

# Developmental Switch in the Transcriptional Activity of a Long-Range Regulatory Element

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#### 27 Abstract

28 Eukaryotic gene expression is often controlled by distant regulatory elements. In developing 29 B lymphocytes, transcription is associated with V(D)J recombination at immunoglobulin loci. 30 This process is regulated by remote *cis*-acting elements. At the immunoglobulin heavy chain 31 (IgH) locus, the 3' regulatory region (3'RR) promotes transcription in mature B cells. This led 32 to the notion that the 3'RR orchestrates the IgH locus activity at late stages of B cell 33 maturation only. However, long-range interactions involving the 3'RR were detected in early 34 B cells, but the functional consequences of these interactions are unknown. Here we show that 35 not only does the 3'RR affect transcription at distant sites within the IgH variable region, but 36 that it conveys a transcriptional silencing activity on both sense and antisense transcription. 37 The 3'RR-mediated silencing activity is switched off upon completion of  $V_{\rm H}$ -DJ<sub>H</sub> 38 recombination. Our findings reveal a developmentally-controlled, stage-dependent shift in the 39 transcriptional activity of a master regulatory element.

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#### 41 Introduction

42 The spatial and temporal control of gene expression in metazoans is effected by regulatory 43 elements that are often located far from gene promoters (1). This pattern of gene expression 44 regulation is a hallmark of antigen receptor loci whose expression involves both transcription 45 and recombination. The mouse IgH locus contains ~195 variable (V<sub>H</sub>) genes subdivided into domain-organized gene families, including the distal  $V_{\text{H}}$ , by far the largest, and the proximal 46 47 V<sub>H</sub> family. The V<sub>H</sub> genes are followed by a dozen of diversity (D) segments, 4 joining (J<sub>H</sub>) 48 segments, and 8 constant ( $C_{\rm H}$ ) genes (2, 3) (Fig. 1A, top scheme). The assembly of an  $I_{gH}$ 49 variable region exon through V(D)J recombination occurs in early developing B cells in an ordered manner, first D to  $J_{\rm H}$  then  $V_{\rm H}$  to  $DJ_{\rm H}.$  While the first recombination step (D-J\_{\rm H}) can 50 51 also be detected in developing T cells,  $V_H$ -DJ<sub>H</sub> recombination is strictly B cell-specific (4).

52 In addition to its B-lineage specificity, V<sub>H</sub>-DJ<sub>H</sub> rearrangement is regulated by allelic 53 exclusion which enables mono-allelic expression of only one IgH locus by a given B cell (4, 54 5). In this process, a productive V(D)J rearrangement on one allele ultimately leads to surface expression of a µ heavy chain which signals arrest of V<sub>H</sub>-DJ<sub>H</sub> recombination on the second 55 allele. If the first rearrangement is not productive (i.e. no µ heavy chain production), then the 56 57 second allele can undergo V<sub>H</sub>-DJ<sub>H</sub> recombination (4, 5). There is considerable evidence in 58 support of the notion that  $V_H$ -DJ<sub>H</sub> rearrangement is the regulated step in IgH allelic exclusion 59 and its maintenance through a feed-back mechanism (4, 5), although the molecular 60 mechanisms through which feed-back signaling inhibits V<sub>H</sub>-DJ<sub>H</sub> recombination remain 61 unclear.

62 Another level of regulation of  $V_H$ -DJ<sub>H</sub> recombination relates to the physical location of  $V_H$ 63 gene segments within the variable domain. Indeed, several gene targeted studies showed that 64 recombination of the distal and the proximal domains is regulated very differently (4, 6, 7).

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Additionally, allelic exclusion of the distal V<sub>H</sub> genes is more stringent than that of the most
proximal V<sub>H</sub> genes (4).

67 In developing B lymphocytes, sense and antisense transcription is associated with V(D)J 68 recombination in a cell-type and developmental-stage specific manner (7). The process is 69 regulated by distant *cis*-acting elements including enhancers, promoters and insulators (6, 7). 70 Three long-range regulatory elements were identified within the *IgH* locus and were shown by 71 targeted deletion studies to regulate the locus activity. The  $E\mu$  enhancer, located between the 72 variable and the constant regions, plays a critical role in V(D)J recombination and associated 73 germ-line transcription (8-12). Additionally, CTCF-binding elements (CBEs) with insulator 74 activity were identified in the  $V_{H}$ -D intergenic region (13-15). This regulatory region (called 75 IGCR1) is important for the order and lineage specificity of V(D)J rearrangements, and for 76 allelic exclusion of proximal  $V_{\rm H}$  genes (16). A locus control region called the 3' regulatory 77 region (3'RR) contains four enhancers (hs3a, hs1-2, hs3b, hs4) (17) and was shown to 78 synergistically promote germ-line transcription of C<sub>H</sub> genes during class switch 79 recombination in mature B cells and  $I_{gH}$  expression in plasma cells (18, 19). Previous 80 targeted deletion studies showed that the 3'RR impacted µ heavy chain gene expression in 81 resting B cells (18, 19), but that it was dispensable for the repertoire diversity in pre-B cells 82 (20). In contrast, its role in allelic exclusion is still unknown.

