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

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*Research article*

# A developmental switch in the transcriptional activity of a long-range regulatory element

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**Short title:** *IgH* 3'RR-mediated silencing activity

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## Abstract

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

## Introduction

The spatial and temporal control of gene expression in metazoans is effected by regulatory elements that are often located far from gene promoters (1). This pattern of gene expression regulation is a hallmark of antigen receptor loci whose expression involves both transcription and recombination. The mouse *IgH* locus contains ~195 variable ($V_H$) genes subdivided into domain-organized gene families, including the distal $V_H$, by far the largest, and the proximal $V_H$ family. The $V_H$ genes are followed by a dozen of diversity (D) segments, 4 joining ($J_H$) segments, and 8 constant ($C_H$) genes (2, 3) (Fig. 1A, top scheme). The assembly of an *IgH* variable region exon through V(D)J recombination occurs in early developing B cells in an ordered manner, first D to $J_H$ then $V_H$ to $DJ_H$. While the first recombination step (D-$J_H$) can also be detected in developing T cells, $V_H$-$DJ_H$ recombination is strictly B cell-specific (4).

In addition to its B-lineage specificity, $V_H$-$DJ_H$ rearrangement is regulated by allelic exclusion which enables mono-allelic expression of only one *IgH* locus by a given B cell (4, 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 $V_H$-$DJ_H$ recombination on the second allele. If the first rearrangement is not productive (*i.e.* no μ heavy chain production), then the second allele can undergo $V_H$-$DJ_H$ recombination (4, 5). There is considerable evidence in support of the notion that $V_H$-$DJ_H$ rearrangement is the regulated step in *IgH* allelic exclusion and its maintenance through a feed-back mechanism (4, 5), although the molecular mechanisms through which feed-back signaling inhibits $V_H$-$DJ_H$ recombination remain unclear.

Another level of regulation of $V_H$-$DJ_H$ recombination relates to the physical location of $V_H$ gene segments within the variable domain. Indeed, several gene targeted studies showed that recombination of the distal and the proximal domains is regulated very differently (4, 6, 7).

65 Additionally, allelic exclusion of the distal $V_H$ genes is more stringent than that of the most
66 proximal $V_H$ 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 Eμ 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 $V_H$ 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 $C_H$ genes during class switch
79 recombination in mature B cells and *IgH* 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.

83 The established role of the 3'RR in *IgH* locus expression in late B cells and the lack of
84 effect on repertoire diversity led to the notion that the 3'RR activity is restricted to the late
85 stages of B cell maturation only (19, 20). However, various studies described long-range
86 interactions between the 3'RR and various upstream sequences, including Eμ enhancer (15,
87 16, 21, 22) though their functional significance is unclear. Strikingly, deletion of either Eμ or
88 the 3'RR had no effect on long-range interactions mediating locus contraction of the *IgH*

89 locus, leading to the proposal that the activity of these elements may be restricted to the ~270
90 kb D-$C_H$ region (22).

91 In this study, we used a mouse line devoid of the 3'RR (19) (hereafter called Δ3'RR) to
92 explore its role in V(D)J recombination and associated germ-line transcription which occurs
93 at distances ~220 kb to megabases far from the 3'RR. Here, we report the striking finding that
94 the 3'RR mediates a transcriptional silencing activity which is switched off after completion
95 of $V_H$-$DJ_H$ recombination.

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## Materials and Methods

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

**Antibodies.** APC-conjugated anti-B220 and FITC-conjugated anti-IgM were from BioLegend. PE-conjugated anti-CD43, FITC-conjugated anti-Igκ, 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. $5x10^5$ cells/assay were stained with anti-B220, anti-CD43 and anti-IgM or anti-Igκ, and gated either on $IgM^-$ or $Ig\kappa^-$ population. Data were obtained on 2.0 x $10^4$ viable cells by using a BD FACSCalibur. FACS acquisitions included isotype controls and exclusion of dead cells by labelling with propidium iodide.

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

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

123 B220$^{+}$IgM$^{-}$CD43$^{low}$) 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 µg/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 *β-actin* and *Gapdh* RNAs as controls.
130 **Statistical analysis.** Results are expressed as mean ± 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 (***).
134

## 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) on genomic DNAs from sorted WT and Δ3'RR pro-B cells and $CD4^+CD8^+$ 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 un-rearranged $D_{Q52}$ 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-$J_H$ 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 $V_HDJ_H$ 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 129Ola ES cells which bear a ~120-kb internal deletion in the proximal $V_H$ domain (22).

