Book Chapter

The *cis*-enhancers 3'RR and 5'Eμ are Independent Motors of IgH Locus Remodelling

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Published **July 05, 2021**

How to cite this book chapter: Melissa Ferrad, Nour Ghazzaui, Hussein Issaoui, Ophélie Alyssa Martin, Jeanne Cook-Moreau, François Boyer, Sandrine Le Noir, Yves Denizot. The *cis*-enhancers 3'RR and 5'Eμ are Independent Motors of IgH Locus Remodelling. In: Hussein Fayyad Kazan, editor. Immunology and Cancer Biology. Hyderabad, India: Vide Leaf. 2021.

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Author Contributions: MF, HI, NG, FB, OAM, JCM, SLN and YD designed and performed experiments and wrote the manuscript. YD obtained financial grants.

Conflicts of Interest: Authors declare no conflict of interest.

Acknowledgements: This work was supported by grants from Ligue Contre le Cancer (Equipe labellisée LIGUE 2018) and Agence Nationale de la Recherche (ANR: projet EpiSwitch-3'RR 2016). N. Ghazzaui was supported by a grant from Association de Spécialisation et d'Orientation Scientifique (Lebanon), the municipality of Khiam (Lebanon) and the Société Française d'Hématologie. H. Issaoui is supported by a fellowship of the University of Limoges. F.B. is supported by Fondation Partenariale de l'Université de Limoges and ALURAD. We thank the genomics platform of Nice Sophia Antipolis for RNAseq experiments and the animal facility of University of Limoges for mice housing.

Abstract

B-cell development is spatially and temporally regulated with the Ig heavy chain (IgH) locus as a conductor. Starting with the first steps of B-cell development, IgH DNA remodeling constantly occurs under the control of the two *cis*-regulatory elements 5'E_μ (during early stages of B-cell development) and 3'RR (during late stages of B-cell development). These enhancers are reported to physically interact via IgH DNA loops of still unclear functional significance at the pro-B cell stage. We thus investigated if 5'E_μ and 3'RR were independent drivers of locus remodelling or if their functions were more intimately intermingled during B-cell ontogeny. RNAseq experiments reported that these two *cis*-enhancers are independent motors of IgH locus remodelling with no mutual *cis*-transcriptional interactions within the IgH locus and no *trans*-transcriptional interaction with the Ig light chain kappa (Igκ) locus. These results validate the hypothesis that the two major enhancers of the IgH locus are independent engines of locus remodelling; the activation of one does not depend on the activation of the other (and vice versa).

Keywords

IgH 3' Regulatory Region; 5'E_μ Enhancer; IgL Locus; Transcriptional Enhancer; Knock-out Mice; RAG-Deficient Mice

Introduction

By their impact on nuclear organisation, enhancers are master regulators of cell fate [1]. The immunoglobulin heavy chain (IgH) locus undergoes numerous changes throughout B-cell differentiation. Among them, transcription and accessibility for V(D)J recombination, class switch recombination (CSR) and somatic hypermutation (SHM) are the most notable [2]. The IgH locus carries two potent enhancers separated by 200 kb (Figure 1A). 5'E_μ and the 3' regulatory region (3'RR), located at both ends of the constant gene cluster, control locus remodelling during B-cell differentiation [2]. Several studies have reported long-range interactions between 5'E_μ and 3'RR enhancers during B-cell maturation [3-5]. The question of mutual transcriptional cross-talk between these two enhancer entities remains open. During B-cell development, the heavy and light chain (IgL) loci are poised for their VDJ and VJ rearrangements, respectively [2]. The IgH locus first rearranges with D-J segments at the pro-B-cell stage followed by V-DJ joining at the pre-B-cell stage. The Ig*k* locus is poised for VJ rearrangements at the pre-B cell stage. A transient association (*trans*-mediated by Igκ enhancer elements) between IgH and Ig*k* loci has been demonstrated at the pre-B cell stage [6,7]. The question of a *trans*-mediated effect of IgH enhancer elements (5'E_μ and 3'RR) on Igκ locus remains open. In this study we examined whether 5'E_μ and 3'RR enhancers were independent motors of locus remodelling or if their functions were more intimately intermingled during B-cell ontogeny. We thus developed ΔE_μ-RAG-deficient and Δ3'RR-RAG-deficient mice to investigate potential transcriptional cross-talk between 5'E_μ and 3'RR enhancers at the immature B-cell stage. We also analysed transcriptomic data in order to identify a putative *trans*-transcriptional effect beyond the Ig*k* locus in IgH enhancer-deficient mice.