The established role of the 3'RR in IgH locus expression in late B cells and the lack of effect on repertoire diversity led to the notion that the 3'RR activity is restricted to the late stages of B cell maturation only (19, 20). However, various studies described long-range interactions between the 3'RR and various upstream sequences, including Eµ enhancer (15, 16, 21, 22) though their functional significance is unclear. Strikingly, deletion of either Eµ or the 3'RR had no effect on long-range interactions mediating locus contraction of the IgH

- locus, leading to the proposal that the activity of these elements may be restricted to the ~270
  kb D-C<sub>H</sub> region (22).
  In this study, we used a mouse line devoid of the 3'RR (19) (hereafter called Δ3'RR) to
  explore its role in V(D)J recombination and associated germ-line transcription which occurs
  at distances ~220 kb to megabases far from the 3'RR. Here, we report the striking finding that
  the 3'RR mediates a transcriptional silencing activity which is switched off after completion
  of V<sub>H</sub>-DJ<sub>H</sub> recombination.
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### 98 Materials and Methods

99 Mice. The generation of 3'RR-deleted mice was described (19). Throughout the study, 100 RAG2-deficient mice used as controls are of 129Sv genetic background. The experiments on 101 mice have been carried out according to the CNRS Ethical guidelines and approved by the 102 Regional Ethical Committee.

Antibodies. APC-conjugated anti-B220 and FITC-conjugated anti-IgM were from
BioLegend. PE-conjugated anti-CD43, FITC-conjugated anti-Igk, PE-conjugated CD4 and
FITC-conjugated CD8 were from BD-Pharmingen.

**FACS analyses.** Bone marrows from 6- to 8-week-old mice were prepared by standard techniques.  $5 \times 10^5$  cells/assay were stained with anti-B220, anti-CD43 and anti-IgM or anti-Ig $\kappa$ , and gated either on IgM<sup>-</sup> or Ig $\kappa$ <sup>-</sup> population. Data were obtained on 2.0 x 10<sup>4</sup> viable cells by using a BD FACSCalibur. FACS acquisitions included isotype controls and exclusion of dead cells by labelling with propidium iodide.

111 V(D)J rearrangement assays. B cells from bone marrows were sorted by using CD19-112 magnetic microbeads and LS columns (Miltenyi) and labelled with anti-B220, anti-CD43, and 113 either anti-IgM or (as a cross-check) anti-Igk. The purity of the sorted pro-B cell populations was checked by FACS (>95%) and by the rearrangement status of the Igk locus. The 114 CD4<sup>+</sup>CD8<sup>+</sup> thymocytes were sorted as described (16). Genomic DNAs from the sorted pro-B 115 cells (B220<sup>+</sup>  $\kappa$ -CD43<sup>high</sup> or B220<sup>+</sup>IgM-CD43<sup>high</sup>) and CD4<sup>+</sup>CD8<sup>+</sup> thymocytes were prepared by 116 117 standard techniques, and diluted for the (q)PCR assays (23). Controls included genomic 118 DNAs from kidney and RAG-2-deficient pro-B cells. The hs5 sequence located downstream 119 of the 3'RR (24) was used for normalization. The primers are listed in table S1.

120 **RT-qPCR.** RAG-2-deficient pro-B cells were sorted using CD19-magnetic microbeads 121 (Miltenyi). RAG-2-deficient thymuses were prepared as described (25). B220<sup>-</sup>, CD4<sup>+</sup>CD8<sup>+</sup> 122 thymocytes were sorted as described (16). Pro-B and pre-B cells  $(B220^+\kappa^-CD43^{low})$  or

123 B220<sup>+</sup>IgM<sup>-</sup>CD43<sup>10w</sup>) were sorted as described above. Unstimulated splenic B cells were sorted 124 by using CD43-magnetic microbeads (Miltenyi), and activated by culturing them for 48 h in 125 the presence of 20  $\mu$ g/ml lipopolysaccharide (Sigma) and 2 ng/ml anti-IgD-dextran (Fina 126 Biosolutions). Total RNAs were reverse transcribed (Fermentas) and subjected to semi-127 quantitative PCR, using SYBR green I (Invitrogen) and Image Quant software as described 128 (26) or to qPCR using Sso Fast Eva Green (BioRad). The relative transcription levels were 129 normalized using  $\beta$ -actin and Gapdh RNAs as controls.

