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Illegitimate V(D)J recombination involving non-antigen receptor loci in lymphoid malignancy

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Abstract

V(D)J recombination of antigen-receptor loci (*IGH*, *IGK*, *IGL*, *TCRA*, *TCRB*, *TCRG*, *TCRD*) is an essential mechanism that confers enormous diversity to the mammalian immune system. However, there are now at least six examples of intra-chromosomal interstitial deletions caused by aberrant V(D)J recombination between non-antigen receptor loci; five of these six are associated with lymphoid malignancy. The *SIL-SCL* fusion, and deletions of *CDKN2A*, *IKZF1*, *Notch1*, and *Bcl11b* are all associated with lymphoid malignancy. These interstitial deletions seem to be species specific, as the deletions seen in mice are not seen in humans; the converse is true as well. Nucleotide sequence analysis of these rearrangements reveals the hallmarks of V(D)J recombination, including site-specificity near cryptic heptamer signal sequences, exonucleolytic “nibbling” at the junction site, and non-templated “N” region nucleotide insertion at the junction site. Two of these interstitial deletions (murine *Notch1* and *Bcl11b* deletions) have been detected, at low frequency, in tissues from healthy mice with no evidence of malignancy, similar to the finding of chromosomal translocations in the peripheral blood or tonsils of healthy individuals. The contention that these are mediated via V(D)J recombination is strengthened by *in vivo* assays using extra-chromosomal substrates, and chromatin immunoprecipitation-sequence (ChIP-Seq) analysis which shows Rag2 binding at the sites of rearrangement. Although the efficiency of these “illegitimate” recombination events is several orders of magnitude less than that at *bona fide* antigen receptor loci, the consequence of such deletions, namely activation of proto-oncogenes or deletion of tumor suppressor genes, is devastating, and a major cause for lymphoid malignancy.

Keywords

lymphoid leukemia; V(D)J recombination; SCL; CDKN2A; IKZF1; NOTCH1; BCL11B

INTRODUCTION

Normal VDJ recombination

During normal B- and T-lymphocyte differentiation, a somatic recombination event leads to a physical reconfiguration of genomic DNA in lymphocytes. Non-contiguous V (variable), D (diversity) and J (joining) gene segments become juxtaposed to one another, and form a single exon which encodes the variable region of an immunoglobulin (Ig) or T-cell receptor (TCR) protein. This process is known as V(D)J recombination, and has been recognized at seven distinct genomic loci in mammals (Oettinger, 1999). These seven loci (*TCRA*, *TCRB*, *TCRG*, *TDRD*, *IGH*, *IGL*, *IGK*) encode immunoglobulin or T-cell receptor proteins;

together, these loci are referred to as antigen receptor loci. The diversity, or D segment is placed in parentheses, since not all antigen receptor loci contain D segments.

V(D)J recombination is initiated by cleavage at specific recombination signal sequences (RSSs) that flank V, D, and J segments (Fig. 1A); these RSSs consist of highly conserved heptamer (consensus sequence 5'-CACA/TGTG-3') and nonamer (consensus sequence 5'-ACAAAAACC-3') motifs that are separated by a spacer of 12 or 23 ± 1 nucleotides (Fig. 1B). The heptamer sequence is considered to be the essential recognition element, and within the heptamer, the four nucleotides closest to the fusion site (boxed in Fig. 1B) are the most important (Hesse et al., 1989; Gellert, 1992). On the other hands, the nanomer sequence is not always required for V(D)J recombination (Gellert, 1992). DNA-double strand cleavage at the RSS is mediated by a lymphocyte specific endonuclease complex, encoded by recombination activating gene (*RAG*) 1 and 2. The DNA double-strand break (DSB) induced by RAG-mediated cleavage is repaired by the action of the non-homologous end joining (NHEJ) DNA DSB repair complex, leading to excision of intervening genomic DNA segments between the D and J segments, and reconfigured genomic DNA that has fused the D and J segments. A second, analogous recombination event takes place between the V and DJ segments, resulting in an exon that contains VDJ sequences (Akira et al., 1987; Hesse et al., 1989; Akamatsu et al., 1994).