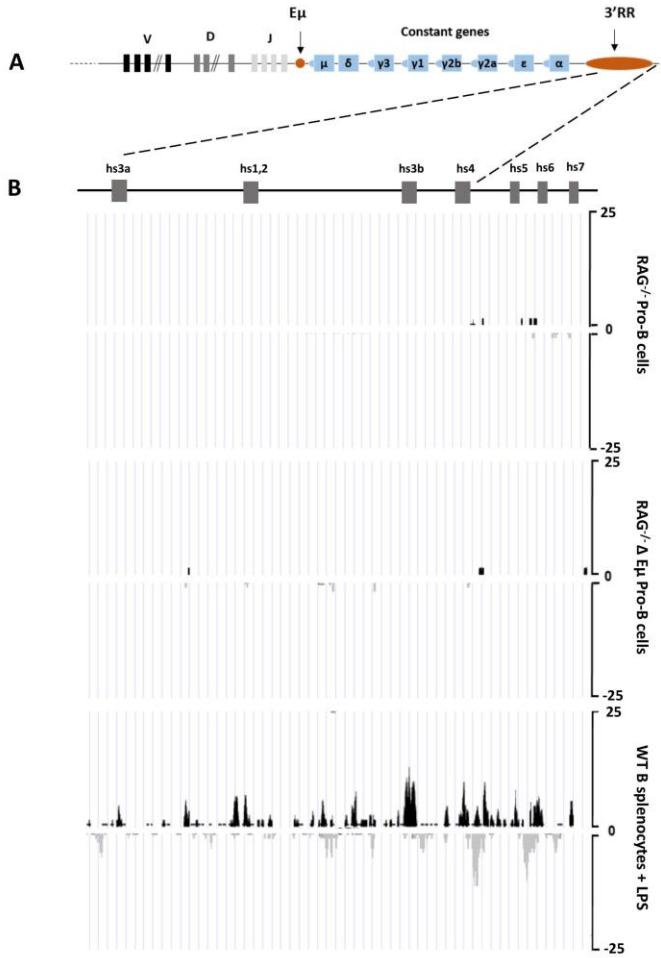

Figure 1: 3'RR eRNA in pro-B cells.
A: Schematic representation of the IgH locus (not to scale). V (variable), D (diversity), J (junctional) and C (constant) segments are located as well as the 5'E_μ element and the 3'RR. The 3'RR contains 4 transcriptional enhancers. Three of them are encompassed in a 25kb palindromic structure. B: Detection of 3'RR eRNAs. Lower panel: *Wt* B-cell splenocytes were stimulated 2 days with 5 μg/ml LPS (8-12 weeks old, males and females). RNAseq experiments were done after depletion of rRNA. One representative experiment out of two is reported with 3 mice per sample. Upper and medium panels: Pro-B cells of RAG-deficient (upper panel) and ΔEμ-RAG-deficient (medium panel) mice. One representative experiment out of two is reported with 4 to 5 mice per genotype.

Material and Methods

Mice

RAG-deficient mice (Janvier Labs, France), ΔE_{μ} [8] -RAG-deficient mice and Δ3'RR [9] -RAG-deficient mice were housed in the EOPS facility of the University of Limoges (France) and procedures were conducted in agreement with European Directive 2010/63/EU on animals used for scientific purposes. The APAFiS≠13855 projet was authorized by the French « Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche ».

Cells for RNAseq Experiments

Femoral pro-B cells were recovered with the EasySep™ mouse B-cell isolation Kit (STEMCELL Technologies, France) designed to isolate B cells from single-cell suspensions by negative selection. Unwanted cells were targeted for removal with biotinylated antibodies directed against non-B cells and streptavidin-coated magnetic particles (RapidSpheres™). Labeled cells were separated using an EasySep™ magnet without the use of columns. Desired cells were collected into a new tube. Cells of RAG-deficient, ΔEμ-RAG-deficient and Δ3'RR-RAG-deficient mice (8-12 weeks old, males and females) were used. In another set of experiments, *wt* B-splenocytes were stimulated with 5μg/ml LPS for two days as a positive control of 3'RR eRNA detection.

RNAseq Experiments

Pro-B cells were obtained from 10 RAG-deficient, 10 Δ3'RR-RAG-deficient mice and 10 ΔE_{μ}-RAG-deficient mice. RNA was extracted using miRNeasy kit from QIAGEN, according to the manufacturer's instructions. Two RNA pools (five samples each) were obtained for each genotype. RNA libraries were prepared using TruSeq Stranded Total RNA with Ribo-Zero Gold (Illumina), according to the manufacturer's instructions. Libraries were sequenced on a NextSeq500 sequencer, using NextSeq 500/550 High Output Kit (Illumina). Illumina

NextSeq500 paired-end 2x150nt reads were mapped with STAR release v2.4.0a versus mm10 with a gene model from Ensembl release 77 with default parameters.10-12 RNAseq experiments were done by the genomics platform of Nice Sophia Antipolis (France). Data were deposited in Gene Expression Omnibus under the accession number GSE117449, GSE169690 and GSE169691.

