Highlights

- Enhancers are critical for the activation of V(D)J recombination
- The small murine *Igλ* locus is ideal for systematically mapping regulatory enhancers
- Using BAC constructs in transgenic mice we identify HS1 as a key enhancer
- HS1 appears to both activate recombination and *restrict* its stage specificity

The Eλ3-1 Enhancer is Essential for V(D)J Recombination of the Murine Immunoglobulin Lambda Light Chain Locus

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Abstract

Enhancers are essential for long range chromatin opening and the activation of V(D)J recombination at the antigen receptor loci. The murine immunoglobulin lambda light chain locus is a duplicated locus and, using a bacterial artificial chromosome spanning the 3' half of the locus to generate transgenic mice, we have identified a critical enhancer element for lambda locus recombination. Four hypersensitive sites had been previously mapped downstream of the $JC\lambda_1$ gene segment (HS1-4). Systematic deletion of these individual hypersensitive sites showed that HS1, which forms the major part of the transcription enhancer, $E\lambda_{3\text{-}1}$, is essential for Igλ recombination and that it also *helps to restrict* Igλ stage-specific recombination.

Introduction

V(D)J recombination generates a highly diverse antigen receptor repertoire but since the reaction involves the breakage and rejoining of DNA, it must be very tightly controlled. Recombination is initiated by just two lymphocyte-specific proteins, RAG1 and RAG2, that bind to conserved recombination signal sequences (RSSs) that lie adjacent to all V, D and J gene segments in the antigen receptor loci. Despite using the same proteins and conserved RSSs, immunoglobulin and T cell receptor genes rearrange specifically in B and T cells, respectively and only at distinct stages of lymphocyte development.

The generation of an accessible chromatin structure at the RSSs is central to this regulation (reviewed by [1]) and knock-out studies have shown that tissue-specific enhancers play an essential role in regulating this accessibility by restricting it to the correct cell and the correct stage of lymphocyte development [2]. Recombination enhancers have been identified for most immunoglobulin and T cell receptor loci and are often identical to the enhancers that upregulate transcription of the rearranged gene [3]. However, since some recombination enhancers function redundantly [4], it is difficult to confidently identify all the regulatory enhancers within a given locus.

The murine *Igλ* light chain locus is the smallest antigen receptor locus and thus is a good model with which to systematically analyse the critical regulatory elements. It spans only 240 kb on chromosome 16 and contains two gene clusters that appear to have arisen by duplication (reviewed by [5]). Each

cluster contains 1-2 variable gene segments followed by two joining/constant (J/C) regions, in the order *Vλ$_2$-Vλ$_x$-JCλ$_2$-JCλ$_4$--Vλ$_1$-JCλ$_3$-JCλ$_1$* [6, 7]; since *JCλ$_4$* is a pseudogene, the locus undergoes only four main rearrangements: Vλ$_1$-Jλ$_1$, Vλ$_2$-Jλ$_2$ Vλ$_x$-Jλ$_2$ and Vλ$_1$-Jλ$_3$ that occur in the ratio 3:2:2:1 [8, 9]. However, *Vλ$_2$* can also recombine with *JCλ$_1$* or *JCλ$_3$*, albeit infrequently [10]. These rare rearrangements suggest that at least some regulatory elements lie either 5' of *Vλ$_2$* or 3' of *JCλ$_1$* since recombination between *Vλ$_2$* and *Jλ$_1$* removes the intervening DNA.

Early studies to identify these regulatory elements relied on DNAse I hypersensitive mapping in B and T cell lines [11]. This identified a single hypersensitive site 15.5 kb downstream of *JCλ$_4$* that appeared to be B-cell specific and coincided with a strong transcriptional enhancer (Eλ$_{2\text{-}4}$). Rearrangement of *Vλ$_2$* to *JCλ$_3$* or *JCλ$_1$* deletes Eλ$_{2\text{-}4}$. However, a second strong enhancer was mapped 35 kb downstream of *Cλ$_1$*, which cannot be eliminated by any *Igλ* gene rearrangements (Figure 1A); this second *Igλ* transcriptional enhancer (Eλ$_{3\text{-}1}$) is 90% homologous to Eλ$_{2\text{-}4}$ [11].

Subsequently, more hypersensitive sites were identified downstream and within 5 kb of Eλ$_{3\text{-}1}$ [12], namely, HS1, HS2, HS3 and HS4 where Eλ$_{3\text{-}1}$ is identical to HS1 and HS2 (Figure 1A). Notably, generation of transgenic mice by co-injection of two overlapping cosmids, which integrated with the same organisation as the endogenous locus, demonstrated that inclusion of the hypersensitive site cluster HS1 to HS4, results in high level expression of *Igλ$_1$*

mRNA in B cells. This suggests that these downstream hypersensitive sites potentially behave as a locus control region (LCR) by conferring position independent, copy number dependent expression of the linked gene [12].

The enhancers that regulate *Igλ* recombination are largely unknown but the transcriptional enhancers ($E\lambda_{2\text{-}4}$ and $E\lambda_{3\text{-}1}$) as well as HS3 and HS4 are good candidates. Most *Igλ* rearrangement occurs between $V\lambda_1$ and $JC\lambda_1$ [13] and, since the *Igλ* locus is duplicated [5], a reasonable hypothesis is that at least some of the regulatory information is present in the 3' half of the locus. Therefore, the regulatory elements were analysed using a BAC construct that encodes the 3' half of the *Igλ* locus from the hypersensitive site 5' of $V\lambda_1$ (hsV_1) to the four hypersensitive sites that lie downstream of $JC\lambda_3$ (HS1-4; Figure 1B). Transgenic mice were generated and recombination examined in mice carrying the wild type BAC and those carrying deletions of each of the downstream hypersensitive sites. These studies identified HS1 as a critical element in the activation of Igλ recombination.

