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Activation Induced Deaminase is required for the chromosomal breaks in *c-myc* that lead to *c-myc/IgH* translocations

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Abstract

Chromosomal translocation requires formation of paired double strand DNA breaks (DSBs) on heterologous chromosomes. One of the most well characterized oncogenic translocations juxtaposes *c-myc* and the immunoglobulin heavy chain locus (*IgH*) and is found in Burkitt's lymphomas in humans and plasmacytomas in mice. DNA breaks in *IgH* leading to *c-myc/IgH* translocations are created by activation induced cytidine deaminase (AID) during antibody class switch recombination or somatic hypermutation. However, the source of DNA breaks at *c-myc* is not known. Here we provide evidence for the *c-myc* promoter region being required in targeting AID-mediated DNA damage to produce DSBs in *c-myc* that lead to *c-myc/IgH* translocations in primary B lymphocytes. Thus, in addition to producing somatic mutations and DNA breaks in antibody genes, AID is also responsible for the DNA lesions in oncogenes that are required for their translocation.

Introduction

Chromosome translocations have been proposed to require formation of pairs of double-strand DNA breaks (DSBs) and trans-chromosomal ligation of the broken ends (Richardson and Jasin, 2000). Among somatic cells, mature B lymphocytes are particularly prone to DSB formation in *Ig* loci during antibody gene diversification reactions and to malignant transformation by *Ig* translocation (Adams et al., 1983; Adams et al., 1982; Adams et al., 1985; Cory et al., 1983; Crews et al., 1982; Dalla-Favera et al., 1983; Erikson et al., 1983; Hamlyn and Rabbitts, 1983; Jankovic et al., 2007; Kuppers and Dalla-Favera, 2001; Leder et al., 1983; Marcu et al., 1983; Stanton et al., 1983; Taub et al., 1982).

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Two different reactions diversify antibody genes in mature B cells: class switch recombination (CSR) and somatic hypermutation (SHM). CSR involves deletion of DNA sequences and replacement of one antibody constant region for another while retaining the specificity of the antigen receptor (Chaudhuri et al., 2007; Stavnezer et al., 2008). In contrast, SHM introduces non-templated nucleotide changes in antibody variable region genes, producing variants with differing affinity for antigen (Di Noia and Neuberger, 2007; Peled et al., 2008). Although these reactions are very different, they are both initiated by AID, an enzyme that is expressed primarily in germinal center B cells (Muramatsu et al., 2000; Muramatsu et al., 1999; Revy et al., 2000). The same enzyme is also essential for *c-myc/IgH* translocations in primary B cells and mouse plasmacytomas (Kovalchuk et al., 2007; Potter, 2003; Ramiro et al., 2006; Ramiro et al., 2004; Takizawa et al., 2008).

AID initiates CSR and SHM by deaminating cytosine residues in single stranded DNA, which is exposed during transcription (Bransteitter et al., 2003; Chaudhuri et al., 2003; Di Noia and Neuberger, 2002; Dickerson et al., 2003; Imai et al., 2003; Petersen-Mahrt et al., 2002; Pham et al., 2003; Rada et al., 2002; Ramiro et al., 2003). The resulting U:G mismatch is recognized by either uracyl DNA glycosylase or mismatch repair proteins and processed by one of several DNA repair pathways to produce point mutations in variable regions or a combination of mutations and DSBs in switch regions (Di Noia and Neuberger, 2007; Peled et al., 2008; Stavnezer et al., 2008). AID introduces multiple DSBs in switch regions during CSR as evidenced by internal deletions (Dudley et al., 2002; Nagaoka et al., 2002; Petersen et al., 2001; Reina-San-Martin et al., 2003) and accumulation of foci of DNA repair factors at the *IgH* locus preceding CSR (Petersen et al., 2001; Reina-San-Martin et al., 2004). Deletions and duplications are less frequent in *Ig* variable regions accounting for 5% of all hypermutations (Goossens et al., 1998; Wilson et al., 1998). *C-myc* translocates to both *Ig* variable and switch regions and in both cases the translocations are AID dependent (Dorsett et al., 2007; Ramiro et al., 2004).

In addition to targeting *Ig* loci, AID also produces mutations in a number of other genes including oncogenes (Gordon et al., 2003; Liu et al., 2008; Muschen et al., 2000; Pasqualucci et al., 1998; Pasqualucci et al., 2001; Shen et al., 1998). However, mutations in *c-myc* are either absent (Pasqualucci et al., 2001; Shen et al., 1998), or occur at a rate that is several orders of magnitude lower than in *IgH* (Liu et al., 2008). In addition the available sequence analysis shows no evidence of AID dependent deletions or duplications in *c-myc* or other oncogenes that might suggest AID dependent DSB formation (Gordon et al., 2003; Liu et al., 2008; Mullighan et al., 2007; Muschen et al., 2000; Pasqualucci et al., 1998; Pasqualucci et al., 2001; Shen et al., 1998). Furthermore, AID induced lesions in genes other than antibody genes appear to be repaired by a pathway that is distinct from and less error prone than lesions in *IgH* (Liu et al., 2008). Indeed, when DSBs have been detected in *c-myc* in human B cells they have been found to be AID independent (Zan et al., 2003).

One potential source for the DSBs in *c-myc* is fragile DNA segments that are prone to form non-B-DNA configurations including Z-DNA, triple helical H-DNA, and G-quadruplex DNA (reviewed in (Liu and Levens, 2006; Marcu et al., 1992; Spencer and Groudine, 1991; Wierstra and Alves, 2008)). These sequences are particularly susceptible to AID independent DNA damage and DSB formation during transcription or replication, but also in response to environmental agents and metabolic stress and they have been implicated as a source of the DSBs in *c-myc* that lead to translocation (reviewed in (Reddy and Vasquez, 2005)).

Here we report on experiments that examine the role of the *c-myc* promoter and AID in producing the lesions in *c-myc* that lead to *c-myc/IgH* translocations in mature B cells *in vivo*.

Results

c-myc is transcribed from several promoters distributed in a region surrounding the non-coding first exon of the gene (Wierstra and Alves, 2008). To determine whether *c-myc* transcription is required for translocation to *IgH*, we used homologous recombination in embryonic stem cells to delete a 2.2 Kbps genomic fragment that includes the region corresponding to *c-myc* promoters P0, P1 and P2 (Figure 1A, and Supplemental Figure S1). *Myc*^{Δ/+} mice were born at nearly Mendelian ratio (Figure 1B) and B cell development and activation in response to mitogenic stimuli were unaltered in heterozygous mice (Supplemental Figure S1).

To examine the transcriptional status of the *Myc*^Δ allele we performed RNA fluorescence in situ hybridization (Osborne et al., 2007). Among the *Myc*^{+/+} B cells with transcriptionally active alleles (n=168), 76% had one active *c-myc* allele and 24% had two active alleles. In striking contrast, 97% of the transcriptionally active *Myc*^{Δ/+} B cells (n=86) had one silent allele, demonstrating that only one of the two alleles was efficiently transcribed in mutant mice (Figure 1C). Consistent with the reported lethality of *c-myc* deletion (Davis et al., 1993), the *Myc*^Δ mutation resulted in intercrosses between *Myc*^{Δ/+} that failed to produce any *Myc*^{Δ/Δ} homozygous mutants (Figure 1B). We conclude that *c-myc* transcription is severely impaired by the *Myc*^Δ mutation.

