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Accessibility
Oncogenic transformation in the absence of Xrcc4 targets peripheral B cells that have undergone editing and switching

Jing H. Wang,1,2,3,4 Frederick W. Alt,1,2,3,4 Monica Gostissa,1,2,3,4 Abhishek Datta,1,2,3,4 Michael Murphy,1,2,3,4 Marat B. Alimzhanov,3 Kristen M. Coakley,1,2,3,4 Klaus Rajewsky,1,5 John P. Manis,2,5 and Catherine T. Yan1,2,3,4

1Howard Hughes Medical Institute, 2The Children’s Hospital, 3Immune Disease Institute, 4Department of Genetics, 5Department of Pathology, Harvard Medical School, MA 02115

Nonhomologous end-joining (NHEJ) repairs DNA double-strand breaks (DSBs) during V(D)J recombination in developing lymphocytes and during immunoglobulin (Ig) heavy chain (IgH) class switch recombination (CSR) in peripheral B lymphocytes. We now show that CD21−cre−mediated deletion of the Xrcc4 NHEJ gene in p53−deficient peripheral B cells leads to recurrent surface Ig−negative B lymphomas (“CXP lymphomas”). Remarkably, CXP lymphomas arise from peripheral B cells that had attempted both receptor editing (secondary V[D]J recombination of Igκ and Igλ light chain genes) and IgH CSR subsequent to Xrcc4 deletion. Correspondingly, CXP tumors frequently harbored a CSR−based reciprocal chromosomal translocation that fused IgH to c−myc, as well as large chromosomal deletions or translocations involving Igκ or Igλ, with the latter fusing Igλ to oncogenes or to IgH. Our findings reveal peripheral B cells that have undergone both editing and CSR and show them to be common progenitors of CXP tumors. Our studies also reveal developmental stage−specific mechanisms of c−myc activation via IgH locus translocations. Thus, Xrcc4/p53−deficient pro−B lymphomas routinely activate c−myc by gene amplification, whereas Xrcc4/p53−deficient peripheral B cell lymphomas routinely ectopically activate a single c−myc copy.

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B lymphocytes are Igκ+, with the remainder being Igλ+. In that context, Igκ assembly usually precedes that of Igλ (9). Thus, most Igκ+ B cells contain Igλ in germline configuration, with Igλ rearrangements occurring in cells in which both Igκ alleles are rearranged out-of-frame or that harbor deletions of the Jκ segments, κ enhancer, and/or Cκ exons (9). Such deletions usually occur via rearrangement of Vκs or an RS heptamer in the Jκ-Cκ intron (IRS) to a bona fide RS 25 kb downstream of Cκ (3’RS) (10). Recent analyses suggest that Igκ deletions via 3’RS rearrangements may play a role in progression to Igλ rearrangement (11).

Expression of complete Ig (IgH/IgL) leads to IgM+ B lymphocytes, which ultimately down-regulate RAG expression to enforce allelic exclusion (1). However, newly generated BM IgM+ B lymphocytes that express autoreactive B cell receptors can maintain RAG expression and continue to rearrange Igλ loci to generate new Igλ chains in a tolerance process termed “receptor editing” (12–14). Receptor editing can replace rearranged Igκ loci with secondary productive Igκ rearrangements, as well as with nonfunctional Igκ rearrangements or Igκ deletions that may lead to Igλ rearrangement (12–14). Thus, Igκ+ B cells can be generated developmentally from pre-B cells with two nonproductive Igκ rearrangements or via receptor editing from immature Igκ+ B cells. Receptor editing is initiated in immature BM B cells (15, 16). Yet, several studies suggested Igλ gene rearrangement, sometimes called “revision,” in mouse and human peripheral B cells, including germinal center B cells (17–21). However, many peripheral mouse RAG+ B lineage cells are pro-or pre-B cells that migrate to the periphery after immunization (22, 23), and knock-in reporter studies suggested that although RAG genes are expressed in B cells that have just migrated from the BM (24, 25), they are not reinduced in peripheral B cells once expression is terminated (25, 26).

After antigen stimulation, mature IgM+ peripheral B cells can undergo IgH class switch recombination (CSR), a recombination/deletion process in which the IgH μ constant region exons (Cμ) are deleted and replaced by one of several sets of downstream Cμ exons (e.g., Cγ, Cε, and Cα; referred to as Cμ genes) (27), leading to switching from IgM to another Ig class (e.g., IgG, IgE, or IgA). The activation-induced cytidine deaminase (AID) initiates CSR (28) by deaminating cytidines in switch (S) regions (29), which are 1–10-kb repetitive sequences located 5’ of each Cμ gene. AID-generated lesions within the donor Spα and a downstream acceptor S region are processed into DSBs, which are end-joined to complete CSR (27). In contrast to V(D)J recombination, substantial CSR occurs in the absence of Xrcc4 or Lig4 (C-NHEJ) via an alternative end-joining (A-Ej) pathway strongly biased to use microhomology (30). However, CSR is significantly impaired in Xrcc4-deficient B cells owing to failure to join broken S regions because up to 20% of Xrcc4-deficient B cells activated for CSR in vitro have IgH chromosomal breaks, with a substantial portion participating in chromosomal translocations (30).

Inactivation of Xrcc4 in mice results in impaired cellular proliferation and ionizing radiation sensitivity. Xrcc4 deficiency also results in extensive apoptosis of newly generated neurons and late embryonic death (6), both of which can be rescued by deficiency for the p53 tumor suppressor (31). In this context, p53 monitors the G1 cell cycle checkpoint, signaling apoptosis of certain cell types, such as neurons and progenitor lymphocytes, which harbor persistent DSBs (32). However, as p53 deficiency does not rescue defective NHEJ associated with Xrcc4 deficiency, Xrcc4/p53–double-deficient mice are still immunodeficient and inevitably succumb to pro-B cell lymphomas that harbor RAG-dependent complex translocations (33). These translocations usually join IgH on chromosome 12 to a region downstream of c-myc on chromosome 15, resulting in dicentric 12;15 translocations and c-myc amplification via breakage–fusion–bridge cycles (34).

