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Published as: *Nature*. 2009 December 10; 462(7274): 803–807.

Long-range Oncogenic Activation of *IgH/c-myc* Translocations by the *IgH* 3' Regulatory Region

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Abstract

B cell malignancies, such as human Burkitt's lymphoma (BL), often harbor translocations that link *c-myc* or other proto-oncogenes to the immunoglobulin heavy chain locus (*IgH*)1. The nature of elements that activate oncogenes within such translocations has been a longstanding question. Translocations within *IgH* involve DNA double strand breaks (DSBs) initiated either by the RAG1/2 endonuclease during V(D)J recombination or by activation induced cytidine deaminase (AID) during class switch recombination (CSR)2-4. V(D)J recombination in progenitor B (pro-B) cells assembles *IgH* variable region exons upstream of μ constant region (Cμ) exons, which are the first of several sets of C_H exons ("C_H genes") within a C_H locus that spans several hundred kilobases5,6. In mature B cells, CSR deletes Cμ and replaces it with a downstream C_H gene6. An enhancer (*iEμ*) between the variable region exons and Cμ promotes V(D)J recombination in developing B cells7. In addition, the *IgH* 3' regulatory region (*IgH3'RR*) lies downstream of the C_H locus and modulates CSR by long-range transcriptional enhancement of C_H genes8-10. Transgenic mice bearing *iEμ* or *IgH3'RR* sequences fused to *c-myc* are predisposed to B lymphomas, demonstrating such elements can confer oncogenic *c-myc* expression11-16. However, in many B cell lymphomas, *IgH/c-myc* translocations delete *iEμ* and place *c-myc* up to 200kb upstream of the *IgH3'RR*1. We now address the oncogenic role of the *IgH3'RR* by inactivating it in two distinct mouse models for B cell lymphoma with *IgH/c-myc* translocations. The *IgH3'RR* is dispensable for pro-B lymphomas with V(D)J recombination-initiated translocations, but required

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature

Author contributions. F.W.A. and M.G. planned studies and interpreted data. M.G. performed experiments, with technical help from J.M.B. C.T.Y generated CXP mice. E.P and M.C. generated *IgH3'RR*-deleted mice and helped interpret data. F.W.A. and M.G. wrote the paper.

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for peripheral B cell lymphomas with CSR-associated translocations. As the *IgH3'RR* is not required for CSR-associated *IgH* breaks or *IgH/c-myc* translocations in peripheral B cell lymphoma progenitors, we conclude this regulatory region confers oncogenic activity via long-range and developmental stage-specific activation of translocated *c-myc* genes.

Individual C_H genes are organized into germline transcription units which consist, from 5' to 3', of a non-coding "I" exon, a switch (S) region and the C_H exons6. CSR to a particular C_H gene requires introduction of AID-initiated DSBs into the donor S region upstream of Cμ (Sμ) and into a downstream acceptor S region6. The I exon is preceded by a germline promoter that is up-regulated by particular activation treatments, with transcription targeting AID to specific S regions17. The *IgH3'RR* contains multiple enhancer elements18-20 and controls germline transcription of certain C_H promoters over distances of 100kb or more8. However, the *IgH3'RR* is not required for V(D)J recombination, expression of rearranged *IgH* genes, or transcription through Sμ or Sγ110. To test potential roles of the *IgH3'RR* in B cell lymphomagenesis, we bred an *IgH3'*RR inactivating mutation, which deletes the key HS3b and HS4 enhancers10 (Supp. Fig. 1), into non-homologous end-joining (NHEJ) and p53 tumor suppressor deficient backgrounds that predispose to either pro-B or peripheral B cell lymphoma.