130 **Statistical analysis.** Results are expressed as mean  $\pm$  SEM (GraphPad Prism) and overall 131 differences between values from WT and mutant mice were evaluated by Student test. The 132 difference between means is significant if *p* value < 0.05 (\*), very significant if *p* value < 0.01 133 (\*\*), and extremely significant if *p* value < 0.001 (\*\*\*).

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## 136 Results

The 3'RR down-modulates V(D)J recombination. To analyze the effect of the 3'RR on
V(D)J recombination, we performed sensitive qPCR-based V(D)J recombination assays (23)

139 on genomic DNAs from sorted WT and  $\Delta 3$ 'RR pro-B cells and CD4<sup>+</sup>CD8<sup>+</sup> thymocytes.

We first quantified the proportion of the  $D_{Q52}$  and  $J_{H1}$  segments that had retained their germ-line configuration in purified pro-B cells (Fig. 1A). The total number of alleles with unrearranged  $D_{Q52}$  and  $J_{H1}$  segments on the mutant alleles was comparable to WT controls (Fig. 1A), clearly indicating that there was no obvious delay in the onset of D-J<sub>H</sub> recombination. Thus, any potential effect of the 3'RR on V(D)J recombination is likely to occur after the onset of the process.

We also quantified recombined  $DJ_H$  segments and fully rearranged  $V_H DJ_H$  segments. We used forward degenerate primers that recognize most of D segments (Fig. 1B, top scheme) or distal  $V_H$  genes (Fig. 1C, top scheme), and specific backward primers located downstream of each  $J_H$  segment (Fig. 1B, 1C, top schemes). We did not analyze recombination of proximal  $V_H$  genes as the mutant allele is derived from 1290la ES cells which bear a ~120-kb internal deletion in the proximal  $V_H$  domain (22).

152 Surprisingly, a mild increase in DJ<sub>H</sub> alleles was detected in mutant pro-B cells, but not in 153 mutant CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (Fig. 1B). Interestingly, a similar increase was also detected 154 for distal  $V_H DJ_H$  alleles (Fig. 1C), which could be due, at least in part, to accumulated  $DJ_H$ 155 substrates. Inspection of  $D-J_H$  and  $V_H-D-J_H$  junction sequences in pro-B cells showed no 156 evidence for an over-representation of a D gene segment family, or an anomaly regarding the 157 number of inserted or deleted nucleotides (Table 1). The increase seen for  $V_H DJ_H$  alleles was 158 not due to a block at the pro-B cell stage either as the pro-B compartment was rather slightly 159 reduced (Fig. 2A). The data suggest an enhanced V(D)J recombination (see discussion).

Wolecular and Cellular Biology 160 161 **The 3'RR does not affect the order of rearrangements.** As mentioned previously (see 162 introduction),  $V_H$ -DJ<sub>H</sub> recombination is strictly B cell-specific and Eµ deletion impairs V(D)J 163 recombination (8, 9, 12); additionally, mutation of IGCR1 CBEs affects the order of V(D)J 164 rearrangements (16). Given the reported CTCF-mediated loop formation between IGCR1 165 CBEs and CBEs downstream of the 3'RR (15, 16, 21, 22), we asked whether deletion of the 166 3'RR, which would disrupt the stable Eµ-3'RR interaction (16) and potentially the 167 architecture of the larger CTCF-mediated domain, would somehow deregulate the order of

V(D)J rearrangements.

169 To this end, we attempted to detect V<sub>H</sub>D amplicons, which result from a direct V<sub>H</sub>-D 170 recombination. Genomic DNAs were extracted from purified pro-B cells and CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, and assayed for V<sub>H</sub>-D recombination by using a forward primer pairing with 171 172 V<sub>H81X</sub> gene and a backward primer downstream of the D<sub>052</sub> segment (Fig. 2B). This sequence 173 is deleted following any D-J<sub>H</sub> rearrangement, but not if the  $V_{H81X}$  segment directly 174 recombines with D<sub>052</sub> segment. As a positive control, we used genomic DNAs from IGCR1  $CBE^{\text{-/-}}$  pro-B cells and  $CD4^{+}CD8^{+}$  thymocytes, which undergo  $V_{H81X}\text{-}D_{Q52}$  recombination 175 176 (16). We found no evidence for  $V_H D$  amplicon in  $\Delta 3$ 'RR pro-B cells or in CD4<sup>+</sup>CD8<sup>+</sup> 177 thymocytes (Fig. 2B). Thus, the 3'RR does not affect the order of rearrangements.

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179 The 3'RR down-regulates sense and anti-sense transcription in the distal variable 180 region. Germ-line transcription in the variable region precedes  $V_H$ -DJ<sub>H</sub> recombination (27, 181 28). To investigate the effect of the 3'RR on germ-line transcription of un-rearranged genes, 182 we first brought the  $\Delta$ 3'RR mutation into RAG-2-deficient background which precludes 183 V(D)J recombination. We mainly focused on the D-C $\mu$  and the distal  $V_H$  domains where high 184 levels of transcription are detected (7, 16).