To examine the effects of the *Myc*^Δ mutation on *c-myc/IgH* translocations we assayed for these events in heterozygous *Myc*^{Δ/+} B cells stimulated with LPS and IL-4, using a previously described PCR assay to detect both derivative 12 and 15 chromosomes (Figure 1D and Supplemental Figure S2). Translocations were detected in *Myc*^{Δ/+} mice, but sequence analysis revealed that 22 of 23 of these were *IgH* fusions to the wild type *Myc*⁺ allele (Figure 1D). Thus, the *Myc*^Δ mutation drastically reduces *c-myc*'s propensity to undergo oncogenic chromosomal translocations to *IgH*.

c-myc/IgH translocations induced by Cre

The *IgH* locus on mouse chromosome 12 and the *c-myc* locus on chromosome 15 co-localize in transcription factories in activated B lymphocytes, and this physical proximity has been proposed to facilitate *c-myc/IgH* translocations (Osborne et al., 2007; Parada et al., 2004; Roix et al., 2003).

To determine whether the *Myc*^Δ allele was accessible for translocation we made use of the phage-derived Cre-loxP recombinase system (Van Duyne, 2001). Cre recognizes 34 nucleotide loxP sites and catalyzes DNA recombination between two such sites. Recombination requires that Cre gain access to the DNA target and the efficiency of the reaction is inversely proportional to the distance between loxP sites (Yu and Bradley, 2001). To test for Cre mediated translocations between the loxP site on the *Myc*^Δ allele and *IgH* we introduced a single loxP site into *IgH*, upstream of the switch μ region (*IgH*^{I/+} mice, Figure 2A and Supplemental Figure S3). As a control for the *Myc*^Δ allele we also introduced a single loxP site into the first intron of intact *c-myc* (*Myc*^{I/+} mice, Figure 2A and Supplemental Figure S4). *IgH*^{I/+} and *Myc*^{I/+} mice showed normal B lymphocyte development and switching in response to LPS and IL-4 stimulation (Supplemental Figure S3, S4 and data not shown). Compound heterozygous B cells bearing either *Myc*^Δ or *Myc*^I and *IgH*^I (*Myc*^Δ/*IgH*^I and *Myc*^I/*IgH*^I respectively) were tested for Cre mediated translocations using a retrovirus expressing Cre. By semi-quantitative analysis, we found that the frequency of *c-myc/IgH* translocations in *Myc*^Δ/*IgH*^I and *Myc*^I/*IgH*^I B cells expressing Cre was indistinguishable (0.5×10^{-5} for der12) (Figure 2B).

To examine the nature of Cre mediated interchromosomal junctions we sequenced 8 candidate translocations. Irrespective of whether the translocations originated from *Myc*^Δ/*IgH*^I or *Myc*^I/*IgH*^I B cells, the breakpoints were always precisely at the loxP site. To corroborate these

results in the absence of retroviral Cre over-expression, we crossed the Myc^{Δ}/IgH^{I} and Myc^{I}/IgH^{I} mice to mice where Cre expression is under the control of regulatory elements of *aicda* ($AID^{Cre/+}$, Supplemental Figure S5). Cre-mediated translocations were readily detected in both $Myc^{\Delta}/IgH^{I}/AID^{Cre}$ and $Myc^{I}/IgH^{I}/AID^{Cre}$ B lymphocytes upon mitogenic stimulation *in vitro* and even in Peyer's Patches *in vivo* (Figures 2C and Supplemental Figure S6). Thus, Cre rescues translocation of the mutant Myc^{Δ} allele in activated B cells *in vitro* and *in vivo*. We conclude that both the active (Myc^{I}) and silent (Myc^{Δ}) alleles reside in Cre-accessible nuclear compartments and remain in sufficient spatial proximity to *IgH* to permit translocation irrespective of *c-myc*'s transcriptional status.

DSBs in *c-myc* catalyzed by I-SceI

AID induced DSBs along the *IgH* locus can be detected by combining immunocytochemistry to detect accumulation of 53BP1 foci, and fluorescence in situ hybridization to localize *IgH* (Figure 3 and (Reina-San-Martin et al., 2004)). Background levels of co-localization of 53BP1 foci and *IgH* were found in AID deficient B cells, and the frequency increased to 42.5% in AID sufficient B cells (Figure 3A and (Reina-San-Martin et al., 2004)). In contrast, when we examined *c-myc* by the same technique we found no AID dependent increase in 53BP1 foci during CSR (Figure 3A). Retroviral over-expression of AID did however result in DNA damage at *c-myc*, as determined by increased co-localization of 53BP1 foci (12.2% vs. 4.3% with control, $p = 0.007$; Figure 3B) and accumulation of mutations in intron 1 (1.2×10^{-4} vs. $<0.07 \times 10^{-4}$ with control, $p = 0.0005$; Supplemental Table 1). Thus, our immuno-FISH experiments are in agreement with the sequencing data and fail to show evidence for AID induced DNA damage on *c-myc* during CSR under physiologic circumstances (Liu et al., 2008; Pasqualucci et al., 2001; Shen et al., 1998). However, *c-myc* can be an AID target when AID is over-expressed.

To examine the consequence of DSB formation at *c-myc*, we used the yeast derived I-SceI endonuclease, which recognizes an 18 bps site that is otherwise absent from the mouse genome (reviewed in (Stoddard, 2005)). DNA breaks were created in the presence or absence of AID using I-SceI to cut at cognate target sites introduced into the Myc^{I} and IgH^{I} alleles (Figure 2A and Supplemental Figures S3 and S4). Similar to AID induced DNA damage, retroviral expression of I-SceI in activated $Myc^{I/+}AID^{-/-}$ or $IgH^{I/+}AID^{-/-}$ B lymphocytes produced frequent DSBs in *c-myc* or *IgH* respectively. As determined by immuno-FISH, 30.8% of $Myc^{I/+}$ B cells expressing I-SceI showed co-localization of *c-myc* and 53BP1 and 21.9% of $IgH^{I/+}$ B cells exhibited *c-myc*/53BP1 co-localization (Figure 3C). Control B lymphocytes infected with a catalytically inactive form of I-SceI (I-SceI*) showed background levels of DNA damage at *c-myc* or *IgH* (Figure 3C). We conclude that I-SceI efficiently targets $Myc^{I/+}$ and $IgH^{I/+}$ in activated B cells.

c-myc/IgH translocations catalyzed by I-SceI

To determine whether DSBs produced by I-SceI can serve as substrates for *c-myc/IgH* translocation we assayed for these events in LPS and IL-4 stimulated $Myc^{I/+}IgH^{I/+}AID^{-/-}$ B cells expressing I-SceI or I-SceI* (Figure 4). Whereas we found no translocation with the retrovirus alone or I-SceI*, I-SceI expression produced der15 translocations at a rate of 10×10^{-5} (Figures 4B, 6, Supplemental Figures S7, S10 and data not shown; Note: while we assayed both der12 and der15 translocations, the close proximity of the binding sites of the der12 primers to the I-SceI sequences and processing of the DNA ends, see below, appears to result in the underestimation of their frequency). Thus, DSBs introduced by I-SceI in *c-myc* and *IgH* result in *c-myc/IgH* transchromosomal rearrangements.

