Transcriptional regulation of the IgH locus during class switch recombination

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Transcriptional regulation of the IgH locus during class switch recombination

Régulation transcriptionnelle du locus IgH lors de la commutation isotypique

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“Desconfía de aquellos que poseen más respuestas que preguntas.”
Rosa Montero - *Historia del rey transparente*

“Distrust those who own more answers than questions.”
Rosa Montero - *The story of the translucent king*

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Table of contents

List of figures

List of abbreviations

3C:	chromosome conformation capture
4C-Seq:	chromosome conformation capture (3C) with high-throughput sequencing
53BP1:	p53 binding protein 1
3'RR:	3' regulatory region
a-EJ:	alternative end joining
AID:	activation-induced cytidine deaminase
APE:	apurinic/apyrimidinic (AP) endonuclease
APOBEC:	apolipoprotein B mRNA-editing catalytic polypeptide
ATM:	ataxia telangiectasia mutated
BCR:	B cell receptor
BER:	base excision repair
C region:	constant region
Cas9:	CRISPR-associated protein-9 nuclease
CBS:	CTCF binding site
ChIP:	chromatin immunoprecipitation
ChIP-Seq:	ChIP followed by deep sequencing
c-NHEJ:	classical non-homologous end joining
CRISPR:	clustered regularly interspaced short palindromic repeats
CSR:	class switch recombination
CTCF:	CCCTC-binding factor
C^{ter}:	carboxy-terminal extremity
D:	diversity genes
dCas9:	Cas9 endonuclease dead (dead Cas9)
DDR:	DNA damage response
DHS:	DNase hypersensitive site
DSB:	double stranded break
eRNA:	enhancer RNA
Exo1:	exonuclease 1
Eμ:	enhancer μ
Fab:	antigen-binding fragment
Fc:	crystallisable fragment
FcR:	receptor of the crystallisable fragment
FO:	follicular B cells
Fv:	variable fragment

GC: germinal center
GFP: green fluorescent protein
GLT: germline transcript
gRNA: guide RNA

HIGM: hyper-Immunoglobulin M
HoxC4: Homeobox C4
hs: DNase hyper sensitive site

I: intervening exon
Id2: inhibitor of DNA binding 2
Ig: immunoglobulin
IGCR1: intergenic control region 1
IgH: immunoglobulin heavy chain
IL4: interleukin 4
IRF4: interferon regulatory factor 4
IRF8: interferon regulatory factor 8

J: joining genes

LCR: locus control region
LPS: lipopolysaccharide
LSR: locus suicide recombination

Mb: mega base pair
Med1: mediator complex subunit 1
MMR: mismatch repair
MSH2: MutS homolog 2
MSH6: MutS homolog 6
MZ: marginal zone B cells

NES: nuclear export signal
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NLS: nuclear localization signal
N^{ter}: NH_2-terminal extremity

PDQ52: promoter of DQ52
Pol II: RNA polymerase II
pre-B: B cell precursor
Pre-BCR: BCR precursor
pro-B: B cell progenitor

RAG1/2: recombination activating genes 1/2
RSS: recombination signal sequences

RT-qPCR: quantitative reverse transcription PCR
RV: retrovirus

S region: switch region
SHM: somatic hypermutation
Smc: structural maintenance of chromosome
ssDNA: single stranded DNA
STAT6: Signal transducer and activator of transcription

TdT: terminal deoxynucleotidyl transferase
TF: transcription factor
TGF-β: transforming growth factor β

UNG: uracil N-glycosylase

V region: variable region

XRCC4: X-ray repair cross-complementing protein 4

YY1: yin-yang 1

INTRODUCTION

Living organisms are exposed to a myriad of foreign bodies that represent a potential threat to their integrity. The **immune system** allows for the recognition of the non-self and protects the organism from possible harm. The basic knowledge acquired over the decades on the field of immunology is now being translated into the treatment of disease through immunotherapies. However, the complexity of the immune system makes its full understanding a daunting challenge and many aspects of its molecular basis remain obscure.

While the innate immune system is broadly conserved throughout evolution, the **adaptive immune system** first appeared in vertebrates (Beck and Habicht, 1996). Its characteristic feature is the presence of T and B lymphocytes, capable of triggering an antigen-dependent immunological memory. Among the adaptive immune cells, the **B lymphocytes** are characterized by the secretion of antibodies, as well as by the production of the membrane-bound form of these antibodies, known as immunoglobulins (Ig's) or B cell receptors (BCRs). These molecules are capable of recognizing with a high affinity specific antigens from foreign bodies and subsequently trigger a series of molecular events in the B cell that will be essential for the adaptive immune response and the generation of memory.

The object of this thesis lays precisely on the molecular mechanisms taking place upon the antigen-antibody interaction that lead to an adapted effector function of the immunoglobulin.

1. The BCR

1.1. Structure and function of the BCR

The BCR or Ig is a membrane-bound antibody with two main functions: binding to the antigen outside the cell membrane, and transmitting the signal to the inside in order to trigger the activation of the B cell. The molecular structure of the BCR **(figure 1)** allows for the fulfillment of both tasks.

BCRs are composed of globular domains, forming two identical light chains and two identical heavy chains linked by disulfide bonds, each one of them comprising a variable (V) and a constant (C) region, located on the NH_2-terminal (N^{ter}) and carboxy-terminal (C^{ter}) of the protein, respectively. There are two types of light chains in mammals, called λ and κ, composed of one variable (V_L) and one constant (C_L) structural subunits. As for the heavy chain, they are composed of one variable (V_H) and several constant (C_H) subunits, the specific number of the later depending on the class of heavy chain.

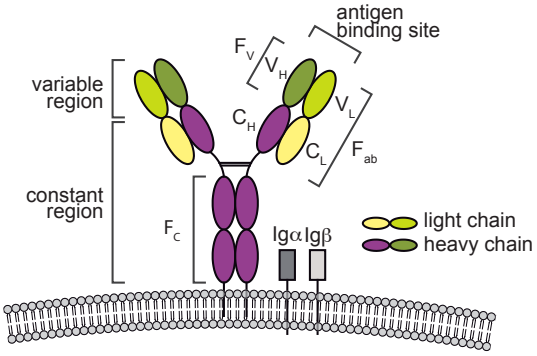


Figure 1: The structure of the BCR. The Ig or BCR is composed of two identical heavy chains and two identical light chains, each one of them composed of a variable region and a constant region. The Fc fragment in the C_H chain determines the effector function of the Ig, whereas the variable region determines the antigen specificity. The Igα/Igβ heterodimer is responsible for the signal transduction to the cytoplasm.

The immunoglobulin region that binds to the antigen is called the antigen-binding fragment (Fab) and is composed of one variable and one constant domain of each heavy and light chains. The variable regions of the light and heavy chains combined form the variable fragment (Fv), which is unique for each B cell and determines the antigen specificity. On the other hand, the structure of the crystallisable fragment (Fc) of the heavy chain differs among isotypes and dictates the effector function of the immunoglobulin.

Finally, the Igβ and Igα subunits form a heterodimer capable of signal transduction through the membrane to the inside of the cell. **(figure 1).**

1.2. The Ig isotypes

There are five main isotypes of immunoglobulins in mammals (IgM, IgD, IgG, IgE, and IgA) **(figure 2)** that differ in the constant region of their heavy chain (Cμ, C∂, Cγ, Cε, and Cα, respectively) and determine the effector function of the molecule. The Fc fragment binds to Fc receptors (FcRs) that are located on diverse effector cells such as mast cells, macrophages, monocytes, and natural killer cells. Specific isotypes bind to different FcRs and this interaction triggers an immune response intended to eliminate the antigen.

Isotype	Form	Characteristics and functions	Fc receptors and their roles
 IgM	Monomer or pentamer (secreted form)	Present in blood and lymph Efficient activation of complement Low affinity, high avidity	FcμR → endocytosis Fcα/μR → phago/endocytosis
 IgG	Monomer	Most abundant isotype Present in blood and extracellular fluid Complement activation Opsonization of pathogens Neutralization of toxins and viruses Mother to fetus transport across placenta	FcγRI (IgG2a,2b,3) → activation FcγRIIB (IgG1,2a,2b) → inhibition FcγRIII (IgG1,2a,2b) → activation FcγRIV (IgG2a,2b) → activation FcRn (IgG1,2a,2b,3) → IgG homeostasis
 IgE	Monomer	Low levels in blood and extracellular fluid Defense against parasites Hypersensitivity reactions	FcεRI → activation FcεRII → regulation FcγRIIB → inhibition FcγRIII → activation FcγRIV → activation
 IgA	Monomer or dimer	Present in mucosal areas and secretions Neutralization of toxins and viruses Newborn immunity through breast milk	Poly-IgR → Ig transport Fcα/μR → phago/endocytosis

Figure 2: The Ig isotypes. The structure of the Ig C_H chain determines its isotype. In the table, the schematic structure of the different isotypes is depicted, together with a short indication of its characteristics, effector functions, and the Fc receptors that can bind them (Bruhns, 2012; Bruhns and Jonsson, 2015; Janeway CA Jr, 2001).

The effector functions, mediated by different immune cells, determine the specificity of the response. Since each isotype can effectively engage only some effector functions, the production of isotypes during the immune response is adapted depending on the stimuli received by the B cell.

2. The murine Igh locus

The BCR isotype is determined by the C region of its heavy chain. The immunoglobulin heavy (IgH) chain is encoded in the *Igh* locus **(figure 3)**, which in mice lays on the long arm of chromosome 12 and spans around 2.8 Mb. Most of its length (approximately 2.5 Mb) is constituted by the 16 families of V_H gene segments on the 5' end of the locus, which can be categorized into distal (5' end), intermediate, or proximal (3' end). These are followed by 8-12 diversity (D_H) gene segments, and 4 joining gene segments (J_H1-4). The V, D, and J gene segments are rearranged in two consecutive recombination events early in the development of B cells known as the V(D)J recombination. Once rearranged, they code for the antigen-binding site of the Ig heavy chain.

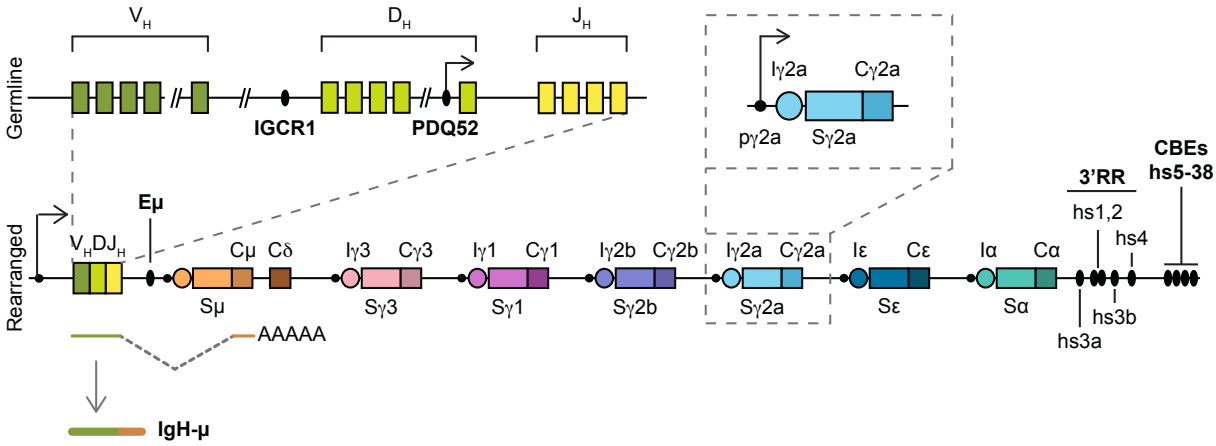


Figure 3: Schematic representation of the murine Igh locus. In mice, the Igh locus is located on chromosome 12. The V, D, and J gene segments, present at the 5' end of the locus, are depicted at the top of the figure. Once reassembled, they constitute the variable part of the Ig and determine the antigen specificity. The C_H regions are encoded as transcriptional units (dashed box) formed by a germline cytokine-inducible promoter that drives transcription through an intervening I exon, an S_X region, and a C_X region. The intronic Eμ enhancer is located downstream from the last J segment. The 3'RR is constituted by a series of enhancers and CBSs acting as insulators. Other regulatory regions are PDQ52 and IGCR1.

All the different constant regions are encoded downstream from the J_H gene segments as transcriptional units, formed by a cytokine-inducible I promoter followed by an intervening exon (I), a highly repetitive switch (S) region, and the exons encoding the constant (C) region (Lennon and Perry, 1985). Traditionally, the exception was C∂, which is not organized as a transcriptional unit but is produced by alternative splicing of Cμ. However, the presence of a rudimentary S∂-like region has also been documented (Saintamand et al., 2015a).

Several cis-regulatory elements are known in the *Igh* locus (Perlot and Alt, 2008): **I)** The intronic Eμ enhancer is located just upstream of Cμ and is known to control V(D)J recombination. **II)** At the 3' end of the locus lays the 3' regulatory region (3'RR), a super-

enhancer region with clustered DNase hypersensitive sites (DHSs) with both enhancer and insulator activities. The DHS hs1,2 is surrounded by the hs3a and hs3b regions, quasi-homologous in sequence but in opposite directions, and hs4 lays directly 3' from hs3b. **III)** Downstream, and constituting the known end of the Igh locus, lay the regulatory elements hs5-7 and hs38, presenting CTCF-binding sites (CBSs) with insulator activity (Garrett et al., 2005; Volpi et al., 2012). **IV)** A promoter upstream of DQ52 (PDQ52), the D segment closest to the J cluster, has been described to have both promoter and enhancer activities in pre-B cells (Kottmann et al., 1994). **V)** Between the VH and DH gene segments lays the intergenic control region 1 (IGCR1) that presents two CBSs (Guo et al., 2011b).

3. Growing up: overview of the B cell development

B cells develop from progenitors in the bone marrow, which differentiate into pro-B cells. It is at this stage when V(D)J recombination of the Igh locus takes place. Pro-B cells expressing a recombined IgH continue developing and produce pre-B cells. At this point, the recombination of the Ig light (IgL) chain takes place. If the V_H and V_L regions fit together, a BCR with a μ constant chain is assembled and displayed on the surface of the immature B cell. Immature B cells leave the bone marrow and enter the periphery, where they will finish their development (Prieto and Felippe, 2017) **(figure 4)**.

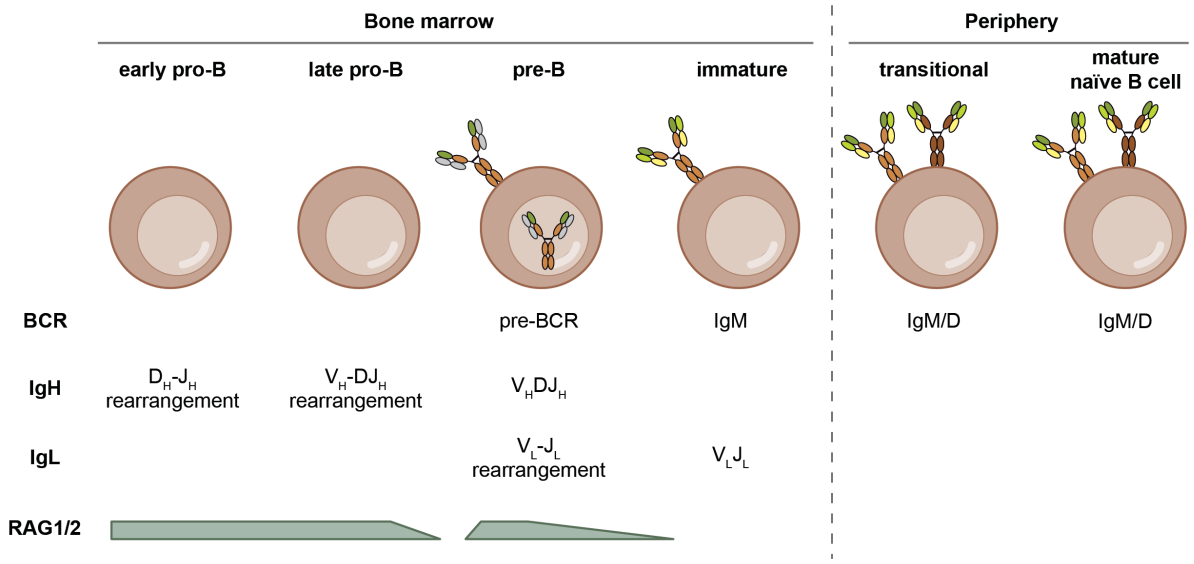


Figure 4: The B cell development. B cells develop from precursors in the bone marrow. During the pro-B stage, they undergo V(D)J recombination on the Igh locus, whereas the light chain is rearranged in the pre-B cell stage. RAG1/2 is expressed in pro-B cells and then silenced, only to be expressed again in the pre-B cell stage to allow the assembly of the V_L genes. The pre-BCR carries a surrogate light chain that is substituted by the mature form once the V_LJ_L rearrangement is complete. Transitional B cells expressing either IgM or IgD isotypes leave the bone marrow and they mature into naïve B cells that are ready to trigger an immune response when they encounter their cognate antigen.

Mature B cells give rise to the main adult B cell populations:

I) **Follicular (FO) B cells** belong to the **B2 lineage** and mediate the adaptive humoral immunity. They are the majoritarian B cell type in the spleen and produce all the different isotypes in response to T cell-dependent stimuli (Prieto and Felippe, 2017).

II) **B1 B cells** are abundant in the pleural and peritoneal cavities. They engage in T cell-independent responses and are an important source of IgM and IgA isotypes (Grasseau et al., 2019; Kaminski and Stavnezer, 2006).

III) **Marginal zone (MZ) B cells** share characteristics with both B1 and B2 B cell lineages. They are located in the marginal zone of the spleen, and are the first line of defense from pathogens carried by the blood. Together with the B1 cells, they are considered part of the innate immunity, since they are involved in early and rapid T cell-independent responses (Cerutti et al., 2013).

There are different hypotheses to explain the development of B1 and B2 lineages. The lineage hypothesis supports the notion of different B cell precursors that give rise to each subset. Supporting this theory, adult B cell precursors were unable to reconstitute the B1 population of an immunodeficient host, while fetal progenitors reconstituted both B1 and B2 populations (Hayakawa et al., 1985) and the bias of a B cell precursor toward the specific generation of the B1 subset (Montecino-Rodriguez et al., 2006). On the other hand, the induced differentiation model … the existence of a common progenitor for B1 and B2 cells and it would be through the signals received by the B cell that the lineage is chosen, depending for example in the strength of the signaling through the BCR (Casola et al., 2004) or the response to T cell-independent antigens (Berland and Wortis, 2002). Finally, the two-pathways model combines both hypothesis and suggests the presence of different progenitors and subsequent selection to give rise to differentiated subsets (Baumgarth, 2011). Altogether, how the B1 and B2 differentiated lineages appear remains to be clarified.

4. The BCR diversification

4.1. V(D)J recombination: the generation of the primary repertoire

The B cell primary repertoire counts with an immense diversity of antigen receptors, which allows for the recognition of the vast number of different pathogens that can potentially invade the organism. The first step in the diversification of the immunoglobulins takes place in developing progenitor (pro-B) and precursor (pre-B) B cells in the bone marrow, when individual V, D, and J gene segments are assembled in a programmed, although arbitrary at the same time, rearrangement process known as the V(D)J recombination **(figure 5)**.

V(D)J recombination in the *Igh* locus is divided in two separated steps; first, the recombination between one D_H and one J_H segment takes place, and this is followed by the assembly of a V_H gene segment with the rearranged DJ_H (Cobb et al., 2006). The D_H to J_H recombination takes place on both alleles, but the V_H to DJ_H recombination is subject to allelic exclusion, ensuring a unique antigen specificity on each B cell (Mostoslavsky et al., 2004). The order of the two recombination steps is regulated through the so-called 12/23 rule. The V_H, D_H, and J_H gene segments are surrounded by recombination signal sequences (RSS) that are composed of a conserved heptamer and nonamer, separated by a non-conserved intermediate sequence or spacer, of 12 or 23 nucleotides long. One can differentiate RSSs with 12 or 23 base pair spacers as 12RSS and 23RSS, respectively. Two gene segments can efficiently synapse only if one of them lays next to a 12RSS and the other one to a 23RSS (Schatz and Swanson, 2011). In the *Igh* locus, the V_H and J_H gene segments are surrounded by 23RSS sequences, while D_H gene segments are next to 12RSS sequences, thus guaranteeing the order of the events and preventing a V_H to J_H recombination.

The V(D)J recombination is initiated by the recombination activating genes (RAG) RAG1 and RAG2 endonucleases, which together form the RAG1/2 recombinase (Alt et al., 2013). First, in combination with DNA-binding proteins, it recognizes RSSs producing double stranded breaks (DSBs) between the RSS and the gene segment. Secondly, the RAG complex interacts with classical non-homologous end joining (c-NHEJ) pathway factors to repair the DNA damage and complete the recombination (Alt et al., 2013).

Even though the two V(D)J recombination successive steps are well regulated, the choice of the individual gene segments involved in the recombination is random. Also, the resolution of the junction by the RAG1/2 recombinase can result in a random loss or gain of some nucleotides at the gene segment added by the enzyme terminal deoxynucleotidyl transferase (TdT) (Alt et al., 2013). These combinatorial stochasticity and junctional diversity lead to a great antigen receptor variability. However, it also causes the final V region sequence to be potentially out of frame. The immune system has evolved to correct this issue and thus B cells that express a BCR are selected, while those bearing non-productive joints are eliminated (Melchers, 2015).

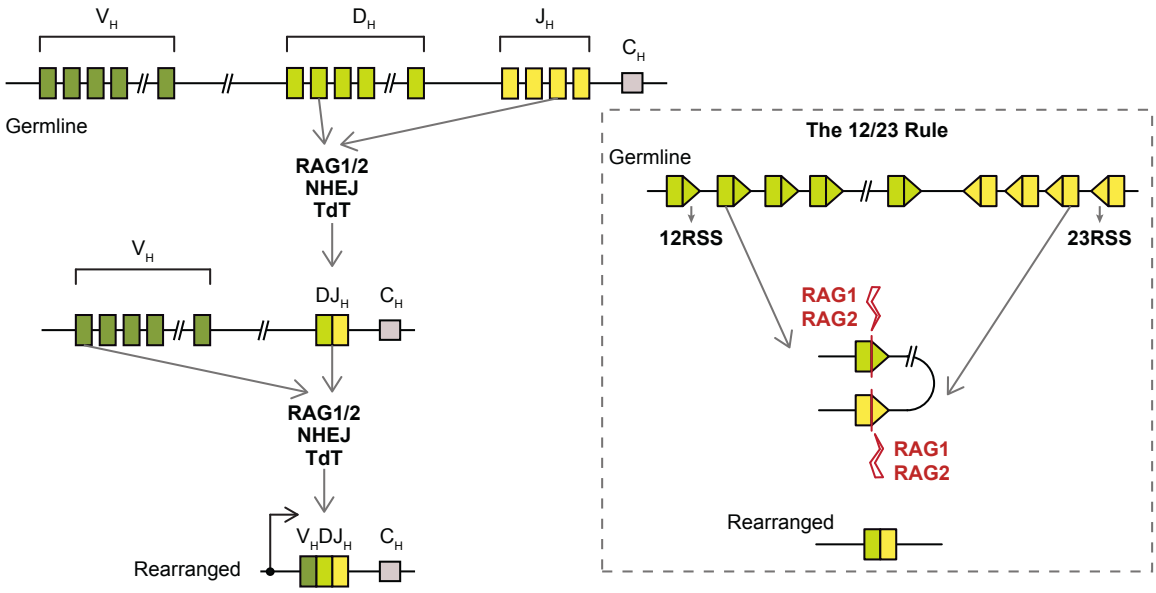


Figure 5: The process of V(D)J recombination of the Igh locus. The V(D)J recombination is temporarily organized. The first step is the D_H to J_H recombination, which is followed by the V_H to DJ_H assembly. Both steps are catalyzed by the recombinase complex RAG1/2, which produces DSBs that are subsequently repaired by the c-NHEJ machinery. Finally, the enzyme TdT causes losses or gains of base pairs in the junction that add variability to the sequence. The order of both rearrangements is orchestrated by the 12/23 rue (dashed box) whereby two RSSs can recombine only if one of them has a 12 bp and the other one a 23 bp long spacer.

4.1.1. The regulation of V(D)J recombination

The random component of the V(D)J recombination process gives necessary and advantageous variability of antigen receptor sites. However, the fact that it involves DNA damage makes it important to be tightly regulated, despite its inherent stochasticity.

There are several **levels of regulation** that control the appropriate functioning of the process (Alicia J. Little, 2015): the tissue where it will take place (only in lymphocytes and not in other cell types); the place (targeted to antigen receptor loci); the time (the specific developmental stage when it has to be active); the order of events (D to J recombination precedes V to DJ); the allelic exclusion (to ensure the expression of an immunoglobulin with a unique antigen specificity in each cell); and the cell cycle stage when it occurs (G0/G1 phase).

The regulation of some of these levels can be explained by the expression pattern of the trigger of V(D)J recombination, the **RAG1/2 recombinase**. Indeed, its expression is restricted to specific developmental stages, only in lymphocytes, and during the G0/G1 cell cycle phase (Alicia J. Little, 2015). However, the RAG1/2 system is not lineage-specific, since it is shared by T and B lymphocytes, and it does not explain either the order of the events, nor the restricted places of action.

The **accessibility model** was the first theory that appeared that could explain other factors (Yancopoulos and Alt, 1985). According to this model, the antigen receptor loci are in a repressive chromatin state by default, thus being inaccessible for the RAG1/2 recombinase, and they become accessible by adopting a more permissive chromatin state at the moment of recombination or just prior to it. Supporting this theory, there is evidence of **germline transcription**, called like this because it does not produce any protein, occurring at the gene segments that are about to be joined (Yancopoulos and Alt, 1985). There has also been documented the antisense transcription of D_H genes during D_H to J_H rearrangement, and V_H antisense transcription after it has completed, explaining the order and the precise localization of the events (Joseph S. Lucas, 2015).

The dynamic location of the locus inside the nucleus, moving away from repressive nuclear compartments when it undergoes recombination (Schatz and Ji, 2011), supports the allelic exclusion initiation and maintenance. Architectural changes of the locus also account for the regulation of V(D)J recombination. Indeed, the locus becomes condensed in pro-B cells and it is decondensed again after the recombination (Schatz and Ji, 2011). The closer proximity between distant regions that this entails, ensures the usage of distal and proximal V_H gene segments in the correct proportions. In this line, the Mediator complex has also been implicated in the regulation of V_H genes usage (Dalloul et al., 2018).

The current **recombination center model** suggests the existence of focal regions of accessible RSSs, with high levels of germline transcription and permissive epigenetic marks, which allow for the recruitment of RAG1 and RAG2 (Ji et al., 2010). Thanks to the physical proximity given by the architectural state of the locus, the RAG1/2 recombinase can find either a proximal or a distal partner RSS and perform the recombination. Altogether, it is noteworthy that the transcriptional regulation of the *Igh* locus is crucial to ensure the accuracy and correct outcome of the recombination process.

4.2. B cells getting ready: the germinal center reaction

Mature, naïve B cells in the periphery that encounter their cognate antigen get activated and migrate to specialized structures within secondary lymphoid organs named germinal centers (GCs), where the affinity maturation process generates highly adapted and specific antibodies through the interaction of antigen-specific B cells with helper T cells and dendritic cells, and the exposure of B cells to cytokines and costimulatory signals **(figure 6)**. With this microenvironment, the GCs constitute the starting point of humoral immunity. Here, the decision between plasma cell or memory B cell commitment is made based on the level of BCR affinity (Ise and Kurosaki, 2019) and the interactions between cognate T and B cells (Biram et al., 2019).

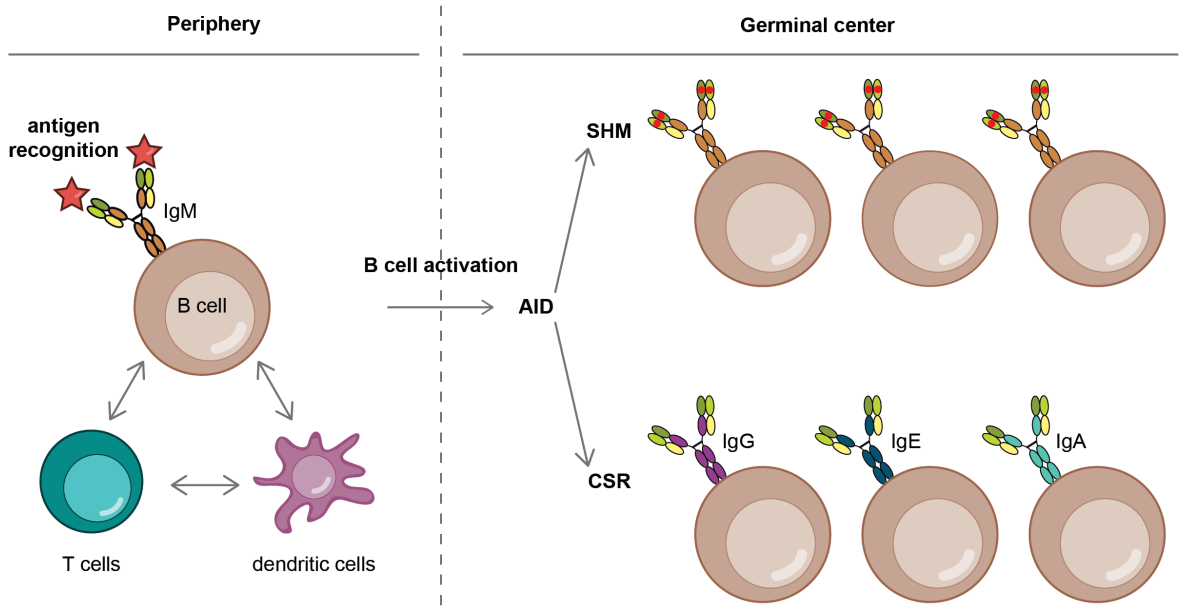


Figure 6: The GC reaction. Mature B cells present in the periphery express IgM/D BCR that can recognize an antigen. Antigen binding, together with extracellular signals received from T cells and dendritic cells, triggers a series of signaling events that lead to B cell activation. When B cells get activated, they migrate into specialized structures called germinal centers (GC) where they diversify their BCR through two independent mechanisms triggered by the enzyme AID. The process of SHM introduces variations in the sequence of V genes, leading to a change in the antigen specificity. BCRs with higher affinity for the antigen are subsequently positively selected. On the other hand, CSR leads to the switch of isotype expressed, from IgM to IgG, IgE, or IgA.

Upon activation, B cells start expressing the enzyme **activation-induced cytidine deaminase (AID)**, which deaminates cytosines into deoxy-uraciles in single-stranded DNA (ssDNA) exposed by transcription. AID triggers two main mechanisms that diversify the BCR and optimize the immune response: on one hand, AID activity introduces modifications in the Ig variable region in a process called **somatic hypermutation (SHM)**, leading to a modified affinity for the antigen. On the other hand, and based on the extracellular signals received by the B cell, it changes the isotype expressed by the B cell, and hence its effector function, through the process of **class switch recombination (CSR)**.

4.2.1. SHM: a tailor-made suit

When B cells get activated upon recognition of their cognate antigen, AID is targeted to the transcribed V_H and V_L regions where it deaminates cytosines into uracils triggering SHM. SHM introduces point mutations and, to a lesser extent, small insertions and deletions in the exons encoding the variable region, causing a change in the antigen-binding capacity of the BCR **(figure 7)**.

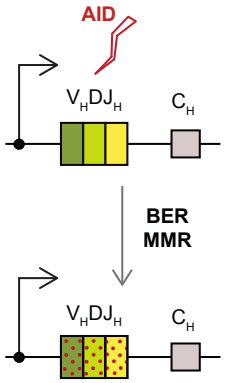


Figure 7: The process of SHM. When B cells get activated, AID is targeted to the V genes, where it generates cytosine deamination. This lesion can either lead to C to T transitions, or be repaired by the BER or MMR pathways. As a result, point mutations and small insertions and deletions are introduced in the V genes.

The U:G mismatches generated by AID activity can directly be resolved as C to T transitions when the replication machinery runs over them (Petersen-Mahrt et al., 2002). Conversely, they can be repaired by the DNA damage response. When targeted by the base excision repair (BER) enzyme uracil DNA glycosylase (UNG), an abasic site is left instead of the U:G and it can be replaced by any nucleotide. In an UNG-deficient mouse model, SHM was biased from transversions towards transitions, meaning that abasic sites were no longer generated (Yokouchi et al., 2017). Also, further processing of U:G mismatches by BER and MMR machinery can generate mutations at A/T sites (Pilzecker and Jacobs, 2019).

These error-prone processing mechanisms lead to the characteristic mutations of SHM, and are crucial for a highly adapted immune response. Indeed, through an affinity maturation process, those B cells bearing mutations that endow their BCR with lower affinity are progressively replaced by negative selection, while those with an enhanced affinity for the antigen are positively selected and proliferate, to finally differentiate into plasma or memory B cells (Hwang et al., 2015; Methot and Di Noia, 2017). Nevertheless, AID must be tightly regulated to avoid potential oncogenic mutations when targeted to non-Ig genes.

4.2.2. The class switch recombination

CSR replaces the isotype expressed from the default IgM/IgD to IgG, IgE, or IgA by a recombination mechanism between the donor Sμ and one of the downstream acceptor S_X (where x is one of the downstream S regions) that precede the C regions (except Cδ) **(figure 8)**. Although constituting a very rare event confined to the mucosal tissues (Choi et al., 2017), a non-conventional recombination between Sμ and an S-like region preceding C_δ named σ_δ has recently been documented, leading to IgM to IgD CSR (Issaoui et al., 2017; Rouaud et al., 2014; Saintamand et al., 2015a).