185 Germ-line transcription within the D-Cµ domain, but not within the distal V<sub>H</sub> domain, is 186 regulated by E $\mu$  enhancer (8-12, 29). In contrast, the effect of the 3'RR is unknown. We 187 found no obvious effect on I $\mu$  or  $\mu 0$  sense transcripts (derived from E $\mu$  enhancer and D<sub>052</sub> 188 promoter respectively), or on D<sub>SP</sub> antisense transcripts (derived from Eµ region and/or an ill-189 known promoter upstream of  $D_{ST4}$  segment (30)) (Fig. 3A). Concordantly, normal levels of Iµ, µ0 and D<sub>SP</sub> GL transcripts were found in RAG-2-deficient thymuses and in RAG-2-190 191 proficient  $CD4^+CD8^+$  thymocytes (Fig. 3A). Thus, within the D-Cµ domain, sense and 192 antisense transcription was not altered in the absence of the 3'RR, indicating that the Eu-193 mediated control of germ-line transcription in the D-Cµ domain does not require the 3'RR.

194 Remarkably, the distal V<sub>H</sub> region yielded an increase of both spliced, sense transcripts, 195 and unspliced, antisense transcripts in  $\Delta 3$ 'RR mice (Fig. 3B). To exclude any bias potentially 196 introduced by the increased primary  $V_{\rm H}$  sense transcripts, we quantified intergenic, antisense 197 germ-line transcript levels within the  $V_{HJ558}$  and  $V_{HJ606}$  clusters. These exclusively antisense 198 transcripts were also increased (Fig. 3C). In contrast, the Pax5-activated intergenic repeat 4 199 (PAIR 4) antisense germ-line transcripts (31) were unaltered (Fig. 3C), suggesting that 200 regulation of these transcripts, derived from the PAIR4 promoter/enhancer element (31), is 201 3'RR-independent. Thus, within the distal  $V_{\rm H}$  domain (except for PAIR elements), the 3'RR 202 affects both sense and antisense transcription.

203 Within the proximal  $V_H$  domain, we also found increased antisense transcription upstream 204 of  $V_{H81X}$  (the most 3' functional  $V_H$  gene segment, which is not encompassed by the ~120 kb 205 deletion in  $\Delta$ 3'RR (22)) (Fig. 3C), suggesting a variable region-wide effect of the 3'RR.

206 Overall, and within the limits of the transcripts analyzed, the 3'RR downregulates sense 207 and antisense germ-line transcription along the remote *IgH* variable domain.

Transcription of some loci could be regulated by elements located on a different chromosome (32). Specifically, it was suggested that the  $Ig\kappa$  locus and its 3' enhancer (on

Molecular and Cellular Richary 210 chromosome 6) were involved in directing the IgH locus (chromosome 12) to a repressive 211 nuclear compartment and inducing IgH locus decontraction (33). To investigate whether the 212 3'RR can act in *trans*, we analyzed germ-line transcription along the  $Ig\kappa$  locus and found it 213 unchanged (Fig. 3D), excluding, at least with regard to the  $Ig\kappa$  locus, any *trans*-effect of the 214 3'RR.

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216 Switching off the 3'RR-mediated silencing activity coincides with the completion of 217 V(D)J recombination. To elucidate precisely at which step the 3'RR-mediated silencing 218 activity is turned off, we quantified the transcript levels of fully rearranged  $\mu$  gene at various 219 developmental stages. To avoid potential bias introduced by cellular selection and/or selective 220 use of distal versus proximal  $V_H$  genes, we also measured I $\mu$  transcript levels. We found 221 normal levels of the distal  $V_H$  exon-containing  $\mu$  transcripts ( $dV_H DJ_H C\mu$ ) in pro-B cells (Fig. 222 3E). These transcript levels were unchanged in pre-B cells, but were clearly decreased in 223 unstimulated splenic B cells (Fig. 3E). Down-regulation of Iµ transcripts was clearly detectable in pre-B cells and was more pronounced in unstimulated cells (Fig. 3E). 224 225 Interestingly, the shift from a silencer (in pro-B cells) to an enhancer (pre-B cells) activity 226 correlated with the appearance of 3'RR enhancers' transcripts (Fig. 3F) (34).

Therefore, the 3'RR-mediated silencing effect appears to be switched off upon completion of V(D)J recombination at the pro-B cell stage and correlates with the onset of 3'RR transcription.