VDJ recombination between TCR or Ig and non-antigen receptor loci

Although V(D)J recombination is normally limited to antigen receptor loci, illegitimate V(D)J recombination events between antigen receptor loci (IG or TCR) and non-antigen receptor loci, resulting in reciprocal chromosomal translocations involving the IG or TCR loci, have been recognized in both B and T-lineage leukemias (Kuppers and Dalla-Favera, 2001; Lieber et al., 2006; Marculescu et al., 2006). Studies of recurrent breakpoint sequences have suggested that some of these translocations may be caused by “illegitimate” recombination between a *bona fide* antigen receptor RSS, and a sequence that resembles an RSS (a “cryptic” RSS or cRSS).

Site-specific V(D)J recombination between non-antigen receptor loci

In addition to chromosomal translocations between an antigen receptor locus and a proto-oncogene, there are now numerous reports implicating “illegitimate” V(D)J recombination between two non-antigen receptor loci, usually in association with a lymphoid malignancy. The result of these recombination events is an intra-chromosomal interstitial deletion typically between 10–200 kb. As mentioned above, although V(D)J recombinase-mediated inter-chromosomal translocations between an antigen receptor locus and a non-antigen receptor locus have been described, there are no examples of chromosomal translocations caused by illegitimate V(D)J recombination involving two non-antigen receptor loci. These illegitimate recombinations include those between *SIL* and *SCL*, as well as interstitial deletions involving the *CDKN2A*, *IKZF1*, *HPRT*, *Notch1*, and *Bcl11b* genes (Table 1). In this review, we discuss these recurrent deletions, their clinical and biological implications, and the evidence that they are mediated by illegitimate V(D)J recombination.

SIL-SCL (STIL-TAL1)—*SCL* (also known as *TAL-1*), which encodes a transcription factor that is required for normal hematopoiesis (Begley, 1994; Orkin, 1995), was originally identified at the site of a t(1;14) chromosomal translocation in a patient with a hematopoietic “stem” cell leukemia (Begley et al., 1989). This translocation juxtaposed the body of the *SCL* gene with the *TCRD* enhancer, leading to unscheduled expression of *SCL* in hematopoietic cells. Subsequent studies demonstrated that *SCL* translocations involving the *TCRB* and *TCRA* loci were also recognized in patients with T-cell acute lymphoblastic leukemia (T-ALL) (Begley and Green, 1999).

A recurrent, site-specific interstitial deletion of 90 kb that disrupted the *SCL* locus was initially identified in several T-ALL cell lines (Aplan et al., 1990; Brown et al., 1990), and confirmed in studies of primary T-ALL patient samples (Brown et al., 1990). This interstitial deletion juxtaposes the 5' regulatory region and exon 1 of the *SIL* (*SCL* interrupting locus) gene to intron 1 of the *SCL* gene (Fig. 2A). A fusion mRNA transcript is produced from this mutant allele, with exon 1 of *SIL* spliced to exon 3 of *SCL*. Since exon 1 of *SIL* and the first two exons of *SCL* are all non-coding exons, the fusion mRNA does not encode a fusion protein, but rather encodes a full-length SCL protein, and the net result of this interstitial deletion is to bring SCL under control of *SIL* regulatory elements, leading to mis-expression of SCL.

Close examination of the nucleotide sequence at and flanking the genomic breakpoints revealed the following features (Fig. 2B). First, there were sequences that were close matches for the consensus heptamer sequence located precisely at the recombination site of both the *SIL* and *SCL* loci. Second, there were non-templated nucleotides added at the junction site, reminiscent of "N" region addition. Third, there was a variable number of nucleotides deleted from both the *SIL* and *SCL* loci, similar to the exonucleolytic "nibbling" seen at normal antigen receptor coding joins. These features, site-specificity, directed by cryptic heptamer sequences, N region addition, and exonucleolytic "nibbling" led to the conclusion that the *SIL-SCL* fusion was mediated by illegitimate V(D)J recombination between two non-antigen receptor loci.

