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3′RR and 5′E$_\mu$ immunoglobulin heavy chain enhancers are independent engines of locus remodeling

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By their impact on nuclear organization, enhancers are master regulators of cell fate.[1] The immunoglobulin heavy chain (IgH) locus undergoes numerous changes as B cells differentiate. Among them, transcription and accessibility for V(D)J recombina-tion, class switch recombination (CSR), and somatic hyper-mutation (SHM) are the most notable.[2] The IgH locus carries two potent enhancers that are separated by 200 kb of distance. E$_\mu$ and the 3′ regulatory region (3′RR), at both ends of the constant gene cluster, control locus remodeling as B cells differentiate.[2] Previous studies reported long-range interactions (of still unclear functional significance) between the E$_\mu$ and 3′RR enhancers during B-cell maturation.[3–5] The question of a mutual transcriptional cross talk between these two enhancer entities remains open. We thus investigated if they were independent engines of locus remodel-ing or if their functions were more intimately intermingled. In this study, we developed ΔE$_\mu$-RAG-deficient and Δ3′RR-RAG-deficient mice to investigate the potential transcriptional cross talk between E$_\mu$ and 3′RR enhancers at the immature B-cell maturation stage.

RAG-deficient mice, double Eμ[6]-RAG-deficient mice, and double 3′RR[7]-RAG-deficient mice were developed in our animal facility (free of specified pathogenic organisms). Our research was approved by our local ethics committee review board (Comité Régional d'Ethique sur l'Expérimentation Animale du Limousin, Limoges, France) and carried out according to the European guidelines for animal experimentation. Femoral pro-B cells were recovered with the EasySep™ mouse B-cell isolation Kit (STEMCELL Technologies, France), which was designed to isolate B cells from single-cell suspensions by negative selection. Cells from RAG-deficient, ΔE$_\mu$-RAG-deficient, and Δ3′RR-RAG-deficient mice (8–12 weeks old, males and females) were used. RNA was extracted using Trizol (ThermoFisher Scientific) according to the manufacturer's instructions. Two pooled RNA (with four to six mice) were obtained for each genotype. RNA libraries were obtained using TruSeq Stranded Total RNA with Ribo-Zero Gold (Illumina), according to the manufacturer instructions. Libraries were sequenced on a NextSeq 500 sequencer, using NextSeq 500/ 550 High Output Kit (Illumina). Illumina NextSeq 500 paired-end 2 × 150 nt reads were mapped with STAR release v2.4.0a versus mm10 with gene model information from Ensembl release 77 with default parameters. RNAseq experiments were done in the genomics platform of Nice Sophia Antipolis, as previously reported.[8–10] Data were deposited in Gene Expression Omnibus under the accession number, GSE117449.

Femoral pro-B cells were isolated from RAG-deficient, ΔE$_\mu$-RAG-deficient, and Δ3′RR-RAG-deficient mice to investigate the potential transcriptional cross talk between E$_\mu$ and 3′RR enhancers in immature B cells. A schematic representation of the IgH locus is reported in Fig. 1a. Non-coding RNAs (ncRNAs) contribute to chromosomal looping.[11] Among these ncRNAs, enhancer RNAs (eRNAs) are transcribed from enhancer DNA sequences, including the 3′RR, and contribute to their enhancer function.[12,13] RNAseq experiments did not highlight any 3′RR eRNA in the pro-B cells of RAG mice (Fig. 1b), confirming results from a previous study with specific reverse trascription-quantitative PCR (RT-QPCR).[14] The absence of 3′RR eRNA in pro-B cells is in agreement with studies reporting that 3′RR has no direct role in V(D)J recombina-tion.[4,15,16] As a positive control, 3′RR eRNA was evident in lipopolysaccharide-stimulated B splenocytes (Fig. 1b). Excepted for C$_\mu$ (Fig. 1c), the RNAseq experiments showed no transcription in the C_γ, C_ε, and C_α constant genes of the IgH locus (data not shown). If genomic deletion of the E$_\mu$ enhancer reduced sense transcription around its location (including C$_\mu$ transcription), genomic deletion of the 3′RR paradoxically enhanced both sense and especially antisense transcription of the D and J segments, as well as E$_\mu$ and C$_\mu$ transcription (Fig. 1c, d). Peak transcription levels were specifically found to originate from the D$_{Q52}$ promotor (D$_{4\text{-}1}$) and the E$_\mu$ enhancer (known as μo and I$_\mu$ sense transcripts, respectively). The concept of the 3′RR-mediated transcriptional silencing activity was first reported by Braikia et al.,[14] with RT-QPCR analysis. In contrast, with the present study, the μo and I$_\mu$ sense transcripts were not reportedly altered by the 3′RR deletion.

Deletion of the 5′Eμ enhancer markedly lowered B-cell V(D)J recombination without affecting SHM and CSR.[6] In contrast, deletion of the 3′RR enhancer affects B2 B-cell fate,[17] SHM,[18] and conventional CSR.[7–9,19] If the 3′RR deletion also affects B1 B-cell fate[16] and SHM,[20] then it has no evident effect on B1 B-cell IgA CSR.[21] If the roles of these two IgH enhancers have been observed during B-cell fate and maturation, then few data were available concerning their synergy, cooperation, and transcriptional cross talk. Analysis of chromatin marks, eRNA, and accessibility in the ΔE$_\mu$ and Δ3′RR mice shows in mature activated B cells that the 3′RR acts in autonomy and controls IgH transcription.[8] The present study shows that despite physical interactions (with a still