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#### 232 Discussion

We reported here the first demonstration of a stepwise shift in the transcriptional activity of a long-range regulatory element in higher eukaryotes. The down-regulating activity of the 3'RR targets multiple upstream sense and anti-sense promoters in the remote IgH variable region, but spares known enhancers/promoters (Eµ and PAIR4). Specifically, the 3'RR does not affect sense and antisense transcription within the D-Cµ domain consistent with the notion that transcription within this domain is mainly controlled by Eµ enhancer (8, 10).

239 As mentioned previously, the  $\Delta 3$ 'RR mouse line is derived from 1290la ES cells which 240 have a 120 kb internal deletion in the proximal  $V_{\rm H}$  domain, which is not the case in the RAG-241 2-deficient mice which were 129Sv-derived. Thus, although we cannot formally exclude the 242 possibility that the 120 kb deletion within the proximal  $V_H$  domain may have affected distal 243  $V_H$  germ-line transcription and V(D)J recombination, we think it is unlikely for various 244 reasons. Multiple studies clearly showed that the proximal and the distal  $V_{\rm H}$  domains were differentially regulated. Thus, recombination of distal but not of proximal  $V_{\rm H}$  genes is 245 246 inhibited in mice deficient in the histone-modifying enzyme EZH2 and different transcription 247 factors involved in V(D)J recombination such as PAX5, YY1, and Ikaros (35-38). Mutations 248 targeting various *cis*-acting elements at the *IgH* locus similarly showed a differential effect on 249 germ-line transcription and recombination of proximal versus distal  $V_{\rm H}$  genes (12, 16, 39-42). 250 Importantly, deletion of the 3'RR in the context of the 120 kb deletion had no effect on long-251 range interactions across the IgH locus in RAG2-deficient pro-B cells (22). Additionally, 252 within the IgH variable region, the viewpoints that were found by 4C-seq analyses to strongly 253 or minimally interact with the 3'RR correlated well with our transcriptional analyses. For 254 instance, antisense transcription upstream of V<sub>H81X</sub> gene (which is intact in 1290la 255 background) was enhanced in the absence of the 3'RR (present study) and was efficiently contacted by hs3b enhancer of the 3'RR (22), whereas PAIR4 whose expression was not 256

affected by the 3'RR deletion (present study) did not significantly interact with the 3'RR (21, 258 22). Moreover, antisense transcription within the J606 family was increased (present study) 259 which correlated well with an interaction of this region with the 3'RR-Eµ loop (21, 22, 260 reviewed in 7). Finally, it is difficult to figure out how the 120 kb deletion would affect the 261 3'RR-mediated effect on D-J<sub>H</sub> recombination which takes place in the stable IGCR1-*I*gH 262 3'CBEs chromatin domain (15, 16, 21, 22).

263 Whether the long-range 3'RR-mediated silencing activity is due to an unidentified 264 developmentally-regulated silencer within the 3'RR itself or is mediated by interacting 265 partners requires further investigations involving combined mutations. The strong correlation 266 between the extinction of the 3'RR-mediated silencing activity and the completion of V(D)J recombination suggests that the interacting partner(s) should be deleted upon  $V_{\mathrm{H}}\text{-}DJ_{\mathrm{H}}$ 267 268 recombination. Likely candidates could be the IGCR1 (16, 21, 22) and/or the newly identified 269 interaction site upstream of IGCR1 (22). This could be a mean through which recombination 270 regulates the 3'RR transcriptional activity. Alternatively, though not-mutually-exclusive, the 271 correlation between the triggering of the 3'RR transcription and its enhancer activity suggests 272 that the long-range activity of the 3'RR during B cell development may be modulated by its 273 enhancers' transcripts.

The B cell-specific down-modulating effect of the 3'RR on D-J<sub>H</sub> recombination suggests that 3'RR/E $\mu$  interaction may affect E $\mu$ -mediated control of recombination rather than transcription. Various studies found that transcription and V(D)J recombination could be mediated by distinct activities of accessibility control elements, including E $\mu$  enhancer (43, reviewed in (44)), and there is some evidence that recombination could be recapitulated *in vitro* in the absence of transcription (45).

By quantifying the proportion of the  $D_{Q52}$  and  $J_{H1}$  segments in their un-rearranged configuration, we found no obvious delay in the onset of D-J<sub>H</sub> recombination clearly

282 indicating that the effect of the 3'RR occurs after the initiation of V(D)J recombination. In 283 contrast, there was a relative accumulation of  $DJ_H$  intermediates and fully recombined  $V_H DJ_H$ 284 alleles with no obvious block at the pro-B cell stage at which V(D)J recombination at the  $I_gH$ 285 locus occurs. Thus, it appears that we are dealing with a specific phenomenon which is 286 restricted to pro-B cells: Increased recombination events in a "fixed" time window. One 287 simple explanation is that, in the absence of the 3'RR, the process runs faster. Recent 288 evidence highlighted the importance of spatial confinement for the kinetics of V(D)J 289 recombination and time encounter with regulatory elements (46). It is tempting to speculate 290 that 3'RR interactions with its partners are a critical component of the mechanisms that 291 regulate the kinetics of V(D)J recombination. Within a newly generated topological domain 292 that forms upon DJ<sub>H</sub> recombination, the 3'RR may for instance contribute to the control of the 293 kinetics of V<sub>H</sub>-DJ<sub>H</sub> recombination by impacting germ-line transcription within the V<sub>H</sub> domain, 294

while  $E\mu$  is focused on  $DJ_H$  transcription (40).