Results and Discussion

Femoral pro-B cells were isolated from RAG-deficient, ΔE_{μ}-RAG-deficient and Δ3'RR-RAG-deficient mice to investigate the potential transcriptional cross-talk between 5'E_{μ} and 3'RR enhancers in immature B-cells. A schematic representation of the IgH locus is reported in Figure 1A. Non coding RNAs (ncRNAs) contribute to chromosomal looping.13 Among these ncRNAs, enhancer RNAs (eRNAs) are transcribed from DNA sequences of enhancers including the 3'RR and contribute to their enhancer function [14,15]. As a positive control of 3'RR eRNA detection, LPS-stimulated *wt* B-splenocytes were used (Figure 1B, lower panel). RNAseq experiments did not highlight any 3'RR eRNAs in pro-B cells of RAG and ΔE_{μ}-RAG-deficient mice (Figure 1B, upper and middle panel, respectively) confirming results from a previous study with specific RT-QPCR [16]. The absence of 3'RR eRNAs in pro-B cells is in agreement with studies reporting that 3'RR has no direct role on V(D)J recombination [17,18].

The 5'E_{μ} enhancer is implicated during DJ recombination [8]. A schematic representation of the variable part of the IgH locus is shown in Figure 2A. Genomic deletion of the 5'E_{μ} enhancer abrogated transcription around its location including J_H and C_{μ} transcription as well as peaks of transcription specifically found to originate from the D_{Q52} promotor ($D_{4\text{-}1}$) and the 5'E_{μ} enhancer (known as μo and I_{μ} sense transcripts, respectively) (Figure 2B, upper and middle panels). As previously reported by Braikia and coll, [16] genomic deletion of the 3'RR had no effect on both μo and I_{μ} transcripts (Figure 2B, lower panel). A small increase in D antisense transcription was found in 3'RR-deficient mice

supporting a 3'RR-mediated transcriptional DJ silencing activity as previously suggested by Braikia and coll, [16] with RT-QPCR analysis. Together with the absence of 3'RR eRNA (Figure 1B), these results strongly suggest no transcriptional interaction of the 3'RR with the 5'E_μ element that transcriptionally control DJ recombination at the pro-B cell stage.

A *trans*-mediated transcriptional effect of the Igκ locus (mediated by Igκ enhancer elements) on the IgH locus has been demonstrated at the pre-B cell stage [6,7]. We next explored the potential transcriptional cross-talk between IgH enhancers on the Igκ locus (schematized in Figure 3A) in pro-B cells. Deletion of 5'E_μ did not affect Igκ locus transcription (Figure 3B, middle panel). Deletion of 3'RR enhancers slightly affected Igκ locus sense and antisense transcription (Figure 3B, lower panel) but this effect was weak and its relevance (if any) remains obscure. In mature B-cells, deletion of 3'RR and 5'E_μ had no *trans* effect on Igκ transcription in LPS-stimulated splenocytes (Figure 3C, middle and lower panels, respectively). These results are expected since no close association between the Ig*k* and IgH loci has been reported in mature B-cells.