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

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

To this end, we attempted to detect $V_HD$ amplicons, which result from a direct $V_H$-D recombination. Genomic DNAs were extracted from purified pro-B cells and $CD4^+CD8^+$ thymocytes, and assayed for $V_H$-D recombination by using a forward primer pairing with $V_{H81X}$ gene and a backward primer downstream of the $D_{Q52}$ segment (Fig. 2B). This sequence is deleted following any D-$J_H$ rearrangement, but not if the $V_{H81X}$ segment directly recombines with $D_{Q52}$ segment. As a positive control, we used genomic DNAs from IGCR1 $CBE^{-/-}$ pro-B cells and $CD4^+CD8^+$ thymocytes, which undergo $V_{H81X}$-$D_{Q52}$ recombination (16). We found no evidence for $V_HD$ amplicon in Δ3'RR pro-B cells or in $CD4^+CD8^+$ thymocytes (Fig. 2B). Thus, the 3'RR does not affect the order of rearrangements.

**The 3'RR down-regulates sense and anti-sense transcription in the distal variable region.** Germ-line transcription in the variable region precedes $V_H$-$DJ_H$ recombination (27, 28). To investigate the effect of the 3'RR on germ-line transcription of un-rearranged genes, we first brought the Δ3'RR mutation into RAG-2-deficient background which precludes V(D)J recombination. We mainly focused on the D-Cμ and the distal $V_H$ domains where high levels of transcription are detected (7, 16).

185 Germ-line transcription within the D-Cμ domain, but not within the distal $V_H$ domain, is
186 regulated by Eμ enhancer (8-12, 29). In contrast, the effect of the 3'RR is unknown. We
187 found no obvious effect on Iμ or μ0 sense transcripts (derived from Eμ enhancer and $D_{Q52}$
188 promoter respectively), or on $D_{SP}$ 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
190 Iμ, μ0 and $D_{SP}$ GL transcripts were found in RAG-2-deficient thymuses and in RAG-2-
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 Eμ-
193 mediated control of germ-line transcription in the D-Cμ domain does not require the 3'RR.
194 Remarkably, the distal $V_H$ region yielded an increase of both spliced, sense transcripts,
195 and unspliced, antisense transcripts in Δ3'RR mice (Fig. 3B). To exclude any bias potentially
196 introduced by the increased primary $V_H$ 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 $V_H$ 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 Δ3'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.
208 Transcription of some loci could be regulated by elements located on a different
209 chromosome (32). Specifically, it was suggested that the *Igκ* locus and its 3' enhancer (on

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

**Switching off the 3'RR-mediated silencing activity coincides with the completion of V(D)J recombination.** To elucidate precisely at which step the 3'RR-mediated silencing activity is turned off, we quantified the transcript levels of fully rearranged μ gene at various developmental stages. To avoid potential bias introduced by cellular selection and/or selective use of distal versus proximal $V_H$ genes, we also measured Iμ transcript levels. We found normal levels of the distal $V_H$ exon-containing μ transcripts ($dV_HDJ_HC\mu$) in pro-B cells (Fig. 3E). These transcript levels were unchanged in pre-B cells, but were clearly decreased in 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). Interestingly, the shift from a silencer (in pro-B cells) to an enhancer (pre-B cells) activity 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.

**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).

As mentioned previously, the Δ3'RR mouse line is derived from 129Ola ES cells which have a 120 kb internal deletion in the proximal $V_H$ domain, which is not the case in the RAG-2-deficient mice which were 129Sv-derived. Thus, although we cannot formally exclude the possibility that the 120 kb deletion within the proximal $V_H$ domain may have affected distal $V_H$ germ-line transcription and V(D)J recombination, we think it is unlikely for various reasons. Multiple studies clearly showed that the proximal and the distal $V_H$ domains were differentially regulated. Thus, recombination of distal but not of proximal $V_H$ genes is inhibited in mice deficient in the histone-modifying enzyme EZH2 and different transcription factors involved in V(D)J recombination such as PAX5, YY1, and Ikaros (35-38). Mutations targeting various *cis*-acting elements at the *IgH* locus similarly showed a differential effect on germ-line transcription and recombination of proximal *versus* distal $V_H$ genes (12, 16, 39-42). Importantly, deletion of the 3'RR in the context of the 120 kb deletion had no effect on long-range interactions across the *IgH* locus in RAG2-deficient pro-B cells (22). Additionally, within the *IgH* variable region, the viewpoints that were found by 4C-seq analyses to strongly or minimally interact with the 3'RR correlated well with our transcriptional analyses. For instance, antisense transcription upstream of $V_{H81X}$ gene (which is intact in 129Ola 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

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

Whether the long-range 3'RR-mediated silencing activity is due to an unidentified developmentally-regulated silencer within the 3'RR itself or is mediated by interacting partners requires further investigations involving combined mutations. The strong correlation 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 $V_H$-$DJ_H$ recombination. Likely candidates could be the IGCR1 (16, 21, 22) and/or the newly identified interaction site upstream of IGCR1 (22). This could be a mean through which recombination regulates the 3'RR transcriptional activity. Alternatively, though not-mutually-exclusive, the correlation between the triggering of the 3'RR transcription and its enhancer activity suggests that the long-range activity of the 3'RR during B cell development may be modulated by its enhancers' transcripts.