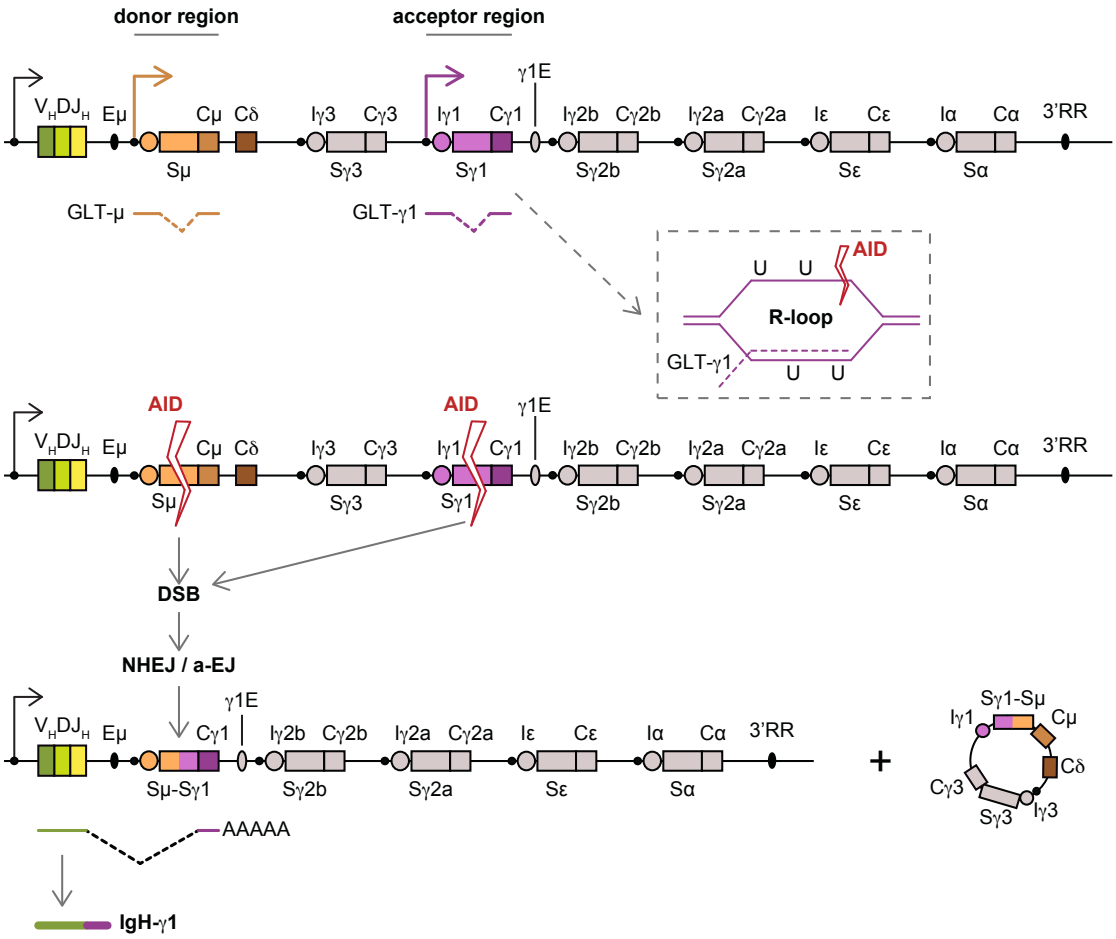


Figure 8: The CSR mechanism. During the process of CSR, AID is targeted to the donor Sμ and an acceptor S_X (Sγ1 in the example) regions. Thanks to the germline transcription from specific cytokine-inducible promoters (γ1 promoter in the example), an R-loop is formed (dashed box) whereby the G-rich non-template strand loops out as ssDNA, providing the substrate for AID activity. The DNA lesions caused on the S regions by AID are repaired through the BER and MMR pathways, resulting in the generation of DSBs that are subsequently joint through c-NHEJ or a-EJ thanks to the dynamic long-range interactions that bring both the donor and acceptor S regions in proximity. As a result, a new C_H region is juxtaposed to the VH genes and a new IgH chain is expressed (IgH-γ in the example).

CSR is transcription-dependent, requires long-range interactions inside the *Igh* locus, and is triggered by AID. When B cells are activated and migrate to the GCs, AID is expressed and targeted to the donor Sμ and an acceptor S regions. Different Ig isotypes provide different protective immune functions and determine the Ig tissue distribution through the binding of their C_H region with Fc receptors on the surface of effector cells. Hence, the choice of isotype is dependent on the combination of antigens and extracellular stimuli received by the B cell (such as different cytokines, CD40L, and lipopolysaccharide) (Nicolas et al., 2018) in order to assure an optimized, adapted response. Through signal transduction from the membrane, individual cytokine-inducible promoters located upstream of the S regions get activated. The transcription from these promoters through the S and C_H regions produces the so-called sterile or **germline transcripts** (GLTs) that do not produce any protein (Chaudhuri et al., 2007) but are essential for the choice of recombination to a determined isotype (Basu et al., 2011; Pavri and Nussenzweig, 2011).

The role of the GLTs is crucial for an effective CSR. Several studies have shown that deletion of I_X promoters, hence suppressing GLT production, reduces CSR to their correspondent isotypes (Bottaro et al., 1994; Jung et al., 1993; Kuzin et al., 2000; Lorenz et al., 1995; Qiu et al., 1999; Seidl et al., 1998; Zhang et al., 1993). GLTs have also been implicated in the introduction of activating epigenetic marks in S regions and repressive marks in C regions, thus reorganizing the topology of the *Igh* locus (Wang et al., 2009). Currently, GLTs are known to help rendering the S regions accessible for AID. Indeed, when the S region is transcribed, the template strand forms a stable RNA-DNA hybrid, while the highly repetitive, G-rich non-template strand loops out, free to form **R-loops** (Roy et al., 2008). Despite the lack of *in vivo* evidence (Pavri, 2017), these R-loops are thought to allow for the liberation of the non-template strand as ssDNA, hence exposing the natural substrate of AID and facilitating its activity (Chaudhuri et al., 2007; Shinkura et al., 2003; Yu et al., 2003). R-loops also constitute a challenge for transcription elongation, and they have been implicated in stalled RNA polymerase II (Pol II) on S regions (Wang et al., 2009), which helps recruiting AID (Kenter et al., 2012) and is thought to enhance its mutational activity (Nambu et al., 2003; Pavri and Nussenzweig, 2011; Rajagopal et al., 2009). The importance of R-loops was evidenced as the inversion of the Sγ1 region, impeding R-loop formation due to the loss of a non-template G-rich strand, led to a significant diminution of CSR efficiency (Shinkura et al., 2003).

Deamination lesions produced by AID inside the S regions trigger the DNA damage response (DDR) and are thus processed by the base excision repair (BER) and the mismatch repair (MMR) machinery. The requirement of these pathways in CSR was made evident in an uracil N-glycosylase (UNG)-deficient mouse model, where CSR was severely impaired (Rada et al., 2002). UNG recognizes the U:G mismatches and generates abasic sites, which are subsequently cleaved by apurinic/apyrimidinic (AP) endonuclease (APE1). The accumulation of nicks along both strands of the S regions facilitates the spontaneous formation of DSBs. Conversely, the MMR can directly generate DSBs from single strand breaks (Schrader et al., 2007).

Thanks to the dynamic long-range interactions taking place in the locus, which bring the participating S regions together, the S-S synapsis that permits recombination is formed. CSR is then resolved by joining of the donor Sμ and the activated acceptor S_X region by means of the classical non-homologous end joining (c-NHEJ) or, in its absence, the alternative end joining

(a-EJ) pathway, and the intermediate sequence is deleted as a circular episome (Dong et al., 2015). The molecular footprint of the junctions differs based on the joining pathway that is used. c-NHEJ leaves blunt or small microhomology joints (Stavnezer et al., 2010), whereas the a-EJ pathway results in longer microhomologies (Chang et al., 2017; Chaudhuri and Alt, 2004; Panchakshari et al., 2018). In the absence of c-NHEJ components, a-EJ takes place instead, as suggests the presence of longer microhomologies in the joints (Panchakshari et al., 2018). The microhomolgoy-mediated end joining pathway (MMEJ) is implicated in the repair through a-EJ, as indicated by the deficit in CSR upon disruption of the MMEJ member poly (ADP-ribose) polymerase (PARP-1) (Robert et al., 2009).

Deficiencies in several DDR and c-NHEJ factors such as ataxia telangiectasia mutated (ATM) (Reina-San-Martin et al., 2004), X-ray repair cross-complementing protein 4 (XRCC-4), ligase 4, ku70, or ku80 produce a defect in CSR and junctions show longer microhomologies, suggesting the usage of a-EJ (Boboila et al., 2012; Han and Yu, 2008; Panchakshari et al., 2018; Soulas-Sprauel et al., 2007; Yan et al., 2007). On the other hand, deletion of 53BP1 or γH2AX abolishes or diminishes, respectively, CSR, and they have been suggested to be implicated in S-S synapsis (Reina-San-Martin et al., 2007; Reina-San-Martin et al., 2003).

p53 binding protein 1 (53BP1) is recruited to DSBs, where it protects the loose ends from resection and biases the repairing choice towards c-NHEJ and against a-EJ (Bothmer et al., 2011; Bothmer et al., 2010; Bunting et al., 2010; Jankovic et al., 2013). 53BP1 has been suggested to have also a structural role in CSR by facilitating the synapses between the Eμ and the hs3b,4 enhancer in stimulated B cells (Feldman et al., 2017; Wuerffel et al., 2007). Altogether, these studies demonstrate the crucial role of DNA repair in CSR and the importance of its components for the efficiency of the process.

As a result of the recombination, a new C_H is brought in proximity of the recombined V_HDJ_H exons and a new constant region is produced, endowing the BCR with novel effector functions while maintaining its antigen affinity intact.

Remarkably, an interesting CSR-like event driven by AID has been described that involves a recombination between Sμ and S-like regions inside the 3'RR (Peron et al., 2012), leading to elimination of all the intervening C_H genes upon repair of the DSBs through the a-EJ pathway (Boutouil et al., 2019). Due to the necessity of BCR expression for B cell survival, this process was named locus suicide recombination (LSR) and it has been suggested to be a regulator of B cell homeostasis both in mice (Peron et al., 2012) and humans (Dalloul et al., 2019).

5. The enzyme AID

The enzyme AID was discovered by the group of Tasuku Honjo thanks to its overexpression in the B cell line CH12 stimulated to undergo CSR, drastically higher than the basal expression displayed by non-stimulated cells (Muramatsu et al., 1999). Also, AID expression was enhanced in the germinal centers of immunized mice. Very soon after, the same group showed that SHM and CSR were dependent on AID and linked the enzyme with the DNA damages of both processes (Muramatsu et al., 2000). At the same time, the human form of AID was found mutated in the hyper-IgM syndrome (HIGM2), displaying abolished CSR and SHM (Revy et al., 2000). Following these discoveries, the action of AID was linked with the initiation steps of CSR, since it was demonstrated that it acted before the apparition of S region mutations (Petersen et al., 2001).

Based on its homology to the enzyme APOBEC1 (apolipoprotein B mRNA-editing catalytic polypeptide), it was initially suggested that AID could act through RNA deamination. However, a DNA deamination model was soon insinuated, as AID was shown to produce transitions at C/G in *E. coli* and these mutations were enhanced in the absence of UNG, which eliminates uracil from the DNA (Petersen-Mahrt et al., 2002).

Not reaching the 200 amino acids, AID is a small protein composed of a nuclear localization signal (NLS) in its N^{ter} domain, a cytidine deaminase motif in the middle region, and a nuclear export signal (NES) in its C^{ter}. The NLS and NES determine the compartmentalization of AID inside the cell (Ito et al., 2004; Patenaude et al., 2009). Besides, the N^{ter} part of the protein has been implicated in the process of SHM, whereas the C^{ter} part appears to be required for CSR (Barreto et al., 2003; Geisberger et al., 2009; Shinkura et al., 2004).

5.1. The regulation of AID

Due to its intrinsic mutagenic activity, AID expression is strictly regulated. Indeed, as stated above, mutations in the *AICDA* gene encoding AID are the cause of the HIGM2 syndrome (Revy et al., 2000). On the other hand, overexpression of AID has been linked to the induction of lupus, concomitant with elevated frequencies of mutation of the V genes and higher percentage of switched cells, normally biased towards the IgG1 isotype (Jiang et al., 2007; van Es et al., 1991; Zan et al., 2009). Moreover, its overexpression also correlates with higher probability of translocations with potential oncogenic effect (Sernandez et al., 2008; Takizawa et al., 2008).

Several levels of regulation are coordinated to control the expression of AID. In the first place, the *AICDA* gene is transcriptionally regulated so its expression is restricted to activated germinal center B cells, and silenced after their differentiation into antibody-secreting cells or memory B cells. A myriad of TFs has been implicated in this regulation through their action on the four cis-regulatory regions present in the *AICDA* locus. For example, signals triggered by CD40 ligand (CD40-L) and IL4 synergize to activate NF-κB and STAT6, respectively, leading to the binding of these factors to 5' regions of *AICDA* and the induction of AID expression (Dedeoglu et al.,

2004). There are several known activator (such as the aforementioned NF-κB and STAT6, but also homeobox C4 (HoxC4), Pax5, interferon regulatory factor 4 (IRF4), CCAAT/enhancer-binding proteins, E-box proteins, and FOXO1) and repressor (such as inhibitor of DNA binding Id2 and Id3, BLIMP, and E2F) factors, whose balanced interaction determine the expression pattern of AID (Matthews et al., 2014b). Similarly, estrogen is thought to activate AID expression, whereas progesterone would repress it (Pauklin and Petersen-Mahrt, 2009; Pauklin et al., 2009).

Beyond transcriptional regulation of the *AICDA* gene, other levels of regulation have been proved to control AID activity. The compartmentalization of the protein, through a balance between the stabilization in the cytoplasm and the nucleus, ensures a mostly cytoplasmic presence of the enzyme and controls the extent of its effect on genomic DNA, while the NLS sequence allows for the active transport of AID to the nucleus (Ito et al., 2004; Orthwein and Di Noia, 2012). Also, some micro-RNAs have been implicated in the stability of the *AICDA* transcript and are known to control the amount of AID that is expressed. Both miR-181b and miR-155 negatively regulate AID (de Yebenes et al., 2008; Teng et al., 2008), and the latter is considered a tumor suppressor, since it prevents Myc-Igh translocations (Dorsett et al., 2008). The post-translational modifications of the protein, mostly phosphorylation, also regulate its activity. Depending on the residue that gets phosphorylated, the outcome varies from inactivating AID (Gazumyan et al., 2011) to enhancing its effect (Basu et al., 2005; Pasqualucci et al., 2006). Finally, there are some known co-factors (importins, heat shock proteins Hsp40 and Hsp90, Dnaja4, YY1, REGγ) that regulate AID targeting and activity, both in the cytoplasm and inside the nucleus, during SHM and CSR (Matthews et al., 2014b).

5.2. AID hits the target

Due to its inherent capacity to generate lesions in the DNA, the correct targeting of AID is crucial. Indeed, off-target DNA damage produced by AID leads to mutations and translocations that are at the base of B-cell lymphomas (Nussenzweig and Nussenzweig, 2010). AID preferentially deaminates cytosines within the specific motif WRCY (Hackney et al., 2009), very abundant in the S regions and in the V genes. However, this sequence preference is not enough to explain AID targeting, since this motif is not absolutely required for CSR (Khamlichi et al., 2004; Shinkura et al., 2003) and the same motif is rarely mutated in regions that are generally not targeted by SHM (Yeap et al., 2015).

In the *Igh* locus, the 3'RR is crucial for AID targeting during both SHM and CSR (Khamlichi et al., 2000a). The deletion of the 3'RR led to reduced AID recruitment during CSR to IgG1 isotype (Saintamand et al., 2015c), although the mechanisms explaining this effect are still not explained. The epigenetic marks also play a role on AID targeting. Indeed, AID targets associate with chromatin marks of active chromatin (Wang et al., 2014). For example, S_X regions are rich in these chromatin marks, whereas the C_X regions appear in a more repressive chromatin state (Wang et al., 2009).

The mutations produced by AID correlate with transcriptionally active sites (Peters and Storb, 1996). The importance of transcription was highlighted when it was observed that AID interacts with Pol II (Nambu et al., 2003), and that Pol II stalling correlates with the mutation pattern of AID and predicts its target sites (Pavri et al., 2010; Rajagopal et al., 2009; Wang et al., 2009; Yamane et al., 2011). The requirement of transcription could be due to the generation of ssDNA that it entails, by enabling the non-template strand to loop out, thereby providing the substrate of AID (Chaudhuri et al., 2003; Ramiro et al., 2003). Similar proportions of AID-driven mutations have been observed on both strands (Milstein et al., 1998; Xue et al., 2006). It has been shown that the exosome complex plays a role on AID targeting to the template strand by degrading the nascent RNA, thus generating ssDNA (Basu et al., 2011; Pefanis et al., 2014).

There are evidences that suggest that AID is targeted to regions with abundant anti-sense transcription, which constitutes a hallmark of active super-enhancers (Meng et al., 2014; Pefanis et al., 2014). In fact, it has been proposed that AID targets enhancers (Wang et al., 2014) and it would exert its activity through the physical interaction with their target regions. This would be supported by the fact that translocation partners and off-target sites mutated by AID are associated with elevated levels of anti-sense transcription (Chiarle et al., 2011; Klein et al., 2011; Liu et al., 2008; Yamane et al., 2011).

Nevertheless, it is of note that, despite being a promiscuous enzyme with numerous known interactions throughout the genome, AID binding does not necessarily imply the generation of mutations (Matthews et al., 2014a).

Altogether, the complexity of AID regulation and targeting, orchestrated by a myriad of coordinated elements, highlights the importance of its control to ensure the physiological activity of the enzyme and prevent potential pathological outcomes.

6. Sailor's knots: Long-range interactions in the Igh locus

The *Igh* locus is not a still structure inside the nucleus. Indeed, it is subjected to diverse conformational changes orchestrated by the Eμ enhancer and the 3'RR super-enhancer.

The locus localizes at the nuclear periphery and appears in a relaxed conformation, and it is actively brought to the center only in the B cell lineage at the stage of pro-B cell, coinciding with the V(D)J recombination (Fuxa et al., 2004; Kosak et al., 2002). The interaction between the Eμ and the 3'RR enhancers occurs early in development in lymphocyte progenitors, preparing the locus in an active state for the V(D)J recombination (Guo et al., 2011a). The *Igh* locus is later decontracted in the pre-B cell stage, probably as a mechanism to help allelic exclusion (Roldan et al., 2005). Transcription factors (TF) like Ikaros (Reynaud et al., 2008), Pax5 (Fuxa et al., 2004), and yin-yang 1 (YY1) (Liu et al., 2007) are known to control the contraction of the locus, which is mediated as well by CCCTC-binding factor (CTCF) and cohesin (Degner et al., 2011; Kenter and Feeney, 2019).

In mature resting B cells, the Eμ and the 3'RR are bound forming a loop that encompasses the C_H genes (Kenter et al., 2012; Wuerffel et al., 2007) **(figure 9 and figure 10)**. This Eμ:3'RR interaction was disrupted in an hs3b,4 deficient context, both in resting and in stimulated B cells, whereas the deletion of Eμ had only a modest effect (Wuerffel et al., 2007), indicating that the loop between the two main regulatory regions of the *Igh* locus is controlled by elements of the 3'RR. Moreover, this interaction is reinforced by 53BP1, as chromosome conformation capture (3C) experiments showed that deletion of this protein led to diminished contact between the two enhancers, while it had little effect on the dynamic long-range interactions occurring in the locus upon B cell stimulation (Feldman et al., 2017). Indeed, for the donor and acceptor S regions to recombine during CSR, they need to be physically near so they can "find" each other, given the long linear distance between them (up to 150kb) and the complex chromatin environment of the nucleus. This problem is overcome through dynamic intra-chromosomal interactions **(figure 9 and figure 10)**. When B cells get stimulated and CSR is triggered, the specific cytokine-inducible promoter involved in the process is also brought to the loop and allows for the formation of the S-S synapsis (Kenter et al., 2012; Pefanis et al., 2015; Thomas-Claudepierre et al., 2016; Wuerffel et al., 2007).

The dynamic interactions in the *Igh* locus of stimulated B cells are stabilized by the Mediator complex and cohesin (Thomas-Claudepierre et al., 2016; Thomas-Claudepierre et al., 2013). Our group showed that the subunits Smc2 and Smc3 of cohesin were dynamically recruited to the donor region and bound AID in stimulated B cells (Thomas-Claudepierre et al., 2013). The interaction mediated by cohesin is required for CSR, since knockdown of cohesin subunits lead to a defect on the efficiency of the process.

Furthermore, Mediator subunits Med1 and Med12 robustly bound the Eμ and 3'RR enhancers in resting B cells, and they were also recruited to the S_X promoters in activated B cells in a stimulation-dependent manner, perfectly correlating with the topology of the *Igh* locus as seen by 4C-Seq experiments (Thomas-Claudepierre et al., 2016). Deficiency of Med1 both in a B cell line and in splenic B cells showed a drop in CSR efficiency, transcription of acceptor S_X regions,

and interactions between Eμ and the acceptor regions (Thomas-Claudepierre et al., 2016), indicating that the mediator complex is required for the dynamic long-range interactions that take place in the *Igh* locus upon activation. Surprisingly, a region downstream the Cγ1 gene (**γ1E** hereafter) was also bound by Med1 and Med12, and it was recruited to the long-range interactions upon B cell activation in a stimulation-dependent manner (Thomas-Claudepierre et al., 2016).

Taking all these studies together, the current model indicates that the *Igh* locus presents a long-range interaction between Eμ and the 3'RR in resting B cells, and that the transcriptionaly active acceptor region promoters are brought to this interaction upon stimulation. These dynamic contacts are regulated by the Mediator complex, whereas the stable association between Eμ and 3'RR is stabilized by 53BP1 (Feldman et al., 2017).

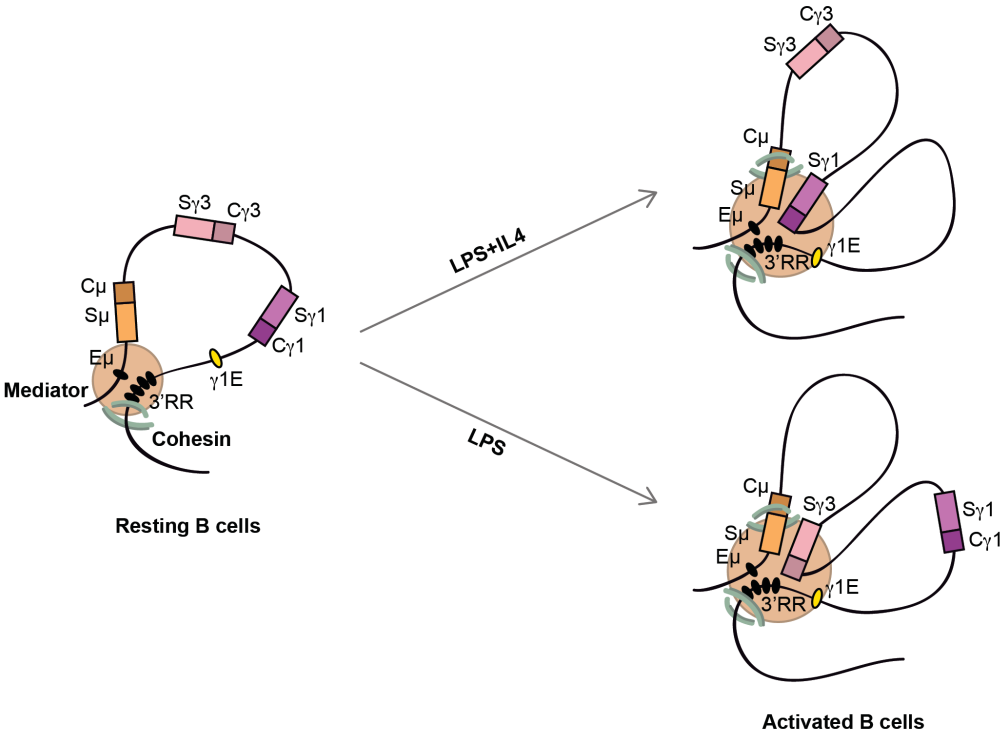


Figure 9: The dynamic conformational changes in the Igh locus upon B cell stimulation. The Igh locus of resting B cells appears in a stable loop conformation in which the Eμ and the 3'RR interact (left side). The Mediator complex localizes on both regions, whereas cohesin is recruited to the 3'RR. When B cells are stimulated and CSR is triggered, a specific cytokine-inducible promoter, together with the S_X region involved in the recombination, are brought to this interaction (right side). For example, if B cells are stimulated with LPS + IL4, which induces CSR to IgG1, the promoter γ1 and the Sγ1 regions interact with the Mediator complex and are dynamically recruited to the loop. If, conversely, B cells are stimulated with LPS, inducing switch towards IgG3, the γ3 promoter and the Sγ3 region are recruited instead. Upon stimulation, the donor Sμ region is also brought in the interaction and is bound by the Mediator complex and cohesin. Moreover, γ1E is dynamically recruited to the interaction after stimulation and binds the Mediator complex (Kenter et al., 2012; Thomas-Claudepierre et al., 2016; Thomas-Claudepierre et al., 2013; Wuerffel et al., 2007).

7. Of "when" and "where" – Transcriptional regulation of the *Igh* locus

Important decisions are taken at the *Igh* locus that will ultimately determine the efficiency of the immune response. Furthermore, if the programmed DNA damage necessary for antibody diversification is not targeted to the Ig genes, it can lead to translocations or other mutations with pathological outcome. The transcriptional regulation ensures the proper functioning of the many different processes that take place in the locus, at defined moments and specific sites; and this complex, but crucial regulation is so far not completely understood.

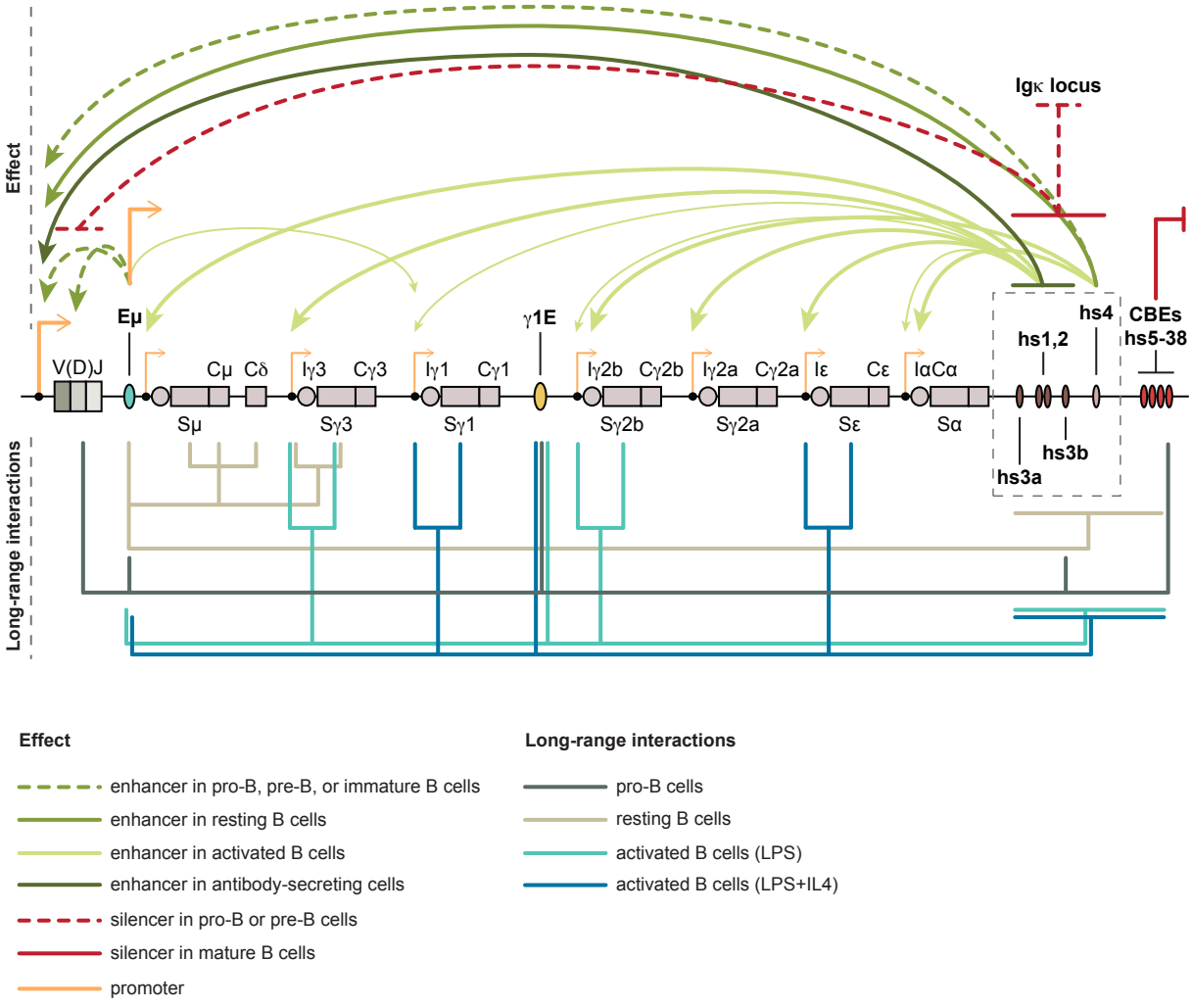
There are several known regulatory elements in the *Igh* locus with known enhancer, promoter, and silencer functions **(figure 10)**, the main two being the intronic **Eμ enhancer** downstream from the J_H gene segments; and the **3'RR**, comprising several individual regulatory regions at the 3' edge of the locus.

The intronic Eμ enhancer has been directly implicated in the control of V(D)J recombination (Afshar et al., 2006; Chen et al., 1993; Perlot et al., 2005; Sakai et al., 1999; Serwe and Sablitzky, 1993) and allelic exclusion (Li and Eckhardt, 2009; Peng and Eckhardt, 2013). More specifically, deletion of Eμ led to defective sense and antisense transcription at specific sites of the V_H region (Perlot et al., 2005). V(D)J recombination is also under the effect of the intergenic control region 1 (IGCR1) and PDQ52 (Afshar et al., 2006; Guo et al., 2011b; Lin et al., 2015; Nitschke et al., 2001). The IGCR1 spans over 4.1 kb within the most D-proximal V_H gene (Guo et al., 2011b) and counts with two CTCF binding sites that act synergistically as insulators to reduce the rearrangement of the more favored proximal V_H regions (Lin et al., 2015). Also, the deletion of IGCR1 or the two CTCF binding sites within this region led to increased GLT and perturbed allelic exclusion (Lin et al., 2015). Hence, IGCR1 is crucial for the ordered and lineage-specific V(D)J recombination (Guo et al., 2011b). On the other side, disruption of PDQ52 led to defective V(D)J recombination and affected the usage of D genes (Nitschke et al., 2001). Later in development, the Eμ enhancer affects IgH chain expression in pre-B cells and modulates B cell fate towards the marginal zone B population (Marquet et al., 2014). Furthermore, the Eμ enhancer promotes Sμ-S_X synapsis in mature, activated B cells, although it is not essential for CSR and only has a slight effect on GLT transcription (Perlot et al., 2005; Wuerffel et al., 2007).

Eμ acts also as a promoter giving rise to the so-called Iμ transcripts that extend through Sμ-Cμ region (Lennon and Perry, 1985; Su and Kadesch, 1990). Indeed, deletion of Eμ region led to a reduction in Iμ transcription levels and a consequent defect in CSR (Bottaro et al., 1998; Perlot et al., 2005).

The 3'RR comprises the regulatory sites hs3b, hs1,2, hs3a (the three of which become hypersensitive in mature B cells), and hs4 (already active in pro-B cell populations) (Garrett et al., 2005; Khamlichi et al., 2000b). Although their individual effect is weak, they act synergistically making the 3'RR a locus control region (LCR). Despite its interaction with the Eμ enhancer in pro-B cells, the 3'RR has no effect on the V(D)J recombination (Rouaud et al.,

2012). However, this region was linked to the process of CSR very early, when murine models in which individual elements of the 3'RR had been replaced with a neomycin gene showed a reduction in CSR that correlated with a default in the corresponding GLTs (Cogne et al., 1994; Manis et al., 1998). However, individual deletions of the 3'RR elements showed a modest effect on GLT production and CSR, if at all (Manis et al., 1998), implying that individual enhancers within the 3'RR had little effect, but the LCR had a strong regulatory capacity as a whole that was disrupted by the insertion of the neomycin cassette. The synergistic effect of the whole region was highlighted in a murine model where the four individual regions had been deleted (Dunnick et al., 2009), in which they found decreased GLT production and CSR to all isotypes. Besides, the non-conventional CSR to IgD is not controlled by the 3'RR (Issaoui et al., 2017; Rouaud et al., 2014), pointing at the existence of potential unknown regulatory regions that could be in charge, or unchecked roles of the already known ones. Interestingly, the control of GLT-α production and subsequent CSR to IgA is exerted by the 3'RR only in B2 B cells (Issaoui et al., 2018; Saintamand et al., 2015c), indicating yet unexplained differential regulatory mechanisms between the B1 and B2 B cell subsets.