Subsequent studies revealed that this *SIL-SCL* rearrangement (also known as the *TAL^d*) was present in approximately 26% of childhood T-ALL patients and 16% of adult T-ALL patients, predominantly in younger adults (Delabesse et al., 1997). A retrospective survey indicated that the *SIL-SCL* fusion does not confer an increased or decreased risk of induction failure or relapse (Aplan et al., 1992). PCR amplification of both genomic DNA and RNA of the *SIL-SCL* fusion have been used as a minimal residual disease (MRD) marker in patients whose leukemic cells contain this fusion (van Dongen et al., 1999). Mice that express a *SIL-SCL* transgene under the control of *SIL* regulatory elements do not show an increased incidence of leukemic transformation, however, mice that express both a *SIL-SCL* transgene and *LMO1* develop T-ALL within 6 months, with almost complete penetrance (Aplan et al., 1997). The T-ALL that develop in these mice is preceded by aberrant thymocyte differentiation (Chervinsky et al., 1999; Herblot et al., 2000), and is likely mediated by a functional inactivation of E-proteins such as E2A and HEB (Chervinsky et al., 1999; Herblot et al., 2000).

CDKN2A and CDKN2B—A segment of chromosome 9p21 contains a 40-kb region encoding the *p16/CDKN2A* tumor suppressor gene and two other related genes, *p14/ARF* and *p15/CDKN2B*, all of which encode factors that are important for cell cycle regulation and/or apoptosis (Kohno and Yokota, 1999; Sherr, 2000). Inactivation of this 9p21 gene cluster by several mechanisms, including deletion, methylation, or point mutation is seen frequently in a wide variety of cancers (Foster et al., 1998; Wiencke et al., 1999; Furonaka et al., 2004). In hematologic malignancies, homozygous deletion of the chromosome 9p21 region is the most common event leading to silencing of these genes, and has been proposed to be a crucial event for the development of ALL, since homozygous deletions of this region have been found in 20–50% of ALL patients (Ohnishi et al., 1995; Drexler, 1998). In many cases, the deletions are very large, sometimes involving the entire chromosome. However, the breakpoint junctions from 15 ALL cell lines with a 9p21 deletion (9 T- and 6 B-lineage) were analyzed and revealed that the majority (11 lines, 73%) of the deletions were mediated by illegitimate V(D)J recombination between *CDKN2A* or *CDKN2B* and *MTAP*, a gene immediately telomeric to these loci, in both the T- and B-lineage cell lines (Kitagawa et al., 2002). Five breakpoint cluster sites in lymphoid leukemia (BCS-LL) and six sporadic sites

were identified and noted to contain sequences similar to V(D)J recombination signal sequences. These sites contain 5'-CAC or 3'-GTG trinucleotide sequences that flanked both the distal and proximal breakpoints. Nucleotide sequence of the breakpoint junctions revealed evidence of exonucleolytic "nibbling" from the heptamer sequences and insertions of 1–10 bp nucleotides (N-nucleotide) (Fig. 3A).

As a consequence of this deletion, the *p16/CDKN2A* and/or *p15/CDKN2B* genes, which encode inhibitors of the cell cycle, become inactivated leading to unopposed RB function and cell cycle progression (Kitagawa et al., 2002). In addition, the *CDKN2A* locus encodes *p14(ARF)*, a protein that inhibits *Mdm2*, indirectly leading to activation of *TP53* (Mullighan et al., 2008; Williams and Sherr, 2008). Thus, deletion of this region results in inactivation of two critical tumor suppressor pathways, those involving RB and TP53.

IKZF1—*IKZF1* encodes IKAROS, a member of a family of zinc finger transcription factors that also includes HELIOS, AIOLOS, EOS and PEGASUS. IKAROS is the best characterized of these, and has key roles in the earliest specification of the lymphoid lineage (Rebollo and Schmitt, 2003; Yoshida et al., 2006). The expressions of aberrant, dominant-negative IKAROS isoforms in B- and T-lineage ALL has been previously reported by several groups (Sun et al., 1999; Sun et al., 1999; Sun et al., 1999; Nakase et al., 2000; Tonnelle et al., 2003), although alternative splicing has been reported to be the underlying mechanism (Klein et al., 2006). In addition to these alternate splicing events, deletions of all or a portion of the *IKZF1* gene has been identified in 60–80% of *BCR-ABL1* positive ALL cases (Mullighan et al., 2008; Martinelli et al., 2009), suggesting that *IKZF1* deletions complement the *BCR-ABL1* fusion. In more than half of these cases, the deletions were confined to a subset of internal *IKZF1* exons, most commonly exons 3–6, less commonly exons 1–6 (Mullighan et al., 2008). The genomic deletions were mapped to highly specific regions within introns 1, 2 and 6. Moreover, heptamer recombination signal sequences were located immediately internal to the deletion breakpoints, there was evidence of exonucleolytic "nibbling" from the heptamer sequence, and a variable number of additional nucleotides were present between the consensus intron 1 or 2 and 6 sequences, suggesting N region nucleotide addition (Mullighan et al., 2008; Martinelli et al., 2009). Together, these data suggest that the *IKZF1* $\Delta 3-6$ and $\Delta 1-6$ deletions arise due to aberrant V(D)J recombination (Fig. 3B).