Translocation breakpoints were found precisely at the I-SceI site and also at distances of up to 2178 bps from the site of the DSB (Figure 4C, and Supplemental Table 2). The average distance

of the breakpoint position from the I-SceI site on *c-myc* was 50 bps for der12 and 1744 bps for der15, and for *IgH* it was 39 bps for der12 and 91 bps (Supplemental Table 2). Out of 21 translocation junctions, 19 involved 1–7 bps of microhomology (average microhomology of 2.9 bps) and 2 of them included insertions (Figure 5 and Supplemental Table 2). We conclude that repair of I-SceI breaks frequently involves processing of the ends and microhomology mediated joining.

Source of DSB in *c-myc* during *c-myc/IgH* translocation

To determine if I-SceI breaks can be joined to AID induced lesions we examined $\text{Myc}^{I/+}$ B cells infected with I-SceI or I-SceI* encoding retroviruses. Baseline levels of AID mediated translocation were enhanced by I-SceI cutting in $\text{Myc}^{I/+}$ B cells (for der15 I-SceI*: 0.2×10^{-5} vs. I-SceI: 0.8×10^{-5} , $p = 0.0002$; Figure 5B and Supplemental Figure S8). Consistent with this enhancement, 15 of 17 translocations produced in $\text{Myc}^{I/+}$ B cells infected with I-SceI were Myc^I to *IgH* translocations, while only 2 involved the wild type Myc^+ allele (Supplemental Table 3). Whereas the breakpoints for *c-myc/IgH* translocations cloned from wild type control B cells were spread over 1.4 Kbps surrounding the first exon of *c-myc*, 10/15 I-SceI induced Myc^I breakpoints were found precisely at the I-SceI site and the remainder mapped within 74 bps of the site (Figure 5C and Supplemental Table 3). In contrast to the breakpoints produced by joining two I-SceI DSB in the absence of AID (Figure 4C, Supplemental Table 2), 7 of the junctions between an I-SceI DSB in *c-myc* and an AID lesion in *IgH* were blunt, 2 had an insertion, and the remaining 6 showed 1–3 bps of microhomology (overall average microhomology: 0.8 bps; Figure 5D, Supplemental Table 2). A similar relationship between direct versus microhomology mediated joins is found in *c-myc/IgH* translocations in wild type B cells (Figure 5D and Supplemental Table 4) and in physiological CSR, where 30–60% of the joints are blunt and the remainder use 1–4 bps of microhomology (reviewed in (Chaudhuri et al., 2007; Stavnezer et al., 2008)).

Whereas I-SceI induced DSBs in *c-myc* enhanced translocation frequency (Figure 5B), we found no difference in translocation frequency between $\text{IgH}^{I/+}$ B cells infected with I-SceI or I-SceI* (for der15 0.14×10^{-5} vs. 0.2×10^{-5} respectively; Supplemental Figure S9). The breakpoint was at the I-SceI cognate site in 2 out of 4 translocations involving the IgH^I allele, confirming that similar to Myc^I , IgH^I was also substrate for I-SceI cleavage and recombination (Supplemental Figure S9). Therefore, although I-SceI generates DSBs at *c-myc* and *IgH* with comparable efficiency (Figure 3C), it enhances the frequency of *c-myc/IgH* translocation in $\text{Myc}^{I/+}$ but not $\text{IgH}^{I/+}$ B cells. We conclude that I-SceI induced breaks in *c-myc* or *IgH* can be joined to AID produced breaks in *IgH* or *c-myc*, respectively, to create *c-myc/IgH* translocations. In addition, we find that DSBs in *c-myc* but not *IgH* are limiting for translocation since a single I-SceI break in *c-myc* but not *IgH* enhances translocation.

To determine whether the source of the DSB alters the frequency of translocation we compared the incidence of *c-myc/IgH* transchromosomal rearrangements in $\text{Myc}^{I/+}\text{IgH}^{I/+}\text{AID}^{-/-}$ B cells infected with AID or I-SceI or Cre expressing retroviruses. We found that the rate of translocation was similar for all three DNA damage-inducing enzymes (for der15 5×10^{-5} for AID, 10×10^{-5} for I-SceI and 3×10^{-5} for Cre, Figure 6 and Supplemental Figure S10). We conclude that the rate of *c-myc/IgH* translocation is similar irrespective of whether the DSBs are induced by AID, I-SceI or Cre.

To determine whether AID is essential to create the breaks in *c-myc* that lead to *c-myc/IgH* translocation we induced DSBs in the *IgH* locus of $\text{IgH}^{I/+}\text{AID}^{-/-}$ or the *c-myc* locus of $\text{Myc}^{I/+}\text{AID}^{-/-}$ B cells using I-SceI. In contrast to $\text{IgH}^{I/+}\text{Myc}^{I/+}\text{AID}^{-/-}$ B cells (Figure 4), and to AID sufficient $\text{Myc}^{I/+}$ and $\text{IgH}^{I/+}$ B cells (Figure 5 and Supplemental Figure S9), I-SceI induced DSBs in either *c-myc* or *IgH* alone, in $\text{Myc}^{I/+}\text{AID}^{-/-}$ or $\text{IgH}^{I/+}\text{AID}^{-/-}$ B cells respectively, failed to produce *c-myc/IgH* translocations (Figure 7). Thus, when I-SceI alone

or I-SceI and AID coordinately generate DSBs on both chromosomes they result in *c-myc/IgH* translocations. In contrast, in the absence of AID, I-SceI-induced DSBs generated solely at *IgH* or *c-myc* do not produce translocations. We conclude that AID is essential for the DSBs in both of the chromosomes involved in *c-myc/IgH* translocation.

Discussion

DSBs are dangerous lesions because they can lead to genomic instability and oncogenic chromosomal translocations. Although DSBs rarely accumulate in somatic cells, they are obligate intermediates in CSR, a reaction that involves introduction of multiple targeted DSBs in the *IgH* locus in mature B lymphocytes. The genome is normally protected from AID dependent *IgH* translocations by ATM-, p53- and p19Arf-dependent pathways that detect and signal DNA damage and oncogenic stress (Ramiro et al., 2006). Nevertheless, these protective mechanisms sometimes fail, as evidenced by involvement of the *IgH* locus in nearly all cancer-associated chromosomal translocations in mature B cell lymphomas and multiple myeloma (Kuppers and Dalla-Favera, 2001).

AID creates the lesions in the *IgH* locus that lead to translocations (Franco et al., 2006; Petersen et al., 2001; Ramiro et al., 2006; Ramiro et al., 2004). However, DSBs in *IgH* alone would not be sufficient for translocation because aberrant joining of heterologous chromosomes requires formation of paired DNA breaks (Richardson and Jasin, 2000), and the source of DNA damage in the oncogenes that are translocated in mature B cell malignancies has not been determined (Kuppers and Dalla-Favera, 2001).

