further gating is indicated directly above the density plots where applicable. In the bone marrow: (B) top row indicates staining for pro/pre-B (B220^{lo}, IgM^{-}), immature (B220^{lo}, IgM^{+}), and mature recirculating (B220^{hi}, IgM^{+}) B cells; middle row shows staining for pro-B cells (IgM^{-}, CD43^{+}, B220^{+}) and pre-B cells (IgM^{-}, CD43^{-}, B220^{-}); bottom row is staining for pre-B (B220^{lo}, CD25^{+}, IgM^{+}) and immature B cells (B220^{lo}, CD25^{-}, IgM^{+}). In the spleen (C): top row shows staining for immature (B220^{lo}, IgM^{+}) and mature (B220^{hi}, IgM^{+}) B cells and the bottom row is staining for transitional type-2 B cells (CD21^{+}, IgM^{+}) and marginal zone (CD23^{-}, CD21^{+}, IgM^{+}) B cells.



Supplementary Figure 3 (Figure 33): KAP1-deficiency results in reduced levels of post-recombination transcripts without affecting germline transcription. Real time quantitative RT-PCR analysis of germline I_H-C_H transcripts (left) and post-recombination Iμ-C_H transcripts (right) in CD19$^{Cre/+}$KAP1$^{F/F}$ (black bars) and control CD19$^{Cre/+}$KAP1$^{F/+}$ (white bars) B cells cultured for 3 days with LPS alone (IgG2b and IgG3) or with LPS + IL-4 (IgG1 and IgE) or LPS + IFN-γ (IgG2a). Expression is normalized to *Cd79b* expression and is presented relative to the expression in CD19$^{Cre/+}$KAP1$^{F/+}$ B cells, set as 1. Mean and s.d. of triplicate samples is shown. p values, Student's *t*-test. Data are representative from three experiments with 2 mice per genotype.



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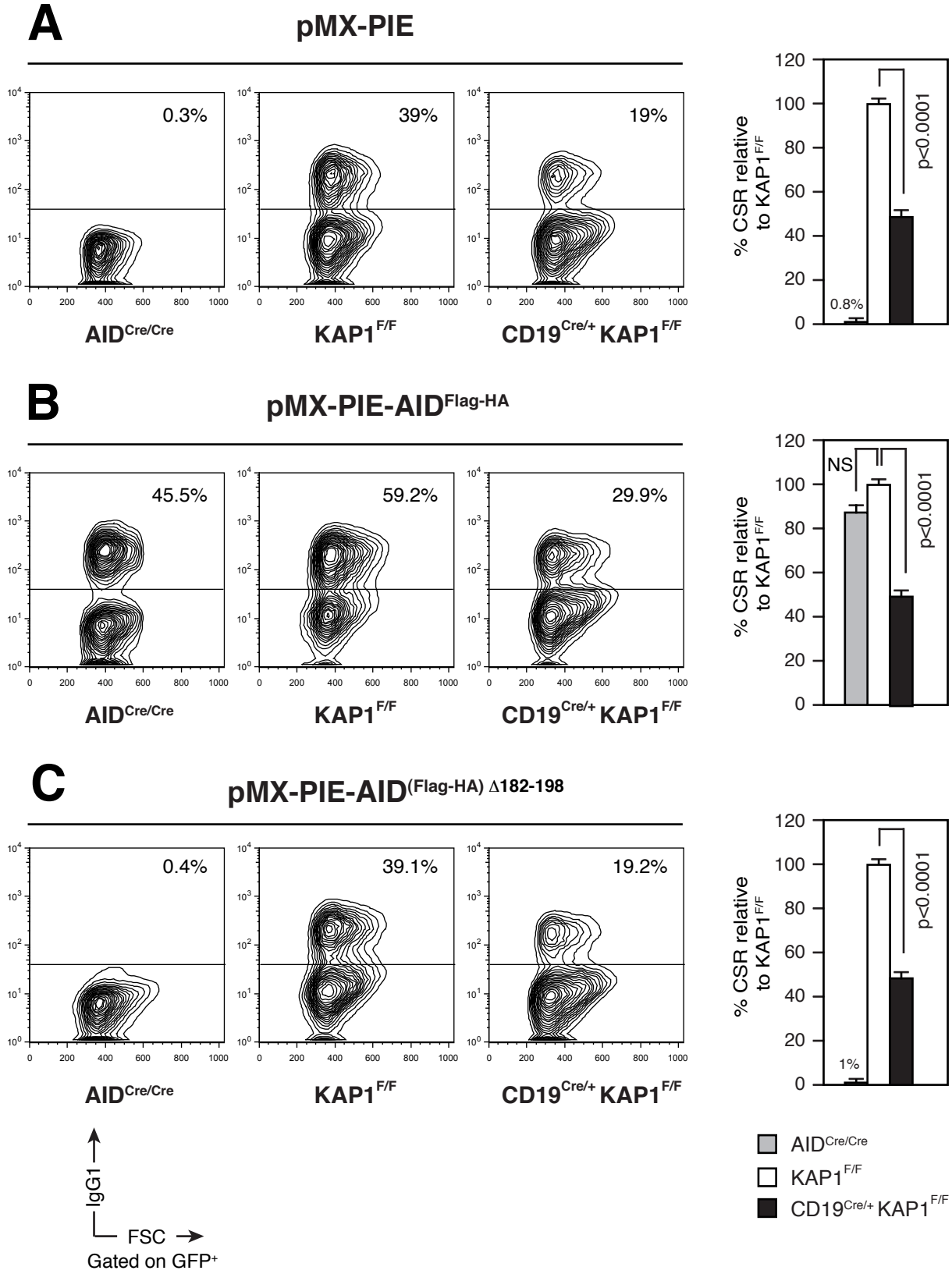
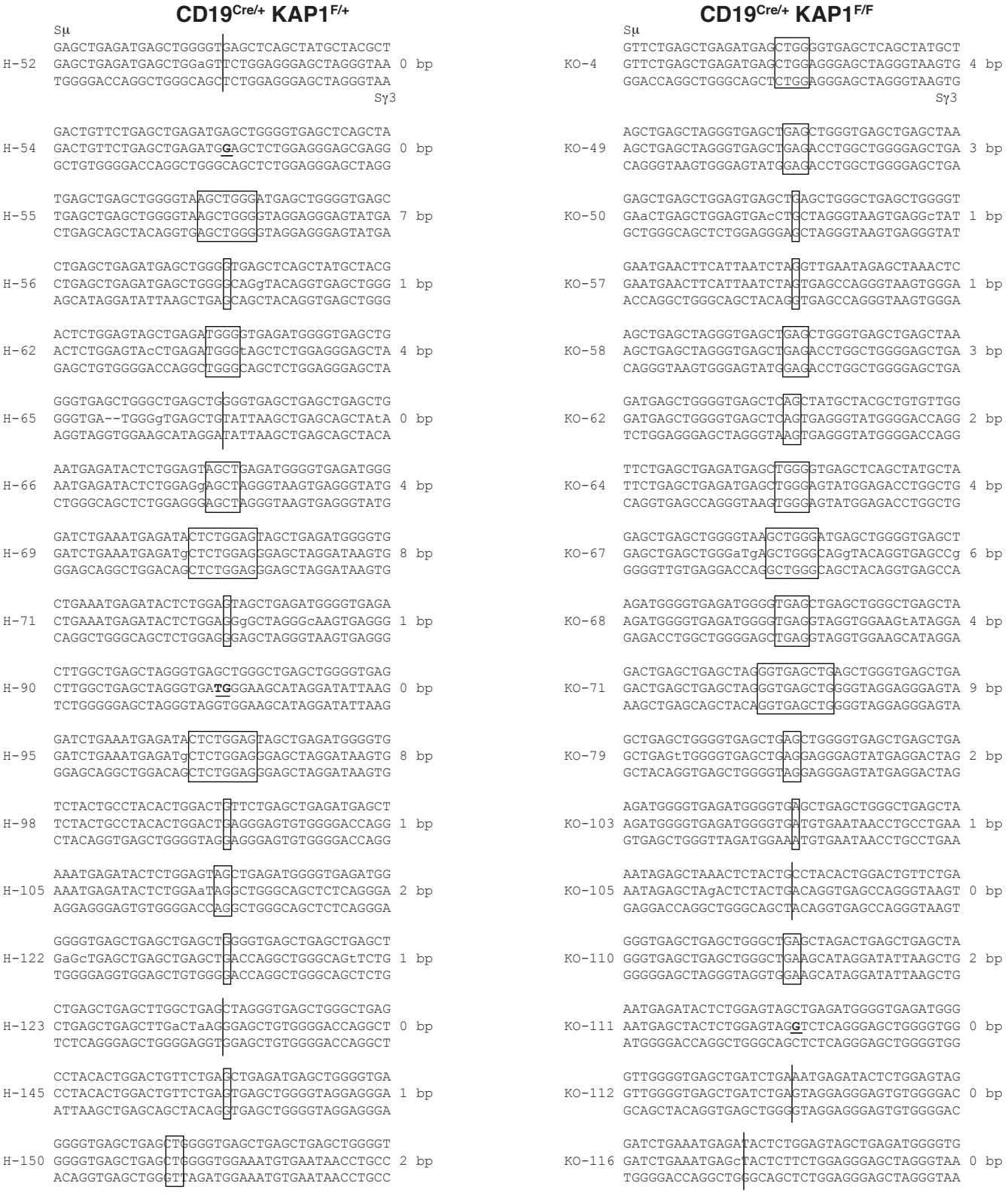
MUTATIONS	CD19Cre/+ KAP1F/+	CD19Cre/+ KAP1F/F	TOTAL # SITES	# SITES Sequenced CD19Cre/+ KAP1F/+	# SITES Sequenced CD19Cre/+ KAP1F/F	Frequency CD19Cre/+ KAP1F/+	Frequency CD19Cre/+ KAP1F/F	P value
G->C	16	17	153	13923	12699	0.001149178	0.001338688	0.7913473
G->A	23	26	153	13923	12699	0.001651943	0.002047405	0.5426855
G->T	13	11	153	13923	12699	0.0009337068	0.00086621	1
sum:G	52	54	153	13923	12699	0.003734827	0.004252303	0.5671627
C->G	6	4	81	7371	6723	0.0008140008	0.0005949725	NA
C->A	2	4	81	7371	6723	0.0002713336	0.0005949725	NA
C->T	11	14	81	7371	6723	0.001492335	0.002082404	0.5279674
sum:C	19	22	81	7371	6723	0.002577669	0.003272349	0.5430073
A->G	29	24	147	13377	12201	0.0021679	0.001967052	0.829631
A->C	16	12	147	13377	12201	0.001196083	0.000983526	0.7458023
A->T	24	17	147	13377	12201	0.001794124	0.001393328	0.5196775
sum:A	69	53	147	13377	12201	0.005158107	0.004343906	0.3935813
T->G	7	9	169	15379	14027	0.0004551661	0.0006416197	0.6639358
T->C	13	17	169	15379	14027	0.0008453085	0.001211948	0.4232451
T->A	16	16	169	15379	14027	0.001040380	0.001140657	0.9335
sum:T	36	42	169	15379	14027	0.002340854	0.002994225	0.3297989
sum:GC	71	76	234	21294	19422	0.003334273	0.003913088	0.3735178
sum:AT	105	95	316	28756	26228	0.003651412	0.003622083	1
sum:ALL	176	171	550	50050	45650	0.003516484	0.003745893	0.592029
Tv:GC	37	36	234	21294	19422	0.001737579	0.001853568	0.8736246
Tr:GC	34	40	234	21294	19422	0.001596694	0.00205952	0.3277425
Tv:AT	63	54	316	28756	26228	0.002190847	0.002058868	0.808162
Tr:AT	42	41	316	28756	26228	0.001460565	0.001563215	0.8417122
Tv:ALL	100	90	550	50050	45650	0.001998002	0.001971522	0.9846667
Tr:ALL	76	81	550	50050	45650	0.001518482	0.001774370	0.3697141

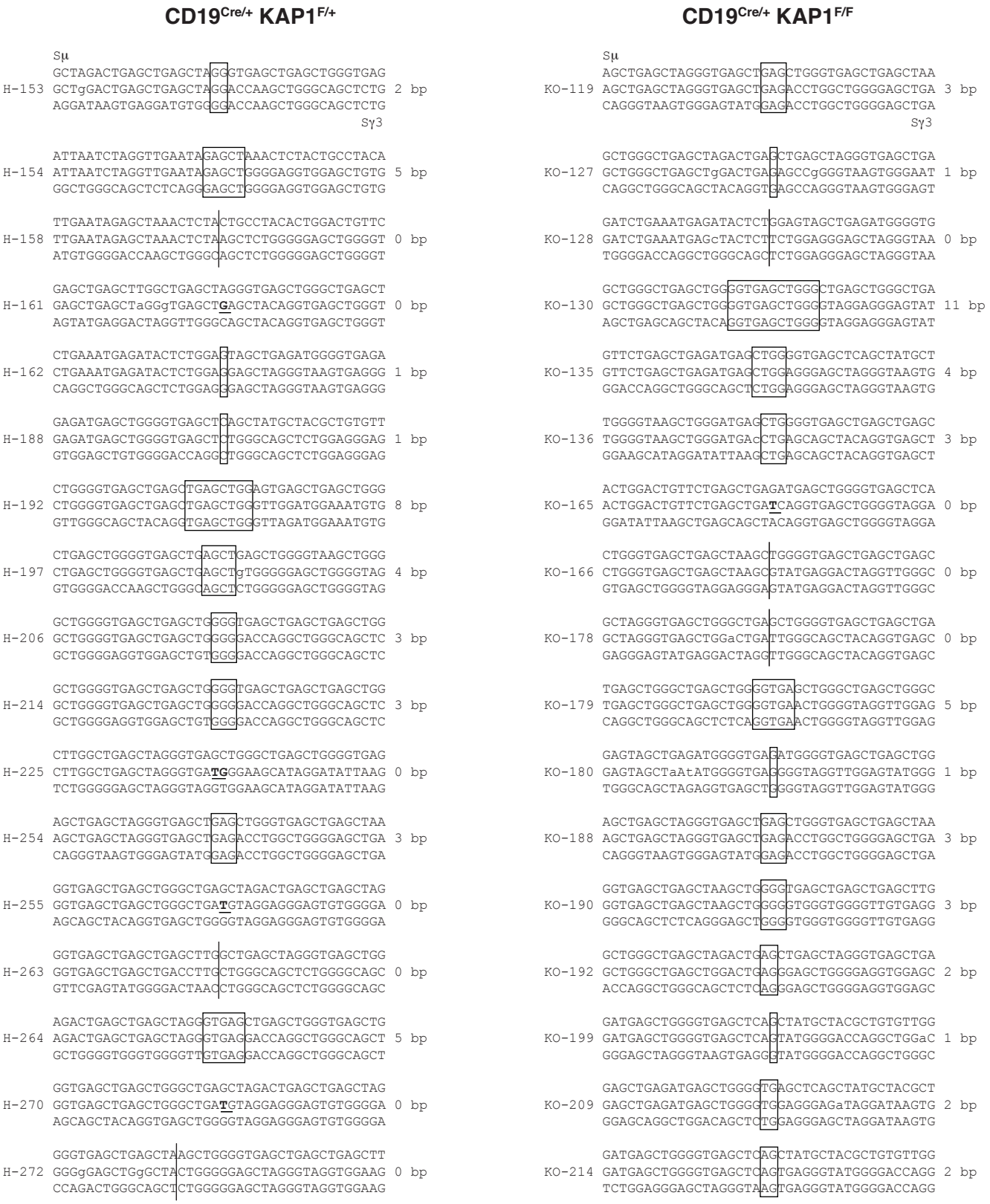
Supplementary Figure 4 (Figure 34): KAP1 is dispensable for Somatic Hypermutation. (A) Flow cytometry analysis of CD19$^{Cre/+}$KAP1$^{F/+}$ and CD19$^{Cre/+}$KAP1$^{F/F}$ germinal centre B cells in the lymph node of NP-CGG immunized mice at day 10. Plots are gated on B220. The percentage of germinal centre B cells is indicated (B220^{+} Fas^{+} GL-7^{+}) above each gate. SHM was analyzed using the SHM-Tool online application. (B) Distribution of mutations at the J_H4 intron comparing CD19$^{Cre/+}$KAP1$^{F/+}$ (above) and CD19$^{Cre/+}$KAP1$^{F/F}$ (below) sequences. The numbers of independent mutations at each nucleotide position are expressed as a percentage of the total mutation database (as analyzed over the entire 550 bp sequence). (C) Table showing mutation type, mutation frequency in CD19$^{Cre/+}$KAP1$^{F/+}$ and CD19$^{Cre/+}$KAP1$^{F/F}$ sequences and the corresponding statistical analysis. Tv: transversions; Tr: transitions.



Supplementary Figure 5 (Figure 35): AID overexpression in KAP1-deficient B cells does not rescue CSR. B cells obtained from $AID^{Cre/Cre}$, $KAP1^{F/F}$ and $CD19^{Cre/+}KAP1^{F/F}$ mice were cultured *in vitro* with LPS+IL-4 and transduced with either (A) empty virus (pMX-PIE) or with viruses encoding (B) $AID^{Flag\text{-}HA}$ or (C) $AID^{Flag\text{-}HA\ \Delta\ 182\text{-}198}$. Representative flow cytometry profiles of two independent experiments with 2 mice per genotype are shown. Plots are gated on GFP^+ cells. The percentage of switched cells is indicated in each plot. A histogram showing the percentage (+ s.d.) of CSR to IgG1 in $AID^{Cre/Cre}$ (grey bars) and $CD19^{Cre/+}KAP1^{F/F}$ (black bars) relative to $KAP1^{F/F}$ (white bars) B cells transduced with the corresponding retroviruses is shown on the right. CSR in KAP1F/F B cells was set to 100%. p values, Student's *t*-test. NS, not significant.



Supplementary Figure 6 (Figure 36): Sμ/Sγ3 switch recombination junctions in CD19$^{Cre/+}$KAP1$^{F/F}$ and control CD19$^{Cre/+}$KAP1$^{F/+}$ B cells. Sμ/Sγ3 switch junction sequences with indicated nucleotide overlap from CD19$^{Cre/+}$KAP1$^{F/+}$ and CD19$^{Cre/+}$KAP1$^{F/F}$ are shown. Overlap was determined by identifying the longest region at the switch junction of perfect uninterrupted donor/acceptor identity. Sequences with insertions at the junction were scored as having no microhomology. The sequenced Sμ/Sγ3 junction sequences are shown in the middle. The Sμ and Sγ3 germline sequences (chromosome 12 genomic sequence: NT-114985) are shown above and below each junction sequence, respectively. Homology at the junctions is boxed and the length of overlap is indicated on the right. Letters in lower-case indicate mutations, insertions are bolded and underligned. Duplicate sequences were discarded. Sequences having identical junctions but differences elsewhere were included.



Supplementary Figure 6 (Figure 36): Sμ/Sγ3 switch recombination junctions in CD19$^{Cre/+}$KAP1$^{F/F}$ and control CD19$^{Cre/+}$KAP1$^{F/+}$ B cells. Sμ/Sγ3 switch junction sequences with indicated nucleotide overlap from CD19$^{Cre/+}$KAP1$^{F/+}$ and CD19$^{Cre/+}$KAP1$^{F/F}$ are shown. Overlap was determined by identifying the longest region at the switch junction of perfect uninterrupted donor/acceptor identity. Sequences with insertions at the junction were scored as having no microhomology. The sequenced Sμ/Sγ3 junction sequences are shown in the middle. The Sμ and Sγ3 germline sequences (chromosome 12 genomic sequence: NT-114985) are shown above and below each junction sequence, respectively. Homology at the junctions is boxed and the length of overlap is indicated on the right. Letters in lower-case indicate mutations, insertions are bolded and underligned. Duplicate sequences were discarded. Sequences having identical junctions but differences elsewhere were included.