Such complex translocations are rare in human peripheral B cell lymphomas, which more frequently harbor reciprocal translocations that fuse IgH, or less frequently IgL loci, just upstream of c-myc, leading to ectopic c-myc activation (35).

In the current study, we have asked whether inactivation of C-NHEJ or p53-deficient peripheral B cells leads to peripheral B cell lymphoma with CSR or V(D)J recombination-associated IgH or IgL locus translocations.

**RESULTS**

**Inactivation of Xrcc4 in p53-deficient peripheral B cells leads to novel B cell lymphomas**

We previously inactivated Xrcc4 specifically in transitional stage peripheral B cells by generating mice that harbored one copy of a loxP-flanked (floxed) Xrcc4 allele (X) and one copy of an Xrcc4-null allele (Xc) plus a transgene that drives Cre recombination expression via a CD21 promoter (termed CXc/– mice) (30). CD21 proteins are expressed during B cell development at the time when immature transitional B cells differentiate into mature long-lived peripheral B cells, including marginal zone B cells, follicular B cells, B1a (CD5+), and B1b cells (36). CD21cre mediates efficient Cre recombination in mature, but not immature, transitional B cells (37). Thus, CXc/– mice have normal IgM+ splenic B cell numbers as Xrcc4 is not deleted during early B cell developmental stages where V(D)J recombination occurs (30). Remarkably, CXc/– mice have a normal life span and are not cancer-prone (Fig. 1 A), despite high levels of general and IgH-specific genomic instability in ex vivo αCD40/IL-4–stimulated peripheral CXc/– B cells (30). Analogous to what we previously found for B lymphoid and neuronal progenitors (31), lack of CXc/– B cell transformation may reflect p53-dependent elimination of cells harboring DSBs and other types of genomic instability. To investigate this possibility, we bred CXc/– mice into a p53-deficient background to generate CXc/–P–/– mice.

The majority of CXc/–P–/– mice become moribund by 3.5–4.5 mo of age, with >50% succumbing to Xrcc4/p53-deficient B lineage lymphomas that present predominantly in the mesenteric lymph nodes and that had deleted Xrcc4 (Fig. 1 A and B). The remaining CXc/–P–/– mice succumbed to thymic lymphomas and sarcomas characteristic of p53 deficiency alone and which had not deleted Xrcc4 (Fig. 1 B),
In contrast, none of the $CP^{-/-}$, $CX^a$, or $CX^i$ littermates developed B lymphomas (Fig. 1, A and B). Of eight $CX^i$/$P^{-/-}$ B lineage lymphomas (termed CXP lymphomas) characterized in detail, all were surface Ig negative ($slg^-$) and all were B220+, CD43low, CD138/Syndecan-1+, and CD19+ (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20082271/DC1). In addition, all had clonal IgH and $Ig_k$ rearrangements and many had clonal $Ig_A$ rearrangements, even though they usually lacked expression of IgH and IgL proteins (Table S1; see below). RNA expression analyses indicated that CXP lymphomas lacked expression of $\lambda_5$ and Vpre-B, two diagnostic early B cell markers, but did show very low-level RAG expression (Fig. S1, B and C, and not depicted). We also analyzed six additional $Xrcc4/p53$-deficient B lymphomas derived from $CX^i$ mice in which the p53 gene was also floxed ($CX^i/P^{-/-}$) and found that the majority had a phenotype similar to $CX^i$/$P^{-/-}$ lymphomas (Fig. S2 and Table S2). Likewise, eight additional $CX^i$/$P^{-/-}$ B lymphomas that were also heterozygous for a mutation in the 3’ IgH regulatory region (CXR lymphomas) had a similar phenotype, including lack of surface Ig expression (unpublished data). As expected, we found little or no evidence of $Xrcc4$ deletion in sorted BM B220+IgM- cells or in sorted HSAhigh/CD21low peripheral immature B cells, but essentially complete $Xrcc4$ deletion in purified HSAlow/CD21high mature splenic B cells (Fig. S3). By PCR, we occasionally observed very low levels of a product corresponding to $Xrcc4$ deletion in BM and immature B cell samples, which theoretically may reflect very low-level deletion in earlier B cell stages, but which also may simply reflect recirculating peripheral B cells in the BM and/or low-level contamination of the sorted populations (unpublished data). Along with the appearance of these B lineage tumors being dependent on $Xrcc4$ deletion via $CD21cre$ expression at transitional B cell stage (Fig. 1 B and Fig. S3) (30), these phenotypic characterizations indicate that CXP lymphomas, despite lack of surface Ig expression, derive from peripheral B cells.