XRCC4 and DNA ligase 4 (Lig4) form a NHEJ ligation complex required for V(D)J recombination21. Mice with germline inactivation of either *Lig4* or *Xrcc4* and the p53 tumor suppressor develop pro-B cell lymphomas with complex translocations ("complicons") involving *IgH* on chromosome 12 (chr12) and a region downstream of *c-myc* on chromosome 15 (chr15)22. These translocations arise from joining RAG1/2-induced DSBs in the *IgH* J_H region to DSBs downstream of *c-myc*, leading to dicentric chr12;15 translocations and *c-myc* amplification via breakage-fusion-bridge cycles22,23. Analysis of Lig4/p53 double-deficient mice ("LP" mice) that harbored *IgH3'RR* inactivating mutations on either one or both *IgH* alleles (referred to as $LPR^{+/-}$ or $LPR^{-/-}$ respectively) revealed that both genotypes succumbed to pro-B cell lymphomas with kinetics similar to those of LP mice22 (Fig. 1a; Supp. Table 1). Likewise, all analyzed $LPR^{+/-}$ and $LPR^{-/-}$ tumors had characteristic chr12 to15 translocations (T(12;15)) and 15;12 complicons (C(15;12)) harboring amplified *c-myc* (Fig. 1b; Supp. Fig. 2; Supp. Table 1). In addition, Southern blotting with a probe that distinguishes *IgH3'RR*-deleted from wild-type (wt) *IgH* alleles (Fig. 1c, top) revealed that *IgH/c-myc* translocations/amplifications involved the wt allele in some $LPR^{+/-}$ tumors and the *IgH3'RR*-deleted allele in others (Fig. 1c, bottom). Thus, the *IgH3'RR* is dispensable for LP pro-B cell lymphomas with *IgH/c-myc* complicons.

Specific inactivation of *Xrcc4* by a loxP/Cre approach in peripheral B cells of p53-deficient mice (referred to as "CXP" mice) leads to surface Ig-negative peripheral B cell lymphomas24,25. CXP B cell lymphomas arise from progenitors that delete or aberrantly rearrange their *Igκ* and *Igλ* light chain loci and routinely harbor a T(12;15) that fuses *IgH* S regions to sequences just upstream of *c-myc*, leading to high level c-myc expression from a translocated single copy *c-myc* gene25,26. Such T(12;15)s lack *iEμ* as they occur downstream of this element24. To test potential roles of the *IgH3'RR* in CXP tumorigenesis, we followed tumor development in cohorts of $CXPR^{+/-}$ and $CXPR^{-/-}$ mice. $CXPR^{+/-}$ mice

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succumbed to the same tumor spectrum as CXP mice25, with 40% developing surface Ig-negative B cell lymphomas that appeared identical to CXP B cell lymphomas (Fig. 2a and Supp. Table 2). The remaining CXPR$^{+/-}$ mice succumbed to thymic lymphomas or other tumors associated with germline p53 deficiency. In contrast, none of 17 analyzed CXPR$^{-/-}$ mice developed B cell lymphoma; instead, most died from thymic lymphoma (Fig. 2a and Supp. Table 2). Thus, homozygous *IgH3'RR* inactivation abrogates CXP B cell lymphomas.

All analyzed CXPR$^{+/-}$ B cell tumors had a clonal T(12;15), based on spectral karyotyping (Fig. 3c, Supp. Fig. 3). As in CXP B cell lymphomas25, most T(12;15) from CXPR$^{+/-}$ B cell lymphomas split *c-myc*, as evidenced by Southern blotting with 5' and 3' *c-myc* locus probes (Fig. 2b, top panels). However, two tumors (393 and 959) had *c-myc* amplification without detectable *c-myc* rearrangements (Fig. 2b), and tumor 796, while harboring a clonal T(12;15) that split *c-myc*, also contained metaphases with a translocation that fused this T(12;15) to chromosome 16, resulting in low level *IgH/c-myc* amplification (Supp. Fig.3; data not shown). Northern blotting revealed elevated *c-myc* expression in all CXPR$^{+/-}$ B cell tumors, with tumors 393, 796 and 959 showing highest levels (Supp. Fig. 4), suggesting that amplification may sometimes be selected secondary to ectopic activation to achieve maximal expression, which may be relevant to certain human lymphomas that acquire *c-myc* amplification during tumor progression12.

To determine which *IgH* allele was involved in CXPR$^{+/-}$ lymphoma T(12;15)s, we analyzed tumor metaphases by fluorescence *in situ* hybridization (FISH) with a probe specific for the deleted portion of the *IgH3'RR* (*3'RR* wt probe, green) and a chr15 paint (red). In this assay, the translocated portion of chr15 (red paint) co-localizes with a green signal if the wt *IgH* allele is translocated, but not if the *IgH3'RR*-deleted allele is translocated (Fig. 3a, left panel). Sequential re-probing of these metaphases with a green chr12 paint (FISH 2, green) plus a 3' *IgH* BAC probe (FISH 2, red) (Fig. 3a, right panel) revealed that all CXPR$^{+/-}$ tumor *IgH/c-myc* translocations involved the wt *IgH* allele (Fig. 3b, c and Supp. Fig. 5). These results, coupled with the absence of B cell tumors in CXPR$^{-/-}$ mice, demonstrate that the *IgH3'RR* is required for peripheral CXP B cell lymphomas via a role in oncogenic *IgH/c-myc* translocations.