CD19$^{Cre/+}$ KAP1$^{F/+}$

```
      Sμ
      GAGCTGGGGTAAGCTGGGATGAGCTGGGGTGAGCTGAGCT
H-281 GAGCTGGGGTAAGCTGGGATGGGAGGTGGAGCTGTGGGGA 1 bp
      GGGCAGCTCTCAGGGAGCTGGGGAGGTGGAGCTGTGGGGA
                                            Sγ3

      GCTGAGCTAAGCTGGGGTGAGCTGAGCTGAGCTTGGCTGA
H-282 GCTGAGCTAAGCTGGGGTGATACAGGTGAGCTGGGGTAGG 0 bp
      AGGATATTAAGCTGAGCAGCTACAGGTGAGCTGGGGTAGG

      TAAGCTGGGATGAGCTGGGGTGAGCTGAGCTGAGCTGGAG
H-283 TAAGCTGGGATGAGCTGGGGTGGGAGTATGGAGACCTGGC 3 bp
      TACAGGTGAGCCAGGGTAAGTGGGAGTATGGAGACCTGGC

      TAAGCTGGGATGAGCTGGGGTGAGCTGAGCTGAGCTGGAG
H-286 TAAGCTGGGATGAGCTGGGGTGGGAGTATGGAGACCTGGC 3 bp
      TACAGGTGAGCCAGGGTAAGTGGGAGTATGGAGACCTGGC

      GGATGAGCTGGGGTGAGCTGAGCTGAGCTGGAGTGAGCTG
H-289 tGATGAGCTGGGGTGATCTGAGTATGGGGACTAACCTGGG 3 bp
      GGGGAGCTGGGGTAGGTTCGAGTATGGGGACTAACCTGGG

      GCTGAGCTAAGCTGGGGTGAGCTGAGCTGAGCTTGGCTGA
H-296 GCTGAGCTAAGCTGGGGTGATACAGGTGAGCTGGGGTAGG 0 bp
      AGGATATTAAGCTGAGCAGCTACAGGTGAGCTGGGGTAGG

      GATCTGAAATGAGATACTCTGGAGTAGCTGAGATGGGGTG
H-303 GATCTGAAATGAGATgCTCTGGAGGGAGCTAGGATAAGTG 8 bp
      GGAGCAGGCTGGACAGCTCTGGAGGGAGCTAGGATAAGTG

      CTGGGCTGAGCTGGGGTGAGCTGAGCTGAGCTGGGGTAAG
H-309 CTGGGCTGAGCTGGGGTGAGCTgCAGGTGAGCTGGGTTAG 4 bp
      ATGAGGACTAGGTTGGGCAGCTACAGGTGAGCTGGGTTAG

      GACTGTTCTGAGCTGAGATGAGCTGGGGTGAGCTCAGCTA
H-310 GACTGTTCTGAGCTGAGATG**G**AGCTCTGGAGGGAGCgAGG 0 bp
      GCTGTGGGGACCAGGCTGGGCAGCTCTGGAGGGAGCTAGG

      AATGAGATACTCTGGAGTAGCTGAGATGGGGTGAGATGGG
H-317 AATGAGATACTCTGGAGTAGCTGTGGGGACCAGGCTGGGC 5 bp
      AGGGAGCTGGGGAGGTGGAGCTGTGGGGACCAGGCTGGGC

      CACTGGACTGTTCTGAGCTGAGATGAGCTGGGGTGAGCTC
H-318 CACTGGACTGcTCTGAGCTGAGGGAGTGTGGGGACCAGGC 3 bp
      TACAGGTGAGCTGGGGTAGGAGGGAGTGTGGGGACCAGGC

      CTGGGGTGAGCTGGGCTGAGCTGGGCTGAGCTGGGCTGAG
H-321 CTGGGGTGAGCTGGGCTGAGCTGGGTTAGATGGAAATGTG 9 bp
      GTTGGGCAGCTACAGGTGAGCTGGGTTAGATGGAAATGTG
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CD19$^{Cre/+}$ KAP1$^{F/F}$

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      Sμ
      GCTGAGCTAAGCTGGGGTGAGCTGAGCTGAGCTTGGCTGA
KO-217 GCTGAGCTAAGCTGGGGTGATGGGGTAGGTTCGAGTATGG 0 bp
      CTGGGCAGCTCTGGGGGAGCTGGGGTAGGTTCGAGTATGG
                                            Sγ3

      AGCTGGGGTGAGCTCAGCTATGCTACGCTGTGTTGGGGTG
KO-218 AGCTGGGGTGAGCTCAGCTA**G**TCTCAGGGAGCTGGGGAGG 0 bp
      GTGGGGACCAGGCTGGGCAGCTCTCAGGGAGCTGGGGAGG

      AGCTAGGGTGAGCTGGGCTGAGCTGGGGTGAGCTGAGCTG
KO-222 AGCTAGGGTGAGCTGGGCTGACCTGCCTGAAGGGCCACAG 1 bp
      GTTAGATGGAAATGTGAATAACCTGCCTGAAGGGCCACAG

      CTGTTCTGAGCTGAGATGAGCTGGGGTGAGCTCAGCTATG
KO-224 CTGTTCTGAGCTGAGATaAGCTGAGCAGATACAGGTGAGC 5 bp
      TGGAAGCATAGGATATTAAGCTGAGCAGCTACAGGTGAGC

      GTGAGCTGAGCTGGGCTGAGCTAGACTGAGCTGAGCTAGG
KO 225 GTGAGCTGACCTGGGCTGAGCTACAGGTGAGCCAGGGTAA 5 bp
      GTGAGGACCAGGCTGGGCAGCTACAGGTGAGCCAGGGTAA

      GTTCTGAGCTGAGATGAGCTGGGGTGAGCTCAGCTATGCT
KO-228 GTTCTGAGCTGAGATGAGCTGGaGgGAGCTAGGGTAAGTG 4 bp
      GGACCAGGCTGGGCAGCTCTGGAGGGAGCTAGGGTAAGTG

      AGCTGGGGTGAGCTGAGCTGAGCTGGGGTGAGCTGGGGTG
KO-229 AGCTGaGcTGAGCTGAGaTGAGCAGCTACAGGTGAGCTGG 5 bp
      AAGCATAGGATATTAAGCTGAGCAGCTACAGGTGAGCTGG

      GATGAGCTGGGGTGAGCTGAGCTGAGCTGGAGTGAGCTGA
KO-236 GATGAGCTGGGGTGAGCTGA**T**GTAAGTGAGGGTATGGGGA 0 bp
      GCAGCTCTGGAGGGAGCTAGGGTAAGTGAGGGTATGGGGA

      GGGTGAGCTGAGCTGGGTGAGCTGAGCTAAGCTGGGGTGA
KO-239 GGGTGAGCTGAGCTGGGcGAGGTTCaAGTATGGGGACTAA 2 bp
      GCTCTGGGGGAGCTGGGGTAGGTTCGAGTATGGGGACTAA

      GGTTGAATAGAGCTAAACTCTACTGCCTACACTGGACTGT
KO-248 GGTTGAATAGAGCTAAACTCTTAaTGGgAGTGTAGGGACC 1 bp
      CAGCTCTGGGGCAGCTGAGGTTAGTGGAAGTGTAGGGACC

      GCTGAGCTAAGCTGGGGTGAGCTGAGCTGAGCTTGGCTGA
KO-249 GCTGAGCTAAGCTGGGGTGATACAGGTGAGCTGGGGTAGG 0 bp
      AGGATATTAAGCTGAGCAGCTACAGGTGAGCTGGGGTAGG

      GAGCTGGGGTGAGCTCAGCTATGCTACGCTGTGTTGGGGT
KO-252 GAGCTGGGGTGAGCTCAGaTAGCTGGGGTAGGAGGGAGTA 1 bp
      AAGCTGAGCAGCTACAGGTGAGCTGGGGTAGGAGGGAGTA

      ATCTAGGTTGAATAGAGCTAAACTCTACTGCCTACACTGG
KO-255 ATCTAGGTTGAATAGAGCTAGGAGACCTGGCTGGGGAGCT 0 bp
      GCCAGGGTAAGTGGGAGTATGGAGACCTGGCTGGGGAGCT

      TGGAGTAGCTGAGATGGGGTGAGATGGGGTGAGCTGAGCT
KO-258 TGGAGTAGCTGAGATGGGGTAGCTCTGGAGGGAGCTAGGA 0 bp
      AGTATAGGAGCAGGCTGGACAGCTCTGGAGGGAGCTAGGA

      AGCTGAGCTAGGGTGAGCTGAGCTGGGTGAGCTGAGCTAA
KO-259 AGCTGAGCTAGGGTGAGCTGAGACCTGGCTGGGGAGCTGA 3 bp
      CAGGGTAAGTGGGAGTATGGAGACCTGGCTGGGGAGCTGA

      AGCTGAGCTAGGGTGAGCTGAGCTGGGTGAGCTGAGCTAA
KO-264 AGCTGAGCTAGGGTGAGCTGAGACCTGGCTGGGGAGCTGA 3 bp
      CAGGGTAAGTGGGAGTATGGAGACCTGGCTGGGGAGCTGA

      GTGAGCTGAGCTGGGTGAGCTGAGCTAAGCTGGGGTGAGC
KO-261 GTGAGCTGAGCTGGGTGAGCTGGAGGGAGCTAGGATAAGT 3 bp
      AGGAGCAGGCTGGACAGCTCTGGAGGGAGCTAGGATAAGT

      GAGCTAGACTGAGCTGAGCTAGGGTGAGCTGAGCTGGGTG
KO-278 GAGCTgGACTGAGCTGACCTGCTACAGGTGAGCTGGGTTG 0 bp
      TATGAGGACTAGGTTGGGCAGCTACAGGTGAGCTGGGTTA
```
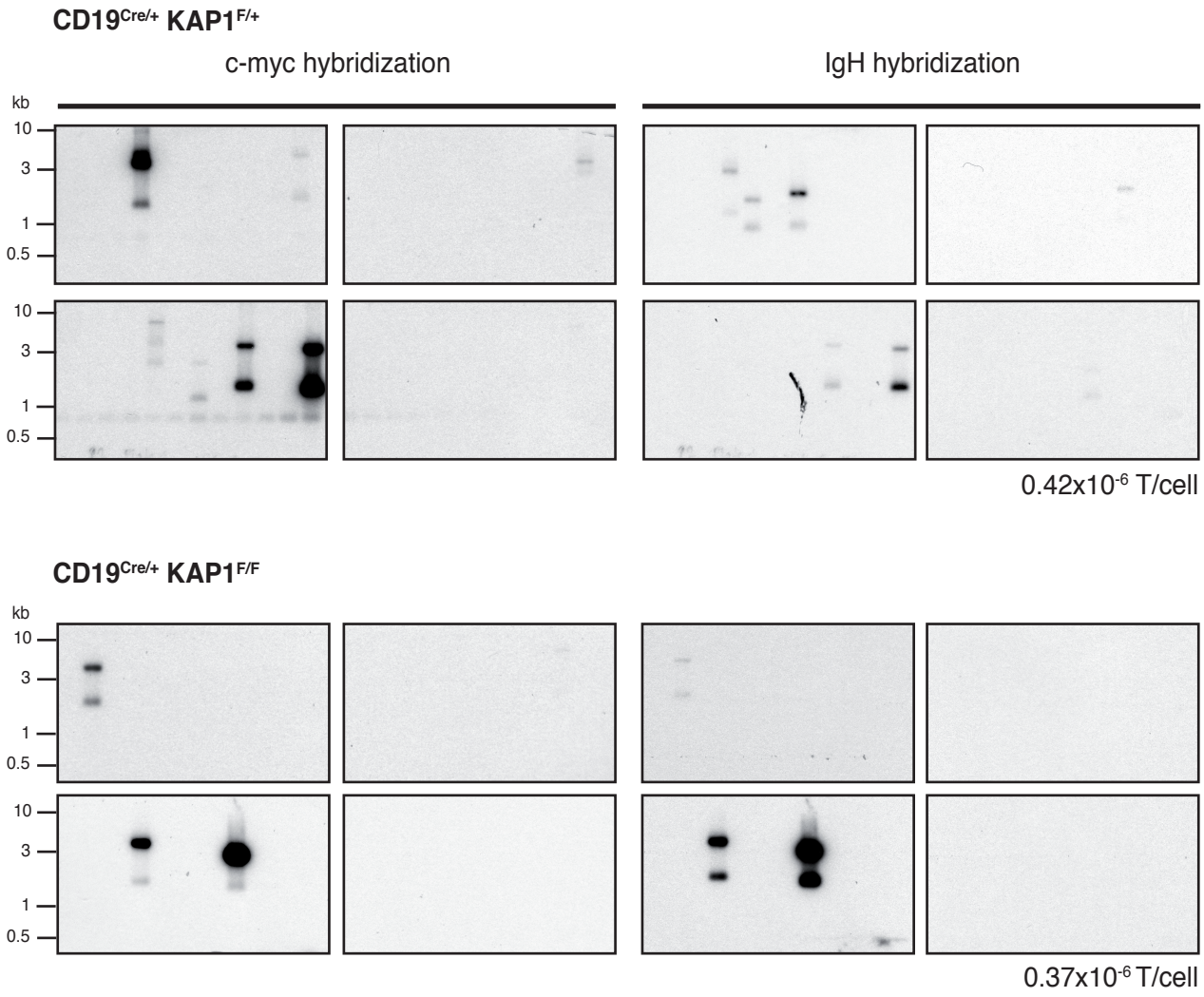
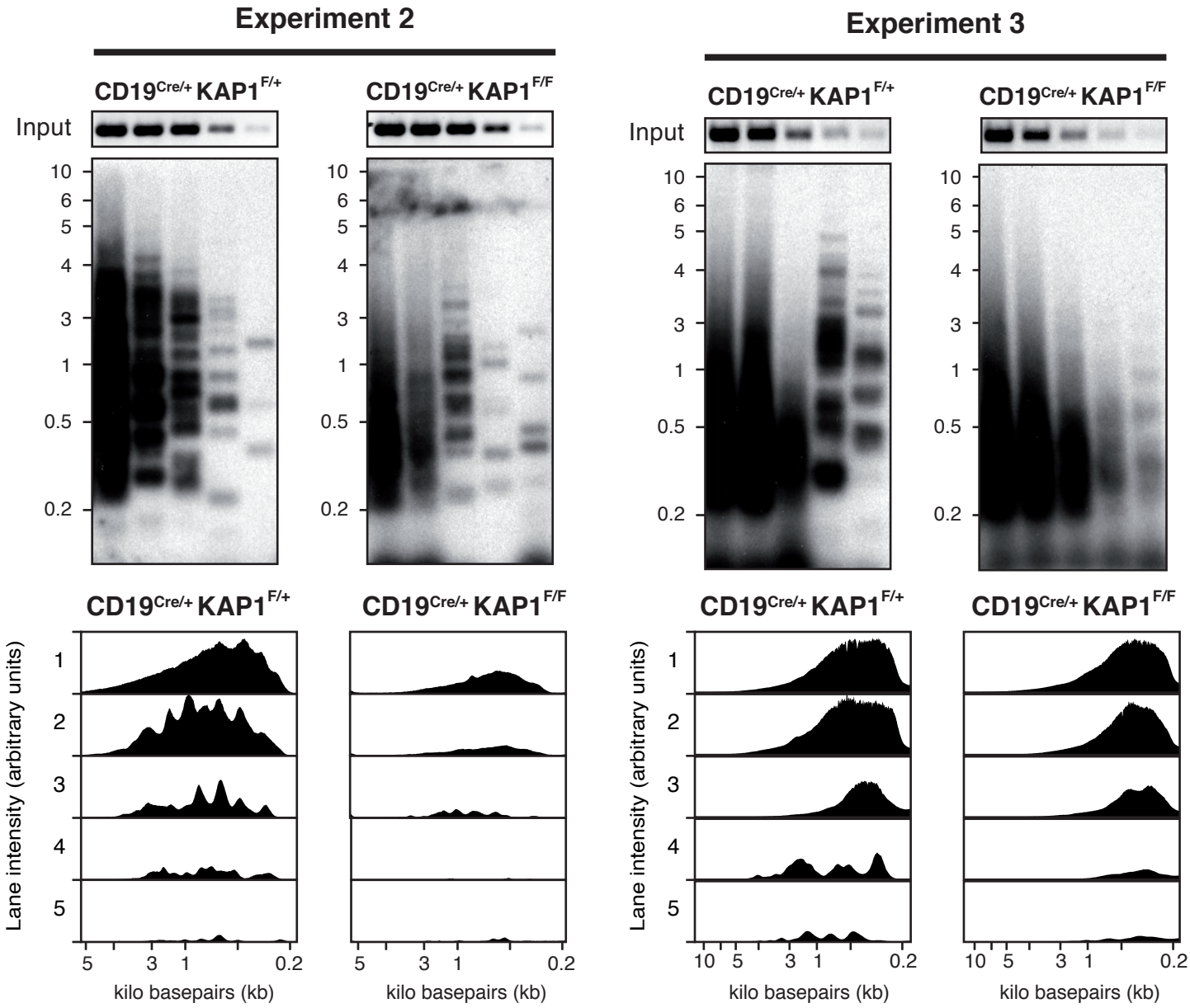
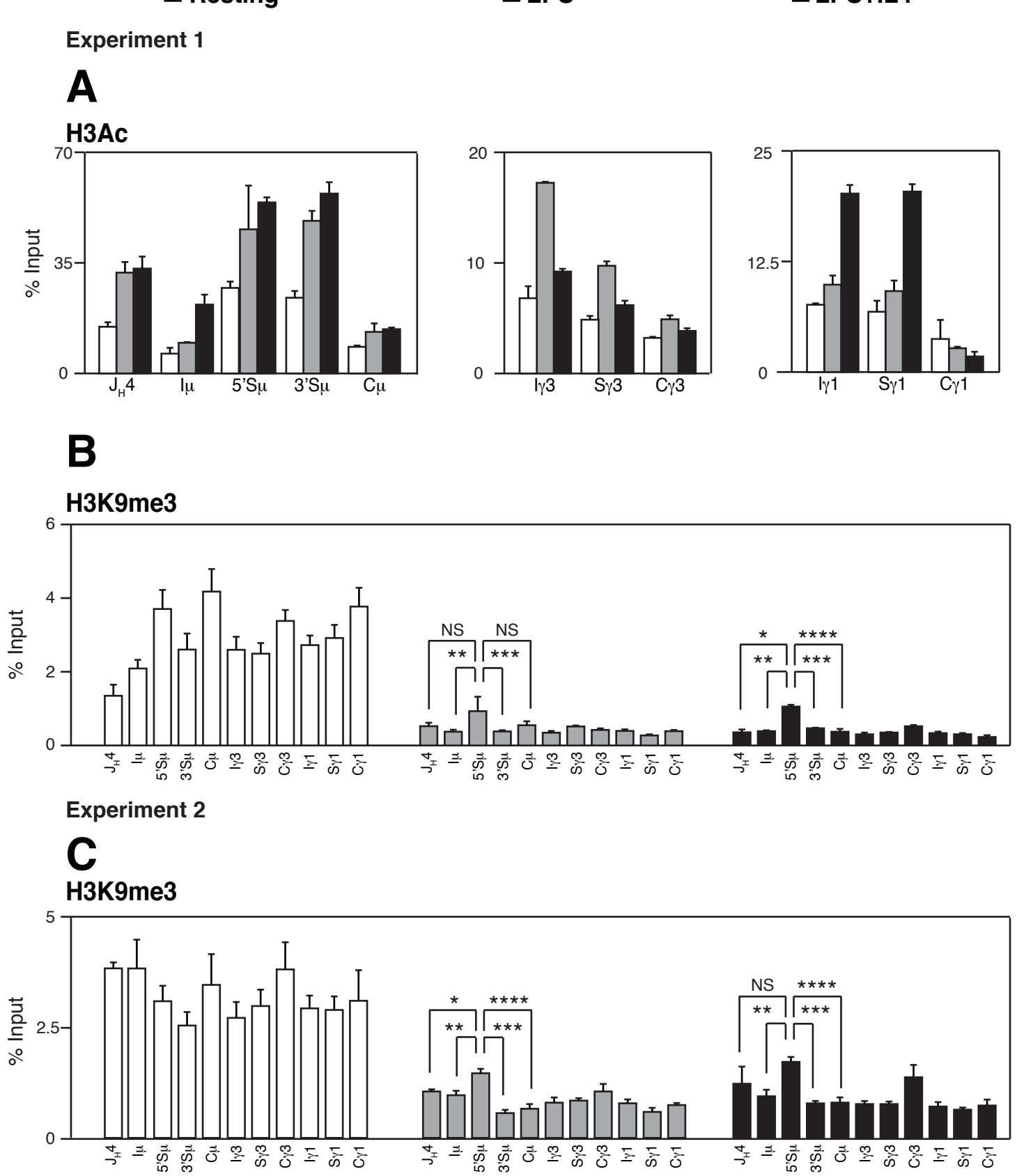

Supplementary Figure 6 (Figure 36): Sμ/Sγ3 switch recombination junctions in CD19$^{Cre/+}$KAP1$^{F/F}$ and control CD19$^{Cre/+}$KAP1$^{F/+}$ B cells. Sμ/Sγ3 switch junction sequences with indicated nucleotide overlap from CD19$^{Cre/+}$KAP1$^{F/+}$ and CD19$^{Cre/+}$KAP1$^{F/F}$ are shown. Overlap was determined by identifying the longest region at the switch junction of perfect uninterrupted donor/acceptor identity. Sequences with insertions at the junction were scored as having no microhomology. The sequenced Sμ/Sγ3 junction sequences are shown in the middle. The Sμ and Sγ3 germline sequences (chromosome 12 genomic sequence: NT-114985) are shown above and below each junction sequence, respectively. Homology at the junctions is boxed and the length of overlap is indicated on the right. Letters in lower-case indicate mutations, insertions are bolded and underligned. Duplicate sequences were discarded. Sequences having identical junctions but differences elsewhere were included.



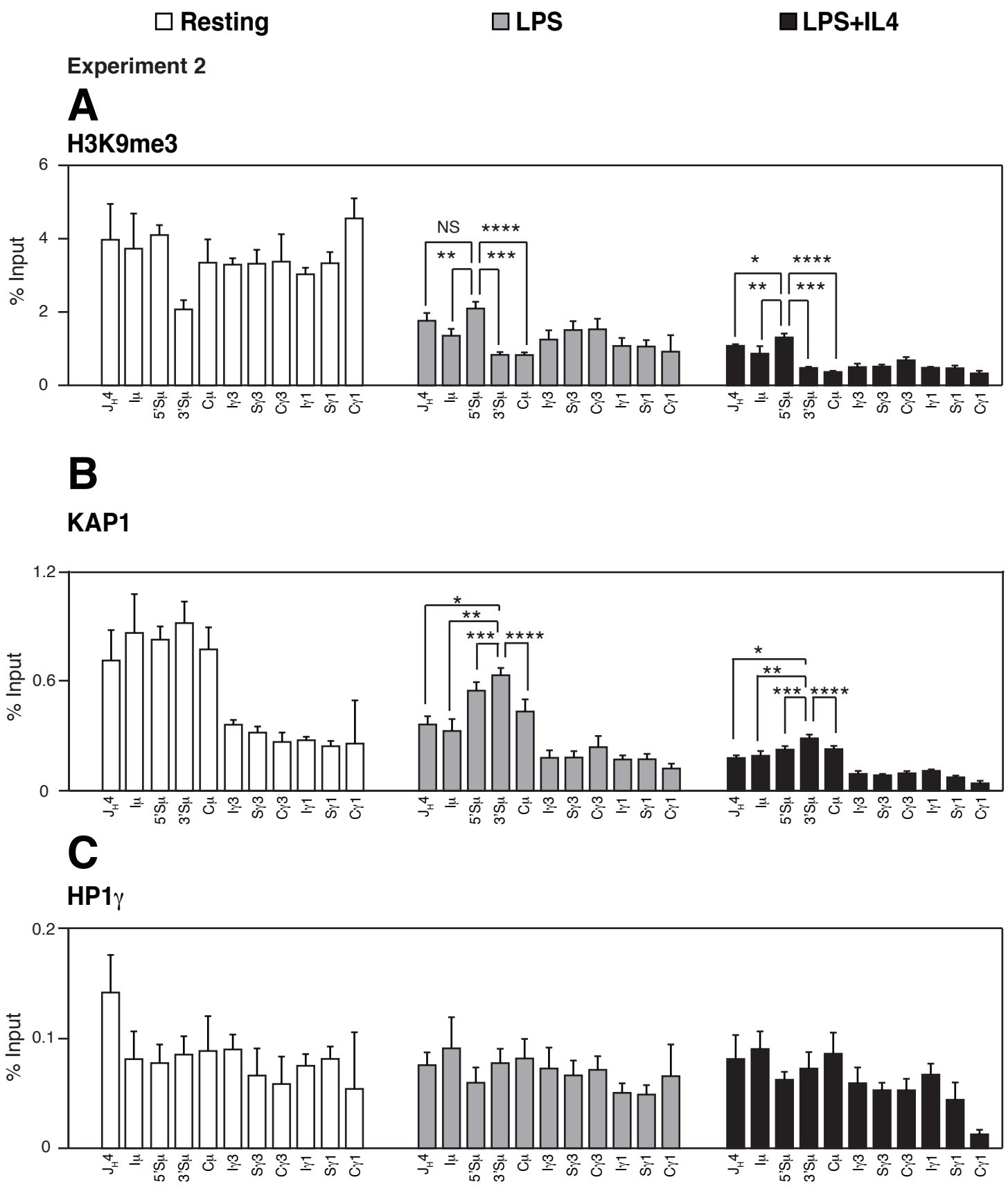
Supplementary Figure 7 (Figure 37): KAP1-deficiency does not result in increased levels of Ig*h*/c-myc translocations. CD19$^{Cre/+}$KAP1$^{F/F}$ and CD19$^{Cre/+}$KAP1$^{F/+}$ splenic B cells were cultured for 3 days with LPS+IL-4 and assayed for Ig*h*/c-myc translocations by long-range PCR. Translocation frequency was determined by Southern blot. Representative Southern blots using c-myc (left) and Ig*h* (right) probes are shown. The number of translocations found (T) with the number of individual PCR assays done (with template DNA corresponding to 105 cells) was: CD19$^{Cre/+}$KAP1$^{F/+}$: T = 7, n = 166 and CD19$^{Cre/+}$KAP1$^{F/F}$: T = 7, n = 190. The corresponding frequency of translocations per cell (T/cell) is indicated underneath each panel and was: CD19$^{Cre/+}$KAP1$^{F/+}$: F = 0.42x10-6 and CD19$^{Cre/+}$KAP1$^{F/F}$: F = 0.37x10-6. Statistical significance was determined using one-tailed exact Fisher's test, p = 0.5038. Molecular weight sizes in kilobasepairs (kb) are indicated on the top panel and are the same for all panels.



Supplementary Figure 8 (Figure 38): KAP1-deficiency results in inefficient DSB formation at switch regions during CSR. Reduced levels of DNA breaks at Sμ in KAP1-deficient B cells. Ligation-mediated PCR was performed on three-fold dilutions of genomic DNA isolated from $CD19^{Cre/+}KAP1^{F/F}$ and $CD19^{Cre/+}KAP1^{F/+}$ splenic B cells cultured for two days with LPS + IL-4. PCR was performed using a primer pair specific for the 5' end of Sμ and the ligated linker. PCR products were blotted and hybridized with an internal Sμ-specific probe. Input genomic DNA was normalized by performing PCR and Southern blotting for *aicda* on the same template dilutions. Two independent experiments are shown. Molecular weights in kilobasepairs (kb) are indicated.



Supplementary Figure 9 (Figure 39): KAP1-deficiency does not alter the pattern of H3K9me3 or H3K9/K14Ac marks at the Ig*h* locus. ChIP performed on chromatin prepared from resting (white bars) or LPS-stimulated (grey bars) and LPS+IL-4-stimulated (black bars) B cells obtained from $CD19^{Cre/+}KAP1^{F/F}$ mice using antibodies specific for (A) H3K9/K14Ac (H3Ac) and (B-C) H3K9me3. Real time quantitative PCR was performed by using primer pairs specific for J_H4, Iμ, Sμ, Cμ, Iγ3, Sγ3, Cγ3, Iγ1, Sγ1 and Cγ1. Fold change over control IgG are expressed as percent of input. Mean (+ s.d) of triplicate samples is shown. p values, One-tailed Student's *t*-test. Experiment 1:H3K9me3, LPS: ** p=0.0320, *** p=0.0326, NS: Not significant; H3K9me3, LPS+IL-4: * p <0.0001, ** p <0.0001, *** p<0.0001, **** p <0.0001. Experiment 2: H3K9me3, LPS: * p=0.0015, ** p <0.0001, *** p=0.0001, **** p=0.0003; H3K9me3, LPS+IL-4: ** p=0.0010, *** p <0.0001, **** p=0.0003. See Supplementary Tables 6 and 8 for detailed statistical analysis and primer sequences respectively. Data are representative of four independent experiments.



Supplementary Figure 10 (Figure 40): H3K9me3 and KAP1 mark the donor switch region (Sμ) before and during CSR. ChIP performed on chromatin prepared from resting (white bars) or LPS-stimulated (grey bars) and LPS+IL-4-stimulated (black bars) B cells obtained from $CD19^{Cre/+}KAP1^{F/+}$ mice using antibodies specific for (A) H3K9me3, (B) KAP1 and (C) HP1-γ. Real time quantitative PCR was performed by using primer pairs specific for J_H4, Iμ, Sμ, Cμ, Iγ3, Sγ3, Cγ3, Iγ1, Sγ1 and Cγ1. Fold change over control IgG are expressed as percent of input. Mean (+ s.d) of triplicate samples is shown. p values, One-tailed Student's *t*-test. H3K9me3, LPS: ** p=0.0041, *** p =0.0002, **** p =0.0002, NS: Not significant; H3K9me3, LPS+IL-4: * p=0.0136, ** p=0.0149, *** p=0.0001, **** p <0.0001; KAP1, LPS: * p=0.0008, ** p=0.0012, *** p=0.0381, **** p=0.0058; KAP1, LPS+IL-4: * p=0.0010, ** p=0.0040, *** p=0.0100, **** p=0.0102. See Supplementary Tables 7 and 8 for detailed statistical analysis and primer sequences respectively. Data are representative of four independent experiments.

Table 1 (Table 4). Two-color Ig*h*-FISH analysis of 4-day anti-CD40/IL4 activated B cells.

Experiment 1

Genotype	Metaphases	Abnormalities	% of IgH abnormalities
$KAP1^{F/+}$	169	5	2.96
$CD19^{Cre/+}$ $KAP1^{F/+}$	128	2	1.56
$KAP1^{F/F}$	161	6	3.73
$KAP1^{F/F}$	172	4	2.33
$CD19^{Cre/+}$ $KAP1^{F/F}$	148	6	4.05
$CD19^{Cre/+}$ $KAP1^{F/F}$	150	6	4.00

Experiment 2

Genotype	Metaphases	Abnormalities	% of IgH abnormalities
$KAP1^{F/F}$	150	5	3.33
$CD19^{Cre/+}$ $KAP1^{F/F}$	150	4	2.67
$CD19^{Cre/+}$ $KAP1^{F/F}$	145	1	0.69

Experiment 1 and 2

Genotype	Metaphases	Abnormalities	% of IgH abnormalities
Controls	780	21	2.69
$CD19^{Cre/+}$ $KAP1^{F/F}$	593	17	2.87

Table 2 (Table 5) – ChIP Statistics (One-tailed Student's *t*-test) – Figure 29

Blue highlight indicates statistical significance.

CD19$^{cre/+}$ KAP1$^{F/+}$

H3Ac

Iμ

	Resting	LPS	LPS+IL4
Resting		<0.0001	0.0024
LPS			0.1587

Iγ3

	Resting	LPS	LPS+IL4
Resting		<0.0001	0.0004
LPS			0.0003

Iγ1

	Resting	LPS	LPS+IL4
Resting		0.0002	<0.0001
LPS			0.0003

5' Sμ

	Resting	LPS	LPS+IL4
Resting		<0.0001	<0.0001
LPS			0.0851

Sγ3

	Resting	LPS	LPS+IL4
Resting		<0.0001	<0.0001
LPS			<0.0001

Sγ1

	Resting	LPS	LPS+IL4
Resting		<0.0001	<0.0001
LPS			<0.0001

3' Sμ

	Resting	LPS	LPS+IL4
Resting		<0.0001	0.0003
LPS			0.0331

Cμ

	Resting	LPS	LPS+IL4
Resting		<0.0001	<0.0001
LPS			0.1003

Cγ3

	Resting	LPS	LPS+IL4
Resting		0.0003	0.0022
LPS			0.0088

Cγ1

	Resting	LPS	LPS+IL4
Resting		0.0607	0.0146
LPS			0.4059

H3K9me3

Resting

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.0247	0.0014	0.0177	0.0047
Iμ		0.0041	0.4208	0.0402
5' Sμ			0.0060	0.0262
3' Sμ				0.0105

	Iγ3	Sγ3	Cγ3
JH4	0.0117	0.0019	0.0007
Iγ3		0.0779	0.0064
Sγ3			0.0062

	Iγ1	Sγ1	Cγ1
JH4	0.0068	0.0025	0.0063
Iγ1		0.1751	0.4510
Sγ1			0.1335

LPS

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.1545	0.0003	0.3496	0.4783
Iμ		<0.0001	0.1922	0.1193
5' Sμ			<0.0001	<0.0001
3' Sμ				0.3358

	Iγ3	Sγ3	Cγ3
JH4	0.3126	0.0246	0.1442
Iγ3		0.0307	0.2280
Sγ3			0.0959

	Iγ1	Sγ1	Cγ1
JH4	0.0464	0.1461	0.0686
Iγ1		0.0998	0.3861
Sγ1			0.1615

LPS + IL4

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.1229	<0.0001	0.0135	0.1051
Iμ		0.0001	0.0381	0.4859
5' Sμ			0.0004	<0.0001
3' Sμ				0.0285

	Iγ3	Sγ3	Cγ3
JH4	0.1611	0.0139	0.3750
Iγ3		0.0056	0.0184
Sγ3			0.0003

	Iγ1	Sγ1	Cγ1
JH4	0.4052	0.0340	0.2333
Iγ1		0.0086	0.2054
Sγ1			0.0666

KAP1

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.0111	0.1130	0.0237	0.0211
Iμ		0.0139	0.1169	0.1748
5' Sμ			0.0601	0.0362
3' Sμ				0.3620

	Iγ3	Sγ3	Cγ3
JH4	0.0693	0.1589	0.0389
Iγ3		0.1871	0.0604
Sγ3			0.0662

	Iγ1	Sγ1	Cγ1
JH4	0.0691	0.1714	0.0629
Iγ1		0.0705	0.4282
Sγ1			0.0565

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.0543	0.0110	0.0040	0.0112
Iμ		0.0383	0.0065	0.0381
5' Sμ			0.0289	0.2344
3' Sμ				0.1628

	Iγ3	Sγ3	Cγ3
JH4	0.1610	0.3125	0.0544
Iγ3		0.0201	0.0044
Sγ3			0.0004

	Iγ1	Sγ1	Cγ1
JH4	0.1087	0.1543	0.1310
Iγ1		0.1234	0.3356
Sγ1			0.2878

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.3200	0.0838	0.0134	0.0159
Iμ		0.1431	0.017	0.0208
5' Sμ			0.0346	0.0465
3' Sμ				0.3472

	Iγ3	Sγ3	Cγ3
JH4	0.0276	0.0148	0.0051
Iγ3		0.4271	0.019
Sγ3			0.0158

	Iγ1	Sγ1	Cγ1
JH4	0.0516	0.0930	0.0297
Iγ1		0.2005	0.2265
Sγ1			0.0754

HP1γ

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.0871	0.0753	0.0935	0.0042
Iμ		0.3742	0.4285	0.0241
5' Sμ			0.4488	0.0453
3' Sμ				0.0389

	Iγ3	Sγ3	Cγ3
JH4	0.0499	0.0326	0.0631
Iγ3		0.1119	0.3920
Sγ3			0.1577

	Iγ1	Sγ1	Cγ1
JH4	0.0076	0.0089	0.0369
Iγ1		0.2438	0.2760
Sγ1			0.1481

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.1160	0.4050	0.2115	0.0327
Iμ		0.2664	0.4884	0.0127
5' Sμ			0.3170	0.0427
3' Sμ				0.0251

	Iγ3	Sγ3	Cγ3
JH4	0.0752	0.2990	0.0115
Iγ3		0.0654	0.0777
Sγ3			0.0164

	Iγ1	Sγ1	Cγ1
JH4	0.2099	0.0845	0.3053
Iγ1		0.3751	0.3486
Sγ1			0.1850

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.1250	0.0331	0.0036	0.0002
Iμ		0.1809	0.0333	0.0005
5' Sμ			0.1517	0.0008
3' Sμ				0.0004

	Iγ3	Sγ3	Cγ3
JH4	0.3684	0.1986	0.0474
Iγ3		0.1542	0.1313
Sγ3			0.0050

	Iγ1	Sγ1	Cγ1
JH4	0.0893	0.0608	0.0447
Iγ1		0.2415	0.1214
Sγ1			0.2280

Table 3 (Table 6) – ChIP Statistics (One-tailed Student's *t*-test) – Figure 39.

Blue highlight indicates statistical significance.

CD19$^{cre/+}$ KAP1$^{F/F}$

Experiment 1

H3Ac

Iμ

	Resting	LPS	LPS+IL4
Resting		0.0163	0.0009
LPS			0.0013

Iγ3

	Resting	LPS	LPS+IL4
Resting		<0.0001	0.0100
LPS			<0.0001

Iγ1

	Resting	LPS	LPS+IL4
Resting		0.0097	<0.0001
LPS			0.0001

5' Sμ

	Resting	LPS	LPS+IL4
Resting		0.0418	<0.0001
LPS			0.1749

Sγ3

	Resting	LPS	LPS+IL4
Resting		<0.0001	0.0063
LPS			0.0002

Sγ1

	Resting	LPS	LPS+IL4
Resting		0.0393	<0.0001
LPS			0.0001

3' Sμ

	Resting	LPS	LPS+IL4
Resting		0.0005	<0.0001
LPS			0.0039

Cμ

	Resting	LPS	LPS+IL4
Resting		0.0196	<0.0001
LPS			0.3071

Cγ3

	Resting	LPS	LPS+IL4
Resting		0.0007	0.0068
LPS			0.0064

Cγ1

	Resting	LPS	LPS+IL4
Resting		0.2249	0.0992
LPS			0.0225

H3K9me3

Resting

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.0138	0.0012	0.0073	0.0010
Iμ		0.0039	0.0733	0.0026
5' Sμ			0.0239	0.1822
3' Sμ				0.0111

	Iγ3	Sγ3	Cγ3
JH4	0.0047	0.0044	0.0006
Iγ3		0.3544	0.0213
Sγ3			0.0103

	Iγ1	Sγ1	Cγ1
JH4	0.0019	0.0021	0.0010
Iγ1		0.2454	0.0170
Sγ1			0.0381

LPS

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.0376	0.0712	0.0345	0.3855
Iμ		0.0320	0.4269	0.0327
5' Sμ			0.0326	0.0829
3' Sμ				0.0307

	Iγ3	Sγ3	Cγ3
JH4	0.0222	0.4503	0.0827
Iγ3		0.0030	0.0548
Sγ3			0.0159

	Iγ1	Sγ1	Cγ1
JH4	0.0489	0.0058	0.0390
Iγ1		0.0077	0.4177
Sγ1			0.0052

LPS +IL4

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.2666	<0.0001	0.0366	0.4064
Iμ		<0.0001	0.0041	0.3741
5' Sμ			<0.0001	<0.0001
3' Sμ				0.0526

	Iγ3	Sγ3	Cγ3
JH4	0.1831	0.4577	0.0143
Iγ3		0.0848	0.0014
Sγ3			0.0007

	Iγ1	Sγ1	Cγ1
JH4	0.3448	0.1745	0.0376
Iγ1		0.1917	0.0235
Sγ1			0.0450

Experiment 2

H3K9me3

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.4976	0.0131	0.0013	0.2066
Iμ		0.0788	0.0180	0.2686
5' Sμ			0.0544	0.2286
3' Sμ				0.0521

	Iγ3	Sγ3	Cγ3
JH4	0.0036	0.0101	0.4758
Iγ3		0.2098	0.0279
Sγ3			0.058

	Iγ1	Sγ1	Cγ1
JH4	0.0040	0.0041	0.0730
Iγ1		0.4478	0.3569
Sγ1			0.3301

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	<0.0001	0.0015	0.0003	0.0022
Iμ		<0.0001	0.0015	0.0015
5' Sμ			0.0001	0.0003
3' Sμ				0.1292

	Iγ3	Sγ3	Cγ3
JH4	0.0133	0.0042	0.4963
Iγ3		0.2854	0.0508
Sγ3			0.0581

	Iγ1	Sγ1	Cγ1
JH4	0.0051	0.0008	0.0007
Iγ1		0.0286	0.2626
Sγ1			0.0298

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.1514	0.0507	0.0610	0.0716
Iμ		0.0010	0.0779	0.1316
5' Sμ			<0.0001	0.0003
3' Sμ				0.4161

	Iγ3	Sγ3	Cγ3
JH4	0.0569	0.0562	0.3116
Iγ3		0.4964	0.0109
Sγ3			0.0106

	Iγ1	Sγ1	Cγ1
JH4	0.0450	0.0303	0.0534
Iγ1		0.1692	0.4107
Sγ1			0.1604

Table 4 (Table 7) – ChIP Statistics (One-tailed Student's *t*-test) –Figure 40.