Materials and methods

Modification of BACs by homologous recombination.

The BAC rp23-247i11 was obtained from CHORI (Children's Hospital Oakland Research Institute) and modified by homologous recombination in DY380 cells as described [14]. Pools containing the targeted clone were screened by PCR using the primers listed in Supplementary Table 1.

T7 and Sp6 deletion cassettes.

Mouse sequences from the T7-proximal region of rp23-247i11 were removed using a deletion cassette that contained approximately 200 bp of homology 5' of the region to be deleted fused to ~200 bp 3' of the deletion site. The cassette was generated by two separate PCR reactions using 100 ng of rp23-247i11 as template and primers T7-HindIII plus T7-NotI and primers T7-3'HS/NotI plus 3'HS (Supplementary Table 1). The amplified products were used as templates in the second round of PCR with primers T7-HindIII and 3'HS. Following homologous recombination in DY380 cells, recombinants were screened by PCR using primers, 3'HSdel and T7 BAC.

DNA was deleted from the SP6 end of the clone, using a deletion cassette generated as described above except primers Sp6-PvuII plus Sp6-NotI and Sp6-5'HS/NotI plus 5'HS were used in the first round and primers Sp6-PvuII plus 5'HS in the second round of PCR. Positive recombinants were screened using 5.3'Vlambda and Vlambda check primers (Supplementary Table 1).

cMyc insertion cassette

The insertion cassette has a 39 bp cMyc tag between ~200 bp regions of homology that lie 5' and 3' of the Xho I site in $C\lambda_1$; it was generated by two rounds of PCR, as described in Supplementary Table 2 and inserted into the ΔT7/ΔSp6 BAC. Recombinants were screened using Cλ1-F2 and cMycR primers (Supplementary Tables 1 and 3).

The HS1, HS2, HS3 and HS4 deletion cassettes

Deletion cassettes for DNAse I hypersensitive sites 1, 2, 3 and 4 were generated by PCR using 100 ng of rp23-247i11 template and primers A + C or B + D for the first round of PCR (Supplementary Tables 1 and 2) and primers A and D for the second round. The PCR reactions used to screen the recombinant BACs are given in Supplementary Tables 2 and 3.

Recombinant BAC DNA was prepared using a Qiagen maxi-prep kit and verified by sequencing.

Isolation of BAC DNA for microinjection

Prior to microinjection, BAC vector sequence was removed by Not I digestion. The insert (~70 kb) was purified via a 5-25% sodium chloride gradient in a Beckman SW40 rotor at 37 000 rpm for 200 minutes at 4°C. The peak DNA fraction was precipitated, washed with 70% ethanol and resuspended in H_2O (~50 μg/□l) prior to microinjection. All transgenic mouse work was performed according to the UK Home Office guidelines with the necessary ethical approval and relevant UK licences.

Detection of Vλ$_1$-JCλ$_1$ rearrangement at endogenous and transgenic loci

Vλ$_1$-Jλ$_1$ products derived from the endogenous and transgenic loci were amplified in the same reaction by semi-quantitative PCR using 500 ng of spleen DNA and primers Vλ1-2/F and Cλ1-2/R (Supplementary Table 4). To distinguish the products from each locus, the PCR products were digested with Xho I. Following separation on a 0.8% agarose gel, the DNA was Southern blotted and hybridised at 60ºC overnight to the *Cλ$_1$* F3-R3 probe.

Detection of Vλ$_1$-JCλ$_1$ rearrangement at transgenic and endogenous *Igλ* loci by nested PCR.

Recombination of BAC constructs was detected via nested PCR, using a primer specific to the transgene, cMyc/F with Vλ1-2/F (Supplementary Table 4) and 50-400 ng of spleen DNA as template in the first round of PCR (15 cycles). An aliquot (0.8 μl) from this first round was used as template in the second round of PCR (25 cycles) using nested primers (Vλ1-Nested-F and Cλ1-Nested-R). The recombination products were detected by Southern blotting, using the Cλ$_1$ F3-R3 probe.

To detect rearrangement of the endogenous locus, the same amount of DNA was amplified by 25 cycles of semi-quantitative PCR using primers Vλ1-int/F and Cλ1-insert/R (Supplementary Table 4). The products were co-electrophoresed with nested PCR products, blotted and hybridized as above.

Analysis of recombination in *CD43+ and CD43- B cells*

CD43+/CD19+ and CD43-/CD19+ cells were purified from femurs of transgenic mice by flow cytometry; DNA was prepared and the levels normalised between samples as described [15]. Recombination of the *Igλ* locus was determined as described above.

Results

Generation of a BAC spanning the 3' half of the *Igλ* locus

To test which elements regulate *Igλ* recombination, mouse genomic sequences were deleted from the original BAC, rp23-247i11, to generate the BAC λΔT7/ΔSP6 that carries the 3' half of the lambda locus from upstream of the Vλ1 promoter to 3 kb downstream of HS4 (Figure 1B). To distinguish this BAC from the endogenous *Igλ* locus, a cMyc tag (39 bp) was introduced into the $C\lambda_1$ constant region so that it inserted into, and destroyed, the Xho I site; this generated the BAC, $C\lambda_1$-T (Figure 1B).

Analysis of recombination in transgenic mice carrying the wild type BAC construct

Hypersensitive sites 1-4 potentially have LCR activity [12] and we first wanted to determine whether collectively they are able to trigger chromatin opening for V(D)J recombination. Therefore, transgenic mice were generated with the tagged BAC construct, Cλ1-T. Since the *Igλ* locus normally rearranges in only 5% of B cells, these founder transgenic mice were crossed with CκT mice, in which the *Igκ* constant region has been deleted [16]. Heterozygous CκT mice have two fold more λ^+ producing cells than *Igκ* wild-type littermates [16] and thus $V\lambda_1$-$JC\lambda_1$ recombination should be more apparent in these crosses.