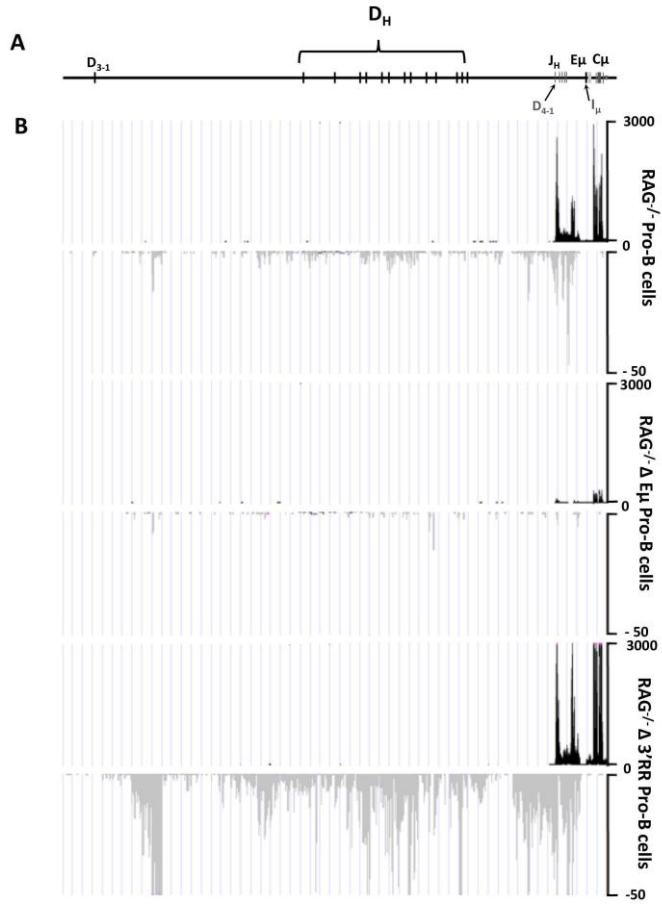

Figure 2: Influence of 5'E_μ and 3'RR deletion on pro-B cell DJ transcription. A: Schematic representation of the IgH locus (not to scale). V, D, J and C_μ segments are located as well as the 5'E_μ element and the 3'RR. B: D, J, 5'E_μ, and C_μ sense and antisense transcription in pro-B cells of RAG-deficient, ΔEμ-RAG-deficient and Δ3'RR-RAG-deficient mice. One representative experiment out of two is reported with 4 to 5 mice per genotype. Locations of D_{4-1} (also known as D_{Q52}) and I_μ promotors are indicated.

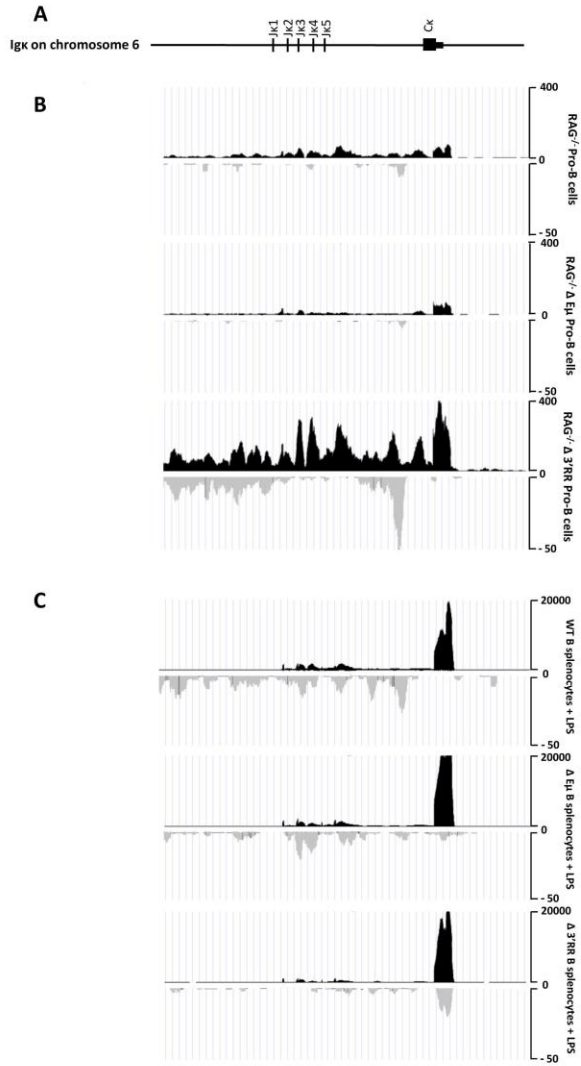

Figure 3: Influence of 5'E_μ and 3'RR deletion on Igκ transcription.
A: Schematic representation of the Igκ locus (not to scale). B: Igκ transcription in pro-B cells of RAG-deficient, ΔEμ-RAG-deficient and Δ3'RR-RAG-deficient mice. One representative experiment out of two is shown with 4 to 5 mice per genotype. C: Igκ transcription in B-cell splenocytes from *wt*, ΔEμ and Δ3'RR mice. B-cell splenocytes were stimulated 2 days with 5 μg/ml LPS (8-12 weeks old, males and female). One representative experiment out of two is shown with 3 mice per sample.

Concluding Remarks

Studies have reported the role of 5'Eμ and 3'RR enhancers during B-cell fate and maturation. Deletion of the 5'Eμ enhancer markedly lowered B-cell V(D)J recombination with no effect on SHM and CSR [8,19]. In contrast, deletion of the 3'RR enhancer affected B-cell fate, [20] SHM, [21] and CSR [9,11,12]. If studies reported the independent roles of 5'E_μ and 3'RR in B-cell maturation, few data were available concerning their synergy, cooperation and transcriptional cross-talk. This study shows that despite strong physical interactions during IgH locus DNA looping at the pro-B cell stage, [4,5] 5'E_μ and 3'RR enhancers are independent drivers of locus remodelling. Their function is not intimately intermingled and their optimal activation does not require physical contact with each other. Analysis of IgH locus transcription in 5'E_μ- and 3'RR-deficient mice reveals unilateral dependence of this pair of enhancers: 5'E_μ and 3'RR autonomously in immature B-cells and mature B-cells, respectively. These results obtained with knock-out (KO) mice are in agreement with previous results obtain with 5'E_μ-GFP, GFP-3'RR and 5'Eμ-GFP-3'RR transgenics [22-24].

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