The B cell-specific down-modulating effect of the 3'RR on D-$J_H$ recombination suggests that 3'RR/Eμ interaction may affect Eμ-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 Eμ 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-$J_H$ recombination clearly

indicating that the effect of the 3'RR occurs after the initiation of V(D)J recombination. In contrast, there was a relative accumulation of $DJ_H$ intermediates and fully recombined $V_HDJ_H$ alleles with no obvious block at the pro-B cell stage at which V(D)J recombination at the *IgH* locus occurs. Thus, it appears that we are dealing with a specific phenomenon which is restricted to pro-B cells: Increased recombination events in a "fixed" time window. One simple explanation is that, in the absence of the 3'RR, the process runs faster. Recent evidence highlighted the importance of spatial confinement for the kinetics of V(D)J recombination and time encounter with regulatory elements (46). It is tempting to speculate that 3'RR interactions with its partners are a critical component of the mechanisms that regulate the kinetics of V(D)J recombination. Within a newly generated topological domain that forms upon $DJ_H$ recombination, the 3'RR may for instance contribute to the control of the kinetics of $V_H$-$DJ_H$ recombination by impacting germ-line transcription within the $V_H$ domain, while Eμ is focused on $DJ_H$ transcription (40).

Why does the 3'RR mediate a transcriptional silencing within the $V_H$ domain? It should be noted that $V_H$-$DJ_H$ recombination is the regulated step in allelic exclusion (4, 5), and that the control of germline transcription is likely the primary event during allelic exclusion (42). In the absence of the 3'RR, we found an increase of germline transcription within the distal $V_H$ domain (with the exception of PAIR promoter/enhancer-derived transcripts) and in the proportion of distal $V_HDJ_H$ alleles, with no obvious block at the pro-B cell stage. This increase could be due, at least in part, to accumulated $DJ_H$ substrates. However, it may also indicate a disruption of allelic exclusion. Thus, our findings of enhanced $V_H$-$DJ_H$ recombination and germ-line transcription may be explained by a speculative model (See Figure 4) in which a productive rearrangement on one allele instructs the 3'RR on the second allele to down-regulate antisense transcription, leading to the inhibition of $V_H$-$DJ_H$ recombination. In the absence of the 3'RR, a productive $V_HDJ_H$ rearrangement on the first





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allele (and subsequent surface expression of μ heavy chain) would not block $V_H$-$DJ_H$ recombination on the second allele, leading to an overall accumulation of $V_HDJ_H$ alleles.

In this regard, our model may fill a gap in the regulated/feed-back inhibition model of allelic exclusion. Indeed, how to explain that a productive rearrangement on the first allele inhibits $V_H$-$DJ_H$ recombination on the second allele? Our interpretation is that surface expression of the μ heavy chain instructs the 3'RR to inhibit germline transcription within the variable domain and therefore $V_H$-$DJ_H$ recombination. Thus, 3'RR-mediated inhibition of $V_H$ germline transcription could be the missing link between surface expression of the heavy chain and effective allelic exclusion. One prediction of this model is that deletion of the 3'RR will result in increased $V_H$ germline transcription and $V_H$-$DJ_H$ frequency: That is what we found in the present study. Another prediction is that if a heavy chain were prematurely expressed (that is, prior to V(D)J recombination), the 3'RR would be instructed to inhibit $V_H$ germline transcription and impairment of $V_H$-$DJ_H$ recombination would be the outcome: That is indeed the case (42).

The wide impact of the 3'RR on sense and antisense germ-line transcription within the variable region raises the question as to whether the 3'RR targets sense and antisense promoters simultaneously. We favor the view that the 3'RR targets primarily antisense promoters and that the silencing of sense transcripts could be a downstream consequence of this primary effect. It should be noted that antisense transcripts are long and extend through multiple $V_H$ genes (28). Thus, the control of a limited number of antisense promoters would be sufficient for a wide transcriptional impact. Nonetheless, the 3'RR must somehow reach its distant target promoters. The possibility of a long-range effect mediated by 3'RR transcripts was ruled out because they were undetectable in pro-B cells. This would rather imply that the 3'RR-mediated silencing activity correlates with the lack of its transcription. A likely 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-$J_H$ 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.

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

In conclusion, our study reveals a hitherto unsuspected function of the 3'RR during early B cell development. The 3'RR emerges as a master regulatory element that mediates a transcriptional silencing activity along the distant and large *IgH* variable region, leading to the inhibition of $V_H$-$DJ_H$ recombination likely to promote allelic exclusion.