295 Why does the 3'RR mediate a transcriptional silencing within the  $V_{\rm H}$  domain? It should 296 be noted that  $V_{H}$ -DJ<sub>H</sub> recombination is the regulated step in allelic exclusion (4, 5), and that 297 the control of germline transcription is likely the primary event during allelic exclusion (42). 298 In the absence of the 3'RR, we found an increase of germline transcription within the distal 299 V<sub>H</sub> domain (with the exception of PAIR promoter/enhancer-derived transcripts) and in the 300 proportion of distal  $V_H DJ_H$  alleles, with no obvious block at the pro-B cell stage. This 301 increase could be due, at least in part, to accumulated  $DJ_H$  substrates. However, it may also 302 indicate a disruption of allelic exclusion. Thus, our findings of enhanced V<sub>H</sub>-DJ<sub>H</sub> 303 recombination and germ-line transcription may be explained by a speculative model (See 304 Figure 4) in which a productive rearrangement on one allele instructs the 3'RR on the second 305 allele to down-regulate antisense transcription, leading to the inhibition of  $V_{H}$ -DJ<sub>H</sub> 306 recombination. In the absence of the 3'RR, a productive V<sub>H</sub>DJ<sub>H</sub> rearrangement on the first 307 allele (and subsequent surface expression of  $\mu$  heavy chain) would not block V<sub>H</sub>-DJ<sub>H</sub> 308 recombination on the second allele, leading to an overall accumulation of V<sub>H</sub>DJ<sub>H</sub> alleles.

309 In this regard, our model may fill a gap in the regulated/feed-back inhibition model of 310 allelic exclusion. Indeed, how to explain that a productive rearrangement on the first allele inhibits  $V_{\mathrm{H}}\text{-}DJ_{\mathrm{H}}$  recombination on the second allele? Our interpretation is that surface 311 312 expression of the  $\mu$  heavy chain instructs the 3'RR to inhibit germline transcription within the 313 variable domain and therefore  $V_H$ -DJ<sub>H</sub> recombination. Thus, 3'RR-mediated inhibition of  $V_H$ 314 germline transcription could be the missing link between surface expression of the heavy 315 chain and effective allelic exclusion. One prediction of this model is that deletion of the 3'RR 316 will result in increased V<sub>H</sub> germline transcription and V<sub>H</sub>-DJ<sub>H</sub> frequency: That is what we 317 found in the present study. Another prediction is that if a heavy chain were prematurely 318 expressed (that is, prior to V(D)J recombination), the 3'RR would be instructed to inhibit  $V_{\rm H}$ 319 germline transcription and impairment of V<sub>H</sub>-DJ<sub>H</sub> recombination would be the outcome: That 320 is indeed the case (42).

321 The wide impact of the 3'RR on sense and antisense germ-line transcription within the 322 variable region raises the question as to whether the 3'RR targets sense and antisense 323 promoters simultaneously. We favor the view that the 3'RR targets primarily antisense 324 promoters and that the silencing of sense transcripts could be a downstream consequence of 325 this primary effect. It should be noted that antisense transcripts are long and extend through 326 multiple V<sub>H</sub> genes (28). Thus, the control of a limited number of antisense promoters would 327 be sufficient for a wide transcriptional impact. Nonetheless, the 3'RR must somehow reach its 328 distant target promoters. The possibility of a long-range effect mediated by 3'RR transcripts 329 was ruled out because they were undetectable in pro-B cells. This would rather imply that the 330 3'RR-mediated silencing activity correlates with the lack of its transcription. A likely 331 explanation is that the 3'RR exploits the developmentally-regulated, 3'RR-independent (22),

mechanisms that allow compaction of the IgH locus. In particular, the large-scale reorganization of the distal variable region into rosette-like structures following D-J<sub>H</sub> recombination and the compaction of the IgH locus in pro-B cells (47) may bring into close proximity the 3'RR and its target promoters.