The level of recombination of the BAC transgene was then compared to that of the endogenous locus using spleen DNA from F_1 mice and PCR primers

specific to $V\lambda_1$ and downstream of the Xho I site in $C\lambda_1$ (Figure 2A). Since the cMyc tag disrupts the Xho I site, PCR products derived from the endogenous and transgene loci were distinguished by digestion with Xho I. We find that the BAC transgene undergoes recombination, albeit at a lower level than that of the endogenous locus by 3 to 6 fold (Figures 2B and 2C). Thus, whilst the regulatory elements within the BAC are sufficient to activate *Igλ* recombination, at least one additional element appears to be required for the full level of *Igλ* rearrangement.

Transgenic mice with deletions of the downstream hypersensitive sites. To determine which of the four downstream hypersensitive sites stimulate *Igλ* recombination, each hypersensitive site was separately deleted from the BAC construct using deletion cassettes that were generated by fusing up to 200 bp of sequence 5' of the region to be deleted with sequences 3' of the region via PCR. The enhancer, $E\lambda_{3\text{-}1,}$ has been previously found to contain two DNAse I hypersensitive sites that lie either side of a Bgl II restriction site ([17] and N. Dillon, personal communication). Therefore, two deletions were made: Firstly, the active transcriptional enhancer region (5' of the Bgl II site), that encompasses all the previously characterised $E\lambda_{3\text{-}1}$ transcription factor binding sites [18], was successfully deleted by removing 564 bp (ΔHS1). A 164 bp deletion of the second hypersensitive site, HS2 was next made to remove DNA 3' of the Bgl II site. In addition, hypersensitive sites HS3 and HS4 were separately eliminated by deleting 626 and 372 bp, respectively. *These deletions correspond to the size required to remove the main transcription factor binding*

sites in each hypersensitive site, as determined from in vitro footprinting studies for HS1 [18] and HS4 (Supplementary Figure 1) and/or the published size of the hypersensitive site [12]. Transgenic mice were generated using these BAC constructs and the founders crossed with CκT mice as above.

Recombination of the transgene determined by nested PCR.

Next, we measured the level of recombination and, to ensure that we could detect recombination even if it is very low, we adopted a more sensitive, nested PCR assay. Since we needed to compare the level of recombination among different samples, we optimised the PCR to ensure that the final product was proportionate to the input DNA and found this is indeed the case for the *wild type BAC* transgenes (Supplementary Figure *2*A and B) and the endogenous locus (Supplementary Figure *2*C) over the range of 50 to 400 ng input DNA. Moreover, we verified that this assay is quantitative at the typical transgene copy number of the mice carrying BACs with deleted hypersensitive sites (Supplementary Figure *2*B and Supplementary Table 5).

HS1 is essential for recombination

Recombination was then analysed in the BAC wild-type and mutant lines, initially using 50 ng of input DNA in the first round of the PCR. In all three transgenic lines in which HS1 had been deleted (ΔHS1), recombination of the transgene was eliminated (Figure 3A, left). To further test if HS1 is indeed critical for recombination, the assay was repeated using increased amounts of input DNA (200 and 400 ng). Once again, no recombination of the transgene

was detected in any of the ΔHS1 transgenic lines (Figure 3A, right); together, these data imply that HS1 is required for recombination.

In contrast, deletion of any of the other three hypersensitive sites resulted in recombination in at least one of the transgenic lines tested using 50 ng of input DNA (Figures 3B and 3C, left and Supplementary Table 5). This suggests that HS1 is the only critical element for recombination. To further verify that recombination was indeed possible in the absence of any of the other hypersensitive sites, we used increased amounts of DNA in the first round of PCR. Recombination of the ΔHS2 transgenic lines, was detectable using either 50 ng (ΔHS2-1) or 100 ng (ΔHS2-3, Figure 3B, right) of input DNA, suggesting that HS2 is not required for recombination. Likewise, recombination was detectable using 50 ng of DNA when HS3 was deleted. Finally, recombination is detectable in all the ΔHS4 lines, except ΔHS4-3 (Figure 3C), suggesting that HS4 also is not essential for recombination. *Since* recombination can be detected in the absence of HS2, 3 or 4, *this* suggests that none of these is essential for recombination.

Notably, mice were obtained with different numbers of copies of the BAC transgene. Importantly, however, recombination can be detected when three or more copies of the transgene are present, except when HS1 is deleted. This is fully consistent with our hypothesis that HS1 is essential for recombination. However, whilst hypersensitive sites 2, 3 and 4 are not essential for recombination, we are unable to conclude whether they contribute quantitatively

to the level of recombination due to the different copy numbers obtained upon deletion of these HS.

HS1 contributes to stage-specific recombination

Immunoglobulin gene rearrangement occurs at distinct stages of B cell development with IgH rearrangement occurring first in CD43 positive cells [19]. RAG proteins are then down-regulated; the IgH-expressing cells undergo rapid proliferation, after which RAG protein expression is up-regulated again in CD43 negative, small pre-BII cells. Most light chain rearrangement occurs in these small pre-BII cells although about 15% occurs in CD43 positive cells. [20]. To test if the BAC constructs properly recapitulate stage-specific recombination, and to determine which hypersensitive site might be responsible for this stage-specificity, primary *CD43 positive and CD43 negative* cells were purified from bone marrow by flow cytometry, *using CD19 as a pan-*B cell marker. The levels of DNA were normalised (Figure 4, lower panels) and rearrangement of the *Igλ* transgene *was then* compared to that of the endogenous locus. Since no recombination is detectable when HS1 is lost, it is not possible to test directly if this hypersensitive site regulates stage-specific recombination. However, deletion of either HS3 or HS4 gives the same stage-specific recombination *pattern* as the endogenous locus, *namely low levels in CD43+ cells that is increased 3-4 fold in CD43- cells* (Figure 4). This suggests firstly that the BAC constructs recapitulate *broad* stage-specific *pattern of* recombination and secondly that neither HS3 nor HS4 is required for this specificity. This further implies that HS1, that contains all known factor binding sites within $E\lambda_{3\text{-}1}$, *helps*