**IgH, Igκ, and Igλ rearrangements in CXP tumors**

Recurrent $slg^-$ peripheral lymphomas have not been observed previously. To elucidate the basis for this striking phenotype, we first assayed for expression of IgH chains via Western blotting of tumor extracts. Most analyzed CXP B lymphomas lacked readily detectable IgH chain expression, although CXP62 expressed cytoplasmic $\gamma$ chains (Fig. 2 B and C). Both normal and C-NHEJ-deficient pro-B lines display rearrangements of both $J_H$ alleles (31). Assays of CXP tumor DNA for $J_H$ rearrangements via Southern blotting revealed that all primary CXP tumors showed $J_H$ rearrangements, along with varying degrees of a germline $J_H$ band (Fig. 2 A). However, the germline band usually occurred in low levels and was often further reduced in passed tumors (unpublished data), indicating derivation from non-B lineage cells within the tumor. Despite lack of detectable IgH expression, molecular cloning demonstrated that CXP tumors contained structurally normal in-frame or out-of-frame V(D)J rearrangements, indicating that they occurred before $Xrcc4$ inactivation (see the following section). Finally, most CXP tumors had rearrangements in or around IgH S regions. At least five out of eight CXP tumors had clonal $Sk/C_\mu$ rearrangements; three had one or more clonal $Sk$ rearrangements of which one was molecularly cloned (from CXP68) and found to be an $Sy2a$ to $Sk$ rearrangement; one CXP tumor had an $Sy2b$ rearrangement, and another had a clonal $Sy3$ rearrangement in addition to the $Sy2a$ to $Sk$ rearrangement (Table S1 and Fig. S4, A and B, available at http://www.jem.org/cgi/content/full/jem.20082271/DC1). Thus, most CXP tumors derived from peripheral B cells that had attempted IgH CSR.

No analyzed CXP tumor expressed readily detectable Igκ and Igλ chains (Fig. 2 B and not depicted). Approximately 60% of normal B cells have one productively rearranged Igκ allele and one germline allele, and ~40% have one productive and one nonproductively rearranged or deleted Igκ allele (38).
We assayed for Jκ rearrangements by Southern blotting and found that all eight analyzed CXP tumors lacked both copies of the Jκ locus (Fig. 3 A and Fig. S5 A, available at http://www.jem.org/cgi/content/full/jem.20082271/DC1). Although there was a variable level of a germline-sized Jκ band, the level was far below that expected for a single germline allele and roughly correlated with that of contaminating nonlymphoid DNA as judged by the Jβ Southern blot (Fig. 2 A). Southern blotting revealed that CXP tumors also lacked both copies of the Cκ exons (Fig. 3 A). We tested for downstream RS rearrangements in CXP tumors via Southern blotting and found rearrangement and/or deletion of the 3’RS sequences in all analyzed tumors (Fig. 3 A and Fig. S5 A). As normal 3’RS rearrangements do not result in deletions larger than a few nucleotides (39), we tested the hypothesis that 3’RS rearrangements in CXP lymphomas had occurred after Xrcc4 inactivation by cloning and sequencing rearrangements. Of three RS rearrangements isolated, two had apparent Vκ and 3’RS region junctions accompanied by large deletions (30–80 bp into Vκ and 26–43 bp beyond 3’RS sequence), whereas the other had a normal Vκ to 3’RS join (Fig. S5 B). In addition, Southern blotting with a 3’RS probe showed CXPR163 had an aberrant RS rearrangement with a large deletion (>4 kb) downstream of the 3’RS sequence (Fig. S5 C). Southern blotting also showed that a significant proportion of CXκ−/−/Pκ−/− B lymphomas (four out of six; Fig. S2) and CXPR B lymphomas (five out of eight; not depicted) had deleted one or both Igκ alleles. We conclude that deletional Igκ 3’RS rearrangements in CXP tumors are reminiscent of those associated with attempted V(D)J joining in the absence of C-NHEJ, suggesting they occurred in progenitors of CXP tumors subsequent to Xrcc4 inactivation via CD21 expression.

Complete Igκ deletion is generally observed in a subset of mouse peripheral Igκ− B cells (39). Therefore, we assayed CXP lymphoma DNA by Southern blotting with probes to detect VAJκ rearrangements within both Igκ clusters (Fig. S5 D). These analyses demonstrated that at least six out of nine assayed CXP or CXκ−/−/Pκ−/− B cell lymphomas had clear-cut Igκ rearrangements, with CXPR62, 162, and 163 showing rearrangements of both alleles (Fig. S5, A and D). Southern blotting analyses also showed that several analyzed CXPR B lymphomas had Igκ rearrangements (unpublished data). To elucidate the nature of the Igκ rearrangements in CXP tumors, we performed PCR using Vκ1/Vκ2- and Jκ1/Jκ2-specific primers to isolate six different rearrangements involving one or the other Jκ cluster (Fig. 3 B and Fig. S5 E). Nearly all isolated Igκ rearrangements involved joining of sequences in or upstream of a Vκ to sequences in or downstream of a Jκ, with the majority of breakpoints leading to deletions larger than the few basepair deletions observed from one or both segments in normal VAJκ joins (Fig. 3 B and Fig. S5 E) (40). As PCR only allows isolation of junctions within the primer boundaries, we cloned several Igκ rearrangements from genomic phage libraries generated from CXPR163 and CXPR162 DNA and identified a deletional rearrangement that went ~2.8-kb and ~800-bp, respectively, beyond the Vκ1 and Jκ1 segments, as well as aberrantly deleted RS rearrangements (Fig. 3 B and Fig. S5 E). Overall, the observed pattern of Igκ rearrangements in CXP lymphomas, namely large deletions from the participating segments on one or both sides of the join, is fully consistent with the types of junctions previously characterized for attempted V(D)J recombination of endogenous loci in C-NHEJ-deficient progenitor B or T lymphocytes (3, 41). Therefore, CXP tumors appear to arise from B cells that attempted Igκ rearrangement subsequent to Xrcc4 deletion via CD21 expression.
C-NHEJ suppresses oncogenic CSR-related translocations between IgH and c-myc

Spectral karyotyping (SKY) revealed that six out of eight characterized CXP tumors (CXP68, 153, 162, 163, 221, and 2152) harbored clonal translocations between chromosomes 12 and 15, possibly involving IgH (Chr12) and c-myc (Chr15) loci (Fig. 4 A, Table S3, and Fig. S6 A, available at http://www.jem.org/cgi/content/full/jem.20082271/DC1). To test this possibility, we performed fluorescence in situ hybridization (FISH) with 5' and 3' IgH BAC probes plus a BAC probe containing c-myc (Fig. 4 A). These analyses demonstrated that 5 out of 6 tumors (CXP68, 153, 162, 163, and 2152) harbored reciprocal t(12;15) and t(15;12) translocations in which the centromeric portion (C\(\text{H}\)) of IgH (5' IgH probe) was fused proximal to the telomeric portion of Chr15 in or around c-myc (c-myc BAC probe) to yield the t(12;15) translocation, and the telomeric (V\(\text{H}\)) portion of IgH (5' IgH probe) was fused proximal to the centromeric portion of Chr15 in or just upstream of c-myc to yield the reciprocal t(15;12) translocation (Fig. 4 A and Fig. S6 A). CXP221 harbored a clonal t(12;15) translocation involving IgH and c-myc loc, but lacked the reciprocal t(15;12) translocation, and instead contained a clonal t(15;7) translocation (Fig. S6 A).