336 How could the 3'RR mediate its silencing activity is presently unknown and may involve 337 a developmental stage-dependent interplay between the topological reorganization of the  $I_{gH}$ 338 locus which may be modulated by CTCF insulators and transcription factors-mediated loops, 339 post-translational modifications of factors such as CTCF, and the 3'RR epigenetic 340 modifications and recruitment of transcription factors and co-repressors (48-51). Interestingly, 341 there is some evidence that the human  $\beta$ -globin locus control region can in some contexts 342 repress gene expression through transcriptional interference potentially involving transcripts 343 initiating in flanking repetitive sequences and running through the  $\beta$ -globin locus (52). This 344 suggests that, besides their established role in gene expression activation, locus control 345 regions have also the potential to mediate transcriptional silencing activity which may depend on the developmental stage, lineage specificity, interacting partners and the chromatin 346 347 context.

348 In conclusion, our study reveals a hitherto unsuspected function of the 3'RR during early 349 B cell development. The 3'RR emerges as a master regulatory element that mediates a 350 transcriptional silencing activity along the distant and large IgH variable region, leading to the 351 inhibition of V<sub>H</sub>-DJ<sub>H</sub> recombination likely to promote allelic exclusion.

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362

#### 363 Author contributions

- 364 FZB and CC performed research and analyzed data; MM, YD and MC contributed new
- 365 reagents or analytic tools; AAK designed research, analyzed data and wrote the paper.

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- 528

## 529 Figure legends

## 530 Fig.1. V(D)J recombination in mutant mice.

531 (A) The top scheme shows the mouse IgH locus (not to scale). CBEs, CTCF-binding 532 elements. Not all CBEs are shown. Genomic DNAs were prepared from sorted WT and  $\Delta 3'RR$  pro-B cells and subjected to qPCR to amplify un-rearranged  $D_{Q52}$  and  $J_{\rm H1}$  gene 533 534 segments. The relative position of the primers is indicated in the upper scheme. Genomic DNA from Rag2<sup>-/-</sup> mice was used as a control. hs5 sequence was used for normalization 535 (n=4). (B) Genomic DNAs from sorted pro-B cells (left) and double-positive thymocytes 536 537 (right) were subjected to qPCR to quantify D-J<sub>H1</sub>, D-J<sub>H2</sub>, D-J<sub>H3</sub> or D-J<sub>H4</sub> recombination events.  $Rag2^{-/-}$  and kidney DNAs were used as negative controls (n≥4). (C) Quantification of distal 538  $(dV_H) V_H$ -DJ<sub>H</sub> recombination events in pro-B cells by qPCR (n≥4). (\*\*, p<0.01; \*, p<0.05; ns, 539 540 not significant; Data are presented  $\pm$  SEM).

541

Fig. 2. Early B cell development and the order of rearrangements. (A). To determine the distribution of pro-B and pre-B cell populations, single-cell suspensions from the bone marrow of WT and  $\Delta 3$ 'RR were stained with anti-B220+anti-CD43+anti-IgM, and gated on IgM<sup>-</sup> population (n=11) (\*, *p*<0.05; ns, not significant; Data are presented ± SEM). (B) Genomic DNAs were prepared from sorted pro-B cells (B220<sup>+</sup>IgM<sup>-</sup>CD43<sup>high</sup>) and CD4<sup>+</sup>CD8<sup>+</sup> thymocytes and were assayed for V<sub>H81X</sub> to D<sub>052</sub> rearrangement (n=2).

548

Fig. 3. Sense and antisense transcription in the IgH variable locus. (A) The top scheme shows the germ-line transcripts analyzed in the D-C $\mu$  domain. The I $\mu$  and  $\mu$ 0 sense transcripts are derived from E $\mu$  and D<sub>Q52</sub> promoter respectively while antisense transcripts originate from E $\mu$  region, and an ill-defined promoter around DST4 segment (26). Dots indicate that the initiation and termination sites of the indicated transcripts were not precisely mapped. pA,

554 polyadenylation site. Germ-line transcripts were quantified by RT-qPCR in RAG-2-deficient 555 pro-B cells (left,  $n\geq 6$ ) and thymuses (middle,  $n\geq 3$ ), and in RAG2-proficient CD4<sup>+</sup>CD8<sup>+</sup> 556 thymocytes (right)  $(n \ge 3)$ . (B) Analysis of distal  $(dV_H)$  germ-line transcripts by semi-557 quantitative RT-PCR (left). Results of two independent experiments are shown (n=4). S, 558 spliced transcripts (sense); US, unspliced (antisense/primary sense) transcripts. Quantification 559 of the bands is displayed in the histograms on the right. (C) The top schemes show the 560 relative position of the primers used along the IgH variable domain. Bottom: The histograms 561 display the antisense transcript levels as measured by RT-qPCR ( $n\geq 6$ ). AS, antisense. (D) The 562 upper scheme indicates the relative position of the analysed germ-line transcripts along the 563  $Ig\kappa$  locus. The histograms display the transcript levels in pro-B and pre-B cells (n=3). (E) RT-564 qPCR analysis of  $\mu$  (V<sub>H</sub>DJ<sub>H</sub>C $\mu$ ) and I $\mu$  transcripts in pro-B, pre-B and unstimulated splenic B 565 cells. Forward primers that bind the distal V<sub>H</sub> (dV<sub>H</sub>) genes or Iµ exon and a reverse primer 566 that pairs with Cµ1 exon were used to quantify  $\mu$  and Iµ cDNAs (n≥6). (F) RT-qPCR analysis 567 of hs3b and hs4 transcripts at various stages of B cell development (n=3). (\*\*\*, p<0.001; \*\*,

568 p < 0.01; \*, p < 0.05; ns, not significant; Data are presented  $\pm$  SEM).