In Burkitt's lymphoma and in murine plasmacytoma, *IgH* is translocated to *c-myc* leading to deregulated expression of *c-myc* and malignant transformation (Adams et al., 1983; Adams et al., 1982; Adams et al., 1985; Crews et al., 1982; Dalla-Favera et al., 1983; Erikson et al., 1983; Hamlyn and Rabbitts, 1983; Marcu et al., 1983; Taub et al., 1982). Recurrent translocation between *c-myc* and *IgH* has been attributed in part to proximity of the two loci in B cells due to the dynamic relocation of the two genes to transcription factories following B cell activation (Osborne et al., 2007; Roix et al., 2003). Consistent with a role for transcription in facilitating *c-myc/IgH* translocation our experiments show that the DNA region containing the *c-myc* promoters is required for efficient translocation in stimulated B cells. However, this effect does not appear to be due to transcriptionally regulated proximity of the two genes or accessibility to recombinase as indicated by equivalent levels of Cre mediated translocation between IgH^I and transcriptionally crippled Myc^{Δ} or active Myc^I alleles. In light of the current discussion about "breakage first" or "contact first" models of chromosomal translocations, our data does not speak directly to the issue of physical proximity, but suggest that proximity is not limiting. Although loss of the *c-myc* promoter region interferes with transcription and *c-myc/IgH* translocation, it does not interfere with *c-myc*'s ability to come into contact with *IgH* or with its accessibility to Cre.

Instead, it appears that the *c-myc* promoter region is essential to recruit AID to initiate DSB formation. This is consistent with the finding that *Ig* transcriptional regulators including promoters, enhancers and the E-box motif CAGGTG (Michael et al., 2003) are involved in targeting AID to *Igs* (Di Noia and Neuberger, 2007; Peled et al., 2008; Stavnezer et al., 2008). Although the elements required for bringing AID to *Ig* or *c-myc* DNA have not yet been precisely defined, *in vitro* experiments with artificial substrates and in *E. coli* show that transcription is essential because it exposes single stranded DNA which is the substrate for AID (Chaudhuri et al., 2003; Dickerson et al., 2003; Pham et al., 2003; Ramiro et al., 2003). Furthermore, the formation of R-loops during transcription has been proposed to play a role in targeting AID (Yu et al., 2003). Thus, our observations suggest a transcriptional requirement

for AID targeting to oncogenes, but the mechanism by which transcription targets AID to any gene remains to be determined.

Although increased AID expression was found to increase *c-myc/IgH* translocation, this effect could have been due to higher levels of DNA breaks on *c-myc* or *IgH* or both (Dorsett et al., 2008; Ramiro et al., 2006; Takizawa et al., 2008). Previous work suggested that DSBs in *c-myc* are AID independent and might be due to increased susceptibility of fragile non-B-DNA to environmental agents like reactive oxygen intermediates or transcriptional or replication-linked DNA damage (Belotserkovskii et al., 2007; Boles and Hogan, 1987; Lieber et al., 2003; Wang and Xu, 2004; Yang and Hurley, 2006). This idea was supported by the finding of AID independent DSBs in *c-myc* in B cell lymphomas (Zan et al., 2003), the relative rarity of somatic mutations in *c-myc* (Liu et al., 2008; Pasqualucci et al., 2001; Shen et al., 1998), and the lack of deletions or duplications in *c-myc* that would mark DSB formation (Gordon et al., 2003; Liu et al., 2008; Mullighan et al., 2007; Muschen et al., 2000; Pasqualucci et al., 1998; Pasqualucci et al., 2001; Shen et al., 1998). Despite these findings, our experiments show that AID can indeed induce lesions and mutations at *c-myc*, and demonstrate that AID is essential for the lesions in *c-myc* that result in *c-myc/IgH* translocation. Nevertheless, the absence of easily detectable DNA damage without AID over-expression suggests that formation of DNA lesions on *c-myc* may be one of the limiting factors in *c-myc/IgH* translocation under physiologic conditions. Consistent with this notion, we find that increasing the frequency of DSB formation by the I-SceI endonuclease at *c-myc* but not *IgH* enhances translocation. Since I-SceI targets *c-myc* and *IgH* with similar efficiency, we can also conclude that AID-induced DSB formation at *c-myc* is lower than at *IgH*. Although the DNA lesions introduced by either I-SceI or AID alone produced *c-myc/IgH* translocation, the extent of DNA processing and the structure of the intermolecular joints differed. The most extensive resection of DNA ends was found at *c-myc* when I-SceI mediated translocations in the absence of AID. Why the same resection was not detected at *IgH* under the same conditions is unclear, but the data is consistent with the existence of locus specific DNA damage processing factors (Liu et al., 2008). Whereas I-SceI induced breaks were resolved by a mechanism biased toward junctional microhomology reminiscent of alternative NHEJ (Richardson and Jasin, 2000), AID breaks produced a combination of blunt and short microhomology joints similar to those found in class switch recombination (Stavnezer et al., 2008). The difference in trans-chromosomal joints between I-SceI and AID may be explained by recruitment of a distinct set of repair enzymes to the two types of DNA damage. In the case of I-SceI a DSB is introduced directly by the yeast endonuclease and must be recognized by the DNA damage recognition and repair system before being processed and joined. In contrast, AID induced U:G mismatches are recognized by mismatch and base excision repair enzymes before recruitment of additional repair factors that then process the lesions to produce DSBs. Thus, the difference in the joints produced by I-SceI breaks and AID lesions may in part be due to the distinct mechanisms that recognize the initial lesion and produce the DSB.

Although the precise mechanism by which AID introduces lesions in DNA is still debated, AID is thought to produce U:G mismatches in DNA by cytidine deamination (Di Noia and Neuberger, 2007; Peled et al., 2008; Stavnezer et al., 2008). In B cells undergoing SHM or CSR, uracyl is detected and removed from antibody genes by error prone mechanisms leading to mutations or DSBs. Other genes, including oncogenes, appear to repair such lesions in a relatively error free manner, which may explain the nearly undetectable level of AID mediated mutation in genes like *c-myc* (Supplemental Table 1 and (Liu et al., 2008; Pasqualucci et al., 2001; Shen et al., 1998)). Our experiments reveal that like the error prone repair pathways that process the lesions at the *IgH* locus, the relatively error free repair of AID dependent lesions in *c-myc* can also lead to oncogene translocation.

Experimental Procedures

Mice

$Myc^{\Delta/+}$, $IgH^{I/+}$, $Myc^{I/+}$, and $AID^{Cre/+}$ mice were generated by homologous recombination in C57BL/6 albino ES cells. Details of the targeting vectors, screening by Southern Blot and genotyping PCR are provided in the legends to the Supplemental Figures. $AID^{-/-}$ mice (Muramatsu et al., 2000) were backcrossed to C57BL/6 for at least 10 generations. All experiments were in agreement with protocols approved by the Rockefeller University and NIH Institutional Animal Care and Use Committee.

Retroviruses

Cre, I-SceI, I-SceI*, and AID were PCR-amplified and cloned into the pMX-IRES-GFP plasmid. In I-SceI*, D44A and D145A substitutions to inactivate the catalytic sites were introduced by PCR (Stoddard, 2005).