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**Author contributions**

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

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**Figure legends**

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

**(A)** The top scheme shows the mouse *IgH* locus (not to scale). CBEs, CTCF-binding elements. Not all CBEs are shown. Genomic DNAs were prepared from sorted WT and Δ3'RR pro-B cells and subjected to qPCR to amplify un-rearranged $D_{Q52}$ and $J_{H1}$ gene segments. The relative position of the primers is indicated in the upper scheme. Genomic DNA from *Rag2*$^{-/-}$ mice was used as a control. hs5 sequence was used for normalization (n=4). **(B)** Genomic DNAs from sorted pro-B cells (left) and double-positive thymocytes (right) were subjected to qPCR to quantify D-$J_{H1}$, D-$J_{H2}$, D-$J_{H3}$ or D-$J_{H4}$ recombination events. Rag2$^{-/-}$ and kidney DNAs were used as negative controls (n≥4). **(C)** Quantification of distal ($dV_H$) $V_H$-$DJ_H$ recombination events in pro-B cells by qPCR (n≥4). (**, $p$<0.01; *, $p$<0.05; ns, not significant; Data are presented ± SEM).

**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 Δ3'RR were stained with anti-B220+anti-CD43+anti-IgM, and gated on IgM$^-$ population (n=11) (*, $p$<0.05; ns, not significant; Data are presented ± SEM). **(B)** Genomic DNAs were prepared from sorted pro-B cells ($B220^+IgM^-CD43^{high}$) and $CD4^+CD8^+$ thymocytes and were assayed for $V_{H81X}$ to $D_{Q52}$ rearrangement (n=2).

**Fig. 3. Sense and antisense transcription in the IgH variable locus. (A)** The top scheme shows the germ-line transcripts analyzed in the D-Cμ domain. The Iμ and μ0 sense transcripts are derived from Eμ and $D_{Q52}$ promoter respectively while antisense transcripts originate from Eμ 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,

polyadenylation site. Germ-line transcripts were quantified by RT-qPCR in RAG-2-deficient pro-B cells (left, n≥6) and thymuses (middle, n≥3), and in RAG2-proficient $CD4^+CD8^+$ thymocytes (right) (n≥3). **(B)** Analysis of distal ($dV_H$) germ-line transcripts by semi-quantitative RT-PCR (left). Results of two independent experiments are shown (n=4). S, spliced transcripts (sense); US, unspliced (antisense/primary sense) transcripts. Quantification of the bands is displayed in the histograms on the right. **(C)** The top schemes show the relative position of the primers used along the *IgH* variable domain. Bottom: The histograms display the antisense transcript levels as measured by RT-qPCR (n≥6). AS, antisense. **(D)** The upper scheme indicates the relative position of the analysed germ-line transcripts along the *Igκ* locus. The histograms display the transcript levels in pro-B and pre-B cells (n=3). **(E)** RT-qPCR analysis of μ ($V_HDJ_HC\mu$) and Iμ transcripts in pro-B, pre-B and unstimulated splenic B cells. Forward primers that bind the distal $V_H$ ($dV_H$) genes or Iμ exon and a reverse primer that pairs with Cμ1 exon were used to quantify μ and Iμ cDNAs (n≥6). **(F)** RT-qPCR analysis of hs3b and hs4 transcripts at various stages of B cell development (n=3). (***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; ns, not significant; Data are presented ± SEM).

**Fig. 4. A speculative model linking the 3'RR-mediated silencing activity to allelic exclusion.** This model stipulates an interplay between *cis*-acting elements and μ heavy chain (HC) signalling. Among the *cis*-regulatory elements which play a role in allelic exclusion, only the interactions between Eμ enhancer and the 3'RR are shown. **(A)**. Upon D-$J_H$ recombination, Eμ enhancer up-regulates $DJ_H$ transcription (Dμ transcripts), and sense and anti-sense germ-line transcripts are detected at the *IgH* variable region. **(B)**. A productive rearrangement on one allele will lead to μ HC surface expression in association with VpreB and λ5 surrogate light chains and Igα/Igβ heterodimer, which will signal to the 3'RR on the second allele to mediate a transcriptional silencing activity within the $V_H$ region, leading to

down-regulation of sense and antisense transcription and $V_H$-$DJ_H$ recombination. Cooperation between Eμ and the 3'RR on the productive allele up-regulates rearranged μ HC gene expression, leading to the enforcement and maintenance of allelic exclusion. **(C)**. If the first rearrangement is not productive (not in frame and therefore no μ HC production), the 3'RR receives no signal to mediate its silencing activity and $V_H$-$DJ_H$ can therefore occur on the second allele. **(D)**. In the absence of the 3'RR, the link between μ HC instruction and germline transcription in the *IgH* variable region is lost, and allelic exclusion is disrupted. $pV_H$, proximal $V_H$ cluster; $dV_H$, distal $V_H$ cluster.