On another note, SHM was severely impaired in B cells from 3'RR-deficient mice (Dunnick et al., 2009). The role of the 3'RR in SHM has been later confirmed, as the process was almost completely abolished in a mouse model deficient for the whole regulatory region, probably due to loss of AID accessibility and recruitment, both in B2 (Rouaud et al., 2013) and B1 (Hussein et al., 2019) B cell populations.

In addition to controlling transcription over the locus of activated B cells, the 3'RR promotes the production of IgH-μ chains in mature B lymphocytes, and predisposes B cell fate in favor of follicular instead of marginal B cells (Saintamand et al., 2015b). Moreover, a silencer effect of the 3'RR has also been documented. Indeed, the 3'RR was found to repress both sense and antisense transcription of the variable region in pro-B cells (Braikia et al., 2015), and it silences in *trans* the Igκ locus on chromosome 6 (Ghazzaui et al., 2019).

Figure 10: The physical and functional relationships among the Eμ, 3'RR, and γ1E regions in the Igh locus over B cell development. The location of the different regulatory regions is indicated in this schematic representation of the Igh locus. The elements of the 3'RR are enclosed in the dashed box. **Above** the representation of the locus, the enhancer, silencer, or promoter effect that each region is known to play on each other in immature and mature B cells are represented by arrows. Overall, Eμ acts both as a promoter and as an enhancer. It induces the expression of Iμ transcripts (Lennon and Perry, 1985; Perlot et al., 2005) and it is required for V(D)J recombination in pro-B cells (Afshar et al., 2006; Chen et al., 1993; Perlot et al., 2005) and IgH expression in pre-B cells (Marquet et al., 2014). The Eμ has been as well proposed to control Iγ1 transcription during B cell activation (Cogne et al., 1994; Wuerffel et al., 2007). The enhancers hs1-3 in the 3'RR regulate GLT production from the different cytokine-inducible promoters (Cogne et al., 1994; Garot et al., 2016; Pinaud et al., 2001; Vincent-Fabert et al., 2010), with a milder effect on Iγ1 transcription (Cogne et al., 1994; Wuerffel et al., 2007). They also promote the expression of the IgH chain in plasma and memory B cells (Garot et al., 2016). The hs4 element of the 3'RR controls IgH expression from the pro-B cell stage, until the mature, resting B cells (Garot et al., 2016; Saintamand et al., 2015b). The 3'RR also plays a silencer role, acting in *cis* in pro-B cells (Braikia et al., 2015) and in *trans* in pre-B cells (Braikia et al., 2015; Ghazzaui et al., 2019). Finally, the CBEs hs5-38 downstream of the 3'RR have an insulator effect, being the known 3' limit of the Igh locus and isolating downstream genes from the effect of the 3'RR (Volpi et al., 2012). **Below** the depicted Igh locus, each line represents a characterized long-range interaction connecting two different regions in immature or mature B cells. In pro-B cells, the locus conformation is organized in a loop that connects the Eμ enhancer to the 3'RR, with the V_H genes and the 3' CBE elements participating in the interaction (Guo et al., 2011a; Medvedovic et al., 2013). In mature, resting B cells, the Eμ is connected to the 3'RR elements and the hs5-38 CBEs, as well as the Cμ and Cδ genes (Kenter et al., 2012; Thomas-Claudepierre et al., 2016; Wuerffel et al., 2007). Moreover, constitutive contact between the Eμ and the Iγ3-Cγ3 region was also revealed in resting B cells (Thomas-Claudepierre et al., 2016). In stimulated B cells, the contact between 3'RR elements hs1-4 and the Eμ enhancer is maintained, and the germline promoters, together with the corresponding S_X region, are dynamically brought to the loop (Thomas-Claudepierre et al., 2016; Wuerffel et al., 2007). In this way, in an LPS stimulation (which drives CSR to IgG3 and IgG2b), the Iγ3-Sγ3 and Iγ2b-Sγ2b regions are recruited to the Eμ:3'RR interaction, whereas in an LPS + IL4 stimulation (driving CSR to IgG1 and IgE), the Iγ1-Sγ1 and Iε-Sε regions are recruited instead (Thomas-Claudepierre et al., 2016). Moreover, the g1E region was also found to participate in these dynamic 3D interactions occurring upon stimulation (Thomas-Claudepierre et al., 2016).

More recently, Garot and colleagues categorized the four individual enhancers that lay on the 3'RR into the proximal (hs3a, hs1,2, hs3b) and the distal (hs4) modules, with distinct functions (Garot et al., 2016). Comparing the pre-existent 3'RR deletion models with a new strain lacking the quasi-palindromic region comprising hs3a, hs1,2, and hs3b, they showed that the distal module is in charge of heavy chain expression in naïve B cells, whereas the proximal module is later activated by antigen stimulation and controls SHM and CSR, while also regulating IgH overexpression in antibody-secreting cells (Garot et al., 2016), which goes in line with preexistent data on the different developmental moments in which the two modules become hypersensitive (Garrett et al., 2005). Interestingly, the proximal module regulated GLT production of γ3, γ1, and γ2a isotypes, while GLT-γ2b and GLT-α were unaffected by the deletion of this module (Garot et al., 2016). Conversely, hs4 would regulate GLT-γ2b and GLT-α production (Pinaud et al., 2001; Vincent-Fabert et al., 2010).

Finally, downstream from hs4, four more sites displaying DNase I hypersensitivity from the pro-B cell stage onward were identified (**hs5-7 and hs38**) (Garrett et al., 2005). Being rich in CTCF- and Pax5-binding sites, these regions appear to have insulator activity. In a study (Volpi et al., 2012), a mouse model with a deletion of hs5-7 led to a mild increase in transcription in the immediately downstream gene, indicating that these CTCF-binding sites (CBSs) could act as a "limit" of the 3'RR super-enhancer effect. Moreover, hs5-7 also seem to be implicated in favoring the usage of more distal V_H genes through an effect on locus contraction. Although there were normal levels of GLT- γ1 transcription, a modest increase in IgG1 CSR was found in hs5-7 deficient B cells, and it was suggested that this region could help the Eμ enhancer in the control of CSR to IgG1 (Volpi et al., 2012). This possibility would be in line with previous studies implicating Eμ, rather than the 3'RR, on the transcriptional control of the γ1 promoter (Cogne et al., 1994; Wuerffel et al., 2007).

Taking all these studies together, we can conclude that the V(D)J recombination is initiated in pro-B cells through the effect of Eμ in coordination with the IGCR1 and the hs5-8 sites. In the pre-B cell stage, Eμ function switches to promoting pre-BCR expression. On the other hand, the 3'RR takes over the control of the locus in mature populations, biasing their fate to follicular B cells. Finally, antigen-dependent transcriptional activation taking place as part of SHM and CSR is orchestrated by the 3'RR, although with isotype- and lineage-specific differences between the B1 and B2 populations **(figure 10)**.

8. More wood to the fire: enhancers, super-enhancers, and the γ1E region

Enhancers are cis-acting regulatory elements ranging in general from 100 to 1000bp in length that control gene expression in a determined developmental stage and in a certain tissue or cell type (Pennacchio et al., 2013). Their mode of action involves the physical interaction between the enhancer and the promoter of their target genes, facilitated by CTCF and cohesin. Enhancers are characterized by the presence of TF binding sites (Dickel et al., 2013), and thus the enhancer-promoter loop aids to the activation of the target genes through the enhancer-bound TFs.

The mediator complex connects the enhancer-bound TFs and the RNA Pol II, acting thus as a nexus between the enhancer and the transcription machinery. The Med1 and Med12 subunits of the mediator complex are associated with cohesin at enhancer and promoters in embryonic stem cells (Kagey et al., 2010), and Med1 binds the TF GATA (Stumpf et al., 2006), which mediates the loop formed in the β-globin gene (Vakoc et al., 2005).

Clusters of enhancers, normally associated with cell identity genes, and with levels of mediator, p300, and TF binding higher than regular enhancers, are termed super-enhancers (Whyte et al., 2013).

There is not a single mark that characterizes enhancers permitting their easy identification. Enhancers can be either upstream or downstream from the genes that they regulate, there is no known enhancer-specific sequence, and they act in an orientation-independent manner. Moreover, they can be located at a considerable distance from their target genes, with non-target genes in the intervening sequence, or even within introns. Altogether, these features make the prediction of enhancers and the identification of their target genes a daunting task.

The most reliable method for identifying potential enhancers is the presence of p300 (Visel et al., 2009), DHSs (Dorschner et al., 2004), and epigenetic marks such as H3K27ac (Creyghton et al., 2010) and H3K4me1 (Heintzman et al., 2007) through chromatin immunoprecipitation (ChIP) experiments. The presence of TF binding sites, as well as the recruitment of the Mediator complex, are other good predictive marks of active enhancers (Dogan et al., 2015).

Interestingly, non-coding transcription emanating from active enhancers has been ubiquitously detected (De Santa et al., 2010; Kim et al., 2010; Li et al., 2016), and the current opinion states that these so-called enhancer RNAs (eRNAs) are a feature and a strong prediction mark of enhancer activity (Andersson et al., 2014; De Santa et al., 2010; Hah et al., 2011; Kim et al., 2010; Li et al., 2013; Melgar et al., 2011; Wu et al., 2014). eRNAs have been shown to facilitate the loop between enhancers and promoters (Hsieh et al., 2014; Lai et al., 2013; Li et al., 2013), but the mechanism of action of eRNAs remains an open question (Li et al., 2016).

However, enhancer activity should not be inferred only based on these known predictive marks, since they not always correlate with gene activation. Traditionally, enhancer validation has been performed with reporter assays. The first description of an enhancer activating

transcription of a reporter gene in an expression vector came with the discovery that the SV40 element enhanced transcription of the β-globin gene (Banerji et al., 1981). Since then, reporter assays have been widely used and they have led to the functional validation of numerous regulatory elements.

The Ig genes have been a broadly used model for enhancer activity and enhancer-promoter interactions. The presence of the Eμ enhancer and the 3'RR super-enhancer, containing several regulatory regions inside, makes of the *Igh* locus an interesting example to study the function of regulatory regions. The relevance of the *Igh* locus enhancers in immunoglobulin production has already been stated in this work. Indeed, the 3'RR binds specific cytokine-inducible promoters in a stimulation-dependent manner, driving the expression of the corresponding C_X genes (Vincent-Fabert et al., 2010). The transcriptional regulation and loop formation in the *Igh* locus, orchestrated by the 3'RR and the Eμ enhancers, are assisted by B cell specific TFs. Binding sites for the B cell lineage TF E2A, known to help targeting AID, have been localized in all *Igh* locus enhancers (Pavri and Nussenzweig, 2011). Other TFs that bind the *Igh* locus enhancers are, for example, PU.1, Med1 (Predeus et al., 2014), Pax5, and IRF4 (Hauser et al., 2016).

A recent work from our lab (Thomas-Claudepierre et al., 2016) showed that a region downstream of the Cγ1 gene, named γ1E, was unexpectedly bound by Med1 and Med12 subunits of mediator, and it was also recruited to the dynamic long-range interactions taking place in the *Igh* locus upon B cell activation in a stimulation-dependent manner.

Interestingly, this region had previously been characterized in two independent studies. Through chromosome conformation capture with high-throughput sequencing (4C-Seq) experiments, Medvedovic et al (Medvedovic et al., 2013) found a novel site involved in long-range interactions between the Cγ1 and Cγ2b genes in pro-B cells. One of the two DHS that it had (which they call Cγ1-2b DHS site 1) presented binding of TFs such as PU.1, Pax5, IRF4 and IRF8, YY1, and E2A. Moreover, it had the marks of active chromatin H3K9Ac and H3K4me2. Independently, Predeus et al. (Predeus et al., 2014) characterized the chromatin profile of Ig loci in pro-B cells based on previous as well as new data of histone modifications, key TF occupancy, DHSs, and transcriptional activity. They described a region downstream of the Cγ1 gene (termed hRE1 in their work) presenting a chromatin state characteristic of regulatory elements (including Pax5, p300, PU.1, and Med1 association, low nucleosome occupancy, and H3K4me1) with positive effect on reporter assays in pro-B cells. In the light of these studies, both authors suggest the potential role of this region as an enhancer controlling V(D)J recombination, SHM, or CSR. Furthermore, a third study described this region as a super-enhancer in pro-B cells, based on the high levels of Med1 binding (Whyte et al., 2013)

WORKING HYPOTHESES AND OBJECTIVES

Both transcription and dynamic conformation of the chromatin are crucial in the regulation of antibody diversification processes in the *Igh* locus. Starting at the beginning of B cell development in the bone marrow, the *Igh* locus in pro-B cells needs to be contracted, long-range interactions need to form, and the *Igh* locus has to be transcribed in order for V(D)J recombination to take place. Later, in mature, activated B cells, the *Igh* locus undergoes AID-dependent SHM and CSR for further diversification, the tight regulation of which is crucial to avoid potential pathological outcomes.

CSR is dependent on a complex coordination between transcription and dynamic long-range interactions involving the well-known Eμ and 3'RR enhancers, and the cytokine-inducible promoters upstream of each C_X gene. Indeed, our group showed that Med1-deficient B cells presented reduced efficiency of CSR, which correlated with a defect in both germline transcription of the acceptor regions and loop formation upon activation. Although knowing that transcription and looping of the *Igh* locus are necessary intermediates for the CSR reaction, we do not know which is the cause or the consequence of the other.

Besides the already known *Igh* locus enhancers, a newly described region downstream of Cγ1 presents DNase I hypersensitivity in pro-B cells, together with other marks of active enhancer (Medvedovic et al., 2013; Predeus et al., 2014). This region, named γ1E, is also bound by Mediator and participates in the dynamic conformational changes in the *Igh* locus upon activation (Thomas-Claudepierre et al., 2016), making it a candidate enhancer in mature B cells. Nevertheless, and despite these evidences, no function has been attributed so far to the γ1E region.

The aim of this thesis is gaining insight into the molecular mechanisms underlying the regulation of transcription and conformational changes taking place in the *Igh* locus during BCR diversification.

To do so, I based my work on the following two **hypotheses**:

I) Given the fact that the γ1E region has marks of active enhancer in pro-B cells and it is bound by Mediator in activated B cells, we believe that the γ1E region acts as an enhancer regulating transcription and conformational changes in the *Igh* locus. In this line, I **characterized the function of the γ1E during the process of CSR**.

II) The reduced CSR efficiency observed in Med1-deficient splenic B cells could be explained either by a decrease in germline transcription or a defect in loop formation. Based on these possibilities, I studied the **coordination between transcription and 3D conformation during CSR**.

Study of the role of γ1E during CSR

Part 1: A novel regulatory region controls Igh locus transcription and switch recombination to a subset of isotypes

In this part, I present our manuscript published in *Cellular & Molecular Immunology* describing the functional characterization of the γ1E enhancer in the process of CSR (Amoretti-Villa et al., 2019).

Part 2: Study of the role of γ1E region as an enhancer during CSR

Here, I present additional experiments further developing our understanding on the role of this region as an enhancer.

Study of the relationship between long-range interactions and transcription during CSR

Part 3: Transcription or loop? Cause versus consequence relationship

The final part corresponds to the study of the relationship between the regulation of transcription and long-range interactions during CSR.

RESULTS

Results - Part 1

A novel regulatory region controls *Igh* locus transcription and switch recombination to a subset of isotypes

CORRESPONDENCE

A novel regulatory region controls IgH locus transcription and switch recombination to a subset of isotypes

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Class switch recombination (CSR) occurs at the IgH locus and replaces the immunoglobulin (Ig) isotype expressed from IgM to IgG, IgE or IgA, endowing the B cell receptor with novel effector functions. CSR is triggered by activation-induced cytidine deaminase (AID),[1] an enzyme that deaminates cytosines to uracils in single-stranded DNA exposed by transcription. The distinct antibody isotypes are encoded in the IgH locus in individual transcription units composed of a cytokine-inducible promoter, an intronic exon, and a switch region (Sx), followed by the exons encoding the constant region (Cx) (Fig. S1a). During CSR, the choice of recombination to a particular isotype is determined by the stimulation-dependent activation of specific promoters, triggering the generation of noncoding germline transcripts (GLTs).[2] Thus far, the transcriptional regulation of the IgH locus is known to be controlled by the Eμ enhancer, located downstream of the variable region and upstream of the donor switch region (Sμ), and the 3′ regulatory region (3′RR) super-enhancer located downstream of Cα.[3]

Because the donor and acceptor switch (S) regions may be separated by up to 200 kb, CSR requires long-range interactions to occur. In mature resting B cells, both Eμ and the 3′RR super-enhancer are located in close proximity.[4,5] Upon B cell stimulation, the locus undergoes 3D conformational changes that bring the two S regions that will recombine near the Eμ and the 3′RR.[5] These conformational changes are in part dependent on the Mediator complex,[5] but the precise mechanism, additional regulatory regions, and factors involved are poorly understood. Through 4C-Seq experiments, we found that a region located downstream of the IgG1 gene (termed here γ1E) dynamically interacts with the Eμ and 3′RR enhancers during CSR.[5] This region is not only bound by the Med1 and Med12 subunits of Mediator[5] but it also bears chromatin marks and features characteristic of enhancers in pro-B cells.[6,7] Moreover, this region is transcribed in activated B cells (Fig. 1a), a feature shared by active enhancers.[8] Based on these observations, we hypothesized that the γ1E region could have a transcriptional regulatory function during CSR. To test this hypothesis, we used a CRISPR/Cas9 knockout strategy in CH12 cells, a murine B cell line that can be efficiently stimulated to perform CSR to IgA.[9] Cells were transfected with a plasmid coexpressing two gRNAs flanking the γ1E region and Cas9 fused to EGFP. One day after transfection, cells were sorted for EGFP expression and cultured under limiting dilutions to generate individual clones that were genotyped by PCR and sequencing (Fig. S1a). Notably, in CH12 cells, only one IgH allele is functional.[10] The other allele has a D-J rearrangement,[11] is prerecombined (between Sμ and Sα) and thus lacks the γ1E region. Therefore, wild-type CH12 cells are denoted $\gamma1E^{+/-}$. Four $\gamma1E^{-/-}$ clones and one $\gamma1E^{+/-}$ clone with comparable levels of AID expression (Fig. S1b, c) were selected for further functional analysis. To determine whether deletion of γ1E has an impact on the efficiency of CSR, these five clones together with the parental cell line (pCH12) were induced to undergo CSR. Three days later, the percentage of IgA^{+} cells was determined by flow cytometry (Fig. 1b, c). The efficiency of CSR was reduced by ~50% in all four $\gamma1E^{-/-}$ clones when compared with the $\gamma1E^{+/-}$ control clone or the pCH12 cells (Fig. 1b, c), showing that the deletion of the γ1E region results in defective CSR to IgA in CH12 cells, independent of differences in AID expression.

To determine whether deletion of the γ1E region has an impact on S region transcription, we measured the levels of Iμ-Cμ and Iα-Cα GLTs by RT-qPCR (Fig. 1d). Surprisingly, deletion of the γ1E region had no negative effect on S region transcription, and both transcripts appeared to accumulate in the $\gamma1E^{-/-}$ clones (Fig. 1d). Hence, the reduced CSR efficiency observed in $\gamma1E^{-/-}$ clones cannot be explained by defects in transcription. A possible explanation is that the γ1E region participates in the 3D conformational changes occurring at the IgH locus during CSR.[4,5] Alternatively, the Sα region may be insensitive to a putative transcriptional effect of the γ1E region due to its close proximity to the 3′RR super-enhancer (2 kb).

To determine whether the γ1E region has a role in CSR to other isotypes, we generated a γ1E knockout mouse model using the CRISPR/Cas9 system and the same gRNAs used for the knockout in CH12 cells. Despite homozygous deletion of the γ1E region, all the different B cell subsets were found to be represented in the bone marrow and in the spleen at normal numbers and proportions (Fig. S2), indicating that γ1E deletion has no obvious effect on B cell development and that it does not lead to a block during differentiation.

To assess the ability of γ1E-deficient B cells to undergo CSR, CFSE-labeled splenic B cells isolated from wild-type and $\gamma1E^{-/-}$ mice were cultured under conditions that induce CSR to different isotypes. After 72 h, we determined the surface expression of the different isotypes by flow cytometry (Fig. 1e, f). While the efficiency of CSR to IgG1 and IgA appeared similar between genotypes, a significant reduction in CSR to IgG3 (−32%), IgG2b (−86.5%), and IgG2a (−24.5%) was observed in $\gamma1E^{-/-}$ B cells compared with control B cells (Fig. 1e, f), independent of AID expression (Fig. S1d). We concluded that the γ1E region has an isotype-specific role in the regulation of CSR.

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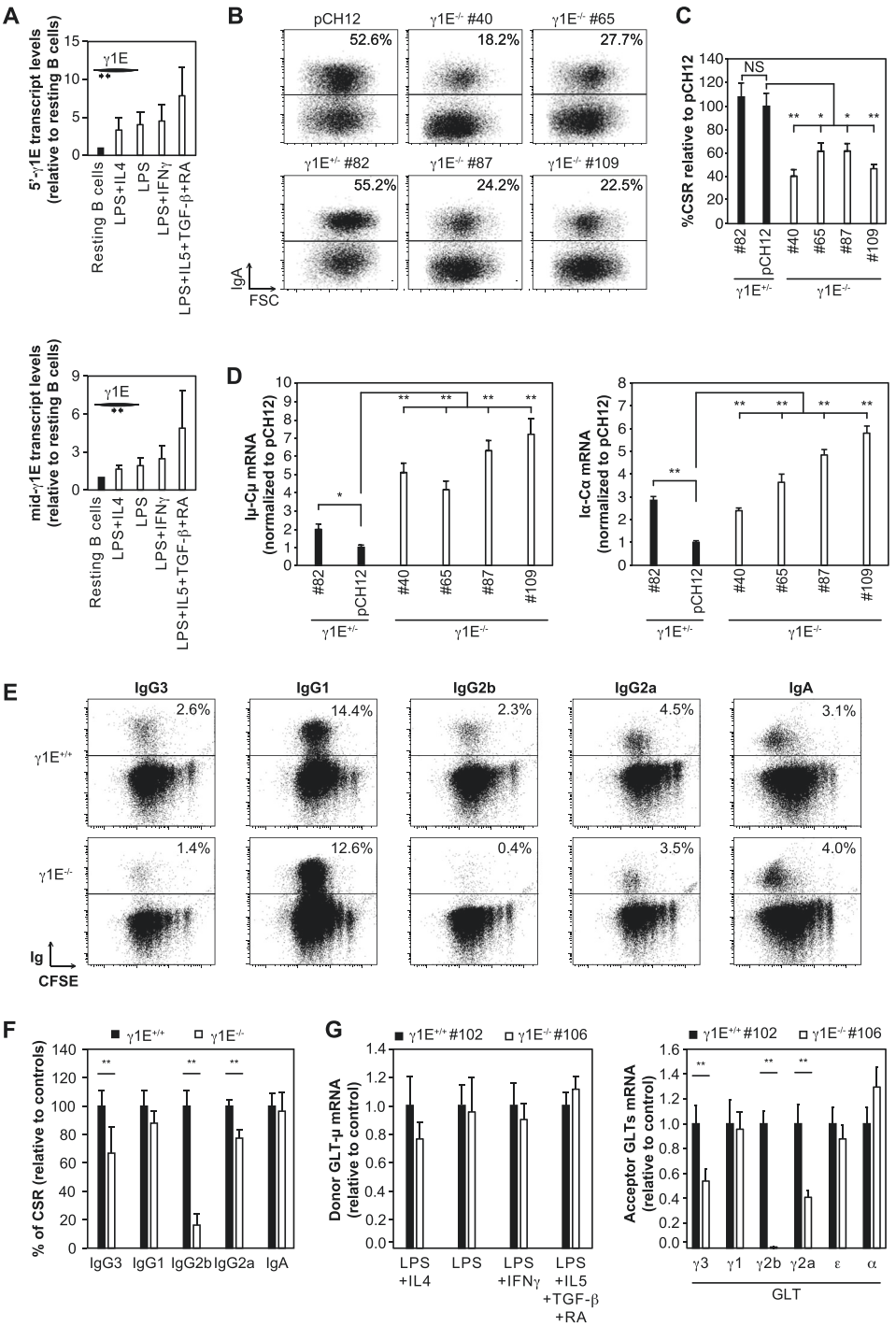

Fig. 1 Role of the γ1E region in CSR and IgH locus transcription in CH12 and primary B cells. **a** RT-qPCR of γ1E transcript levels using primers located in the 5′ (top) or middle (bottom) region of the γ1E. **b** Surface expression of IgA analyzed by flow cytometry in $\gamma1E^{+/-}$ and $\gamma1E^{-/-}$ clones and pCH12 cells after 3 days in culture with TFG-β, IL-4, and anti-CD40 antibody. The percentage of switched cells is indicated. Representative dot plots of six experiments are shown. **c** Percentage of CSR in $\gamma1E^{+/-}$ and $\gamma1E^{-/-}$ clones relative to pCH12 cells. Data are pooled from six independent experiments. Statistical significance was determined by two-tailed Student's *t*-test. **d** RT-qPCR for Iμ-Cμ (left panel) and Iα-Cα (right panel) transcripts. Triplicates were normalized to the abundance of Igβ, set as 1. Statistical significance was determined by a two-tailed Student's *t*-test. (*p-value < 0.05; **p-value < 0.005). Data are representative of six experiments. **e** Flow cytometry analysis of surface Ig expression in CFSE-labeled primary B cells purified from $\gamma1E^{+/+}$ or $\gamma1E^{-/-}$ mice and cultured in vitro for 3 days with LPS (CSR to IgG3 and IgG2b), LPS + IL4 (CSR to IgG1 and IgE), or LPS + IFNγ (CSR to IgG2a) or for 4 days with LPS + IL5 + TGFβ + retinoic acid (RA) (CSR to IgA). The percentage of switched cells is indicated. Representative dot plots from six experiments are shown. **f** CSR efficiency in primary B cells obtained from $\gamma1E^{-/-}$ ($n = 13$) or $\gamma1E^{+/+}$ ($n = 12$) mice, shown as the percentage of CSR. The mean + SD from six experiments is presented relative to $\gamma1E^{+/+}$ control B cells. Statistical significance was determined by two-tailed Student's test (*p-value < 0.05; **p-value < 0.005). **g** RT-qPCR for donor (Iμ-Cμ; left panel) and acceptor (Iγ3-Cγ3, Iγ1-Cγ1, Iγ2b-Cγ2b, Iγ2a-Cγ2a, Iε-Cε, and Iα-Cα; right panel) GLT transcripts in primary B cells purified from $\gamma1E^{+/+}$ or $\gamma1E^{-/-}$ mice and cultured as in **e**. Mean + SD of triplicate values was normalized to the abundance of Igβ and is shown relative to $\gamma1E^{+/+}$ B cells, set as 1. Statistical significance was determined by a two-tailed Student's *t*-test. (*p-value < 0.05; **p-value < 0.005). For biological replicates, refer to Fig. S1e

Consistent with the reduction in CSR observed for IgG3, IgG2b, and IgG2a, we found that the level of the corresponding GLTs (Iγ3-Cγ3, Iγ2b-Cγ2b, and Iγ2a-Cγ2a) was significantly decreased in γ1E$^{-/-}$ B cells compared with controls (Figs. 1g and S1e), while no differences were found for the Iγ1-Cγ1, Iε-Cε, and Iα-Cα transcript levels (Figs. 1g and S1e). We concluded that γ1E deletion impairs sterile transcription at the γ3, γ2b and γ2a genes, perfectly correlating with the isotype-specific deficiencies observed in CSR.

Given the defect in CSR to IgA revealed in CH12 cells, it was surprising to find that CSR to IgA was not affected in primary γ1E$^{-/-}$ B cells. Notably, CH12 cells present a B1 lineage-like phenotype, and B1 B cells are known to be biased towards CSR to IgA.[12] Interestingly, the 3′RR super-enhancer did not regulate CSR to IgA in B1 cells.[13] In this regard, a lineage-specific regulatory role of the 3′RR and the γ1E region could be a plausible explanation for the disparities observed in the transcriptional regulation of γ1E$^{-/-}$ CH12 and primary B cells.

The role of insulator regions in the transcriptional regulation of the IgH locus is also notable. In this regard, a recent study showed that the 5′hs1RI region acts as an insulator, restraining the 3′RR super-enhancer effect, and that it contributes to the regulation of CSR to IgG3, IgG2b, and IgG2a by blocking the premature activation of their corresponding promoters.[14] Therefore, upon activation, the γ1E region may counteract the insulator function of the 5′hs1RI region. Hence, deletion of the γ1E region could result in unchecked 5′hs1RI insulator activity, resulting in defective transcription and reduced CSR to these isotypes.

Although we found an effect of γ1E in the transcriptional regulation of the switch region promoters, which could explain the defect in IgG3, IgG2b, and IgG2a CSR, we cannot exclude the possibility that the γ1E region is involved in the dynamic conformational changes of the IgH locus that occur during CSR. Indeed, this region is brought to the Eμ enhancer and the 3′RR super-enhancer during CSR after B cell stimulation.[5] Therefore, deletion of this region might affect IgH locus looping and consequently transcription.

Altogether, our results are consistent with a model in which the γ1E region regulates IgH locus transcription and CSR in an isotype-specific manner.

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AUTHOR CONTRIBUTIONS

Conceptualization, B.R.-S.-M., R.A.-V., M.R., and I.R. Methodology, B.R.-S.-M., R.A.-V., and I.R. Investigation, R.A.-V., M.R., I.R., and V.H. Writing—original draft, R.A.-V. Writing—review and editing, B.R.-S.-M. and I.R. Supervision, B.R.-S.-M. and I.R. Funding Acquisition, B.R.-S.-M.

ADDITIONAL INFORMATION

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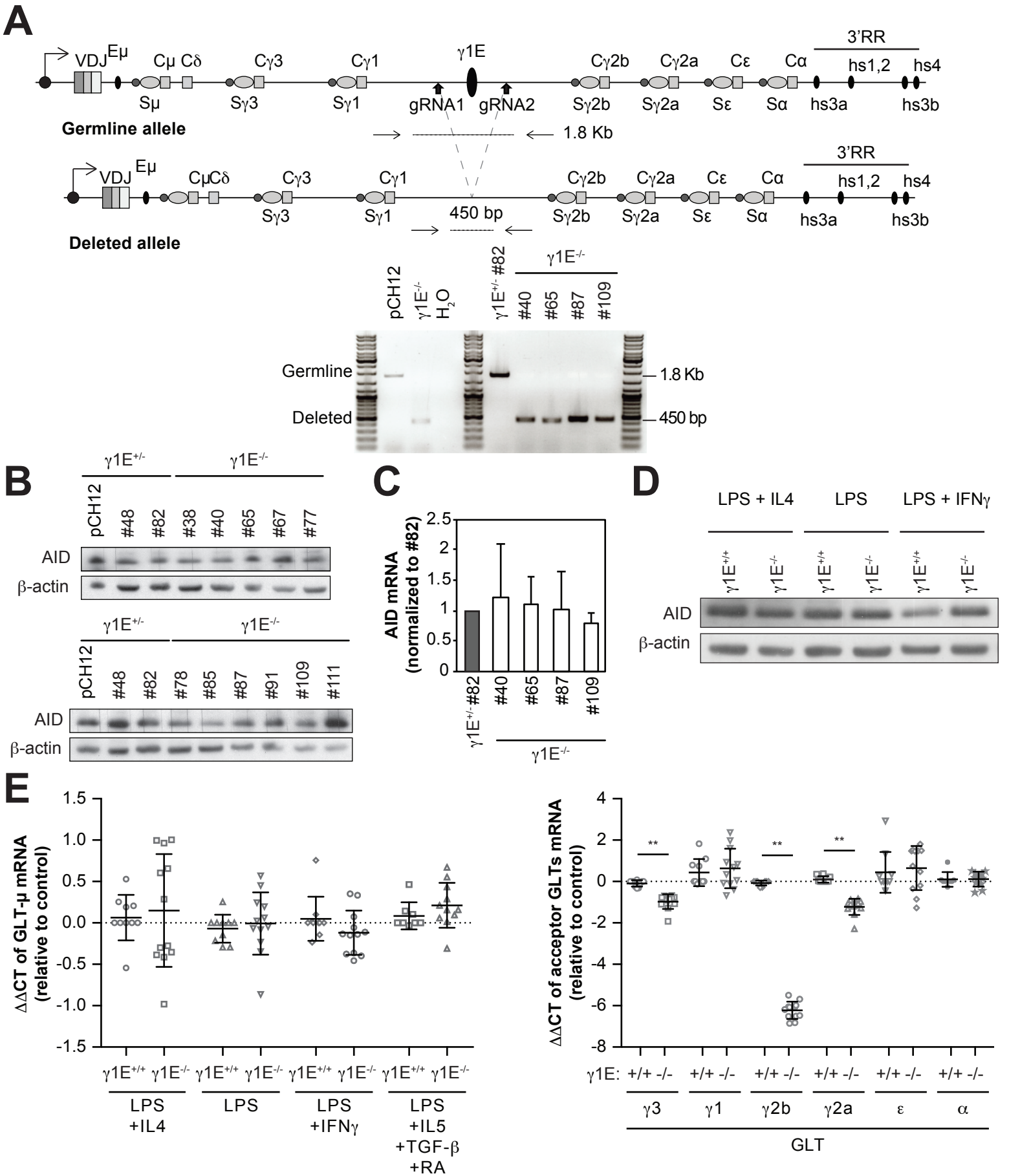

Figure S1. Generation and characterization of γ1E-/- CH12 cells and γ1E-/- mouse model. A (top). Schematic representation of the IgH locus before and after CRISPR/Cas9-mediated deletion of the γ1E region. gRNAs and PCR primers for genotyping are indicated. A (bottom) Representative agarose gel of genotyping by PCR. The bands corresponding to the germline or the deleted alleles are indicated. B. Western blot analysis for AID and β-Actin of CH12 cell clones of different genotypes after 72h in culture with TFG-b, IL-4 and anti-CD40 antibody. Theoretical molecular weights (kDa) are indicated. C. RT-qPCR for AID expression in selected CH12 clones after 72h in culture with TFG-β, IL-4 and anti-CD40 antibody. Mean+SD of AID values normalized to Igβ and relative to γ1E+/- clone #82 from 4 independent experiments is shown. D. Western blot analysis for AID and β-Actin of primary B cells from γ1E+/+ and γ1E-/- mice cultured for 72h with LPS+IL4, LPS, or LPS+IFNγ. E. RT-qPCR of the donor (left) and acceptor (right) GLTs of γ1E+/+ and γ1E-/- B cells cultured as in Fig. 1E. Mean±SD of biological replicates from 5 independent experiments were normalized to the abundance of Igβ. Two γ1E+/+ mice were used for each experiment, and one of them was randomly set as control. Statistical significance was determined by a one-way ANOVA (**p-value < 0.005).