B cell cultures and retroviral transduction

Resting B lymphocytes were isolated from mouse spleens by immunomagnetic depletion with anti-CD43 MicroBeads (Miltenyi Biotech), and cultured at 0.5×10^6 cells/ml in R10 medium: RPMI supplemented with L-glutamine, sodium pyruvate, antibiotic/antimycotic, 50 μ M 2-mercaptoethanol (all from GIBCO-BRL), and 10% fetal calf serum (Hyclone). B cells were stimulated in the presence of 25 μ g/ml lipopolysaccharide (LPS, E. coli serotype 0111:B4, Sigma) and 5 ng/ml mouse recombinant IL-4 (Sigma), and analyzed after 96 hours. For the analysis of translocations in $Myc^{\Delta/+}$ mice (Figure 1D) cultures were supplemented with 50ng/ml mouse recombinant BAFF (R&D Systems) to boost the translocation frequency. For infection experiments, retroviral supernatants were prepared by co-transfecting BOSC23 cells with pCL-Eco and pMX-IRES-GFP-derived plasmids (encoding for Cre, I-SceI, I-SceI*, or AID) using Fugene 6, 72 hours before infection. At 20 and 44 hours of lymphocyte culture, retroviral supernatants were added and B cells spinoculated at 1150g for 1.5 hours in the presence of 10 μ g/ml polybrene. After 4 hours at 37C, supernatants were replaced with LPS and IL4 supplemented R10. B cells were analyzed at 96 hours from the beginning of their culture.

Flow cytometry and cell sorting

For FACS analysis, spleen cell suspensions were stained with fluorochrome-conjugated anti-CD19, anti-CD3, anti-IgM, anti-IgD, and anti-IgG1 (Pharmingen). CFSE-labeling for cell division was at 37C for 10 min in 5 μ M carboxyfluorescein succinimidyl ester. In all experiments, retrovirally infected GFP⁺ cells were sorted to >95% purity with a FACSVantage SE or FACS Aria instruments (Becton Dickinson).

RNA fluorescence *in situ* hybridization (RNA FISH)

Stimulated splenic B cells were fixed on slides and analyzed by RNA FISH as described (Gribnau et al., 1998; Gribnau et al., 2000; Chakalova et al., 2004).

Immuno-FISH

Immunocytochemistry staining followed by fluorescence *in situ* hybridization (Immuno-FISH) was as described (Petersen et al., 2001; Difilippantonio et al., 2000).

PCR assay for translocations

PCR reactions were performed on genomic DNA from 10^5 cells, unless otherwise indicated. Using Expand Long Template PCR System nested reactions were performed with the following

primers: for derivative chromosome 12 translocations first round, 5-TGAGGACCAGAGAGGGATAAAAAGAGAA-3 and 5-GGGGAGGGGGTGTCAAATAATAAGA-3; for derivative chromosome 12 translocations second round, 5-CACCCTGCTATTTCTTGTTGCTAC-3 and 5-GACACCTCCCTTCTACTCTAAACCG-3; for derivative chromosome 15 translocations first round, 5-ACTATGCTATGGACTACTGGGGTCAAG-3 and 5-GTGAAAACCGACTGTGGCCCTGGAA-3; for derivative chromosome 15 translocations second round, 5-CCTCAGTCACCGTCTCCTCAGGTA-3 and 5-GTGGAGGTGTATGGGGTGTAGAC-3; for derivative chromosome 12 translocations (upstream of switch μ) first round, 5-GGCAACTTCAAATTCATTAACACAT-3 and 5-GGGGAGGGGGTGTCAAATAATAAGA-3; for derivative chromosome 12 translocations (upstream of switch μ) second round, 5-AAATGTGAGTGACCCAGACA ACG-3 and 5-GACACCTCCCTTCTACTCTAAACCG-3; PCR conditions were according to manufacturer instructions with 29 cycles for the first round (10 cycles at 92C, 10 s; 60C, 30 s; and 68C, 7 min; followed by 19 cycles at 92C, 15 s; 60C, 30 s; and 68C, 7 min with 20 s of additional extension time per cycle) and 25 cycles for the second round (10 cycles at 92C, 10 s; 60C, 30 s; and 68C, 4 min; followed by 15 cycles at 92C, 15 s; 60C, 30 s; and 68C, 4 min with 20 s of additional extension time per cycle). PCR products were gel-purified and sequenced.

Southern Blotting

Southern blot analysis was performed as described (Ramiro et al., 2006). Oligoprobes were: for der15 *c-myc* 5-GCCGCCACTTTACTGGACTGCGCAGG-3; for der15 IgH 5-GAGGGAGCCGGCTGAGAGAAGTTGGG-3; for der12 *c-myc* 5-GCAGCGATTCAGCACTGGGTGCAGG-3; for der12 IgH (3' of switch μ) 5-CCTGGTATACAGGACGAACTGCAGCAG-3; for der12 IgH (5' of switch μ) 5-TTCAGTCATTGCTTTAGGGGGAG-3. P value was calculated using the 2-Tail Fisher's exact test.

Mutational analysis

Primers 5-TGGTCTTTCCCTGTGTTCTTTCTG-3 and 5-GACACCTCCCTTCTACTCTAAACCG-3 were used to amplify a portion of *c-myc* intron 1 with Pfu polymerase and 25 cycles of amplification (95C, 10 s; 58C, 30 s; and 72C, 90 s), prior to cloning in TOPO vector and sequencing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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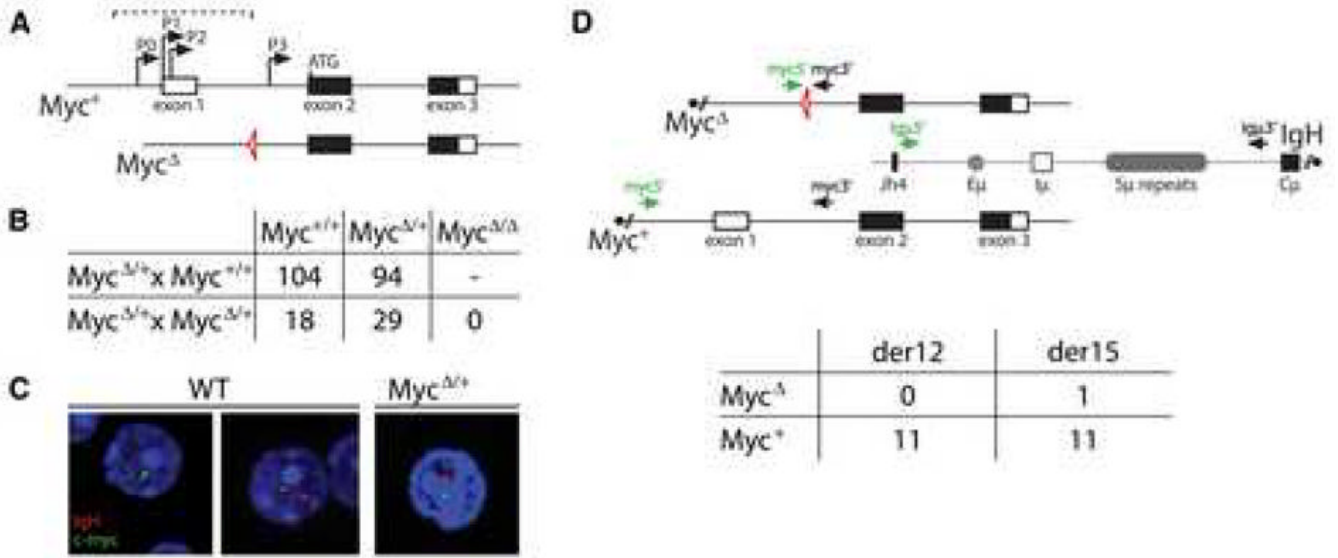


Figure 1. The *Myc*^Δ allele is protected from translocating to the IgH
 (A) Schematic representation of wild type (*Myc*⁺) and mutant (*Myc*^Δ) *c-myc* alleles. The dashed bracket indicates the region deleted and replaced by a loxP site (triangle) in the *Myc*^Δ allele.
 (B) Genotyping summary from the indicated *Myc*^{Δ/+} cross. (C) RNA-FISH showing monoallelic vs. biallelic *c-myc* transcription in wild type (WT) vs. *Myc*^{Δ/+} cells, respectively.
 (D) Diagram showing the location of the primers used in the PCR assay to detect derivative chromosome 12 (der12, black arrows) and derivative chromosome 15 (der15, green arrows) *c-myc/IgH* translocations. The table summarizes the number of allele-specific der12 and der15 *c-myc/IgH* translocations in activated *Myc*^{Δ/+} B cells, as determined by sequencing. Three independent experiments.