Fig. 1

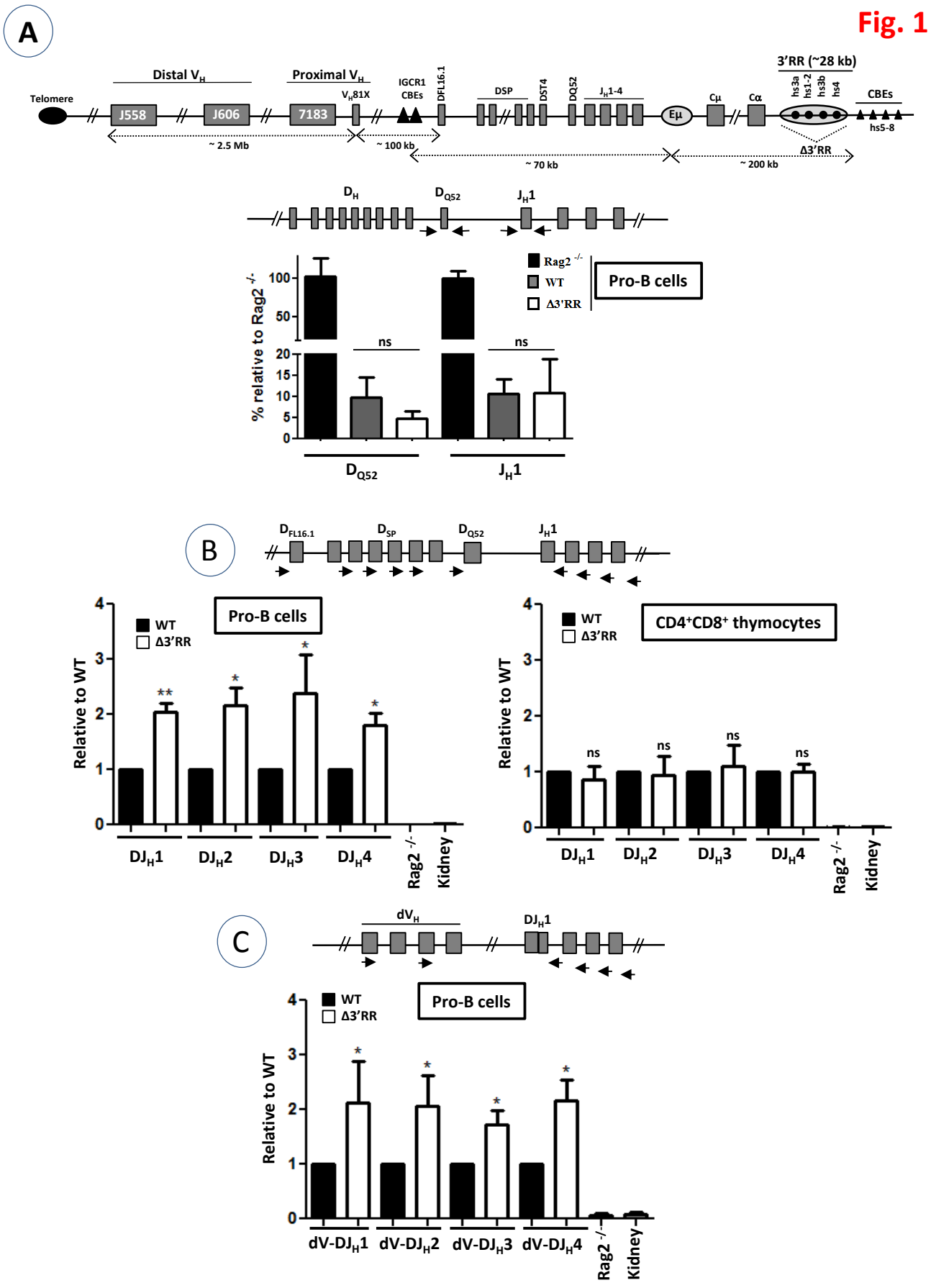

**Fig. 2**

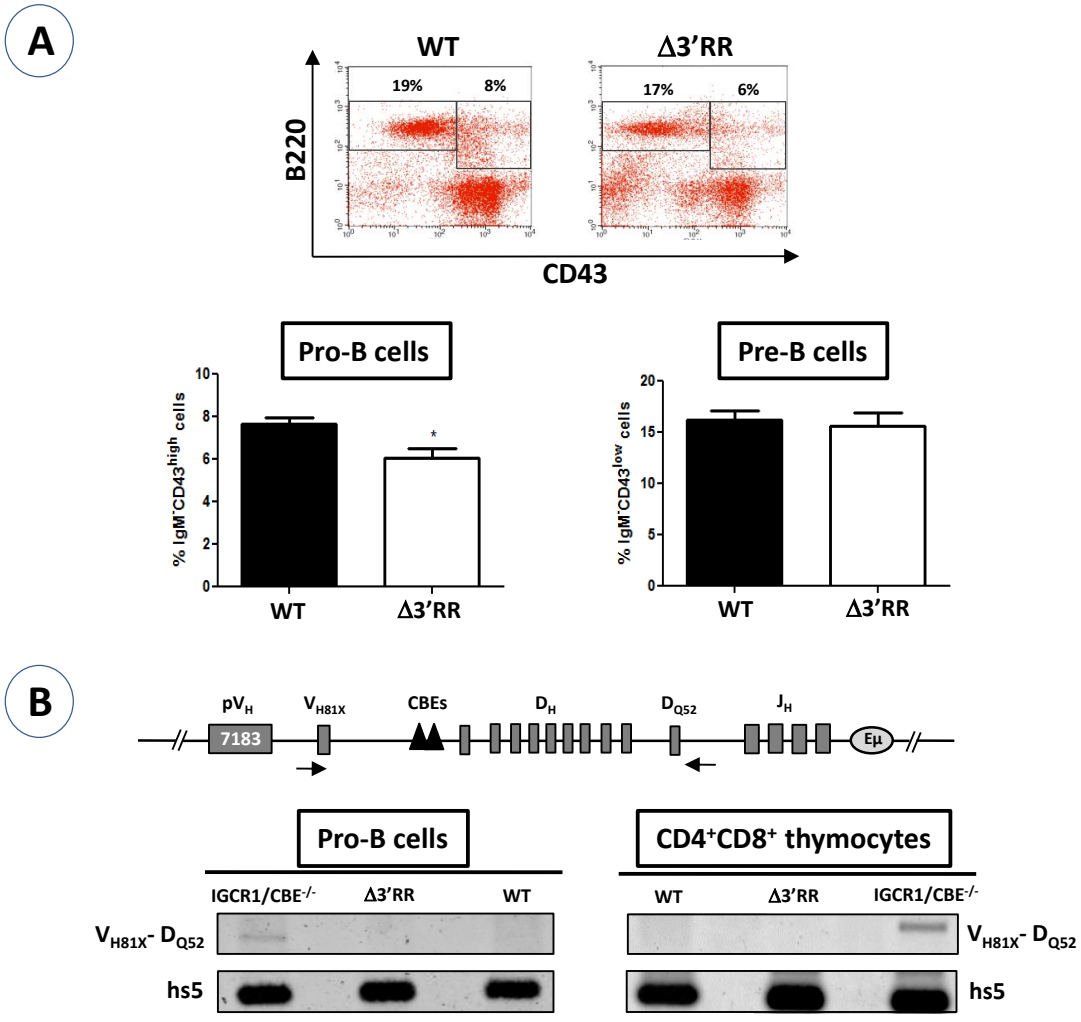

Fig. 3

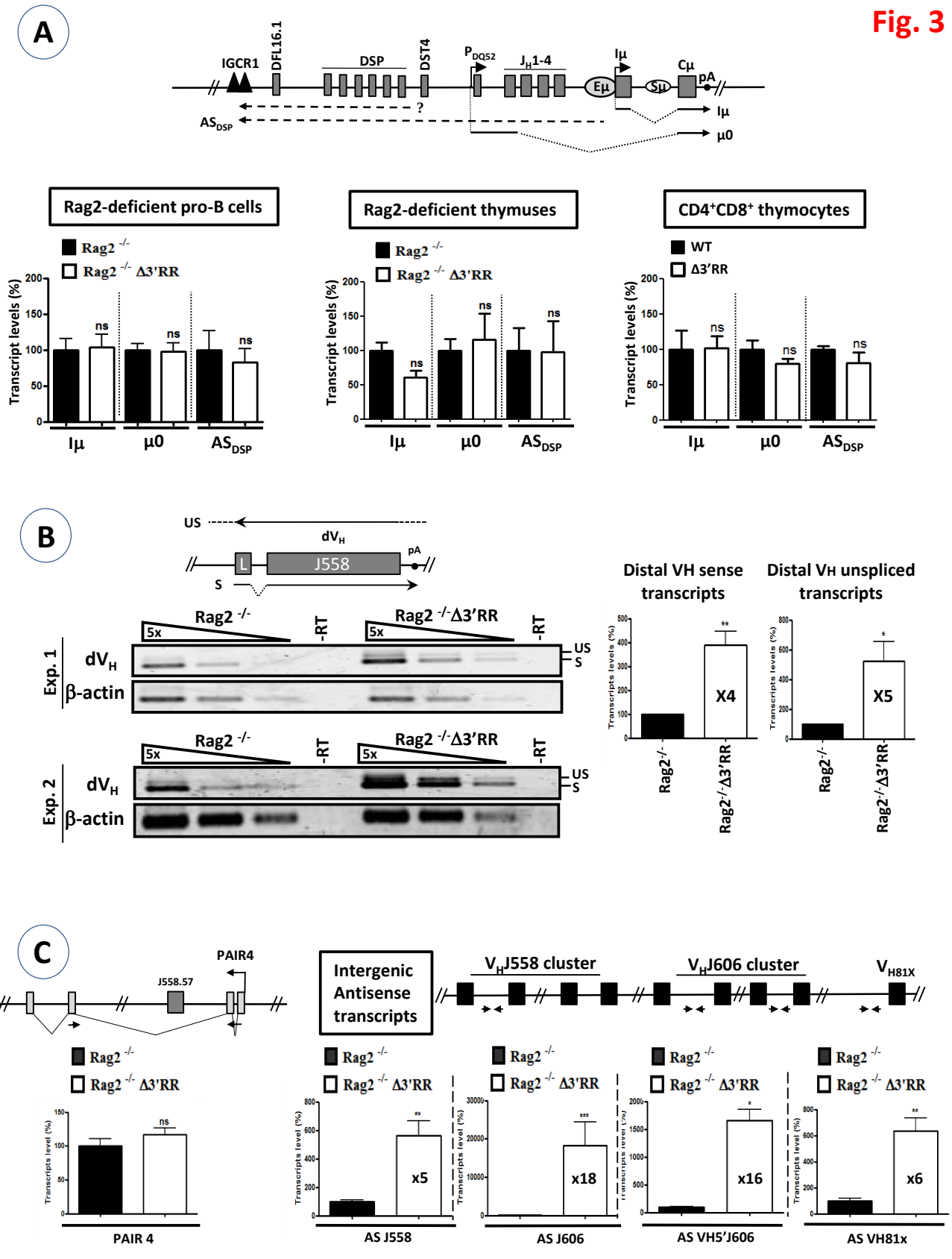