Amoretti-Villa *et al*.

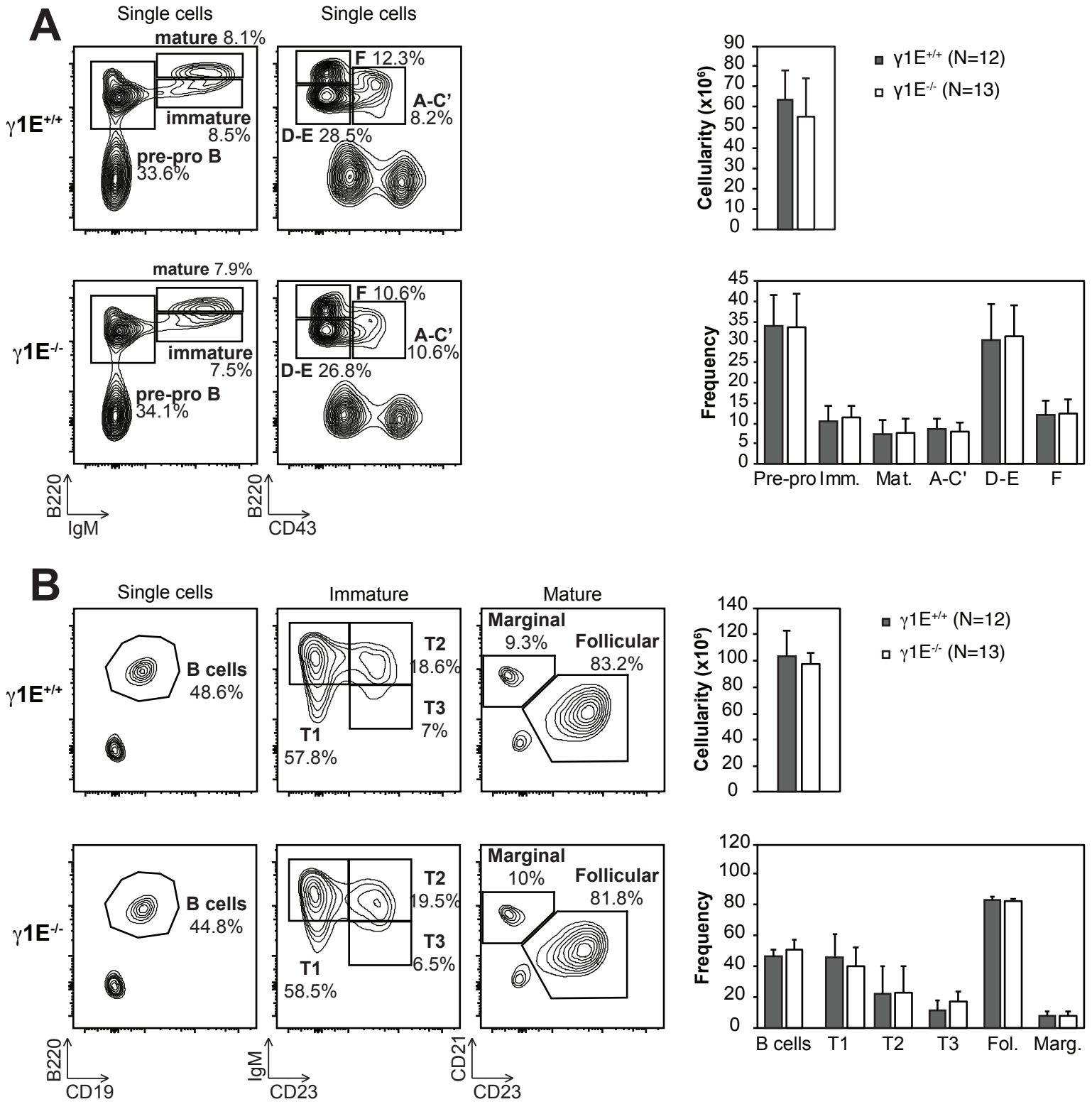

Figure S2. Robust B cell development in γ1E-/- mice. Flow cytometry analysis of B cell development in the bone marrow (A) and the spleen (B) of γ1E+/+ (n=12) and γ1E-/- (n=13) mice. Representative plots from 6 experiments are shown. When necessary, gating is indicated above the plot. The frequency of the different B cell fractions is represented by the mean+SD for each population from γ1E-/- relative to γ1E+/+ mice, set as 1. The cellularity represents the absolute number of cells (mean+SD).

Materials and Methods

Mice

The $\gamma 1E^{-/-}$ mutant mouse line was established at the MCI/ICS (Mouse Clinical Institute - Institut Clinique de la Souris-, Illkirch, France; http://www.ics-mci.fr/en/). Briefly, *in vitro* transcribed Cas9 mRNA (10 ng/µl) and 2 gRNAs (10 ng/µl) were co-injected into 803 C57BL/6N fertilized oocytes. Seventy pups were born. Two founders with the expected deletion were identified, one of which gave germline transmission. The $\gamma 1E^{-/-}$ line was maintained and bred under specific pathogen-free conditions. All animals used in this study were 8 to 12 weeks old. All animal work was performed under protocols approved by an ethics committee.

Cell culture and flow cytometry

CH12 cells were induced to undergo CSR with IL-4 (5 ng/ml; Sigma), TGF-β (3 ng/ml; R&D Systems), and anti-CD40 antibody (100 ng/ml; eBioscience) for 72h. Resting B cells were isolated from the spleen using CD43 Microbeads (Miltenyi Biotec), labeled with 5 µg/ml CFSE (Invitrogen) for 10 minutes at 37°C, and cultured with 50µg/ml LPS (Sigma-Aldrich), 5ng/ml IL-4 (PeproTech), 100 ng/ml IFN-γ (PeproTech), 5ng/ml IL-5 (BD), 3 ng/ml TGF-β (R&D Systems), or 0.3 ng/ml retinoic acid (Sigma-Aldrich). Cells were stained with biotin-anti-IgG1 (BD), biotin-anti-IgG3 (BD), biotin-anti-IgG2b (BioLegend), biotin-anti-IgG2a (BD), PE-anti-IgA (SouthernBiotech) antibodies and PE-Streptavidin (Beckman Coulter). ToPro-3 (Invitrogen) was used to exclude dead cells in the analysis. For B cell development, bone marrow and splenic lymphocytes were stained with anti B220-PECy7 (e-biosciences), anti IgM-Cy5 (Southern Biotech), anti CD43-BB515 (BD Pharmingen), anti CD93-Biotin (e-biosciences), Streptavidine-BUV395 (BD Pharmingen), anti CD21-FITC (BD Biosciences), anti CD23-PE (BD Biosciences), and anti IgM-Cy5 (Southern Biotech) antibodies. All flow cytometry data were acquired in a Fortessa (BD Biosciences) flow cytometer and analyzed using the FlowJo software.

Generation of $\gamma 1E^{-/-}$ CH12 cells.

$5x10^6$ CH12 cells were electroporated with 0.5 µg of a plasmid co-expressing 2 gRNAs and Cas9 fused to EGFP using a Neon Transfection System (Thermo Fisher Scientific). 24h later, EGFP+ cells were sorted with a BD FACSAria (BD Biosciences) and plated under limiting dilution conditions to obtain single-cell clones, which were cultured for approximately 2 weeks. Genomic DNA was extracted and individual clones were genotyped by PCR and sequencing. See table S1 for gRNAs and primer sequences.

Western blot

Proteins were fractionated on 4-12% gradient SDS-PAGE gels (Invitrogen), transferred to Immobilon PVDF membranes (Millipore) and stained with anti-AID (Strasbg9, AID-2E11; IGBMC) or β-actin (Sigma) antibodies.

RT-qPCR

RNA and cDNA from CH12 or primary B cells were obtained following standard protocols. qPCR was performed in triplicates using the Universal Probe Library system (Roche) or SyberGreen (QIAGEN) and a LightCycler 480 (Roche). Transcript quantities were calculated relative to standard curves and normalized to Igβ mRNA. PCR primers and probes used are shown in Table S1.

Because the article was published as a *Correspondence* in the journar *Cellular & Molecular Immunology*, it was adapted to a short format. To provide a longer version, I include in the next pages the manuscript that we had previously submitted to the *Journal of Experimental Medicine* presenting the same results.

A novel regulatory region controls IgH locus transcription and switch recombination to a subset of isotypes.

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Abstract

Immunoglobulin (Ig) class switch recombination (CSR) is the long-range recombination event that takes place at the immunoglobulin heavy chain (IgH) constant locus. CSR occurs upon B cell activation and results in the expression of novel antibody isotypes with specific effector functions. CSR is triggered by activation-induced cytidine deaminase (AID) and is dependent on inducible long-range enhancer/promoter looping and on switch transcription, which is controlled by the Eμ enhancer and the 3' regulatory region (3'RR) superenhancer. Here, we characterize the role on switch transcription and recombination of γ1E, a region located downstream of the Cγ1 gene, that bears marks of active enhancers and that interacts dynamically with both IgH enhancers upon B cell activation. Upon deletion of this region in the CH12 cells, we find a 50% reduction in the efficiency of CSR to IgA. Moreover, inactivation of this region in a murine model leads to a selective defect in transcription at the IgG3, IgG2b, and IgG2a genes and a corresponding defect in CSR to these isotypes. Our results suggest that γ1E regulates transcription of the IgH locus and CSR in an isotype-specific manner.

Introduction

During immune responses, the B cell receptor (BCR) is diversified in an antigen-dependent manner through the mechanisms of somatic hypermutation (SHM) and class switch recombination (CSR). SHM introduces point mutations in the variable (V) region of both heavy and light chain genes, modulating the affinity of the BCR for its cognate antigen. CSR replaces the immunoglobulin (Ig) isotype expressed from IgM to IgG, IgE or IgA through a recombination event taking place at the IgH locus that endows the BCR with novel effector functions. Together, SHM and CSR are essential to promote highly specific and adapted antibody responses (Methot and Di Noia, 2017). Both SHM and CSR are triggered by activation-induced cytidine deaminase (AID) (Hwang et al., 2015; Muramatsu et al., 2000; Revy et al., 2000), an enzyme that deaminates cytosines to uracils in single stranded DNA exposed by transcription. The resulting dU:dG mismatches are processed by base excision repair and mismatch repair pathways, leading to the generation of mutations in V regions and double stranded DNA breaks (DSBs) at switch (S) regions during SHM and CSR, respectively.

The distinct antibody isotypes are encoded in the IgH locus in individual transcription units composed of a cytokine-inducible promoter, an intronic exon (Ix), a switch region (Sx) followed by the exons encoding the constant region (C_X). During CSR, the choice of recombination to a particular isotype is determined by the stimulation-dependent activation of specific promoters (Basu et al., 2011; Pavri and Nussenzweig, 2011), triggering the generation of non-coding germline transcripts (GLTs) (Chaudhuri et al., 2007). Germline transcription occurs at both the donor (Sμ) and acceptor (Sx) switch regions (the latter upon activation), and it precedes and is required for recombination (Chaudhuri et al., 2007). So far,

the transcriptional regulation of the IgH locus is known to be controlled by two enhancers. The Eμ enhancer, located downstream of the variable region and upstream of the donor switch region (Sμ), and the 3' regulatory region (3'RR) super-enhancer located downstream of Cα (Chaudhuri et al., 2007; Pavri and Nussenzweig, 2011). The 3'RR controls the initial steps of CSR (Saintamand et al., 2015). It is composed of four enhancers (hs3a, 3b, 1–2, and 4) that can be functionally divided in two modules. The distal module (hs4) would be in charge of heavy chain expression in naïve B cells, whereas the proximal module (comprising hs3a, hs1-2, and hs3b) would be later activated by antigen stimulation and control SHM and CSR in antigen-activated B cells and hyper antibody production in plasma cells (Garot et al., 2016).

Because the donor and acceptor switch (S) regions may be separated by up to 200 kb, CSR requires long-range interactions to occur. In mature resting B cells, both Eμ and the 3'RR super-enhancer are located at close proximity (Thomas-Claudepierre et al., 2016; Wuerffel et al., 2007). Upon B cell stimulation, the locus undergoes 3D-conformational changes that bring the two S regions that will recombine to the proximity of Eμ and the 3'RR (Thomas-Claudepierre et al., 2016) in order to be transcribed and become the substrate for AID-dependent cytidine deamination. AID's activity, both on- and off-target, is preferentially found in regions of high tridimensional complexity, such as bi-directionally transcribed superenhancers that engage in long-range interactions (Meng et al., 2014; Qian et al., 2014). These conformational changes are in part dependent on the Mediator complex (Thomas-Claudepierre et al., 2016) but nevertheless, the precise mechanism, additional regulatory regions, and factors involved are poorly understood. Through 4C-Seq experiments, we have found that a region located downstream of the IgG1 gene (termed here γ1E) dynamically interacts with the Eμ and 3'RR enhancers during CSR (Thomas-Claudepierre et al.,

2016). This region is not only bound by the Med1 and Med12 subunits of Mediator (Thomas-Claudepierre et al., 2016), but in addition, it bears chromatin marks and features characteristic of enhancers (i.e. DNaseI hypersensitive sites, transcription factor binding, epigenetic marks of active chromatin, H3K27Ac, etc.) in pro-B cells (Medvedovic et al., 2013; Predeus et al., 2014). Based on this, we hypothesized that the γ1E region could have a transcriptional regulatory function or that it could be involved in IgH locus looping during CSR. Here, we explore the functional role of this region in B cell development and in CSR by deleting this region in CH12 cells and in mice.

Results and Discussion

γ1E deletion impairs CSR to IgA in CH12 cells without reducing switch region transcription.

Different marks of active enhancers were found in the γ1E region of pro-B cells (Medvedovic et al., 2013; Predeus et al., 2014). Since this region was also found to recruit Med1 and Med12, and interact with Eμ and the 3'RR in activated mature B cells (Thomas-Claudepierre et al., 2016), we hypothesized that this region could act as an enhancer also in mature B cells. Recent work has proven that most, if not all, active enhancers are transcribed (Li et al., 2016). These non-coding transcripts are called enhancer RNA (eRNA) (Kim et al., 2010) and they have been proposed as a reliable prediction/annotation mark of active enhancers (Andersson et al., 2014; De Santa et al., 2010; Hah et al., 2011; Kim et al., 2010; Melgar et al., 2011; Wang et al., 2011). Thus, we performed RT-qPCR analysis of the γ1E region (defined by a strong peak of Med12 binding in activated primary B cells through ChIP-Seq (Wang et al., 2014)) using two different combinations of primers in order to detect the potential presence of eRNA in *in vitro* activated murine B cells. Indeed, we found that the γ1E region is transcribed in activated B

cells, and that the transcript levels are consistently increased in B cells after 72 or 96 hours in culture with LPS, LPS + IL-4, LPS + IFN-γ, or LPS + IL-5 + TGF-β + retinoic acid, when compared to non-activated B cells (**Fig. 1B**), further suggesting the role of γ1E as an enhancer.

To determine whether the γ1E region has a functional role in the regulation of CSR, we undertook a CRISPR/Cas9 knockout strategy in CH12 cells, a murine B cell line that can be efficiently stimulated under controlled CSR from IgM to IgA (Nakamura et al., 1996). Cells were transfected with a plasmid co-expressing two gRNAs flanking the γ1E region (defined by the peak of Med12 binding as determined by ChIP-Seq) and Cas9 fused to EGFP. One day after transfection, cells were sorted for EGFP expression and cultured under limiting dilutions to generate individual clones. Note that in CH12 cells, only one IgH allele is functional (Arnold et al., 1988). The other allele has a D-J rearrangement (Ono et al., 2000), is pre-recombined (between Sμ and Sα) and thus lacks the γ1E region. Therefore, wildtype CH12 cells are denoted $\gamma1E^{+/-}$. Individual clones were genotyped by PCR using primers located upstream and downstream of the two gRNAs (**Fig. 1A and S1**). The expected deletion was obtained in 29 out 72 clones analyzed (40.3 %) and was confirmed by sequencing (data not shown). Since CH12 cells are known to display clonal heterogeneity in terms of AID expression (Ramachandran et al., 2016), and because CSR efficiency is sensitive to AID levels (Dorsett et al., 2008; Takizawa et al., 2008; Teng et al., 2008), we determined AID levels by Western blot and RT-qPCR after CSR induction (**Fig. S1B and S1C**). Four $\gamma1E^{-/-}$ and one $\gamma1E^{+/-}$ clones with comparable levels of AID expression were selected for further functional analysis. To determine whether deletion of the γ1E has an impact on the efficiency of CSR, these five clones together with the parental cell line (pCH12) were induced to undergo CSR. Three days later, the percentage of cells expressing cell surface IgA was determined by flow cytometry (**Fig. 1C and 1D**). We found that the efficiency of CSR was reduced by approximately 50% in all four $\gamma1E^{-/-}$ clones, when

compared to the γ1E$^{+/-}$ control clone or the parental cell line (**Fig. 1C and 1D**). We conclude that the deletion of the γ1E region results in defective CSR to IgA in CH12 cells, independently of differences in AID expression.

To determine whether deletion of the γ1E region has an impact on switch region transcription, we measured the levels of Iμ-Cμ and Iα-Cα switch region transcripts by RT-qPCR (**Fig. 1E**). Surprisingly, deletion of the γ1E region has no negative effect on switch region transcription and both transcripts appear to accumulate in the γ1E$^{-/-}$ clones (**Fig. 1E**). Hence, the reduced CSR efficiency observed in γ1E$^{-/-}$ clones cannot be explained by defects in transcription. A possible explanation for the defect observed in CSR to IgA in γ1E$^{-/-}$ clones is that the γ1E region plays a structural role and that it participates in the 3D conformational changes occurring at the IgH locus during CSR (Thomas-Claudepierre et al., 2016; Wuerffel et al., 2007). Alternatively, it is possible that the Sα switch region is insensitive to a putative transcriptional effect of the γ1E region due to its close proximity to the 3'RR superenhancer (2kb).

Robust B cell development in γ1E$^{-/-}$ mice.

To determine whether the γ1E region has a role in B cell development and in CSR to other isotypes, we generated a γ1E knockout mouse model using the CRISPR/Cas9 system and the same gRNAs used for the knockout in CH12 cells. Despite homozygous deletion of the γ1E region, all the different B cell subsets were found to be represented in the bone marrow and in the spleen at normal numbers and proportions (**Fig. S2**). We conclude that deletion of the γ1E region has no obvious effect on B cell development and that it does not lead to a block during differentiation.

Transcription and CSR to IgG3, IgG2b, and IgG2a are defective in γ1E$^{-/-}$ primary B cells.

To assess the ability of γ1E-deficient B cells to undergo CSR, we cultured CFSE-labeled splenic B cells isolated from wildtype and γ1E$^{-/-}$ mice under conditions that induce CSR to different isotypes. After 72h, we determined the surface expression of the different isotypes by flow cytometry (**Fig. 2A and 2B**). While the efficiency of CSR to IgG1 and IgA appeared similar between genotypes, a significant reduction in CSR to IgG3 (-32%), IgG2b (-86,5%), and IgG2a (-24,5%) was observed in γ1E$^{-/-}$ B cells when compared to control B cells (**Fig. 2A and 2B**). As expected, AID expression levels were not affected by deletion of the γ1E region (**Fig. S1D**). We conclude that the γ1E region has an isotype-specific role in the regulation of CSR and that the CSR defect observed is B cell-intrinsic and independent of defects in proliferation or AID expression.

To determine whether switch region transcription is affected in γ1E$^{-/-}$ B cells, we measured the level of switch region transcripts (Ix-Cx) by RT-qPCR (**Fig. 2C**). Consistent with the reduction in CSR observed to IgG3, IgG2b, and IgG2a, we found that the level of the corresponding switch region transcripts (Iγ3-Cγ3, Iγ2b-Cγ2b, Iγ2a-Cγ2a) was significantly decreased in γ1E$^{-/-}$ B cells when compared to controls (**Fig. 2C**). Conversely, the transcript levels of Iγ1-Cγ1, Iε-Cε, Iα-Cα were not different between γ1E$^{-/-}$ and γ1E$^{+/+}$ B cells (**Fig. 2C**). We conclude that γ1E deletion impairs sterile transcription at γ3, γ2b and γ2a genes, and that these defects in switch region transcription perfectly correlate with isotype-specific deficiencies in CSR.

Given the defect in CSR to IgA revealed in CH12 cells, it was surprising to find that CSR to IgA was not affected in primary γ1E$^{-/-}$ B cells. It is worth noting that CH12 cells present a B1 lineage-like phenotype (Li et al., 2018) and that B1 B cells are known to be biased towards CSR to IgA

(Kaminski and Stavnezer, 2006). Interestingly as well, the 3'RR superenhancer was found not to regulate CSR to IgA in B1 cells (Issaoui et al., 2018) and CSR to IgA was reduced but not abolished by deletion of the 3'RR in CH12 cells (Kim et al., 2016). A possible explanation for the different effect of the deletion of the γ1E region between CH12 cells and primary B cells could be a differential regulatory function of the 3'RR and the γ1E region between the B1 and B2 lineages.

It is also to note the role of insulator regions in the transcriptional regulation of the IgH locus. In this regard, it was recently shown that the 5'hs1RI region acts as an insulator, restraining the 3'RR's superenhancer effect, and that it helps in the regulation of CSR to IgG3, IgG2b, and IgG2a by blocking the premature activation of their corresponding promoters (Braikia et al., 2017). Therefore, it is possible that upon activation, the γ1E region counteracts the insulator function of the 5'hs1RI region. Hence deletion of γ1E region could result in unchecked 5'hs1RI insulator activity, resulting in defective transcription and reduced CSR to these isotypes. Furthermore, it has been recently described that Eμ is subjected to the influence of the 3'RR superenhancer in mature B cells in a hierarchic manner (Saintamand et al., 2017). In this regard, we cannot exclude the possibility that these well-characterized regulatory regions, together with the γ1E region, could interact and regulate each other's functions.

Although we find an effect of γ1E in the transcriptional regulation of the switch region promoters; which could *per se,* explain the defect in IgG3, IgG2b, and IgG2a CSR, we cannot exclude the possibility that the γ1E region is involved in the dynamic conformational changes of the IgH locus that occur during CSR. Indeed, this region is brought to the proximity of the Eμ enhancer and the 3'RR superenhancer during CSR after B cell stimulation (Thomas-

Claudepierre et al., 2016). Therefore, it is possible that deletion of this region might affect IgH locus looping and consequently transcription.

Altogether, our results are consistent with a model in which the γ1E region regulates IgH locus transcription and CSR in an isotype-specific manner.

Materials and Methods

Mice

The $\gamma 1E^{-/-}$ mutant mouse line was established at the MCI/ICS (Mouse Clinical Institute - Institut Clinique de la Souris-, Illkirch, France; http://www.ics-mci.fr/en/). Briefly, *in vitro* transcribed Cas9 mRNA (10 ng/µl) and 2 gRNAs (10 ng/µl) were co-injected into 803 C57BL/6N fertilized oocytes. Seventy pups were born. Two founders with the expected deletion were identified, one of which gave germline transmission. The $\gamma 1E^{-/-}$ line was maintained and bred under specific pathogen-free conditions. All animals used in this study were 8 to 12 weeks old. All animal work was performed under protocols approved by an ethics committee (Authorization N° 67-343).

Cell culture and flow cytometry

CH12 cells were induced to undergo CSR with IL-4 (5 ng/ml; Sigma), TGF-β (3 ng/ml; R&D Systems), and anti-CD40 antibody (100 ng/ml; eBioscience) for 72h. Resting B cells were isolated from the spleen using CD43 Microbeads (Miltenyi Biotec), labeled with 5 µg/ml CFSE (Invitrogen) for 10 minutes at 37°C, and cultured with 50µg/ml LPS (Sigma-Aldrich), 5ng/ml IL-4 (PeproTech), 100 ng/ml IFN-γ (PeproTech), 5ng/ml IL-5 (BD), 3 ng/ml TGF-β (R&D Systems), or 0.3 ng/ml retinoic acid (Sigma-Aldrich). Cells were stained with biotin-anti-IgG1 (BD), biotin-anti-IgG3 (BD), biotin-anti-IgG2b (BioLegend), biotin-anti-IgG2a (BD), PE-anti-IgA (SouthernBiotech) antibodies and PE-Streptavidin (Beckman Coulter). ToPro-3 (Invitrogen) was used to exclude dead cells in the analysis. For B cell development, bone marrow and splenic lymphocytes were stained with anti B220-PECy7 (e-biosciences), anti IgM-Cy5 (Southern Biotech), anti CD43-BB515 (BD Pharmingen), anti CD93-Biotin (e-biosciences),

Streptavidine-BUV395 (BD Pharmingen), anti CD21-FITC (BD Biosciences), anti CD23-PE (BD Biosciences), and anti IgM-Cy5 (Southern Biotech) antibodies. All flow cytometry data were acquired in a Fortessa (BD Biosciences) flow cytometer and analyzed using the FlowJo software.

Generation of γ1E$^{-/-}$ CH12 cells.

5x10^6 CH12 cells were electroporated with 0.5 μg of a plasmid co-expressing 2 gRNAs and Cas9 fused to EGFP using a Neon Transfection System (Thermo Fisher Scientific). 24h later, EGFP+ cells were sorted with a BD FACSAria (BD Biosciences) and plated under limiting dilution conditions to obtain single-cell clones, which were cultured for approximately 2 weeks. Genomic DNA was extracted and individual clones were genotyped by PCR and sequencing. See table S1 for gRNAs and primer sequences.

Western blot

Proteins were fractionated on 4-12% gradient SDS-PAGE gels (Invitrogen), transferred to Immobilon PVDF membranes (Millipore) and stained with anti-AID (Strasbg9, AID-2E11; IGBMC) or β-actin (Sigma) antibodies.

RT-qPCR

RNA and cDNA from CH12 or primary B cells were obtained following standard protocols. qPCR was performed in triplicates using the Universal Probe Library system (Roche) or SyberGreen (QIAGEN) and a LightCycler 480 (Roche). Transcript quantities were calculated relative to standard curves and normalized to Igβ mRNA. PCR primers and probes used are shown in Table S1.

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The authors have no conflicting financial interests.

Figure Legends

Figure 1. Deletion of the γ1E region results in defective CSR in CH12 cells. A. Schematic representation of the IgH locus before and after CRISPR/Cas9-mediated deletion of the γ1E region. gRNAs and PCR primers for genotyping are indicated. **B.** RT-qPCR of γ1E transcript levels (γ1E eRNAs) using primers located in the 5' (top) or middle (bottom) region of the γ1E. **C.** Surface expression of IgA analyzed by flow cytometry in $\gamma1E^{+/-}$, $\gamma1E^{-/-}$ clones and parental CH12 cells (pCH12) after three days in culture with TFG-β, IL-4 and anti-CD40 antibody. The percentage of switched cells is indicated. Representative dot plots of 6 experiments are shown. **D.** Percentage of CSR in $\gamma1E^{+/-}$and $\gamma1E^{-/-}$ clones, relative to pCH12 cells. Data are pooled from 6 independent experiments. Statistical significance was determined by two-tailed Student's t test. **E.** RT-qPCR for Iμ-Cμ (left panel) and Iα-Cα (right panel) transcripts. Triplicates were normalized to the abundance of Igβ, set as 1. Statistical significance was determined by a two-tailed Student's t-test. (*p-value < 0.05; **p-value < 0.005; NS: Not significant). Data are representative of 6 experiments.

Figure 2. Transcription and CSR to IgG3, IgG2b, and IgG2a are defective in $\gamma1E^{-/-}$ primary B cells. A. Flow cytometry analysis of surface Ig expression in CFSE-labeled primary B cells purified from $\gamma1E^{+/+}$ or $\gamma1E^{-/-}$ mice and cultured *in vitro* for 3 days with LPS (CSR to IgG3 and IgG2b), LPS+IL4 (CSR to IgG1 and IgE), LPS+IFNγ (CSR to IgG2a) or for 4 days with LPS+IL5+TGFβ+RA (CSR to IgA). The percentage of switched cells is indicated. Representative dot plots from 6 experiments are shown. **B.** CSR efficiency in primary B cells obtained from $\gamma1E^{-/-}$ (n=13) or $\gamma1E^{+/+}$ (n=12) mice, shown as percentage of Ig expression (left) and percentage of CSR (right). Mean+SD from 6 experiments is presented relative to $\gamma1E^{+/+}$ control B cells.

Statistical significance was determined by two-tailed Student's test (*p-value < 0.05; **p-value < 0.005). **C.** RT-qPCR for Iμ-Cμ, Iγ3-Cγ3, Iγ1-Cγ1, Iγ2b-Cγ2b, Iγ2a-Cγ2a, Iε-Cε and Iα-Cα transcripts in primary B cells purified from $\gamma1E^{+/+}$ or $\gamma1E^{-/-}$ mice and cultured as in **A**. Mean+SD of triplicates values were normalized to the abundance of Igβ, and are shown as relative to $\gamma1E^{+/+}$ B cells, set as 1. Statistical significance was determined by a two-tailed Student's t-test. (*p-value < 0.05; **p-value < 0.005).

Figure S1. Generation and characterization of $\gamma1E^{-/-}$ CH12 cells. A. Representative agarose gel of genotyping by PCR. The bands corresponding to the germline or the deleted alleles are indicated. **B.** Western blot analysis for AID and β-Actin of **γ1E** CH12 cell clones of different genotypes after 72h in culture with TFG-β, IL-4 and anti-CD40 antibody. Theoretical molecular weights (kDa) are indicated. **C.** RT-qPCR for AID expression in selected CH12 clones after 72h in culture with TFG-β, IL-4 and anti-CD40 antibody. Mean+SD of normalized AID values to Igβ and relative to clone #82 $\gamma1E^{+/-}$ from 4 independent experiments is shown.

Figure S2. Robust B cell development in $\gamma1E^{-/-}$ mice. Flow cytometry analysis of B cell development in the bone marrow **(A)** and the spleen **(B)** of $\gamma1E^{+/+}$ (n=12) and $\gamma1E^{-/-}$ (n=13) mice. Representative plots from 6 experiments are shown. When necessary, gating is indicated above the plot. The frequency of the different B cell fractions is represented by the mean+SD for each population from $\gamma1E^{-/-}$ relative to $\gamma1E^{+/+}$ mice, set as 1. The cellularity represents the absolute number of cells (mean+SD).