to promote Igλ recombination in CD43-/CD19+ cells. Although the different stages of CD43- B cells were not separated, it is clear that this enhancer, like the Igκ 3' enhancer [21], is sufficient to prevent premature Ig rearrangement in CD43+/CD19+ cells. Moreover, since RAG proteins are primarily expressed in small pre-BII cells in the CD43- population, it is likely that $E\lambda_{3\text{-}1}$ helps to activate recombination at this stage of B cell development.

Discussion

The *Igλ* locus is the smallest antigen receptor locus. Its small size, together with the fact that the locus is duplicated, enabled us to generate transgenic mice with a BAC encompassing all the unique, potential regulatory elements. Using this system, we could not only maintain the correct genomic organisation but also, since the hypersensitive sites had been extensively mapped, we could methodically analyse their role in regulating lambda locus recombination. We find that the BAC transgene undergoes V(D)J recombination with the same ***broad*** cell stage specificity as the endogenous locus. Systematic analysis of the known hypersensitive sites then identified HS1, the 5' part of $E\lambda_{3\text{-}1}$, as being critical for *Igλ* recombination.

Notably, recombination of the BAC occurs at a lower level than the endogenous locus. Thus, although the BAC carries enough information to trigger recombination, at least one additional regulatory element seems to be required for full Igλ recombination. A good candidate is the $E\lambda_{2\text{-}4}$ enhancer, which is 90% homologous to HS1 and is bound by the same transcription factors [18]. Indeed, the unrearranged *Igλ* locus is organised: $V\lambda_2$-$V\lambda_x$-$JC\lambda_2$-$JC\lambda_4$-$E\lambda_{2\text{-}4}$-$V\lambda_1$-$JC\lambda_3$-$JC\lambda_1$-$E\lambda_{3\text{-}1}$ [5]. Thus, whilst each cluster of two *JCλ* genes has its own downstream enhancer, two enhancers surround $V\lambda_1$-$JC\lambda_3$-*JCλ1*. Consistent with a role of $E\lambda_{2\text{-}4}$ in stimulating rearrangement of $V\lambda_1$-$JC\lambda_1$ and $V\lambda_1$-$JC\lambda_3$, these rearrangements occur at a higher frequency than that of $V\lambda_2$-$JC\lambda_2$ [22]. These data also raise the possibility that cross talk between $E\lambda_{3\text{-}1}$ and $E\lambda_{2\text{-}4}$, that map >100 kb apart, is required for full chromatin opening and activation of *Igλ* recombination.

The BAC system offered a significant advantage in that it enabled us to unequivocally determine a role for HS1 in activating recombination. Indeed, had this analysis been attempted within the full locus (via knock-out studies), or even using BAC constructs where $E\lambda_{2\text{-}4}$ is present, then the high conservation between $E\lambda_{2\text{-}4}$ and HS1 [18] would likely have resulted in a reduction in recombination upon deletion of HS1 rather than its complete loss. Instead, by analysing just the 3' half of the locus, the essential role of HS1 could be uncovered.

The critical role of HS1 in triggering recombination meant that it was not possible to directly test its role in the regulation of stage-specificity of recombination. However, deletion studies of HS3 and HS4 showed that neither of these hypersensitive sites measurably affects *the broad* stage-specific recombination *pattern*. *Importantly, loss of HS3 or HS4 does not result in premature Igλ recombination*, implying that $E\lambda_{3\text{-}1}$ could *direct recombination to CD43- stages of B cell development*. Only two B cell-specific transcription factors bind to $E\lambda_{3\text{-}1}$, PU.1 and IRF4 [23, 24] but notably, the levels of these proteins increase by two to three-fold *between CD43+ and CD43- B cells* [25]. Therefore, a plausible hypothesis is that this increase plays an important role in activating *Igλ* recombination *in CD43- cells*.

Together, our data highlight the utility of BAC constructs in transgenic mice to identify the elements that regulate *Igλ* V(D)J recombination. Our studies demonstrate that for the smallest antigen receptor locus, a single enhancer, HS1 is necessary and sufficient to trigger locus opening and recombination over 70 kb. These studies now pave the way to examine the mechanism by which this single enhancer triggers long range locus activation and V(D)J recombination.

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References

1. Bassing, C.H., W. Swat, and F.W. Alt, *The mechanism and regulation of chromosomal v(d)j recombination.* Cell, 2002. **109 Suppl**: S45-55.
2. Cobb, R.M., et al., *Accessibility control of V(D)J recombination.* Adv Immunol, 2006. **91**: 45-109.
3. Oltz, E.M., et al., *A V(D)J recombinase-inducible B-cell line: role of transcriptional enhancer elements in directing V(D)J recombination.* Mol Cell Biol, 1993. **13**(10): 6223-30.
4. Inlay, M., et al., *Essential roles of the kappa light chain intronic enhancer and 3' enhancer in kappa rearrangement and demethylation.* Nat Immunol, 2002. **3**(5): 463-8.
5. Eisen, H.N. and E.B. Reilly, *Lambda chains and genes in inbred mice.* Annu Rev Immunol, 1985. **3**: 337-65.
6. Dildrop, R., et al., *A new V gene expressed in lambda-2 light chains of the mouse.* Eur J Immunol, 1987. **17**(5): 731-4.
7. Storb, U., et al., *Physical linkage of mouse lambda genes by pulsed-field gel electrophoresis suggests that the rearrangement process favors proximate target sequences.* Mol Cell Biol, 1989. **9**(2): 711-8.
8. Nadel, B., P.A. Cazenave, and P. Sanchez, *Murine lambda gene rearrangements: the stochastic model prevails over the ordered model.* Embo J, 1990. **9**(2): 435-40.
9. Sanchez, P., B. Nadel, and P.A. Cazenave, *V lambda-J lambda rearrangements are restricted within a V-J-C recombination unit in the mouse.* Eur J Immunol, 1991. **21**(4): 907-11.