Blue highlight indicates statistical significance.

CD19$^{cre/+}$ KAP1$^{F/+}$

H3K9me3

Resting

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.3451	0.4182	0.0156	0.1654
Iμ		0.0440	0.0002	0.1807
5' Sμ			0.0003	0.0658
3' Sμ				0.0161

	Iγ3	Sγ3	Cγ3
JH4	0.1511	0.1706	0.2241
Iγ3		0.4612	0.4321
Sγ3			0.4568

	Iγ1	Sγ1	Cγ1
JH4	0.0880	0.1698	0.2094
Iγ1		0.1065	0.0051
Sγ1			0.0139

LPS

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.0332	0.0544	0.0010	0.0010
Iμ		0.0041	0.0056	0.0053
5' Sμ			0.0002	0.0002
3' Sμ				0.4728

	Iγ3	Sγ3	Cγ3
JH4	0.0271	0.1212	0.1606
Iγ3		0.1343	0.1369
Sγ3			0.4638

	Iγ1	Sγ1	Cγ1
JH4	0.0086	0.0057	0.0212
Iγ1		0.4643	0.3092
Sγ1			0.3206

LPS + IL4

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.0760	0.0136	<0.0001	<0.0001
Iμ		0.0149	0.0157	0.0070
5' Sμ			0.0001	<0.0001
3' Sμ				0.0067

	Iγ3	Sγ3	Cγ3
JH4	0.0003	<0.0001	0.0011
Iγ3		0.4160	0.0334
Sγ3			0.0228

	Iγ1	Sγ1	Cγ1
JH4	<0.0001	0.0001	<0.0001
Iγ1		0.3971	0.0125
Sγ1			0.0384

KAP1

Resting

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.1938	0.1686	0.0783	0.3177
Iμ		0.3954	0.3598	0.2790
5' Sμ			0.1593	0.2739
3' Sμ				0.1065

	Iγ3	Sγ3	Cγ3
JH4	0.0112	0.0079	0.0057
Iγ3		0.0764	0.0239
Sγ3			0.1115

	Iγ1	Sγ1	Cγ1
JH4	0.0054	0.0043	0.0047
Iγ1		0.0879	0.4499
Sγ1			0.4610

LPS

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.2412	0.0039	0.0008	0.1013
Iμ		0.0044	0.0012	0.0598
5' Sμ			0.0381	0.0360
3' Sμ				0.0058

	Iγ3	Sγ3	Cγ3
JH4	0.0033	0.0027	0.0237
Iγ3		0.4819	0.1215
Sγ3			0.1179

	Iγ1	Sγ1	Cγ1
JH4	0.0014	0.0018	0.0007
Iγ1		0.4788	0.0350
Sγ1			0.0456

LPS + IL4

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.2584	0.0153	0.0010	0.0110
Iμ		0.0739	0.0040	0.0592
5' Sμ			0.0100	0.4357
3' Sμ				0.0102

	Iγ3	Sγ3	Cγ3
JH4	0.0012	0.0003	0.0008
Iγ3		0.2526	0.3988
Sγ3			0.1302

	Iγ1	Sγ1	Cγ1
JH4	0.0010	0.0002	0.0002
Iγ1		0.0039	0.0009
Sγ1			0.0140

HP1γ

Resting

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.0347	0.0219	0.0312	0.0602
Iμ		0.4236	0.4129	0.3839
5' Sμ			0.3023	0.3122
3' Sμ				0.4406

	Iγ3	Sγ3	Cγ3
JH4	0.0360	0.0182	0.0137
Iγ3		0.1092	0.0640
Sγ3			0.3616

	Iγ1	Sγ1	Cγ1
JH4	0.0163	0.0221	0.0351
Iγ1		0.2623	0.2626
Sγ1			0.2102

LPS

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.2176	0.1004	0.4297	0.3223
Iμ		0.0803	0.2488	0.3291
5' Sμ			0.0893	0.0824
3' Sμ				0.3788

	Iγ3	Sγ3	Cγ3
JH4	0.4138	0.2084	0.3450
Iγ3		0.3326	0.4658
Sγ3			0.3273

	Iγ1	Sγ1	Cγ1
JH4	0.0198	0.0164	0.3034
Iγ1		0.4211	0.2155
Sγ1			0.1952

LPS + IL4

	Iμ	5' Sμ	3' Sμ	Cμ
JH4	0.2923	0.1086	0.2963	0.3927
Iμ		0.0253	0.1163	0.3901
5' Sμ			0.1741	0.0577
3' Sμ				0.1955

	Iγ3	Sγ3	Cγ3
JH4	0.1051	0.0457	0.0527
Iγ3		0.2612	0.2824
Sγ3			0.5000

	Iγ1	Sγ1	Cγ1
JH4	0.1765	0.0359	0.0027
Iγ1		0.0506	0.0005
Sγ1			0.0146

Table 5 (Table 8): Primers Used

Primer	Sequence (5'-3')
SHM	
VH588/FR3-Fwd	GGAATTCGCCTGACATCTGAGGACTCTGC (Jolly et al., 1997)
JH4 Intron-Rev	GACTTTTGCAGGCTCCACCAGACC (Jolly et al., 1997)
JH4 Probe	TATGCTATGGACTACTGG (Jolly et al., 1997)
JH3 Probe	CCTGGTTTGCTTACTGG (Jolly et al., 1997)
GLTs	
CD79b-Fwd	CCACACTGGTGCTGTCTTCC (Park et al., 2009)
CD79b-Rev	GGGCTTCCTTGGAAATTCAG (Park et al., 2009)
Iμ-Cμ-Fwd	ACCTGGGAATGTATGGTTGTGGCTT (Park et al., 2009)
Iμ-Cμ-Rev	TCTGAACCTTCAAGGATGCTCTTG (Park et al., 2009)
Iγ3-Cγ3-Fwd	AACTACTGCTACCACCACCACCAG (Park et al., 2009)
Iγ3-Cγ3-Rev	ACCAAGGGATAGACAGATGGGG (Park et al., 2009)
Iγ1-Cγ1-Fwd	GGCCCTTCCAGATCTTTGAG (Park et al., 2009)
Iγ1-Cγ1-Rev	ATGGAGTTAGTTTGGGCAGCA (Park et al., 2009)
Iγ2b-Fwd	CCAACCAGGAAGAGTCCAGAG (Sellars et al., 2009)
Cγ2b-Rev	ACAGGGATCCAGAGTTCCAAGT (Sellars et al., 2009)
Iγ2a-Cγ2a-Fwd	GCTGATGTACCTACCGAGAGA (Park et al., 2009)
Iγ2a-Cγ2a-Rev	GCTGGGCCAGGTGCTCGAGGTT (Park et al., 2009)
Iε-Cε-Fwd	ACTAGAGATTCACAACG (Park et al., 2009)
Iε-Cε-Rev	AGCGATGAATGGAGTAGC (Park et al., 2009)
PSTs	
Iμ-Cγ3-Fwd (Iμ2)	CTCGGTGGCTTTGAAGGAAC (Park et al., 2009)
Iμ-Cγ3-Rev	ACCAAGGGATAGACAGATGGGG (Park et al., 2009)
Iμ-Cμ-Fwd (Iμ1)	ACCTGGGAATGTATGGTTGTGGCTT (Park et al., 2009)
Iμ-Cγ1-Rev	ATGGAGTTAGTTTGGGCAGCA (Park et al., 2009)
Iμ-Cμ-Fwd (Iμ1)	ACCTGGGAATGTATGGTTGTGGCTT (Park et al., 2009)
Iμ-Cγ2b-Rev	CGGAGGAACCAGTTGTATC (Park et al., 2009)
Iμ-Cμ-Fwd (Iμ1)	ACCTGGGAATGTATGGTTGTGGCTT (Park et al., 2009)
Iμ-Cγ2a-Rev	GCTGGGCCAGGTGCTCGAGGTT (Park et al., 2009)
Iμ-Cγ3-Fwd (Iμ2)	CTCGGTGGCTTTGAAGGAAC (Park et al., 2009)
Iμ-Cμ-Rev	AGCGATGAATGGAGTAGC (Park et al., 2009)
Switch Junctions	
μ3H3	AACAAGCTTGGCTTAACCGAGATGAGCC (Schrader et al., 2002)
g3-2	AACAAGCTTACCCTGACCCAGGAGCTGCATAAC (Schrader et al., 2002)
LM-PCR	
LM-PCR.1	GCGGTGACCCGGGAGATCTGAATTC (Guikema et al., 2007)
LM-PCR.2	GAATTCAGATC (Guikema et al., 2007)
5' Sμ-Fwd	GCAGAAAATTTAGATAAAATGGATACCTCAGTGG (Guikema et al., 2007)
5' Sμ Probe	AGGGACCCAGGCTAAGAAGGCAAT (Guikema et al., 2007)
AID^{cre} 2	CACTCGTTGCATCGACCGGTAATG (Robbiani et al., 2008)
AID^{cre} 3	CCTCTAAGGCTTCGCTGTTATTACCAC (Robbiani et al., 2008)
AID Probe	ACGCTGGAGACCGATATGGACAG*
Sμ Sequencing	
5μ3	ATCGAATTCAATGGATACCTCAGTGGTTTTTAATGGTGGGTTTA (Reina-San-Martin et al., 2003)

Table 8

continued:

SμR5	GCGGCCCGGCTCATTCCAGTTCATTACAG (Reina-San-Martin et al., 2003)
ChIP	
J_H4-Fwd	AGGGACTTTGGAGGCTCATT*
J_H4-Rev	GCGGAACATTCCTCACAAAT*
Iμ Exon-Fwd	GGTCTCCATTCAATTCTTTTCCAATACC (Kuang et al., 2009)
Iμ Exon-Rev	ACCAACCAGCATGTTCAACCGAA (Kuang et al., 2009)
5' Sμ-Fwd	TAAAATGCGCTAAACTGAGGTGATTACT (Kuang et al., 2009)
5' Sμ-Rev	CATCTCAGCTCAGAACAGTCCAGTG (Kuang et al., 2009)
3' Sμ-Fwd	CTGAATGAGTTTCACCAGGCC (Wang et al., 2006)
3' Sμ-Rev	GCCTGTCCTGCTTGGCTTC (Wang et al., 2006)
Cμ-Rev	CATACACAGAGCAACTGGACACCC*
Iγ3 Exon-Fwd	AGATCCCAAAGCTAAGCTCCTG (Wang et al., 2006)
Iγ3 Exon-Rev	CAGAGAGACCCCCTCCACAGT (Wang et al., 2006)
Sγ3-Fwd	GCTGAGAGTATGCACAGCCA (Wang et al., 2006)
Sγ3-Rev	GGATCATGGAAACTCCTCCG (Wang et al., 2006)
Cγ3-Fwd	CCATACCCCCACCTCGTGAACA (Wang et al., 2006)
Cγ3-Rev	GGTTCTTCTGTGTGTGGTGGTTATGG (Wang et al., 2006)
Iγ1 Exon-Fwd	CCCACTGTCAATCCTGTTCTTAGTCAA (Kuang et al., 2009)
Iγ1 Exon-Rev	CTCCCGTGATAGGATGACTCAAAGAT (Kuang et al., 2009)
Sγ1-Fwd	GGAGGTCCAGTTGAGTGTCTTTAG (Li Z, 2004)
Sγ1-Rev	TTGTTATCCCCCATCCTGTCACCT (Li Z, 2004)
Cγ1-Fwd	GCACACAGCTCAGACGCAACC (Wang et al., 2006)
Cγ1-Rev	TGGTCAGCACAGAGGTCACGGA (Wang et al., 2006)
Translocations	
Igμ 3'	TGAGGACCAGAGAGGGATAAAAGAGAA (Ramiro et al., 2004)
c-Myc 3'	GGGGAGGGGGTGTCAAATAATAAGA (Ramiro et al., 2004)
Igμ 3'nested	CACCCTGCTATTTCCTTGTTGCTAC (Ramiro et al., 2004)
c-Myc 3'nested	GACACCTCCCTTCTACACTCTAAACCG (Ramiro et al., 2004)
derChr12 c-Myc Probe	GCAGCGATTCAGCACTGGGTGCAGG (Ramiro et al., 2004)
derChr12 Igh Probe	CCTGGTATACAGGACGAAACTGCAGCAG (Ramiro et al., 2004)
AID^{cre} 1	GGACCCAACCCAGGAGGCAGATGT (Robbiani et al., 2008)
AID^{cre} 3	CCTCTAAGGCTTCGCTGTTATTACCAC (Robbiani et al., 2008)
HTS Amplicon Sequencing-Fwd	ADAPTOR TAG
A1-5μ3	CCATCTCATCCCTGCGTGTCTCCGACTCAG**aaca**AATGGATACCTCAGTGGTTTTTAATGGTGGGTTTA*
A2-5μ3	CCATCTCATCCCTGCGTGTCTCCGACTCAG**tacg**AATGGATACCTCAGTGGTTTTTAATGGTGGGTTTA*
A5-5μ3	CCATCTCATCCCTGCGTGTCTCCGACTCAG**ttgt**AATGGATACCTCAGTGGTTTTTAATGGTGGGTTTA*
HTS Amplicon Sequencing-Rev	
B1-SμR2-Rev	CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGCCATGCTTTTTAGATCCTCGCTTAC*

Primers designed in this study are marked with an *

Table 6 (Table 9): Antibodies Used.

Antibody	Clone	Source
IP and WB		
anti Flag-HRP	M2	Sigma
anti AID	Strasbg 9	IGBMC
anti KAP1	1TB 1A9	IGBMC
anti γ-KAP1	n.a	Bethyl Labs
anti HP1-α	2HP 2G9	IGBMC
anti HP1-β	1MOD 1A9	IGBMC
anti HP1-γ	2 MOD 1G6	IGBMC
anti β-actin	AC 15	Sigma
Flow Cytometry		
anti IgM-PE	n.a	Jackson ImmunoResearch
anti IgM-Cy5	n.a	Jackson ImmunoResearch
anti CD21-Biotin	7G6	IGBMC
anti B220-Cy5	RA3-6B2	IGBMC
anti B220-FITC	RA3-6B2	Caltag
anti CD43-PE	S7	BD Pharmingen
anti CD25-Biotin	7D4	BD Pharmingen
anti IgG1-Biotin	A 85-1	BD Pharmingen
anti IgG2a-Biotin	Igh-1b 5.7	BD Pharmingen
anti IgG2b-Biotin	RMG2B1	Biolegend
anti IgG3-Biotin	R40-82	BD Pharmingen
anti CD95-PE	Jo2	BD Pharmingen
anti GL7-FITC	GL7	BD Pharmingen
ChIP		
anti histone H3	n.a	Abcam ab1791
anti tri-methyl H3 (K9)	n.a	Abcam ab8898
anti acetyl H3 (K9,K14)	n.a	Upstate-Millipore 06-599
anti KAP1	PF 64	IGBMC
anti HP1-γ	2 MOD 1G6	IGBMC
anti rabbit IgG	n.a	Bethyl Labs
anti mouse IgG	n.a	IGBMC

III: Complementary results

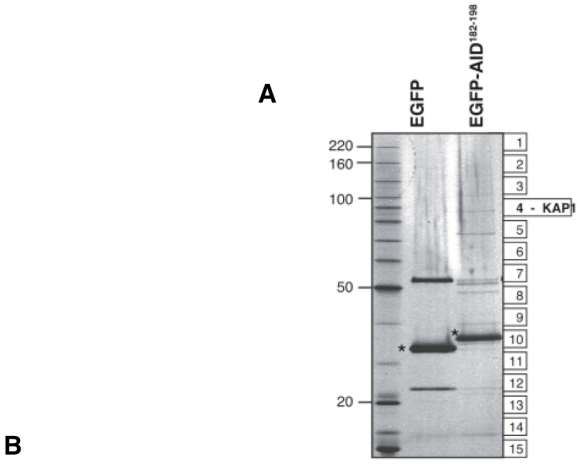
We have shown that KAP1 interacts with AID and that AID forms a complex with HP1 and KAP1 that binds H3K9me3 *in vitro*. Furthermore, we show that the conditional inactivation of KAP1 results in a B cell intrinsic CSR-specific defect and that the *in vivo* association between KAP1 and HP1 is required for efficient CSR.
This section elaborates on these findings and includes experiments aimed at further supporting the data presented in the previous section. It is broadly drawn into two tangents:

> one is aimed at understanding better the interaction between KAP1 and AID.

> the second is directed towards explaining better the partial CSR phenotype in $CD19^{Cre/+}$ $KAP1^{F/F}$ B cells.

1. KAP1 associates with the C-terminus of AID

To identify nuclear proteins associating with AID through its C-terminus, we performed sequential immunoprecipitations and identification by mass spectrometry, using extracts prepared from B cell lines expressing tagged EGFP fused to the C-terminus of AID (EGFP-AID$^{182-198}$) or EGFP alone. Among the identified proteins (Figure 41), we found known AID partners and also identified KAP1. KAP1 was selected for analysis (as discussed before). Association between AID and KAP1 was confirmed by reciprocal immunoprecipitations and western blotting (Figure 1, [Figure 24]). Although KAP1 was precipitated by the C-terminal domain of AID (Figure 41), we found that AID C-terminal deletions did not disrupt the association between AID and KAP1 (Figure 1C, [Figure 24C]). We conclude that endogenous KAP1 associates with tagged AID through interactions that involves but are not restricted to its C-terminus.



B

KAP1
Transcription intermediary factor 1beta OS=Mus musculus GN=Trim28 PE=1 SV=3 - [TIF1B_MOUSE] Q62318)

Position	Peptide Sequence	Activation type	Xcorr	z	MH$^+$(Da)
129-142	DIVENYFMRDSGSK	CID	1.69	2	1660.92
409-428	IVAERPGTNSTGPGPMAPPR	CID	3.18	3	2006,29
751-770	LSPPYSSPQEFAQDVGRMFK	CID	1.67	2	2284.14

Figure 41: KAP1 associates with the C-terminus of AID. (A) Silver stain, after Flag and HA immunoprecipitations on nuclear extracts from CH12 cells expressing EGFP or EGFP$^{AID182-198}$. Approximate locations of gel slices submitted to mass spectrometry analysis are indicated. KAP1 was identified in bands 4 and 5 depending on the experiment. *, positions of the baits. **(B)** The peptides identified by mass spectrometry from EGFP$^{AID182-198}$ are indicated, corresponding peptide position, sequence, fragmentation method (CID or ETD), cross correlation score of the identification by Sequest (Xcorr), charge state of the precursor (z) and corresponding monocharged (MH+) ion are shown.

2. KAP1 does not interact directly with AID by yeast-two hybrid

Having identified KAP1 as a protein that associates with AID by affinity purification and mass spectrometry experiments, CoIPs, as well as gel filtration, we used yeast two-hybrid

experiments to determine if this interaction was direct. In this system, we used a genetically engineered strain of yeast (L40a) and expressed AID fused either to the C-terminus of the activation domain of the herpes virus protein VP16, or the DNA binding domain of the bacterial repressor LexA. VP16-AID or LexA-AID and KAP1 (again fused to either LexA or VP16) were used to analyze their interactions in yeast. As a positive control for transactivation of the β-galactosidase reporter we co-expressed LexA-RFC3 with VP16-RFC4, while cells expressing the empty plasmids alone served as negative controls. In addition, we expressed VP16-AID with LexA-RPA2, as a positive control for AID because RPA2 is a known interacting partner of AID (Chaudhuri and Alt, 2004) and LexA-KAP1 with VP16-HP1α as a positive control for KAP1 (Le Douarin et al., 2001).

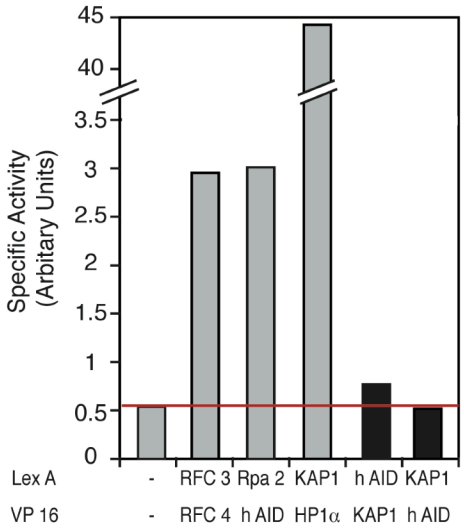


Figure 42: KAP1 does not interact with AID in yeast. Yeast L40a cells were transformed with vectors expressing the indicated fusion proteins. Bar graph represents specific activity of β-galactosidase (liquid assays). Red line indicates the level of background of specific activity from cells expressing the empty vectors alone. Grey bars indicate positive controls, and black bars indicate specific activity of LexA-hAID with VP16-KAP1 or VP16-hAID with LexA-KAP1.

We performed liquid β-galactosidase reporter assays (Figure 42), and as expected, we found that KAP1 and HP1α as well as AID and RPA2 interact directly with each other (Figure 42), thus indicating that the systems as well as the fusion proteins were functional. However, cells co-expressing either VP16-AID and LexA-KAP1 or LexA-AID and VP16-KAP1 did not show any specific activity above background levels. We thus concluded, that AID and KAP1 do not interact directly in yeast.

3. Deletion of Tif1α does not affect CSR

We have shown, that the inactivation of KAP1 in B cells results in a 50% reduction in CSR to all isotypes tested. This partial reduction in CSR upon deletion of KAP1 could be due to functional

redundancy within members of the Tif family of proteins. To address this question we analyzed CSR efficiency in B cells from Tif1$\alpha^{-/-}$ and age matched littermate controls. Resting B cells were purified, stained with CFSE to track proliferation, cultured *in vitro* with LPS, LPS+IL-4 or LPS+INFγ and 72 hours later assayed for IgG surface expression by FACS. We found that Tif1$\alpha^{-/-}$ B cells proliferate as efficiently as B cells from controls and that there is no significant difference in Tif1$\alpha^{-/-}$ B cells to undergo CSR to all the isotypes tested, relative to the controls (Figure 43). This suggests, that Tif1α is not required for CSR and that the CSR defect in KAP1-deficient B cells is due to the deletion of KAP1 alone.

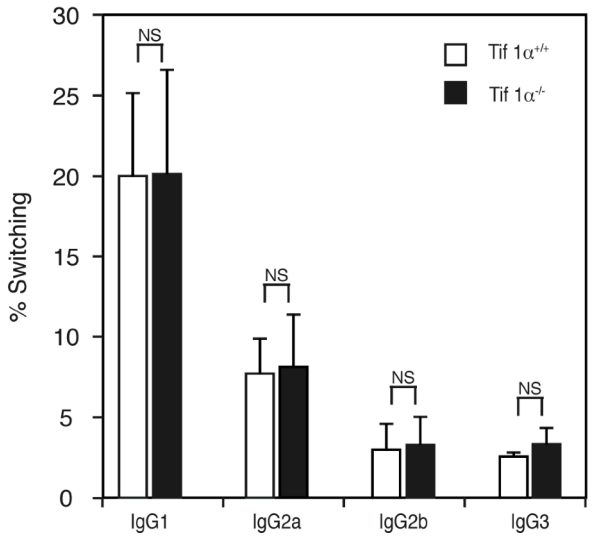


Figure 43: Deletion of Tif1α does not affect CSR. Resting splenic B cells were stained with CFSE and cultured for 3 days with LPS (CSR to IgG3 and IgG2b); LPS+IL-4 (CSR to IgG1); LPS+IFN-γ (CSR to IgG2a). Immunoglobulin surface expression was determined by flow cytometry within the live cell population (gated using ToPro-3 exclusion) in 2 independent experiments, with 3 mice per genotype in each experiment. Cell division was measured by CFSE dye dilution. Histogram indicates the percentage (+ s.d.) of CSR in Tif1$\alpha^{-/-}$ (black bars) relative to Tif1$\alpha^{+/+}$ (white bars) for the different isotypes tested. Statistical significance Student's *t*-test, NS – not significant.

4. HP1α deficiency does not alter CSR

We have demonstrated that KAP1 and AID$^{Flag\text{-}HA}$ are specifically precipitated *in vitro*, along with all HP1 isoforms by an H3K9me3 peptide. We have also shown, that HP1γ reciprocally co-immunoprecipitates with AID$^{Flag\text{-}HA}$ in the CH12 B cell line and is present at the Ig*h* locus in B cells before and during CSR. Furthermore, the integrity of the HP1BOX of KAP1 is essential for efficient CSR.

In order to determine if other members of the heterochromatin protein family were required for or directly involved in CSR, we analyzed the efficiency of HP1$\alpha^{-/-}$ B cells to undergo CSR *in vitro*. As described previously, resting B cells were purified, stained with CFSE, cultured *in vitro* and analyzed 72 hours later for IgG surface expression. In two independent experiments we

found that HP1$\alpha^{-/-}$ B cells proliferate as efficiently as B cells from control mice and underwent efficient CSR to all isotypes tested (Figure 44), suggesting that B cells from HP1α-deficient mice have no intrinsic defect in CSR and that indeed HP1α is not required for CSR.

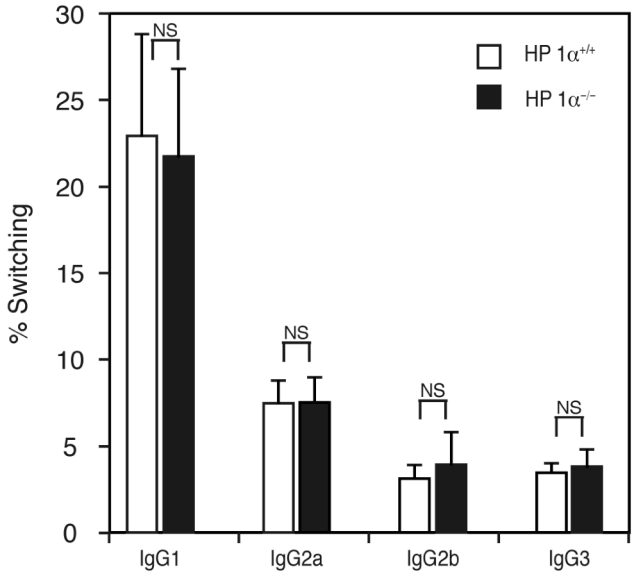


Figure 44: HP1α deficiency does not alter CSR. Resting splenic B cells were stained with CFSE and cultured for 3 days with LPS (CSR to IgG3 and IgG2b); LPS+IL-4 (CSR to IgG1); LPS+IFN-γ (CSR to IgG2a). Immunoglobulin surface expression was determined by flow cytometry within the live cell population (gated using ToPro-3 exclusion) in 2 independent experiments, with 3 mice per genotype in each experiment. Cell division was measured by CFSE dye dilution. Histogram indicates the percentage (+ s.d.) of CSR in HP1$\alpha^{-/-}$ (black bars) relative to HP1$\alpha^{+/+}$ (white bars) for the different isotypes tested. Statistical significance Student's *t*-test, NS- not significant.

5. γH2AX at the Ig*h* locus does not correlate with DSB induction in KAP1-deficient B cells

To determine if KAP1-deficiency results in reduced levels of DSBs, we performed chromatin immunoprecipitation experiments using a γH2AX-specific antibody. We prepared chromatin from resting or *in vitro* stimulated (with LPS or LPS+IL-4) CD19$^{Cre/+}$ KAP1$^{F/F}$, or control B cells, and γH2AX enrichment at Sμ was evaluated by ChIP-qPCR. Following 48 hrs of stimulation, γH2AX was enriched across Sμ in both KAP1-deficient and WT cells (Figure 45), indicating that *Igh* locus specific, AID-induced DNA damage could be detected. However, we found that γH2AX levels were similar across Sμ after stimulation, and did not show any statistically significant differences between CD19$^{Cre/+}$ KAP1$^{F/F}$ and control B cells. We concluded, that analysis of γH2AX levels was not the optimal method to detect a decrease in DSB induction.