Table S1. Primers, probes and gRNAs

RT-qPCR

Primer	Sequence (5'-3')	Probe and Reference
Igβ–F	TGGTGCTGTCTTCCATGC	UPL Probe 18 (Thomas-Claudepierre et al., 2013)
Igβ–R	TTGCTGGTACCGGCTCAC	UPL Probe 18 (Thomas-Claudepierre et al., 2013)
Iμ-Cμ-F	ACCTGGGAATGTATGGTTGTGGCTT	(Jeevan-Raj et al., 2011)
Iμ-Cμ-R	TCTGAACCTTCAAGGATGCTCTTG	(Jeevan-Raj et al., 2011)
Iμ-Cμ2-F	CCCAGACCTGGGAATGTATG	UPL Probe 29 (Robert et al., 2015)
Iμ-Cμ2-R	GGAAGACATTTGGGAAGGACT	UPL Probe 29 (Robert et al., 2015)
Iγ3-Cγ3-F	GCAGAAATCTGCAGGACTAACA	UPL Probe 71 (Robert et al., 2015)
Iγ3-Cγ3-R	ACCGAGGATCCAGATGTGTC	UPL Probe 71 (Robert et al., 2015)
Iγ2b-Cγ2b-F	TGGGCCTTTCCAGACCTAAT	UPL Probe 88 (Robert et al., 2015)
Iγ2b-Cγ2b-R	GGGCTGATCTGTCAACTCCT	UPL Probe 88 (Robert et al., 2015)
Iγ2a-Cγ2a-F	CAGCCTGGGATCAAGCAG	UPL Probe 109 (Robert et al., 2015)
Iγ2a-Cγ2a-R	TGGGGCTGTTGTTTTGGT	UPL Probe 109 (Robert et al., 2015)
Iγ1-Cγ1-F	GGCCCTTCCAGATCTTTGAG	(Park et al., 2009)
Iγ1-Cγ1-R	ATGGAGTTAGTTTGGGCAGCA	(Park et al., 2009)
Iμ-Cε-F	CCCAGACCTGGGAATGTATG	UPL Probe 29 (Thomas-Claudepierre et al., 2016)
Iμ-Cε-R	GGGTAGAGCTGAGGGTTCCT	UPL Probe 29 (Thomas-Claudepierre et al., 2016)
Iμ-Cα-F	GGAGACTCCCAGGCTAGACA	UPL Probe 27 (Thomas-Claudepierre et al., 2013)
Iμ-Cα-R	CGGAAGGGAAGTAATCGTGA	UPL Probe 27 (Thomas-Claudepierre et al., 2013)

Genotyping primers (CH12 cells and $\gamma 1E^{-/-}$ mice)

Primer	Sequence (5'-3')
g1E-F	GCTGAGCAAAACACCACCTG
g1E-R	GACCTCTCCAGTTTCGGCTG

gRNAs

Primer	Sequence (5'-3')	PAM	Strand
gRNA1	AACATTGGCCTCCCAACA	TGG	-
gRNA2	CCAATGGCATTGGTAACC	AGG	+

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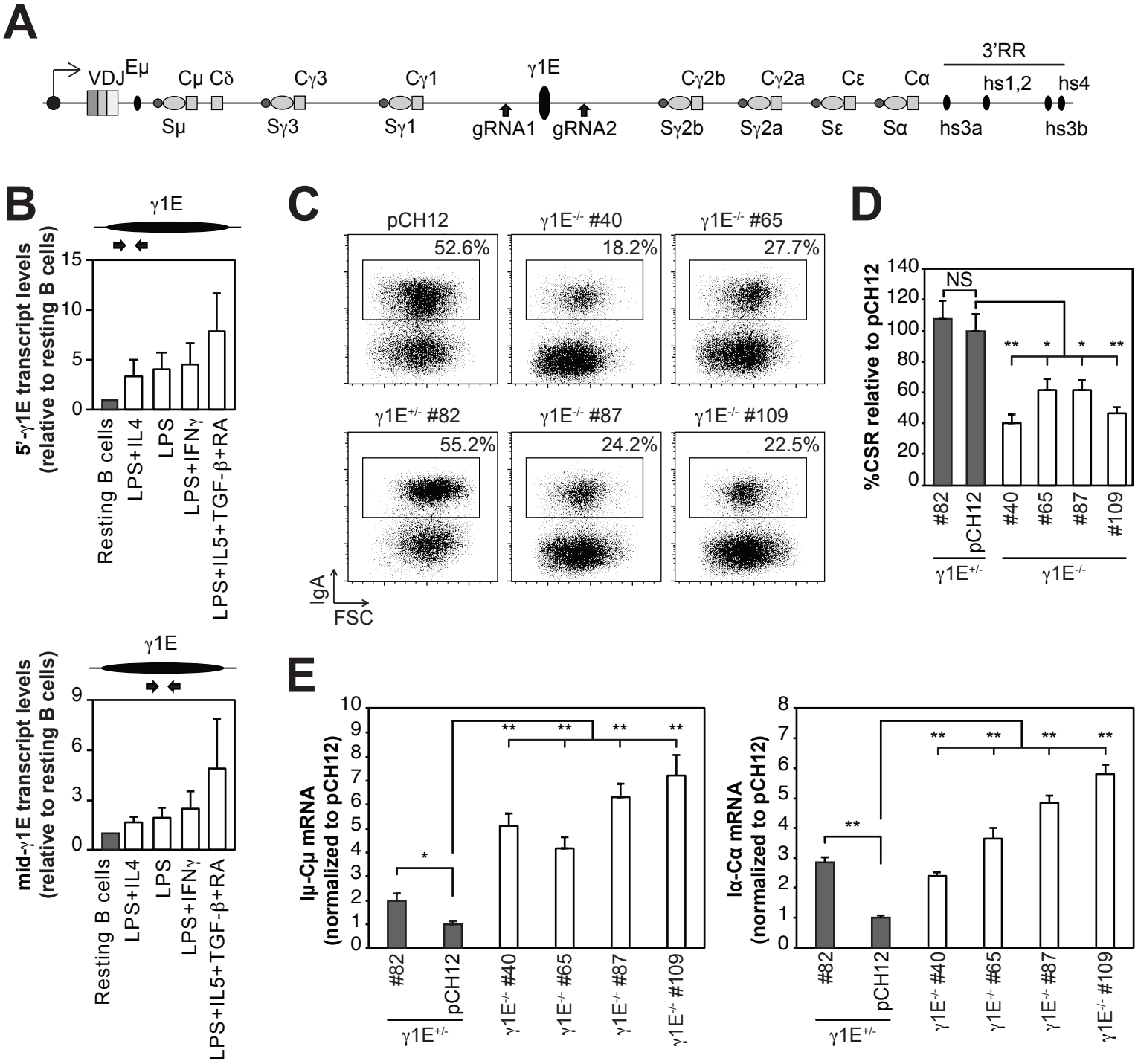
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Sμ
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Sγ1
gRNA1
gRNA2
Sγ2b
Sγ2a
Sε
Sα
hs3a
hs3b
B
γ1E
5'-γ1E transcript levels (relative to resting B cells)
Resting B cells
LPS+IL4
LPS
LPS+IFNγ
LPS+IL5+TGF-β+RA
mid-γ1E transcript levels (relative to resting B cells)
C
pCH12
γ1E-/- #40
γ1E-/- #65
52.6%
18.2%
27.7%
γ1E+/- #82
γ1E-/- #87
γ1E-/- #109
55.2%
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IgA
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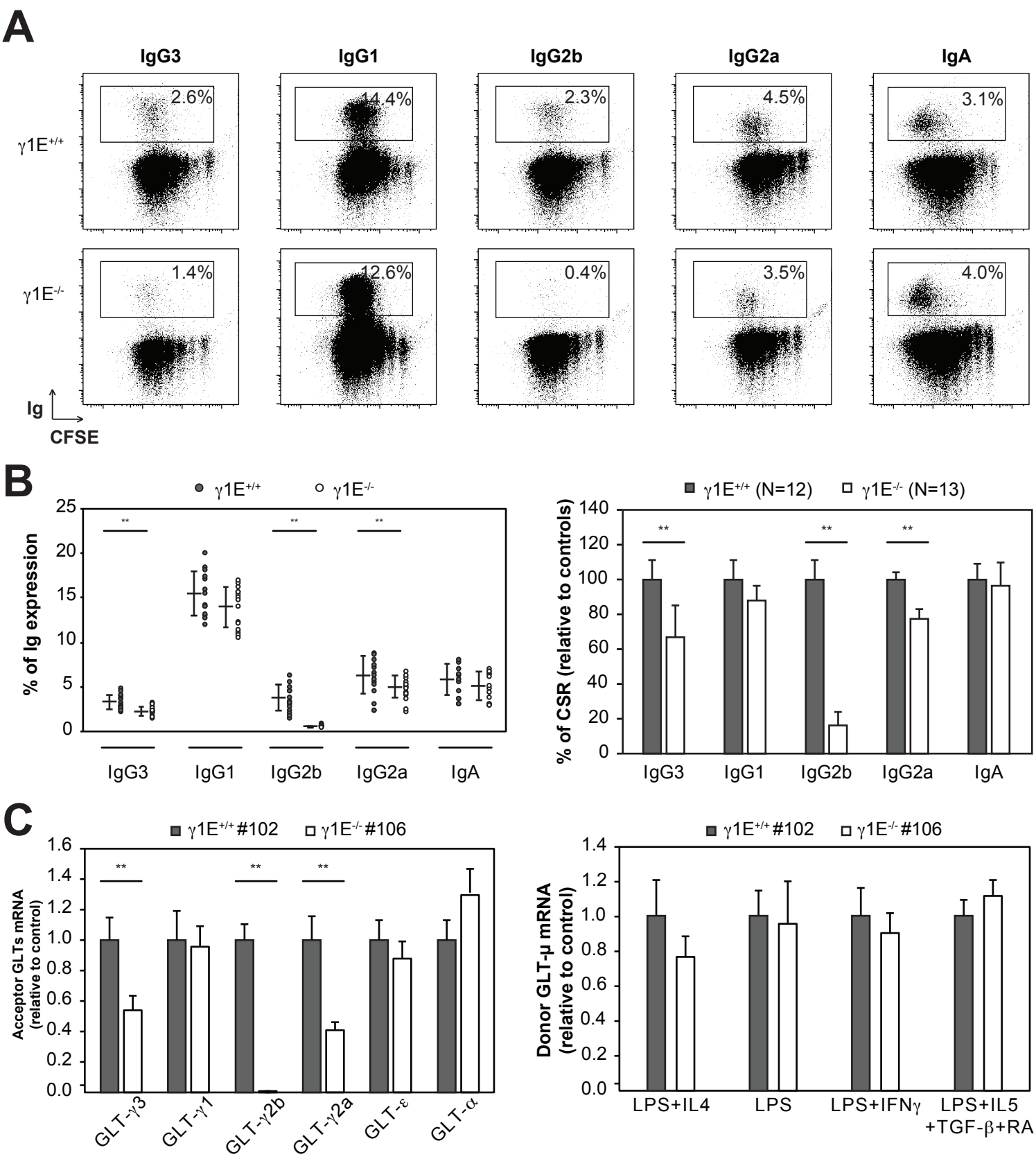
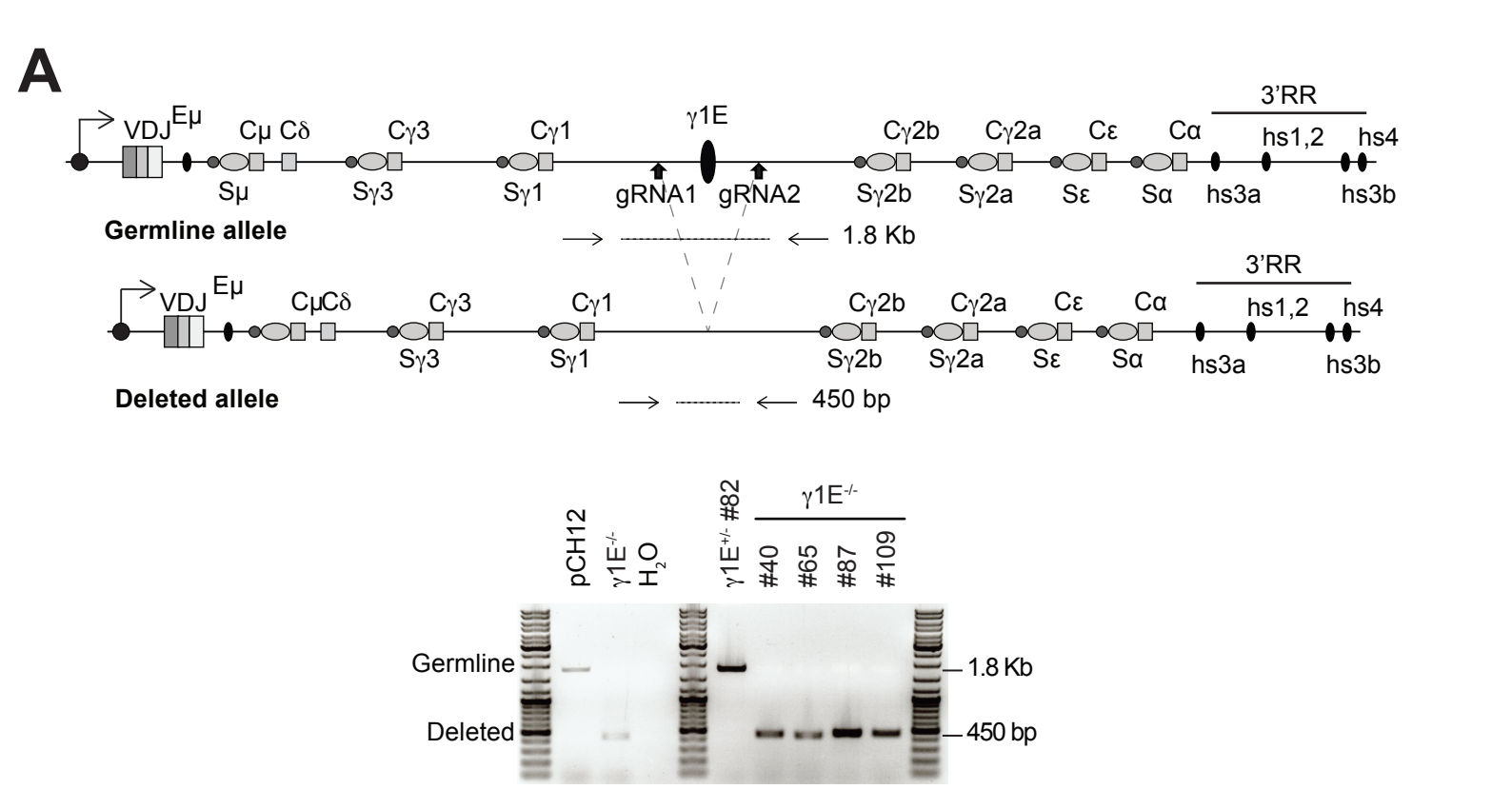
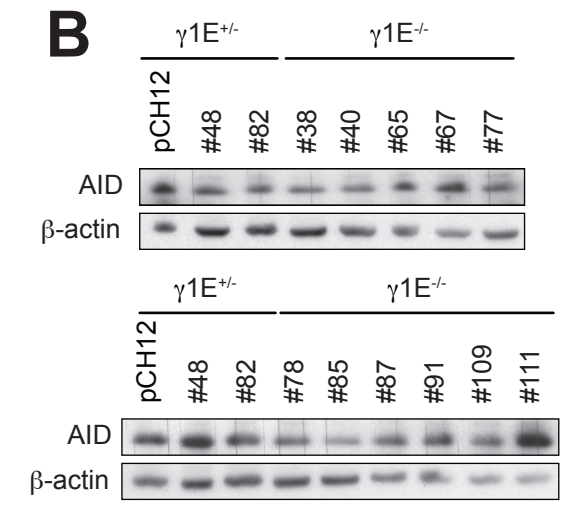
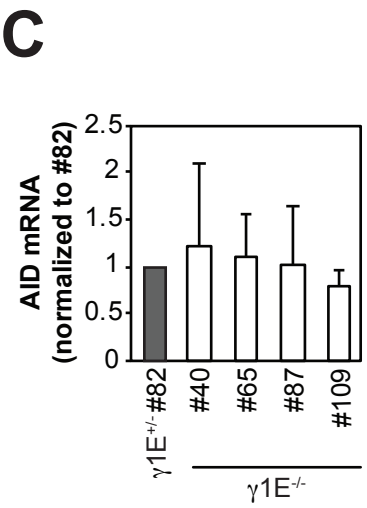
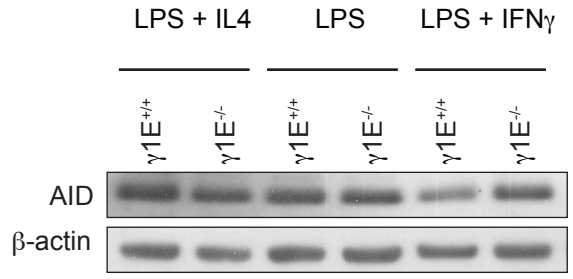


Amoretti-Villa *et al.* Figure 2

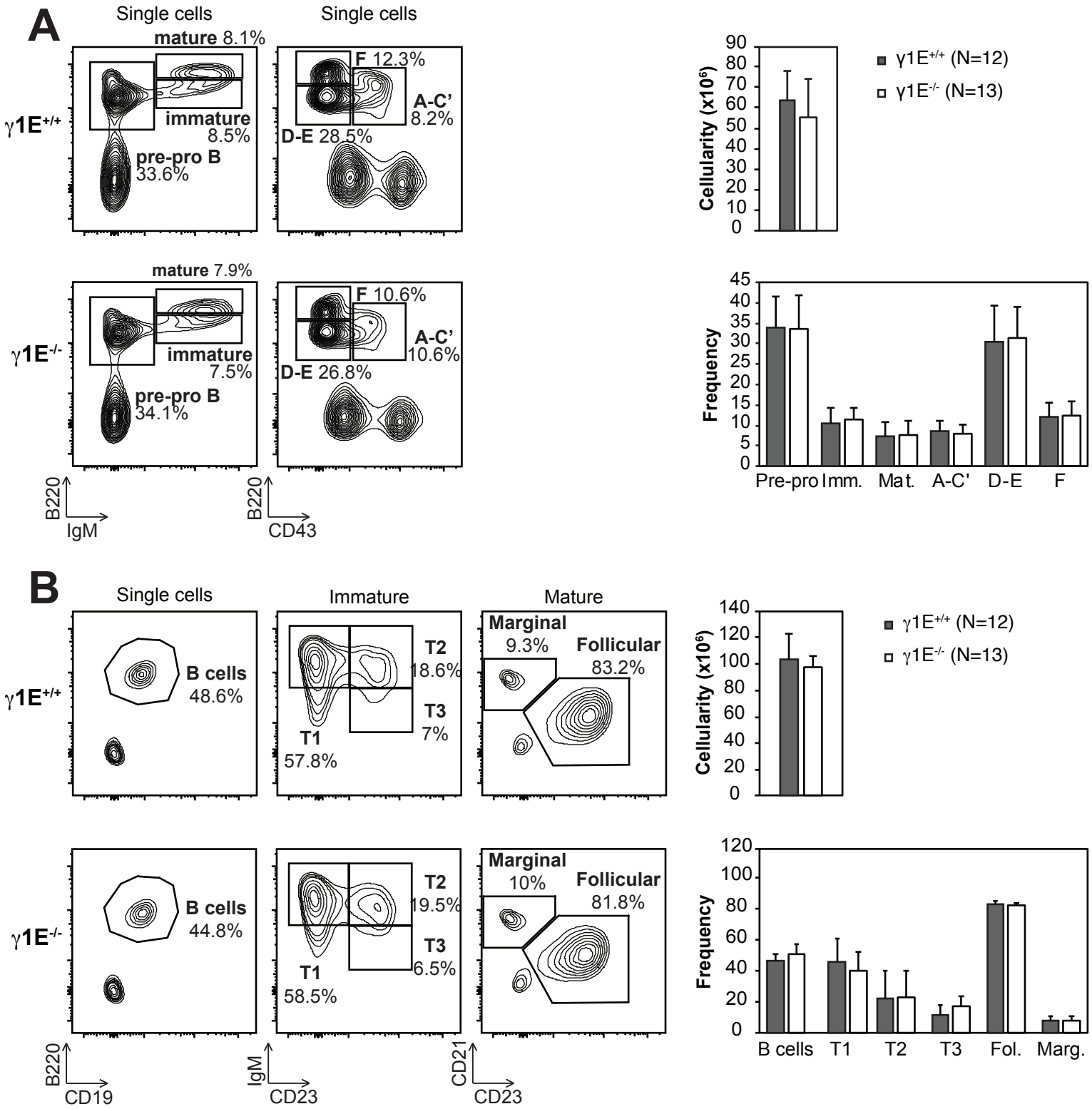








Amoretti-Villa *et al.* Figure S1



Amoretti-Villa *et al*. Figure S2

Results - Part 2

Study of the role of the γ1E region as an enhancer during CSR

In the first part, I presented the functional characterization of the γ1E in CH12 cells and primary B cells, and demonstrated that this region regulates GLT transcription in an isotype-specific manner, affecting the CSR efficiency to the corresponding isotypes (Amoretti-Villa et al., 2019). However, based on these results alone, we cannot conclude that the transcriptional activation of the B cell germline promoters is directly exerted by the γ1E region. In this part, I will present additional experiments performed in order to test if the γ1E region has a transcriptional activator effect in activated CH12 cells.

On the other hand, γ1E binds the 3'RR on activated B cells (Thomas-Claudepierre et al., 2016) and the 3'RR elements are known to control transcription from the germline promoters (Garot et al., 2016; Pinaud et al., 2001; Vincent-Fabert et al., 2010). Hence, I present in this part results on the study of the potential effect of γ1E on the transcription of 3'RR elements.

1. Expression of a reporter gene was not induced by γ1E under the tested conditions

The γ1E region has marks of active enhancer in pro-B cells as shown through 4C-seq and ChIP followed by deep sequencing (ChIP-seq) experiments. Indeed, Medvedovic and colleagues found on this region DNase I hypersensitivity, binding of Pax5, and YY1, and active chromatin marks such as H3K4me2 and H3K9ac (Medvedovic et al., 2013). Moreover, it was attributed to a chromatin state characteristic of enhancers because of its enrichment for features of regulatory regions including H3K4me1, H3K4me2, H3ac, and binding of Pax5, p300, E2A, PU.1, and Med1; and it acted as a B cell-specific enhancer in reporter assays (Predeus et al., 2014). Independently, it was described as a super-enhancer due to its high levels of Med1 and PU.1 binding (Whyte et al., 2013).

Intriguingly, it also shows marks of active enhancer in mature B cells, since it is bound by Med1 and Med12 both in LPS and LPS + IL4 activated B cells (Thomas-Claudepierre et al., 2016). This activity could be stimulation-dependent, since resting B cells didn't show significant binding of mediator subunits to the region (Thomas-Claudepierre et al., 2016). Importantly, the pattern of mediator binding on the γ1E region perfectly correlated the dynamic 3D conformation adopted by the *Igh* locus in activated B cells. Indeed, it was recruited to the loop formed by the Eμ enhancer and the 3'RR in an activation-dependent fashion, together with the specific cytokine-inducible promoters (Thomas-Claudepierre et al., 2016). Hence, we hypothesized that the γ1E region could act as an enhancer during the process of CSR.

To test this possibility, I undertook a reporter assay using the dual-luciferase system. Briefly, inside a reporter vector that expresses the firefly luciferase gene under the SV40 promoter (pGL3-P), I cloned different fragments that covered the whole γ1E sequence (pGL3- γ1E-1 to 4). As a positive control, I used the pGL3-C vector, which differs from the pGL3-P in an additional SV40 enhancer element downstream of the luciferase gene. For normalization purposes and as a transfection control, I employed the pRL vector expressing a *Renilla* luciferase gene under the CMV enhancer/promoter element **(figure 11)**.

First, I tested the capacity of the different cloned γ1E fragments for the induction of luciferase expression in the 3T3 murine fibroblast cell line. For this purpose, I transfected 3T3 cells either with a pGL3-γ1E or with the negative and positive control plasmids pGL3-P and pGL3-C, respectively. In all conditions, the pRL normalization control plasmid was co-transfected. 24 hours after transfection, I measured the firefly and the *Renilla* luciferase activity.

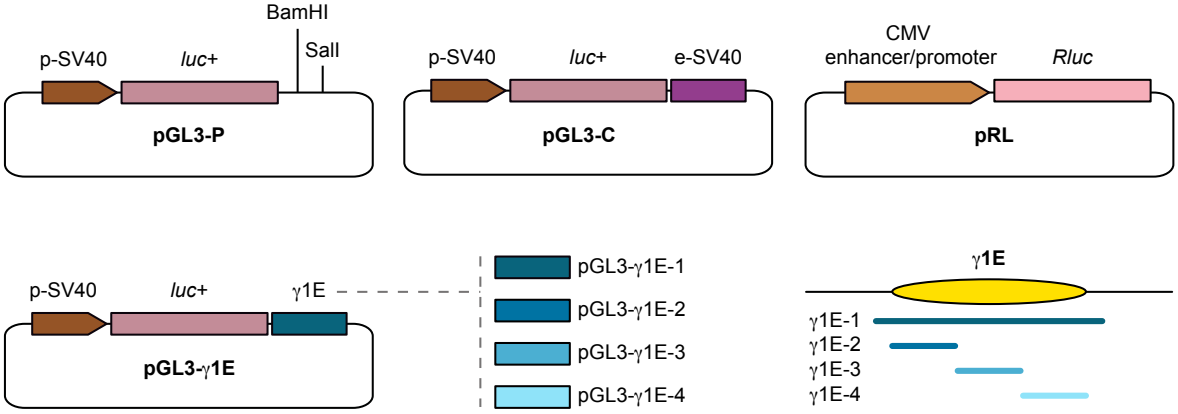


Figure 11: Reporter vectors for the dual luciferase assay. Schematic representation of the firefly (pGL3) and the *Renilla* (pRL) luciferase reporter vectors. The whole g1E region (γ1E-1) or three different fragments (γ1E-2 to 4) were cloned inside the pGL3-P vector.

Despite the 4.5-fold luciferase activity of the pGL3-C positive control compared to pGL3-P, no one of the 4 different fragments of γ1E showed an induction effect compared with the pGL3-P negative control **(figure 12)**.

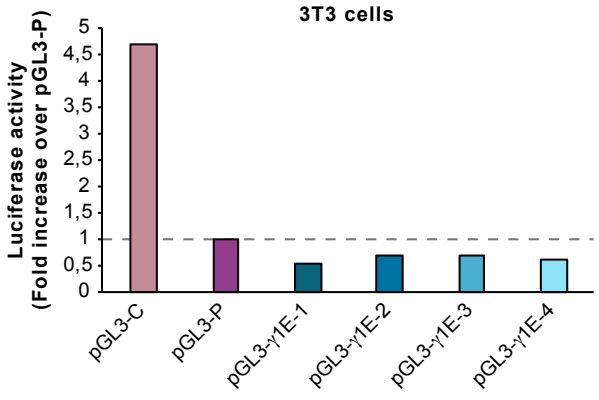


Figure 12: The cloned vectors do not potentiate the luciferase signal in 3T3 cells. 3T3 cells were collected 24 hours after transfection with the different reporter vectors. The results are presented as a ratio of the firefly luciferase luminescence versus the *Renilla* luciferase luminescence, and normalized to the value corresponding to cells transfected with pGL3-P vector (set as 1). Results are representative of 3 independent experiments.

As discussed above, B-cell specific TFs bind the γ1E region in pro-B cells (Medvedovic et al., 2013; Predeus et al., 2014), and Mediator subunits are recruited to γ1E upon B cell activation (Thomas-Claudepierre et al., 2016). Hence, it would be reasonable to think that the γ1E has a lineage-specific effect, which would explain the lack of effect observed in 3T3 cells.

In order to test if the γ1E had an enhancer effect in the B cell lineage, I undertook the reporter assay on the CSR-competent B cell line CH12. The CH12 cell line is a murine B lymphoma that is broadly used as a model for CSR, since it can be induced *in vitro* to switch from IgM to IgA at high efficiency when cultured with a stimulation medium containing interleukin 4 (IL4), anti-CD40 antibody, and transforming growth factor β (TGF-β) (Nakamura et al., 1996). Comparison of the CSR-induced cells with the non-stimulated condition served to test if the γ1E activity is stimulation-dependent.

CH12 cells were transfected with the same combinations of vectors as in the experiments on the 3T3 cell line, and directly seeded under stimulation conditions or not. The luciferase activity measurements were taken either at 24 hours (when we would expect higher luciferase expression) or 48 hours after transfection (to allow for the induction of CSR).

As expected, the CSR efficiency 24 hours after transfection was very low, and only 5-8% of cells were IgA+ as shown by flow cytometry analysis of the samples. Nevertheless, at 48 hours the efficiency was of ~37% for all the different transfection conditions **(figure 13A)**. However, the pGL3-γ1E vector did not cause any increase in the luciferase activity compared with the pGL3-P control, neither at 24 nor at 48 hours after transfection. Moreover, there was no difference in luciferase activity between the stimulated and the non-stimulated cells **(figure 13B)**.

To summarize, neither the whole sequence nor the smaller fragments of the γ1E regions showed an increased luciferase activity compared with the vector without enhancer (pGL3-P) in the 3T3 nor in the CH12 cell lines. Moreover, the stimulation of the CH12 cells did not cause an induction in luciferase expression. However, it is of note the low transfection efficiency of the CH12 cells, which could affect the results. Since the luciferase expression vectors do not carry any fluorescent reporter, it was impossible to accurately measure the transfection efficiency and we can only rely on the *Renilla* luciferase measurements, which were higher in the 3T3 than in the CH12 cells. Altogether, being the CH12 cells difficult to transfect, and relying only on non-B cell lines, the optimal conditions for the experiment were impossible to achieve, and I cannot conclude on a positive enhancer effect of the γ1E region in activated B cells based on this reporter assay.

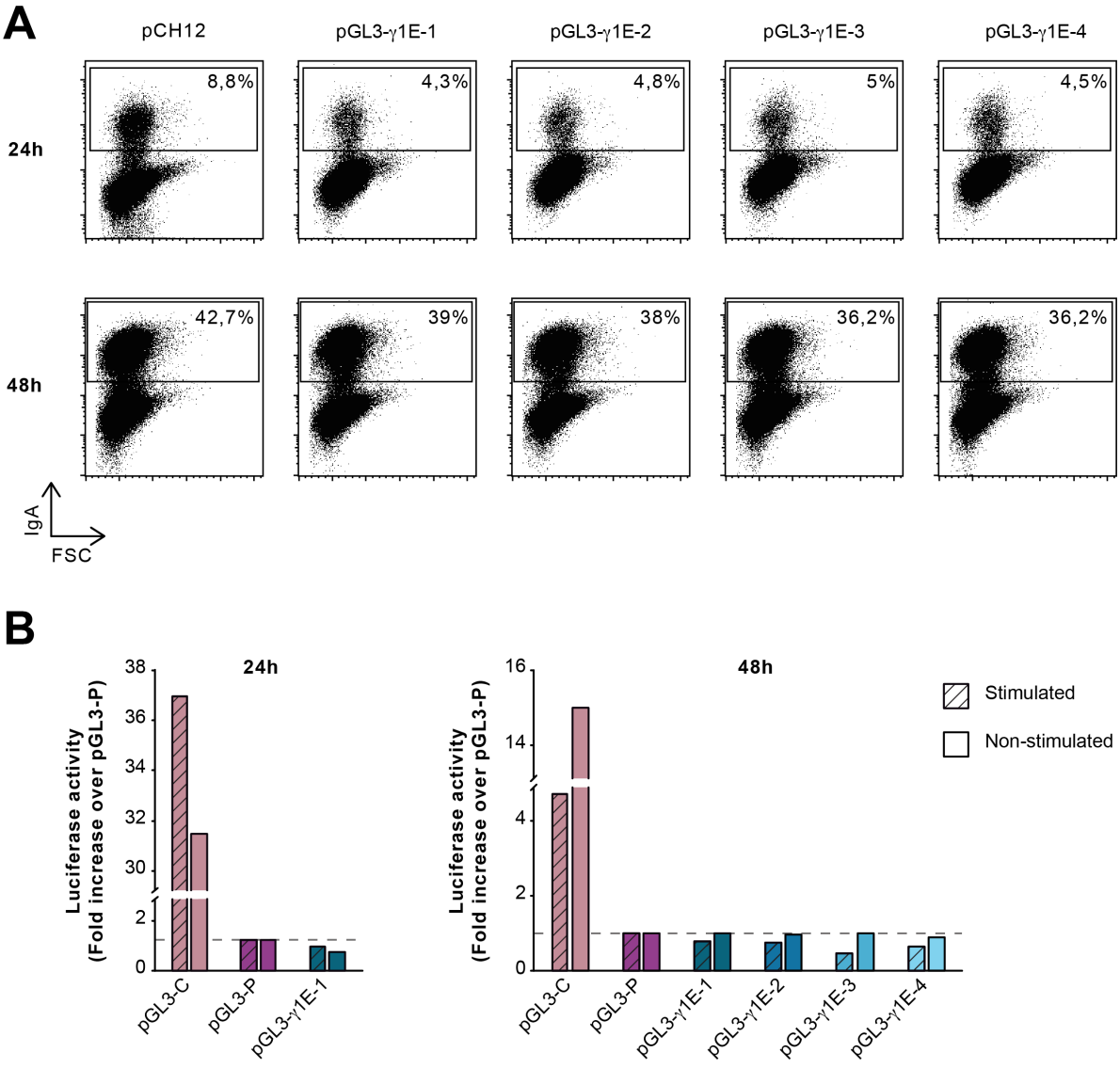


Figure 13: The cloned vectors do not potentiate the luciferase signal in CH12 cells. A. Flow cytometry plots of a representative experiment showing the IgA expression of CH12 cells stimulated for 24 or 48 hours with IL4 + anti-XΔ40 antibody + TGF-β. **B.** Luminescence signal 24 or 48 hours after transfection of CH12 cells with the reporter vectors, cultured as in **A**. Luciferase activity was calculated as in figure 12.

2. Deletion of the γ1E region does not affect transcription of 3'RR elements

The first studies that described the γ1E showed that there exists an interaction between this region and the 3'RR. Indeed, it was found to bind hs3b in pro-B cells (Medvedovic et al., 2013). Moreover, the γ1E region is dynamically recruited to the loop that is formed between the Eμ and the 3'RR upon B cell stimulation, as shown through 4C-Seq experiments (Thomas-Claudepierre et al., 2016). Furthermore, the $\gamma1E^{-/-}$ B cells show a deficient GLT expression, which is known to be controlled by the 3'RR. Altogether, the physical contact existent between the γ1E and the 3'RR could suggest a functional relationship between both enhancer regions. To address the possibility of a potential effect of the γ1E region on the 3'RR, I compared the levels of transcription of the 3'RR in $\gamma1E^{+/+}$ and $\gamma1E^{-/-}$ B cells.

Using RNA samples from B cells stimulated *in vitro* to undergo CSR to the different isotypes, I performed quantitative reverse transcription PCR (RT-qPCR) of the hs1,2 and the hs4 enhancers, known to be part of different functional modules of the 3'RR (Garot et al., 2016) and also known to interact with the γ1E region and bind Mediator subunits in activated B cells (Thomas-Claudepierre et al., 2016). Nevertheless, the transcription levels of both enhancers were comparable between $\gamma1E^{+/+}$ and $\gamma1E^{-/-}$ B cells cultured under different stimulation conditions **(figure 14)**. I concluded that the γ1E region does not directly affect the transcription of individual 3'RR enhancers. Even though there was no effect on 3'RR transcription in the γ1E-deficient B cells, there could still be a functional relationship between both regions. In this regard, a potential hierarchical relationship in which the 3'RR affects the γ1E, similar to the 3'RR control over Eμ (Saintamand et al., 2017), should be addressed.

