AID produces DNA double-strand breaks in non-*Ig* genes and mature B cell lymphomas with reciprocal chromosome translocations

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Summary

Cancer initiating translocations such as those associated with lymphomas require the formation of paired DNA double-strand breaks (DSBs). Activation-induced cytidine deaminase (AID) produces widespread somatic mutation in mature B cells; however the extent of "off-target" DSB formation and its role in translocation-associated malignancy is unknown. Here we show that deregulated expression of AID causes widespread genome instability, which alone is insufficient to induce B cell lymphoma; transformation requires concomitant loss of the tumor suppressor p53. Mature B cell lymphomas arising as a result of deregulated AID expression are phenotypically diverse and harbor clonal reciprocal translocations involving a group of *Immunoglobulin (Ig)* and non-*Ig* genes which are direct targets of AID: this group includes *miR-142*, a previously unknown micro-RNA target which is translocated in human B cell malignancy. We conclude that AID produces DSBs throughout the genome, which can lead to lymphoma associated chromosome translocations in mature B cells.

Introduction

Chromosome translocations are characteristic features of several different forms of B cell cancers in mice and humans, including lymphomas and myelomas (Kuppers, 2005; Kuppers and Dalla-Favera, 2001; Potter, 2003). These karyotypic abnormalities are etiologically important in malignancy because they either deregulate oncogene expression, or create novel oncogenes by bringing together disparate transcription units (Rabbitts, 2009). Chromosome

translocations are believed to be particularly frequent in mature B lymphocytes because, in addition to V(D)J rearrangements early in development, these cells undergo two forms of programmed DNA damage in their immunoglobulin (*Ig*) loci during immune responses: somatic hypermutation and class switch recombination (Peled et al., 2008; Stavnezer et al., 2008; Teng and Papavasiliou, 2007).

Somatic hypermutation introduces non-templated point mutations in antibody variable genes and class switch recombination replaces one constant region for another by a deletional recombination reaction (Peled et al., 2008; Stavnezer et al., 2008; Teng and Papavasiliou, 2007). Although the two reactions are mechanistically distinct, both are initiated by a single enzyme, AID, which is believed to act by deaminating cytosine to produce U:G mismatches in target DNA (Di Noia and Neuberger, 2007; Honjo et al., 2002; Muramatsu et al., 2000). Diverse and overlapping DNA repair pathways process these lesions to produce somatic mutations, or DNA double-strand breaks (DSB), which are obligate intermediates in the class switch reaction (Peled et al., 2008). These same DSBs can also become substrates for chromosome translocations (Jankovic et al., 2007; Ramiro et al., 2007; Ramiro et al., 2006b). However, a single DSB at *Ig* alone is not sufficient for translocation (Robbiani et al., 2008). To account for the DSBs in non-*Ig* genes it has been proposed that the enzymes that create the breaks in *Ig* loci, RAG1/2 and AID, can also cause damage in non-*Ig* genes (Lieber et al., 2008; Tsai et al., 2008). Consistent with this idea we found that the oncogene *c-myc* is a target of AID, although the frequency of DSB formation at *c-myc* is significantly lower than at *IgH* (Robbiani et al., 2008).

Burkitt's lymphoma, diffuse large B cell lymphoma, and multiple myeloma represent different types of mature B cells that carry clonal translocations and may have expressed AID (Kuppers, 2005; Shaffer et al., 2002). A role for AID in inducing translocations in these cancers has been suggested by studies of activated B cells *in vitro* and plasmacytosis in IL-6 transgenic and pristane-treated mice, all of which develop AID dependent *c-myc/IgH* translocations (Dorsett et al., 2007; Ramiro et al., 2006a; Ramiro et al., 2004; Unniraman et al., 2004). In addition, AID expression accelerates the rate of tumor development in *Bcl6* transgenic mice (Pasqualucci et al., 2008). Finally, transgenic AID produces mutations in *c-myc* and T cell receptor genes as well as epithelial and T cell malignancies (Okazaki et al., 2003).