In addition to the frequent co-occurrence of *IKZF1* deletions with *BCR-ABL1* positive B-lineage ALL, *IKZF1* deletions were also detected in 28% of high risk pediatric *BCR-ABL*-negative B-lineage ALL (Mullighan et al., 2009). In this cohort, alteration of *IKZF1* was identified as a predictor of poor outcome (Mullighan et al., 2009). As noted above, *IKZF1* deletions are common in *BCR-ABL* positive ALL and also in lymphoid blast crisis CML (66%), but not reported in chronic-phase CML, suggesting that the *IKZF1* deletion is an important progression event in lymphoid blast crisis (Mullighan et al., 2008; Iacobucci et al., 2009; Martinelli et al., 2009). In pediatric B-cell progenitor ALL, *IKZF1* deletions correlated significantly with an increased frequency of relapse at 5 and 10 years and with resistance to chemotherapy (Mullighan et al., 2009). However, the prognostic value of *IKZF1* deletions in adult patients with *BCR-ABL*-positive ALL is still lacking (Martinelli et al., 2009).

The *IKZF1* deletions result in haploinsufficiency, expression of a dominant-negative IKAROS isoform, or complete loss of IKAROS expression. Within these, the $\Delta 3-6$ isoform of IKAROS (also known as Ik6) has been shown to act as a dominant-negative inhibitor of the transcriptional activity of IKAROS and related family members (Sun et al., 1996; Sun et al., 1999). Mice homozygous for either an *IKZF1* null mutation (Wang et al., 1996) or a dominant-negative *IKZF1* mutation (Georgopoulos et al., 1994) exhibit profound defects in

lymphoid development, and mice heterozygous for a dominant-negative *IKZF1* mutation develop clonal T-cell expansions and lymphoproliferative diseases, (Winandy et al., 1995; Dumortier et al., 2006). Interestingly, although IKAROS inactivation leads to T-cell ALL in murine models, loss of IKAROS appears to be rare in human T-cell ALL (Kastner and Chan, 2011).

Notch1—Notch1 signaling is essential for T-lineage commitment as well as for survival and proliferation of normal thymocytes, and *Notch1* mutations have been identified in both human and mouse TALL samples (Aster et al., 2008). Deletions of the 5' portion of the Notch1 gene were originally identified in murine T-ALLs arising after irradiation (Tsuji et al., 2003). Nucleotide sequence analysis revealed the presence of cryptic heptamer or heptamer- and nonamer-like elements, separated by 12-bp or 23-bp spacers, in the vicinity of the genomic breakpoints (Tsuji et al., 2004). In addition, exonucleolytic “nibbling” and nontemplated “N-region” nucleotides were identified at the breakpoint junctions, suggesting that these deletions were also mediated by illegitimate V(D)J recombination. Further support for the hypothesis that these specific 5'-*Notch1* deletions were mediated by illegitimate V(D)J recombination, came from the observation that these deletions were significantly less frequent in thymic lymphomas from *Scid* mice, and were undetectable in *Rag2*^{-/-} lymphoma. Interestingly, 5' *Notch1* deletions did occur in irradiated *Scid* or *Rag2*^{-/-} mice, but there was no evidence these were V(D)J mediated, since they were not site specific and had no flanking cryptic heptamer sequences (Tsuji et al., 2004; Tsuji et al., 2009). Recently, these 5' *Notch1* deletion mutations were also identified in murine TALL cell lines derived from genetically engineered mice (Ashworth et al., 2010). V(D)J recombinase-mediated 5' *Notch1* deletions (type 1) were observed in 11 of the 14 cell lines and non-V(D)J recombinase-mediated 5' *Notch1* deletions (type 2) were observed in 3 of the 14 cell lines. Interestingly, these 5' *Notch1* deletion frequently accompanied PEST domain mutation (8 of the 14 cell lines). Another group independently reported that spontaneous deletion of 5' *Notch1* sequences were observed in approximately 75% of T-ALLs that developed in *Ikaros* knock down mice (Jeannet et al., 2010). Despite the high frequency of 5' *Notch1* deletions in murine T-ALL, such lesions were not detected in a large panel of human samples (Ashworth et al., 2010).