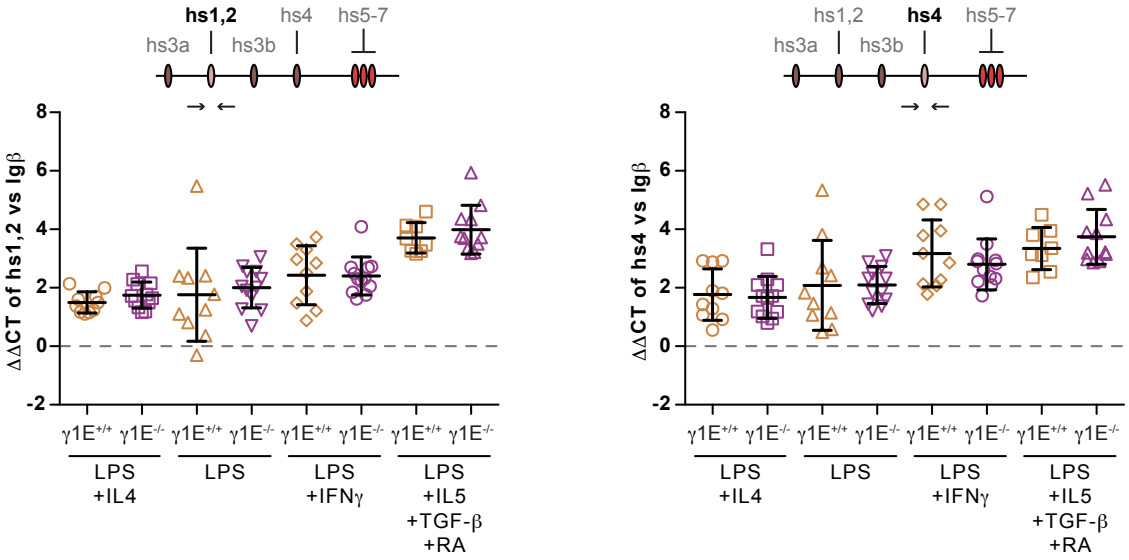


Figure 14: Transcription levels of the 3'RR regions hs1,2 and hs4 in B cells are comparable between $\gamma1E^{+/+}$ and $\gamma1E^{-/-}$ B cells under all stimulation conditions. RT-qPCRs of the hs1,2 (left) and hs4 (right) elements of the 3'RR are presented. The localization of the analyzed regions is depicted in the schematic representation of the 3'RR above each graph. Splenic $\gamma1E^{+/+}$ (orange) and $\gamma1E^{-/-}$ (purple) B cells were cultured under different stimulation conditions (as indicated in the x axis) for 72h before extracting the total RNA. Results are presented as the mean ΔΔCT normalized to Igβ from 5 independent experiments. RA: retinoic acid. Error bars represent standard deviation.

Results – Part 3

Transcription and DNA looping: a chicken-and-egg situation

There is a clear relationship between the activation of transcription and the long-range interactions that take place between enhancers and promoters. There are different hypotheses that explain this relationship, suggesting that looping promotes the recruitment of the transcriptional machinery, that there is a transference of polymerase from enhancer to promoter thanks to the physical proximity between them, and that chromatin looping favors the movement of the promoter towards a more transcription-favorable nuclear environment (Krivega and Dean, 2012). However, the question whether the looping is a casualty or a consequence of transcription initiation remains to be cleared.

So far, several studies have revealed that looping precedes and facilitates transcription in different models. Lef1 interacts with TFs bound to the promoters of target genes to mediate an IL-1β stimulation-dependent loop that facilitates transcriptional regulation in chondrocytes (Yun et al., 2009). Moreover, tethering the self-association domain of Ldb1 both to the LCR and promoter of β-globin gene induced chromatin looping and reactivated transcription in a GATA1-depleted model (Deng et al., 2012), and forcing chromatin looping between the locus control region and the promoter of an embryonic globin gene led to transcriptional reactivation in adult erythroblasts, bypassing the developmental silencing (Deng et al., 2014). Also, through a high-resolution map of enhancer contacts in Drosophila, it was suggested that the interactions between enhancers and promoters, mediated by TFs, are stable during development and help recruit Pol II, which remains poised until additional TFs or enhancers are recruited to the chromatin loop and activate transcription through Pol II release (Ghavi-Helm et al., 2014). Altogether, these studies suggest a mechanism whereby gene looping, mediated by TFs, drives transcriptional activation in eukaryotic cells.

Mediator is a multiprotein complex composed of 31 subunits in mammals organized in the head, middle, and tail stable modules, which can reversibly interact with the kinase module. It is implicated in transcriptional regulation through different mechanisms. Mediator is required for transcription through the RNA pol II (Holstege et al., 1998; Myers et al., 1999). In this line, it has been shown that mediator helps in the recruitment of RNA Pol II (Asturias et al., 1999; Bernecky et al., 2011; Davis et al., 2002) and facilitates the assembly of the pre-initiation complex (Baek et al., 2006; Baek et al., 2002; Esnault et al., 2008; Jiang et al., 1998). Besides, Mediator has been implicated in the pausing and release of RNA Pol II and the elongation of transcription (Kremer et al., 2012; Malik et al., 2002; Meyer et al., 2010; Wang et al., 2005) and, through its interaction with the histone acetyltransferase p300, it is thought to induce transcription through the regulation of the chromatin state (Acevedo and Kraus, 2003). Moreover, mediator has a pivotal role in loop formation and maintenance.

In our lab, it was shown that Mediator plays a role in CSR, through an effect on acceptor S region transcription and the dynamic long-range interactions taking place upon B cell activation. Indeed, Med1-deficient CH12 and primary B cells displayed a defect in CSR, concomitant with deficient GLT transcription and reduced interaction frequency between the acceptor S region and the *Igh* locus enhancers (Thomas-Claudepierre et al., 2016). However, it was not explained whether the looping is the cause or the consequence of the transcription. It could be the case that chromatin looping facilitates the necessary GLT transcription; but there also exists the non-mutually exclusive possibility that transcription initiation constitutes a mechanism whereby chromatin loop can be formed and facilitate CSR.

To test this possibility, we decided to force transcription of GLT-γ1 in a Med1-deficient background, where both transcription and looping are defective, with the idea that CSR could be restored through loop formation upon artificial transcriptional activation.

To do so, I took advantage of the targeting capability of the clustered regularly interspaced short palindromic repeat (CRISPR)-Cas system. Specifically, I used the catalytic mutant dCas9 (Qi et al., 2013), which has mutations in the two nuclease domains of wildtype Cas9 and thus constitutes a convenient tool for tethering fused peptides to the regions of interest without causing DNA damage. In this study, I used the transcriptional activator VP64 fused to dCas9. VP64 is the tetrameric repeat of the minimal activation domain VP16 of herpes simplex virus (Beerli et al., 1998). The dCas9-VP64 fusion protein has been previously demonstrated to be a powerful tool for enhancing transcription of endogenous genes in combination with guide RNAs (gRNAs) that target the region of interest (Maeder et al., 2013).

In this study, four gRNAs were designed to tether the dCas9-VP64 fusion protein to the Iγ1 region and thus drive GLT-γ1 transcription **(figure 15)**. We hypothesized that this artificial transcriptional activation could potentially restore CSR efficiency in Med1-deficient B cells, which would imply the formation of the loop as a necessary intermediate.

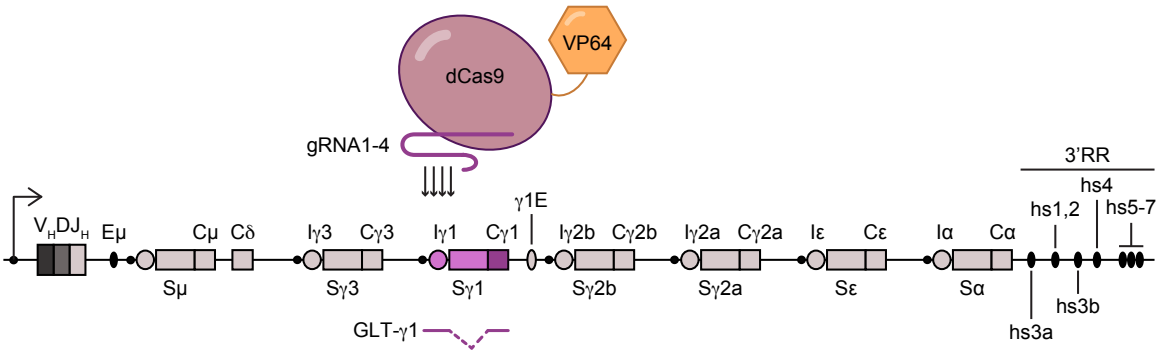


Figure 15: Strategy for the induction of transcription of GLT-γ1 with the dCas9-VP64 system. The dCas9 was fused to the VP64 transcriptional activator and tethering of the complex was achieved through the design of 4 different gRNAs targeting the Iγ1 region.

1. Efficient induction of GLT-γ1 transcription in non-stimulated CH12 cells

With the objective of unraveling the relationship between transcriptional activation and formation of the loop, I planned on forcing GLT-γ1 transcription through the tethering of the dCas9-VP64 induction system to Iγ1 region in a background devoid of the capability of looping, and studying the IgG1 switching efficiency promoted by the forced transcription.

To test the efficacy of the approach I used the CH12 cell line, which is committed to perform CSR only to IgA (Nakamura et al., 1996). Hence, transcription of Iγ1 region was not expected in this cell line. GLT transcription is induced upon B cell activation and, for this reason, I tested the efficiency of the system to activate GLT-γ1 transcription in non-stimulated CH12 cells. Cells were transfected with either one or two plasmids, each one expressing two different gRNAs targeting the Iγ1 region, together with the dCas9-VP64 protein fused to a fluorescent marker **(figure 16A)**. Fluorescent cells were sorted 24 hours after transfection and RNA extraction was performed on the sorted cells and 72 hours after transfection.

RT-qPCR of the Iγ1-Cγ1 region showed consistently higher transcription levels in the transfected cells than in the parental CH12 line **(figure 16B)**. Moreover, the effect was stronger in the presence of four gRNAs in contrast to cells transfected with only one vector and hence expressing only two gRNAs. The highest transcription was achieved at 24h post-transfection, when the levels reached those of B cells stimulated with LPS + IL4 (condition that induces CSR to IgG1) in cells transfected with one vector, and largely overpassed them in the combination of four gRNAs. Therefore, the dCas9-VP64 system efficiently induces transcription of the targeted region and its effect is exacerbated in the presence of four gRNAs compared with only two.

In order to test if the transcriptional activation effect was targeted to other regions than Iγ1, I analyzed the transcription levels of the rest of the isotypes. In the case of GLT-γ3, GLT-γ2b, and GLT-γ2a, transcription reached the levels of non-transfected CH12 cells or even exceeded them in some transfection conditions, although without reaching the levels of the optimal stimulation condition in any of the regions. On the other hand, transcription was highly induced to GLT-α reaching the levels of stimulated CH12 cells. However, in this case the transcription in the presence of four gRNAs was not higher than with two gRNAs and the levels of transcription at 72 hours were comparable to those at 24 hours post-transfection. This pattern was very different from the GLT-γ1 region, suggesting that this effect could be due to something else than the dCas9-VP64 system *per se*.

Overall, with the exception of GLT-α, in any case other than GLT-γ1 did the transcript levels reach those of activated B cells in the optimal stimulation conditions that induce CSR to the corresponding isotypes. Altogether, I concluded that the dCas9-VP64 system efficiently and exclusively forces transcription of GLT-γ1 in non-stimulated CH12 cells.

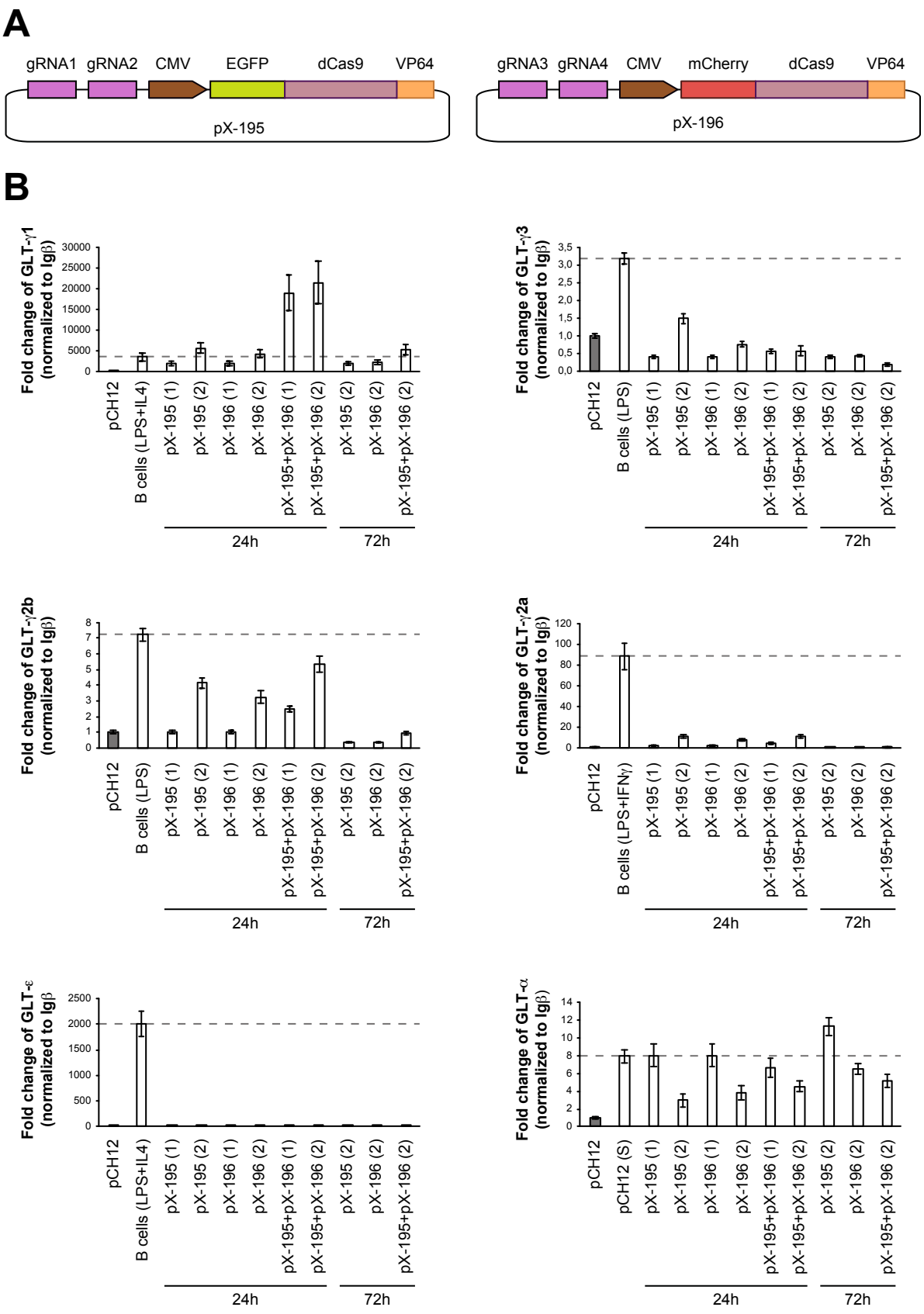

A
gRNA1
gRNA2
CMV
EGFP
dCas9
VP64
pX-195
gRNA3
gRNA4
CMV
mCherry
dCas9
VP64
pX-196
B
Fold change of GLT-γ1 (normalized to Igβ)
Fold change of GLT-γ3 (normalized to Igβ)
Fold change of GLT-γ2b (normalized to Igβ)
Fold change of GLT-γ2a (normalized to Igβ)
Fold change of GLT-ε (normalized to Igβ
Fold change of GLT-α (normalized to Igβ)
pCH12
B cells (LPS+IL4)
B cells (LPS)
B cells (LPS+IFNγ)
pCH12 (S)
pX-195 (1)
pX-195 (2)
pX-196 (1)
pX-196 (2)
pX-195+pX-196 (1)
pX-195+pX-196 (2)
24h
72h

Figure 16: The dCas9-VP64 system correctly targets Iγ1 and induces specific transcription of GLT-γ1. A. Schematic representation of the vectors used for expressing dCas9-VP64 in the CH12 cells. Each vector carries two different gRNAs targeting Iγ1 region and the EGFP-dCas9-VP64 complex. **B.** RT-qPCRs of the different GLTs 24 or 72 hours after transfection with one or the two vectors. Data from two replicates (indicated as 1 or 2 inside parentheses) is presented. Results are presented as the fold change of non-stimulated parental CH12 (pCH12) cells and normalized to Igβ, for the different transfections (indicated on the x axis). For comparison purpose, dashed lines indicate the fold change value of the optimal stimulation condition driving activation of the corresponding I_X promoter. Error bars represent standard deviation of technical triplicates.

2. CSR to IgG1 is not restored with the dCas9-VP64 induction system in Med1$^{-/-}$ B cells

Med1$^{-/-}$ B cells show a deficit both in germline transcription and formation of the dynamic loop upon stimulation, which leads to a defect in CSR efficiency (Thomas-Claudepierre et al., 2016). Hence, Med1$^{-/-}$ B cells offer an appropriate setting to study whether the induction of germline transcription is enough to rescue CSR to the corresponding isotype.

In order to express the dCas9-VP64 induction system in Med1$^{-/-}$ B cells, I cloned different retroviral (RV) vectors carrying either the four gRNAs and the fluorescent marker mCherry (in the pQCXIX backbone vector), or the dCas9-VP64 fusion protein together with EGFP (in the pMX-PIE, pQCXIP, and pQCXIX backbone vectors) **(figure 17A)**.

First, I tested the infection potential of the different vectors in the CH12 B cell line. BOSC 23 cells were transiently transfected with each RV vector to generate the viral particles that were subsequently used to infect CH12 cells. Expression of the dCas9-VP64 fusion protein in BOSC 23 cells from all the different vectors was confirmed by western blot **(figure 17B)**.

24 hours after infection, I analyzed the percentage of GFP+ and mCherry+ cells by flow cytometry **(figure 17C)**. The efficiency of infection was variable among the different vectors, with the highest being of around 10%. Infection with the vector expressing the gRNAs did not yield good efficiency. Antibiotic selection increased the percentage of pMX-PIE infected cells by 7-fold, but negatively affected the viability of pQCXIP infected cells **(figure 17C)**. Because it does not carry an antibiotic resistance cassette, no selection could be made on cells infected with vectors containing pQCXIX backbone.

Altogether, the cloned RV vectors did not show high infection potential in CH12 cells, although the pMX-PIE backbone was the most effective and infected cells could be efficiently selected.

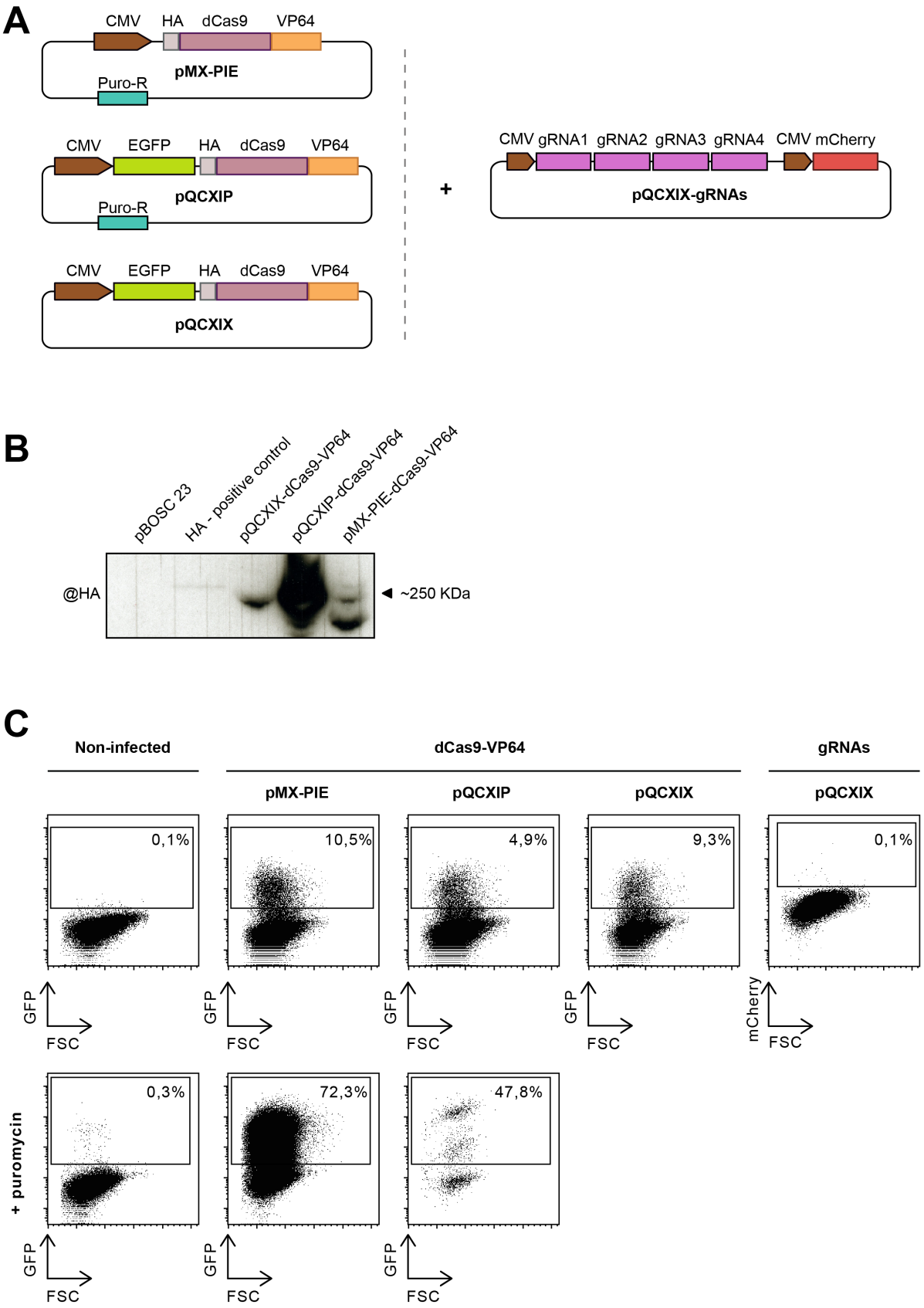


Figure 17: Retroviral infection of CH12 cells for the expression of the dCas9-VP64 system. A. Schematic representation of the different retroviral vectors carrying either the EGFP-dCas9-VP64 fusion protein or the four gRNAs in different RV backbones (pMX-PIE, pQCXIP, pQCXIX). Fluorescent reporter markers allowed for the

double infection with a vector expressing dCas9-VP64 (EGFP marker) and the gRNAs (mCherry marker). The pMX-PIE and pQCXIP backbones express a puromycin resistance gene (puro-R), which permits antibiotic selection. **B.** Western blot of transfected BOSC 23 cells using an anti-HA antibody. As an HA-positive control, protein extracts from BOSC 23 cells transfected with a non-relevant vector containing HA-dCas9 were used. Protein extracts from parental BOSC 23 cells (pBOSC 23) were used as negative control. The approximate expected molecular weight of the HA-dCas9-VP64 fusion protein is indicated by a black arrowhead. **C.** The CH12 infection efficiency is variable among the different retroviral vectors and the percentage of infected cells can be highly increased with puromycin selection in the case of pMX-PIE vector. In the top line, flow cytometry plots show the infection efficiency of the different cloned vectors, as indicated by the percentage of GFP or mCherry positive cells. On the bottom line, the plots show the selection efficiency after 5 days of culture with puromycin. Since pQCXIX backbones do not carry a puromycin resistance gene, cells infected with these vectors could not be subjected to selection.

Once the infection efficiency of the different vectors had been tested, I focused on the pMX-PIE as the backbone to express dCas9-VP64 based on its higher performance compared with the rest. Subsequently, in order to test if CSR to IgG1 could be restored in Med1-deficient B cells by the induction of transcription, I used a $Med1^{-/-}$ mouse model.

I isolated splenic B cells from $Med1^{+/+}$ and $Med1^{-/-}$ mice and co-infected them with the viral particles carrying dCas9-VP64 and the gRNAs, present in the supernatant of previously transfected BOSC 23 cells. B cells were cultured under LPS alone, driving CSR to IgG3; or LPS + IL4, which induces CSR to IgG1. At the end of the infection, I analyzed by flow cytometry the percentage of infected cells, as well as the CSR efficiency **(Figure 18)**.

The staining of surface IgG1 and IgG3 allowed for quantification of switched cells. As expected (Thomas-Claudepierre et al., 2016), Med1-deficient cells switched with a lower efficiency than wild-type cells, both when cultured with LPS alone or LPS + IL4 **(Figure 18)**. Not surprisingly, IgG1-positive cells were very scarce in the LPS-stimulated population **(Figure 18)**. Gating on the double-infected cells (GFP+ mCherry+ population) under LPS stimulation, the IgG1 positive cells did not reach levels higher than the ungated cells **(Figure 18)**, meaning that the dCas9-VP64 system could not induce CSR to IgG1 under LPS stimulation, neither in $Med1^{+/+}$ nor in $Med1^{-/-}$ cells. However, in the LPS + IL4 stimulation, a higher percentage of switched cells was observed in the double-infected cells (GFP+ mCherry + population) than in the ungated population.

To test if the system had been correctly targeted to Iγ1 and if it had induced transcription, I sorted GFP+ mCherry+ double-positive cells and performed RT-qPCR of the Iγ1-Cγ1 region **(Figure 19)**. For $Med1^{-/-}$ cells cultured with LPS, I found a 5-fold increase in the transcript levels of double-infected when compared with non-infected cells. However, this increase was probably not enough to induce CSR. Indeed, the cells cultured with LPS+IL4 displayed levels of GLT-γ1 transcription of almost 300-fold over the non-infected cells under LPS stimulation **(Figure 19)**, probably indicating that the necessary transcription to induce CSR to IgG1 under the optimal stimulation conditions is much higher than the levels that are artificially obtained with the dCas9-VP64 induction system. Hence, although the dCas9-VP64 system had been properly targeted and did induce GLT-γ1 transcription, it did so at levels not high enough to produce CSR to IgG1 in LPS-stimulated B cells.

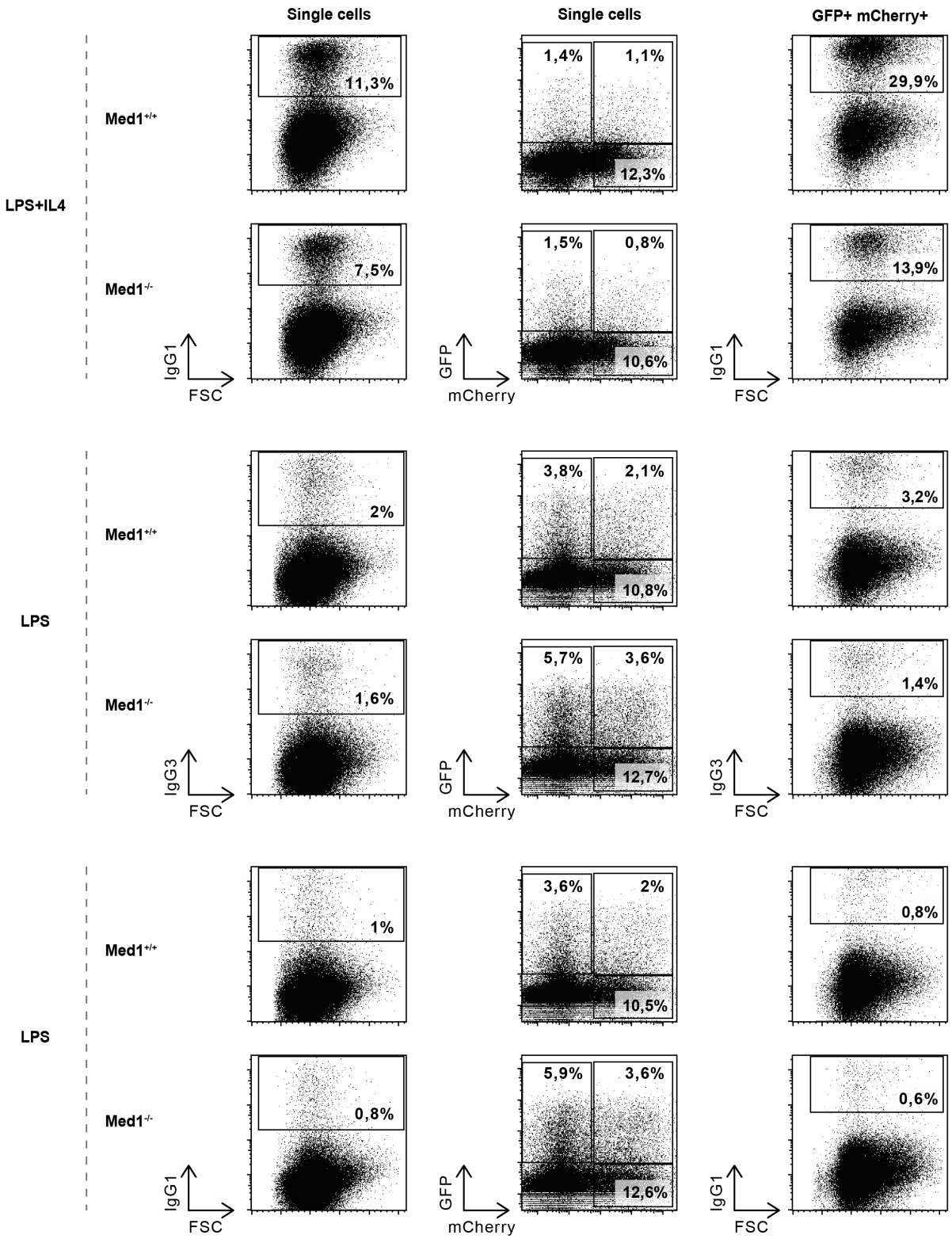


Figure 18: There is no CSR to IgG1 in LPS-stimulated B cells expressing dCas9-VP64 and the gRNAs. Splenic B cells from Med1$^{+/+}$ and Med1$^{-/-}$ mice were isolated and cultured under LPS + IL4 (CSR to IgG1) or LPS (CSR to IgG3) stimulation. After 72h in culture, cells were analyzed by flow cytometry for the surface expression of IgG1 or IgG3 to test efficiency of switching. The presence of GFP and mCherry allowed for the quantification of CSR efficiency in the double-infected cells. Results are representative of 3 independent experiments.

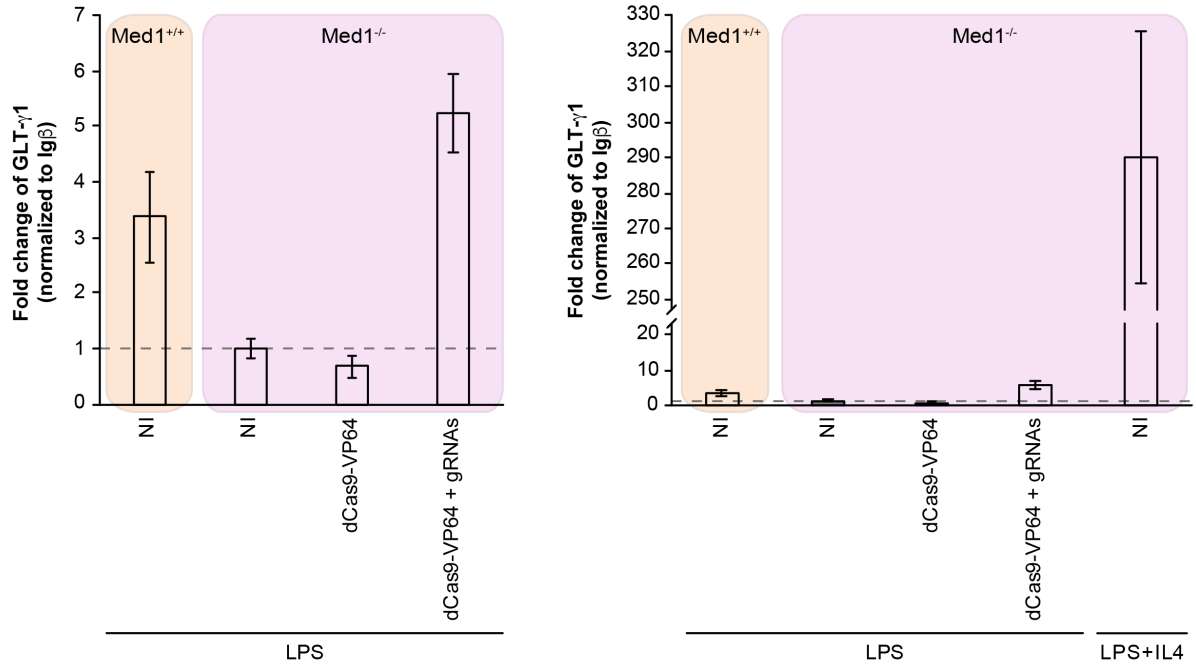


Figure 19: The dCas9-VP64 system induces GLT-γ1 transcription in Med1$^{-/-}$ B cells stimulated with LPS at levels above non-infected cells, but below LPS + IL4 stimulated cells. GLT-γ1 transcript levels of Med1$^{-/-}$ and Med1$^{+/+}$ B cells infected either with only one vector expressing dCas9-VP64 as a negative control, or with both the dCas9-VP64 vector and the vector expressing the gRNAs. B cells were cultured for 72h with LPS or LPS + IL4. Values are presented as the fold change of non-infected (NI) cells stimulated with LPS (set as 1, marked by the dashed line), and normalized to Igβ.