569

570 Fig. 4. A speculative model linking the 3'RR-mediated silencing activity to allelic 571 **exclusion.** This model stipulates an interplay between *cis*-acting elements and  $\mu$  heavy chain 572 (HC) signalling. Among the *cis*-regulatory elements which play a role in allelic exclusion, 573 only the interactions between E $\mu$  enhancer and the 3'RR are shown. (A). Upon D-J<sub>H</sub> 574 recombination, Eµ enhancer up-regulates  $DJ_H$  transcription (Dµ transcripts), and sense and 575 anti-sense germ-line transcripts are detected at the IgH variable region. (B). A productive 576 rearrangement on one allele will lead to  $\mu$  HC surface expression in association with VpreB 577 and  $\lambda 5$  surrogate light chains and Ig $\alpha$ /Ig $\beta$  heterodimer, which will signal to the 3'RR on the 578 second allele to mediate a transcriptional silencing activity within the  $V_{\rm H}$  region, leading to 588

579	down-regulation of sense and antisense transcription and $V_{\mathrm{H}}\text{-}DJ_{\mathrm{H}}$ recombination. Cooperation
580	between $E\mu$ and the 3'RR on the productive allele up-regulates rearranged $\mu$ HC gene
581	expression, leading to the enforcement and maintenance of allelic exclusion. (C). If the first
582	rearrangement is not productive (not in frame and therefore no $\mu$ HC production), the 3'RR
583	receives no signal to mediate its silencing activity and $V_{\text{H}}\text{-}DJ_{\text{H}}$ can therefore occur on the
584	second allele. (D). In the absence of the 3'RR, the link between $\mu$ HC instruction and
585	germline transcription in the IgH variable region is lost, and allelic exclusion is disrupted.
586	$pV_{H}$ , proximal $V_{H}$ cluster; $dV_{H}$ , distal $V_{H}$ cluster.
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dVDJ-Cµ

(A)

WT //\_

dVн

**Productive VHDJH** 

(VDJ+ → µHC)

рVн



≻Dμ **D-JH recombination** Iμ DJH4 WT //dVн D 3'RR Cμ ⇒Dμ (B) Iμ DJH2 Excluded DJH allele dVн рѴн D Cμ 3'RR ⇒Dμ 3'RR-mediated silencing activity μ HC-instruction μ 3'RR-mediated silencing **VH-DJH recombination Allelic exclusion** ĺμ VHDJH4 3'RR Productive VHDJH dVн Cμ (VDJ+ **→** μ HC) (C) Iμ DJH1 dVн D Cμ 3'RR Ongoing VH-DJH ⇒Dμ No µHC instruction μ **Vн-DJн recombination** No 3'RR-mediated silencing No allelic exclusion Îμ VнDJнз Non productive VHDJH dVн 3'RR Cμ (VDJ- → no µHC) (D) Iμ DJH1 **Ευ** Cμ Δ3'RR)-Ongoing VH-DJH dVн D рVн ⇒Dμ No 3'RR-mediated μ **VH-DJH recombination** silencing No allelic exclusion

Iμ

ĺμ

Δ3'RR -

Cμ

VнDJнз

dVн

3'RR

Сμ

DJH2

D

Junction	Nb seq	D <sub>FL16</sub>	DSP	D <sub>Q52</sub>
$D-J_{H}(WT)$	39	9	29	1
$D-J_{H}(\Delta 3'RR)$	42	10	31	1
$V_{H}$ -D- $J_{H}$ (WT)	22	9	11	2
$V_{H}$ -D- $J_{H}(\Delta 3'RR)$	23	8	14	1

### Table 1: D segment usage

**Table 1.** Genomic DNAs were purified from sorted WT and  $\Delta 3$ 'RR pro-B cells, and subjected to PCR using degenerate primers that amplify  $DJ_H$  or  $V_HDJ_H$  segments. Amplicons were cloned and sequenced. The junctional diversity was used to check for the clonality of the sequences. Thus, sequences with identical insertions/deletions were considered as one. Within the limits of our data set, there is no obvious anomaly with regard to the D gene segments usage or to the number of inserted or deleted nucleotides.