The NOTCH1 receptor functions as a ligand-activated transcription factor which transduces extracellular signals from the cell surface to the nucleus (Ferrando, 2009; Paganin and Ferrando, 2011). NOTCH receptors are important for cell fate specification during development and participate in numerous vital biological processes. In the hematopoietic system NOTCH1 activation plays a critical role at several stages of T-cell differentiation (Tanigaki and Honjo, 2007). *Notch1* mutations occur in many human and murine T-ALL samples, however, the most common mutations differ between the 2 species (Weng et al., 2004; Lin et al., 2006). In human T-ALL, the most frequent *NOTCH1* mutations are point substitutions or small in-frame insertions or deletions in the NOTCH1 heterodimerization domain (HD) which normally holds NOTCH receptors in an inactive state in the absence of ligand. Thus, these HD mutations result in ligand-independent activation of NOTCH1. In contrast, the most common *Notch1* mutations in murine T-ALL samples are frameshift mutations that result in premature stop codons and deletion of the C-terminal PEST domain (Weng et al., 2004). Deletion of the PEST domain, which is required for proteasomal degradation of the activated NOTCH1 by the FBXW7/SCF complex, leads to persistent activation of downstream targets of the activated NOTCH1 protein (Thompson et al., 2007). PEST mutations are less frequent in human T-ALL samples, and are often in *cis* with HD mutations in the same allele. PEST mutations only weakly activate *Notch1* signaling in reporter assays and are poorly leukemogenic in mice (Chiang et al., 2008), possibly because *Notch1* activation remains ligand-dependent in those assays.

In samples with the 5' *Notch1* deletion mutations, the deletion removes the 5' *Notch1* promoter as well as the initiator ATG codon within exon1, leading to aberrant *Notch1* mRNA initiated from a cryptic promoter within *Notch1* exon 25 (Fig. 3C). This leads to production of a Notch1 protein which lacks the ligand-binding extracellular domain and results in the production of an active, intracellular Notch independent of ligand binding and γ -secretase cleavage. The 5' *Notch1* deletion mutations, which render *Notch1* activation ligand-independent, are often accompanied by PEST mutations in mice. Therefore 5' *Notch1* deletions in mice is considered to function similar to the HD mutation in human T-ALL, both mutations leading to ligand independent activation of *Notch1*. Thus, the combination of HD + PEST mutation or 5' deletion + PEST mutation are both considered to be synergistic, whereas a HD + 5' deletion mutation would be considered redundant in mechanism and have not been described to occur in the same leukemic sample.

Bcl11b—*Bcl11b* encodes a zinc-finger transcription factor that plays an important role in normal T-cell development. In murine thymocytes, Bcl11b inactivation leads to developmental arrest at the DN2-3 stage and aberrant self-renewal activity (Wakabayashi et al., 2003; Ikawa et al., 2010; Li et al., 2010). Mouse thymic lymphomas induced by γ -irradiation exhibited frequent homozygous deletions of the *Bcl11b* tumor suppressor gene on chromosome 12 (Sakata et al., 2004). DNA sequences flanking the breakpoint junction sites contained a consensus heptamer sequence, CACAGTG, in intron 3 and a cryptic heptamer sequence, CACACAC, in intron 1. Inspection of the breakpoint junctions again revealed exonucleolytic “nibbling” and random “N” region nucleotide insertion (Fig. 3D). As a result of the interstitial deletion, an in-frame transcript fusing exons 1 and 4 is produced, encoding an alternately spliced form of Bcl11b referred to as the gamma isoform (Wakabayashi et al., 2003).