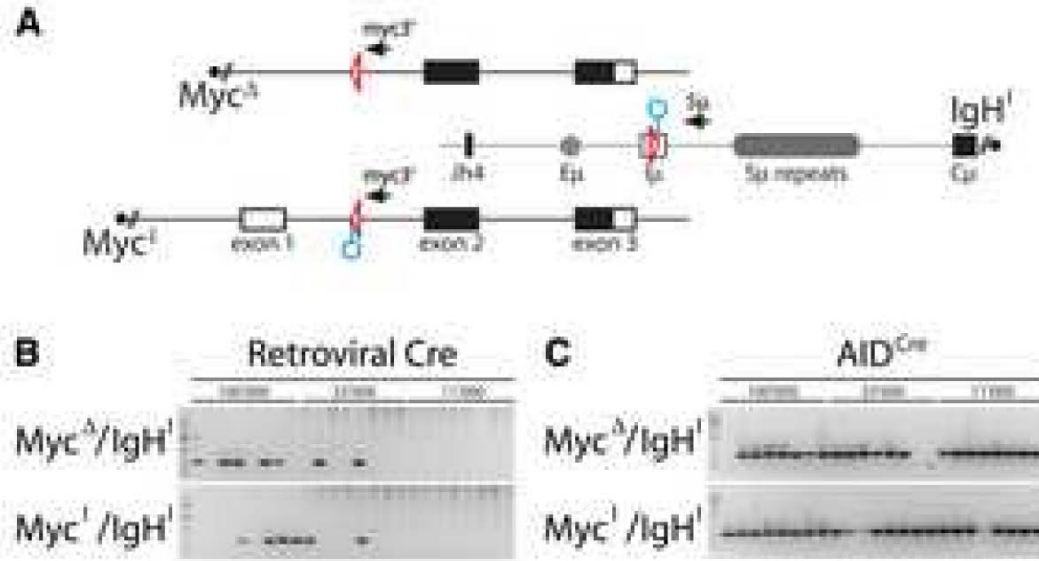


Figure 2. Cre-mediated *c-myc/IgH* translocations

(A) Schematic representation of the *Myc*^Δ, *Myc*^I and *IgH*^I alleles with the PCR primers for detecting der12 *c-myc/IgH* translocations. Triangles represent loxP sites, circles point to recognition sequences for I-SceI. (B) Translocations by retroviral Cre. Ethidium bromide stained agarose gel with 0.5kb PCR products corresponding to precise loxP-to-loxP *c-myc/IgH* translocations (as verified by sequencing). B lymphocytes of the indicated genotypes were stimulated with LPS and IL-4 and infected with retroviruses that direct expression of Cre. The number of cells that were assayed in each reaction is indicated. Two independent experiments. (C) Translocations in AID^{Cre} B cells. Agarose gel with 0.5kb PCR products corresponding to precise loxP-to-loxP *c-myc/IgH* translocations (as verified by sequencing). AID^{Cre} B cells of the indicated genotypes were stimulated with LPS and IL-4. The number of cells assayed in each reaction is indicated. Two independent experiments.