The *IgH3'RR* might influence appearance of oncogenic *IgH/c-myc* translocations by mechanistically promoting them through induction of CSR at certain S regions and/or by long-range activation of translocated *c-myc* expression. To distinguish between these potential mechanisms, we asked whether the *IgH3'RR*-deleted allele is a substrate for AID-induced DSBs. As one measure, we activated CXPR$^{+/-}$ and CXPR$^{-/-}$ B cells for 4 days with αCD40/IL4 and found that both genotypes switched to IgG1, as expected by the fact that the *3'IgHRR* is not required for CSR to this IgH isotype8,10 (Supp. Fig. 6a). We also analyzed metaphases from αCD40/IL4-stimulated CXPR$^{+/-}$and CXPR$^{-/-}$ B cells via two-color FISH27 and found that the increased level of *IgH* breaks in the absence of XRCC424 was not markedly affected by the *IgH3'RR* mutation (Fig. 4a). CSR to IgG3 is severely impaired in mice homozygous for *IgH3'RR* inactivating mutations due to inhibition of Iγ3 transcription8,10. However, LPS/αIgD-dextran stimulation to induce IgG3 CSR led to similarly increased *IgH* breaks in CXPR$^{+/-}$ and CXPR$^{-/-}$ B cells (Supp. Fig. 6b; Fig. 4a), likely reflecting unimpeded AID activity on Sμ, which is transcribed independently of the

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*IgH3'RR*10. Southern blotting further revealed that most CXPR$^{+/-}$ B cell tumors had Sμ rearrangements or deletions on both alleles, again indicative of substantial AID activity on *IgH3'RR*-deleted alleles (Fig. 4b). We conclude that introduction of AID-induced *IgH* lesions is not markedly impaired by the *IgH3'RR* deletion.

While AID-induced *IgH* breaks occur at high frequency on *IgH3'RR*-deleted alleles, *IgH/c-myc* translocations still might be inhibited, for example by a different distribution of *IgH* DSBs or by effects on proximity of the two loci26. Therefore, we employed a PCR assay28 to directly evaluate potential effects of the *IgH3'RR* deletion on *IgH/c-myc* translocation frequency and found *IgH/c-myc* translocations, indeed, occurred at similar frequencies in αCD40/IL4-stimulated CXPR$^{+/-}$ and CXPR$^{-/-}$ B cells (Fig. 4c). Moreover, one CXPR$^{+/-}$ B cell tumor had a T(12;11) involving the *IgH3'RR*-deleted allele in addition to its T(12;15), again indicating the *IgH3'RR*-deleted allele is a translocation target (Fig. 3c, Supp. Fig. 3). We conclude that the *IgH3'RR* is dispensable for generation of *IgH/c-myc* translocations in XRCC4-deficient B cells.