**Fig. 3**

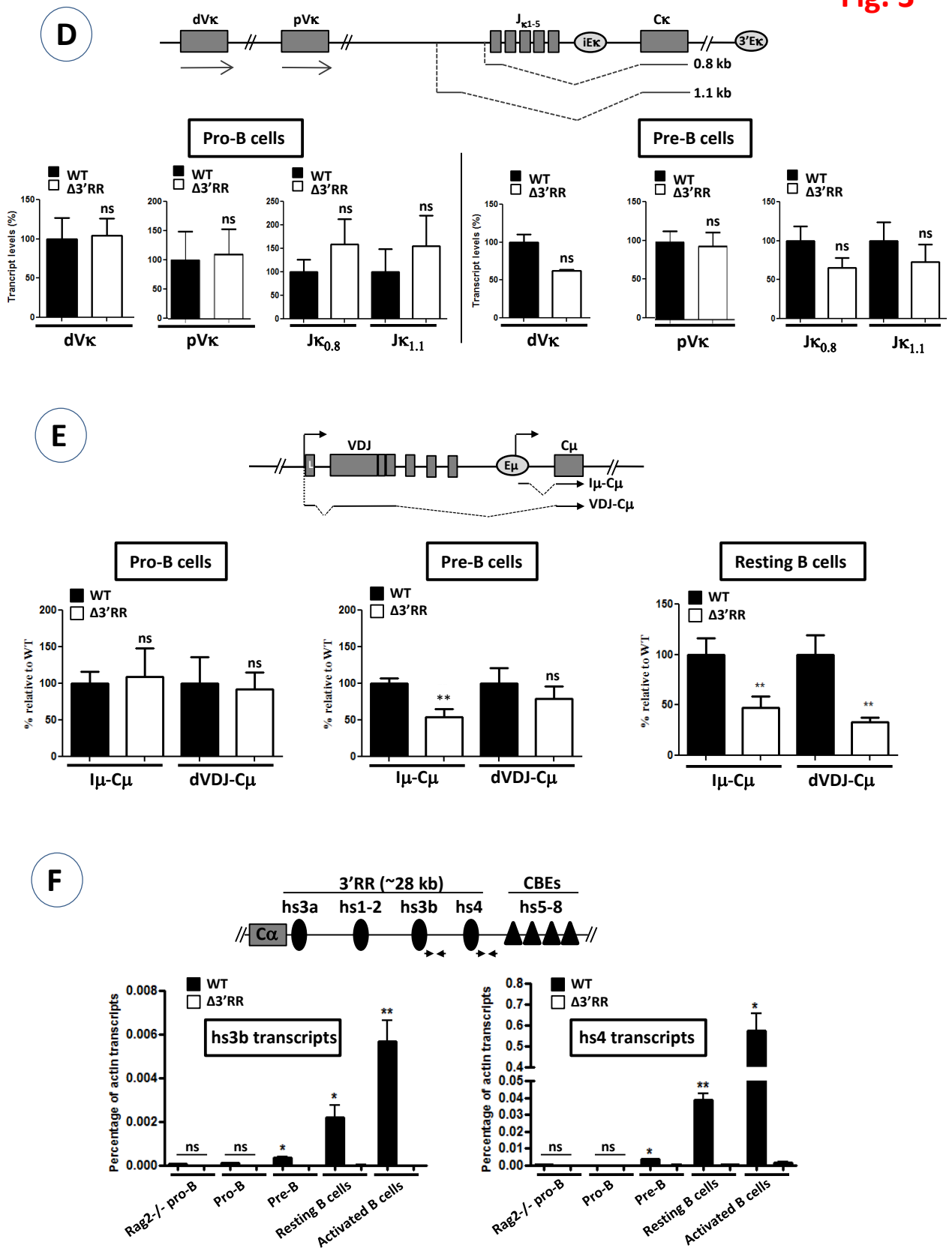

Fig. 4

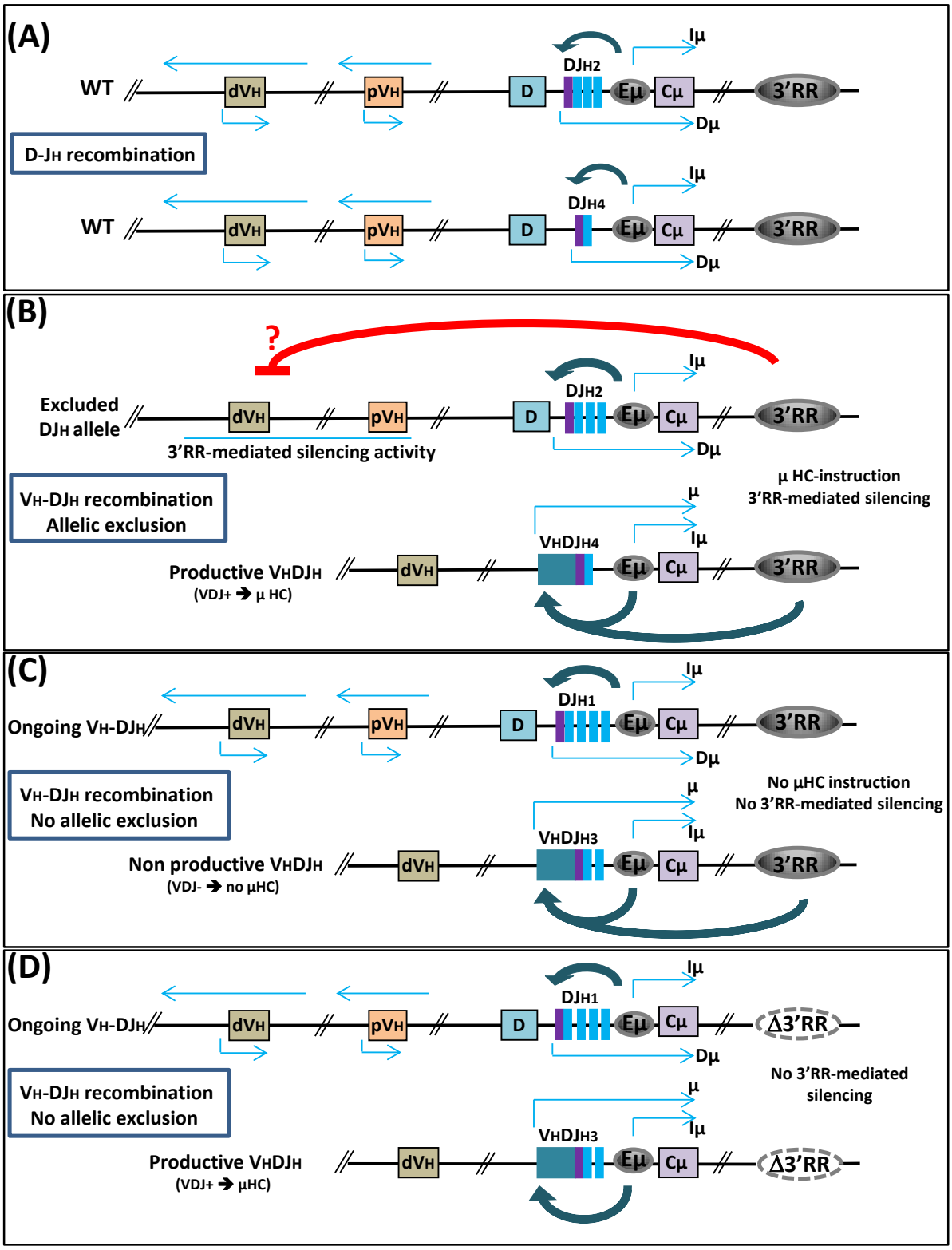

**Table 1: D segment usage**

| Junction | Nb seq | $D_{FL16}$ | DSP | $D_{Q52}$ |
|---|---|---|---|---|
| D-$J_H$ (WT) | 39 | 9 | 29 | 1 |
| D-$J_H$ (Δ3'RR) | 42 | 10 | 31 | 1 |
| $V_H$-D-$J_H$ (WT) | 22 | 9 | 11 | 2 |
| $V_H$-D-$J_H$ (Δ3'RR) | 23 | 8 | 14 | 1 |

**Table 1.** Genomic DNAs were purified from sorted WT and Δ3'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.