Curiously, however, deregulated AID does not cause malignancy or translocation-associated cancer in B cells (Muto et al., 2006; Okazaki et al., 2003; Shen et al., 2008). In addition, despite its obligate role in *c-myc/IgH* translocation, AID is not required for the development of plasmacytosis or plasmacytoma in IL-6 transgenic or pristane-treated mice, respectively (Kovalchuk et al., 2007; Ramiro et al., 2004). Finally, most human B cell lymphoma associated translocations do not involve *c-myc* and many do not involve *Ig* genes (Kuppers, 2005). Thus, the issue of whether AID can induce a sufficiently varied genomic damage to account for *Ig* and non-*Ig* gene translocation associated B lymphocyte malignancy *in vivo* remains to be determined.

Results

Increased switching, mutation and *c-myc/IgH* translocation in AID transgenic mice

To determine whether deregulated AID expression can destabilize the genome and induce B cell malignancy *in vivo*, we produced transgenic mice that express AID under the control of *Igκ* regulatory elements (*Igκ*AID, Figure 1A). AID is expressed at low levels in resting B cells in *Igκ*AID mice, and increases in activated and germinal center B cells (Figure 1B and 1C and Supplemental Figure S1). Nevertheless, with the exception of the expected decrease in germinal center cells (Figure 1D and (Dorsett et al., 2008; Muramatsu et al., 2000)), B cell development and distribution is normal in *Igκ*AID mice and serum Ig isotypes are not increased

(Supplemental Figures S2 and S3). Consistent with the increase in AID expression in stimulated B cells *in vitro*, *Igκ*AID B cells switched at higher frequency to IgG1 and IgG3 (Figure 1E and 1F). Thus, although deregulated AID expression does not significantly alter B cell development *in vivo*, it does lead to increased levels of class switch recombination *in vitro*. This effect is consistent with the observation that AID concentrations are limiting for this reaction (Dorsett et al., 2008; McBride et al., 2006; Takizawa et al., 2008).

To determine whether transgenic AID expression might alter its targeting, we examined the extent of somatic mutation in documented AID target (*IgV$_H$*, *Sµ*, *c-myc*) and non-target genes (*Taci*, *Whsc1*, *H2Eα*, *A20*), all of which are expressed in stimulated B cells (Supplemental Figure S4 and (Liu et al., 2008)). *IgV$_H$* mutation was measured by combining the B1-8^{hi} *IgV$_H$* knock-in allele (Casellas et al., 2001) with *Igκ*AID (*Igκ*AID/B1-8^{hi}) or AID$^{-/-}$ controls (AID$^{-/-}$/B1-8^{hi}). Consistent with constitutive AID expression in naïve B cells, resting *Igκ*AID/ B1-8^{hi} B cells showed high levels of mutation in *IgV$_H$* and *Sµ*, and only a modest increase upon *in vitro* activation with LPS and IL-4 (Figure 2A). *IgV$_H$* and *Sµ* mutation was not significantly detectable in controls, but was found at rates of 7.5×10^{-4} and 51×10^{-4}, respectively in naïve AID transgenic B cells. This rate of mutation is one order of magnitude lower than the rates normally found in germinal center B cells (Liu et al., 2008; McKean et al., 1984). In contrast, the increased levels of AID did not result in hypermutation in any of the non-target genes tested (Figure 2A). Also consistent with a physiologic pattern of target selection, *c-myc*, which has a propensity to error-free DNA repair (Liu et al., 2008), shows only a modest increase in mutation rate in activated *Igκ*AID B cells (1.43×10^{-4} vs. 0.64×10^{-4}). We conclude that deregulated expression of AID induces hypermutation in resting B cells at levels close to those normally found in germinal center cells; however the specificity of target selection is maintained since there is no significant DNA damage in non-target genes.

Even though *c-myc* is a *bona fide* target of AID, DSBs at *c-myc*, and *c-myc/IgH* translocations are difficult to detect under physiological conditions (Robbiani et al., 2008). Despite constitutive AID expression in *Igκ*AID B cells, we found no *c-myc/IgH* translocations in resting transgenic B cells (Figure 2B). In contrast, *Igκ*AID B cells stimulated with LPS and IL-4 showed an increase in translocation frequency when compared to controls (Figure 2B). Therefore, transgenic B cell expression increases the frequency of *c-myc/IgH* translocations in activated B cells.