10. Reilly, E.B., A.R. Frackelton, Jr., and H.N. Eisen, *Synthesis of lambda 1, lambda 2, and lambda 3 light chains by mouse spleen B cells.* Eur J Immunol, 1982. **12**(7): 552-7.

11. Hagman, J., et al., *A novel enhancer in the immunoglobulin lambda locus is duplicated and functionally independent of NF kappa B.* Genes Dev, 1990. **4**(6): 978-92.

12. Eccles, S., et al., *Enhancer sequences located 3' of the mouse immunoglobulin lambda locus specify high-level expression of an immunoglobulin lambda gene in B cells of transgenic mice.* New Biol, 1990. **2**(9): 801-11.

13. Boudinot, P., et al., *Various V-J rearrangement efficiencies shape the mouse lambda B cell repertoire.* Eur J Immunol, 1995. **25**(9): 2499-505.

14. Liu, P., N.A. Jenkins, and N.G. Copeland, *A highly efficient recombineering-based method for generating conditional knockout mutations.* Genome Res, 2003. **13**(3): 476-84.

15. Grange, S. and J. Boyes, *Chromatin opening is tightly linked to enhancer activation at the kappa light chain locus.* Biochem Biophys Res Commun, 2007. **363**(1): 223-8.

16. Zou, Y.R., S. Takeda, and K. Rajewsky, *Gene targeting in the Ig kappa locus: efficient generation of lambda chain-expressing B cells, independent of gene rearrangements in Ig kappa.* Embo J, 1993. **12**(3): 811-20.

17. MacNeil, A. PhD Thesis (2000) University of London

18. Rudin, C.M. and U. Storb, *Two conserved essential motifs of the murine immunoglobulin lambda enhancers bind B-cell-specific factors.* Mol Cell Biol, 1992. **12**(1): 309-20.

19. *Osmond, D.G., A. Rolink, and F. Melchers, Murine B lymphopoiesis: towards a unified model. Immunol Today, 1998.* ***19****(2): 65-8.*

20. Novobrantseva, T.I., et al., *Rearrangement and expression of immunoglobulin light chain genes can precede heavy chain expression during normal B cell development in mice.* J Exp Med, 1999. **189**(1): 75-88.

21. *Hiramatsu, R., et al., The 3' enhancer region determines the B/T specificity and pro-B/pre-B specificity of immunoglobulin V kappa-J kappa joining. Cell, 1995.* ***83****(7): 1113-23.*

22. Persiani, D.M., J. Durdik, and E. Selsing, *Active lambda and kappa antibody gene rearrangement in Abelson murine leukemia virus-transformed pre-B cell lines.* J Exp Med, 1987. **165**(6): 1655-74.

23. Eisenbeis, C.F., H. Singh, and U. Storb, *PU.1 is a component of a multiprotein complex which binds an essential site in the murine immunoglobulin lambda 2-4 enhancer.* Mol Cell Biol, 1993. **13**(10): 6452-61.

24. Eisenbeis, C.F., H. Singh, and U. Storb, *Pip, a novel IRF family member, is a lymphoid-specific, PU.1-dependent transcriptional activator.* Genes Dev, 1995. **9**(11): 1377-87.

25. Johnson, K., et al., *Regulation of immunoglobulin light-chain recombination by the transcription factor IRF-4 and the attenuation of interleukin-7 signaling.* Immunity, 2008. **28**(3): 335-45.

Figure legends

Figure 1: Map of the locus and construct. A) Map of the whole *Igλ* locus [11] and B) Map of the BAC construct. A cMyc tag was inserted into *Cλ1*. Downward arrows identify the DNAse I hypersensitive sites. HsC_1 was shown previously not to affect Igλ transcription [11]. All Apa LI, Not I, Sna BI and Sph I restriction sites are shown, but only relevant Bam HI, Eco RI, Bgl II, and Kpn I sites are indicated. Only two Rsa I sites, are shown (in grey) since these were used to locate HS4. The Not I site shows the boundary with the BAC sequence. The diagram is largely to scale.

Figure 2: Recombination of the endogenous *Igλ* locus and wild type BAC transgene. A) Positions of the PCR primers used to simultaneously detect recombination of the endogenous locus and transgenic locus ($Vλ_1$-2/F and $Cλ_1$-2/R). The cMyc tag is indicated by a black box. The primers used to generate the Southern probe (Cλ1-R3 and Cλ1-F3) are indicated. B) $Vλ_1$-$JCλ_1$ recombination products, derived from the endogenous locus (lower band) and the transgenic wild type BAC (upper band), were amplified by semi-quantitative PCR. 1-3 mice from each transgenic line were analysed. The DNA was increased by two-fold for one sample (wedged arrow) and the product increases proportionately. The uncut PCR product is shown. C) Calculated fold difference in recombination of the endogenous *Igλ* locus and the wild type BAC.