Based on FISH, neither c-myc nor IgH appeared amplified in CXP tumors. Similar to CXP tumors, three out of four analyzed CX c/\(-/\text{P}\) c/c B lymphomas and six out of six CXPR tumors analyzed had translocations involving chromosomes 12 and 15 that involved the IgH and c-myc loci (Fig. S6 A, Table S4, and not depicted).

To further analyze CXP B lymphoma t(12;15) and t(15;12) translocation junctions, we performed Southern blotting with probes within or just upstream of c-myc (Fig. S6 B). These analyses demonstrated that six CXP lymphomas with t(12;15) translocations had clonal rearrangements in or around c-myc (c-myc BAC probe) and the telomeric (V\(\text{H}\)) portion of IgH (5' IgH probe) was fused proximal to the centromeric portion of Chr15 in or just upstream of c-myc to yield the reciprocal t(15;12) translocation.
around c-myc (Fig. S6 B and not depicted), with one additional tumor that lacks a t(12;15) translocation (CXP62; detailed in the following section), also showing a clonal c-myc rearrangement (Fig. S6 B). Southern blotting confirmed that none of the CXP tumors had amplified c-myc (Fig. S6 B). Northern blotting with a c-myc probe further demonstrated that all CXP tumors with t(12;15) translocations had greatly increased c-myc expression, indicating ectopic overexpression from a single copy of the translocated c-myc gene (Fig. S6 C). Of note, only two CXP tumors lacked t(12;15) translocations; however, one (CXP62) activated c-myc via an IgH/c-myc translocation (see below) and the other (CXP35) had a complex Chr12 translocation that resulted in N-myc amplification and overexpression (Fig. S6 C and Fig. S7 A, available at http://www.jem.org/cgi/content/full/jem.20082271/DC1) reminiscent of that observed in Artemis/p53-deficient pro–B cell tumors (42). Thus, all CXP tumors overexpressed a myc family gene.

To further elucidate IgH/c-myc translocations in CXP tumors, we isolated translocation junctions by screening genomic DNA libraries from individual tumors. Nucleotide sequence analyses showed that all five characterized junctions corresponded to translocations between IgH and c-myc (Fig. 4 B and Fig. S6 D). All junctions on Chr12 (Fig. 4 B, red), including those associated with both t(12;15) and t(15;12) occurred within IgH, most in the C_H region within or around S regions consistent with derivation via aberrant CSR. The CXP153 t(12;15) junction linked S_μ to sequences just 5′ of c-myc (junction 1, CXP153), whereas the t(15;12) linked S_μ to sequences in the first intron of c-myc (Fig. 4 B and Fig. S6 D).

The CXP163 t(12;15) junction linked I to sequences around c-myc (Fig. S6 B and not depicted), with one additional tumor that lacks a t(12;15) translocation (CXP62; detailed in the following section), also showing a clonal c-myc rearrangement (Fig. S6 B). Southern blotting confirmed that none of the CXP tumors had amplified c-myc (Fig. S6 B). Northern blotting with a c-myc probe further demonstrated that all CXP tumors with t(12;15) translocations had greatly increased c-myc expression, indicating ectopic overexpression from a single copy of the translocated c-myc gene (Fig. S6 C). Of note, only two CXP tumors lacked t(12;15) translocations; however, one (CXP62) activated c-myc via an IgH/c-myc translocation (see below) and the other (CXP35) had a complex Chr12 translocation that resulted in N-myc amplification and overexpression (Fig. S6 C and Fig. S7 A, available at http://www.jem.org/cgi/content/full/jem.20082271/DC1) reminiscent of that observed in Artemis/p53-deficient pro–B cell tumors (42). Thus, all CXP tumors overexpressed a myc family gene.

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The CXP163 t(12;15) junction linked I to a sequence

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**Figure 4.** Reciprocal IgH/c-myc translocations in CXP lymphoma. (A) SKY, FISH, and chromosome paint analyses (Chr12 pink, Chr15 green) for IgH/c-myc translocations in CXP163 and CXP153. A 3′ IgH BAC (light green) and a c-myc BAC (light green) were used in combination with chromosome paints to detect t(12;15) translocations. The t(15;12) translocation, not detectable by SKY, was visualized using the 5′ IgH BAC (pink) and a Chr15 paint (green). At least 15 metaphase spreads were analyzed for SKY, FISH, and chromosomal paints, respectively, for each CXP tumor. Translocations shown were observed in the majority of metaphases, and were scored as clonal if observed in >80% of metaphases. (B) Schematic of IgH/c-myc translocation breakpoint junctions cloned and sequenced from λ-phage tumor genomic DNA libraries. At least two independent phage clones were isolated from each genomic DNA library, and breakpoint junctions identified within them were further independently confirmed by PCR and sequencing from the appropriate tumor DNA samples. The numbers on the top indicate t(12;15) translocation breakpoints and the same numbers containing a ′ on the bottom indicate the “reciprocal” t(15;12) translocation breakpoints in the corresponding tumor: CXP153 (junction 1 and ′1), 163 (junction 2 and ′2), and 68 (junction 3′).
translocations of Chr6 (which contains Igk) in one of eight CXP tumors and in two of four characterized CX<sup>C</sup>/<sup>P</sup>-c/B lymphomas (Tables S3 and S4). Notably two tumors (CXP162 and CX<sup>C</sup>/<sup>P</sup>-c/B 1893) harbored clonal t(6;16) translocations that fused Igk to Chr6. However, cytogenetic analyses indicated that these t(6;16) translocations did not harbor Igk (Fig. S7 A), although Igk may still have been involved and deleted during joining (see the following section).