3. The dCas9-VP64 system induces CSR to IgG1 in CH12 cells

The induction system did not force GLT-γ1 transcription in splenic B cells at a sufficient efficiency to induce CSR to IgG1. Because the system had proven efficient in forcing GLT-γ1 transcription in CH12 cells, I tried to induce CSR to IgG1 in this cell line. CH12 cells are committed to switch to IgA (Nakamura et al., 1996), and thus is a system where, in normal conditions, there is no CSR to IgG1. Hence, I hypothesized that if the transcription that is forced to Iγ1 with the induction system was enough to provoke the formation of the loop between the donor Sμ and acceptor Sγ regions, CH12 cells could artificially switch to IgG1.

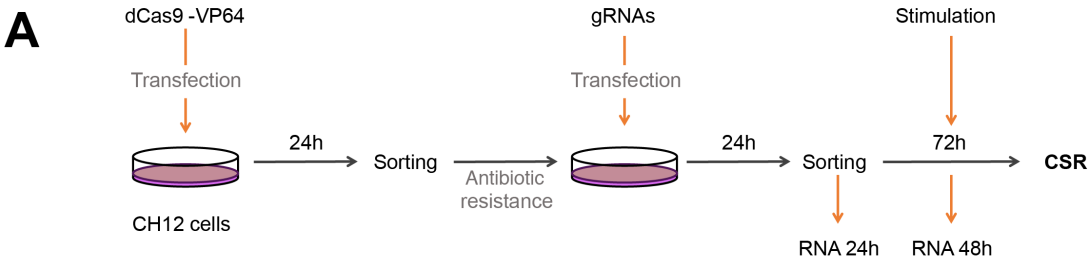
I transfected CH12 cells with the pMX-PIE vector expressing dCas9-VP64 and sorted the GFP-positive cells 24 hours after transfection **(figure 20A)**. Then, I cultured the sorted cells under antibiotic resistance to generate a CH12-dCas9-VP64 stable cell line and I subsequently transfected it with the RV vector carrying the gRNAs. After the antibiotic selection and the transfection with the gRNAs vector, the percentage of cells expressing both the dCas9-VP64 and the gRNAs was of 26,8% **(figure 20B)**, which allowed for an efficient sorting of the double-positive population. I cultured sorted cells with IL4, anti-CD40 antibody, and TGF-β, constituting the classical stimulation conditions to induce CSR to IgA in CH12 cells. RNA samples were collected directly from the sorted cells (24 hours after the transfection of the gRNAs) and one day after. The efficiency of CSR was measured by the surface Ig expression after three days of stimulation.

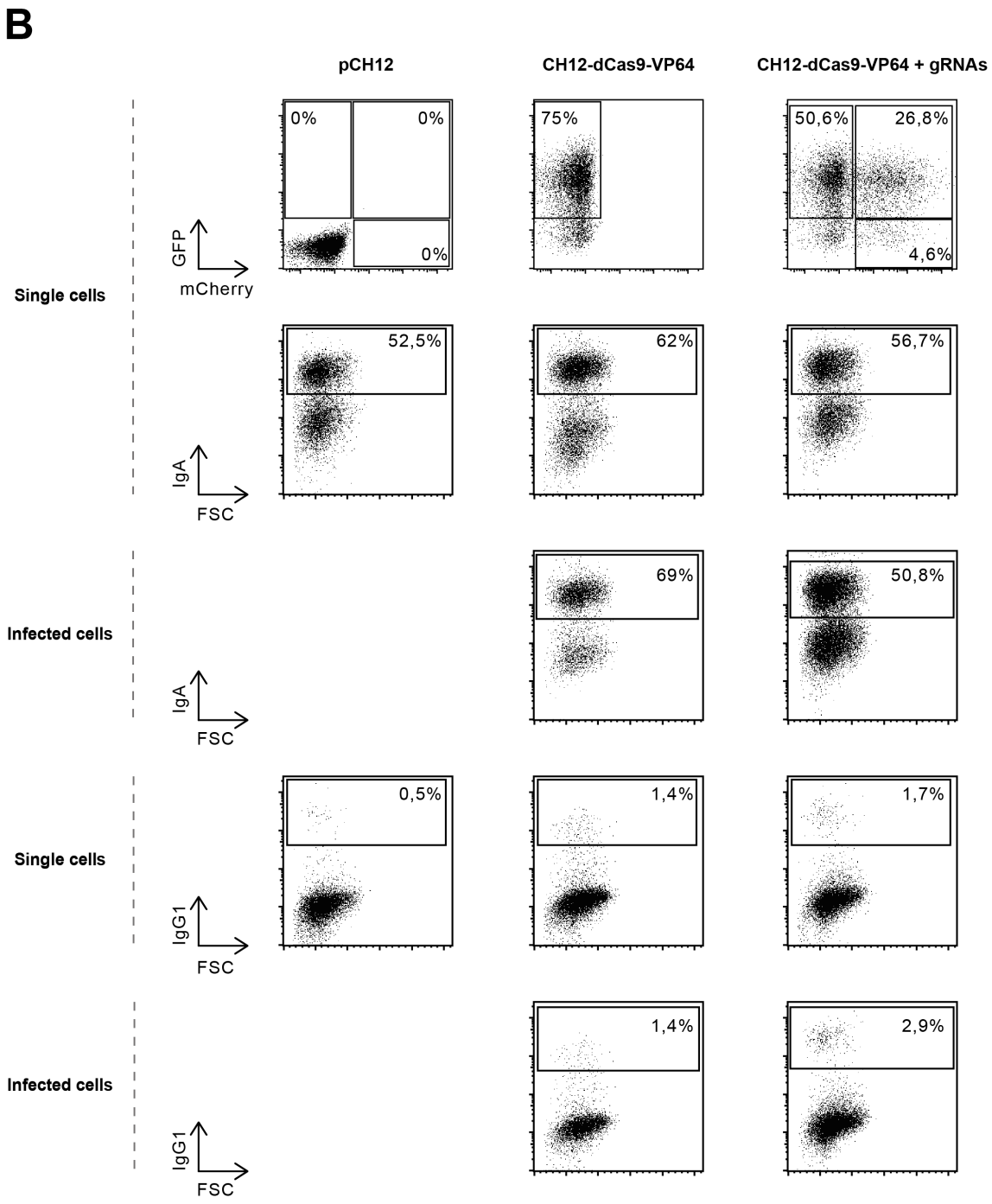
Although constituting only the 1,7%, there was a distinguishable IgG1+ population in the stimulated CH12-dCas9-VP64 cells transfected with the gRNAs vector. Interestingly, the percentage of IgG1+ cells increased to almost 3% in the GFP+ mCherry+ gated cells, while this was not the case for the GFP+ population in CH12-dCas9-VP64 cells that lacked the gRNAs. Moreover, this effect was not an artifact caused by the vectors, since CSR to IgA was not increased in the GFP+ mCherry+ population compared with ungated single cells.

Importantly, RT-qPCR of the Iγ1-Cγ1 region from GFP+ mCherry+ sorted cells showed transcription levels that equaled those of B cells cultured with LPS+IL4 in the cells expressing dCas9-VP64 and the gRNAs under stimulation conditions, whereas no transcription was observed when only the dCas9-VP64 was present **(figure 21)**. This transcriptional activation was enhanced in the stimulated cells compared to the non-stimulated condition, suggesting that other factors intrinsic to B cell activation affect the transcription of GLT-γ1 in CH12 cells.

Hence, under stimulation conditions, the dCas9-VP64 system was able to induce transcription to Iγ1 in CH12 cells, and this correlated with a modest induction of CSR to IgG1.

Figure 20: The dCas9-VP64 system promotes a slight induction of CSR to IgG1 in CH12 cells. A. Workflow for the induction of CSR in the CH12 cell line. CH12 cells were first transfected with a retroviral vector expressing dCas9-VP64 and sorted for GFP+ cells 24 hours later. After 5 days of puromycin selection, a homogenous population expressing dCas9-VP64 (CH12-dCas9-VP64) was achieved. These cells were subsequently transiently transfected with the retroviral vector expressing the four gRNAs and, 24 hours later, GFP+ mCherry+ cells were sorted and cultured under stimulation conditions (with IL4, TGF-β, and anti-CD40 antibody) for 72 hours before measuring the CSR efficiency to IgA and IgG1. RNA samples were collected at 24 and 48 hours after the transient transfection of the vector expressing the gRNAs. **B.** Flow cytometry plots from pCH12 cells and CH12-dCas9-VP64 cells infected or not with the vector expressing the four gRNAs (CH12-dCas9-VP64 + gRNAs) after 72 hours of culture with IL4, TGF-β, and anti-CD40 antibody. Whether the showed population was gated on single cells or infected cells (GFP+ in CH12-dCas9-VP64 cells or GFP+ mCherry+ in CH12-dCas9-VP64 + gRNAs cells) is indicated.


A
dCas9 -VP64
Transfection
CH12 cells
24h
Sorting
Antibiotic resistance
gRNAs
Transfection
24h
Sorting
RNA 24h
Stimulation
72h
RNA 48h
CSR


B
pCH12
CH12-dCas9-VP64
CH12-dCas9-VP64 + gRNAs
Single cells
GFP
mCherry
0%
0%
0%
75%
50,6%
26,8%
4,6%
IgA
FSC
52,5%
62%
56,7%
Infected cells
IgA
FSC
69%
50,8%
Single cells
IgG1
FSC
0,5%
1,4%
1,7%
Infected cells
IgG1
FSC
1,4%
2,9%

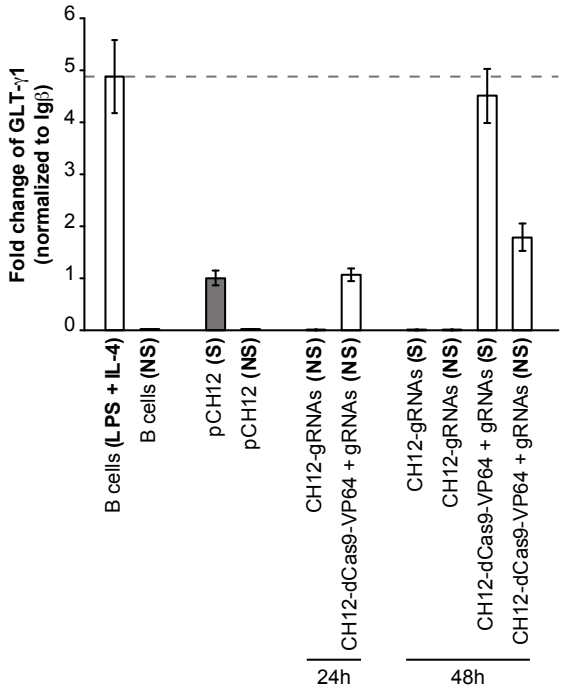


Figure 21: Transcription of GLT-γ1 is induced in CH12 cells expressing dCas9-VP64 and the four gRNAs. GLT-γ1 transcription levels from primary B cells (cultured with LPS + IL4 or not), and stimulated (S; cultured with IL4 + anti-CD40 antibody + TGF-β) or non-stimulated (NS) CH12 cells, expressing only the gRNAs as negative control or both the dCas9-VP64 and the gRNAs. Samples were collected at 24 or 48 hours after transfection of CH12 cells with the vector encoding the gRNAs. Results are presented as the fold change of pCH12 (S) cells, set as 1, and normalized to Igβ. For comparison reasons, the dashed line marks the level of GLT-γ1 transcription in B cells cultured with LPS + IL4, as the optimal stimulation conditions to induce switching (and hence transcription of the corresponding GLT) to IgG1.

SUPPLEMENTARY
MATERIALS AND METHODS

This section is intended to complement the materials and methods included as supplementary information of the article presented in the Part 1 of Results (Amoretti-Villa et al., 2019). Therefore, I include here the materials and methods corresponding to Parts 2 and 3.

Luciferase assay

The different γ1E fragments were cloned in the BamHI and SalI sites of pGL3-P vector (Promega) by digestion/ligation reactions. The primers corresponding to each cloned region of γ1E were the following:

F-γ1E-1	5'-GGATCCCGTGTACACGAGTGAAGG-3'
R-γ1E-1	5'-GTCGACCCTGACAGCTTTTCTCC-3'
F-γ1E-2	5'-GGATCCGGTTACCAATGCCATTGGG-3'
R-γ1E-2	5'-GTCGACGGTTCTCTGTAACTGGCC-3'
F-γ1E-3	5'-GGATCCCGTGTACACGAGTGAAGG-3'
R-γ1E-3	5'-GTCGACCCTCTGTAGGACGGTTGG-3'
F-γ1E-4	5'-GGATCCGGCTTTGGTGCTGGG-3'
R-γ1E-4	5'-GTCGACCCATGTTGGGAGGCC-3'

3T3 cells were co-transfected using Lipofectamine 2000 (Invitrogen), and CH12 cells electroporated with the Neon Transfection System (Thermo Fisher Scientific), with pRL-CMV and either pGL3-P, pGL3-C, or pGL3-γ1E vectors (Promega) at a ratio of 1:100. Electroporated CH12 cells were directly cultured under stimulation conditions (IL4 + TGF-β + anti-CD40 antibody). 3T3 and CH12 cells were lysed one day after transfection and luminescence was measured on fresh lysates on an LB 960 luminometer (Berthold) following instructions for the Dual-Luciferase Reporter Assay System kit (Promega). CH12 cells were also lysed 2 days after electroporation and luminescence measured on fresh lysates. Firefly luciferase luminescence was normalized to *Renilla* luciferase luminescence to obtain the value of luciferase activity.

Retroviral infection of CH12 cells

Retroviral vectors were constructed by digestion/ligation reactions from modules of the Universal Expression System (Reina lab). BOSC 23 cells were transiently transfected (FuGENE; Promega) with the retroviral vectors to produce infectious viral particles. Two days later, CH12 were spin-infected with viral supernatants supplemented with polybrene (10 µg/ml; Sigma-Aldrich). One day later, infection efficiency was measured by flow cytometry by the expression of fluorescent markers.

B cells were isolated from Med1$^{-/-}$ or Med1$^{+/+}$ mice as described (Amoretti-Villa et al., 2019) and spin-infected with the supernatant of previously transfected BOSC 23 cells supplemented with polybrene (10 µg/ml; Sigma-Aldrich) on two consecutive days. They were cultured with 50µg/ml LPS (Sigma-Aldrich) with or without 5ng/ml IL4 (PeproTech) and fresh medium was added daily. One day later, infection and CSR efficiencies were measured by flow cytometry by the expression of fluorescent markers or surface immunoglobulin, respectively.

DISCUSSION

Until now, the transcriptional regulation of the *Igh* locus during CSR was known to relay on the 3'RR and the Eμ enhancers. Both enhancers interact in the three-dimensional chromatin context and the participating S regions are recruited to this interaction upon B cell stimulation, facilitating the synapse (Kenter et al., 2012). Moreover, for the targeting of the CSR machinery to specific S regions, transcription from the germline promoters is necessary (Haddad et al., 2011; Yewdell and Chaudhuri, 2017).

In this section, I will first discuss the implication of the newly described γ1E region on CSR based on the results corresponding to Parts 1 and 2, and I will try to connect the observed effect of this region with the current knowledge of the *Igh* locus transcriptional regulation. Secondly, I will discuss the results corresponding to Part 3 on the relationship between chromatin looping and gene activation. Although being preliminary results, I will try to include them in the current opinion based on other models than the *Igh* locus.

1. γ1E as an enhancer

γ1E appears on the map

The γ1E region was first described in a study that provided through 4C-seq a high-resolution map of the three-dimensional interactions taking place in the *Igh* locus of Rag2$^{-/-}$ and Rag2$^{-/-}$ Pax5$^{-/-}$ pro-B cells (Medvedovic et al., 2013). Here, they found two DHSs in the region encompassed between the Cγ1 and Cγ2b genes that interacted with IGCR1, Eμ, and the 3'RR elements hs3b and hs38. Both sites were bound by the TF Pax5 and this interaction was necessary for the appearance of the DHSs and the presence of the epigenetic marks of active chromatin H3K4me2 and H3K9ac (Medvedovic et al., 2013). Besides Pax5, the most Cγ1-proximal of the two DHSs sites also bound IRF4, IRF8, YY1, and E2A. In their work, the authors suggested that these two DHS sites could act as enhancers regulating V(D)J recombination or CSR.

Shortly after this first report on this region, a new study identified a set of candidate regulatory regions in pro-B cells through their classification based on chromatin stage profiling (Predeus et al., 2014). One of the chromatin states that they described was characterized by enrichment of the TFs Pax5, E2A, p300, PU.1, and Med1, as well as DNase hypersensitivity and both methylation and acetylation of the lysine 4 on histone 3 (H3K4). Incidentally, most of the already known Ig loci enhancers fell into this chromatin category, irrespectively of their active, poised, or inactive state in pro-B cells (Predeus et al., 2014).

Interestingly, the authors identified the same two sites in the region between Cγ1 and Cγ2b as in the study by Medvedovic and colleagues. However, they attributed the most Cγ2b-proximal to a different chromatin state characteristic of promoters, and suggest that it acts as the promoter of the Cγ2b gene. On the other hand, the most Cγ1-proximal site was subjected to a reporter assay and showed enhancer activity in pro-B cells, but not in pro-T or plasma cell lines.

Altogether, these first reports suggest the role of this region as an enhancer that could control some aspects of antigen diversification. In this work, I aimed at characterizing this newly described enhancer and finding its involvement in CSR.

Activity of γ1E in B cell development

A first analysis of B cells isolated from the bone marrow and the spleen of γ1E-deficient mice revealed the presence of mature B cells, as well as B cell populations corresponding to different developmental stages, at comparable proportions as in the γ1E-wildtype animals. As the V(D)J recombination takes place during the pro-B cell stage, if this process was affected by the absence of the γ1E a blockage in B cell development would have been observed. There are two possibilities to explain the lack of phenotype that we observed based on B cell developmental markers:

I) The γ1E does not control V(D)J recombination. Despite the fact that the γ1E presented marks of active enhancer in pro-B cells (Medvedovic et al., 2013), suggesting its potential involvement in V(D)J recombination, it was characterized alongside other known B-cell enhancers that were either active, paused, or inactive in pro-B cells (Predeus et al., 2014). Hence, the γ1E could be paused or inactive in the pro-B cells where it was initially characterized, and thus not exerting any regulatory function in this developmental stage. Supporting this possibility, RNA-seq of the *Igh* locus did not show a peak corresponding to the γ1E region in pro-B cells (Predeus et al., 2014). A hallmark of active enhancers being their active transcription and consequent production of non-coding enhancer RNAs (eRNAs) (Li et al., 2016), this could mean that the γ1E is not active as an enhancer in pro-B cells, although low levels of transcription could also account for this observation. However, it showed enhancer activity in reporter assays in pro-B cells (Predeus et al., 2014), suggesting that it might control other processes than V(D)J recombination at this developmental stage.

II) The γ1E controls V(D)J recombination in a qualitative instead of a quantitative fashion. The region could be involved in the control of the balance between the usage of proximal and distant V_H genes. With the cytometry analysis of developmental markers that I present in this work this potential effect could not be addressed, and further experiments will be required to address this possibility. The balance in the usage of distal and proximal gene segments during V(D)J recombination is dependent on locus contraction (Fuxa et al., 2004; Jhunjhunwala et al., 2008; Kosak et al., 2002; Roldan et al., 2005). Since individual deletion of IGCR1, Eμ, or 3'RR did not affect the contraction of the locus in pro-B cells (Medvedovic et al., 2013), it would be interesting to study if γ1E is in control of this process. Conversely, there could be a compensatory mechanism among the different regulatory regions.

Activity of γ1E in mature B cells

Effect of γ1E deletion on GLT production and CSR

Recently, our group showed by 4C-seq experiments that γ1E was brought to the dynamic long-range interactions formed upon B cell stimulation, and ChIP-seq of the region revealed that it bound Med1 and Med12 subunits of Mediator in a stimulation-dependent manner recapitulating the long-range contacts formed upon B cell activation (Thomas-Claudepierre et al., 2016) and suggesting that the γ1E could be an active enhancer in mature, stimulated B cells.

To investigate this possibility, I generated γ1E knock-out models both in the CH12 B cell line and in mice, and stimulated the cells to undergo CSR. I found that γ1E deficiency led to a defect on CSR to IgA in CH12 cells and to IgG3, IgG2b and IgG2a in primary B cells. Consistently with this, I found decreased efficiency of transcription to the corresponding γ3, γ2b, and γ2a GLTs in activated B cells. Hence, the γ1E region plays a role in the transcriptional regulation of CSR in an isotype-specific manner.

Isotype-specific transcriptional regulation of the Igh locus

Mature B cells express IgM as the default isotype and they switch to IgG, IgE, or IgA upon activation in a stimulation-dependent manner. The choice of isotype determines the effectiveness of the immune response that follows, and its regulation is thus crucial. As already mentioned above, CSR is dependent on the transcription from the cytokine-inducible promoters upstream of each C_X gene, and this germline transcription is controlled by the 3'RR elements (Vincent-Fabert et al., 2010). However, there is an isotype-specific regulation, since the 3'RR was shown to have little effect on regulating CSR to IgG1 isotype (Pinaud et al., 2001; Vincent-Fabert et al., 2010). The γ1E being immediately downstream of Cγ1, it was suggested as a candidate regulatory region controlling CSR to IgG1 (Predeus et al., 2014). However, the results presented in this study show that CSR to IgG1 remains unaffected in γ1E-deficient B cells, indicating that this region is not involved in μ to γ1 switching. An explanation could be that the Iγ1 region itself acts as a regulator of CSR to IgG1 through the recruitment of necessary factors. Indeed, a capacity of the I_X regions to regulate CSR has been proposed before (Manis et al., 2002). On the other hand, other regions could be responsible for the regulation of transcription and switching to this isotype. For example, Eμ has been proposed to regulate GLT-g1 transcription upon B cell activation (Cogne et al., 1994; Wuerffel et al., 2007).

Another example of isotype specificity is the active transcription of GLT-γ3 in resting B cells, which is enhanced by LPS stimulation but diminished with the addition of IL4 (Wuerffel et al., 2007). The GLT-γ3 transcription pattern correlates with a constitutive interaction between the Eμ enhancer and the Iγ3 promoter and Sγ3 in resting B cells, which is lost with LPS+IL4 stimulation but maintained with LPS alone (Thomas-Claudepierre et al., 2016).

IgG2b is also transcribed before B cell stimulation. Indeed, the Iγ2b is transcribed as early as the pro-B cell stage (Predeus et al., 2014). CSR to IgG2b and IgE has been documented in pro-

B cells, correlating with long-range contacts between both regions and both Eμ and 3'RR elements, and preceding V(D)J recombination (Kumar et al., 2013). Interestingly, the pattern of CSR in mature B cells is biased to IgG3 in LPS stimulated cells and IgG1 in the case of LPS+IL4 stimulation; whereas in pro-B cells the pattern would be complementary and be biased towards IgG2b and IgE instead (Kumar et al., 2013).

Altogether, there is a developmental stage-dependent regulation of CSR whereby immature B cells perform CSR to IgG2b and IgE, while mature, stimulated B cells preferentially switch to IgG3 and IgG1 instead. How this developmental switch in terms of CSR preference is regulated remains to be elucidated and further study of the involvement of the IgH regulatory regions, including the γ1E, will be crucial. Indeed, the γ1E region does not only, as argued above, show marks of active enhancer in stimulated B cells, but it also appears as an active enhancer in pro-B cells (Medvedovic et al., 2013; Predeus et al., 2014), coinciding with the stage when CSR to IgG2b and IgE occurs.

Interestingly, the transcription of IgG3, IgG2b, and IgG2a isotypes was found to be specifically silenced by the CTCF-binding site 5'hs1RI, located within Cα, since the GLT levels of these isotypes, but not IgG1, IgE, nor IgA, was increased in resting B cells after 5'hs1RI deletion (Braikia et al., 2017). The 3'RR is in charge of the transcriptional regulation of the germline promoters. However, no effect on the 3'RR eRNA was found in the 5'hs1RI mutants. These results are consistent with a study showing that CTCF prevents premature activation of CSR by the silencing of the germline promoters before B cell activation (Marina-Zarate et al., 2017). In this work, the authors report CTCF binding to 5'hs1RI site in resting B cells, while the signal became weaker upon B cell stimulation (Marina-Zarate et al., 2017).

An interesting model would be that the γ1E counteracts the silencing effect of CTCF binding to the 5'hs1RI region on the 3'RR, resulting in the specific induction of transcription of Iγ3, Iγ2b, and Iγ2a promoters.

B1 vs B2 subset regulation

The γ1E-deficient CH12 cells showed a defect on CSR to IgA isotype of around 50%. However, this phenotype was not conserved in splenic B cells induced to switch to IgA, which showed deficient CSR to IgG3, IgG2b, and IgG2a isotypes instead. Moreover, the transcription of GLT-α was not reduced in the $\gamma1E^{-/-}$ CH12 cells, whereas GLT-γ3, GLT-γ2b, and GLT-γ2a transcription was reduced in primary B cells deficient for γ1E. These results were unexpected and showed a differential regulation of CSR between the CH12 cell line and the splenic B cells.

It is noteworthy that the CH12 cells present a B1-like phenotype, while splenic B cells are mainly from the B2 lineage (Kaminski and Stavnezer, 2006). Moreover, CSR to IgA is controlled by the 3'RR in B2 B cells (Saintamand et al., 2015c; Vincent-Fabert et al., 2010), whereas this is not the case in the B1 lineage (Issaoui et al., 2018).

Interestingly, the B1, as well as the marginal zone B cells, are characterized by the generation of early, rapid immune responses that precede in time the T cell-dependent responses mediated

by follicular B cells, which are from the B2 B cell lineage (Cerutti et al., 2013; Grasseau et al., 2019; Prieto and Felippe, 2017). B1 and marginal zone B cells are biased towards IgA switching above the rest of isotypes (Kaminski and Stavnezer, 2006). Altogether, this shows that the regulation of CSR, from the BCR stimulation to the downstream signaling events, differs between B1 and B2 B cells (Prieto and Felippe, 2017). Hence, it would be reasonable to infer that this distinct regulation of CSR could be differentially orchestrated by the *Igh* locus enhancers between both lineages. In this line, the γ1E, and not the 3'RR (Issaoui et al., 2018), would control CSR to IgA in the B1 lineage, whereas it would regulate CSR to IgG3, IgG2b, and IgG2a isotypes in B2 B cells.

In the future, it will be interesting to test the *in vivo* CSR efficiency to the different isotypes in the B1 lineage to study whether the phenotype is similar to that of the CH12 cells, as opposed to that of B2 cells and the γ1E-deficient mouse model that we generated will be useful for these studies.

So, is γ1E actually an enhancer in mature, activated B cells?

Study of γ1E activity through the expression of a reporter gene

To examine the enhancer activity of the γ1E region, I undertook a dual-luciferase reporter assay. Vectors with three different fragments of γ1E or the complete region were cloned inside the pGL3-P vector, which expresses the firefly luciferase under the control of SV40 promoter. Cells were transfected with one of these vectors together with a normalization vector expressing *Renilla* luciferase. However, no effect on the luciferase luminescence levels was observed when 3T3 cells were transfected. Arguing that the effect of γ1E might be lineage-specific, I undertook the experiment using CH12 cells both under stimulation conditions or not. However, none of the vectors caused an increase in luciferase expression in this cell line.

With the negative results obtained in the reporter assay, we cannot confirm that the γ1E holds an enhancer activity in resting nor stimulated CH12 cells. However, the technical constraints could also be an explanation for these results. Indeed, the CH12 cell line presents very irregular and low transfection efficiency, whilst a high percentage of transfected cells is necessary for a reliable luciferase assay (Schagat, 2007). The lack of a fluorescent marker in the reporter vectors made it impossible to measure an accurate transfection efficiency, although based on the low levels of *Renilla* luciferase luminescence we can infer that the co-transfection might have yielded a low efficiency. This inefficient transfection could be tarnishing a potential enhancer effect of the γ1E region.

On the other hand, we cannot obviate the fact that, with the reporter assay, the potential enhancer region is removed from its endogenous position, subtracting it from the chromatin environment where it may exert its function. Thus, another explanation could be that the endogenous context of the γ1E region could be crucial to perform its function and we necessarily eliminate this variable with the reporter assay.

Another possibility would be that the SV40 promoter, from which the luciferase gene is expressed in the reporter vectors, is not efficiently activated by γ1E. Cloning one of the promoters that was found affected upon γ1E deletion in replacement of SV40 in the pGL3-P vector could be a way of testing the enhancer effect of the region in a more physiological manner.

γ1E transcripts: eRNAs?

Non-coding transcripts, called enhancer RNAs (eRNAs), emanate from active enhancers (De Santa et al., 2010; Kim et al., 2010). Whether they are mere byproducts or they mediate some function in the regulation of target genes is still an open question, but enhancer transcription has become a hallmark of active enhancers, and it has been used to predict enhancer activity (Li et al., 2016). This is also the case in the *Igh* locus, where the 3'RR is specifically transcribed in activated B cells, coinciding with its active state (Braikia et al., 2015; Peron et al., 2012).

To investigate if the γ1E region is transcribed, I performed quantitative polymerase chain reaction with reverse transcription (RT-qPCR) using two different combinations of primers targeting the 5' and the middle regions of the enhancer. By comparing γ1E transcription between resting B cells and B cells stimulated to switch to the different isotypes, we can study if γ1E transcription is stimulation-dependent. Interestingly, all stimulated B cells showed higher levels of transcription than the non-stimulated cells. Without any known coding region nor promoter annotated within the γ1E, these results suggest an active regulatory capacity of the γ1E region.

Literature: TF binding

TF binding is a common characteristic of active enhancers and a good predictor of enhancer activity (Dogan et al., 2015; Visel et al., 2009). Interestingly, several TFs bind the γ1E region in different developmental stages.

The E-proteins E2A and E2-2 were described to play a role in the induction and maintenance of DHSs over B cell development (Wohner et al., 2016). Indeed, E2A was found to bind all four enhancers of the 3'RR and the DNase hypersensitivity of hs3a, hs1,2, and hs3b was dependent on the presence of E2A (Wohner et al., 2016). E2A and E2-2 control the 3'RR enhancers, hence regulating CSR through germline transcription. Interestingly, a peak of E2A binding was found in LPS+IL4 stimulated B cells downstream of Cγ1, which co-localized with DNase I hypersensitivity and the mark of active chromatin H3K27ac (Wohner et al., 2016). The fact that there are E2A binding sites in the γ1E region could mean that the γ1E-dependent germline transcription could be mediated through the TF E2A. Moreover, E2A binds the 3'RR enhancers, which are as well connected to the γ1E in stimulated B cells (Thomas-Claudepierre et al., 2016). Thus, the E-proteins are good candidates to mediate the γ1E roles during B cell activation.

The B-cell lineage TF E2A, together with Pax5 and IRF4, forms a complex with AID in activated B cells that localizes on S regions and SHM targets (Hauser et al., 2016). The different chromosomal localizations of this complex within the *Igh* locus are reminiscent of the three-dimensional contacts taking place during CSR, and suggest a role of the complex on directing CSR to the appropriate classes. More recently, the complex formed by E2A, Pax5, IRF4, and ETS1 together with AID was found to help recruit AID to Pax5 binding sites in the *Igh* locus, driving selective antibody diversification (Grundstrom et al., 2018). Since the DHSs in the γ1E region are dependent on Pax5 (Medvedovic et al., 2013; Nicolas and Chaudhuri, 2013), this TF could also be considered a candidate mediator of the γ1E function.

Another TF that binds the γ1E is YY1 (Medvedovic et al., 2013), which controls enhancer-promoter interactions (Weintraub et al., 2017) and also binds Eμ enhancer (Liu et al., 2007). Therefore, the involvement of γ1E in long-range interactions mediated by YY1 could be envisioned.

Altogether, these studies suggest that TFs such as E2A, Pax5 and YY1 are good candidates to mediate the γ1E function during antibody diversification processes, and it will be of interest to study the TFs that bind the γ1E region upon B cell stimulation.

Relationships between γ1E and Igh locus enhancers

The Eμ intronic enhancer and the 3'RR constitute the two main regulatory regions of the *Igh* locus and they have complementary windows of activity. While the Eμ enhancer acts mainly during the pro- and pre-B cell stages and controls V(D)J recombination (Afshar et al., 2006; Perlot et al., 2005), the 3'RR becomes active in mature B cells and regulates CSR and SHM (Vincent-Fabert et al., 2010). Both regions interact in the three-dimensional conformation of the locus, and it was revealed that the 3'RR controls the Eμ eRNA transcription and epigenetic marks of active chromatin, while the Eμ itself had little effect on the 3'RR (Saintamand et al., 2017). Moreover, the direct contact between both regions is not disrupted upon Eμ deletion (Wuerffel et al., 2007), indicating that the long-range interaction is dependent on the 3'RR in a unidirectional manner.

Since the γ1E region also participates in the long-range interaction upon B cell stimulation and it contacts both Eμ and 3'RR regions, I studied the potential effect of γ1E on 3'RR eRNA transcription. The RT-qPCRs of hs1,2 and hs4 elements of 3'RR showed no difference in the transcript levels between $\gamma1E^{+/+}$ and $\gamma1E^{-/-}$ B cells cultured under different stimulation conditions. Hence, I concluded that the γ1E does not control 3'RR eRNA transcription. However, it will be interesting to test whether the 3'RR acts as a major regulator of the locus and, as it does with the Eμ enhancer, it controls the γ1E activity in mature B cells. Also, analyzing the relationship among these three regions in early stages of development will shed light on the windows of action of the *IgH* locus enhancers from pro-B to mature B cells.