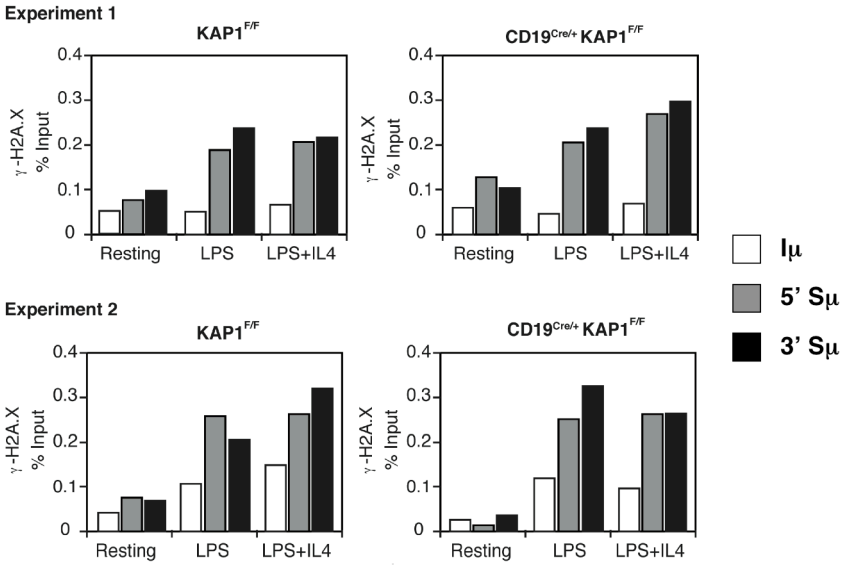


Figure 45: γH2AX at the Ig*h* locus does not correlate with DSB induction in KAP1-deficient B cells. ChIP from resting, LPS or LPS+IL-4 stimulated B cells from KAP1$^{F/F}$ and CD19$^{Cre/+}$ KAP1$^{F/F}$ mice, using an antibody specific for γH2AX. Real time quantitative PCR was performed using primers specific for the 5' ends of Iμ (white bars), and either the 5' or 3' ends of Sμ (grey and black bars respectively). Results were normalized to control IgG and are expressed as percent of input. Data for two individual experiments are shown.

6. Overexpression of KAP1^{S824A} but not KAP1$^{V488A-L490A}$ rescues the CSR defect in KAP1-deficient B cells

To determine if the ATM-dependent phosphorylation of KAP1 and/or an intact HP1 interaction domain (HP1BOX) was required for CSR, we used a retroviral system and transduced *in vitro* stimulated CD19$^{Cre/+}$KAP1$^{F/F}$ B cells to express several KAP1 retroviral constructs: an epitope tagged wild type KAP1 protein (Flag-KAP1), a serine 824 to alanine mutant (Flag-KAP1^{S824A}, that cannot be phosphorylated by ATM) or a mutant protein bearing mutations in the HP1BOX (Flag-KAP1$^{V488A-L490A}$), and empty vector. Resting B cells were purified, cultured *in vitro* with LPS+IL-4, transduced 24 hours post stimulation and analyzed 48 hours later for IgG1 surface expression by FACS. We found, that retroviral over-expression of full-length KAP1 (Flag-KAP1) in KAP1-deficient B cells rescued the CSR defect (Figure 46 B), whereas a mutant that is unable to bind HP1 proteins (Flag-KAP1$^{V488A-L490A}$, Figure 46 C) did not. Interestingly, we also found that the phospho-mutant of KAP1 - Flag-KAP1^{S824A} behaves like a WT protein and rescues the CSR defect in KAP1-deficient B cells (Figure 46 D).

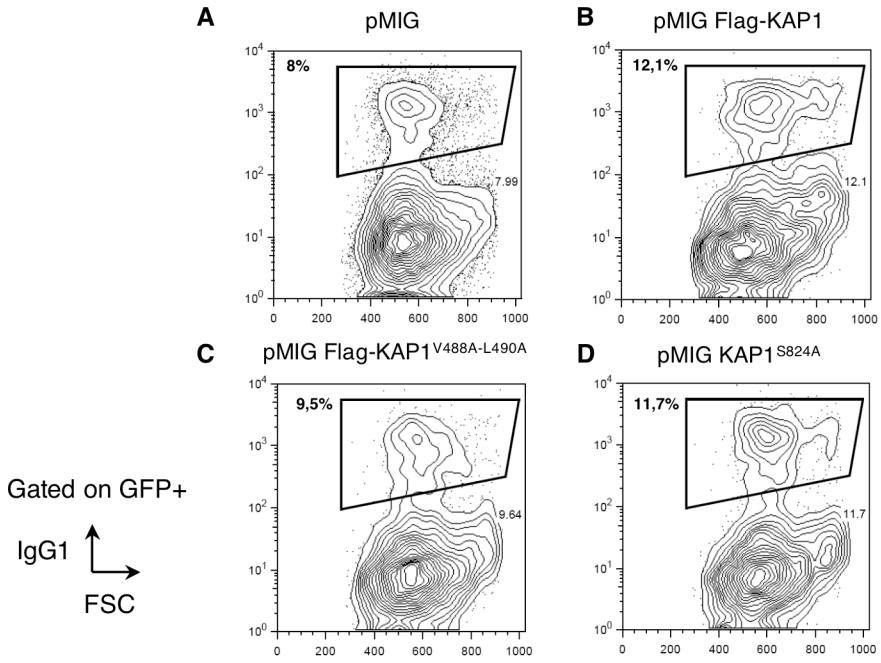


Figure 46: Overexpression of KAP1^{S824A} but not KAP1$^{V488A-L490A}$ rescues the CSR defect in KAP1-deficient B cells. B cells from CD19$^{Cre/+}$KAP1$^{F/F}$ mice were cultured *in vitro* with LPS+IL-4 and transduced with either **(A)** empty virus (pMIG) or with viruses encoding **(B)** Flag-KAP1 **(C)** Flag-KAP1$^{V488A-L490A}$ or **(D)** Flag-KAP1^{S824A}. FACS profiles from one experiment are shown and are gated on GFP^{+} cells. Percentage of switched cells is indicated in each plot.

These experiments support our conclusions that CSR is independent of the phosphorylation status of KAP1 at S824 and that the interaction between KAP1 and HP1 is required for efficient CSR.

DISCUSSION

I have considered this chapter as an extension of the discussion of the manuscript presented in the previous chapter. So, while some of the issues have already been addressed in the manuscript, I have attempted to not only discuss these issues in detail, but also critically analyze my data (the manuscript and complementary results) and include a few experimental ideas, so as to draw a bigger picture and put my findings into a broader perspective.

Understanding the interactions between the C-terminal of AID and KAP1

We identified that KAP1 associates with both $AID^{Flag\text{-}HA}$ and $EGFP^{AID182\text{-}198}$ in our MS expereiments. Considering its role in the DNA damage response (White et al., 2006; Ziv et al., 2006), we hypothesized that KAP1 could be required for CSR by responding to AID-initiated DNA breaks through its association with the C-terminal domain of AID. However, though we found that KAP1 associates with EGFP-$AID^{182\text{-}198}$ in our MS experiments, this interaction is not mediated exclusively through the C-terminus of AID, as KAP1 co-immunoprecipitates with a C-terminal deletion of AID ($AID^{Flag\text{-}HA\Delta 182\text{-}198}$; Figure 1C, [Figure 24C]) as well. Taken together, this suggests that the interaction between AID and KAP1 is mediated through, though not exclusively by the C-terminal domain of AID. How can We explain this?

One main possibility is, that "bridging" proteins within the same complex could also contribute to the interaction. Consistent with this, we have found that KAP1, AID and HP1γ exist in a complex of ~670 Kda (Figure 5B, [Figure 28B]) and MS analysis of this complex has revealed several novel proteins. This is further supported by the fact that KAP1 and AID do not interact directly in our yeast-two hybrid experiments (Figure 42). Knowing that KAP1, AID and HP1γ independently are part of several other protein-protein interactions, whether any of these proteins are required for or involved in the interaction between these 3 proteins, remains to be elucidated. In addition, we do not know the stoichiometry of KAP1, AID or HP1γ within this complex. Considering that we find a majority of HP1γ eluting in low molecular weight fractions in the gel filtration experiments suggests that only a small fraction of the complex contains HP1γ or that the *in vitro* association of HP1γ within the complex is weaker. Our PPD experiments suggest that the presence of an H3K9me3 peptide could in a way stabilize HP1γ within the complex, and may be even the α and β isoforms of HP1, and could be one explanation to why we do not find HP1α and β reciprocally co-precipitating with $AID^{Flag\text{-}HA}$. It therefore would be interesting to subject a complex that is precipitated by an H3K9me3 peptide to MS analysis in order to see if this is indeed the case. Furthermore, the HP1 proteins have multiple isoform-specific functions in different nuclear environments (Kwon and Workman, 2011), while HP1α and β mostly localize to HC, HP1γ is found in euchromatin (Fanti et al., 2003; Minc et al., 2000). Thus, it is highly possible that only HP1γ plays a role in CSR while the other isoforms do not – a possibility that is supported by the fact that $HP1\alpha^{-/-}$ B cells are proficient for switching (Figure 44). In order to better understand the role of the HP1 isoforms in CSR, the $HP1\beta^{-/-}$ and $CD19^{Cre/+}$ $HP1\gamma^{F/F}$ mice are currently being analyzed in the laboratory of Régine LOSSON. What would also be interesting is to perform these studies on endogenous proteins as well, and specifically in B cells undergoing CSR.

In addition to this, understanding the interaction between KAP1 and AID is complicated by the nature of the C-terminal domain of AID. Based on mutation analysis of the AID NES and AID chimeras built with heterologous NESs, Geisberger et al. showed that the nuclear export function of the NES is required, but not sufficient, for CSR. While one class of NESs support CSR and nuclear export, the other essentially supports only export and that these two classes of NES sequences have an effect on the stability of AID. They suggest, that both CSR and AID stabilization depends not just the sequence, but also the nature of the NES (Geisberger et al., 2009). Therefore, analyzing or identifying a protein that interacts with the C-terminal domain of AID could be a challenging issue. However, considering that the AID NES appears to have features that allows it to sustain CSR, the nature of the NES is critical for AID stabilization and CSR function (Geisberger et al., 2009), and that the KAP1-AID interaction could be partially mediated by the C-terminus of AID; it might be interesting to determine if an AID protein housing a mutant NES that affects CSR still binds KAP1.

Can we exclude a role for KAP1 in DNA repair during CSR?

KAP1 has been described to play an important role in responding to DNA damage (White et al., 2006; Ziv et al., 2006). KAP1 accumulates in foci that co-localize with γ-H2AX in response to γ-irradiation, is phosphorylated by ATM at serine 824 (S824), and its knock-down results in chromatin decondensation and radiosensitivity (Ziv et al., 2006). Changes in chromatin structure surrounding a DSB have been suggested to play a role in permitting efficient DNA damage responses (Downs et al., 2007; Goodarzi et al., 2010). DDR proteins like ATM, H2AX, 53BP1 and Nbs1 are required for CSR (Ramiro et al., 2007), probably by promoting long-range recombination between S regions (Reina-San-Martin et al., 2004; Reina-San-Martin et al., 2003). Interestingly, additional ATM kinase substrates (uniquely phosphorylated by ATM) have been suggested to play a role in CSR during switch region synapsis (Reina-San-Martin et al., 2004). Based on this, our hypothesis was that KAP1 could be required in the ATM-dependent pathway and that it could mediate efficient repair of AID-induced lesions during CSR.

We were surprised when we found that the role of KAP1 in CSR was independent of its function in the DDR, a conclusion based on several experiments: (a) Analysis of ATM-dependent KAP1 phosphorylation during CSR. We found that almost equal levels of γ-KAP1 were detected in both WT and AID-deficient B cells, and concluded that KAP1-phosphorylation was not dependent on AID expression, and that the role of KAP1 in CSR is independent of its phosphorylation status at serine 824. Furthermore, given that S824 phosphorylation in response to DSBs does not affect the interaction between KAP1 and HP1 (Ziv et al., 2006) and that we have shown that the interaction between KAP1 and HP1 was required for CSR, gave us a reason to believe that γ-KAP1 does not contribute towards the repair of DSBs during CSR. (b) As a complementary approach, a phospho-mutant of KAP1 (Flag-KAP1^{S824A}) overexpressed in

$CD19^{Cre/+}$ $KAP1^{F/F}$ B cells by retroviral transduction behaves like a WT protein and rescues the CSR defect in these cells. (c) In collaboration with Frederick ALT, using either two-color Ig*h*-FISH or telomere FISH (Franco et al., 2006; Yan et al., 2007), Jing WANG saw that there was no significant increase in Ig*h*-specific abnormalities, or overall genomic instability (data not shown) in KAP1-deficient B cells. (d) We analyzed the frequency of AID-dependent Ig*h*/c-myc translocations by long-range PCR and Southern blotting (Ramiro et al., 2004), to find that frequency of these translocations were not increased in KAP1-deficient B cells (Figures 26G and 37). (e) Drawing similarities with the NHEJ components, we analyzed IgG3 CSR junctions and found that DNA ends are repaired normally by NHEJ in the absence of KAP1.

Thus as we detect similar levels of γ-KAP1 in WT and AID-deficient B cells and no obvious effect on genomic stability in the absence of KAP1, these results suggest that role of KAP1 in CSR was independent of its function as a DNA damage response protein, and that γ-KAP1 does not contribute towards the repair of DSBs during CSR. This conclusion turned our hypothesis around, and we then hypothesized that KAP1 could function upstream of AID-initiated DNA breaks during CSR.

Finally, recent evidence suggests that the SUMOylation of KAP1 mediates it's transcriptional co-repressor function (Lee et al., 2007; Li et al., 2007), and interestingly a "switch" between KAP1-SUMOylation and KAP1-Ser 824 phosphorylation has been proposed to be a regulatory circuit in mediating the de-repression of several KAP1-repressed genes like p21, Gadd45α, Bax, Puma and Noxa (Lee et al., 2007; Li et al., 2007). Thus, although both the "classical" functions of KAP1 seem to be dispensable for CSR, we cannot rule out the possibility that an interplay of functional regulation like that between ATM activation and KAP1 SUMOylation does not exist and could play a role in mediating CSR.

Is there a functional redundancy with other members of the Tif family?

In trying to better understand the partial phenotype of KAP1-deficient B cells, the possibility of functional redundancy within members of the same protein family arises. Interestingly, Tif1γ does not contain the HP1 binding PxVxL motif (Yan et al., 2004) and the expression of Tif1δ is restricted to the testis (Khetchoumian et al., 2004), ruling out their possible involvement in CSR according to our hypothesis. On the other hand, at the molecular level while loss of Tif1α gene function results in hepatocarcinogenesis, it does not cause changes in the gross morphology of the lymphoid organs like the spleen and thymus (Khetchoumian et al., 2007). To ask if Tif1α was required for CSR, we analyzed the efficiency of B cells from Tif1α-deficient mice to undergo CSR *in vitro*. As shown in Figure 43, $Tif1\alpha^{-/-}$ B cells undergo efficient CSR to all the isotypes tested, suggesting that Tif1α is not required for CSR and that the CSR defect in KAP1-deficient

B cells is due to the deletion of KAP1 alone. However, the actual answer to the question of redundancy between Tif1α and KAP1 would be the generation of a Tif1α-KAP1 double knockout mouse or deletion of Tif1α on a KAP1-deficient background using a shRNA retroviral approach.

Is the tethering of AID to downstream S regions rate-limiting?

CSR initiates at Sμ and involves the downstream S regions (Schrader et al., 2003). AID-dependent mutations are introduced at the 5' Sμ segment, but rarely at downstream S regions in B cells stimulated to undergo CSR. In addition, donor and not acceptor S regions sustain internal deletions in cells that have been activated to, but have not undergone successful CSR (Reina-San-Martin et al., 2003; Schrader et al., 2003). Taken together, this suggests that DNA lesions are possibly introduced first at Sμ and only then at downstream S regions. So, while on one hand DSBs at Sμ are not limiting (Guikema et al., 2007; Schrader et al., 2007); the recruitment of AID, deamination and subsequent DNA lesions at acceptor S regions are considered a rate-limiting step during CSR (Reina-San-Martin et al., 2003; Schrader et al., 2003).

We have shown that KAP1, H3K9me3 and HP1γ mark the Ig*h* locus in resting B cells that is reduced upon activation (Figures 29 and 40). However, both H3K9me3 and KAP1 are retained specifically over the donor S region, forming a binding motif for a KAP1/HP1/AID complex at Sμ. As a result, KAP1-deficiency possibly does not affect the retention or activity of AID at acceptor S regions during CSR. This could also be one reason why the CSR defect in $CD19^{Cre/+}$ $KAP1^{F/F}$ B cells is not more pronounced.

In order to show that KAP1 deletion does not affect DSB induction at acceptor S regions we need to perform LM-PCR, TdT-end labeling PCR experiments and/or sequencing of the downstream S regions. Although we tried, owing to technical problems we have not managed to successfully do these experiments. Of course, the direct proof would be that binding of AID to Sγ1 or Sγ3 is not affected in KAP1-deficient B cells upon activation. This can be shown by an AID ChIP experiment. However, as all the anti-AID antibodies that we have tried so far have failed to work in a ChIP, we have not been able to show this.

Finally, just as there are multiple mechanisms that operate to recruit AID to S regions during CSR, we cannot exclude that in addition to H3K9me3/KAP1-mediated tethering of AID there are other mechanisms that control the tethering and retention of AID at S region(s) in B cells undergoing CSR. This could be either different chromatin marks, or proteins such as RPA, PKA, PTBP2 or the 14-3-3 isoforms (Basu et al., 2005; Chaudhuri et al., 2004; Nowak et al., 2011; Xu et al., 2010), or even a combination of both, and remains to be determined.

Consistent with this, the interaction of AID with genes across the genome is biased towards genes associated with an open chromatin configuration, as shown by the presence of RNA PolI and H3K4me3, but not H3K27me3, that correlates with the binding of AID to the myc locus in activated B cells (Yamane et al., 2010). In addition, dynamically modified histone methylation patterns produced by transcription, is thought to permit S region accessibility to allow AID action (Wang et al., 2009b). Therefore, it might be interesting to extend our studies to other epigenetic modifications and analyze if they could be required for, or involved in, the tethering of AID to S region(s). Furthermore, from our gel filtration experiments using nuclear extracts from CH12 cell lines expressing $AID^{Flag\text{-}HA}$, in addition to identifying KAP1 and HP1γ, we also found several other proteins. One such protein that was identified appears an interesting candidate to study, and we have initiated experiments to characterize the role(s) of this protein in CSR and/or SHM.

Why are our results different from that of the Scharff lab?

While discussing the binding of H3K9me3, KAP1 and HP1γ, it is important to address the following issues. Kuang et al. showed that in resting cells and throughout CSR, Sμ is both methylated and acetylated at H3K9. On the other hand in stimulated cells, H3K9me3 is induced and is associated predominantly with Sμ as well as the recombining acceptor S region (Kuang et al., 2009). Contrary to this, we found that H3K9me3 is already present all across the Ig*h* locus in resting B cells, perhaps to setup the locus for changes that will follow post stimulation. Neither is H3K9me3 further induced at Sμ, nor did we observe specific induction at acceptor Sγ3 or Sγ1 on stimulation (Figure 6B, [Figure 29B]). Furthermore, we find that H3K9me3 is reduced across the locus and retained specifically only at Sμ. This binding pattern is different from that published by Kuang et al, and could be attributed to (i) differences in stimulation conditions, (ii) the time point at which the analysis was done, or (iii) antibody used. However, this is probably not the explanation, as we have used the same antibody as well as primers in their study. I have also performed these experiments at a 72 hr time point and see the same pattern as at 48 hr. More importantly, our experimental conditions work, as consistent with previous reports (Wang et al., 2009b), we found that H3K9/K14Ac is associated with the pair of recombining switch regions. Possible experimental differences are that Kuang et al. used densitometry (ImageQuant) and the local average method to analyze the ChIP DNA, while we use a more quantitative SYBR green qPCR detection, and our ChIP conditions are more stringent than theirs.

Why is there a difference between KAP1/HP1 and H3K9me3 enrichment at the Igh locus?

In addition to these differences, we also see a disconnect in the binding patterns between

H3K9me3-KAP1 and HP1γ at the Ig*h* locus, as HP1γ binding is distributed throughout the locus compared to H3K9me3, and KAP1 that are enriched at Sμ. However, an abundant protein like HP1γ could have several physiological roles, and its association with the Ig*h* locus could also be independent of its binding to H3K9me3, as suggested for its role in the DDR (Ball and Yokomori, 2009).

And finally, if KAP1/HP1/AID generally bind to regions of high H3K9me3 density, it would support the idea that this mark is sufficient to recruit the complex, and could provide potential mechanistic insight into the off target activities of AID. To address this, we have recently initiated ChIP followed by sequencing (ChIP-Seq) experiments on activated WT B cells with antibodies against H3K9me3, KAP1, HP1γ and AID. We expect that these experiments would provide interesting insights into questions like: Do KAP1, HP1γ and AID binding sites overlap always with H3K9me3 and vice versa? If not (and this is most probably the case), are there other factors/modifications in addition to H3K9me3 that permit the recruitment of AID? If so, what are they?

Why does KAP1 dictate AID tethering to Sμ?

What exactly determines this specific pattern of KAP1/H3K9me3 at Sμ? - H3K9me3 has been found at repetitive elements (Rice et al., 2003) and the sequence of the Ig*h* locus is known to partly determine AID specificity (Chaudhuri et al., 2007). More specifically, the S region's 5'-AGCT-3'- rich core is necessary and sufficient to mediate CSR (Luby et al., 2001; Zarrin et al., 2004), and the fact that the frequency of 5'-AGCT-3' is concentrated at Sμ (Milstein et al., 1998) probably reflects the essential role of this S region in mediating CSR to all downstream isotypes.

Interestingly, a recent (ChIP)-on-chip analysis performed in the human testicular carcinoma cell line Ntera2, used high-density oligonucleotide arrays of 26,000 human promoters, for H3K9me3 and H3K27me3 binding revealed, that ~40% of promoters bound by H3K9me3 were also bound by KAP1 and enriched for ZNF transcription factors (TFs, O'Geen et al., 2007). Furthermore, studies on human TFs have indicated that though transcription complexes are often bound near promoter regions, many of them also bind throughout the genome (Bieda et al., 2006; Carroll et al., 2005; Cawley et al., 2004). Then, using genome-wide (ChIP)-on-chip O'Geen et al. identified ~7000 KAP1 binding sites several of which were located within the core promoter regions, while the rest of the sites are distributed in regions between 5-50 Kb up/downstream of the start sites (O'Geen et al., 2007). By analogy, one could imagine that the sequence of Sμ and its surrounding regions has KAP1 recognition motifs, which are also binding sites for the H3K9me3 mark that could eventually recruit a KAP1/AID/HP1 complex to these sites. However,

as these sequence motifs are not very well described or determined, it could be difficult to define them along the Ig*h* locus.

Could we be underestimating the requirement of KAP1 in CSR?

Finally, could we be underestimating the effect of KAP1 in CSR, in a conditional knockout? It is likely that some B cells that do undergo CSR express residual levels of KAP1. Thus, there is a possibility that such "wild type" cells exist (in a pool of $CD19^{Cre/+}$ $KAP1^{F/F}$ B cells), which either escape Cre activity or have not undergone efficient deletion and are proficient for CSR. This is substantiated by a functional heterozygous allele, which undergoes CSR at wild type frequencies. If this is the case, we are actually under estimating the effect of KAP1 on the efficiency of CSR. This however is very unlikely as KAP1 deletion in $CD19^{Cre/+}$ $KAP1^{F/F}$ B cells is very efficient both at the DNA level (data not shown) as well as at the protein level (Supplementary Figure 1A, [Figure 31A]).

Is AID activity affected?

AID activity, like many other proteins, is regulated by its sub-cellular localization as well as posttranslational modifications (Delker et al., 2009; Maul and Gearhart, 2010), and mice expressing ~ 50% of normal AID levels have impaired CSR and SHM, indicating that AID gene dose is limiting for its physiologic function (Sernandez et al., 2008). Interestingly, depletion of CtIP – a protein involved in transcriptional repression and DNA repair (Chinnadurai, 2006; Yu et al., 1998; Yun and Hiom, 2009), in the CH12 B cell line results in a CSR defect as well as a decrease in AID expression (Lee-Theilen et al., 2011).

Could the defect in CSR be a result of reduced AID levels or defective function in the absence of KAP1? As shown in Figures 26B and 35B, neither does inactivation of KAP1 affect AID protein levels, nor does overexpression of AID with a retrovirus rescue the CSR defect in KAP1-deficient B cells. This suggests that the deletion of KAP1 does not change the "quantity" of AID. Furthermore, the function of AID is regulated by PKA-mediated phosphorylation at S38 (Basu et al., 2005) and mutation of this site (AID^{S38A}) diminishes CSR and SHM (Basu et al., 2005; Cheng et al., 2009; McBride et al., 2006; McBride et al., 2008; Pasqualucci et al., 2006). Therefore, although total protein levels of AID are not reduced on KAP1 deletion, the possibility that its phosphorylation and subsequent activation is inefficient does exist. To rule out this possibility, I would have to assay for the phosphorylation status of AID in KAP1-deficient B cells but, we currently have no phospho-AID specific antibody. However, although active AID constitutes only about 5-10% of the AID in B cells, undergoing CSR and contributes disproportionately to CSR and hypermutation activity (McBride et al., 2006), it is only fair to

conclude that since SHM appears normal in the absence of KAP1, the activation of AID, its deaminase activity and downstream processing of the U:G mismatches per se remain unaffected during CSR.

Is it targeting or tethering?

We have known that the targeting of AID to Ig loci is determined by transcription (either enhanced or modulated by AID's interaction with RPA) as well as primary sequence structure of the Ig locus (Chaudhuri et al., 2007). However, it is counterintuitive that such specificity is provided only by the sequence and transcription of a locus, and the involvement of other mechanisms in dictating AID target specificity have been proposed (Ramiro et al., 2007). Consistent with this, recent reports from the Nussenzweig, Alt and few other laboratories have led to a better understanding of these mechanisms. Since targeting of AID to S regions is dependent on its interactions with RNA Pol II, Spt5 and the RNA exosome (Basu et al., 2011; Pavri et al., 2010) as well as adaptor proteins, 14-3-3 isoforms and PTBP2 (Nowak et al., 2011; Xu et al., 2010), we think that the access of AID to its target S regions is not compromised on deletion of KAP1. Thus, while AID is targeted to S regions by binding RNA Pol II, Spt5 and the RNA exosome, its retention and subsequent initiation of DNA lesions at the donor Sμ is dependent on its interaction with KAP1/HP1 and the H3K9me3 mark.

We have proposed a model, in which AID forms a complex with HP1 and KAP1 that is tethered to the donor S region marked by H3K9me3, during CSR. However, we need to directly demonstrate that AID is recruited to S regions and that KAP1 inactivation results in reduced AID levels at Sμ in switching B cells. We have tried to perform ChIP experiments using several anti AID antibodies (a monoclonal that we have generated in the lab, one that was a gift from another lab and 2 from commercial sources), but have never obtained enrichment in AID binding in WT B cells compared to chromatin from activated $AID^{Cre/Cre}$ B cells. We are however trying to modify these experimental conditions and complete this experiment, as it is crucial to the hypothesis to show that AID is tethered to S regions and the efficiency is reduced in the absence of KAP1. We have shown that the KAP1-HP1 interaction is required to sustain CSR. So, does the expression of a mutant KAP1 $^{V488A\ L490A}$, which cannot bind the HP1 proteins also result in inefficient tethering of AID at Sμ? To answer this question, we are performing ChIP experiments on resting and activated B cells from $CD19^{Cre/+}KAP1^{V488A\ L490A/F}$ mice, and results from these experiments would strengthen our hypothesis.

Is AID present at S regions bearing H3K9me3?

Simultaneous occupancy of AID, KAP1 and HP1γ at S regions specifically enriched with H3K9me3 or vice versa, could be tested by well-controlled and quantitative ChIP, followed by Re-ChIP of enriched fractions. Furthermore, in an *in vitro* culture only ~20-25% of cells undergo switching, thus within a population of cells the chromatin can be either recombinationally active or inactive, with a differential display of chromatin marks and associated proteins. Thus, the specificity of the KAP1/HP1/AID complex for H3K9me3 can also be verified by performing ChIP on chromatin bound by H3K9me3, as well as other epigenetic marks like H3Ac or H3K27me3, followed by a Re-ChIP for KAP1, AID and HP1γ.

Are the transcriptional co-repressor functions of KAP1 intact during CSR?

As KAP1 is a bonafide transcriptional co-repressor, and we know that CSR requires transcription of S regions, one would possibly expect to find an increase in S region transcription at the Ig*h* locus on stimulation of KAP1-deficient B cells. While we find no reduction in the level of sterile transcripts in KAP1-deficient B cells, we do detect occasional increases in the levels of γ3, γ1, γ2b and γ2a transcripts (Figures 26A and 33A), and this could be an outcome of KAP1 function elsewhere than the Ig*h* locus during CSR (see below). However, the fact to be highlighted here is that despite these slight increases in GLT's, KAP1-deficient B cells display a CSR defect, similar to what is observed in $ATM^{-/-}$, $H2AX^{-/-}$ or $53BP1^{-/-}$ B cells (Reina-San-Martin et al., 2004; Reina-San-Martin et al., 2003; Ward et al., 2004). Thus, germline transcription at S regions seems unaffected by the absence of KAP1. However, as KAP1 is a global regulator, we cannot rule out the possibility that the phenotype of the KAP1 conditional knockout is a reflection of the regulation of other factors that either function (or not) in the CSR mechanism by KAP1, and we would have to analyze the transcriptomic profile of KAP1-deficient versus KAP1-proficient B cells to address this issue.

The KRAB-KAP1-HP1-H3K9me3 repression complex directs the assembly of a highly organized chromatin environment that interferes with the binding of transcriptional activators and RNA Pol II recruitment, and consequently transcriptional repression (Ayyanathan et al., 2003; Riclet et al., 2009; Schultz et al., 2002; Sripathy et al., 2006; Urrutia, 2003). As mentioned above, although we cannot rule out that the defect in CSR on deletion of KAP1 is due to the global de-repression of genes that are normally silenced by KAP1, this is most probably not the case – why? KAP1 is the only member of the Tif family that silences transcription through its interaction with HP1 and it requires an "intact" HP1BOX motif for full repression activity, indicating that the formation of a complex with the HP1 proteins is instrumental in KAP1-mediated transcriptional repression (Cammas et al., 2004; Nielsen et al., 1999; Sripathy et al.,

2006). Furthermore, (a) We have overexpressed Flag-tagged wildtype KAP1 (Flag-KAP1) and a Flag-KAP1$^{V488A-L490A}$ mutant in activated CD19$^{Cre/+}$ KAP1$^{F/F}$ B cells by retroviral transduction. In this experiment, while overexpression of the Flag-KAP1 rescues CSR to wildtype levels in KAP1-deficient B cells, the mutant Flag-KAP1$^{V488A-L490A}$ protein fails to do so (Figure 46, one experiment). (b) B cells from CD19$^{Cre/+}$ KAP1$^{V488A-L490A/F}$ mice (Cammas et al., 2004; Herzog et al., 2010) on a KAP1-deficient background, failed to restore CSR to WT levels compared to B cells from mice expressing the wild-type and mutant protein (Figure 7, [Figure 30]). This underscores the fact that the *in vivo* association between KAP1 and HP1 is required to mediate efficient CSR.