The *IgH* locus has long been speculated to have cis-acting elements that activate *c-my*c or other oncogenes in the context of translocations. We now demonstrate that the *IgH3'RR* is required for oncogenicity of *IgH/c-myc* translocations that ectopically activate *c-myc* in mouse CXP B cell lymphomas. As a substantial proportion of CXPR$^{+/-}$ (Supp. Fig. 7) and CXP lymphomas25 have translocations that fuse c-*myc* to Sμ, oncogenic *IgH3'RR* activity extends at least 200kb upstream. Thus, our findings define a major oncogenic role for the *IgH3'RR* in activating *c-myc* subsequent to *IgH/*c-myc translocations; although we do not rule out an additional role in promoting translocations by enhancing AID-mediated lesions in certain S regions regulated by this element. Although high-copy *iEμ* transgenes predispose to pro-B cell lymphoma in mice12, the role of *iEμ* in *IgH*/oncogene translocations remains to be determined. In this context, knock-in of *c-myc* into the *IgH* J_H region led to peripheral B cell lymphomas, as opposed to pro-B cell lymphomas12, suggesting *iEμ* alone may not always be sufficient to activate *c-myc* in the endogenous setting. In this regard, our finding that the *IgH3'RR* is dispensable for LP pro-B cell lymphomas may explain why *c-myc* is amplified in these tumors and ectopically activated in peripheral CXP B cell tumors. Specifically, the *IgH3'RR* is not active in pro-B cells9 and would not activate *IgH* translocations upstream of a single-copy *c-myc*, favoring selection for translocations downstream of *c-myc* that promote gene amplification. Given the similar organization of mouse and human *IgH*9, our findings suggest that the *IgH3'RR* also supports activated oncogene expression in human B cell tumors with *IgH* S region translocations that eliminate *iEμ* (e.g. sporadic BL29) and, potentially, even in some with *IgH* J_H region translocations that leave *iEμ* intact (e.g. endemic BL29) In this regard, targeted inhibition of the B cell-specific *IgH3'RR* could theoretically provide a therapeutic strategy for such human B lymphomas.

METHODS SUMMARY

Mouse strains

IgH3'RR-deleted mice were generated previously10 and crossed into Lig4$^{+/-}$/p53$^{+/-}$30 or CD21-Cre/XRCC4$^{c/c}$/p53$^{+/-}$25 mice to obtain triple or quadruple heterozygous animals,

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which were appropriately crossed to obtain the experimental cohorts. Mice were analyzed as outlined in the text at 8–30 weeks of age. The Institutional Animal Care and Use Committee of Children's Hospital (Boston, Massachusetts) approved all animal work.

Splenic B-cell purification, activation in culture, and CSR assays

$CD43^-$ B cells were isolated and cultured as previously described27. Cells were processed at day 4 of stimulation with αCD40/IL4 or at day 5 of stimulation with LPS/αIgD-dextran for DNA isolation, metaphase preparation and flow cytometry analysis (see Methods).

Two-color FISH

Metaphase spreads were prepared and FISH experiments performed according to standard protocols27. FISH probes are detailed in Methods. Whole chromosome paints specific for mouse chromosome 12 and 15 were used according to the manufacturer's instructions (Applied Spectral Imaging).

PCR assay to detect *IgH/c-myc* translocations

IgH/c-myc translocation junctions were amplified by PCR from genomic DNA prepared from splenic B cells activated for 4 days with αCD40/IL4, using primers and conditions previously described28 (see Methods). DNA corresponding to 50000 or 100000 cells was analyzed in separate reactions. PCR products were run on agarose gel, blotted and hybridized with an internal oligonucleotide probe in the *c-myc* locus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Lynn G. Jiang for help with cloning translocation breakpoints. This work was supported by NIH grant CA92625 and the Leukemia and Lymphoma Society of America (LLS) SCORE grant to F.W.A. M.G. was a Special Fellow of LLS and C.T.Y. was supported by an NCI training grant. M.G. and C.T.Y were also supported by the de Villiers International Achievement Award of the LLS to F.W.A. E.P. was a Fellow of the Fondation pour la Recherche Medicale. F.W.A. is an Investigator of the Howard Hughes Medical Institute.