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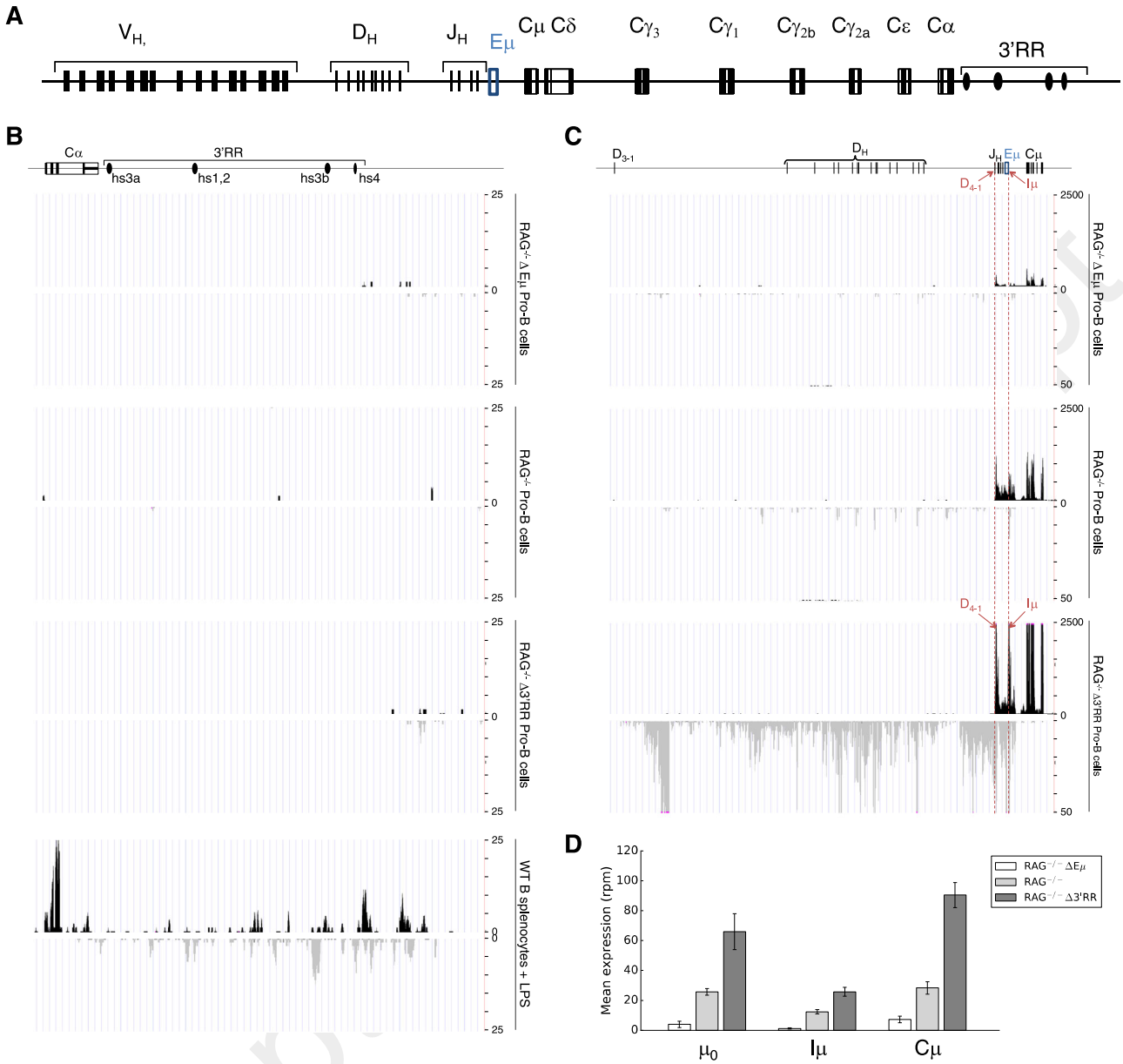

Fig. 1 Influence of the E_μ and 3′RR enhancers on IgH transcription in pro-B cells. **a** Schematic representation of the IgH locus (not to scale). V (variable), D (diversity), J (junctional), and C (constant) segments are shown as well as the E_μ enhancers and the 3′RR. The 3′RR contains four transcriptional enhancers. Three of them are encompassed in a 25 kb palindromic structure. **b** Detection of 3′RR eRNA in the pro-B cells of RAG-deficient, ΔEμ-RAG-deficient, and Δ3′RR-RAG-deficient mice (8–12 weeks old, males and females). RNAseq experiments were done after depletion of rRNA. Data represent the mean of two independent experiments with four to six mice per genotype. 3′RR eRNA from LPS-stimulated B splenocytes from wt mice are reported as positive control.[8] **c** D-J-E_μ-C_μ sense and antisense transcription in pro-B cells of RAG-deficient, Eμ-RAG-deficient, and Δ3′RR-RAG-deficient mice. Locations of the $D_{4\text{-}1}$ (also known as D_{Q52}) and I_μ promotors are indicated. The same mice were utilized as in **a**. **d** Quantitative representation of $D_{4\text{-}1}$, I_μ, and C_μ transcription (in reads per million). The mean of two independent experiments are shown (error bars show extreme values)

hypothetical meaning) during IgH locus DNA looping,[3–5] the 5′Eμ and 3′RR enhancers are independent engines of locus remodeling, and their function is not intimately intermingled and their optimal activation does not require physical contact with each other. Our results reinforce the concept that E_μ-3′RR interactions may affect E_μ-mediated recombination control rather than transcription.[14] Finally, they also highlight that if the 3′RR acts as a transcriptional enhancer in mature B cells, it acts as a transcriptional silencer at the immature B-cell stage. Clearly, determining how the 3′RR mediates its transcriptional silencing within the D and J domains will be an exciting challenge to resolve.

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AUTHOR CONTRIBUTIONS

H.I., N.G., A.S., F.B., O.A.M., and Y.D. designed and performed the experiments and wrote the manuscript. Y.D. obtained financial grants.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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