In human T-ALL, *BCL11B* is involved in the recurrent t(5;14)(q35;q32) translocation with the *TLX3* locus, in which *BCL11B* gene regulatory elements drive aberrant overexpression of the *TLX3* oncogene (Nagel et al., 2007). In addition, somatic mutations of *BCL11B* have been reported in most of the major molecular subtypes of human T-ALL. Heterozygous deletions involving *BCL11B* were identified in 6% (3 of 47) of primary T-ALL specimens; each deletion pattern was unique and no site-specificity was observed (Gutierrez et al., 2011). Heterozygous missense *BCL11B* mutations were observed in 6% (7 of 117) of primary T-ALL samples; the deletion and missense mutations of *BCL11B* were mutually exclusive. Thus mutations of *BCL11B* are a recurrent abnormality in both human and mouse T-ALL samples. Although the most commonly observed mutations in mice are homozygous deletions leading to production of an alternately spliced *Bcl11b* isoform, the most common mutations in human T-ALL are heterozygous deletions or missense mutations.

The mechanism(s) by which *Bcl11b* mutations might lead to malignant transformation remain unclear. *Bcl11b*-null mice die shortly after birth, exhibiting profound apoptosis of thymocytes (Wakabayashi et al., 2003). Mice which are haplo-insufficient for *Bcl11b* (*Bcl11b*^{+/-}) did not develop lymphoma, but were more susceptible to thymic lymphoma than *Bcl11b*^{+/+} mice when irradiated or in combination with the p53^{+/-} genotype (Kamimura et al., 2007). Taken together, these preliminary findings suggest, but do not prove, that *Bcl11b* may function as a haplo-insufficient tumor suppressor gene.

HPRT—*HPRT*, which encodes hypoxanthine phosphoribosyl transferase, is a constitutively expressed but nonessential gene located at Xq26 in humans. HPRT converts 6-TG to 6-MP, which is a toxic nucleoside analog; thus, loss of HPRT function leads to 6-TG resistance. Investigators have used the loss of HPRT function as a sensitive assay to estimate mutation frequency (Albertini, 2001). The loss of HPRT function can be due to deletions of all or a portion of the gene, frameshift mutations, or single nucleotide substitutions. While

investigating the mechanism(s) which might lead to genomic deletions of the *HPRT* gene, investigators identified a deletion which was consistent with illegitimate V(D)J recombination (Fusco et al., 1991). These investigators analyzed 18 mutant lymphocyte clones isolated from 13 normal newborns and identified an identical intron 1 breakpoint and 3 clustered intron 3 breakpoints (Fig. 3E) (Fusco et al., 1991). Heptamer-like sequences and unique N-nucleotide insertion were identified at the breakpoint site, supporting the notion that these deletions were mediated by illegitimate V(D)J recombination.

Although, this *HPRT* deletion has not been described in primary ALL patients, and is not thought to be oncogenic, this specific *HPRT* deletion appears to be inducible by treatment with etoposide in the CEM cell line. Treatment of the human T-cell leukemia cell line CEM with 5uM etoposide for 4hr led to the generation of clones that had a site-specific fusion between intron 1 and 3 (Chen et al., 1996; Chen et al., 1998).

Experimental evidence for illegitimate VDJ recombination

The nucleotide sequence characteristics described for each of these interstitial deletions, including site-specificity, the presence of cryptic heptamers, addition of non-templated “N” region nucleotides, and exonucleolytic “nibbling” strongly implicate that these deletions are mediated by illegitimate V(D)J recombination between non-antigen receptor loci. To obtain direct experimental evidence that these recombination events were consistent with V(D)J recombination events, several laboratories have applied an extra chromosomal V(D)J recombination assay to assess the functionality of various cryptic recombination signal sequences (cRSSs) in cell culture. The cRSSs present at the breakpoint sites flanking the *SIL-SCL* fusion were shown to be active *in vivo* using an extra chromosomal V(D)J rearrangement assay (Raghavan et al., 2001), and reinforced by EMSA assays that demonstrated binding of RAG proteins to oligonucleotide encompassing the *SIL* and *SCL* cRSSs (Zhang and Swanson, 2008). An extra chromosomal V(D)J recombination assay using the most frequent recombination site associated with the *MTAP/CDKN2A* deletion demonstrated a recombination potential 150-fold less than the consensus RSSs (Kitagawa et al., 2002). Analysis of cRSSs from the *LMO2*, *LMO1*, *HOX11*, *SIL* and *SCL* loci revealed V(D)J recombination at levels from 30-fold to over 20,000-fold less than a consensus RSS (Zhang and Swanson, 2008).