B cell lymphoma

Despite high levels of *c-myc/IgH* translocations in stimulated B cells expressing deregulated AID, B cell lymphomas did not develop in *Igκ*AID or in three other previously reported strains of AID transgenic mice (Figure 3A and (Muto et al., 2006; Okazaki et al., 2003; Shen et al., 2008)). All eight of the *Igκ*AID mice survived beyond 40 weeks (Figure 3A). One potential explanation for this unexpected result is that B cells are protected from AID mediated DNA damage induced transformation by gatekeepers such as p53, which trigger cell cycle arrest or apoptosis (Bassing et al., 2003; Green and Kroemer, 2009; Kruse and Gu, 2009; Lowe et al., 2004; Ramiro et al., 2006a). Consistent with this idea, gatekeeper genes, or genes that control them such as *Bcl6* (Phan and Dalla-Favera, 2004), are frequently deleted or mutated in mature human B cell lymphomas (Kuppers, 2005).

To determine whether p53 is protective against AID-induced B cell lymphomas *in vivo* we produced *Igκ*AID/p53$^{-/-}$ mice. In contrast to *Igκ*AID mice, all 30 of the *Igκ*AID/p53$^{-/-}$ mice died within 28 weeks and they succumbed more rapidly to malignancy than p53$^{-/-}$ controls (P<0.0001, Figure 3A and 3B). In addition, tumors that developed in *Igκ*AID/p53$^{-/-}$ mice differed from those in the p53$^{-/-}$ controls: the former were predominantly mature B cell lymphomas, whereas the p53$^{-/-}$ controls developed thymic lymphomas and sarcomas (Figure 3B and (Jacks et al., 1994)). The mature B cell lymphomas in *Igκ*AID/p53$^{-/-}$ mice represent

a broad spectrum of the different stages of B cell development; from naïve B cells that express high levels of IgM and little or no IgD to plasmacytomas that express CD138 and no surface Ig (Table 1 and Supplemental Figure S5). We conclude that deregulated AID expression in a p53 deficient background leads to the emergence of a broad spectrum of mature B cell lymphomas.

To determine whether *Igκ*AID/p53$^{-/-}$ lymphomas were associated with chromosome translocations we performed multicolor-FISH (M-FISH) analysis on tumor metaphase spreads. We found that 11 out of the 12 *Igκ*AID/p53$^{-/-}$ B cell lymphomas tested contained a clonal reciprocal translocation. In all cases additional non-clonal chromosome aberrations were present (Figure 2C and Table 1). In contrast, clonal reciprocal translocations were not observed in the p53$^{-/-}$ control T cell lymphomas (data not shown and (Liao et al., 1998)). Thus, in the absence of p53, deregulated AID can produce B cell lymphomas that harbor clonal reciprocal chromosome translocations, similar to those that are characteristic of human B cell lymphomas.

Tumor associated translocations induced by AID *in vivo*

Nine of the eleven *Igκ*AID/p53$^{-/-}$ B cell lymphomas analyzed by M-FISH showed a clonal T (11;15) translocation; the other two exhibited a T(12;15), or T(5;6) translocation, with a variety of additional clonal and non-clonal chromosomal abnormalities (Table 1). The tumor with T (12;15) was a typical mouse plasmacytoma that expressed CD19 and high levels of CD138 but not surface Ig (Table 1 and Supplemental Figure S5). PCR analysis was used to determine that the translocation involved *c-myc* and *IgH* and sequence analysis revealed that the breakpoint on *c-myc* was in the promoter region, and that the breakpoint at the *IgH* locus was in the IgH switch μ region (Supplemental Table 1). Such reciprocal translocation is similar to those observed in human Burkitt's lymphomas and mouse plasmacytomas, and are consistent with *IgH* and *c-myc* being targets of AID activity (Robbiani et al., 2008).