Taken together, these results show that the association of KAP1 and HP1 is necessary during CSR. Since this interaction is absolutely required for KAP1-dependent repression, we conclude that the CSR defect in CD19$^{Cre/+}$ KAP1$^{F/F}$ B cells is not due to the disruption KAP1's function as a transcriptional co-repressor.

How do we re-enforce the hypothesis that DSB formation if affected?

By dissecting the various steps in the CSR reaction, we pinpoint a role for KAP1 downstream of AID expression, and hypothesized that if AID is inefficiently retained at the Sμ in the absence of KAP1, it would result in decreased DSBs.

Gaining insight into the mechanism of AID's target specificity and DSB induction requires a reliable cleavage assay. We used more than one strategy to determine the induction of DSBs at Sμ: (a) Analysis of mutation frequency in the 5' end of Sμ by conventional Sanger sequencing (Figure 4B, [Figure 27B]); (b) Mutation analysis by high-throughput amplicon sequencing (Figure 4C, [Figure 27C]); (c) Ligation-mediated PCR to detect DSB frequency in the 5' end of Sμ (Figures 27A and 38); and (d) As AID-induced DSBs result in γ H2AX foci at S regions and are important for CSR (Petersen et al., 2001; Reina-San-Martin et al., 2003), we also analyzed histone H2AX phosphorylation at Sμ by ChIP (Figure 45). We found that the induction of DSBs is inefficient in the absence of KAP1. However, there are caveats in detecting small differences in AID-induced DNA damage during CSR by measuring γH2AX by ChIP. Although, DNA cleavage has been previously measured by γH2AX focus formation (Begum et al., 2004a), this assay is unlikely to reveal quantitative differences in DSB induction at S regions, as γH2AX can spread over large stretches of DNA sometimes extending upto megabases (Rogakou et al., 1999; Shroff et al., 2004). Therefore, the γH2AX ChIP was not ideal to determine subtle reductions in DSB induction at S regions that we see in KAP1-deficient B cells by other approaches like LM-PCR and sequencing. In an attempt to support this data with a more quantitative experiment, I tried to quantify AID-induced DSBs in CD19^{Cre} KAP1$^{F/F}$ versus control

B cells by terminal deoxynucleotidyl transferase (TdT)–mediated PCR - a very sensitive method to detect breaks in DNA (Doil et al., 2009; Ju et al., 2006), which unfortunately due to technical issues, did not work.

We have however, shown by three different experimental approaches that DSBs are reduced at Sμ in $CD19^{Cre/+}$ $KAP1^{F/F}$ B cells undergoing CSR, and this is probably because AID is inefficiently retained close to its substrate in the absence of KAP1 during CSR resulting in a compromise on the efficiency of DSB induction, and all of these findings with arguments presented allows us to propose a model.

Working model for CSR

Trimethylation of H3 at K9 has always been regarded as an epigenetic mark associated with silenced genes and heterochromatin (Bannister et al., 2001; Barski et al., 2007; Rice et al., 2003). More recently, however, the generality of this concept has changed as the coding regions of several active genes were found to be associated with H3K9me3 and HP1γ (Vakoc et al., 2005), and H3K9me3 was shown to be associated with promoters bound by RNA Pol II (Squazzo et al., 2006). This suggests, that H3K9me3 could have dual roles in maintaining chromatin structure, possibly by its association with other epigenetic modifications (Berger, 2007).

The role of chromatin remodeling and accessibility in the context of AID mediated Ig diversification has gained interest, and it has been proposed that containment and accurate targeting of mutator enzymes like AID undoubtedly involve chromatin accessibility and interaction with relevant transcription factors. Consistent with this, is the association of inducible histone marks including γ-H2AX, H3 and H4 hyperacetylation, (Li et al., 2004a; Nambu et al., 2003; Petersen et al., 2001; Wang et al., 2006b). Acetylation and trimethylation on K9 of H3 with S regions during CSR have been suggested to play a role in targeting the CSR machinery (Chowdhury et al., 2008; Kuang et al., 2009). More specifically, H3K9me3 has been detected at actively transcribing S regions in mouse and human B cells undergoing CSR (Chowdhury et al., 2008; Kuang et al., 2009). Moreover, while H3K9me3 was found to be associated with the γ and ε S and C_H regions in human B cells in response to stimulation with IL-4/anti CD40 (Chowdhury et al., 2008), H3K9me3 and H3K9Ac mark recombining donor and acceptor S regions and accumulate prior to AID expression (Kuang et al., 2009). Taken together, this strong association of H3K9me3 with the Ig*h* locus has been proposed to increase S region accessibility and/or play a role in the specific targeting of AID to the Ig*h* locus during CSR (Kuang et al., 2009).

Furthermore, G9a or G9a-related protein, two related methyltransferases, cooperate to mediate the bulk of H3K9 methylation euchromatic regions (Tachibana et al., 2005). On the other hand, SUV39H1/H2 seem to repress expression only during terminal differentiation (Ait-Si-Ali et al.,

2004) and SUV39H1-mediated H3K9me is known to serve as a binding site for HP1 at promoter regions (Tachibana et al., 2005). The proposition that H3K9me could be required for CSR is reiterated by either human diseases or mice deficient for three methyltransferases: SETDB1 deficiency (in humans) leads to reduced serum IgE levels and dermatitis (Jang et al., 2005; Zhang et al., 2003), mice deficient for G9a show reduced CSR to IgG1 and IgE (Thomas et al., 2008), and Suv39h1 deletion in mice is linked to a decrease in switching to IgA (Bradley et al., 2006). On the other hand, it is important to remember that all PTMs are reversible. The two broad classes of lysine demethylases (KDMTs) are, (a) LSD1/BHC110 that remove H3K4me1 and me2, and (b) the Jumonji class, which remove H3K4me2 and me3, H3K9me2 and 3 and H3K36me2 and 3 (Berger, 2007). It therefore might be of interest to overexpress an H3K9me3-specfic KDMT in WT B cells, to see if removal of the methylation at H3K9 results in a CSR phenotype similar to the KAP1-deficient B cells.

Finally, as discussed above, we have shown that KAP1 forms a complex with AID and HP1, which binds an H3K9me3 peptide *in vitro* and that the *in vivo* association of KAP1 and HP1 is required for CSR. Furthermore, KAP1, HP1 and H3K9me3 mark the Ig*h* locus in naïve B cells. KAP1 and H3K9me3 more precisely mark the donor S region, both before and after stimulation perhaps serving to identify as a common donor for all downstream regions, while HP1 is distributed across the locus.

Evolutionary forces generating very similar solutions to the same problem from very different starting points or “convergent evolution” is probably an interesting explanation to our findings. Mating-type switching in two distantly related yeast species, *Saccharomyces cerevisiae (S. cerevisiae)* and *Schizosaccharomyces pombe (S. pombe)* involves a highly ordered recombination event which involves replacing genetic information at the expressed *mat1* locus with sequences copied from one of the two silent donor loci, *mat2-P* or *mat3-M*, located within a 20Kb heterochromatin domain (Haber, 1998). It is believed that heterochromatin at the *mat2/3* locus preferentially brings one donor close to the *mat1* locus in a cell-specific manner. HC plays a role in imposing structural changes that are important for donor choice mechanisms and in long-range regulatory interactions by acting as a platform for the recruitment of other proteins (Jia et al., 2004). Consistent with this, heterochromatin seems to control the spreading of a recombination promotion complex, which influences the silent locus that is selected for recombination (Figure 47, Grewal and Jia, 2007; Jia et al., 2004).

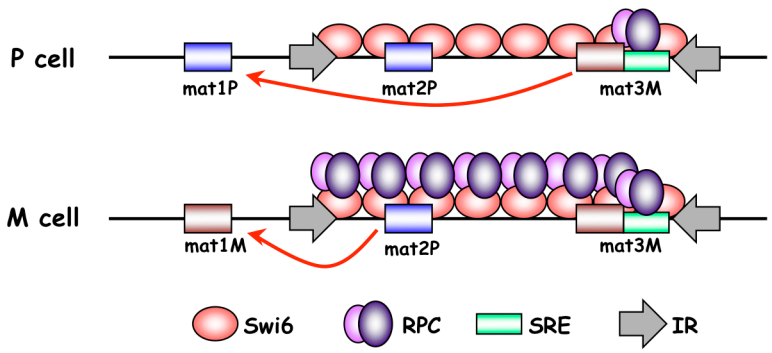


Figure 47: Heterochromatin regulates cell-type specific spreading of a protein complex: A recombination-promoting complex (RPC) that is involved in mating-type switching binds specifically to an enhancer element (SRE), located near mat3M locus in the P mating-type cells. However, in the M mating-type cells, the RPC spreads across a 20-kb region, including the mat2P locus. This cell-type-specific differential spreading of the RPC is regulated by heterochromatin, in particular Swi6 - the yeast homolog of HP1 in mammals, and is involved in donor choice during mating-type switching. IR, inverted repeat boundary elements. Adapted from (Grewal and Jia, 2007).

Interestingly, the distribution pattern of H3K9me3, KAP1 and HP1γ across the Ig*h* locus before and during CSR appears to be analogous to the heterochromatic signature at the mating-type switching locus in the fission yeast *S. pombe*, which in turn is important for the choice of the recombination donor site (Jia et al., 2004). We show that H3K9me3, KAP1 and HP1 are present at the Ig*h* locus in resting B cells, and that upon activation H3K9me3 and KAP1 are retained over Sμ. This specific enrichment of KAP1 binding around the donor S region probably prepares Sμ to serve as the donor in response to CSR stimuli. Based on these observations, we propose a model where H3K9me3 can serve as a docking motif for an AID/KAP1/HP1 complex *in vivo* at the donor switch region during CSR and that this complex is thus “epigenetically tethered” to Sμ in B cells during CSR (Figure 48).

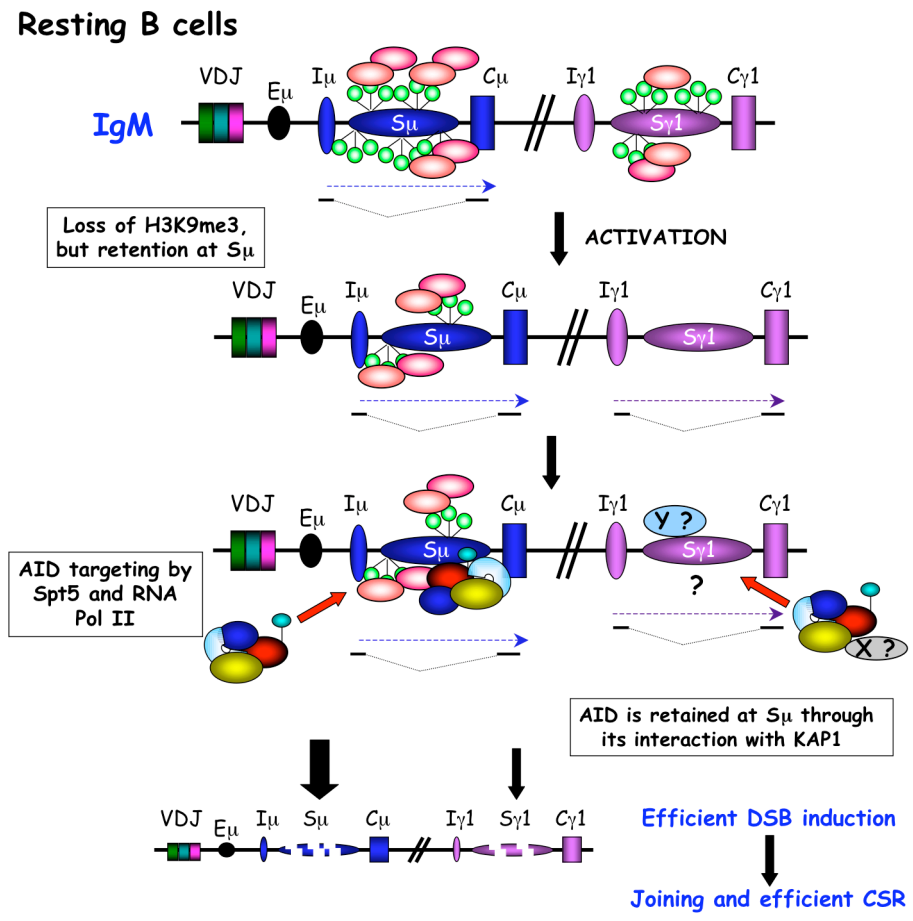


Figure 48: Working model for CSR: Describes the various steps in the CSR reaction. In our model the H3K9me3 modification can serve as a docking motif for an AID/KAP1/HP1 complex *in vivo* at the donor switch region during CSR to tether AID at Sμ in order to keep it close to its target and initiate deamination that will lead to efficient DSBs at the donor S regions in B cells during CSR.

General conclusions:

Although AID was discovered just about a decade ago, substantial progress has been made towards understanding its roles in antibody diversification. However, as in most new fields, there are pieces missing from the puzzle and despite this fair understanding it is still unclear how CSR is regulated and particularly how AID is targeted to the Ig genes. That CSR requires transcription, RNA splicing, AID-induced DSB formation, recombination, and repair and that each of these events are known to be or are likely accompanied by epigenetic modifications, these changes in chromatin structure have been suggested to regulate and/or target the CSR machinery. Our results provide a mechanism linking AID to chromatin modifications associated with the Ig*h* locus during CSR. We have extended earlier findings (from other labs) and thus fitted one more piece into the puzzle of revelation into the patterns of epigenetic modifications and regulatory mechanisms that could govern the specific targeting of a protein like AID.

Considering the recent burst of information on the role(s) of AID partner proteins, the field is seeing just the beginning of a better understanding of the importance and influence of such cofactors in AID function. In the coming years, progress in this area will shed more light on how AID is initially brought to the loci, how it interacts with the plethora of partners and most importantly, on how AID localizes to and mutates Ig loci.

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APPENDIX I

B CELL STIMULATION

Date :

Spleen Isolation :

- Mice are sacrified on dry ice with absorbant paper soaked in 70% EtOH for about 5-10 mins.
- Surface sterilzed with 70% EtOH and placed with the head towards the left, as the spleen is on the right side of the abdomen.

IF THE OTHER ORGANS LIKE THYMUS OR LN's ARE TO BE REMOVED ITS BETTER TO LAY THE ANIMAL VENTRAL SIDE UP.

- Make an incision (all instruments should be sterilized with alcohol) at acute angles to the body and tear open the skin, at all times make sure that the animal is cleaned well, as hair is a major contaminant.
- Take out the spleen carefully, taking care to tease away extra tissue and put it in a tube with 5mL of cold, sterlie PBS 0,5% BSA and leave on ice. (From now on called just PBS- BSA)

Purification of Spleenocytes:

STERILE CONDITIONS, UNDER THE HOOD

- The spleen is teased out using two 18/23 gauge needles bent at right angles into a 5 cm culture dish containing PBS-BSA. (As an alternate you can smash the spleen, using the piston of a syringe in a cell strainer into a 10 cm culture dish containing PBS-BSA).
- The suspension is then pipetted up and down in order to obtain a homogenous suspension.
- Transfer the cells to a ____ mL tube, add _____ mL of PBS-BSA and leave on ice.

The size of the tube and volume of buffer varies with the number of samples.

Usually one 14 mL tube and 13mL buffer for one spleen.

- Centrifuge 5 min/1200 RPM/4°C.
- Apsirate the supernatant and tap the pellet well, the pellet is usually red at this stage.

Lysis of RBC's and Purification of Primary B Cells:

- Add to this cell pellet 1mL of ACK, tap the tube and leave at RT for 1 min.
- Quench the reaction IMMEDIATELY by adding 13mL of PBS BSA to the tube and invert well. Allow the tube to stand till the debris settles to the bottom.

- Remove the supernatant taking care to not aspirate the debris and centrifuge the cells 5 min /1200 RPM/4°C.

(The pellet should be white, which is an indication of proper RBC lysis)

- Aspirate the supernatant, resuspend the pellet and add 10mL of PBS-BSA.
- Count the cells, (centrifuge 5 min/1200 RPM /4°C) and resuspend the pellet in 900μL of PBS BSA. (Cells should usually be at ~ 100M cells/mL)
- Add the MACS CD43 beads (Miltenyi Biotec, Ref**:**130-049-801) to this cell suspension and incubate at 4^0C for 30mins with mixing.

(~100μL of beads for 100M cells, but we now use a 100μL/spleen)

- Prepare the affinity columns during this time :
  - Columns should be placed on the magnetic racks and equlibrated with 900μL (for MS; Miltenyi Biotec, Ref**:**130-042-201) or 3mL (for LS; Miltenyi Biotec, Ref**:** 130-042-401) of PBS-BSA, discard this buffer.

(Use one MS column/spleen, and one LS if upto 5 spleens are being pooled)

- After 30mins incubation, add 10mL of PBS BSA to the cell-bead suspension and centirifuge 5 min/1200 RPM/4°C.
- Resuspend the pellet in _____ of buffer with pipetting (usually 500μL/10^8 cells, take care at this step to not get too much debris, as it can clog the columns).
- Place the collection tubes below the columns and add the cell-bead suspension to the columns. Collect the flow-though. These are $CD43^-$, resting B cells.
- 3X washes of the column with 500μL of PBS-BSA (MS columns) or 1mL of PBS-BSA (LS columns).
- Wash the cells with 14 mL of PBS-BSA, centirifuge 5 min/1200 RPM/4°C and count.
- If you are doing a CFSE staining (see below) add 37°C, pre-warmed incomplete RPMI, else add complete RPMI to have 2.4x10^6 cells/mL.
- Count the cells and plate them at 2.4x10^6 cells/well (if its a 6 well plate) in 1mL with complete RPMI and add the 2X stimulation medium as needed.

I now plate cells in a 10 cm dish, so I plate upto 6 mL of cells and add 6 mL of 2X stimulation medium.

CFSE Staining :

This is done after cells are purified on the column and washed with PBS-BSA.

Remember to keep an aliquot of cells for a CFSE- control.

- Add 37°C, pre-warmed incomplete RPMI upto 10 mL to the cells after the PBS-BSA wash.

- Count the cells, keep an aliquot for a $CFSE^-$ control both stimulated and non stimulated.
- Centrifuge 5 min/1200 rpm/RT.
- Aspirate the supernatant and resuspend the pellet well @ 10^7/mL in pre warmed incomplete RPMI.
- Add CFSE (1000X) upto 1X into these cells, and incubate the cells @ 37°C/10mins, with circulation. (I keep tapping the tube every 2 mins so as to keep the concentration of the CFSE close to uniform all the time).
- Wash the cells, count and resuspend them in complete RPMI @ $2.4x10^6$/mL.
- Cells are diluted to $2.4x10^6$/mL and plated 1mL/well with 2X induction medium

Medium :

Incomplete RPMI: RPMI + 10 mM HEPES + PS

Complete RPMI: Incomplete RPMI + 10% iFCS (K3399) + goodies.

- β-ME 50 µM (250 µl of 100 mM for 500 mL, i.e 1:2000 dilution)
- Sodium Pyruvate 1 mM (5 mL of 100 mM for 500 mL of media, i.e 1:100 dilution)
- Non essential AA's 1 X (5 mL of 100 X for 500 mL of media, i.e 1:100 dilution)
- L-Glu 1 X (5 mL of 100 X for 500 mL of media, i.e 1:100 dilution)

2X INDUCTION medium:

- Complete RPMI + the corresponding stimulants added to a final 2X concentraion.
- Add 1mL of this media to the cells, to give a final concentration of 1,2M cells/mL.

The LPS (LPS from *E.coli* SEROTYPE 0111:B4, Ref**:** L-2630, 100mg. Stock @ 25mg/mL in complete RPMI/-80°C), IL4 (Recombinant murine IL-4, Peprotech, Ref: 214-14,10µg. Stock @ 5µg/µL in PBS-1%BSA/-80°C) and IFNγ (Recombinant murine IFNγ, Peprotech, Ref: 315-05,10µg. Stock @ 100µg/µL in RPMI+10%FCS/-80°C) are titrated in the lab and are added to the 2X induction media according to each batch.

B CELL STIMULATION

Date :

Mice :

Dissection and Purification :

Spleen dissection

Lysis of red blood cells

CD43- cell purification

Cell number

Cells in spleen

_____

CD43-cells

_____

CFSE+ cells

_____

Volume

Sufficient for $2,4x10^6$/mL

_____

Number of wells

Vol of RPMI				
LPS (250 X)				
LPS+IL4 (500 X)				
LPS+IFNγ (500 X)				
LPS+IL5+TGFβ				

FACS

ANTIBODIES	**No of Samples**	**Total Volume**	**FACS Buffer (µl)**	**Antibody (µl)**	**Fc Block Diluted 1 :50 (µl)**
IgG1 Biotin (A 85-1, BD) diluted 1:500					
IgG2a Biotin (Igh-1b 5.7, BD) diluted 1:250					
IgG2b Biotin (RMG2B1,Biolegend) diluted1/500					
IgG3 Biotin (R40-82,BD) diluted 1/250					
Streptavidin-PE (Immuno D4d) diluted 1/500					

B CELL STIMULATION-TRANSDUCTION

Date :

Day 1 :

- Count and plate BOSC 23 (or 293T) cells, in DMEM (1g/L glucose) + 10% FCS 332 + PS @ 0.3-0.4x10^6/2 mL/ transfection.
  I usually split my cells 1/2 the day before to have them fresh for the transfection
  Cells should be well trypsinized with no clumps to get a homogeneous confluency

Required Density : x10^6/mL Cell Count : x10^6/mL

# of wells : Dilution:

Day 2 : Prepare the following mix per construct, per well:

PS: Quality of DNA matters - MaxiPrep DNA works much better than MiniPreps !!

Optimum Medium (94 µl)					
FUGENE (6 µl)					
Construct (1.4ug/mL)					
pCL Eco (0.6ug/mL)					

- Reagents are mixed in this order, vortexed and incubated at RT/15 min.
- Added slowly 100µL/well of BOSC23/293T cells (**from here on consider the cells and supernatants as potentially dangerous**) and incubate 37°C/5% CO_2 in the virus room.

Day 3 : (Protocol as described for B cell stimulation)

Sacrifice mice

Stimulation with LPS + Il 4 (IgG1)

Plate at a density of $2.4x10^6$ cells/well (i.e $1.2 x10^6$/mL)

Day 4 : (48 hrs after Transfection)

- Viral supernatants were recovered and filtered witha 5mL syringe through a 0.45μM syringe filter, to remove all cellular debris. (I keep these cells to FACS and see what the % of GFP was and put the rest in one volume of 2X Laemilli buffer, just in case).
- Volume was adjusted to 4mL with DMEM (1g/L glucose) + 10% FCS 332 + PS
- To this add 80mL HEPES (1M) and 10mL Polybrene (4mg/mL) and mix well by pipetting up and down.
- B cell medium is carefully aspirated and replaced with 2mL of the viral sup`s.

> This is tricky, so I usually take my cells and leave them in the virus room incubator in the morning the day I have to transduce them so that they settle to the bottom and its easier to pipette out the medium.

> When I remove the medium, I tilt the plate at an angle and try not to put it back down until I finish with it, to keep all the cells at one end.

Spinfection :

- Centrifuge at 2500 RPM/ RT/1.5 hr/ without breaks.
- Add LPS+IL4 to the cells and leave at 37°C / 5% CO_2.

> Here again, I DO NOT add the LPS+IL4 during the spinfection, I leave the cells to recover for about an hour or two and then add the goodies.

Day 5 : (24 hrs after transduction)

- The viral sup`s are carefully replaced with 2mL of B cell medium and leave at 37°C/5% CO_2.

Day 6 an d 7: (48 & 72 hrs after transduction, 72 & 96 hrs after stimulation)

- Harvest the cells , wash 1X in ice-cold PBS and prepare for FACS (same as before).
- FACS for IgG1.
- Keep an aliquot of cells for Western blot and/or RNA depending on the experiment.

B CELL STIMULATION-AMAXA TRANSFECTION

Date :

Mouse B cell Nucleofector® kit (Lonza, Cat. No. VPA-1010, 25 reactions)

Kit contains:

- Mouse B Cell Nucleofector® Solution 2.25 mL
- Supplement 500mL
- pmaxGFP® Vector (0.5 μg/μL in 10 mM Tris pH 8.0) 30 μg
- 25 Certified sterile cuvettes
- 25 Plastic sterile pipettes

Storage and stability: Store Nucleofector® Solution and Supplement at 4°C. For long-term - pmaxGFP® Vector is stored at -20°C.

Once the Nucleofector® Supplement is added to the Nucleofector® Solution it is stable for three months at 4°C. The supplement has to be added at one shot, it cannot be done in aliquots, so make sure that the entire supplement is added to the Nucleofector® Solution.

Things that you will need:

- Nucleofector® Device (I use the one from the HG lab – this is the NuFe II. SC/PK also have the same machine)
- Supplemented Nucleofector® Solution at RT (I add the Supplement and store it at 4°C, so I take it out about 3 hr before I have to start the transfection. The cells don't like cold stuff!)
- Supplied cuvettes and plastic pipettes
- pmaxGFP® Vector (this is the positive control and it always gives me ~ 90% GFP+ and 70-80% survival. I also use one of my EGFP constructs as a second postive control, to have a plasmid that I have made and not a commercial one!)
- The plasmid that you need to transfect, purified (all my MaxiPreps are made with the Qiagen endotoxin free kit).
- 12-well culture dish (you can use smaller wells if you want, but with the amount of death post-transfection it becomes too crowded, so I use the 12-well plates – this I take from the SC/PK lab as we don't buy them in tissue culture).

Plating medium:

RPMI 1640 with 2mM GlutaMAX^{TM} supplemented with 10% iFCS (I use the same K3399 serum that I use for my B cell cultures), 50 μM β-mercaptoethanol, 1% ITS (Insulin-Transferrin-Selenium, we get this in the cell culture service) and LPS+IL-4 (as per our conditions).

Pre Nucleofection® medium:

RPMI 1640 with 2mM GlutaMAX^{TM} supplemented with 10% iFCS (I used the K3399 serum that I use for my B cell cultures), 50 μM β-mercaptoethanol and LPS+IL-4 (as per our conditions).

The cell culture service does not buy RPMI1640+GlutaMAX, so we need to order it through them.
PS: Now Lonza sells the media themselves:
Culture medium I: RPMI1640 (Lonza; Cat. No. 12-167F) supplemented with 10% FCS, 2mM UltraGlutamine I (Lonza, Cat. No. 17-605E/U1), 50 μM β-mercaptoethanol, 1% ITS (Sigma) and LPS (50 μg/mL) if desired.
Culture medium II: RPMI 1640 (Lonza; Cat. No. 12-167F) supplemented with 10% FCS, 2mM UltraGlutamine I (Lonza, Cat. No. 17-605E/U1), 50 μM β-mercaptoethanol and 50 μg/mL LPS (Sigma).

Day 1: B cell isolation and Pre Nucleofection:

The protocol is the same as our B cell isolation, except that cells processed for Amaxa should not be lysed with ACK – this is important as the shock of lysis can affect the efficiency of transfection! – so NO RBC LYSIS.

- Sacrifice mice, isolate $CD43^-$ B cells in PBS-BSA and count.
- Plate at a density of $2.4x10^6$ cells/well (i.e $1.2\ x10^6$/mL) in pre-Nucleofection media with LPS+IL-4 for 24 hours (as per our conditions, here I plate them in 6 well or 10cm plates depending on the number of transfections I am doing).
- On day 2, the B cells should have formed visible clusters, showing the blast formation has been induced successfully.

Day 2: Nucleofection:

Before I start, I prepare the cuvettes, set the program in the machine and pre-warm 1.5mL of plating media (WITHOUT LPS+IL-4) in 6-well plates as well as a FALCON tube with the same media to recover the cells post-Nucleofection.

- Harvest the cells count and make $5x10^6$ cell aliquots into 14mL FALCON tubes per transfection. (I use $5x10^6$ cells though the protocol says $3x10^6$, but you loose a lot of cells. Also, don't pipette too much and use Trypan-blue to count).
- Centrifuge at 90xg/ 10 min/ RT.
- Aspirate the supernatant (Make sure that no residual medium covers the cell pellet).
- Break the pellet very gently, add 100μL of Nucleofection® Solution and pipette very carefully. (Avoid storing the cell suspension longer than 15 minutes in Nucleofector® Solution, as this may reduce cell viability. I do one reaction at a time).
- Combine 100 μl of cell suspension with 2-4 μg DNA and transfer cell/DNA suspension into a cuvette (sample must cover the bottom of the cuvette without air bubbles). Close the cuvette with the cap.

One reaction contains:

5 x10^6 cells

100 μL Mouse B Cell Nucleofector® Solution

2-4 μg plasmid DNA (my plasmids are all @ 1 μg/μL in the Qiagen Endofree TE buffer)

or 2 μg pmaxGFP® Vector (control)

- Apply the Nucleofector® Program **X-001** (the kit says Z-001, but I used the **X-001** after several tests – this one gives optimal transfection efficiency and viability).
- Take the cuvette out of the holder once the program is finished (the cuvette holder automatically turns around when you apply the program and when its finished).
- Add 500μl of the pre-warmed plating medium to the cuvette and gently transfer the sample immediately into the 6-well plate (final volume 2mL/sample) using the supplied pipettes and avoid repeated pipetting of the cells. (I DO NOT add LPS+IL-4 into the medium at this stage).
- Incubate the cells for about 2-3 hrs and let them recover. Then count again with Trypan-blue, centrifuge at 90xg/5min/RT and re-plate them at 1.2x10^6 cells/mL in plating medium with LPS+IL-4 in 12-well plates (as per our conditions, I also take an aliquot here to do a quick FACS to check transfection efficiency and viability).
- FACS for GFP, IgG1 with ToPro3 (Molecular probes) at 48, 72 and 96 hrs, keep cells for WB.