Figure 3: Deletion of HS1 eliminates *Igλ* recombination. A) Rearrangement of the transgene (Tg) was analysed by nested PCR using 50 ng (left) or 200-400 ng (right) of spleen DNA from mice carrying wild type (Lanes 1 and 2) or a BAC where HS1 was deleted. At least two mice were analysed for each line although only a single

sample is shown. B) Rearrangement of the transgene using 50 ng (left) or 100 ng (right) of spleen DNA from two mice of the ΔHS2 and ΔHS3 lines. C) Rearrangement of the transgene using 50 ng (left) or 100-200 ng (right) of spleen DNA from two mice of the ΔHS4 lines. The endogenous locus (E) was separately amplified by PCR for each sample; it was co-electrophoresed with the transgene PCR as a control.

Figure 4: *Loss of HS3 or HS4 does not trigger premature Igλ rearrangement* A) *CD43+/CD19+ and CD43-/CD19+* cells were purified from ΔHS3 and ΔHS4 transgenic lines; the levels of DNA were normalized via PicoGreen and verified by amplification of the β-globin locus (lower panel). Upper panel: Nested PCR to amplify Igλ recombination; increasing amounts of sample were used to verify that the product increased proportionately to the input DNA. Two to four mice from each transgenic line were analysed; representative blots with the average fold increase and standard deviations are shown. B) Representative blot of recombination of the endogenous Igλ locus. A single round of PCR was used to amplify the endogenous locus using the same samples as above.

Figure
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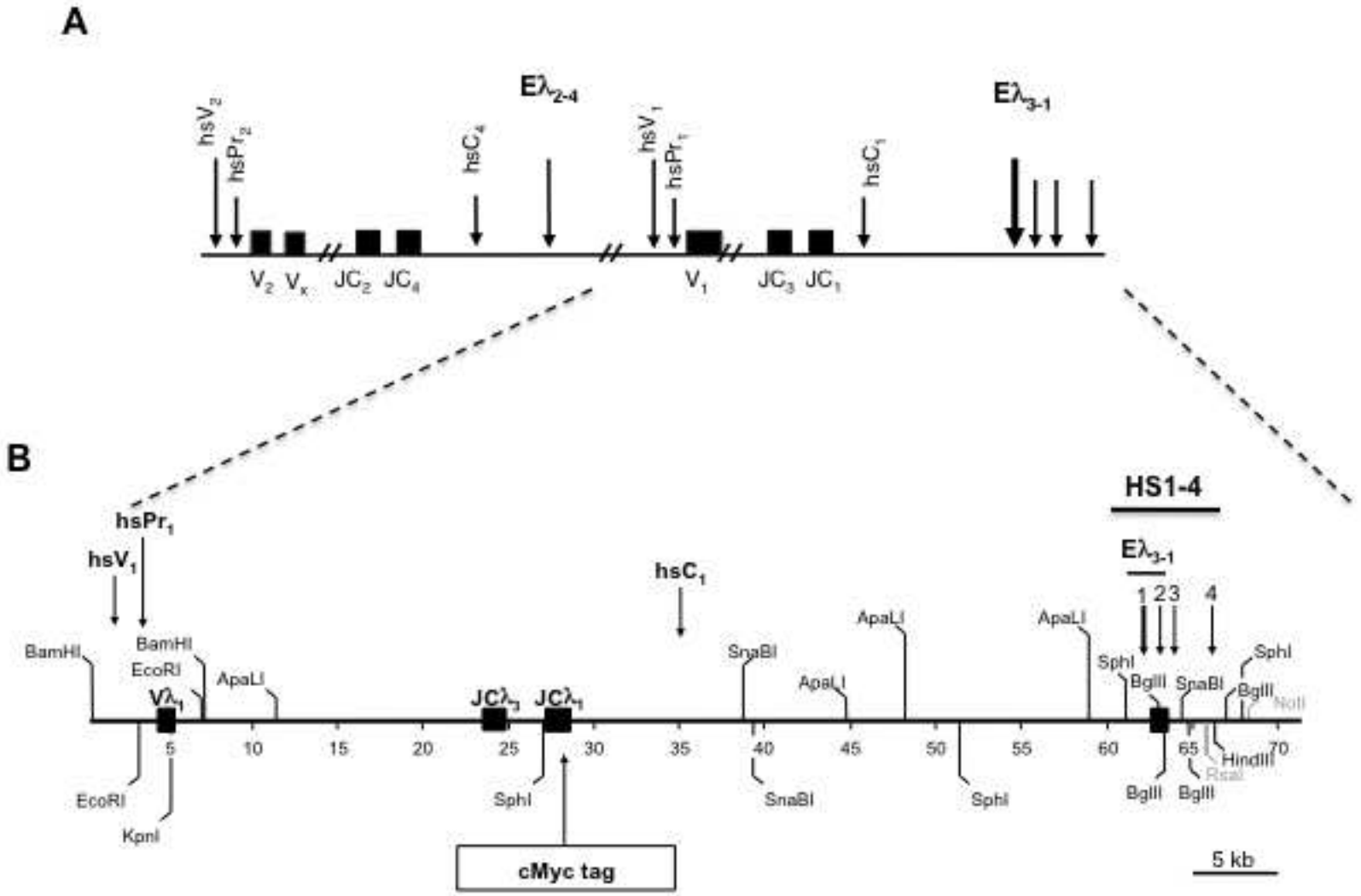