To identify the translocation breakpoints in tumors containing T(11;15) and T(5;6), we performed high resolution array painting. Because the translocations in *Igκ*AID/p53$^{-/-}$ B cell lymphomas appeared to be balanced as determined by M-FISH (Figure 3C), we sorted derivative chromosomes from the tumor samples to compare with normal reference DNA (Gribble et al., 2007). Translocation breakpoints and copy number changes were revealed as discontinuities (high-low or low-high ratio) in the hybridization ratio of sorted derivative chromosomes compared to unsorted reference DNA. We found that the breakpoints in the T (11;15) translocation were in the region of *micro-RNA 142 (miR-142)* and *c-myc* and in T(5;6) at *Anxa4* and *Wdfy3* (Figure 4A and 4B and Supplemental Figure S6). The apposition of *miR-142* on chr11 with *c-myc* on chr15 was confirmed by Southern blotting (Figure 4C). The high resolution mapping by array painting allowed for the design of PCR primers to characterize fourteen T(11;15) translocations molecularly. The *miR-142* breakpoints were clustered in 1.8kbps surrounding the micro-RNA, while the breakpoints at *c-myc* were in a 1.5kbps region centered in intron 1 (Figure 4D). The resulting *miR-142/c-myc* fusion genes on der11 chromosome contained the *miR-142* promoter and the coding region of *c-myc.* These genes were associated with the production of fusion transcripts between *miR-142* and *c-myc* (der11), leading to the over-expression of *c-myc* at levels similar to those found in the S107 plasmacytoma, which carries a *c-myc/IgH* translocation (Figure 4E and 4F). Importantly, a closely related clonal translocation between *miR-142* and *c-myc* has been reported in human B cell leukemia (Gauwerky et al., 1989). We conclude that the T(11;15) translocation found in the vast majority of *Igκ*AID/p53$^{-/-}$ B cell cancers results in deregulated expression of *c-myc* by juxtaposition to regulatory elements from *miR-142*, one of the most highly expressed micro-RNAs in hemaetopoetic cells (Chen et al., 2004; Landgraf et al., 2007). Based on the B cell developmental stages as well as karyotype and high resolution analysis of the T(12;15)

and T(11;15) translocations, it appears that the *Igκ*AID/p53$^{-/-}$ mouse provides a relevant model for studying the development of human B cell lymphoma *in vivo*.

AID might induce lymphoma-associated translocations by producing direct genomic damage or indirectly by compromising the genes that maintain genomic stability (Jankovic et al., 2007). The T(11;15) translocation junction sequences revealed either blunt end joining or small amounts of microhomology (Figure 4G and Supplemental Table S1). In eight cases, we were able to characterize both derivative chromosomes. Surprisingly, six of these showed duplications in one or both of the translocated genes. In agreement with this observation, duplications were also present in the lymphomas with T(12;15) and T(5;6) translocation. In this setting, duplication could only be produced by filling in a break created by two staggered single-strand nicks, one on each DNA strand. This type of DSB is consistent with a staggered pair of lesions introduced by AID (Figure 4G, Supplemental Table S2, and Supplemental Figure S7).

To determine if any of the translocated genes are direct targets of AID, we obtained DNA sequences from primary resting and activated B cells (Figure 5A). We found a high rate of mutation in *miR-142* in resting (4.5×10^{-4}) and activated (7.8×10^{-4}) *Igκ*AID B cells (P=0.0004 and P=0.0007, respectively, Figure 5A and Supplemental Figure S8), but not in *Anxa4* or *Wdfy3*. Hypermutation indicative of AID activity at *miR-142* was also detected in the region surrounding the translocation breakpoints as well as, more significantly, in the non-translocated allele of T(11;15) lymphomas (Figure 4G, Supplemental Table 1 and Supplemental Figure S9). Finally, we confirmed the *in vitro* results by analyzing *c-myc* and *miR-142* mutations in germinal center B cells. Both *c-myc* and *miR-142* were hypermutated in *Igκ*AID germinal center B cells at levels similar to those found in activated B cells *in vitro* (Figure 5B). In addition, both *c-myc* and *miR-142* mutations were enhanced in the absence of UNG in cells with endogenous levels of AID (Figure 5B). Therefore, *miR-142* resembles *c-myc* in that AID-induced lesions have a propensity to error free repair, which is UNG dependent (Figure 5B and (Liu et al., 2008)). Thus, the majority of the B cell lymphomas induced by deregulated AID expression involves direct AID target genes (a total of 15 T(12;15) and T(11;15)) while a minority (1 T(5;6)) may result indirectly from other genome destabilizing mutations.