SORTING GC B CELLS TO ANALYZE SHM

Date :

MICE:

Mice No. & Genotype	Total Cell No.	B220+ Cell No.

IMMUNIZATIONS :

- Inject mice with NP-CGG (Biosearch Technologies Inc, Ref : N-5055-5). – Stock of NP-CGG is made in 1X PBS @ 1.5 mg/mL and stored at -80°C.
- For each mouse, make an emulsion of 1 volume of NP-CGG with 1 volume complete Freunds adjuvant (CFA) and inject 25μL/footpad/mouse (75μg/mouse).

  (Michael does the injections and I always bleed my mice one day before the immunizations and a day before I sacrifice them – i.e : Day -1 and Day 9).

ISOLATION OF GC B CELLS AND CELL SORTING :

Day 10 post-immunization

ALL PROCEDURES ARE DONE UNDER NON-STERILE CONDITIONS

- Sacrifice mice and place belly up, isolate the lymph nodes (and depending on the experiment, the spleen as well)
- Pool and smash the LN's (& spleen's) into a 10mm culture dish with 10mL PBS – BSA through a cell strainer 0.5μM and make a single cell suspension.

- Centrifuge 1200 RPM/5mins/RT
- Lysis of RBS's with ACK (as per standard conditions - 1 min/RT), leave standing for a few mins and take supernatant, count total cell number.

(KEEP AN ALIQUOT FOR FACS ON TOTAL LN's AND SPLEEN)

- Pellets are resuspended in ____ μL of B220 beads (Miltenyi Biotech, Ref : 130-049-501) & incubated at 4°C /20mins with mixing. (bead volume depends on cell number)
- Wash 1X with 14 mL of PBS – BSA, resuspend pellet in ____ μL of PBS-BSA.
- Equilibrate one MS column (for LN's) and one LS column (for spleen's) for each genotype. Pass the cell-bead suspension through the column and discard the flow through.
- Use piston and collect the bound fraction of cells, these should be enriched B200+ cells.
- Count the cells.

Staining of cells for sort, in 100μL :

GL7 – FITC (BD Pharmingen, GL7)- 1/50
FAS 95 – PE (BD Pharmingen, Jo2) - 1/100
B220 Cy5 (IGBMC, RA3-6B2) - 1/50)

- Wash 2X in the eppendorf, then transfer to a 14mL FALCON tube and wash 1X with 10mL of PBS-BSA.
- Resuspend the pellet in the required volume for sort (~30x10^6 cells/mL)

Sort for : B220+ GL7+ FAS 95+ cells (GC B Cells)
B220+ GL7- FAS 95- cells (Control)

- Wash cells well in 1X PBS (cold) and resuspend in 200uL of PK buffer O/N at 55°C to prepare gDNA. (Back extract the gDNA, see below).

PCR, CLONING AND SEQUENCE ANALYSIS:

- Genomic DNA from sorted germinal center ($B220^+$ $GL7^+$ Fas^+) B cells is amplified by PCR using Pfu Turbo DNA polymerase (Agilent Technologies, Ref : 600250) from 5,000 sorted cell equivalents.
- I did eight reactions that were pooled for cloning. Cloned PCR products into the pCR TOPO 4 (Invitrogen, TOPO® TA Cloning® Kit for Sequencing).

- Positive clones should be identifed by screening with the J_H3 and J_H4 probes and sequence using M13 universal primers.

Method :

94°C - 2 min
94°C - 30 sec
60°C - 30 sec | 25X
72°C - 2 min
72°C - 5 min
4°C - HOLD

PRIMERS :

BR 448 - VH588/FR3-Fwd – GGAATTCGCCTGACATCTGAGGACTCTGC

BR 451 - J_H4 Intron-Rev 2 – GACTTTTGCAGGCTCCACCAGACC

BR 452 - J_H4 Probe - TATGCTATGGACTACTGG

BR 453 - J_H3 Probe – CCTGGTTTGCTTACTGG

PCR protocol for IgH/c-myc translocations detection
(derivatives of chromosome 12)

Date :

Protocol from Isabelle Robert

- Genomic DNA is prepared from stimulated B lymphocytes, purified by phenol/chloroform extraction and ethanol-precipitated (as per our conditions, see below).
- Genomic DNA (500 ng per assay) is submitted to a first round of long expand PCR (Expand long template PCR system kit, Roche, Ref : 1681834).

PCR1, total volume : 50 µL

MIX	1X	X
10X Buffer S3 Roche *(long expand kit)	5µL	
10 mM dNTP's	2.5µL	
50 µM (P1) - BR175	0.3µL	
50 µM (P2) - BR176	0.3µL	
50 µM (P3) – AID Fwd	0.3µL	
50 µM (P4) – AID Rev	0.3µL	
genomic DNA 100 ng/µL	5µL	
DNA pol Long Expand Roche	0.4µL	
Water	35.9µL	

* pre-warmed to 56°C

Method - touchdown PCR :

96°C	5 min	
94°C	15 sec	25 X
62°C	15 sec	
68°C	7 min + 20 sec/cycle	
68°C	15 min	
4°C	HOLD	

- Nested PCR is then set up using 1 µl from PCR1.

PCR2 (nested), total volume : 25 μL

MIX	1X	X
10X Buffer S3 Roche *(kit long expand)	2.5μL	
10 mM dNTP's	12.5μL	
50 μM (P1) - BR177	0.15μL	
50 μM (P2) - BR178	0.15μL	
DNA from PCR 1	1μL	
Taq VH	0.5μL	
Water	19.45μL	

* pre-warmed to 56°C

Method:

96°C	5 min	
94°C	15 sec	25 X
62°C	15 sec	
72°C	8 min	
72°C	15 min	
4°C	HOLD	

- In general, we load 20μL from PCR2 on a 0,8% agarose gel and blot onto GeneScreen Nylon membranes (Perkin Elmer, Ref : NEF1017001PK) with 0.4N NaOH.
- For Southern blot, we sequentially probe membranes using radiolabeled myc and IgH primers in CHURCH buffer.

PRIMERS

PCR1

BR175	5'-TGAGGACCAGAGAGGGATAAAAGAGAA-3' (Igmu3')
BR176	5'-GGGGAGGGGGTGTCAAATAATAAGA-3' (Myc3')
AID Fwd	5'-GGACCCAACCCAGGAGGCAGATGT-3'
AID Rev	5'-CCTCTAAGGCTTCGCTGTTATTACCAC-3'

PCR2

BR177	5'-CACCCTGCTATTTCCTTGTTGCTAC-3' (Igmu3' nested)
BR178	5'-GACACCTCCCTTCTACACTCTAAACCG-3' (Myc3' nested)

Southern Blot

- The membranes are pre-hybridized O/N at 60°C with 500 μg of sheared salmon sperm in 15mL of CHURCH buffer.
- Probe synthesis :

MIX	1X	X
10X PNK buffer	2μL	
50 μM primer	1μL	
γATP	1μL	
Water	15μL	
Polynucleotide kinase	1μL	

  - Incubate 37°C/1hr (in the radioactivity chamber), quench the reaction with 180μL of TE buffer (we use the eltuion buffer from the Qiagen MaxiPrep kit) and purify the probes with the SIGMASpin™ post-reaction clean-up columns (SIGMA Aldirch, Ref : S-5059).
  - Add the probes to the pre-hybridized membranes and leave O/N at 60°C.
- Wash the membranes with ~200mL of the following buffers in the order given below:

  2X SSC + 0.1% SDS – two washes/15min/60°C

  0.2X SSC + 0.1% SDS– two washes/15min/60°C
- After the washes, mount the membranes onto a clean surface and expose either with a film between two intensifier screens at -80°C or using the phosphor imaging plate and reveal using the Typhoon™ imager (GE healthcare, on the 2nd floor).
- We use a thin layer of cardboard covered tightly in SARAN wrap and taped down to have a neat surface for exposure and to avoid any bubbles.

SOUTHERN BLOT PROBES :

BR185 5'-GCAGCGATTCAGCACTGGGTGCAGG-3' (Myc)

BR186 5'-CCTGGTATACAGGACGAAACTGCAGCAG-3' (Igmu)

MEMBRANE STRPIPPING :

- Boil 0.5% SDS and to the membranes when hot. Leave the membranes (with slow shaking) until the SDS reaches RT (usually O/N), expose again to check and the blots can be re-hybridized.

BUFFERS :

20X SSC: (pH 7.0)

20X SSC	**1 litre**
3M NaCl	175 g
0.3M Na_3-citrate.2H_2O	88 g

CHURCH BUFFER: (pH 7.0)

CHURCH	**1 litre**
1M Sodium phosphate (pH 7.2)	450 mL
20% SDS	350 mL
0.5M EDTA	2 mL
dd H_2O	198 mL

Sμ MUTATION ANALYSIS

Date :

- Sacrifice mice, isolate $CD43^-$ B cells in PBS-BSA and label with CFDA-SE for 10 min at 37°C, as described before.
- Plate at a density of $2.4x10^6$ cells/well (i.e $1.2 x10^6$/mL, as per our conditions, I plate them in 10cm plates with LPS+IL-4).
- Cells are harvested 72 hr post-stimulation for the sort.

CELL SORTING:

- Harvest cells from culture, centrifuge 1200 RPM/5mins/RT and count.
- Wash 1X with 14 mL of PBS and stain for sort.

Staining of cells for sort, in 200μL of PBS-BSA :

anti IgM-Cy5 (Jackson ImmunoResearch) - 1/50

anti IgG1-Biotin (BD Pharmingen, A 85-1) - 1/500

Streptavidin-PE (Immuno D4d) - 1/500

- Wash 2X in PBS-BSA and resuspend the pellet in the required volume for sort (~$30x10^6$ cells/mL)

Sort for : IgM^+ $IgG1^-$ cells

IgM^- $IgG1^+$ cells (Control)

- Wash cells well in 1X PBS (cold) and resuspend in 200μL of PK buffer O/N at 55°C to prepare gDNA. (Back extract the gDNA).

PCR, CLONING AND SEQUENCE ANALYSIS – SANGER SEQUENCING:

- 250ng of genomic DNA from sorted cells is amplified by PCR using the Expand long template PCR system (Roche, Ref : 11681834001) in a 50μL reaction. – I used the expand long template, because I had problems with the Pfu Turbo doing these experiments.
- I did eight reactions that were pooled for cloning. Cloned PCR products into the pCR TOPO 4 (Invitrogen, TOPO® TA Cloning® Kit for Sequencing).
- Positive clones should be identifed by screening and sequence using M13 universal primers.

  PRIMERS :

  BR 377 - 5μ3– ATCGAATTCAATGGATACCTCAGTGGTTTTTAATGGTGGGTTTA

  BR 540 - SμR5– GCGGCCCGGCTCATTCCAGTTCATTACAG

Method :

94°C - 2 min
94°C - 10 sec
60°C - 30 sec — 10X
68°C - 1 min
94°C - 10 sec
60°C - 30 sec — 15X + 20 sec increase/cycle
68°C - 1 min
68°C - 7 min
4°C - HOLD

PCR, CLONING AND SEQUENCE ANALYSIS – HIGH THROUGHPUT SEQUENCING:

- 250ng of genomic DNA from sorted cells is amplified by PCR using the Platinum Taq DNA polymerase high fidelity (Invitrogen, Ref : 11304-011) in a 25μL reaction.
- I did eight reactions that were pooled and purified with AmPure beads (Agencourt), quantified using Quan-iT Pico Green (Invitrogen) – the quantification had to be done at the 1st floor with Bernard JOST.
- Mix the PCRs in equimolar ratios prior to sequencing with a 454 GS FLX sequencer (Roche; GATC).

PRIMERS :

BR 629 – A1
CCATCTCATCCCTGCGTGTCTCCGACTCAGaacaAATGGATACCTCAGTGGTTTTTAATGGTGGGTTTA

BR 630 – A2
CCATCTCATCCCTGCGTGTCTCCGACTCAGtacgAATGGATACCTCAGTGGTTTTTAATGGTGGGTTTA

BR 631 – A3
CCATCTCATCCCTGCGTGTCTCCGACTCAGctgaAATGGATACCTCAGTGGTTTTTAATGGTGGGTTTA

BR 632 – A4
CCATCTCATCCCTGCGTGTCTCCGACTCAGtgccAATGGATACCTCAGTGGTTTTTAATGGTGGGTTTA

BR 633 - A5
CCATCTCATCCCTGCGTGTCTCCGACTCAGttgtAATGGATACCTCAGTGGTTTTTAATGGTGGGTTTA

BR 634 - B1
cctatcccctgtgtgccttggcagtctcagGCCATGCTTTTTAGATCCTCGCTTAC

Method :

94°C - 2 min, 30 cycles of 94°C - 15 sec, 60°C - 15 sec, 68°C - 1 min.

Sμ-S$_X$ JUNCTION ANALYSIS

Date :

- Sacrifice mice, isolate CD43$^-$ B cells in PBS-BSA and plate at a density of 2.4x10^6 cells/well (i.e 1.2 x10^6/mL, as per our conditions, I plate them in 10cm plates with LPS+IL-4).
- Cells are harvested 72 hr post-stimulation.
- Wash cells well in 1X PBS (cold) and resuspend in 200μL of PK buffer O/N at 55^{o}C to prepare gDNA. (Back extract the gDNA).

PCR, CLONING AND SEQUENCE ANALYSIS – SANGER SEQUENCING:

- 250ng of genomic DNA from sorted cells is amplified by PCR using the Expand long template PCR system (Roche, Ref : 11681834001) in a 50μL reaction.
- I did eight reactions that were pooled for cloning. Cloned PCR products into the pCR TOPO 4 (Invitrogen, TOPO® TA Cloning® Kit for Sequencing).
- Positive clones should be identifed by screening and sequence using M13 universal primers.

PRIMERS :

Sμ-Sγ1 junctions

BR 377 - 5μ3– ATCGAATTCAATGGATACCTCAGTGGTTTTTAATGGTGGGTTTA

BR 378 - γ1R– gtcgaattCAATTAGCTCCTGCTCTTCTGTGG

Sμ-Sγ3 junctions

BR 379 - μ3H3– AACAAGCTTGGCTTAACCGAGATGAGCC

BR 380- γ3-2 – aacaagctTACCCTGACCCAGGAGCTGCATAAC

Method :

94°C - 2 min

94°C - 10 sec
60°C - 30 sec
68°C - 1 min
} 10X

94°C - 10 sec
60°C - 30 sec
68°C - 1 min
} 15X + 20 sec increase/cycle

68°C - 7 min

4°C - HOLD

Cross link Chromatin IP (X-ChIP) protocol

Date :

Adapted from the Upstate-Millipore protocol with modifications from Mac and myself - for mouse B cells (either resting or stimulated).

- Sacrifice mice, isolate $CD43^-$ B cells in PBS-BSA and count. – if you need resting cells proceed with the crosslinking directly on Day 0.
- Plate at a density of $2.4x10^6$ cells/well (i.e 1.2 $x10^6$/mL, as per our conditions, here I plate them in 6 well or 10cm plates depending on the number of ChIPs I am doing).
- Cells are harvested either at 48 or 60-72 hr post-stimulation and then crosslinked.

Crosslinking and sonication:

Things that you will need to prepare before hand for crosslinking:

- Pre equlilibirate the water bath to 37°C
- Pre-warm 14 mL FALCON tubes with 5 mL PBS-BSA or 50 mL with 10 mL PBS BSA.
- Prepare the 16% PFA (ideally should be under the chemical hood, but I do it on the bench).
- Cool down centrifuges.
- Get liquid nitrogen.
- Prepare ice-cold PBS 1X PIC 1X (for washes post crosslink).
- Timers set to 10min, each.

Procedure – crosslink:

NOTE: As resting cells are already processed in PBS BSA and counted just spin and keep the pellet. For stimulated cells, wash 1X with ice-cold PBS, resuspend in 1X ice-cold PBS, count cells and aliquot. I try to NOT crosslink and sonicate more than 50-70 $x10^6$ cells in the same tube, so if you have more than this number – try and split the cells in such a way that you have equivalent cell numbers in each experiment, this makes the sonication more homogenous.

Volumes used to crosslink:

1-70 $x10^6$ cells in 5 mL PBS BSA (in 14 mL FALCON tube)

> 70 $x10^6$ cells in 10 mL PBS BSA (in 50 mL FALCON tube)

- Harvest and count cells (either resting or from culture, at time point required).
- Centrifuge at 1200 RPM/5min/ 4°C (depending on the number of cells, use either a 14 or 50 mL FALCON tube).

- Wash 1X with ice-cold 1X PBS, if you are dealing with stimulated cells (I always count after the wash). If they are resting cells, they are already in PBS-BSA (see NOTE above).

  DO NOT tap the pellet, and all steps from here on need to be done quickly.
- Use 1 mL of the pre-warmed PBS BSA to resuspend the cells and transfer the cells into the remaining PBS BSA.
- Add **immediately** 320μL of 16% PFA/5 mL, or 640μL of 16% PFA/10 mL (Electron microscopic sciences, Ref: 15710), start timer for 10 min, vortex for 5 sec, stop and vortex again for 5 sec, mix by inversion 10 times and put the tube in the water bath for 10 min.
- Quench the reaction with 550μL of Glycine 1.25 M (RT, pH is not adjusted)/5mL, or 1100μL/10 mL (final concentration 125 mM). Vortex for 5 sec, stop and vortex again for 5 sec, add ice-cold PBS 1X (+PIC 1X) up to max volume and mix by inversion.
- Centrifuge at 1200 RPM/5min/ 4°C (do this immediately, DO NOT leave the tube standing until you have finished all your samples).
- Repeat this wash and at this stage you can sonicate directly or aspirate the PBS, snap freeze the pellets and store at -80°C for later use. (I have always frozen cell pellets and sonicated the next day).

I always space my samples by two mins each, to have enough time to process them.

Procedure – sonication (Vibracell 75043, H. Gronemeyer's lab) :

- Thaw cell pellets on ice.

While the pellets thaw, prepare the sonication ice bucket with the sonicator rack (HG's lab, can hold upto 8x1.5mL Eppendorf tubes), fill the bucket with ice and a little water so that the rack is horizontal.

- Resuspend cell pellets in 480μL of Lysis Buffer (with PIC 1X, see below for buffer compositions), pipette up and down carefully and transfer the Eppendorf tubes to the sonicator rack.
- Leave for 10min on ice (lysis, during this incubation I clean the sonicator probes and set the parameters).
- Place the rack in a such a way that the probes do not touch the bottom or the walls of the tubes (avoid splashing into the tube covers).

Parameters for sonication :

For resting cells - 30 + 30 cycles (with a break in the middle, where I change the ice)

For stimulated cells - usually 45 cycles

Amplitude - 30%, timer at 5 min (30 cycles) or 7 min 30 sec (45 cycles)

Pulser - 10 sec ON 50 sec OFF

- Once sonication is done, centrifuge at 14000 RPM/10min/ 4°C.
- Transfer the "sonicated chromatin" into a new tube, snap-freeze in liquid nitrogen and store at -80°C.
- Keep aside the volume required for 1 million cell equivalents (or simply 10μL) to test sonication efficiency.

Procedure – de-crosslinking:

- To the sonicated chromatin, add 8μL of 5M NaCl (final conc 200mM) + ChIP lysis buffer upto 200μL and incubate O/N at 65°C.
- Add 1μL of PK (20 mg/mL) for 2 h at 45°C
- Extract DNA either by phenol/chloroform and O/N ethanol precipitation or the Qiaquick PCR purification columns from Qiagen.
- Resuspend the final DNA pellets in 25μL of $T_{10}E_1$ (1:6 diluted) and load on a 2.5 % agarose gel to check chromatin size. (I run the gel for not too long to be able to see the size of bulk of sheared chromatin).
- This chromatin is ready to now immunoprecipitate.

I have used chromatin that is ~300-500 bp in size for all my ChIP experiments. If you sonicate for ChIP-Seq, the size has to be between 200-300 bp.

Chromatin Immunoprecipitation (ChIP) :

For ChIP, all tubes are precoated with PBS-BSA (minimum 2 hrs/RT or O/N @ 4°C)

Things that you will need to prepare before hand for the IP:

- Precoat 14 mL tubes and 2 mL tubes with PBS-BSA.
- Thaw the sonicated chromatin on ice (takes 2-3 hours).
- Make Protein A/G bead slurry FRESH (Sigma-Aldirch, Ref: P-9424, GE Healthcare, Ref : 17-0618-06) and wash 3X in ChIP IP buffer – the beads used at every step are a 50% slurry.
- Block Protein A/G beads with 0.2 mg/mL sheared salmon sperm DNA+0.5 mg/mL BSA, for 2 h to O/N at 4°C, prepare sufficient volume for preclearing and IP.

(Beads + 20μL BSA [NEB] + 50 μl ssDNA [@10mg/mL, sheared] + ChIP IP buffer to 1 mL)

Procedure - Chromatin Immunoprecipitation (ChIP) :

PRE-CLEARING

- Dilute 2X the sonicated chromatin with ChIP dilution buffer - 500μL of sonicated chromatin + 500μL of ChIP dilution buffer (ideally it should be diluted 1:10, but Mac used to use these conditions, I followed them and they worked).

- Pre-clear the entire volume with 40μL of blocked Protein A + 40μL of Protein G - 2hr/4°C (IntelliMixer, C1/20RPM).
- Depending on the antibody, take the volume corresponding to 3-5x10^6 cells/IP, transfer it to a 2 mL Eppendorf and make up the final volume with ChIP IP buffer. (I usually do my IP in 1 or 1.5mL).
- Keep 1/10^{th} of the IP (in terms of cell number) as inputs (i.e: 0.3-0.5x10^6 cells) and adjust the volume up to 500μL with ChIP dilution buffer and freeze at -20C°. (I process them along with the IP DNA).

IP, WASHES AND DNA EXTRACTION

- To the pre-cleared chromatin, add corresponding Ab's (see below for details) and leave O/N at 4°C (IntelliMixer, C1/20RPM).
- Add 60μL of blocked Protein A or G beads (depending on the source of Ab), incubate 5 hr/4°C (IntelliMixer, C1/20RPM).

NOTE: All washes are done in the cold room, with ice-cold buffers (+1X PIC)

Spinning faster than 900RPM can break the beads and give a lot of background.

- Wash the protein A or G agarose/antibody/protein complex by mixing with 900μl of the following buffers in the order given below for 5 min each:

  Low Salt Immune Complex Wash Buffer, one wash
  High Salt Immune Complex Wash Buffer, one wash
  LiCl Immune Complex Wash Buffer, one wash
  TE ($T_{10}E_1$), two washes

I do the washes in the cold room on the IntelliMixer, C1/20RPM and centrifuge 900RPM/4ºC/2 min after each wash.

- After the last TE wash, samples can be processed for either PCR or WB:

For WB - wash with 1X PBS and resuspend in 1X bead volume of 2X Laemmli, heat 95°C/10 min and store for further use.

For PCR - elute the immune complexes with 250μL of elution buffer at RT 15 min on the IntelliMixer (C1/80-85 RPM), repeat twice and pool the elutions.

(Make the elution buffer FRESH each time)

Follow the de-crosslinking protocol as described above:

- Add 20μL of 5M NaCl (final concentration 200 mM) and incubate O/N at 65°C, do not forget to treat the inputs in parallel.
- Add 1μL of PK (@20 mg/mL)/2hr/45°C and purify the DNA by phenol/chloroform extraction.

- Resuspend the final pellets, in 100µL $T_{10}E_1$ (diluted 1:6) – I used 100µL based on the number of PCRs I had to do in the end, people usually resuspend the DNA in 50-80µL.
- Resuspend the pellet for 15 min on the IntelliMixer (60°/70RPM) – I do this only to dissolve the pellet really well and I leave the DNA at 4°C O/N or a few hours on ice before I set a PCR.
- After this, you can directly use the DNA for PCR (I use 2µL of DNA in a 10µL PCR reaction).

ANTIBODIES

@H3 (Rabbit, Abcam ab1791): 3μg/3x10^6 cells
@H3K9K14Ac (Rabbit, Millipore 06-599): 3μg/3x10^6 cells
@H3K9me3 (Rabbit, Abcam ab8898): 3μg/3x10^6 cells
@KAP1 (Rabbit, IGBMC PF 64): 8μL of Ab/5x10^6 cells
@HP1γ (Mouse, IGBMC 2MOD1G6): 8μL of Ab/5x10^6 cells
Control IgGs:
rIgG (Bethyl Labs, stock 5 mg/mL, diluted at 1 mg/mL in PBS) : 3μL/5x10^6 cells
mIgG (IGBMC, stock 35 mg/mL, diluted at 1 mg/mL in PBS): 3μL/5x10^6 cells

ChIP BUFFERS

SDS Lysis Buffer
1% SDS, 50 mM Tris (pH 8.1), 10 mM EDTA (pH 8.1), 1x PIC
(Add PIC FRESH)

ChIP dilution buffer (I make 5X stock and dilute to 1X every time)
0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl

ChIP buffer (used to do the IP)
0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl

Wash buffers (I store all of them in the cold room)

(i) Low Salt Immune Complex Wash Buffer – (add SDS and PIC FRESH)
0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl

(ii) High Salt Immune Complex Wash Buffer – (add SDS and PIC FRESH)
0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl.

(iii) LiCl Immune Complex Wash Buffer – (add only PIC FRESH)
0.25 M LiCl, 1% IGEPAL CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris, pH 8.1.

(iv) TE Buffer – (does not need PIC)
10 mM Tris-HCl, pH 8.0, 1 mM EDTA

Elution buffer – (I dilute a 0.5M stock of $NaHCO_3$ with 20% SDS FRESH each time)

1%SDS, 0.1M $NaHCO_3$

Crosslink quenching solution

Glycine 1.25 M

NOTE:

To test/titrate a new Ab:

- Test different cell numbers and antibody quantities.
- I usually test 2 quantities : 5 and $10x10^6$ cells and 2 different antibody quantities : recommended amount in paper/by colleagues/Ab data sheet and twice the quantity.
- Control are ChIP with beads alone or with rIgG/mIgG on the highest cell number and highest quantity of Ab.
- Resuspend beads in Laemmli, after 2nd TE wash and analyze by WB.

BIOTIN END LABELING & TdT DEPENDENT PCR

Date:

Adapted from Doi et al, PNAS, 2009/Ju BG et al, Science, 2006 with my modifications - for mouse B cells (either resting or stimulated).

- Sacrifice mice, isolate $CD43^-$ B cells in PBS-BSA and count. – if you need resting cells proceed directly on Day 0.
- Plate at a density of $2.4x10^6$ cells/well with LPS+IL-4 (i.e 1.2 $x10^6$/mL, as per our conditions, here I plate them in 10cm plates depending on the number of reactions I am doing).
- Cells are harvested either at 48 hr post-stimulation.

IT IS IMPORTANT TO DO ALL THE CELL COUNTS WITH TRYPAN-BLUE

- Harvest and count cells (48 hrs post stimulation).

  Cell Number: 1. _____
  2. _____
  3. _____
  4. _____

- Wash cells with 1X ice cold PBS and adjust concentration to 1-$2x10^7$ cells/ml.

  Buffer Volume: 1. _____
  2. _____
  3. _____
  4. _____

- Add 5 mL of lympholyte-M (Cedarlane CL-5030) to a 15 mL FALCON tube.
- Carefully layer 5 mL of the cell suspension over the lympholyte-M with as little mixing as possible, a distinct interface will be formed.(I do this with a Pasteur pipette and the pipette aid set on “S”)
- Centrifuge 1000-1500xg /20 min/RT. (I centrifuge at 1250xg)
- Using a Pasteur pipette remove the cells from the interface and transfer to a new tube.
- Wash cells with 1X ice cold PBS and count.

  Cell Number: 1. _____
  2. _____
  3. _____
  4. _____

- Resuspend 5X10^6 cells in 1mL of Fixation Buffer/15mins/RT/rotation. (I do it in a 14 mL FALCON)

  Buffer Volume: 1. _____
  2. _____
  3. _____
  4. _____

NOTE : At this stage, with the fixation buffer the cells will flake out, look like they have lysed and stik to the sides of the tube, but don't worry – they are fine (I have taken an aliquot to look at them under a microscope) and will pellet down with the washes.

- Wash cells with 1X ice cold PBS.
- Resuspend cells in 5mL of ice cold Buffer A
- Centrifuge 1200RPM /5 min/4°C.
- Resuspend cells in 5mL of ice cold Buffer B
- Centrifuge 1200RPM /5 min/4°C.
- Permeabilize nuclei in 5mL of ice cold Buffer C/30mins/on ICE.
- Centrifuge 1200RPM /5 min/4°C.
- Wash cells with 1X ice cold PBS and make aliquots of 5x10^6 cells equivalents.
- Wash cells with 1mL 1X ice cold TdT buffer (from NEB, comes with the enzyme).
- Resuspend each 5x10^6 cells aliquot in 100uL of 1X TdT buffer with 3μL of 1mM Biotin-16-dUTP (Roche, Ref : 11388908910) and 60U of TdT (NEB, Ref : M0315S). Incubate 37°C/ 1hr.
- Wash cells (1X) in 1mL Buffer D
- Resuspend cells in 250μL lysis buffer and incubate at 55°C O/N.
- Extract genomic DNA by Phenol-chloroform.
- Digest 20μg of genomic DNA with HindD III (NEB)/ 37°C O/N.

  10X NEB Buffer : 5uL
  10X BSA : 5uL
  gDNA (20μg) :
  H_2O :

- Resuspend the digested DNA in 50μL of TE buffer (Total volume 100μL) and capture biotinylated fragments with 20μL of streptavidin magnetic beads (Streptavidin MagneSphere paramagnectic particles, Promega) at RT/30 mins/ rotation.