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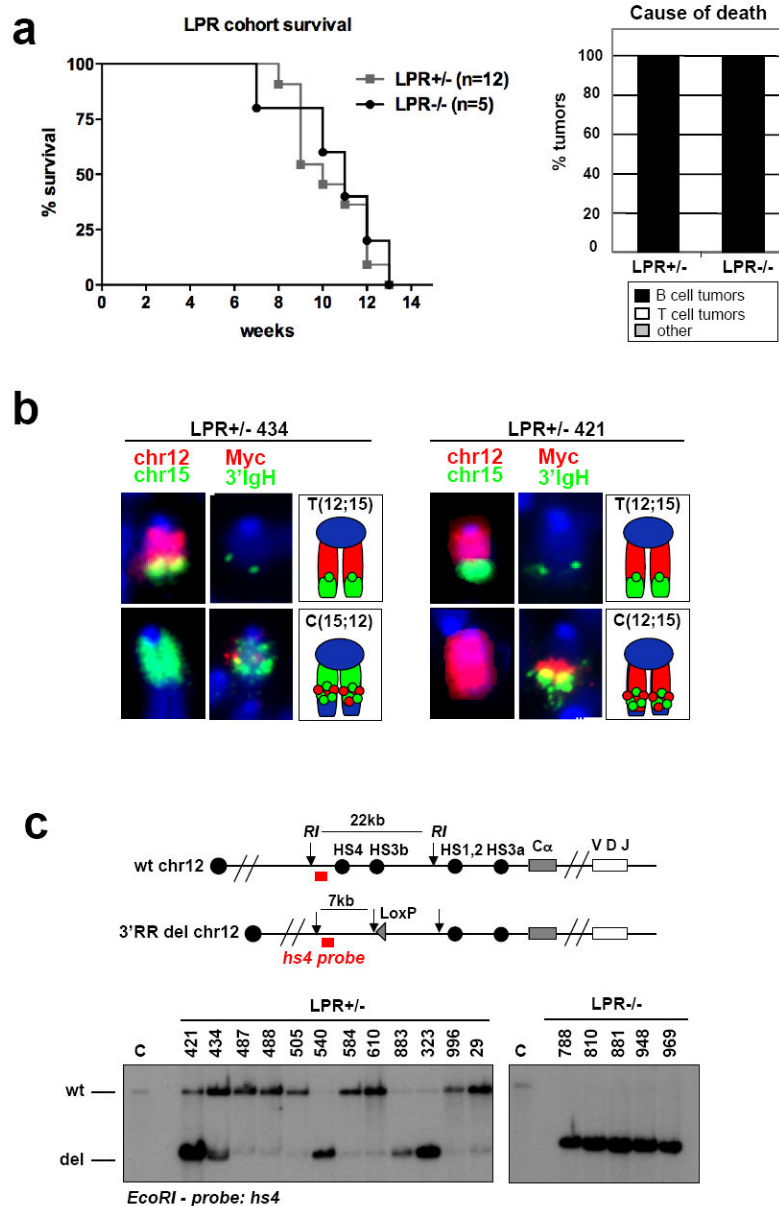

Figure 1.

Deletion of the Igh3'RR does not affect development of pro-B-cell lymphomas. a, Left, Kaplan–Meier curve of the LPR1+/– (n=12) and LPR–/– (n=5) cohorts. Curves represent total survival. Right, the percentage of mice in the LPR+/– and LPR–/– cohorts succumbing to B-cell lymphomas, thymic lymphomas or other causes of death. b, Examples of cytological aberrations in representative LPR+/– tumours with translocations to the wild-type (mouse number 434) or the 3'RR-deleted (mouse number 421) Igh alleles. In each set of panels: left, paints specific for chr12 (red) and chr15 (green); middle, FISH analysis on separated metaphases with 3'Igh (green) and c-myc (red) probes; right, graphic representation. Only chromosomes involved in translocations are shown. Whole metaphases are presented in Supplementary Fig. 2. c, Southern blot analysis of LPR+/– (left) and LPR–/– (right) tumour DNA with a probe downstream of hs4, which distinguishes the wild-type

(WT) and 3’RR-deleted (del) Igh alleles. A schematic of the wild-type and del Igh locus, with the position of the probe, is on the top. Numbers refer to individual mice in the cohort (see Supplementary Table 1). C, control, total spleen DNA from wild-type mouse; RI, EcoRI.