Genomic DNA damage

To examine the spectrum of genomic DNA damage induced by AID deregulation in primary non-transformed B cells, we measured chromosome translocations by M-FISH (Figure 6A and 6B). We found that 4.4% of all *Igκ*AID/p53$^{-/-}$ B cells showed translocations in LPS, IL-4 and RP105 stimulated B cells (n= 250; Figure 6B). Metaphases containing translocations were rare in wild type B cells (3/500 metaphases, Supplemental Table S3 and (Callen et al., 2007; Ramiro et al., 2006a; Wang et al., 2009)). Interestingly, *Igκ*AID, although tumor-free, showed a similar frequency of translocation to *Igκ*AID/p53$^{-/-}$ (6%, n=500 metaphases; Figure 6B and Supplemental Table S3). Thus, p53 does not impact on the formation of translocations, and this factor must act downstream of translocation to prevent malignancy. Significantly, out of 500 metaphases from AID$^{-/-}$ mice none showed chromosome aberrations, indicating that the translocations in *Igκ*AID mice are completely AID-dependent (Figure 6B). Every chromosome (except Chr5, Chr14, Chr18 and ChrY) was found translocated in at least one metaphase from *Igκ*AID B cells. The distribution of translocations was similar between *Igκ*AID/p53$^{-/-}$ and *Igκ*AID mice (Figure 6C). Remarkably some of the chromosomes were found at an extremely high frequency including Chr12 (21/45 translocations), Chr16 (9/45 translocations), Chr 11 and Chr13 (both 7/45 translocations)(Figure 6C and Supplemental Table S3). We conclude that in addition to *c-myc/IgH* translocations, AID induces a large number of translocations in B cells and that loss of p53 does not increase the frequency of translocations.

Translocations are products of DNA breaks. To directly determine the frequency of DNA breaks we examined DAPI-stained metaphase spreads that were hybridized with a telomere-repeat specific peptide nucleic acid probe marking chromosome ends (Figure 6A and 6B and (Callen et al., 2007)). The level of chromosome and chromatid breaks was similar in *Igκ*AID (9%) and *Igκ*AID/p53$^{-/-}$ (8%) B cells, and this level of DNA damage was 8–9 fold higher than found in AID$^{-/-}$ mice (Figure 6A and 6B). Thus, deregulated AID expression produces widespread DNA damage which is independent of p53 and which promotes chromosomal translocations in mature B cells.

Discussion

Lymphoid malignancies account for 5% of all human cancers; the majority of these involve mature B cells at various stages of development. Chromosome translocations between *Ig* and non-*Ig* genes feature prominently in these cancers; however, they can also occur between two non-*Ig* genes. For example, Burkitt's lymphomas typically contain *c-myc/IgH* translocations; whereas in MALT lymphomas the translocation commonly involves API2 and MALT (Inagaki, 2007). Multiple origins have been suggested for the paired DNA lesions that are obligate intermediates in lymphoid translocations, including the RAG1/2 V(D)J recombinase (Bassing et al., 2002; Callen et al., 2009; Dudley et al., 2005; Schatz, 2004; Schlissel et al., 2006; Tonegawa, 1983), AID (Ramiro et al., 2007; Ramiro et al., 2006a; Ramiro et al., 2004), reactive oxygen species, fragile sites and combinations of the above (Callen et al., 2007; Greaves and Wiemels, 2003; Lieber et al., 2008; Lieber et al., 2006; Tsai et al., 2008; Wang et al., 2009). However, none of these is directly demonstrated to produce the necessary initiating lesions on multiple chromosomes in lymphoid cells. Our experiments show that transgenic over-expression of AID in mice produces DSBs on most chromosomes and indicate that expression of this enzyme may be sufficient to account for many of the lesions that lead to mature B cell lymphoma.

AID is known to produce translocations between *c-myc* and *IgH* in B cells *in vitro* as well as in IL-6 transgenic plasmacytes and pristane-induced plasmacytomas (Dorsett et al., 2007; Ramiro et al., 2006a; Ramiro et al., 2004; Unniraman et al., 2004). However, a number of laboratories have shown that deregulated AID expression is not sufficient to induce B cell lymphoma *in vivo* (Muto et al., 2006; Okazaki et al., 2003; Shen et al., 2008). Our data resolve this apparent disparity and reveal that AID is indeed a cancer susceptibility gene that causes a broad spectrum of B cell malignancies. In contrast to gatekeeper genes that control cell proliferation and cell death, or caretakers that regulate DNA repair, AID is a tumor promoter that destabilizes the genome by inducing DNA breaks and chromosome translocations.