Deep sequencing techniques have recently enabled investigators to determine preferred binding sites for Rag2 throughout the mouse genome. Rag2 ChIP-Seq data demonstrated binding of Rag2 at the 5' end of the *Notch1* locus, near the exon 1 transcription start site, in wild-type thymocytes (Fig. 4A) (Ashworth et al., 2010; Ji et al., 2010). The major Rag2 binding peak overlapped with the ChIP-Seq peak for histone H3K4 trimethylation (H3K4-me3), an activation mark that may be recognized by the plant homology domain of Rag2. Similar to the findings with *Notch1*, analysis of the publicly available Rag2 ChIP-Seq data for the *Bcl11b* locus (Ji et al., 2010) demonstrated Rag2 binding peaks at the recombination sites; the 5' peak also co-localizes with a peak for H3K4-me3 (Fig. 4B). Taken together the combination of nucleotide sequence features, *in vivo* recombination using extra-chromosomal substrates, and *in vivo* localization of Rag2 binding strongly support the hypothesis that illegitimate V(D)J recombination activity is involved in generation of these interstitial deletions.

Difference in illegitimate V(D)J recombination between species

With the exception of the *HPRT* interstitial deletions, all of the interstitial deletions discussed above are known or suspected to contribute to leukemic transformation. Intriguingly, these deletions seem to be species-specific, as the V(D)J recombinase-mediated interstitial deletions involving *SIL* and *SCL*, *CDKN2A/CDKN2B*, and *IKZF1* have been

reported only in human samples, and those involving *Notch1* and *Bcl11b* have been reported only in mouse T-ALL. However, *SCL* activation, *CDKN2A* deletion, and *IKZF1* deletion have all been shown to be oncogenic in mice, indicating a potential role for leukemic transformation of each of these genes in mice. Conversely, *Notch1* and *Bcl11b* mutations are observed in 60% and 10% of human patients with T-ALL, strongly suggesting the oncogenic potential of these genes in humans as well as mice. Although the reason for this species difference is unknown, we suspect that the difference is due to the random placement of cryptic heptamer sequences, as the cryptic heptamers identified in each of these genes (typically present in non-conserved exonic sequences) are not conserved between human and mouse.

Both *Notch1* and *Bcl11b* deletions have been identified in wild-type mouse thymus, at a low frequency (*Notch1*, 7.1×10^{-7} ; *Bcl11b*, 5.6×10^{-6}) (Tsuji et al., 2004). Although the *Bcl11b* deletion was initially identified in thymic lymphomas induced by γ -irradiation or *N*-methyl-*N*-nitrosourea (MNU) treatment, the frequency of the deletion was not increased by γ -irradiation (Sakata et al., 2004), suggesting that these deletions were not triggered by DNA damage. Similar to findings with the 5'-*Notch1* deletion, *Bcl11b* deletions were not detected in the thymus of Rag2-knockout mice and were noted at low frequencies in *Scid* mice, again reinforcing the hypothesis that these deletions are mediated by V(D)J recombinase activity (Sakata et al., 2004; Tsuji et al., 2004).

Summary

V(D)J recombination is essential for the proper development of the mammalian immune system. However, mistakes in normal V(D)J recombination can lead to deletion of tumor suppressor genes or activation of proto-oncogenes. These illegitimate recombination events have thus far been detected only in lymphoid cells, as would be expected for V(D)J recombination. Although these recombinations can be useful clinical markers for disease, the important challenge now is to devise means by which these aberrant recombination events can be prevented.

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Figure 1. V(D)J recombination at antigen receptor loci

A) Normal V(D)J recombination, with one of many V segments (blue) fusing to a D segment (red) and a J segment (orange). Discreet V, D, and J segments are flanked by heptamer/nonamer sequences (purple triangles). Splicing of the recombined VDJ segment to the C segment occurs at the RNA level, as depicted. Transcription is regulated by an enhancer region (green). **B)** Consensus recombination signal sequences. The RSSs normally consists of a highly conserved heptamer motif (consensus sequence 5'-CACA/TGTG-3') and a conserved nonamer sequence (consensus sequence 5'-ACAAAAACC-3') separated by a poorly conserved spacer sequence of 12 or 23 ± 1 bp. The boxed nucleotides closest to the coding sequence are thought to be the most critical for signal specification (see text).