Characterization of several Igα breakpoints by FISH and/or molecular cloning revealed partner loci and showed that sequences around translocation junctions lacked somatic hypermutation (SHM; Fig. S7 B and not depicted). By SKY, the CXP62 tumor, which was the only one that did not activate a myc-family gene by an IgH translocation, contained reciprocal t(16;15) and t(15;16) translocations, which juxtaposed the Igα P12 BAC probe to the telomeric portion of Chr15 in

CXP lymphomas frequently harbor Igα translocations

SKY analyses demonstrated that five out of eight characterized CXP B lymphomas (CXP35, 62, 68, 162, and 163), many of the same ones that harbored translocations between chromosomes 12 and 15, harbored Chr16 translocations to various other chromosomes with two others (CXP153 and 2152) harboring nonclonal Chr16 translocations (Fig. 6 A, Table S3, and Fig. S7 A). In contrast to the Chr12 translocations, all but one Chr16 translocation (i.e., CXP62) appeared nonreciprocal (Fig. 6 A and Fig. S7 A), similar to RAG-initiated oncogenic IgH translocations in Xrcc4<sup>C</sup>/p53<sup>-</sup>-deficient pro-B lymphomas (34). As the Igα locus is on Chr16, we used FISH with three different BAC probes covering the Igα locus to determine whether the Chr16 breakpoints involved Igα (Fig. 6 A; P12 is centromeric to Igα, E14 spans both Igα clusters, P9 is telomeric to Igα). These analyses revealed that all five Chr16 breakpoints occurred directly within various regions of Igα (Fig. 6 A and Fig. S7 A). We also observed clonal

288 bp upstream of c-myc exon 1, and the t(15;12) linked sequences between J<sub>N</sub> and J<sub>H</sub> to sequences 289 bp upstream of c-myc exon 1 (Fig. 4 B and Fig. S6 D); notably, the CXP163 t(15;12) junction occurred downstream of a nonproductive V<sub>i</sub>H(D)J<sub>H</sub> rearrangement (Fig. 5 B), indicating that it did not happen during V(D)J recombination and, rather, may have resulted in association with a large deletion from a breakpoint downstream in µ-Sµ region. Finally, the t(15;12) CXP68 junction linked sequences near Iγ3 to sequences 1.4-kb upstream of c-myc exon 1 (Fig. 4 B), again, likely resulting from attempted CSR to Syγ3. Four of these five cloned junctions contained junctional microhomology suggestive of A-EJ (Fig. S6 D). Overall, CXP IgH/c-myc translocations are remarkably similar to those of human sporadic Burkitt’s lymphoma that fuse IgH S regions upstream of c-myc.

Because of the location of the characterized translocation junctions downstream of the IgH<sub>H</sub> region, we considered the possibility that many CXP tumors were sIg<sup>-</sup> because they separated a productive V<sub>i</sub>H(D)J<sub>H</sub> exon from the C<sub>H</sub> region. In this context, two out of three analyzed CXP tumors with IgH/c-myc translocations (CXP68 and CXP153) contained an in-frame (productive) V<sub>i</sub>H(D)J<sub>H</sub> rearrangement fused to the c-myc locus on Chr15; whereas the other (CXP163) involved a translocated nonproductive V<sub>i</sub>H(D)J<sub>H</sub> rearrangement (Fig. 5, A–C). In CXP163, which has the nonproductive V<sub>i</sub>H(D)J<sub>H</sub> translocated to c-myc, the other Chr12 was involved in a t(12;16) translocation that may have resulted in loss of the presumably productive V<sub>i</sub>H(D)J<sub>H</sub> exon, as the breakpoint is centromeric to IgH (Fig. 7 A and Fig. S7 A). Of the four V<sub>i</sub>H(D)J<sub>H</sub> junctions and downstream flanks sequenced (3,884 bp), one carried two somatic point mutations and the other three lacked mutations (unpublished data). Finally, cloned V<sub>i</sub>H(D)J<sub>H</sub> rearrangements appeared normal in respect to junctional deletions (Fig. 5, A–D), indicating they occurred in C-NHEJ-proficient cells. Therefore, primary V(D)J recombination at the IgH locus was normal in B lineage cells that gave rise to CXP lymphomas.

CXP lymphomas harbor normal V(D)J rearrangements in IgH locus

Nucleotide sequence analysis of primary V<sub>i</sub>H(D)J<sub>H</sub> rearrangements cloned from the following: CXP153 by both λ-phage library and PCR (A); CXP163 by λ-phage library (B); CXP68 by PCR (C); and CXP2152 by PCR (D). Additional sequence analyses revealed that CXP153 had a productive VT183-D-JH2 rearrangement juxtaposed into to the first intron of c-myc and CXP163 has a nonproductive VH12-D-JH2 rearrangement juxtaposed to the sequence upstream of c-myc exon 1. V regions are shown in blue and J regions in red. The open reading frame is indicated under the DNA sequence. PCR analyses were performed at least three times from one or multiple independent tumor DNA samples. PCR fragments were subcloned into pGEM vector, 10–20 subclones were sequenced, and identical rearrangements observed in >80% clones were scored as clonal rearrangements.
Comparative genomic hybridization analyses of CXP tumors