AID creates DNA lesions by cytosine deamination (Petersen-Mahrt et al., 2002). Like other DSBs, AID breaks are recognized by proteins that mediate the DNA damage response including H2AX, Nbs1 and 53BP1 (Manis et al., 2004; Petersen et al., 2001; Reina-San-Martin et al., 2005; Ward et al., 2004), and ATM (Difilippantonio et al. 2005; Reina-San-Martin et al., 2004), which in turn phosphorylates and recruits a canonical cascade of additional factors to the breaks (Jankovic et al., 2007). Although AID's activity is focused on antibody genes, where it produces high rates of mutation, its specificity is not absolute and its mechanism of targeting is still to be defined. The lack of absolute specificity results in low frequency off-target mutation of oncogenes such as *Bcl6* and *c-myc* (Pasqualucci et al., 1998; Shen et al., 1998). However, the rate of AID-induced mutations in genes like *c-myc* is reduced by error free repair by a yet to be determined UNG-dependent pathway resulting in mutation rates that are difficult to detect (Liu et al., 2008). Therefore the complete list of genes that can be mutated by AID is not known.

Even less is understood about the extent to which the genome might be susceptible to AID-induced DSBs. Our experiments demonstrate that although deregulating AID expression is not

sufficient to overcome its targeting specificity, it induces a wide spectrum of chromosome aberrations, including reciprocal and non-reciprocal translocations, dicentric chromosomes as well as chromosome deletions in primary B cells.

AID deregulation leads to increased somatic mutation and increases genomic instability, but is not sufficient to cause malignant transformation. Conversely, loss of p53 was required for malignancy but it did not significantly increase the level of translocation or DSBs. Thus, the presence of p53 did not prevent translocation per se, but p53 was required for transformation of cells bearing translocations. This would be in keeping with p53's ability to respond to oncogenic stress and induce the death of cells that over-express oncogenes (Green and Kroemer, 2009; Hoffman and Liebermann, 2008; Kruse and Gu, 2009; Lowe et al., 2004). For example, p53 dependent cell death is triggered when *c-myc* is deregulated by the *Ig* enhancer in the *c-myc/IgH* translocation or by the *miR-142* promoter in the *c-myc/miR-142* translocation. Of note, p53 or its regulators are frequently mutated in human B cell malignancies including diffuse large-B cell lymphoma, Burkitt's lymphoma and chronic lymphocytic leukemia (reviewed in (Kuppers, 2005)). Finally, finding that there was no difference in the number of DSBs or chromosome abnormalities in the presence or absence of p53 indicates that the majority of the damage created by deregulated AID involves genes that do not activate p53. Based on our observations in mice, we speculate that p53's role in human lymphomas is to eliminate cells bearing AID induced translocations that activate oncogenes.

Human B cell lymphomas can carry reciprocal translocations between two non-*Ig* genes; however, whether AID produces the lesions that lead to the malignancies associated with this class of translocations was not known. We found one example of a translocation where neither of the two aberrantly joined non-*Ig* genes was an AID target as measured by somatic hypermutation (*Anxa4/Wdfy3*). However, the remaining non-*Ig* translocations in the B cell malignancies that arose in *Igκ*AID/p53$^{-/-}$ mice (n=14) were associated with *c-myc/miR-142* translocation, which is also found in human B cell leukemia (Gauwerky et al., 1989). Both of these genes are AID targets. Consistent with bilateral initiation of these non-*Ig* translocations by AID, most showed junctional duplications that can only arise when the initiating lesions are staggered nicks on two DNA strands including the *Anxa4/Wdfy3* translocation. Therefore, in addition to translocations involving *Ig*, AID can also induce cancer-associated translocations between two non-*Ig* genes. It is important to note that somatic mutation as currently measured is not a sensitive indicator of whether or not a gene is an AID target simply because the rates of mutation sometimes approach the error rates of the polymerases used for PCR amplification. Thus, the lesions that lead to *Anxa4/Wdfy3* translocation, which show junctional duplication but no evidence of somatic mutation may still be due to AID. Given its destabilizing effects on the genome and oncogenic potential *in vivo*, it is not surprising that the expression of this enzyme and its residence in the nucleus are tightly regulated by a combination of cytoplasmic retention signals (Patenaude et al., 2009), nuclear export (McBride et al., 2004), phosphorylation (Cheng et al., 2009; McBride et al., 2006; McBride et al., 2008), micro-RNAs (Dorsett et al., 2008; Teng et al., 2008) and ubiquitylation (Aoufouchi et al., 2008).