Wash the beads 3X in 500μL of TE before use. DO NOT centrifuge. Prepare 30mins before it needs to be used, else the beads are destabilized.

- Wash beads (3X) with 300μL of TE (Tris-EDTA, pH 8)
- Resuspend the beads in 20μL of Tris-HCl (pH 8.0).
- Use 3μL of beads for a PCR reaction, with 6ng of genomic DNA as INPUT.

BUFFERS

Fixation Buffer: 3 g of bronopol, 3 g of diazolidinyl urea, 1.2 g of zinc sulfate heptahydrate, 0.29 g of sodium citrate dihydrate and 50 mM EDTA in 100 mL)

!!! Bronopol is a hazardous chemical – handle in the chemical hood and always store it in the cardboard box.

Buffer A: 0.25% TritonX-100, 10 mM EDTA, 10 mM Hepes (pH 6.5)

Buffer A	**250 ml**
1M HEPES (NaOH) pH 6.5	2.5 ml
500 mM EDTA (NaOH) pH 8	5 ml
20% TritonX-100	3.1 ml

Buffer B: 200 mM NaCl, 1 mM EDTA, 10 mM Hepes (pH 6.5)

Buffer B	**250 ml**
1M HEPES (NaOH) pH 6.5	2.5 ml
500 mM EDTA (NaOH) pH 8	500 µl
5M NaCl	10 ml

Buffer C: 100 mM Tris-HCl (pH 7.4), 50 mM EDTA, 1% TritonX-100

Buffer C (Permeablization)	**250 ml**
1M Tris-Cl pH 7.4	25 ml
500 mM EDTA (NaOH) pH 8	25 ml
20% TritonX-100	12.5 ml

Buffer D: 100 mM Tris-HCl (pH 7.4), 150 mM NaCl

Buffer D	**250 ml**
1M Tris-Cl pH 7.4	25 ml
5M NaCl	7.5 ml

Lysis buffer: 10 mM EDTA, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS,

(Add FRESH - 0.2mg/mL of proteinase K)

Lysis Buffer	**500 ml**
1M Tris-Cl pH 8	5 ml
500 mM EDTA (NaOH) pH 8	10 ml
20% SDS	5 ml
5M NaCl	15 ml

TE Buffer: ($T_{10}E_1$) 10mM Tris-cl (pH 8.0), 1mM EDTA

Tris-Cl(pH8.0)

* Buffers A, B and C are all used at 4°C .

pH 7.4 @ 4°C is pH 6.8 @ Room temperature, 1M HEPES pH 6.5 is set with NaOH on ice.

LIGATION-MEDIATED PCR

Date:

Protocol from Carol Schrader (Guikema., 2007, J Exp Med, Schrader., 2005. J Exp Med)

Isolation of viable cells by flotation on Lympholyte-M.

- Wash cells with PBS and adjust concentration to 1-2x10^7 cells/ml.
- Add 5 mL of lympholyte-M (Cedarlane CL-5030) to a 15 mL FALCON tube.
- Carefully layer 5 mL of the cell suspension over the lympholyte-M with as little mixing as possible, a distinct interface will be formed.
- Centrifuge 1000-1500xg /20 min/RT (I centrifuge at 1250xg).
- Using a Pasteur pipette remove the cells from the interface and transfer to a new tube.
- Wash the cells with PBS and adjust to 10^6 cells / 25μL (for LMPCR).

Isolation of Genomic DNA in Low Melting Point Agarose Plugs

- Prepare 1% LMP agarose in 40mM EDTA and cool at 37-42°C.
- Add 1X volume of LMP agarose (final agarose concentration 0.5%), mix 6-8 times with a wide-bore pipette and incubate on ice for 5'.
- Overlay the plug with 1 mL of lysis buffer (50 mM Tris pH8, 20mM EDTA; 1% SDS ; 1 mg/mL proteinase K) and incubate overnight @ 55°C.
- Incubate tubes on ice for 5 min.
- Spin 1 min @ 10 000 rpm/4°C before removing supernatant.
- Wash with 1 mL of 50 mM Tris pH8, 20mM EDTA by rotating for 1 hr/ RT°.
- Wash three times with 1 mL of 50 mM Tris pH8, 20mM EDTA by rotating for 5 min/RT.
- Repeat proteinase K treatmeant overnight @ 55°C.
- Wash three times with 1 mL of 50 mM Tris pH8, 20mM EDTA by rotating for 5 min/RT.
- Add 1 mL of 2mM PMSF (in 50 mM Tris pH8, 20mM EDTA) and rotate for 30 min/ 37°C.
- Wash three more times with 1 mL of 50 mM Tris pH8, 20mM EDTA by rotating for 5 min/4°C.
- Overlay the plug with 10μl of 1X Ligase Buffer and store at 4°C (DNA can also be stored in 1mL of TE).

Linker annealing

- Resuspend each linker @ 200μM in 10 mM Tris.
- Dilute each linker to 50μM in 1X Ligase Buffer.
- Mix 100μL of each linker (5 nmol) then add 30μL 10X ligase buffer and 70μL H_2O (16.7 μM final).

- Boil for 5 min.
- Cool down the heating block @ RT (approximately 2h).
- Store annealed linker @ -20°C.

Linker ligation

- Remove supernatant from agarose plug.
- Add 50μL of fresh 1x Ligase Buffer and heat @ 70°C for 10 min to melt agarose.
- Take 20μL (10^5 cell equivalents) of plug and transfer to a tube containing 3.5μL of 10X ligase buffer, 10μL of annealed linker (16.7μM ; 2.5μM final), 29.5μL of H_2O and add 2μL of T4 DNA ligase (2 Weiss units or 400 cohesive end ligation units). Final volume = 65μL.
- Incubate overnight @ 18°C using the PCR machine.

Linker ligation with T4 DNA polymerase fill-in

- Remove supernatant from agarose plug.
- Add 50μL of fresh 1x Ligase Buffer and heat @ 70°C for 10 min to melt agarose.
- Take 20μL (10^5 cell equivalents) of plug and transfer to a tube containing 3μL of 10X ligase buffer, 4μL of 2.5 mM dNTPs (200μM final), 21μL of H_2O and 2μL of T4 DNA polymerase. Final volume = 50μL.
- Incubate for 20 min/11°C then @ 75°C/20 min using the PCR machine.
- Add 1.5μL of 10X ligase buffer, 10μL of annealed linker (16.7μM ; 2.5μM final), 1.5μL of H_2O and 2μL of T4 DNA ligase. Final volume = 65μL.
- Incubate overnight @ 18°C using the PCR machine.

PCR

- Heat ligated DNA samples @ 70°C for 10 min.
- Dilute ligated DNA 1/5 in H_2O.
- Setup PCR for AID using 3-fold dilutions of ligated DNA (1/5) and starting with 5μL.
- If loading is homogeneous setup touchdown PCR with the following conditions and using primers listed below. For Sμ LM-PCR start with 5μL of ligated DNA (1/5) per 50μL PCR reaction and do 3X 3-fold dilutions. For downstream switch regions (less breaks) start with 15μL of ligated DNA (1/5). For the first experiment also test 5μL (Sμ) or 15μL (Sγ3 and Sγ1) of undiluted ligated DNA.
- Run PCR on a 1.3% agarose gel and transfer to nylon membranes (GeneScreen Plus; PerkinElmer) with 0.4 M NaOH.
- Hybridize with specific probes @ 55°C in Church buffer.

(these conditions did not work for me, so I changed them – see below)

PCR – My conditions

- Heat ligated DNA samples @ 70°C for 10 min.
- Dilute ligated DNA 1/5 and 10 more dilutions in H_2O.
- Setup PCR for AID or Sμ using DNA from dilution # 4 or 5 onwards until # 10 (i.e: the DNA diluted 1:135 times onwards).
- For downstream Sγ1 (less breaks) start with a lesser dilution or undiluted ligated DNA (this however never worked for me).
- Run PCR on a 1.3% agarose gel and proceede as above.

MIX	1X	X
10X Buffer S1 Roche *(kit long expand)	5μL	
10 mM dNTP's	1.75μL	
50 μM (P1)	0.3μL	
50 μM (P2)	0.3μL	
genomic DNA 100 ng/μL	5μL	
DNA pol Long Expand Roche	0.75μL	
Water	36.9μL	

* pre-warmed to 37°C

Method :

94°C - 2 min
94°C - 10 sec
60°C - 30 sec — 10X
68°C - 1 min
94°C - 10 sec
60°C - 30 sec — 25X – 30X + 20 sec increase/cycle
68°C - 1 min
68°C - 7 min
4°C - HOLD

NOTE: For the Sμ and Sγ1 PCRs I used the expand long template system, while for AID I used our home-made Taq (Taq VH) and Vincent's genotyping conditions.

PRIMERS AND PROBES:

Target sequence	Primers
5' of Sμ	BR 512 GCGGTGACCCGGGAGATCTGAATTC BR 514 GCAGAAAATTTAGATAAAATGGATACCTCAGTGG
3' of Sμ	BR 512 GCGGTGACCCGGGAGATCTGAATTC BR 515 GCTCATCCCGAACCATCTCAACCAGG
5' of Sγ3	BR 512 GCGGTGACCCGGGAGATCTGAATTC BR 518 AACATTTCCAGGGACCCCGGAGGAG or BR 512 GCGGTGACCCGGGAGATCTGAATTC
5' of Sγ1	BR 512 GCGGTGACCCGGGAGATCTGAATTC BR 521 CAGGGGAGTGGGCGGGGAGGAGATAT or BR 512 GCGGTGACCCGGGAGATCTGAATTC
AID^{cre} 2	BR 39 CACTCGTTGCATCGACCGGTAATG
AID^{cre} 3	BR 40 CCTCTAAGGCTTCGCTGTTATTACCAC

Target sequence	Probes
5' of Sμ	BR 516 AGGGACCCAGGCTAAGAAGGCAAT
3' of Sμ	BR 517 GGGCTGGCTGATGGGATGCCCC
5' of Sγ3	BR 520 CCTGGGGGATTATGGAAACCT
5' of Sγ1	BR 522 CTGCAGGGAAAGAGAAAGTAGGA
AID Probe	BR 51 ACGCTGGAGACCGATATGGACAG

NOTE: I have done PCR only for the 5' of Sμ (BR 512+ BR 514) and 5' of Sγ1 (BR 512+ BR 521) PCRs, the other primer pairs did not work very well.

PROTEIN EXTRACTION

Date :

Method 1: Single-step lysis for extraction of total cellular protein. ***Adapted from Klenova E, Methods 26(2003) 254-259.***

> This is the protocol I used for all my WBs and also for the γ-KAP1 immunoprecipitations.

- Count and harvest cells, wash 1X in 1mL ice-cold PBS.
- Resuspend the cells in ice-cold lysis buffer (I usually lyse $1x10^6$ cells in 100μΛ of lysis buffer).

Lysis buffer :

NP-40 lysis buffer:
20 mM Tris–HCl (pH 7.4),
150 mM NaCl
10 mM $MgCl_2$
2 mM EDTA
10% glycerol
1% NP-40
1 mM dithiothreitol (DTT, add FRESH)
1 mM phenylmethylsulfonyl fluoride (PMSF, add FRESH)
Complete protease inhibitors (Roche, Ref : 11873580001, add FRESH)
1 mM NaF (SIGMA-Aldirch, Ref : S-7920, add FRESH, only if doing phosphorylation studies)
1 mM Na_3VO_4 (SIGMA-Aldirch, Ref : S-6508 add FRESH, only if doing phosphorylation studies).

The exact concentration of NaCl can be varied (150–500 mM)

- Incubate the lysate on ice for 15–20 min with occasional gentle mixing (I mix by pipetting up and down, when I add the buffer and again after 10min).
- Centrifuge the lysate at 10,000g for 10 min at 4°C and recover the supernatant for subsequent WB or IP, if not store at -80°C for later use.

Method 2: Nuclear and cytoplasmic protein extraction. Adapted *from Roeder's Lab.*

> We grow cells either on the 2nd floor in the 37°C room in big ballon flasks for large volumes or in the T175 flasks for 1 or 1.5 L cultures.

> CH12 cells are usually maintained at ~1x10^6 cells/mL throughout and the first centrifugation is done at the 2nd floor in the tissue culture Sorvall centrifuge – so you have to make sure the bottles are sterilized and kept ready.

Day 1 : (Not counting the days taken for culture, which is one week if you do a 12L culture of CH12 cells)

- Count and harvest cells, centrifuge at 2000 RPM/15 min/4°C.
- Resuspend with 5x cell volume of ice cold PBS.
- Centrifuge cells at 3000 RPM/10 min/4°C and record cell volume.
- Resuspend cells with 4x cell volume of ice-cold buffer A.
- Incubate 10 min on ice.
- Centrifuge cells at 2000 RPM/10 min/4°C.
- Record swollen cell volume (should be between 1.5x - 2x),
- Resuspend cells with 2x initial cell volume of ice-cold buffer A.
- Dounce slowly 10 times on ice (Use rod B).
- Centrifuge cells at 3000 RPM/10 min/4°C.
- Recover supernatant containing cytoplasmic proteins (S-100).

Make up the buffer concentration of the S-100 to BC-100 at this stage, aliquot and snap freeze on dry ice. Store at -80°C for later use.

- Wash the pellet 1X with ice-cold buffer A and record nuclear pellet volume.
- Add equal nuclear pellet volume of ice-cold buffer C.
- Dounce slowly 10 times on ice (Use rod B).
- Invert for 45-60 min in cold room on the Itelli-mixer, C1 20-25 RPM.
- Centrifuge cells 14000 RPM/30 min/4°C in 2mL Eppendorf tubes.
- Recover and pool supernatant containing nuclear proteins (NE).
- Recover nuclear pellet containing proteins attached to chromatin.
- Dialyze overnight NE against buffer BC-100 in the dialysis casettes (Thermo scientific, Ref : 66455 or 66453).

Day 2 :

- Centrifuge cells 14000 RPM/30 min/4°C in 2mL Eppendorf tubes.
- Recover extracts, snap freeze on dry ice and store extracts at -80°C for later use.

See below for buffer compositions

Nuclear Extracts Flow Sheet

Date :

Volume of cell suspension: _____

Cells/ mL: _____

Remarks: _____

1. Centrifuge 2000 RPM/15 min/4°C.
2. Resuspend with 5x cell volume of ice cold PBS.
3. Centrifuge 3000 RPM/10 min/4°C.

   Cell volume: 1. _____
   2. _____
   3. _____
   4. _____
4. Resuspend with 4x cell volume of ice cold buffer A.
5. 10 min on ice.
6. Centrifuge 2000 RPM/10 min/4°C.

   Swollen cell volume: 1. _____
   2. _____
   3. _____
   4. _____

7. Resuspend with 2x cell volume (from 3) of ice cold buffer A.
8. Dounce 10 times.
9. Centrifuge 3000 RPM/10 min/4°C.

   Recover supernatant (S-100), make up final buffer concentration to BC-100.
10. Wash 1X with ice cold buffer A.

    Nuclear pellet volume: 1. _____
    2. _____
    3. _____
    4. _____
11. Add equal pellet volume of ice cold buffer C.
12. Dounce 10 times.
13. Invert 45-60 min @ 4° C.
14. Centrifuge 14000 RPM/30 min/4°C.
15. Recover supernatant (NE).
16. Dialyze NE against buffer BC-100.
17. Centrifuge 14000 RPM/30 min/4°C, freeze and store @ -80° C.

[#] PMSF and DTT should be added to buffers A and C before use.
[*] All buffers are stored and used at 4°C.

Buffer A: (10 mM Tris pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF)

Buffer A*#	**500 ml**	**1 L**
500 mM Tris pH 7.9	10 ml	20 ml
1M $MgCl_2$	750 µl	1.5 ml
3M KCl	2.5 ml	5 ml

Buffer C: (20 mM Tris pH 7.9, 25% Glycerol, 1.5 mM MgCl2, 500 mM KCl, 1mM EDTA, 1mM EGTA, 1 mM DTT, 0.5 mM PMSF)

Buffer C*#	**500 ml**	**1 L**
500 mM Tris pH 7.9	20 ml	40 ml
50% Glycerol	250 ml	500 ml
1M $MgCl_2$	750 µl	1.5 ml
3M KCl	125 ml	250 ml
500 mM EDTA (KOH) pH 8	1 ml	2 ml
500 mM EGTA (KOH) pH 8	1 ml	2 ml

Buffer 2X BC-0:

2x BC-0*	**1 L**	**10 L**
500 mM Tris pH 7.9 #	80 ml	800 ml
100% Glycerol	400 ml	4 L
500 mM EDTA (KOH) pH 8	1 ml	10 ml
500 mM EGTA (KOH) pH 8	500 µl	5 ml

BC-100: (20 mM Tris pH 7.9 (4° C), 100 mM KCl, 20% Glycerol, 0.25 mM EDTA, 0.125 mM EGTA, 0.5 mM PMSF, 1 mM DTT, 0.025% NP-40 (or Triton X-100).

BC-100*#	**1 L**	**10 L**
2x BC-0	500 ml	5 l
3M Kcl	50 ml	500 ml
10% NP40	2.5 ml	

Buffer exchange from Buffer A to BC100				
Extract:				
1M Tris pH 7.3				
87% Glycerol				
3M KCl				
500 mM EDTA				
250 mM EGTA				
10%NP40				
1M DTT				
250mM PMSF				
Final Vol				

Volume of DTT and PMSF:

Add:	**for 10 ml**	**for 20 ml**	**For 30 ml**	**for 40 ml**	**for 50 ml**	**for 100 ml**	**for 250 ml**
1M DTT	10 µl	20 µl	30 µl	40 µl	50 µl	100 µl	250 µl
250 mM PMSF	20 µl	40 µl	60 µl	80 µl	100 µl	200 µl	500 µl

IMMUNOPRECIPITATION

Date :

SERIAL IMMUNOPRECIPITATION : Flag-HA on CH12 NE for MS

Stock	Final conc				
Input (NE)					
3M KCl	**150 mM**				
10% NP40	**0,1%**				
25X PIC	**1X**				
1M DTT	**1 mM**				
250mM PMSF	**0,5 mM**				
Benzonase (25U/μL)					
2X BC 0	**1X**				
H_2O					

- Thaw extracts on ice
- Centrifuge 14000 RPM/20 min/4°C
- Recover supernatant and adjust salt, detergent and buffer concertrations depending on which buffer (BC-150 or BC-300) you do the IP in.

(WASH ALL BEADS IN BC-150 OR BC-300 3-4 TIMES BEFORE USE, CUT THE TIP TO AVOID DAMAGE AND TO ALLOW EXACT BEAD VOLUME)

Day 1 : PRE CLEARING:

- Add normal mouse/rabbit serum (1μL/mL, @ approximately 30mg/mL) and incubate 1hr/4°C with mixing.
- Add 80μL (for NE) 100μL (for S100) of Protein A/G Agarose beads (50% Slurry) incubate 1hr/4°C with mixing. (This volume changes with the volume of extract and number of IPs).
- Centrifuge 1000 RPM/3 min/4°C and recover supernatant. (Repeat twice to remove all Protein A/G beads). Save aliquot for Input. (I usually keep 1/10th).
- Add **100μL** of Flag-M2 beads (SIGMA-Aldirch, Ref : A-1205, 50% Slurry) to the supernatant incubate O/N @ 4°C with mixing.

Day 2 : WASHING AND 1st IP RECOVERY:

- Centrifuge 1000 RPM/3 min/4°C, recover the supernatant that can be frozen at -80°C for future use.
- Wash beads (7X) with 1mL of BC-300. (If using the EGFP constructs, the beads may even turn green sometimes !)
- Wash 1X with BC 100.
- **3X ELUTION's – 30mins/4°C with 100µL 2X Bead Vol of Flag peptide (0,2 mg/mL) or if your doing only 1 IP, you can directly put the beads in 1 bead volume of 2X Laemilli buffer (for normal gels) or 2X LDS buffer (for pre-cast gels).**
    - Save an aliquot from EACH elution for Western's - (Input for the 2nd IP).
    - Pool elutions for 2nd IP and adjust salt/detergent conc upto BC-150 or BC-300 and vol upto **500µL.**

Stock	**Final conc**			
Input (Elution of 1st IP)				
3M KCl	**300 mM**			
10% NP40	**0,1%**			
25X PIC	**1X**			
1M DTT	**1 mM**			
250mM PMSF	**0,5 mM**			
Benzonase				
2X BC 0	**1X**			
H_2O				

- Add **50µL** HA beads (SIGMA-Aldirch, Ref : A-2095, 50% Slurry) and incubate O/N @ 4°C with mixing. Again, slurry volume changes with the amount of starting material.

(WASH BEADS IN BC-150 or BC-300 3-4 TIMES BEFORE USE)

Day 3 : WASHING AND 2nd IP RECOVERY:

- Centrifuge 1000 RPM/3 min/4°C, recover the supernatant and freeze at -80°C, could be used again.
- Wash beads (min 7X) with 1mL of BC-300.
- Wash 1X with 4mM Tris-Cl (pH 7.9) 10 mM NaCl.
- **2X ELUTION's – 30mins/RT with 20µL 100 mM Glycine-HCl (pH 2 .5) FRESH.**

- Neutralize with 2μL 1M Tris (pH 8), save an aliquot from EACH elution for Western's, snap freeze and store at -80°C.

Or you can directly put the beads in 1 bead volume of 2X Laemilli buffer (for normal gels) or 2X LDS buffer (for pre-cast gels).

CELL TYPE : DATE :

CONSTRUCT/EXTRACT :

CONC (mg/ml) Vol Required

S100

NE

S100

NE

S100

NE

YEAST-TWO-HYBRID

Date:

Protocol from Régine LOSSON's lab.

Things that you will need, if you want to set up yourself:

- Glassware (specially treated and washed – from the ground floor stock).
- Yeast incubators – I used the ones in Régine's lab.
- All of the reagents mentioned below.
- A large plastic tub to recycle the glassware – this needs to be filled with water+7X PF.
- The strain of yeast we used yeast - L40a (*MATa,his3Δ200,trp1-901,leu2-3,112,ade2, LYS::(lexAop)4-HIS3, URA3::(lexAop)8-LacZ*).
- All the plasmids cloned into the yeast expression vectors – pASV3 and pBTM-mod. (I have all of the constructs cloned into both vectors).
- Once you have positive transformants, make glycerol stocks (in 15% glycerol) and store at -80°C.

Yeast-two hybrid – transformation:

- The day before you need to do a transformation, start an O/N culture/220RPM/28°C in yeast extract/peptone/dextrose (YPD) medium in 5 mL.
- From this culture, start a fresh one in yeast extract/peptone/dextrose (YPD) medium 3-4 hr/220RPM/28°C in 25mL and these cells are used to make FRESH competent cells (I used 25mL, but the volume varies with the number of transformations).
- Cells are grown in 25mL of YPD to an OD_{600} of 0.6-0.8.
- Centrifuge 3000 RPM/5min/4°C and resuspend the pellet in 20mL of sterile water.
- Centrifuge 3000 RPM/5min/4°C and resuspend the pellet in 1mL of sterile water.
- Centrifuge 6000 RPM/1min/RT and resuspended in 250μL of 100mM LiAc.
- Incubate 15min/30°C to make fresh competent yeast cells (for each experiment).

(these cells can be stored at 4°C for upto 3 days and used the next day – but the efficiency of the transformation will be lesser)

- 25μL of these cells is mixed with 2μg of DNA along with a transformation mix. A transformation mix (for 1 reaction) contains 30% PEG 3350, 90mM 1M LiAc, 125μg/ml sheared salmon sperm DNA in a final volume of 200μL.

(I usually make a mix containing the PEG, LiAc and salmon sperm, distribute 173μL of this into each tube, then add the different plasmids and finally the competent cells).

- Vortex this well and incubate 30 min/30°C followed by a heat shock for 20 min/42°C.
- Centrifuge at 14000 RPM/30 sec/4°C and resuspend in 100μL of sterile water and plated on selective media (SD media).
- SD media with appropriate supplements - depending on the plasmid used pASV3 is grown on HTA media, while pBTM is grown on HLA media. (I plate between 5-20μL of the transformation on the appropriate selective plates, and keep the rest just in case, I discard the cells only after I have positive transformants)
- Incubate at 28°C, single transformants (takes 3-5 days) are selected from these plates check expression of proteins by Western using either an @LexA or @VP16 antibody. (available in-house).
- Re-transformed positive clones with the second plasmid – follow same protocol as above.
- Double transformants are selected by growing on HA media, checked for protein expression and positive clones are used in β-galactosidase liquid assays.

@ LexA antibody (IGBMC, Lp560), rabbit polyclonal – used 1 :1000 in WB
@ VP16 antibody (IGBMC, 2GV4), mouse monoclonal – used 1 :1000 in WB

Yeast-two hybrid – transactivation assays:

- Cells are grown in selective media, O/N 220RP /28°C and harvested.
- Wash the cells in 10mL water (does not need to be sterile) and pellets are resuspended in 1/10th volume of the culture of breaking buffer.
- Centrifuge 14000 RPM/30 sec/4°C and resuspend in 1/100th volume of breaking buffer.
- Incubate on ice/15min and vortex for 3 mins along with glass beads (G1145, Sigma-Aldirch).
- This vortex step is crucial as it is the only form of mechanical rupture to the cells) Vortex parameters : ON HIGH SPEED, WITH THE EDGE OF THE TUBE ALONG THE RIM OF THE VORTEX - 1 min – cool on ice for 1 min (repeat this thrice).
- Incubated the lysed cells on ice/15min, centrifuge 14000 RPM/30 mins/4°C and measure protein quantity by Bradford method. (the same protocol upto here is followed to make extracts for WB also).
- Mix 5-20 μg of protein (in 50μl) with 450μl of Z buffer and incubate RT/5min before addition of 100μL of 4μg/μL ortho-nitrophenyl-β-galactoside (ONPG, in Z buffer).
- Stop the reaction (the yellow color should not be allowed to saturate) with 250μL of 1M $Na_2.CO_3$.

- Note the time difference between the start and stop of each reaction.
- OD is read at 420nm in order to calculate the specific activity. (I use the reader on the 4th floor in JME's lab).

Specific Activity – (OD420*0.85)/(0.0045*Protein conc*volume*time)

where :

OD420 : OD read at 420nm after the reaction is stopped

Protein conc : the amount of protein in the reaction (in mg/mL)

Volume : volume of extract used

Time : time difference between the start and stop of each reaction

BUFFERS AND SOLUTIONS REQUIRED :
(ALL SOLUTIONS ARE AUTOCLAVED AT ½ ATM)

Synthetic complete liquid broth (SD medium)

Bacto yeast nitrogen base – 6.7g

Glucose – 20g

Water – upto 1 L

SD medium with supplements

HTA – SD + histidine,tryptophan and alanine

HLA – SD + histidine,leucine and alanine

HA – SD + histidine and alanine

Alanine @ 1mg/mL, histidine @ 10mg/mL, tryptophan @ 10mg/mL – made in water. (all these amino acid powders are from the Losson lab, **FILTER STERILIZE**).

Yeast extract/peptone/dextrose (YPD) medium

Bacto yeast extract – 10g

Bacto peptone – 20g

Glucose – 20g

Water – upto 1L

To make Agar plates :

Liquid broth – 500mL

Agar – 10g

1M LiAc – dilute to 0.1M before use - FILTER STERILIZE THIS AS IT IS DEGRADABLE

LiAc – 10.2g

Water – upto 100mL

50% PEG 3350

PEG 3350 – 50G

Water – upto 100mL

Breaking buffer :

50 mM Tris–HCl (pH 8, at 4°C), 400mM KCl, 1 mM PMSF and complete protease inhibitor cocktail

Z buffer:

60mM $Na_2.HPO_4.7H_2O$, 40mM $NaH_2PO_4.H_2O$, 10mM KCl, 1mM $MgSO_4.7H_2O$ and 50mM β-mercaptoethanol – FRESH

Ortho-nitrophenyl-β-galactoside (ONPG): 4mg/mL

ONPG – 200mg

Z buffer – 50 mL

Can be made and stored @ -20°C

15% glycerol, sterile MQ, carrier sheared salmon sperm (@10mg/mL).

GENOMIC DNA (gDNA) EXTRACTION

Date :

Proteinase K (PK)/Digestion Buffer: (for 500mL)

100mM Tris-Cl pH8.0 (50mL of 1M Tris-Cl)

200mM NaCl (20mL of 5M NaCl)

5mM EDTA (5mL of 0.5M EDTA, pH8.0)

0.2% SDS (5mL of 20 % SDS)

Add 1μL proteinase K @ 20mg/mL per 100μL of buffer, FRESH

- Add 500μL (or required volume) of PK buffer per sample. and incubate O/N at 55 °C.

gDNA Extraction from tails for genotyping :

Tails were cut (Michael does this, as our mice are in the SPF) at ~4-5 weeks of age.

- Digest tails in 500μL of PK buffer (with protease K), O/N at 55°C.
- Mix well by inversion and centrifuge 14000RPM/15 min/RT.
- Transfer supernatant to a new tube and precipitate with 500μL of isopropanol.
- Centrifuge 14000RPM/15 min/4°C.
- Wash 1X with 70% ethanol and air-dry the pellet.
- Resuspend the pellet in 300μL of 10mM Tris-Cl, pH 8.0 and dissolve the pellet 30min-1hr/65°C. Use 1-2μL of this as PCR template.

PCR was done with the following conditions:

MIX	1X	X
10X TPT	3μL	
dNTP's	0.6μL	
50 μM YD 208 (P1) BR 228	0.2μL	
50 μM VR 211 (P2) BR 229	0.2μL	
50 μM TV 210 (P3) BR 230	0.2μL	
Taq VH	0.3μL	
Water	24.5μ	
Tail DNA	1μL	

Primers for KAP1 genotyping:

Primer	Lab code	Sequence (5'-3')
YD 208	BR 228	GGAATGGTTGTTCATTGGTG
VR 211	BR 229	ACCTTGGCCCATTTATTGATAAAG
TV 210	BR 230	GCGAGCACGAATCAAGGTCAG

Method (KAP1 genotyping):

95°C – 5 min
95°C – 30 sec
60°C – 30 sec — 35X
72°C – 1 min
72°C – 5min
15°C – Hold

gDNA Extraction from cells/ChIP DNA preparation:

> I use the same protocol to extract gDNA from cells and to process my ChIP DNA.