Here, I have shown that the γ1E is required for efficient transcription of specific GLTS. Deletion of the region led to reduced GLT-γ3 and GLT-γ2a, and abolished GLT-γ2b transcription.

Strikingly, the production of these transcripts was already known to be regulated by the 3'RR, suggesting a redundancy in function between both enhancer elements. Nevertheless, the 3'RR has been shown to control GLT to all isotypes, whereas γ1E effect is isotype-specific. This raises the intriguing possibility that the γ1E fine-tunes the existent control on GLT production and adapts it to a subset of isotypes.

Understanding the mechanisms underlying γ1E activity will be crucial to comprehend how the seemingly overlapping functions of 3'RR and γ1E are orchestrated. For this, it will be interesting to study the long-range contacts involving the γ1E. In the future, understanding the hierarchic relationship among all *Igh* locus enhancers along B cell development and in mature B cells will be essential to broaden our understanding on the generation of antibody diversity.

Future directions for γ1E

The results presented in this work raised many exciting questions to be addressed in the future about how the γ1E exerts its control on *Igh* locus transcription.

Both the mouse and human *Igh* loci function through similar regulatory mechanisms (Hwang et al., 2015). Hence, it would be reasonable to speculate that an enhancer driving isotype-specific transcriptional regulation, like the γ1E in mice, could exist in the human locus. Some studies indicate that the sequence conservation of enhancers among species is rather poor, while their function as enhancer is conserved (Blow et al., 2010; Pennacchio et al., 2013; Schmidt et al., 2010). For example, the accurate targeting of SHM among different species is controlled by shared mechanisms involving the coordination of Ig enhancers (Buerstedde et al., 2014). Interestingly, although not the general sequence of the enhancers, the TF binding motifs present within revealed high inter-species conservation (Blomberg et al., 1991; Buerstedde et al., 2014; Combriato and Klobeck, 2002).

It is to note that this work focused specifically on the transcriptional regulation promoted by the γ1E. Future studies on the three-dimensional contacts in the absence of γ1E will indicate if this region is important for the establishment of the long-range interactions of the *Igh* locus. Moreover, potential functions of this regulatory region on other processes such as SHM and AID targeting should be considered.

2. Transcription: cause or consequence of loop formation?

What do we know about it?

While both transcription and loop precede gene activation, whether one is the cause or the consequence of the other is still under debate. Several studies point at the loop as the cause of the transcriptional activation through the mediation of TFs. For example, Lef1 was found to interact with other TFs bound to distant DNA regions and facilitate the formation of a loop between regulatory regions and target genes, which subsequently led to transcriptional activation (Yun et al., 2009). This was consistent with previous work in yeast that suggested that the looping is involved in early stages of transcriptional activation (O'Sullivan et al., 2004).

In another study, forcing the formation of a loop between the endogenous LCR and the promoter of the β-globin gene in a background depleted of the TF GATA led to transcription initiation (Deng et al., 2012). Although the loop was completely formed, the transcription did not reach optimal activation levels, suggesting that full transcription is not a requisite for the formation of the loop (Deng et al., 2012).

Recently, a new approach that used prediction models revealed that the process of transcription itself generates DNA supercoiling that leads to a higher frequency of local contacts (Benedetti et al., 2017). Soon after, a more detailed model suggested that cohesin moves along the DNA molecule pushed by the supercoiling generated by transcription, leading to the formation of loops that precede gene activation (Racko et al., 2018). In this model, CTCF would stabilize cohesin, facilitating the formation of loops (Racko et al., 2018). As a candidate cause of DNA supercoiling, the authors point to the eRNAs (Racko et al., 2018). Indeed, enhancer transcription precedes activation of target genes (Arner et al., 2015). However, experimental validation of these models is still needed. Taking together all these studies, it would be interesting to test this model in the *Igh* locus. Indeed, the onset of the enhancer role of the 3'RR correlates with the production of high levels of eRNA (Braikia et al., 2015).

Forcing transcription: does it induce loop formation and lead to CSR?

Efficient CSR requires transcription from the germline promoters and long-range interactions involving the IgH enhancers and the participating S regions. In this work, I showed that GLT-γ1 transcription is efficiently forced by the dCas9-VP64 induction system in non-stimulated CH12 cells. This effect is maintained in Med1$^{-/-}$ B cells, although the level of induction was probably not enough to cause cells to switch to IgG1 isotype under LPS stimulation. Indeed, when cultured with LPS, cells that had successfully incorporated the dCas9-VP64 system showed a 5-fold increase in GLT-γ1 transcript levels compared to non-infected Med1$^{-/-}$ B cells. However, B cells cultured with LPS + IL4 (which induces CSR to IgG1) presented levels of transcription up to almost 3 times those of non-infected cells. Although the system was correctly targeted and induced transcription, the strength of this induction may not have been sufficient

to rescue the loop in Med1-/- cells, which would explain the lack of CSR. Therefore, we cannot conclude whether forcing the transcription in a Med1$^{-/-}$ background can rescue the formation of the loop and, hence, the CSR efficiency. For this, a higher induction efficiency is required.

The dCas9-VP64 system efficiently induced GLT-γ1 transcription in CH12 cells. Since this cell line is committed to switching to IgA (Nakamura et al., 1996), transcription of GLT-γ1 and interaction of Iγ1 with the *Igh* locus enhancers would not be expected in this model. Hence, I decided to test whether an induction in transcription was enough to cause CSR to IgG1 in CH12 cells stimulated with IL4 + TGF-β + anti-CD40 antibody. This cocktail provides the optimal conditions for isotype switching to IgA, whereas no CSR to IgG1 is obtained (Arnold et al., 1988).

Strikingly, CH12 cells modestly switched to IgG1 when stimulated, and induction of transcription from Iγ1 promoter was confirmed by RT-qPCR. The CSR efficiency was measured after 72 hours of stimulation. It is known that multi-step switching events can occur in the same locus (Hwang et al., 2015; Wu and Zarrin, 2014). Therefore, it is possible that cells that had switched to IgG1 underwent a second round of CSR to IgA, which would be leading to an underestimation of the percentage of IgG1 switching. For this, it will be interesting to test the CSR efficiency at different time points before 72 hours.

Since chromatin looping is necessary for CSR (Kenter et al., 2012; Thomas-Claudepierre et al., 2016), long-range contact involving Sγ1 must have been generated in order to promote switching to IgG1. However, the analysis of chromatin conformation under these conditions will be necessary to confirm loop formation. Moreover, a Med1-defficient CH12 line would provide a convenient background devoid of transcription and loop capability (Thomas-Claudepierre et al., 2016) whereby this approach could be used to test the potential rescue of looping through the induction of transcription.

Altogether, these preliminary results could suggest that the induction of transcription is enough to provide at least modest switching. Although analyzing the long-range interactions remains a necessary step to confirm it, it would mean that transcription can precede loop formation. As mentioned, some studies have found that looping can induce transcriptional activation (Deng et al., 2012; Yun et al., 2009). Nevertheless, both possibilities are not mutually exclusive. Even though complete restoration of looping did not yield total transcriptional activation, meaning that full transcription would not be required for loop formation (Deng et al., 2012), a partial effect should be envisioned.

CONCLUSION

The *Igh* locus forms an intricate system of transcriptional activation, long-range interactions, and dynamic conformational changes that are mediated by numerous coordinated factors. The understanding of its temporal and spatial functioning is essential to explain both physiological and pathological aspects of the immune system. Besides, from a broader point of view, the *Igh* locus provides an excellent model to study transcriptional regulation and chromatin conformation in an endogenous environment.

With the present work, I demonstrate that the recently identified regulatory region **γ1E controls germline transcriptional and CSR in an isotype-specific manner**. Moreover, the isotype specificity observed in splenic B cells was distinct than in the CH12 cell line.

An intriguing model would be that the γ1E region regulates CSR to the isotypes involved in early immune responses triggered by T cell-independent stimulations. Several evidences support this hypothesis:

I) The γ1E has marks of active enhancer in pro-B cells (Medvedovic et al., 2013; Predeus et al., 2014; Whyte et al., 2013), moment when premature, LPS-dependent germline transcription and CSR to IgG2b occurs in the bone marrow (Braikia et al., 2017; Kumar et al., 2013; Predeus et al., 2014).

II) B1 B cells switch preferentially to IgA during T cell-independent responses (Kaminski and Stavnezer, 2006), and the γ1E showed to control CSR to IgA in the B1-like CH12 cells.

III) Finally, the germline promoters Iγ3, Iγ2b, and Iγ2a are activated in the rapid, T-cell independent immune responses triggered by LPS stimulation in B2 and marginal zone B cells (Cerutti et al., 2013) and here I showed that they are under the control of γ1E in B2 cells undergoing CSR.

How this isotype-specific regulation of germline transcription and CSR is developmentally controlled remains an open question that will be interesting to address in the future, but TFs such as E2A and Pax5 are potential candidates to mediate this regulation. More research on the interaction of the γ1E with other regulatory regions and its potential involvement in other antibody-diversification processes will be of great significance. Finally, it will be interesting to investigate if a γ1E counterpart, holding similar isotype-specific regulatory patterns, exists in the human locus.

On another note, whether transcription precedes or follows looping to induce CSR is still unknown. To address this question, we targeted a transcriptional induction system based on the CRISPR/Cas9 technology to Iγ1 in a background deficient for transcription and looping to study whether CSR could be restored. Using this approach, **transcription was successfully forced to Iγ1 in CH12 cells and B cells**, although the levels of the latter proved not to be sufficient for a phenotypic effect on CSR efficiency.

Through the transcriptional induction of Iγ1, **CSR to IgG1 was forced in CH12 cells**, a model in which isotype switching is committed to IgA (Nakamura et al., 1996). Although final conclusions cannot be drawn from these preliminary results, we could infer that the three-dimensional contacts that are required for CSR have been formed, and that the Sγ1 region must have participated to some extent in these contacts. Med1-depleted CH12 cells, where germline

transcription and looping are impaired, will be a useful tool to prove if forcing transcription can rescue the formation of the loop and, subsequently, induce CSR. Overall, the dCas9-VP64 induction system has proved to be a useful tool to study the effect of transcription in a single-locus level in CH12 cells and further optimization of the technique will be needed to improve the outcome in primary cells.

Altogether, this work supports the notion that CSR should not be viewed as a process occurring homogeneously along the *Igh* locus. Switching has isotype specificities with physiological implications in the immune response that should be addressed in the future. Studying the effect that the regulatory regions exert on each other, as well as the three-dimensional interactions among them, will be crucial in this task.

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Régulation Transcriptionnelle du Locus Igh Lors de la Commutation Isotypique

Résumé en Français

Au cours du développement de la moelle osseuse, les cellules B assemblent un ensemble divers de récepteurs de cellules B fonctionnels (BCR) par recombinaison V(D)J, qui se diversifie davantage au cours de la réponse immunitaire dans les organes lymphoïdes secondaires par deux mécanismes : l'hypermutation somatique (HS) et la commutation isotypique (CI). L'HS introduit des mutations ponctuelles dans la région variable (V) des gènes des chaînes lourdes et légères, modifiant ainsi l'affinité du BCR pour son antigène apparenté. La CI remplace l'isotype exprimé d'IgM à IgG, IgE ou IgA par un événement de recombinaison au niveau du locus de la chaîne lourde des Ig (IgH), conférant à la BCR de nouvelles fonctions effectrices [1] (*figure 1*).

L'HS et la CI sont tous deux déclenchés par AID, une enzyme qui génère des mutations dans les régions V et des cassures double brin de l'ADN au niveau des régions de commutation (S) au cours de l'HS et la CI, respectivement. En raison de son potentiel mutagène, AID doit être étroitement régulée et ciblée. Cependant, les mécanismes moléculaires précis qui sous-tendent la régulation et le ciblage de l'AID restent à élucider[2].

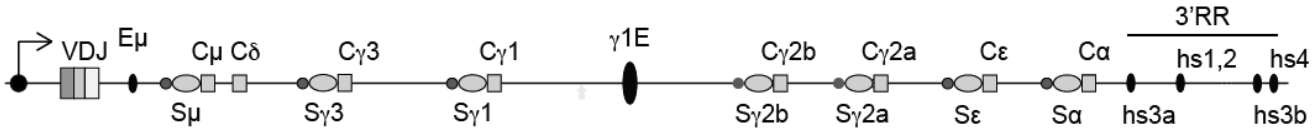

Figure 1 : (A) *Représentation schématique du locus IgH murin.*

La CI est initiée par le recrutement d'AID couplé à la transcription dans le locus IgH. L'activation transcriptionnelle du locus IgH pendant la CI est contrôlée par l'enhancer Eμ et par la région régulatrice 3'RR [3, 4]. Étant donné que les régions de recombinaison donneuse et accepteuse (S) peuvent être distantes de 200 kb, la CI nécessite également des interactions à longue portée. En effet, il a été montré que le locus IgH dans les cellules B au repos forme une boucle impliquant la région Sμ du donneur, l'enhancer Eμ et le récepteur 3'RR, et que la région S de l'accepteur est également recrutée dans la boucle dans une manière cytokine-dépendante dans les cellules B stimulées [5]. Les mécanismes et les facteurs impliqués dans la formation et le maintien de boucles d'ADN, ainsi que leur rôle dans la diversification des anticorps, sont mal compris.

Notre laboratoire a récemment montré que Med1 et Med12, deux sous-unités du complexe Mediator, sont recrutés de manière dynamique dans les enhancers du locus IgH et la région accepteuse au cours de la CI et qu'ils ont un effet sur l'efficacité de la transcription stérile, la formation de boucles et la CI [6]. Nous avons constaté que pendant la CI, les enhancers Eμ et 3'RR interagissent de manière dynamique avec une région située en aval du gène IgG1 (γ1E) [6]. Cette région est non seulement liée par les sous-unités Med1 et Med12 de Mediator, mais porte en outre de marques de la chromatine caractéristiques des enhancers [7, 8]. Comme la transcription seule ne peut expliquer le ciblage et l'action d'AID sur les gènes des Ig, d'autres facteurs, tels que la structure de la chromatine, pourraient être importants [1]. En effet, certaines études soulignent l'importance des séquences d'amplification dans le recrutement d'AID sur les loci des Ig [9, 10].

Sur la base de ces observations, mon hypothèse de travail est que la région γ1E est un nouvel enhancer putatif du locus IgH et qu'elle pourrait jouer un rôle dans la régulation de la recombinaison V(D)J, de la HS et de la CI chez les cellules B. L'objectif principal de ma thèse est de caractériser fonctionnellement le rôle de la région γ1E récemment décrite lors de la diversification des anticorps.

Par ailleurs, comme indiqué précédemment, la boucle et la transcription sont nécessaires pour la CI. Plusieurs études ont indiqué que la transcription était le processus déclencheur qui induit la boucle dans d'autres modèles [11, 12]. Ainsi, un autre objectif de ma thèse est de déterminer si la transcription stérile des régions S est suffisante pour induire la CI.

Caractérisation fonctionnelle de γ1E

La région γ1E régule la CI et l'activation de la transcription du locus IgH

Pour étudier le rôle de la région γ1E dans la CI, je l'ai supprimé à l'aide de la technologie CRISPR/Cas9 dans des cellules CH12, une lignée de cellules B compétente pour la CI. Nous avons constaté une réduction significative de l'efficacité de la CI chez les clones $\gamma1E^{-/-}$ par rapport aux témoins (*figure 2*). Ainsi, le CI est altéré en l'absence de la région γ1E dans les CH12.

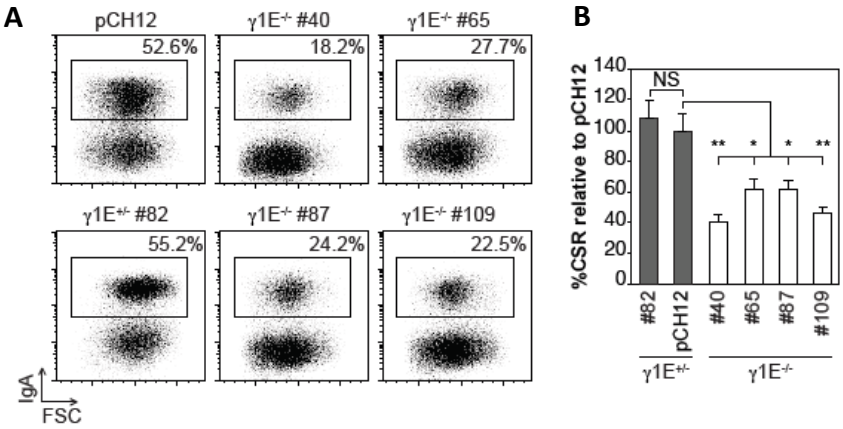

Figure 2 : La CI vers IgA est compromise dans les cellules γ1E$^{-/-}$ CH12. (A) *Expression en surface d'IgA après 72h de stimulation, montré par cytométrie en flux.* ***(B)*** *Efficacité moyenne de la CI sur 5 expériences indépendantes.*

Comme la CI est un processus dépendant de la transcription, j'ai vérifié si l'absence de l'activateur putatif affecte la transcription au niveau des régions S donneur et accepteur par RT-qPCR. Néanmoins, aucune réduction de la transcription n'a été observée au niveau des régions S donneuse et accepteuse dans les cellules CH12 $\gamma1E^{-/-}$.

Mise en place d'un modèle murin knock-out γ1E et sa caractérisation fonctionnelle

Pour étudier l'impact de la suppression de la région γ1E sur la CI vers d'autres isotypes, nous avons généré un modèle de souris knock-out pour γ1E.

J'ai recueilli la moelle osseuse et la rate de souris $\gamma1E^{-/-}$ et $\gamma1E^{+/+}$ et étudié différents marqueurs du développement des lymphocytes B par cytométrie en flux. Aucune différence majeure n'a été observée et toutes les populations de cellules B étaient présentes en nombre et en proportions normaux chez les souris $1E^{-/-}$ par rapport aux témoins, ce qui indique que la région γ1E n'est pas absolument requise pour le développement des cellules B et suggère que la recombinaison V(D)J n'est pas affectée quantitativement par la suppression de cette région.

Pour déterminer si la suppression de la région γ1E entraîne une CI défectueuse, j'ai cultivé des cellules B spléniques marquées au CFSE in vitro, isolées de souris témoins $\gamma1E^{-/-}$ et $\gamma1E^{+/+}$ dans des conditions induisant le CI sous différents isotypes. Tandis que le CI aux IgG1 n'est pas affecté, le CI aux IgG3, IgG2a et IgG2b est réduit dans les cellules $\gamma1E^{-/-}$ B par rapport aux témoins (*figure 3A*). En accord avec cela, les transcrits GLT-γ3, GLT-γ2a et GLT-γ2b sont réduits (*figure 3B*). Ces données suggèrent que la région γ1E est requise pour la transcription et la CI vers un sous-ensemble d'isotypes.

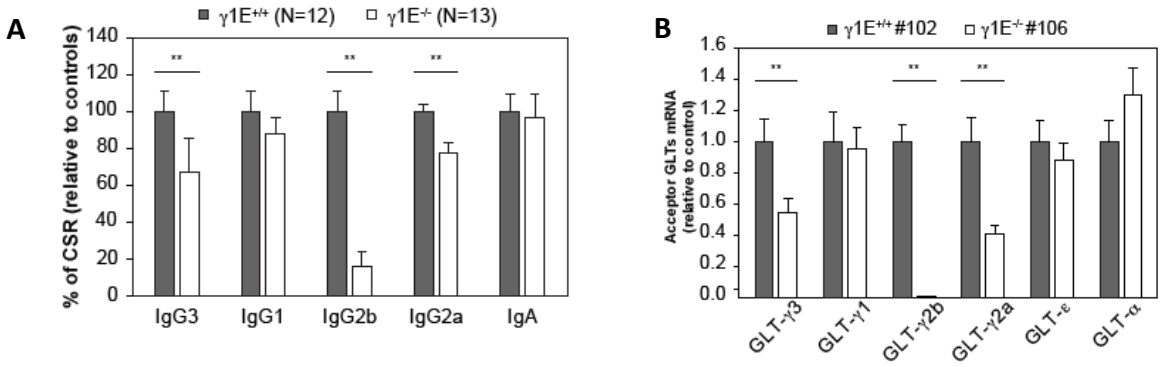

Figure 3 : La transcription et la CI en IgG3, IgG2b et IgG2a sont défectueuses dans les cellules B primaires γ1E$^{-/-}$. (A) *Expression de surface des dilutions IgG3, IgG1, IgG2b et IgG2a et CFSE, telle que déterminée par cytométrie en flux dans les cellules γ1E$^{-/-}$ et E B stimulées pendant 72h. Le pourcentage de cellules commutées est indiqué.* ***(B)*** *Les niveaux de transcription des différents GLT mesurés par RT-qPCR après 72h de stimulation.*

Transcription ou boucle ; lequel vient en premier ?

L'efficacité réduite de la CI dans les cellules B spléniques Med1 KO pourrait être expliquée par la diminution de la transcription de la lignée germinale ou par le défaut d'efficacité de la formation de boucles [6]. Afin de répondre à cette question, nous avons voulu forcer la transcription de GLT-γ1 dans des cellules KO B Med1 avec l'idée que cela permettrait de corriger le défaut de la boucle et d'augmenter l'efficacité de la CI jusqu'à atteindre les niveaux WT.

Forcer la transcription de GLT-γ1 dans les cellules CH12

Nous avons fusionné le mutant catalytique dCas9 au peptide activateur de la transcription VP64 [13]. Quatre différents ARNg ciblant Iγ1 ont été conçus. Comme preuve de principe, nous avons transfecté des cellules CH12 et trié celles qui exprimaient la protéine de fusion dCas9-VP64 et 2 ou 4 ARNg. La RT-qPCR sur des cellules triées 24h après la transfection a montré une induction de la transcription de GLT-γ1 par rapport aux cellules B spléniques qui ont été stimulées pour subir un CI en IgG1 (*figure 4*). En effet, les taux de transcription GLT-γ1 des cellules CH12 non stimulées exprimant 2 gARN étaient similaires aux taux de GLT-γ1 des cellules B spléniques stimulées. Fait important, aucune induction n'a été observée dans les autres GLT (*figure 4*). Ainsi, le système dCas9-VP64 force efficacement et exclusivement la transcription de GLT-γ1.

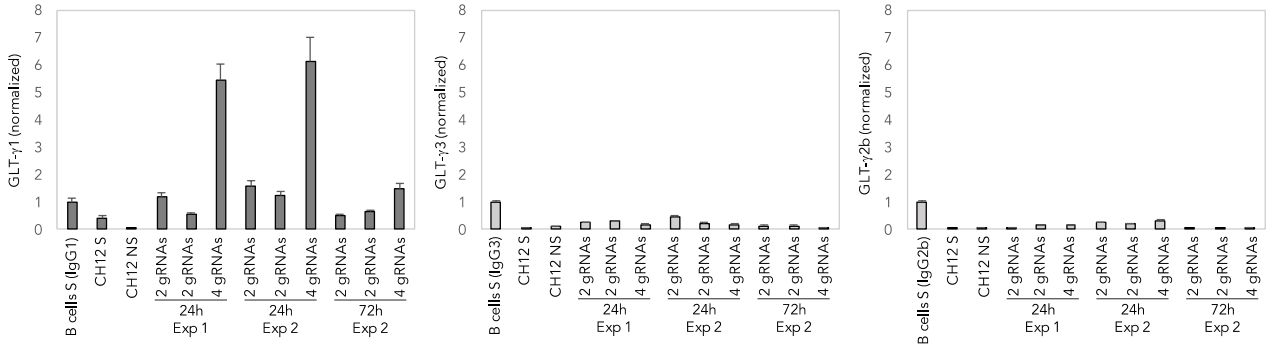

Figure 4 : Niveau de différents GLTs dans des CH12 non stimulées exprimant dCas9-VP64 et les ARNg. *Normalisé par le gène ménage IgB. Dans tous les graphiques, deux expériences différentes ont été réalisées dans lesquelles des échantillons d'ARN ont été prélevés 24 h ou 72 h après la transfection de cellules CH12 avec des vecteurs portant 2 ou 4 gARN.* ***(Gauche)*** *Effet du système dCas9-VP64 ciblé sur Iγ1 dans la transcription de GLT-γ1.* ***(Milieu et droit)*** *Les niveaux de transcription de GLT-3 et GLT-2b sont montrés à titre d'exemple du reste des isotypes (S = stimulé ; NS = non stimulé).*

Forcer la transcription de GLT-γ1 dans les cellules B spléniques Med1 KO et tester le CI

Nous avons infecté des cellules B spléniques Med1 KO avec de vecteurs rétroviraux exprimant dCas9-VP64 et les quatre ARNg ciblant Iγ1. Ensuite, nous les avons stimulées avec LPS (induisant le CI vers IgG3) ou LPS + IL4 (CI vers IgG1). Aucune CI vers IgG1 n'a été observée dans les cellules B Med1 KO infectées (*figure 5A*). Un subséquent analyse RT-qPCR a montré une induction d'environ 5 fois de la transcription de GLT-γ1 par rapport aux cellules KO non infectées cultivées avec du LPS. Cependant, le niveau d'induction de la transcription de GLT-γ1 est faible et pourrait ne pas être suffisant pour promouvoir la CI. En effet, les cellules Med1 KO non infectées cultivées avec LPS + IL4 présentaient une transcription de GLT-γ1 environ 300 fois supérieure par rapport aux cellules cultivées avec du LPS seul (*figure 5B*). Ainsi, le système dCas9-VP64 ne force pas la transcription de GLT-γ1 dans les cellules B à des niveaux suffisants pour induire un effet sur la CI.

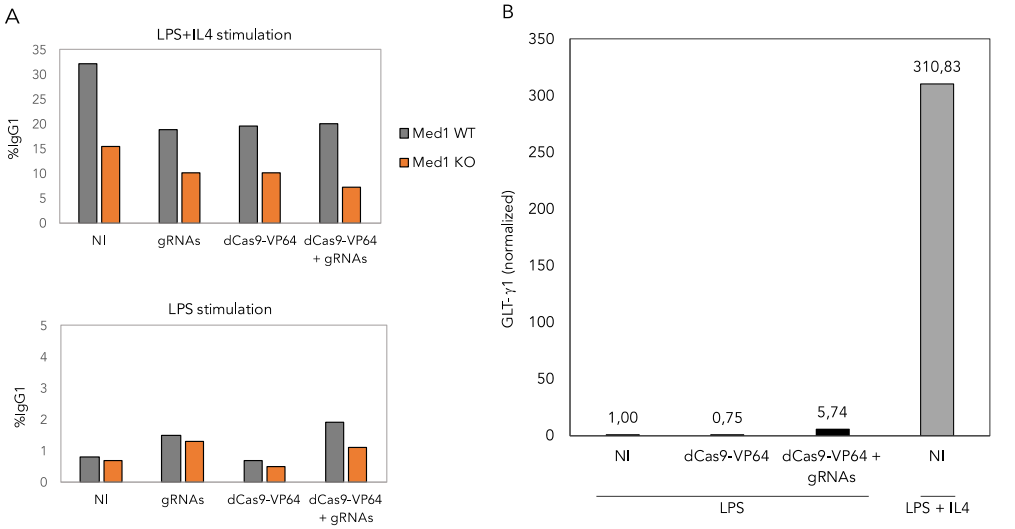

Figure 5 : L'activation de la transcription par dCas9-VP64 n'est pas suffisante pour induire une CI vers IgG1 dans les cellules B. ***(A)*** *Efficacité de CI vers IgG1 chez les cellules B Med1 WT et Med1 KO exprimant ou non dCas9-VP64 et les ARNg, dans différentes conditions de stimulation.* ***(B)*** *Niveaux de transcription de GLT-γ1 dans les cellules B stimulées Med1 KO, exprimant ou non dCas9-VP64 et les ARNg. (NI = cellules non infectées).*

Induction de la CI vers IgG1 dans les cellules CH12

Bien que les cellules CH12 ne peuvent subir la CI que vers IgA [14], et puisque les CH12 ont montré une plus grande induction de transcription que les cellules B, nous avons voulu tester si les cellules CH12 dans lesquelles la transcription est forcée à Iγ1 pourraient montrer un CI vers IgG1. Ainsi, nous avons établi une lignée CH12 exprimant dCas9-VP64. Ensuite, nous l'avons transfectée avec un vecteur portant les quatre ARNg. Les cellules transfectées ont été stimulées et analysées par cytométrie en flux. Nous avons constaté que 2,9% des cellules exprimant dCas9-VP64 et les ARNg avaient subi une CI vers IgG1, contre 0,5% des cellules non infectées et 1,6% des cellules exprimant uniquement dCas9-VP64 comme contrôle (*figure 6*). Par conséquent, nous avons réussi à induire légèrement la CI vers IgG1 dans les cellules CH12 en exprimant le système dCas9-VP64 ciblé sur Iγ1.

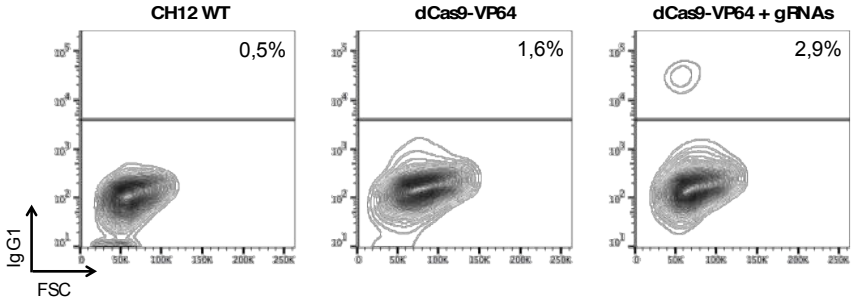

Figure 6 : Induction de CI à IgG1 en ciblant dCas9-VP64 à Iγ1 chez les CH12. *Pourcentage de CI à IgG1 dans les cellules CH12 non infectées ou infectées après 72h de stimulation (@CD40, IL4 et TGF-β), montré par cytométrie en flux.*

Conclusions and discussion

J'ai montré que la région γ1E joue un rôle dans la CI dans les cellules CH12. J'ai confirmé son rôle dans la régulation transcriptionnelle et la CI dans un modèle KO murin. Globalement, nos résultats sont cohérents avec un modèle dans lequel la région γ1E régule la transcription du locus d'IgH et la CI d'une manière spécifique à l'isotype.
Nous avons également prouvé que le système dCas9-VP64 est capable de forcer efficacement et exclusivement la transcription de GLT-γ1 lorsqu'il était ciblé sur Iγ1 dans les cellules CH12, et qu'il était capable de forcer la CI à IgG1 dans ce modèle. Bien que nous n'ayons pas obtenu d'induction de la transcription à des niveaux suffisamment élevés pour provoquer un effet phénotypique sur la CI dans les cellules B, nous avons pu observer une légère induction de la CI à IgG1 chez les CH12 exprimant dCas9-VP64 et 4 gRNA ciblés sur Iγ1. Ainsi, en forçant la transcription, nous pourrions induire la CI, ce qui signifie probablement que la région Sγ1 est impliquée dans les modifications conformationnelles 3D du locus.

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Rocío AMORETTI VILLA

Transcriptional regulation of the IgH locus during class switch recombination

Résumé

La commutation isotypique (CI) des immunoglobulines (Ig) a lieu au locus constant de la chaîne lourde (IgH) de l'immunoglobuline lors de l'activation des cellules B et entraîne un changement de l'isotype exprimé. La CSR est déclenchée par l'enzyme AID et dépend des boucles à longue portée entre enhancers et promoteurs et de la transcription non-codante, qui sont contrôlés par l'enhancer Eμ et le super-enhancer de la région régulatrice 3' (3'RR). Ici, nous caractérisons le rôle sur la transcription non-codante et la CI de γ1E, une région située en aval du gène Cγ1 qui porte de marques d'enhancers actifs et qui interagit dynamiquement avec les deux enhancers du locus lors de l'activation des cellules B. Nous montrons que la suppression de γ1E réduit l'efficacité de la CI vers IgA dans les cellules CH12 et affecte la transcription non-codante et la CI d'une manière spécifique à l'isotype chez la souris. D'autre part, si la transcription précède ou suit la formation de la boucle pour induire la CI est encore inconnue. Pour répondre à cette question, nous avons ciblé un système d'induction transcriptionnelle basé sur la technologie CRISPR/Cas9 au promoteur Cγ1 dans un contexte dépourvu de transcription et boucle pour étudier si la CI pouvait être restaurée.

Mots clés : Commutation isotypique; locus IgH; transcription; enhancer.

Abstract

Immunoglobulin (Ig) class switch recombination (CSR) takes place at the immunoglobulin heavy chain (IgH) constant locus upon B cell activation and results in a change of the isotype expressed. CSR is triggered by activation-induced cytidine deaminase (AID) and is dependent on inducible long-range enhancer/promoter looping and on germline transcription, which are controlled by the Eμ enhancer and the 3' regulatory region (3'RR) super-enhancer. Here, we characterize the role on switch transcription and recombination of γ1E, a region located downstream of the Cγ1 gene that bears marks of active enhancers and that interacts dynamically with both IgH enhancers upon B cell activation. We show that γ1E deletion reduces CSR efficiency to IgA in CH12 cells and affects germline transcription and CSR in an isotype-specific manner in mice. On the other hand, whether transcription precedes or follows looping to induce CSR is still unknown. To address this question, we targeted a transcriptional induction system based on the CRISPR/Cas9 technology to the Cγ1 promoter in a background deficient for transcription and looping to study whether CSR could be restored.

Keywords: Class switch recombination; IgH locus; transcription; enhancer