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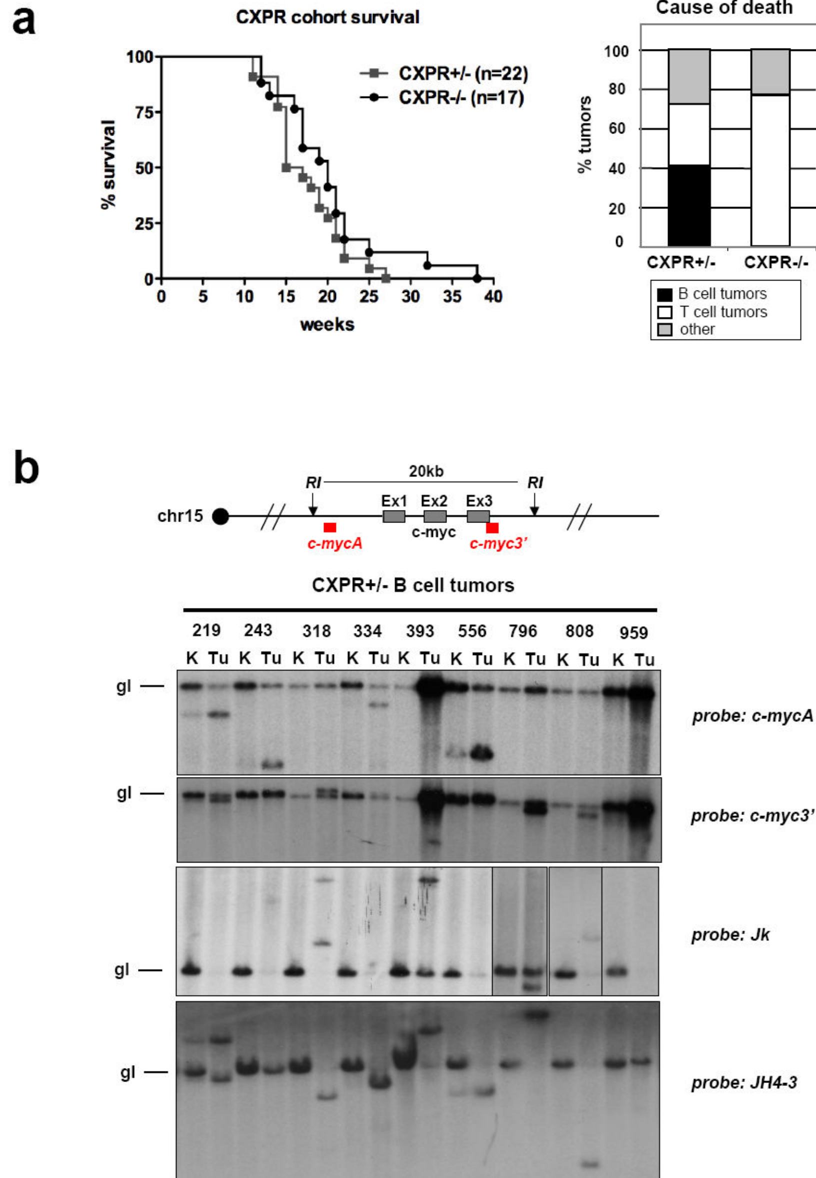
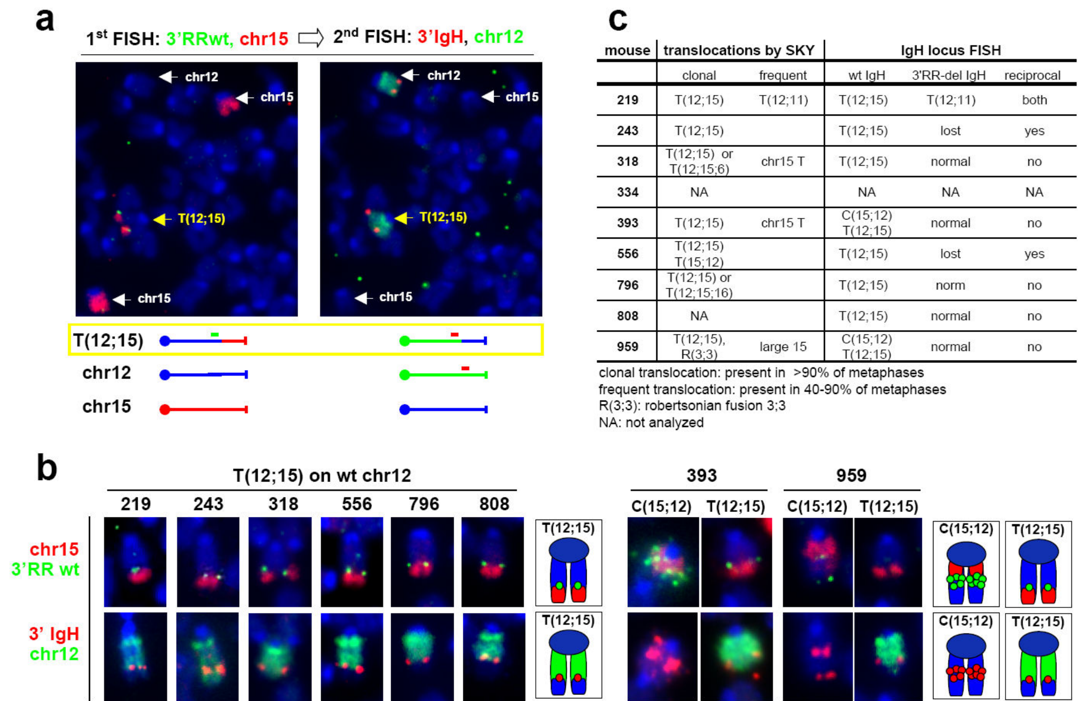