Figure 1

Figure
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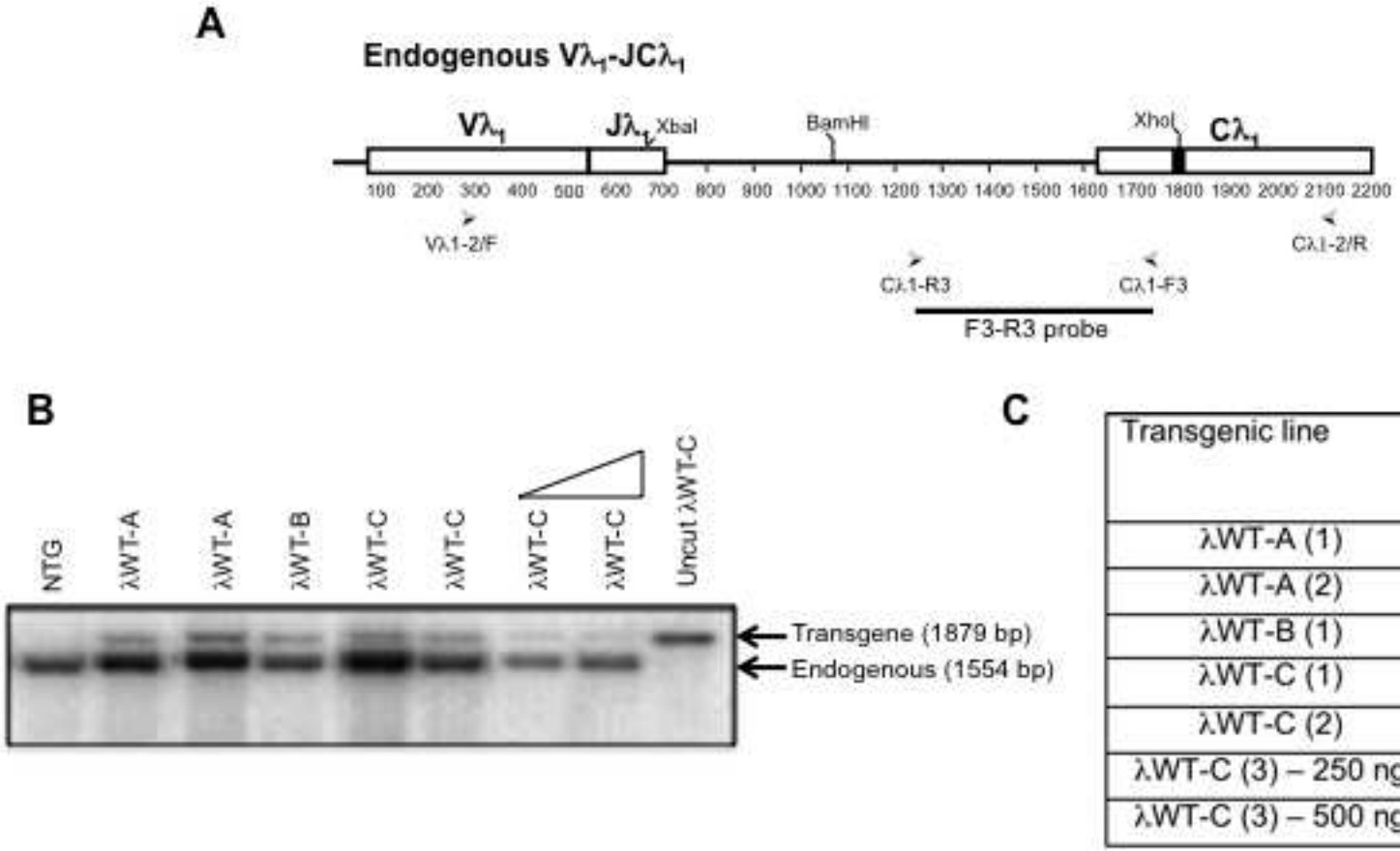

C

Transgenic line	Fold difference in recombination from endogenous
λWT-A (1)	4
λWT-A (2)	3.9
λWT-B (1)	2.9
λWT-C (1)	5.4
λWT-C (2)	4.6
λWT-C (3) – 250 ng	6.3
λWT-C (3) – 500 ng	6.5

Figure 2

Figure
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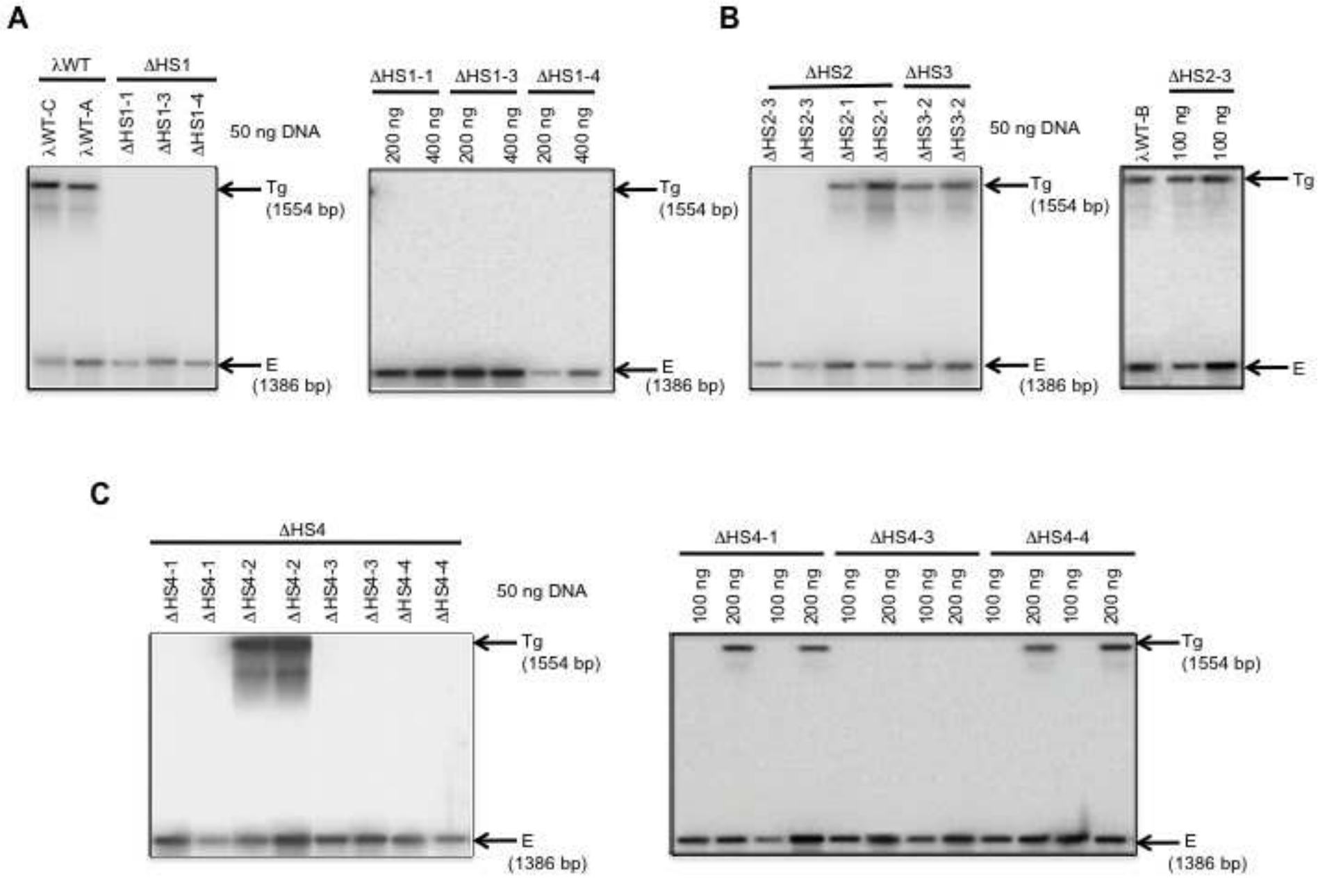