Figure 2. *SIL-SCL* fusion produced by illegitimate V(D)J recombination

A) Intra-chromosomal deletion between *SIL* (red exons; only exons 1, 2, and 18 are shown for clarity) and *SCL* (blue exons). Cryptic heptamers within the *SIL* and *SCL* loci are depicted by purple triangles. The reconfigured genomic DNA produces a *SIL-SCL* fusion mRNA, controlled by *SIL* regulatory elements. **B)** Nucleotide sequence of germline *SIL*, germline *SCL*, and the breakpoints from T-ALL cell lines. *SIL* and *SCL* germline sequences are in capital letters; *SIL* sequences are in bold type. Identical nucleotide sequences are indicated by vertical lines. Non-templated nucleotides at the *SIL-SCL* breakpoints are indicated in lowercase type. Heptamer sequences are boxed.

Figure 3. V(D)J recombination-mediated interstitial deletion of the indicated gene

A) Human *CDKN2A/p16* region. Exons of *MTAP*, *CDKN2A*, *CDKN2B*, and *ARF* are shown, cryptic heptamers are shown as triangle. Centromeric (cen) and telomeric (tel) orientation are indicated. Deletions lead to a loss of *CDKN2A* and *ARF*, some deletions also lead to a loss of *CDKN2B*. **B)** Human *IKZF1*. Interstitial deletion leads to production of the $\Delta 3-6$ isoform, and the $\Delta 1-6$ deletion does not produce a detectable protein. **C)** Murine *Notch1*. The interstitial deletion leads to production of an alternative transcript that begins within exon 25 and initiates translation at a conserved methionine residue, M1727, within the Notch1 transmembrane domain. **D)** Murine *Bcl11b*. The interstitial deletion leads to an in-frame transcript, producing an alternate isoform of Bcl11b. **E)** Human *HPRT1*. The interstitial deletion leads to production of a non-functional protein lacking amino acid residues encoded by exons 2 and 3.

Figure 4. ChIP-Seq demonstrates Rag2 binding nearby *Notch1* and *Bcl11b* breakpoints

A) Binding of Rag2 near the transcriptional start site in exon 1 on *Notch1* was absent in thymocytes from Rag2-deficient mice (Rag2 $-/-$), but readily detectable in wild-type thymocytes. Transcription start sites co-localize with H3K4me3 binding. Known clustered deletion sites are indicated by arrows. *Notch1* genomic structure is as shown. **B)** Binding of Rag2 near the 5' and 3' breakpoint sites of *Bcl11b* was readily detectable in wild-type thymocytes; 3' deletion site shows a lesser, more diffuse signal. Known clustered deletion sites are indicated by arrows. *Bcl11b* genomic structure is as shown. (Data from Ji et al., 2010; GEO accession: GSE21207.)

Table 1

illegitimate V(D)J recombination mediated deletion

Gene	Species	Deletion length	Disease	consequences	Cell lines which have this mutation	Ref
<i>SIL-SCL</i>	Human	80–82kb	T-ALL	Aberrant expression of SCL	RPMI8402, CEM, HSB2, MOLT-16	Aplan 1990
<i>CDKN2A and CDKN2B</i>	Human	160–201kb	T-, B-ALL	Loss of function	H-SB2, TAKEDA, KOPN-55bi, CCRF-CEM, KOPN-K1, L-SMY, KOPN-30, SKW-3, KOPN-K, PEER, MOLT-4	Kitagawa 2002
<i>IKZF1</i>	Human	51–116kb	B-ALL (especially Ph+)	Dominant negative form of IKAROS ($\Delta 3-6$)	$\Delta 3-6$: SUP-B15, OPI, $\Delta 1-6$: 380	Mullighan 2008
<i>HPRT</i>	Human	18–20kb	None	Loss of function		Fuscoe JC 1991
<i>Notch1</i>	Mouse	12kb	T-LBL/ALL	Gain of function		Tsuji 2004
<i>Bcl11b</i>	Mouse	64kb	Thymic lymphoma	Loss of function?		Sakata 2004