Figure 2. Deletion of the *IgH3'RR* abrogates development of peripheral B cell lymphomas

a. Left: Kaplan-Meier curve of the CXPR$^{+/-}$ (n=22) and CXPR$^{-/-}$ (n=17) cohorts. Curves represent total survival. **Right**: Percentage of mice in the CXPR$^{+/-}$ and CXPR$^{-/-}$ cohorts succumbing to B cell lymphomas, thymic lymphomas or to other cause of death. **b**. Southern blot analysis of CXPR$^{+/-}$ tumor DNA with probes indicated on the right of each panel. Numbers refer to individual mice in the cohort (see Supp. Table 2). K, kidney, used as control; Tu, tumor. A schematic of the *c-myc* locus, indicating the 5' and 3' probes used to detect *c-myc* rearrangements is on the top.



mouse	translocations by SKY		IgH locus FISH		
	clonal	frequent	wt IgH	3'RR-del IgH	reciprocal
219	T(12;15)	T(12;11)	T(12;15)	T(12;11)	both
243	T(12;15)		T(12;15)	lost	yes
318	T(12;15) or T(12;15;6)	chr15 T	T(12;15)	normal	no
334	NA		NA	NA	NA
393	T(12;15)	chr15 T	C(15;12) T(12;15)	normal	no
556	T(12;15) T(15;12)		T(12;15)	lost	yes
796	T(12;15) or T(12;15;16)		T(12;15)	norm	no
808	NA		T(12;15)	normal	no
959	T(12;15), R(3;3)	large 15	C(15;12) T(12;15)	normal	no

clonal translocation: present in >90% of metaphases
frequent translocation: present in 40-90% of metaphases
R(3;3): robertsonian fusion 3;3
NA: not analyzed

Figure 3. T(12;15) in CXPR$^{+/-}$ B cell tumors always involves the wt *IgH* allele

a. Example of FISH and chromosome paints on CXPR$^{+/-}$ tumor metaphases. The same metaphase was sequentially analyzed with the different set of probes indicated at the top of each panel. A schematic of the different chromosomal species detected is shown at the bottom. **b**. Summary of FISH and chromosome paint analyses on all analyzed CXPR$^{+/-}$ tumors. Numbers refer to individual mice in the cohort. Sequential hybridization with the set of probes indicated on the left was performed. Only chromosomes involved in translocations are shown, along with a graphic representation. The whole metaphases are presented in Supp. Fig. 4. **c**. Table summarizing SKY and FISH data for CXPR$^{+/-}$ B cell tumors.