Figure 3

Figure
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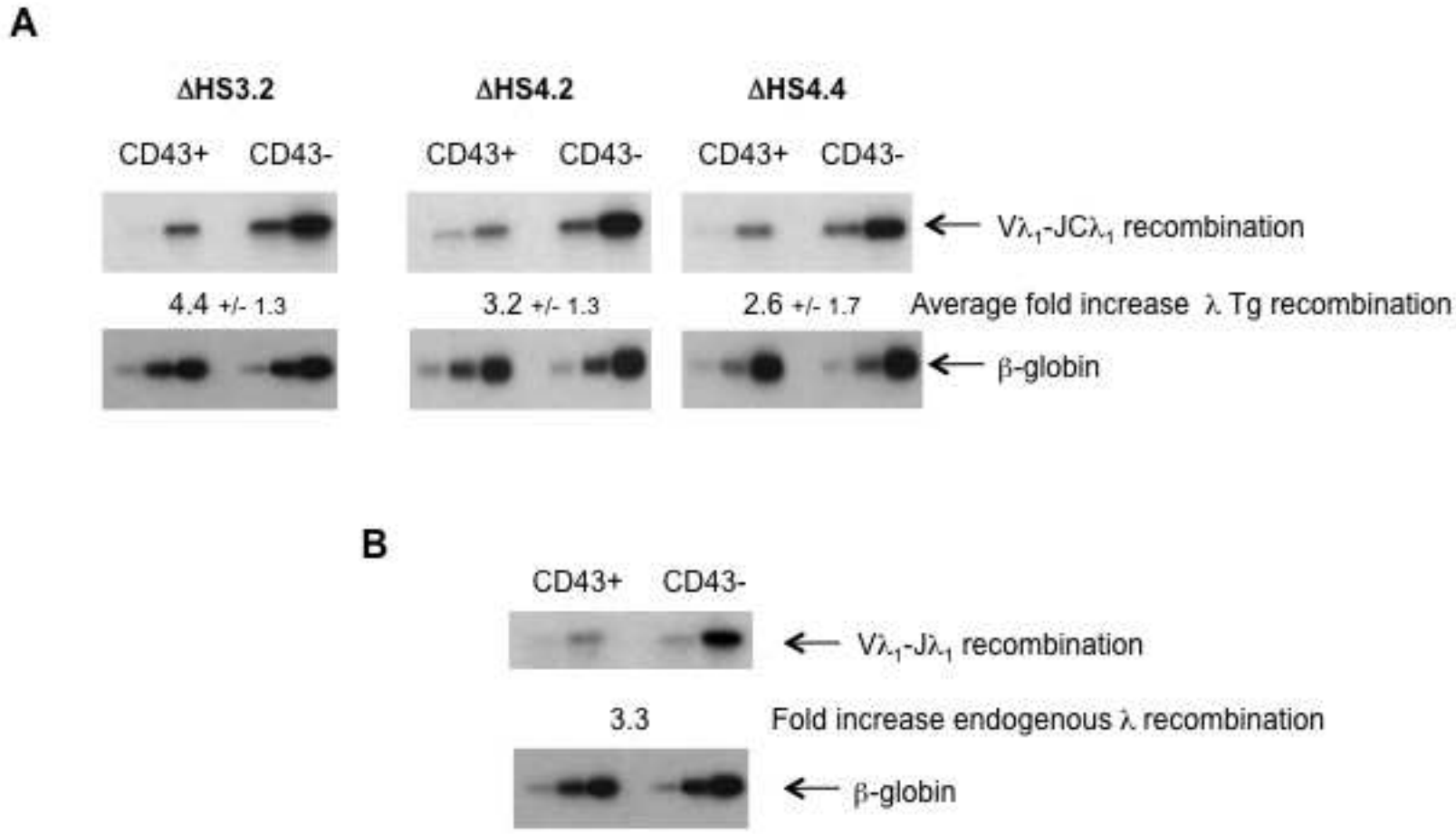

Figure 4

Electronic Supplementary Material (online publication only)

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