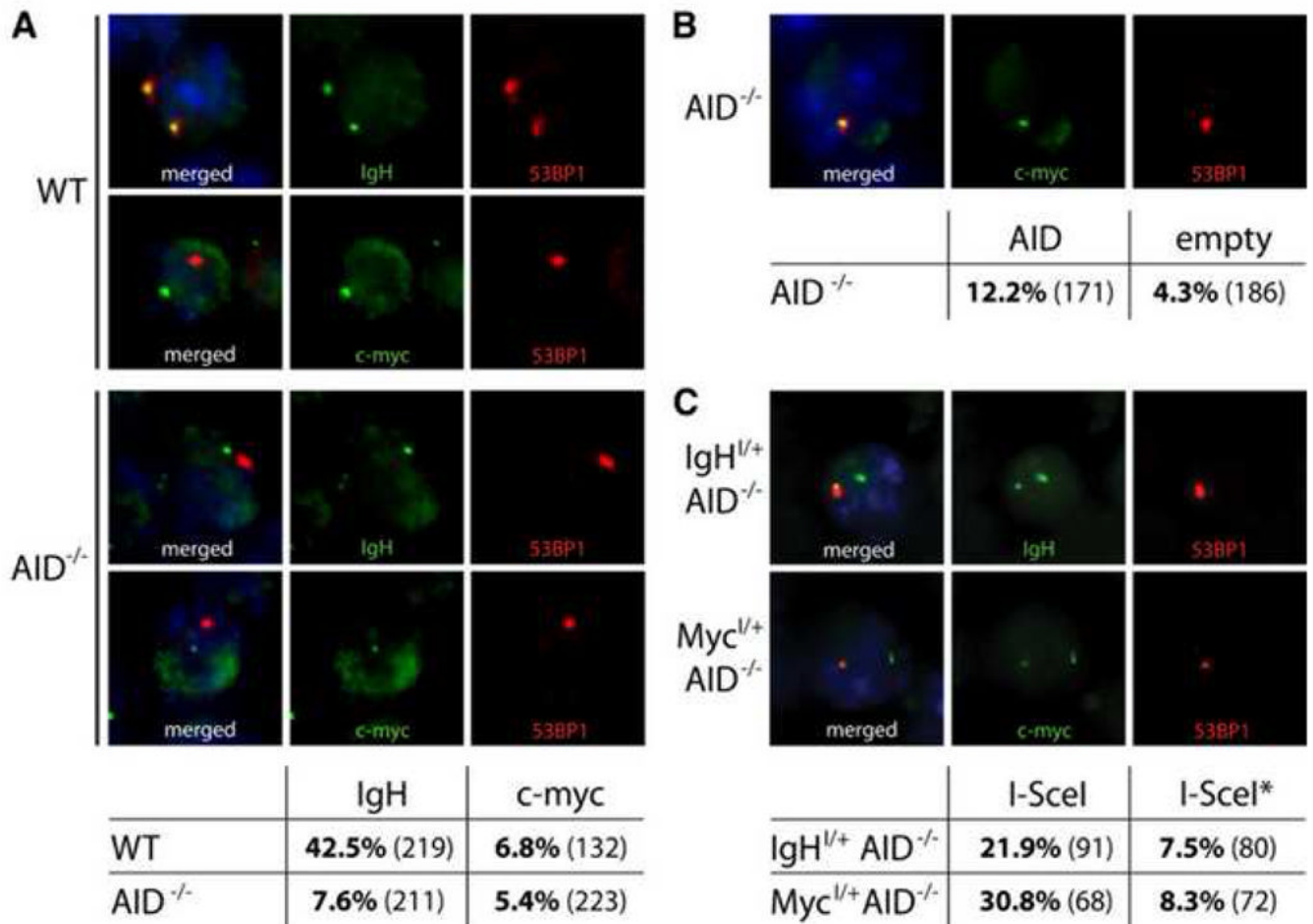


Figure 3. I-SceI induced breaks

(A) Immuno-FISH analysis of 53BP1 DNA damage foci at *IgH* or *c-myc* loci in activated B cells. Representative images and table showing co-localization frequencies as percentage of cells analyzed in LPS and IL-4 stimulated wild type (WT) or AID^{-/-} B lymphocytes. The number of cells analyzed is shown in parentheses. Three independent experiments. (B) Immuno-FISH analysis of AID induced 53BP1 DNA damage foci in AID^{-/-} B cells infected with AID or empty virus control. Representative images and table showing co-localization frequencies at *c-myc* ($p = 0.007$ with Student's T-Test). (C) Immuno-FISH analysis of I-SceI induced 53BP1 DNA damage foci in IgH^{I/+}AID^{-/-} (top) or Myc^{I/+}AID^{-/-} B cells (bottom). Representative images and table showing co-localization frequencies at *IgH* or *c-myc* (as indicated) in I-SceI or I-SceI* expressing B lymphocytes.

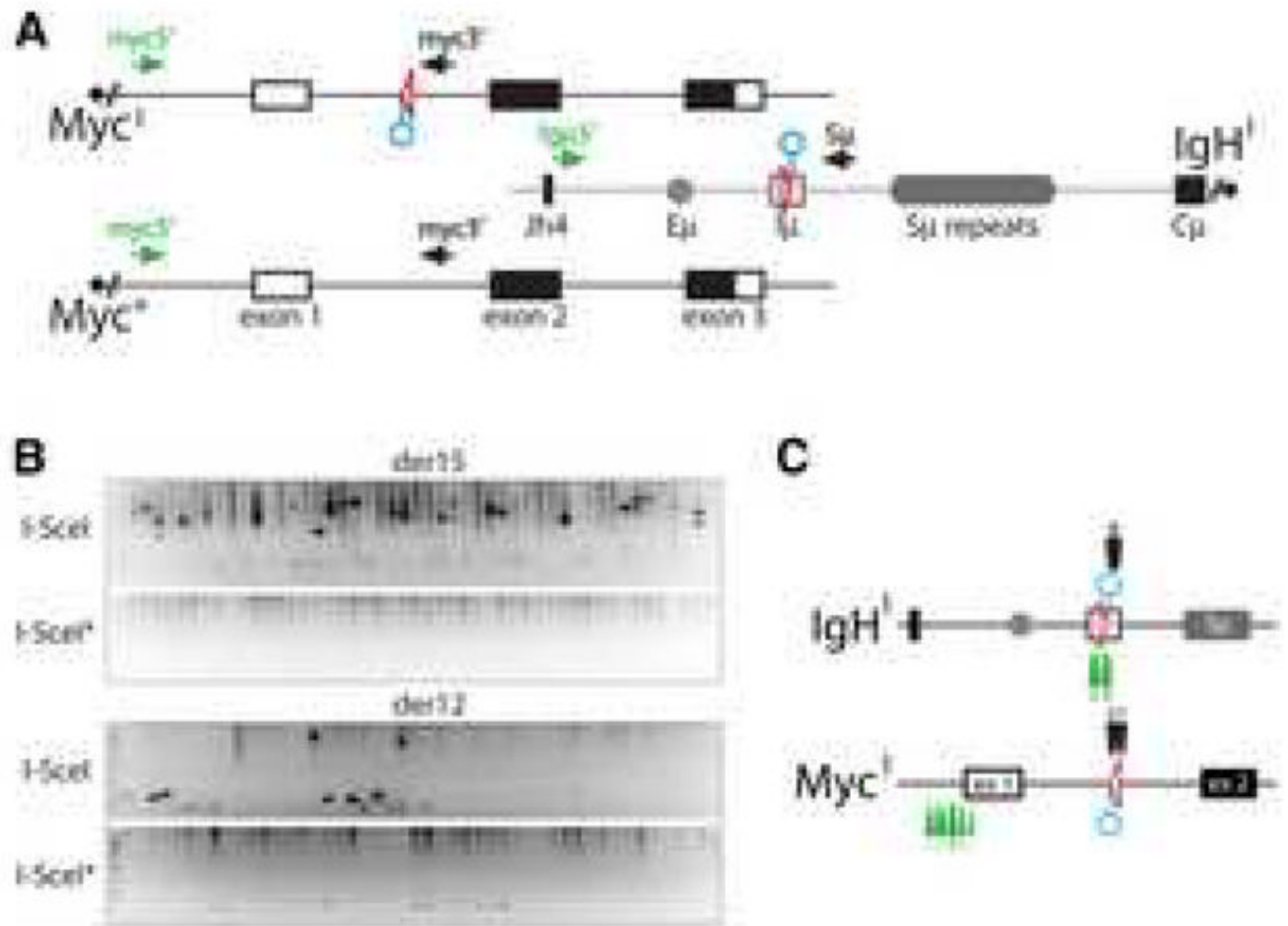


Figure 4. I-SceI induced translocations in AID deficient B lymphocytes

(A) Schematic representation of the *Myc*^I, *Myc*⁺ and *IgH*^I alleles with the PCR primers for detecting *der12* and *der15* *c-myc/IgH* translocations. Circles point to the position of the I-SceI sites. (B) I-SceI rescues translocations in the absence of AID. Representative agarose gels with PCR products corresponding to *c-myc/IgH* translocations (as verified by sequencing and/or Southern Blot, see Supplemental Figure S7). *Myc*^{I/+}*IgH*^{I/+}*AID*^{-/-} B cells were stimulated with LPS and IL-4 and infected with I-SceI or I-SceI* control retroviruses. 100,000 cells were assayed each in lane. Three independent experiments. (C) Map of translocation breakpoints from I-SceI expressing *Myc*^{I/+}*IgH*^{I/+}*AID*^{-/-} B cells (see also Supplemental Table 2). Arrows point to *c-myc/IgH* translocation breakpoints for *der12* (black) or *der15* (green).