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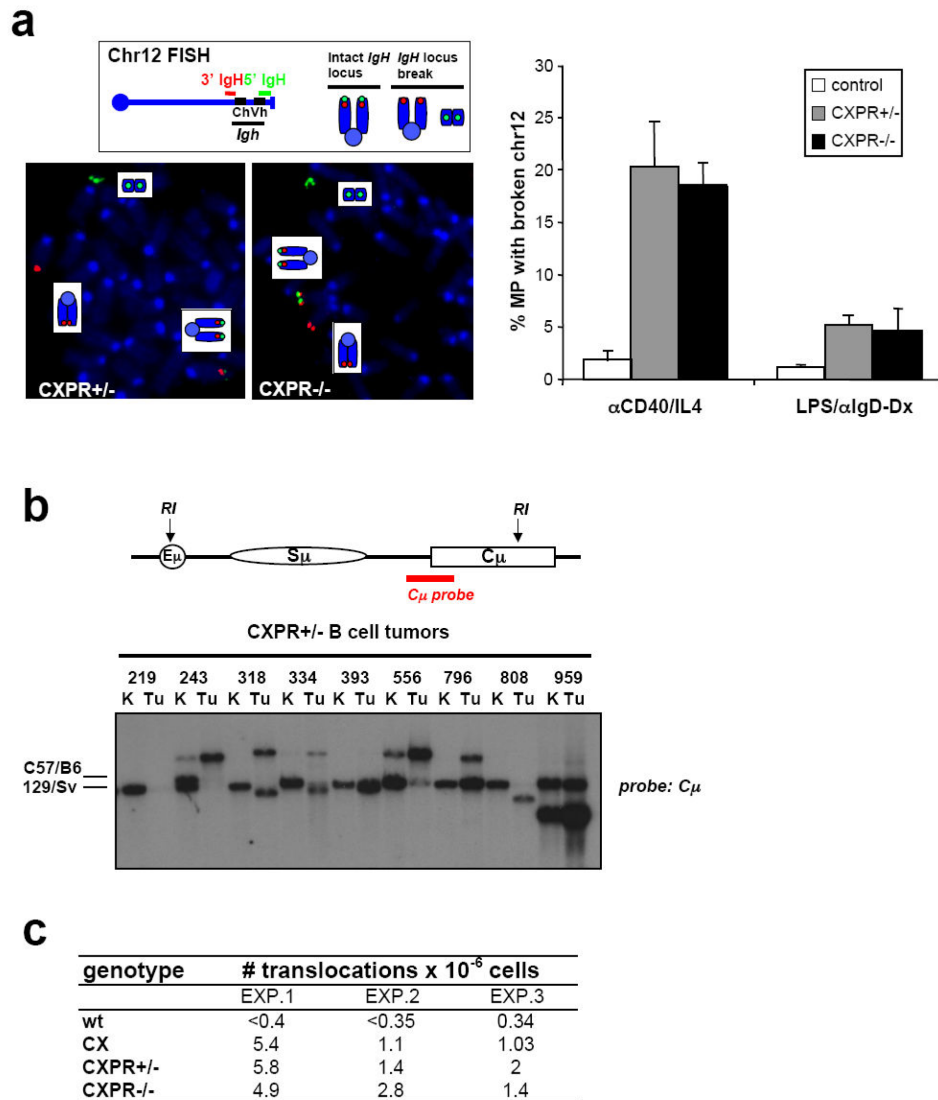

genotype	# translocations x 10^{-6} cells		
	EXP.1	EXP.2	EXP.3
wt	<0.4	<0.35	0.34
CX	5.4	1.1	1.03
CXPR+/-	5.8	1.4	2
CXPR-/-	4.9	2.8	1.4

Figure 4. Deletion of the *IgH3'RR* does not affect *IgH* locus breaks and *IgH/c-myc* translocations

a. Left, top: Diagram showing location of 3' *IgH* (red) and 5' *IgH* (green) BAC probes used for FISH. An intact *IgH* locus on chr12 shows colocalized red and green signals, a broken locus shows split red and green signals. **Left, bottom**: Examples of metaphases from $CXPR^{+/-}$and $CXPR^{-/-}$ αCD40/IL4-stimulated splenic B cells showing *IgH* breaks. **Right**: Quantification of *IgH* locus breaks in day 4 αCD40/IL4-activated or day 5 LPS/αIgD-dextran-activated B cells from control, $CXPR^{+/-}$, and $CXPR^{-/-}$ mice. At least three mice per each genotype and at least 60 metaphases per mouse were analyzed; data are presented as average ± sd. **b**. Southern blot analysis of $CXPR^{+/-}$ tumor DNA with a Cμ probe that detects Sμ rearrangements. A schematic of the *IgH* locus, with position of the probe is on the top. Position of germline bands in C57/B6 and 129Sv backgrounds is indicated on the left; the 3'RR-deleted allele is from 129Sv background. Numbers refer to individual mice in the cohort. K, kidney, used as control; Tu, tumor. Note that in some cases kidneys contained infiltration of tumor cells, as judged by tumor-specific rearranged J_H and *c-myc* bands (Fig. 2b). **c**. Frequency of *IgH/c-myc* translocations was measured by PCR assays using Sμ and *c-*

myc primers. DNA samples were isolated from day4 αCD40/IL4-activated wt, CX, $CXPR^{+/-}$ and $CXPR^{-/-}$ splenic B cells.

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