We used comparative genomic hybridization (CGH) to assay for sequence copy number differences among tumor and control tissues samples. Among the four samples derived from the CXP or CX^{c/-P^{c/-}} cohort, three had clearly lost one copy of either the entire Chr6 or a large region of Chr6, with the boundary of the loss occurring near or spanning IgH (Fig. S8, available at http://www.jem.org/cgi/content/full/jem.20082271/DC1, and not depicted). Out of nine CXPR B lymphoma DNAs analyzed, seven again had a clear loss of one copy of the entire Chr6 or a large region that in two extended directly from IgH (Fig. S8 and not depicted). CGH also showed focal deletion of IgH in most analyzed CXP, CX^{c/-P^{c/-}}, or CXPR tumors (Fig. S8 and not depicted), which is consistent with results of Southern blotting.

Figure 6. Recurrent chromosome 16 translocations. (A) SKY/FISH analyses of IgA translocations in CXP lymphomas. (A, top) schematic map of IgA locus in germline configuration and the location of the three BACs used for FISH (P9, E14, and P12). (bottom) SKY revealed a t(12;16) translocation in CXP68, and a reciprocal t(16;15) and t(15;16) translocation in CXP62. FISH and chromosome paints (Chr12 pink, Chr16 light blue or white, Chr15 green) showed the breakpoints of Chr16 translocations occurred in different regions of the IgA locus, indicated by yellow lightning symbols over metaphases hybridized with different IgA BACs. 3’ IgH (pink) and P9 (light blue) BACs were colocalized at the junction of t(12;16) in CXP68. CXP62 has a reciprocal translocation with P12 BAC (white) on the der(16)t(16;15) and E14 and P9 (white) BACs on the der(15)t(15;16). At least 15 metaphase spreads were analyzed for each of the cytogenetic analyses, and representative clonal translocations observed in >80% of the metaphases are shown. (B, top) Cloning of t(12;16) breakpoints from a genomic library made from CXP68 DNA. Nucleotide sequence analyses of cloned junction (shown in Fig. S7 B) indicated breakpoints occurred between C6 in IgH and Jα2 in IgA locus. (bottom) Cloning of t(16;15) breakpoints from a genomic library made from CXP62 DNA. Nucleotide sequence analyses indicated breakpoints occurred between sequences upstream of c-myc exon 1 and Jα1 with 3’ IgA enhancer in close proximity. At least two independent phage clones were isolated from each genomic library and the junctions found in phage clones were independently confirmed as clonal junctions in a given tumor by PCR analyses of tumor DNA sample. Fig. S7 is available at http://www.jem.org/cgi/content/full/jem.20082271/DC1.
analyses. In contrast, three thymic lymphomas and two forebrain tumors, most likely of glial origin, analyzed did not show loss of Chr6 or a large region proximal to Igk (Fig. S8 and not depicted).

**DISCUSSION**

Xrcc4 deficiency in peripheral B cells does not predispose to B cell lymphoma, despite dramatic IgH instability in Xrcc4-deficient B cells activated for CSR by treatment with αCD40 plus IL-4 (30). In this context, αCD40 plus IL-4-activated splenic CXP B cells had at most a very modest increase in IgH breaks compared with activated Xrcc4-deficient peripheral B cells (28 ± 13% versus 17 ± 7% of metaphases containing IgH breaks). Yet, CD21cre deletion of Xrcc4 in p53-deficient peripheral B cells, or deletion of both genes in peripheral B cells, recurrently led to slg- B lymphomas that harbor activated myc, likely reflecting the critical role of p53 in eliminating B cells with activated c-myc expression and/or eliminating B cells with high genomic instability (33, 43, 44). CXP B lymphomas are remarkable in several ways. First, most CXP lymphomas arise from B cells that have attempted IgH CSR and a majority appear to arise from B cells that have undergone receptor editing, with both processes occurring after Xrcc4 inactivation by CD21Cre. Second, many CXP tumors harbored two separate, clonal translocations that, regardless, involved IgH and IgL loci. Occurrence of clonal IgH and IgL translocations in the same tumor may reflect dual CSR and editing activities of B cells from which they arose. In this context, some CXP lymphomas had clonal translocations that fused IgH and Igα, clearly indicating simultaneous presence of breaks in both loci in tumor progenitors. As outlined below, these unusual features of CXP tumors have novel implications for B cell developmental processes and for oncogenic translocation mechanisms.

Germline Xrcc4 plus p53 deficiency in mice leads to pro-B lymphomas with RAG-dependent translocations that fuse IgH sequences to sequences far downstream of c-myc, resulting in dicentric chromosomes and c-myc activation via breakage-fusion-bridge amplification (34). Thus, it is remarkable that inactivation of same two genes in mature B cells recurrently leads to ectopic activation of a single copy c-myc gene by fusion of IgH into its upstream region, analogous to similar IgH/c-myc translocations in human Burkitt’s lymphoma and diffuse large B cell lymphomas (35). In pristane- or IL-6-induced mouse plasmacytomas, similar IgH/c-myc translocations occur and are initiated by AID (45, 46). In this context, CXP lymphoma IgH/c-myc translocations occur in and around IgH S regions and downstream of normal VH(D)JH exons, indicating their likely selection from the abundant pool of IgH translocations that result from aberrant CSR in activated Xrcc4-deficient peripheral B cells (30). As the intrinsic IgH enhancer is generally absent from CXP IgH/c-myc translocations, long-range 3’ IgH regulatory region activity (47), or that of unknown IgH regulatory elements, may ectopically activate c-myc. The recurrent differences in IgH/c-myc translocations in Xrcc4/p53-deficient pro-B versus CXP peripheral B cell tumors might reflect RAG versus AID-dependent DSB initiation in IgH and/or c-myc, other differential stage-specific mechanisms that lead to DSBs in and around c-myc, stage-specific differences in nuclear localization of IgH and c-myc loci, or mechanisms that differentially activate c-myc after translocation, including stage-specific IgH enhancer activities. As both Xrcc4/p53-deficient pro-B and CXP peripheral B cell lymphomas frequently have IgH/c-myc junctions that appear to be mediated by A-EJ, repair pathway choice is not likely to be a major factor in generating a particular form of translocation. Given the uniform and recurrent IgH/c-myc translocations, the CXP B lymphoma system should allow putative roles of cis elements and other factors to be tested.