Phenol:chloroform extraction (done under the fume hood)

- Add half the volume each of phenol and chloroform to the DNA solution. (Could be either cells digested with PK buffer O/N or ChIP DNA samples. I usually do my extraction in 500μL, so that makes 250μL phenol+250μL of chloroform).
- Mix by voxtexing for about 10sec and centrifuge 14000 RPM/15 min/4°C. (Use out Eppendorf microfuges in the cold room. DO NOT use Evi's 4°C microfuge, it gets to cold and precipitates the SDS!! Esp in the ChIP samples!)
- Transfer the aqueous layer (top layer) to a new tube. (I usually keep the bottom layer until my extraction is done, just in case).
- To the aqueous phase add an equal volume of chloroform.
- Mix by voxtexing for about 10sec and centrifuge 14000 RPM/15 min/4°C.
- Transfer the aqueous (top) layer to a new tube. (I leave this open on the bench for ~10 min just to let any residual chloroform evaporate).

You can also do a back-extraction of the phenolic phase by adding an equal volume of 10mM Tris-Cl (pH 8.0) and chloroform and proceed as above. I do this when the starting material is very less as in the case of sorted cells in the Sμ sequencing and SHM experiments.

Ethanol Precipitation

- Add 1/10th the volume of the aqueous phase of 3M sodium acetate (final concentration to 0.3M), 1μL of the glycogen carrier (Applied Biosystems, Ref: AM9510) and two volumes 100% ethanol.
- Mix by inverting and incubate O/N at -20°C. (This is absolutely necessary especially for ChIP samples, else the yields are low).
- Centrifuge 14000 RPM/30-45 min/4°C.

- Remove supernatant carefully the pellets can be very loose and get easily aspirated!!
- Wash 1X with 70% ethanol, centrifuge 14000 RPM/30-45 min/4°C.
- Carefully aspirate or decant supernatant, the pellets are even looser and get easily aspirated!! But there will be a clear pellet at the bottom.
- Repeat wash if you think there's too much salt – this could happen sometimes and its important especially for the ChIP samples that the DNA is as clean as possible for the PCR to be efficient.
- Dry the pellet by placing the tube upside down on a rack or under the lamp. It shouldn't take longer than 10-20 min – just until all residual ethanol has evaporated.
- Dissolve pellet in appropriate amount of TE or 1:6 diluted TE buffer (for ChIP samples).

- Depending on the experiment 2-5μL of this is used as PCR template.

RNA EXTRACTION-TRIzol METHOD

Date :

Total RNA Isolation with TRIzol Reagent (Invitrogen, Ref : 15596-026)

Extraction from CH12 or B cell cultures:

- Harvest cells, add an appropriate volume of TRIzol and resuspend by pipetting. Use 750μL of TRIzol per 5-10x10^6 cells. (I do not wash the cells in PBS before, samples can be stored at this stage at -80°C).
- Incubate the samples at room temperature for 5 minutes. (If you are working with frozen samples, leave them to thaw at RT and proceede).
- Add 200μL of chloroform, shake vigorously (not vortex) for 15 seconds to mix well and let samples stand at RT for 15 min.
- Centrifuge 14000RPM/15 min/4°C to separate phases.
- Transfer the upper (clear) aqueous layer to a fresh tube.
- Add 500μL of isopropanol to the aqueous layer to precipitate, mix thoroughly by shaking for 15 seconds, and incubate at RT/10 min.
- Centrifuge 14000RPM/10 min/4°C.
- Carefully remove the supernatant and add 1mL 75% DEPC-ethanol and vortex on low for 5-10 seconds to wash the pellet thoroughly. (I usually cool the ethanol before I use it).
- Centrifuge 14000RPM/10 min/4°C and air-dry the pellet at room temperature for 5-10 minutes.
- Dissolve the pellet in DEPC (or dd)-dH_2O (10-15μL, depending on number of cells) by gentle pipetting and incubate at 55°C for 5-10 minutes.
- Measure the concentration on the Nanodrop.

cDNA SYNTHESIS- SuperScript™ II RT

Date :

First-Strand cDNA Synthesis Using SuperScript™ II RT (Invitrogen, Cat no : 18064014)

A 20-µL reaction volume can be used for 1 ng–5 µg of total RNA or 1–500 ng of mRNA.

Make the following mix in a 10µL final volume:

- 1µL of random hexamer (@300 ng/ µL)
- 1-5µg of RNA

(You can also use Oligo(dT)12-18 (500 µg/mL) or 1 µL or 2 pmole gene-specific primer, but I have used only random hexamer in the lab)

- Denature the RNA 2 min/94°C and cool on ice – immediately. (If I have few samples I do this in the PCR machine, else I use a "V" bottomed plate to make my cDNA – this makes it easier to dilute and pipette for PCR later).

Prepare RT mix and add 10µL of this mix to each sample:

10 mM dNTP Mix - 1 µL

5X First-Strand Buffer - 4 µL

20mM DTT - 1µL

RNAsin (40U/µL) – 0.5µL

SuperScript™ II RT (200 units) - 0.5µL

Sterile, distilled water - 3 µL

(remember to keep a RT- control before you add the enzyme and make up the volume with 1.5µL of water).

- Mix contents of the tube gently and incubate at 25°C (RT) for 10 min.
- Incubate at 42°C for 50 min.
- Inactivate the reaction by heating at 70°C for 15 min.
- Dilute the cDNA 1:5 with water and store at -20°C (that is 20µL of cDNA + 80µL of water) and use 5µL of this for each PCR either to clone or to do qRT-PCR experiments.

NOTE : If you use a plate to do the cDNA you have to be very careful to not cross contaminate between samples. I always leave 1 row and 1 column inbetween, seal the plate with parafilm and centrifiuge the plate inbetween every step !!

WESTERN BLOT

- Immunoprecipitates, whole cell, cytoplasmic or nuclear extracts were separated either on a SDS-PAGE gel, NuPAGE Novex 10% or 4-12% Bis-Tris gels (Invitrogen, Ref : NP0321/22/01).
- Transfer onto a polyvinylidene difluoride (PVDF, Millipore, Ref : IPVH00010) membrane was done on the semi-dry transfer system with the parameters: (this is for one membrane measuring 9x7 cm)
    - mA – 130 (13x10 on our power pack)
    - mV- 25V
    - for 45-50 min
- The membrane was blocked O/N @ 4°C or at RT for 1 hour with blocking buffer, incubated with primary antibody at 4°C over night or at RT for 1 hour. (I prefer to block for an hour at RT and put the Ab O/N, especially for the @ KAP1 and @ AID antibodies this works better).
- 3X washes with TBST/ 20min each.
- The membrane was incubated with 1:10000 dilution of horseradish peroxidase conjugated secondary antibody (Ref:) at RT for 1-2 hr.
- 3X washes with TBST/ 20min each.
- Revealed with Western Pico (Pierce, Ref:) or the Immobilon Western (Millipore). (I use the Pierce for @ actin and @ KAP1 Western blots, as they are very strong, while an @ AID WB would need the Immobilon reagent).

TBST buffer: 0.1% Tween-20 in 1x TBS buffer
Blocking buffer: 5% non-fat milk in TBST buffer

Western blot stripping by Acidic pH

- Place the blot in stripping solution and agitate for 30 minutes.
- 2X washes with TBST/ 10min each.
- Proceed to the blocking step for the next round of detection.

* Stripping solution: 25 mM glycine-HCl (pH 2), 1% (w/v) SDS – make the strip buffer FRESH each time.

COLONY SCREEN

Date :

	1X	X
10X TPT -	1.5μL	
10mM dNTP's -	0.3μL	
50μM (P1) -	0.1μL	
50μM (P2) -	0.1μL	
Taq -	0.3μL	
Water -	2.7μL	
	5μL	

Method :

95°C – 10 min
95°C – 30 sec
60°C – 30 sec } 40X
72°C –
72°C – 5min
15°C – Hold

Template : bacterial colonies in 10μL of sterile MQ water.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Date :

		1X	X
2X SYBR (Qiagen/SIGMA)	-	5μL	
100μM primer mix	-	0.06μL	
Water	-	2.94μL	
		8μL	

Method :

95°C – 5 min

95°C –

°C – 50X

72°C –

Template : 2μL/PCR

Standard:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

APPENDIX II

Résumé de thèse

***INTRODUCTION**:*

Lors des réponses immunitaires, le répertoire des lymphocytes B est diversifié par les mécanismes d'hypermutation somatique (HMS) et de commutation isotypique (CI)[1] afin d'établir des réponses spécifiques et adaptées, ainsi qu'une immunité durable. L'HMS modifie l'affinité des anticorps pour l'antigène en introduisant des mutations ponctuelles dans la région variable des chaînes lourdes (IgH) et légères (IgL) des immunoglobulines (Ig)[2]. La CI est un évènement de recombinaison qui remplace la région constante exprimée ou isotype (de IgM à IgG, IgA ou IgE) tout en préservant la région variable et donc la spécificité de l'anticorps. Elle permet de changer la fonction effectrice des anticorps[3].

En dépit de différences claires dans leur mécanisme, ces deux processus sont dépendants de l'expression d'« Activation Induced Cytidine Deaminase » (AID)[4,5]. AID initie la CI par désamination de cytosines en uracyles dans le locus IgH au niveau de régions spécifiques hautement répétitives, appelées régions de « switch » ou régions S (longueur de 1 à 12 kb). Les lésions ainsi générées dans l'ADN sont des mésappariements (dU : dG)[6] qui sont processés par des protéines de la voie de réparation des mésappariements et par excision de bases, pour donner lieu à des cassures double-brins dans le locus IgH ou des mutations lors de la CI[7,8].

La présence de cassures double-brins active la voie de réponse aux dommages à l'ADN[9] incluant ATM, H2AX, le complexe MRN (Mre11, Nbs1 et Rad50), MDC1 et 53BP1 pour promouvoir une recombinaison efficace et la réparation par la voie de réparation par jonction d'extrémités non homologues (« non homologous end joining », NHEJ).

Cependant, malgré les nombreuses voies et les multiples facteurs impliqués dans la reconnaissance et la réparation des dommages de l'ADN, les cassures générées par AID peuvent être également processées de manière aberrante et mènent alors à des translocations chromosomiques impliquant le locus IgH et des oncogènes tels que c-Myc[10-13]. C'est pourquoi de robustes mécanismes de régulation et de ciblage de AID sont requis afin de permettre et limiter les dommages à l'ADN au locus IgH dans les cellules B et afin de prévenir les dommages collatéraux qui peuvent résulter en une tumorogenèse étendue. Les cellules B ont de multiples niveaux de régulation de l'enzyme AID, tels que la régulation de son expression par la dégradation de son ARNm médiée par microARN, de sa localisation subcellulaire par son signal d'export nucléaire ou encore de sa phosphorylation[14].

Il apparaît qu'AID distingue les régions variables des régions de switch par son association avec des facteurs tels que la protéine de réplication A (RPA)[15], la protéine kinase A (PKA)[16], CTNBBL1[17], SLIP-GC[18], RNF8/RNF168[19] ou encore par la formation de structures

tridimensionnelles de l'ADN (boucles R, G-quadruplexes, tiges-boucles)[3]. De plus, des modifications inductibles des histones comprenant la phosphorylation de H2AX, l'hyperacétylation des histones H3 et H4[20-23], ainsi que l'acétylation et la tri-méthylation de la lysine 9 de l'histone H3 au niveau des régions de switch ont été démontrées lors de la CI. Il a été proposé que ces modifications épigénétiques pourraient augmenter l'accessibilité des régions de switch et/ou jouer un rôle dans le ciblage spécifique d'AID au niveau du locus IgH au cours de la CI[24]. Cependant, un lien direct entre ces modifications et la fonction d'AID au cours de la CI n'est toujours pas établi. Par ailleurs, le domaine C-terminal d'AID contient un signal d'export nucléaire (NES) qui contrôle sa localisation subcellulaire[25-28]. De plus, ce domaine est requis spécifiquement pour la réaction de CI car la troncation du domaine C-terminal des protéines AID murines et humaines résulte en un défaut de CI. Il a donc été suggéré que le domaine C-terminal soit responsable de l'association d'AID avec des facteurs ayant un rôle spécifique pour la CI. Ces facteurs pourraient médier potentiellement le ciblage spécifique d'AID au niveau des gènes des Ig et/ou promouvoir une réparation efficace de l'ADN[29-32].

Cependant, la question du ciblage spécifique d'AID aux loci Ig au cours des réponses immunitaires reste ouverte. Aussi afin d'appréhender les mécanismes moléculaires qui sous-tendent le ciblage d'AID dans les loci Ig, avons-nous entrepris tout d'abord d'identifier les facteurs qui interagissent avec la protéine AID, et en particulier avec son domaine C-terminal, et étudier dans un deuxième temps leur(s) rôle(s) fonctionnel(s) au cours des processus d'HMS et de CI.

RESULTATS:

Identification de cofacteurs d'AID et de son domaine C-terminal

Dans un premier temps, afin d'identifier les facteurs qui interagissent avec la protéine AID, nous avons utilisé une stratégie de purification d'affinité en tandem suivie d'identification par spectrométrie de masse. En utilisant des extraits nucléaires de cellules CH12 qui expriment la protéine AID murine comportant les épitopes Flag et HA en N-terminal ($AID^{Flag\text{-}HA}$), nous avons spécifiquement coimmunoprécipité et identifié différentes protéines, dont certaines sont déjà connues pour être impliquées dans la CI et/ou comme partenaires d'AID. D'autres n'ont pas encore de rôle décrit. Pour identifier des partenaires spécifiques du domaine C-terminal d'AID, la même stratégie a été utilisée avec des extraits nucléaires exprimant les 17 derniers acides aminés d'AID fusionnés à la protéine eGFP ($eGFP\text{-}AID^{182\text{-}198}$). De façon intéressante, la protéine KAP1 ou « KRAB-domain associated protein » (aussi connue sous le nom de TRIM28, TIF1β ou KRIP1) a été identifiée comme partenaire d'AID et comme s'associant également avec le domaine C-terminal. Le facteur KAP1 est connu comme corépresseur transcriptionnel requis pour le développement embryonnaire[33] et est également un effecteur dans la voie de réponse des dommages à l'ADN[34]. Ces données suggèrent un rôle important de KAP1 dans les processus de CI et/ou d'HMS.

KAP1 s'associe à AID, est requis spécifiquement pour la CI mais n'est pas nécessaire pour l'HMS

Afin de confirmer l'interaction entre KAP1 et AID, nous avons réalisé des expériences d'immunoprécipitation et avons montré que KAP1 et AID interagissent *in vivo*.

Pour tester le rôle de KAP1 lors de la CI et l'HMS, nous avons croisé des souris comportant un allèle floxé du gène KAP1 ($KAP1^{F/F}$)[33] à des souris transgéniques $CD19^{Cre/+}$ [35] pour générer les mutants conditionnels $CD19^{Cre/+}$ $KAP1^{F/F}$. Ceci permet d'inactiver spécifiquement le gène codant pour KAP1 dans les cellules B immatures. Nous avons ensuite testé la capacité intrinsèque des lymphocytes B $CD19^{Cre/+}$ $KAP1^{F/F}$ à réaliser la réaction de commutation isotypique *in vitro*. Nous avons observé que l'inactivation conditionnelle de KAP1 dans les cellules B résulte en un défaut de CI pour les différents isotypes testés. La réduction est de l'ordre de 50 à 60% par rapport au niveau de commutation des cellules contrôles. De plus, ce défaut est indépendant de la prolifération et de la survie des cellules.

Puis pour déterminer le rôle de KAP1 lors de l'HMS, nous avons analysé les profils de mutations dans les séquences de la région variable du locus IgH dans les cellules B de souris déficientes pour KAP1 et contrôles après immunisation. Nous n'avons pas observé de différences significatives dans la fréquence de mutations ou dans le profil de substitutions des nucléotides dans les cellules B déficientes pour KAP1 par rapport aux cellules contrôles. Nous en concluons que l'hypermutation somatique n'est pas altérée dans les souris $CD19^{Cre/+}$ $KAP1^{F/F}$. Le facteur KAP1 n'est donc pas nécessaire pour l'HMS et est requis exclusivement pour médier la réaction de CI.

Afin de comprendre les causes du défaut de commutation isotypique et les mécanismes qui sous-tendent ce défaut, nous avons entrepris de tester les différentes étapes du processus de CI afin de déterminer lesquelles sont altérées dans les souris déficientes pour KAP1 par un ensemble d'expériences.

La fonction de KAP1 lors de la CI est en aval de l'étape d'expression d'AID mais en amont de la génération des dommages à l'ADN

Sachant que la CI est dépendante d'une première étape de transcription dite stérile au niveau des régions de switch donneuse et acceptrices[3] et étant donné que KAP1 est connu comme corépresseur transcriptionnel[33], nous avons testé l'expression des transcrits stériles dans les lymphocytes B déficients pour KAP1. Par des expériences de RT-PCR quantitatives, nous n'avons pas détecté d'altérations significatives des niveaux d'expression de ces transcrits dans les cellules B $CD19^{Cre/+}$ $KAP1^{F/F}$ par rapport aux cellules contrôles. Nous en concluons donc que la transcription et son effet sur l'accessibilité aux régions de switch ne sont pas altérés en l'absence de KAP1. La fonction de KAP1 lors de la CI se situe donc en aval de l'étape de

transcription des régions de switch. En accord avec les résultats obtenus lors de l'analyse de l'HMS dans les souris déficientes pour KAP1, nous avons également observé que le niveau d'expression de AID n'est pas altéré par l'inactivation de KAP1 et nous en concluons que le défaut de CI dans les cellules B $CD19^{Cre/+}$ $KAP1^{F/F}$ est en aval de l'étape d'expression de AID.

Par ailleurs, la CI requiert la génération de cassures double-brins dans les régions de switch donneuse et acceptrices et leur résolution par les voies classique et alternative de la réparation par jonction d'extrémités non homologues[36,37]. Il a été montré récemment que KAP1 a un rôle dans la réparation de l'ADN. Aussi nous avons testé l'étape de réparation des cassures, et pour cela nous avons analysé les jonctions recombinées dans les cellules B mutantes et avons déterminé qu'elles ne sont pas affectées par l'inactivation du gène KAP1. Les extrémités d'ADN sont donc processées et réparées normalement lors de la CI en absence de KAP1. De plus, sachant que l'inactivation des gènes codants pour les facteurs de la voie de réponse des dommages à l'ADN ou des composants du NHEJ résulte en une instabilité génomique globale et spécifique du locus IgH[13,36,38], nous avons également testé la présence de cassures dépendantes d'AID non réparées par des expériences de FISH spécifiques du locus IgH. De façon surprenante, nous avons observé qu'en l'absence de KAP1, les cassures de l'ADN générées par AID dans les régions de switch sont réparées efficacement, et que la fréquence des aberrations chromosomiques n'est pas augmentée. De même, grâce à des essais testant l'occurrence de translocations chromosomiques entre le locus IgH et l'oncogène c-myc, nous n'avons pas observé d'augmentation de la fréquence de ces translocations. L'ensemble de ces résultats montre que l'inactivation de KAP1 n'altère pas l'étape de réparation ni le maintien de la stabilité génomique globale et liée au locus IgH. La fonction de KAP1 dans la CI se situe donc en amont de l'étape de réparation des cassures générées par AID dans les régions de switch.

L'inactivation de KAP1 résulte en un défaut d'induction des cassures de l'ADN dans les cellules B au cours de la CI

Afin de détecter l'induction des cassures double-brins de l'ADN dans les régions de switch et pour déterminer si elles sont générées de façon moins efficace en l'absence de KAP1, nous avons réalisé des expériences de PCR médiée par ligation (ligation-mediated PCR, LM-PCR). Nous avons observé de façon reproductible une diminution du nombre de cassures dans l'ADN des cellules B déficientes pour KAP1 par rapport aux cellules B contrôles. Ceci suggère que KAP1 est requis pour l'induction des dommages à l'ADN dépendants d'AID et la génération des cassures double-brins au niveau du locus IgH pour permettre une CI efficace.

Pour évaluer le niveau de dommages à l'ADN induits par AID, nous avons analysé les mutations dans la région de switch donneuse. Ces mutations sont dépendantes de l'activité d'AID et permettent d'analyser quantitativement la fréquence des dommages dépendants d'AID[29]. Par séquençage de la région de switch donneuse dans les lymphocytes B stimulés,

nous avons observé que les mutations dans cette région sont moins fréquentes dans les cellules B inactivées pour le gène KAP1 par rapport aux cellules contrôles. L'ensemble de ces résultats montre que la formation des cassures double-brins au niveau des régions de switch est moins efficace en absence de KAP1, et suggère qu'AID ne peut accéder efficacement aux régions de switch ou qu'elle n'est pas retenue de manière efficace au niveau des régions de switch lors de la CI.

AID en complexe avec KAP1 et HP1 est retenue aux régions de switch par H3K9me3 lors de la CI

Il a été démontré que des modifications épigénétiques incluant la phosphorylation de l'histone H2AX, l'hyperacétylation des histones H3 et H4, l'acétylation et la triméthylation de la lysine 9 de l'histone H3 (H3K9me3)[20-24] sont associées avec les régions de switch lors de la CI. Leur rôle précis n'est pas déterminé et il a été proposé qu'elles augmenteraient l'accessibilité des régions de switch et/ou joueraient un rôle pour le ciblage spécifique d'AID au locus IgH durant la CI[24].

Notre hypothèse est qu'AID pourrait être retenue aux régions transcrites de switch porteuses de la modification H3K9me3 par l'association entre AID, KAP1 et la protéine de l'hétérochromatine HP1, et la capacité de cette dernière de se lier spécifiquement aux résidus H3K9me3[39].

Afin de tester l'hypothèse qu'AID est recrutée via le complexe KAP1-HP1 à la modification H3K9me3, nous avons réalisé des expériences de peptide-pulldown, et nous avons montré que le peptide H3K9me3 précipite spécifiquement KAP1, les différents isoformes de HP1 ainsi que la protéine AID. Nous en concluons que KAP1, HP1 et AID existe en un complexe qui lie la modification H3K9me3 *in vitro*. Ainsi cette marque pourrait servir de motif retenant le complexe formé par AID, KAP1 et HP1 au niveau des régions de switch lors de la CI *in vivo*.

Pour déterminer si les protéines KAP1 et HP1 sont récrutées au niveau des régions de switch lors de la CI *in vivo,* et tester si ce recrutement corrèle avec la présence des modifications H3K9me3, nous avons réalisé des expériences d'immunoprécipitation de la chromatine (ChIP) sur les cellules B $CD19^{Cre/+}$ $KAP1^{F/F}$ et contrôles. Nous avons déterminé que la modification H3K9me3 est présente dans les régions de switch dans les cellules B déficientes pour KAP1 et dans les cellules contrôles, montrant que l'inactivation de KAP1 n'altère pas la structure ni les modifications de la chromatine. De même, nous avons montré que KAP1 et HP1γ sont présentes au niveau des régions de switch donneuse et acceptrices dans les cellules contrôles lors de la CI, corrélant avec le profil de rétention de la modification H3K9me3. Donc, en étant en complexe avec KAP1 et HP1γ, AID peut être retenue au niveau des régions de switch portant la modification H3K9me3.

Enfin pour tester si la formation du complexe AID/KAP1/HP1 est requise *in vivo* pour médier la CI, nous avons utilisé un modèle de souris mutantes qui expriment uniquement une forme de la

protéine KAP1 porteuse de mutation bloquant l'interaction avec la protéine HP1. De manière intéressante, nous avons mis
en évidence le même défaut de CI que celui observé dans les cellules B déficientes pour KAP1. Nous concluons que l'association *in vivo* entre KAP1 et HP1 est requise pour médier une CI efficace, et que le complexe formé par AID, KAP1 et HP1 est retenu au niveau des régions de switch des immunoglobulines comportant la modification H3K9me3 *in vivo*.

CONCLUSION ET PERSPECTIVES:

Au cours de ce travail de thèse, nous avons montré que la protéine AID forme un complexe avec les protéines KAP1 et HP1 et que la dissociation *in vivo* de ce complexe résulte en un défaut de formation des cassures double-brins au niveau des régions de switch et en un défaut concomitant de la CI, l'HMS n'étant pas altérée. Nous concluons que ce complexe est retenu au niveau des régions de switch porteuses de la modification H3K9me3, et proposons pour la première fois un modèle reliant AID aux modifications épigénétiques induites au cours de la CI.
Notre projet permet une avancée significative dans la compréhension des mécanismes qui contrôlent le ciblage spécifique d'AID aux gènes des immunoglobulines. Nous pensons que ces résultats élargissent les connaissances sur le contrôle de l'accès de la protéine mutagène AID au locus IgH et fournissent une vue nouvelle des mécanismes de régulation de l'activité d'AID. La compréhension des mécanismes qui préviennent les dommages collatéraux d'AID dans le génome et limitent ainsi les mutations généralisées, les translocations chromosomiques ainsi que la transformation cancéreuse permettra d'ouvrir de nouvelles pistes de recherche et pourra déboucher sur de nouvelles orientations thérapeutiques pour la lutte contre le cancer.

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Résumé français:

Lors des réponses immunitaires, le répertoire des lymphocytes B est diversifié par les mécanismes d'hypermutation somatique (HMS) et de commutation isotypique (CI) afin d'établir des réponses spécifiques et adaptées, ainsi qu'une immunité durable. L'HMS modifie l'affinité des anticorps pour l'antigène en introduisant des mutations ponctuelles dans la région variable des chaînes lourdes (Ig*h*) et légères des immunoglobulines (Ig). La CI est un évènement de recombinaison qui remplace la région constante exprimée permettant de changer la fonction effectrice des anticorps. Ces deux processus sont dépendants de l'expression d'« Activation Induced Cytidine Deaminase » (AID). En effet, AID initie ces processus par déamination des bases cytosines en uraciles dans l'ADN résultant en des mésappariements dU:dG. Lors de la CI, les lésions générées sont reconnues et processées par différents facteurs de la voie de réponse des dommages de l'ADN, pour donner lieu à des cassures double-brins. Celles-ci sont ensuite réparées par la voie de réparation par jonction d'extrémités non homologues (« non homologous end joining », NHEJ). Cependant, malgré les nombreuses voies et les multiples facteurs impliqués dans la reconnaissance et la réparation des dommages de l'ADN, les cassures générées par AID peuvent être également processées de manière aberrante et mènent à des événements de recombinaisons illégitimes. C'est pourquoi de robustes mécanismes de régulation et de ciblage d'AID sont requis afin de permettre et limiter les dommages à l'ADN au locus Ig*h* dans les cellules B et afin de prévenir les dommages collatéraux qui peuvent résulter en une tumorogenèse étendue. Parmi ces mécanismes de régulation et de ciblage, il a été proposé que des modifications épigénsétiques du locus Ig*h* qui corrèlent avec le processus de CI pourraient être importantes pour le ciblage spécifique d'AID au locus Ig*h*. Aussi afin d'appréhender les mécanismes moléculaires qui sous-tendent le ciblage d'AID dans les loci Ig, avons-nous entrepris d'identifier les facteurs qui interagissent avec la protéine AID, et étudier dans leur(s) rôle(s) fonctionnel(s) au cours des processus d'HMS et de CI.
Au cours de ce travail de thèse, nous avons montré que la protéine AID forme un complexe avec les protéines KAP1 et HP1 qui est retenu au locus Ig*h*, spécifiquement au niveau de la région de switch µ, qui est porteuse de la modification de l'histone H3 H3K9me3 *in vivo*. De plus, la dissociation *in vivo* de ce complexe résulte en un défaut de formation des cassures double-brins au niveau de la région de switch Sµ et en un défaut concomitant de la CI, l'HMS n'étant pas altérée. Nous concluons que KAP1 et HP1 retiennent AID aux résidus H3K9me3 qui marquent la région de switch Sµ afin de permettre une CI efficace et proposons pour la première fois un modèle reliant AID aux modifications épigénétiques induites au cours de la CI.

Summary:

B lymphocytes diversify their antibody repertoire through somatic hypermutation (SHM) and class switch recombination (CSR). SHM introduces single base pair substitutions in the immunoglobulin (Ig) heavy and light variable region genes while CSR is a recombination reaction that replaces the heavy chain constant region exons thus switching the antibody isotype expressed. Both these reactions are initiated by a single enzyme, Activation induced cytidine deaminase (AID). AID deaminates cytosines to uracils in DNA to produce dU:dG mismatches that are recognized and processed by alternative DNA repair pathways to result in SHM or CSR. AID-induced DNA lesions are processed to form DNA double-stranded breaks (DSBs) during CSR, recognized by components of the cellular DNA damage response pathway and repaired through non-homologous end joining. Aberrant processing of AID-induced DNA breaks leads to increased levels of illegitimate recombination events, therefore tight regulatory and targeting mechanisms are required to restrict this potential to the appropriate cell type and loci. Although, CSR correlates with epigenetic modifications at the Ig*h* locus, and these modifications have been proposed to target the CSR machinery, the relationship between these and AID remains unknown. To understand the molecular mechanisms underlying the mechanisms of AID targeting to Ig loci, we set out to identify factors that interact with AID, and study their functional role(s), if any during SHM and/or CSR. My results provide a mechanism linking AID to epigenetic modifications during CSR. We show that during CSR, AID forms a complex with KAP1 and HP1 that is tethered to the Ig*h* locus (specifically to the donor switch region-Sµ), bearing trimethylation at lysine 9 (H3K9me3) *in vivo*. Furthermore, *in vivo* disruption of this complex results in inefficient DSB-formation at Sµ and a concomitant defect in CSR but not in somatic hypermutation. We thus propose a model in which KAP1 and HP1 tether AID to H3K9me3 residues that mark Sµ in order to permit efficient CSR.