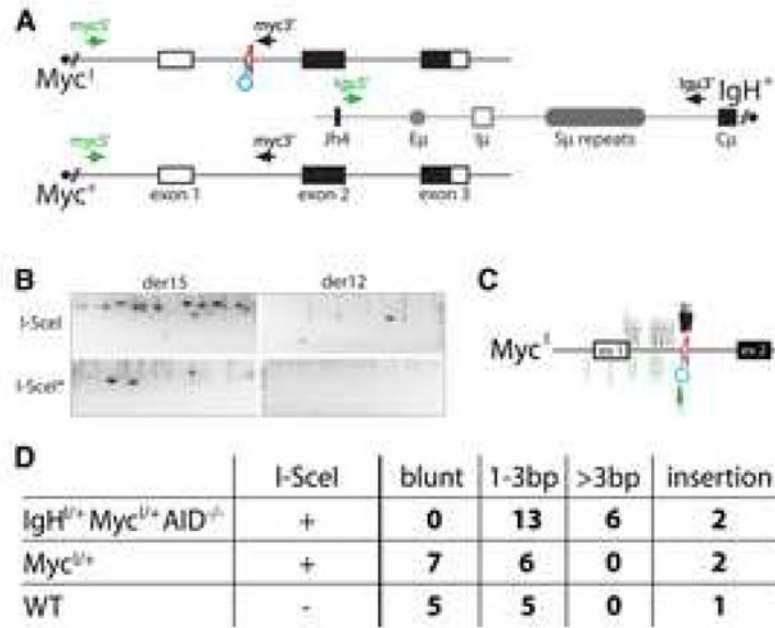


Figure 5. I-SceI induced translocations in AID sufficient B lymphocytes

(A) Schematic representation of the Myc^I, Myc⁺ and IgH⁺ alleles with the PCR primers for detecting der12 and der15 *c-myc/IgH* translocations. Circle points to recognition sequence for I-SceI. (B) I-SceI directed translocations in the presence of AID. Representative agarose gels with PCR products corresponding to *c-myc/IgH* translocations (as verified by sequencing and/or Southern Blot, see Supplemental Figure S8). Myc^{I/+} B cells were stimulated with LPS and IL-4 and infected with I-SceI (three independent experiments) or I-SceI* control. 100,000 cells were assayed in each lane. (C) Map of translocation breakpoints from stimulated Myc^{I/+} B cells infected with I-SceI (see also Supplemental Table 3). Filled arrows point to *c-myc/IgH* translocation breakpoints from der12 (black) or der15 (green). Empty arrows indicate the distribution of breakpoints obtained in the absence of I-SceI. (D) Table shows summary of the extent of junctional microhomology from *c-myc/IgH* translocations (see Supplemental Tables 2, 3, and 4).

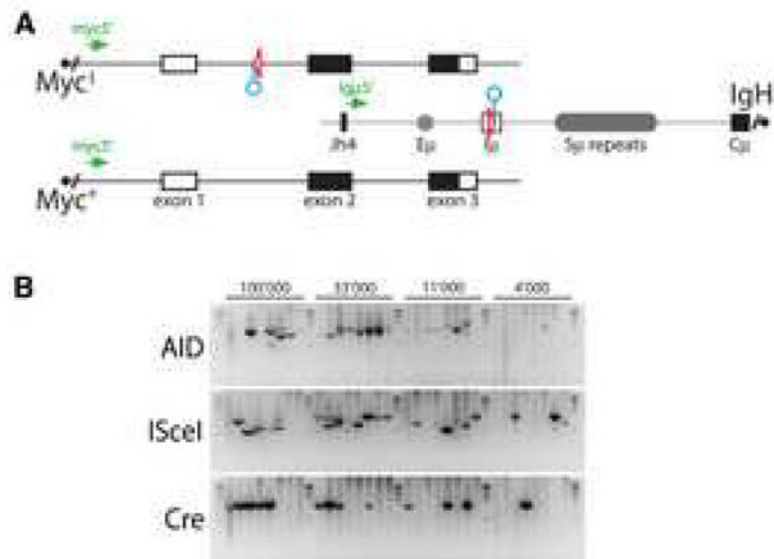


Figure 6. Translocations induced by AID, I-SceI or Cre in *AID*^{-/-} cells

(A) Schematic representation of the *Myc^I*, *Myc⁺* and *IgH^I* alleles with the PCR primers for detecting der15 *c-myc/IgH* translocations. Triangles represent loxP sites, circles point to recognition sequences for I-SceI. (B) Representative agarose gels with PCR products corresponding to *c-myc/IgH* translocations (as verified by sequencing and/or Southern Blot, see Supplemental Figure S10). *Myc^{I/+} IgH^{I/+} AID^{-/-}* B cells were stimulated with LPS and IL-4 and infected with AID, I-SceI, or Cre, as indicated. DNA equivalents for the indicated number of cells were assayed in each reaction. Two independent experiments.

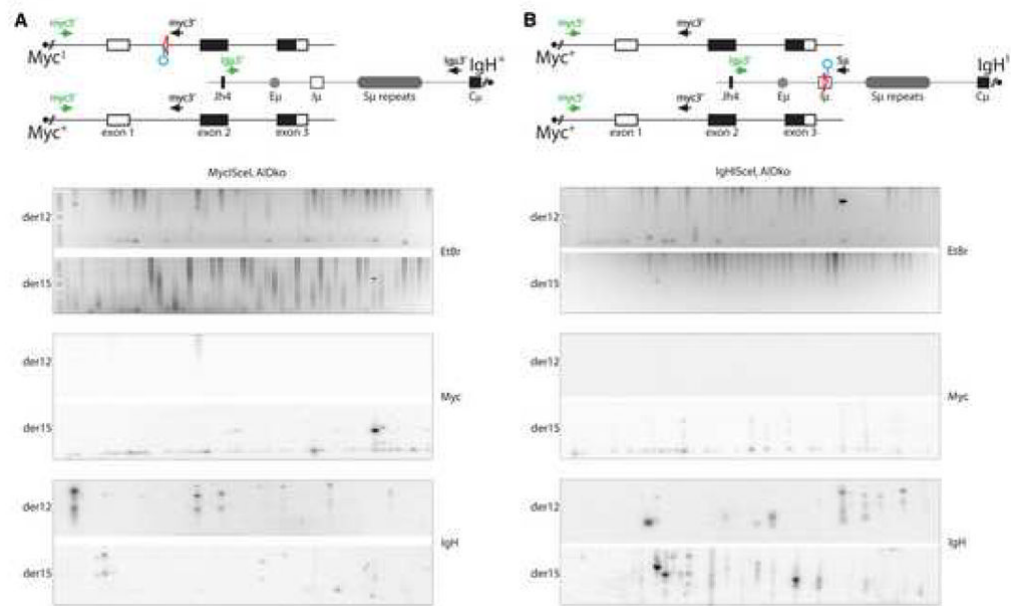


Figure 7. AID is essential for the lesion in *c-myc* that leads to *c-myc/IgH* translocation

(A) Top: schematic representation of the *Myc*^L, *Myc*⁺ and *IgH*⁺ alleles with the PCR primers for detecting der12 and der15 *c-myc/IgH* translocations. Circle indicates I-SceI site. Bottom: a representative ethidium bromide (EtBr) stained agarose gel was also Southern blotted and probed for *c-myc* and *IgH*, as indicated, to verify translocations. *Myc*^{L/+}*AID*^{-/-} B cells were stimulated with LPS and IL-4 and infected with an I-SceI encoding retrovirus. 100,000 cells were assayed in each lane. No translocations were identified out of 3.4×10^7 cells analyzed. Three independent experiments. (B) Top: schematic representation of the *Myc*⁺ and *IgH*^I alleles with the PCR primers for detecting der12 and der15 *c-myc/IgH* translocations. Circle indicates I-SceI site. Bottom: a representative ethidium bromide (EtBr) stained agarose gel was also Southern blotted and probed for *c-myc* and *IgH*, as indicated, to verify translocations. *IgH*^{I/+}*AID*^{-/-} B cells were stimulated with LPS and IL-4 and infected with I-SceI. 100,000 cells were assayed in each lane. No translocations were identified in 6.9×10^7 cells. Seven independent experiments.