CXP lymphomas also frequently harbor chromosome 16 translocations that involve aberrant V(D)J recombination of Igα after Xrcc4 deletion. At least one CXP Igα translocation was oncogenic. CXP62 had reciprocal t(15;16) and t(16;15) translocations, the latter of which fused the downstream portion of Igα to sequences just upstream of c-myc, resulting in ectopic c-myc activation, reminiscent of Igα/c-myc translocations observed in certain human B lymphomas (35). Characterization of additional CXP Igα translocations may lead to identification of additional oncogenes. Notably, we also observe loss of all or part of one copy of chromosome 6 with some large deletions extending from the vicinity of Igk. The latter findings suggest that rearrangements of Igk, likely in association with aberrant editing, may also lead to translocations or chromosome loss. In the latter context, unrepaired DSBs can lead to chromosome loss in yeast (48). The frequent loss of all or a large portion of chromosome 6 in CXP tumors is striking and suggests a potential role in etiology of these tumors, perhaps by deletion of a tumor suppressor sequence.

More than 95% of mouse peripheral B cells express Igk and, correspondingly, have VkJκ joints on one or both Igk alleles and Igk in germline configuration (9). A small percentage of mouse B cells express Igλ, and these have Igk alleles that are nonproductively rearranged or deleted by 3’RS or related rearrangements (39, 49). Notably, Igk-expressing mouse B cells frequently arise via receptor editing, often in the context of Igk deletion via 3’RS rearrangement (39). Thus, it is striking that CXP lymphomas frequently had clonal Igk deletions via aberrant Vk to 3’RS rearrangements and aberrant Vλ to λ rearrangements, with both types of rearrangements occurring subsequent to CD21cre expression. Based on these findings, we speculate that a common CXP tumor progenitor derives from an Igk-expressing B cell that attempted receptor editing subsequent to Xrcc4 deletion. Attempted editing in the absence of Xrcc4 would lead to aberrant rearrangement/deletion of Igk and progression to Igk rearrangement, which also would be aberrant. Thus, editing Xrcc4-deficient B cells would lose IgL expression caused by inability to undergo productive IgL editing.

As a working model, we suggest that aberrant receptor editing in CXP tumor progenitors generates an initial transforming event and/or activates aberrant CSR through loss of parts of chromosome 6 or translocations of Igk. Aberrant
CSR in the absence of Xrcc4 would lead to a high level of IgH translocations (30), including IgH/c-myc translocations that would complete the transformation process. In the case of the CXP tumor that harbors an IgH/c-myc translocation, the former may have been sufficient to fully transform the cells. This scenario provides a potential role for chromosome 6 losses, and also could explain why all CXP Igα translocations are not clearly oncogenic, why some CXP Igα translocations have IgH as a partner, and why CXP IgH translocations can involve either productive or nonproductive IgH alleles.

Receptor editing is generally thought to occur in immature B cells in the BM (16); yet a substantial fraction of CXP tumor progenitors appear to have both breaks associated with attempted editing of the Igα locus and breaks associated with attempted IgH CSR. In this regard, BM immature B cells in quasimonoclonal (QM) mice can express both AID and RAG1/2 (50), although other studies of developing B cells in WT mice concluded that AID is not expressed in BM B cells (51). QM mice harbor a productive IgH knock-in V(D)J allele and a homozygous gene-targeted deletion of both Jκ alleles and, thus, only have Igκ-producing B cells (52). The striking similarities between immature QM B cells and proposed CXP tumor progenitors suggest a potential relationship. In this regard, CXP tumor progenitors might derive from an undetected, low-level population of CD21+ expressing BM immature B cells that delete Xnc4 and undergo aberrant editing and CSR, before migration to the periphery. However, we note that CXP tumors arise in the periphery, have features of peripheral B cells, and harbor clonal Igκ and Igλ rearrangements, as well as IgH CSR-associated rearrangements that occurred subsequent to CD21+ expression. Conceivably, the Igλ breaks that lead to translocations found in CXP tumor progenitors might reflect RAG-initiated breaks in BM developing B cells that persist because of p53 deficiency, as observed for RAG-initiated IgH breaks in ATM-deficient or 53BP1/p53–/- mice (53, 54). However, we also observed dual IgH and Igα translocations in a CXβ/cδ/cε tumor in which both p53 and Xnc4 were deleted peripherally. Therefore, we must consider the alternative possibility that CXP tumors arise from peripheral B cells driven into editing. Such cells might also participate in extrafollicular responses in which CSR occurs with limited SHM (55), a possibility consistent with most CXP tumor IgH variable region exons lacking SHM.

 Transitional B cell populations express very low levels of RAG upon arrival in the periphery, with RAG expression being essentially undetectable in more mature splenic B cell populations (24, 25). Thus, if the RAG breaks in CXP tumor progenitors, indeed, are generated in the periphery, it remains to be determined in what cell type they occur and how apparently low-level RAG expression could generate them. Given that CXP progenitors nearly all arise from cells that harbor IgH/c-myc translocations, it is quite striking that CXP tumors do not arise from slgκ+ B cells simply in the context of aberrant CSR. Indeed, the recurrent occurrence of slgκ+ lymphomas with aberrant Igκ and Igλ rearrangements and translocations indicates that inability to productively complete receptor editing confers a major predisposition to transformation of peripheral CXP B cells. Perhaps editing CXP B cells represent a larger fraction of the CD21+ expressing B cell pool than anticipated or are enriched in certain locations. For example, receptor editing might especially contribute to antibody diversification in B cells of the gut-associated lymphoid tissue (e.g., mesenteric lymph nodes), where the CXP tumors appear to arise most frequently and in which lymphocytes are chronically activated and driven into immune responses by products of the gut microflora.

**MATERIALS AND METHODS**

**Mice.** CD21int/Xrcc4–/– mice were generated as previously described (30) and crossed into p53 germline-deficient mice (56) or mice carrying conditional p53 alleles (57). Animal work was approved by the Institutional Animal Care and Use Committee of Children’s Hospital Boston.

**Southern, Northern, and Western blot.** Genomic DNA was isolated from tumor masses or normal tissues from control mice, and Southern blotting was performed as previously described (58). 3′/5′ probe was a 1-kb XbaI–HindIII fragment from plasmid pSPIg8 containing the Jκ and Cκ region (59), or generated by PCR using the following primers: 3′ Jκ5, 5′-CATCCAAAGAGAGTTGGATCGGAATAAGCA-3′; MA35, 5′-AAC-ACGGATAAACGCAGTTATGCCTTTC-3′; and plasmid pSPIg8 as template. Cκ probe was a 1.7-kb HindIII–BglII fragment covering the Cκ region. RS probes were previously described (60). RNA samples were extracted from tumor masses or normal tissues from control mice using TriPure Isolation Reagent (Roche). Northern blotting was performed following a standard protocol. Western blotting was performed using HRP-conjugated antibodies at 1:1,000 dilution (HRP goat anti–mouse IgM [μ chain–specific antibody], HRP goat anti–mouse λ, HRP goat anti–mouse κ, and HRP goat anti–mouse IgG [γ chain–specific antibody]; SouthernBiotech). Additional primers used for generating probes are detailed in the Supplemental materials and methods (available at http://www.jem.org/cgi/content/full/jem.20082271/DC1).

**Phage and PCR cloning.** Tumor DNA samples were digested to completion with EcoRI, and fragments were cloned into either λZAP II vector (Stratagene) or λDASH II vector (Stratagene) according to the size of the fragments. Libraries were screened according to standard protocols (Stratagene) using CA1 or Jκ2 probe for λ rearrangements or translocations; c-myc probe A or c-myc probe B for c-myc translocations; Cκ, Cy1, and Syl probes for S region rearrangement and translocations (see details for probes in the Supplemental materials and methods). Single plaques were purified, subcloned, and sequenced. In the case of λZAPIII clones, the inserts were excised according to the protocol provided (Stratagene). Positive clones were verified by restriction analysis and hybridization. Sequencing of subcloned inserts was performed using T7 or T3 primers (DF-HCC DNA Resource Core). To obtain junctional sequences, internal primers were required for some tumors. For PCR cloning of Igκ rearrangements: Vλ lambda1int–5′–tggtgacctgaggtctgca–3′ and Lämumu5′–ggagcagtctgaaatgagacaaagcat–3′ were used. Additional primers used for PCR cloning of junctions are detailed in Online supplemental material.

**SKY and FISH analyses.** Preparation of metaphase chromosomes, SKY analyses, FISH, and whole-chromosome painting using single-chromosome-specific paints were performed as previously described (61). FISH probes were as follows: a BAC that covered the 3′ region of the IgH locus encompassing 3′ IgH enhancer and 100 kb downstream (3′ IgH BAC), a BAC just upstream of the IgH Vλ1 region (5′ IgH BAC) as described previously (62), and a BAC that contained the c-myc gene (RP23-307D14, c-myc BAC). BACs for Igκ regions are RP23-3829P1, RP23-60E14, RP23-374P12, and BACs for Igκ regions are RP23-84F6 and RP23-64F9. All the BACs were obtained from BACPAC CHORI database, except for the IgH BACs.
Online supplemental material. Fig. S1 shows the FACS, Northern blot, and RT-PCR data for assays used to characterize indicated surface protein and transcript expression by CXP lymphomas. Fig. S2 shows a Kaplan-Meier curve and summary of tumors that develop in CX<sup>−/−</sup>P<sup>−/−</sup> lymphomas. Fig. S3 shows the evidence for specific X chromosome deletion via CD21 cre. Fig. S4 shows assays for IgH S region rearrangements in CXP lymphomas. Fig. S5 shows Southern blot data for Igk and Igk rearrangements and sequences of aberrant V<sub>α</sub>A<sub>α</sub> joins in CXP lymphomas. Fig. S6 shows various characterizations of translocations in CXP tumors, including representative cytogenetic data (SKY, FISH, and chromosome paints) for analyses that were used to characterize Igk-c-myc translocations, Southern blots for characterization of c-myc rearrangements, Northern blot assays for c-myc expression, and sequences of translocation breakpoints. Fig. S7 displays cytogenetic data (SKY, FISH, and chromosome paints) for Igk translocations, and sequence data for translocation breakpoints from CXP lymphomas. Fig. S8 displays CGH array data for analysis of genetic amplifications and deletions in CXP lymphomas. Table S1 summarizes the phenotypes of CXP lymphomas. Table S2 summarizes the phenotypes of CX<sup>−/−</sup>P<sup>−/−</sup> lymphomas. Table S3 summarizes the characterized clonal translocations in CXP lymphomas. Table S4 summarizes the characterized clonal translocations in CX<sup>−/−</sup>P<sup>−/−</sup> lymphomas. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082271/DC1.

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REFERENCES