In conclusion, AID is a potent endogenous tumor promoter as it mutates oncogenes like *Bcl6*, and creates DSBs in *Ig* and non-*Ig* genes that can serve as substrates for chromosome translocation. These lesions may account for a significant fraction of the mature B cell lymphomas that frequently arise in humans.

Experimental Procedures

Mice

The *Igκ*AID transgene is based on VkMYC (Robbiani et al., 2005) and includes the coding sequence of mouse AID (split over two exons) embedded in regulatory elements of the *κ* light

chain gene. The plasmid backbone was removed with MluI and NotI prior to pronuclear injection into C57BL/6 x (C57BL/6 x CBA) oocytes. Transgenic males were backcrossed to C57BL/6 for at least ten generations. $AID^{-/-}$, B1-8^{hi} and p53$^{-/-}$ mice were backcrossed to C57BL/6 (Casellas et al., 2001; Jacks et al., 1994; Muramatsu et al., 2000). UNG$^{-/-}$ mice were previously described (Endres et al., 2004). All experiments were performed in accordance with protocols approved by the Rockefeller University and National Institutes of Health (NIH) Institutional Animal Care and Use Committee.

B cell cultures

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 as previously described (Robbiani et al., 2008). For class switch recombination to IgG3, cells were stimulated with LPS (25μg/ml, Sigma) and RP105 (0.5 μg/ml, Pharmingen). For class switch recombination to IgG1, IL-4 (5 ng/ml, Sigma) was supplemented in addition to LPS and RP105. Cells were analyzed after three days of culture.

Flow cytometry and cell sorting

For flow cytometric analysis, single-cell suspensions were stained with the indicated fluorochrome-conjugated antibodies (all from Pharmingen). CFSE-labeling for cell division was at 37C for ten minutes in 5μM carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes). Data were acquired on a FACSCalibur or LSRII instrument (Becton Dickinson) and analyzed with FlowJo software.

Lymphoid populations were sorted to >95% purity with a FACSVantage SE with DIVA option or FACSAria instruments (Becton Dickinson). Follicular B cells (CD19$^+$CD23$^+$CD21$^+$) from naïve spleens and bone marrow pre-B cells (B220$^+$CD43$^{low/int}$IgM$^-$IgD$^-$) were enriched with anti-CD19 microbeads (Miltenyi Biotech). Splenic germinal center B cells (CD19$^+$GL7$^+$CD95$^+$) from *P. chabaudi* infected mice (day 14) were enriched with anti-CD19 microbeads prior to sorting.

Germinal center induction

Plasmodium chabaudi chabaudi clone AS (MRA 429) was obtained through the MR4 (Peters and Robinson, 2000). Parasites were maintained as frozen stocks and passaged in mice as described previously (Meding et al., 1990). For experiments, mice were injected intra-peritoneally with 10^5 infected red blood cells. Infections were monitored by microscopic examination of Giemsa-stained thin blood smears.

Western Blot

AID expression was analyzed as previously described (McBride et al., 2008). Lymphoid cells were purified as described above from AID$^{-/-}$, wild type or *Igκ*AID mice. Activated splenic B cells were stimulated in vitro with LPS and IL-4 for four days.

PCR assay for translocations

DNA was extracted from resting splenic B cells or B cells that were stimulated *in vitro* for four days with LPS and IL-4. PCR reactions were performed on genomic DNA from 10^5 cells, using the Expand Long Template PCR System (Roche). Nested reactions were performed as detailed and confirmed by Southern Blot as described (Robbiani et al., 2008).

Mutational analysis

The following primers were used to amplify the indicated genes with Pfu polymerase (Stratagene) and 25 cycles of amplification, prior to cloning in TOPO vector (Invitrogen) and

sequencing. V_H(B1-8^{hi}): 5-CCATGGGATGGAGCTGTATCATCC-3 and 5-
GAGGAGACTGTGAGAGTGGTGCC-3. *Sµ*: 5-GACCCAGGCTAAGAAGGCAATC-3
and 5-GCGGCCCGGCTCATTCCAGTTCATTACAG-3. *c-myc*: 5-
TGGTCTTTCCCTGTGTTCTTTCTG-3 and 5-
GACACCTCCCTTCTACACTCTAAACCG-3. *Taci*: 5-
GTCAGGTCAGACAACTCAGGAAGG-3 and 5-GTTTGCCACCCACATCAAGC-3.
Whsc1: 5-ACGACGAAACGGTATGAAATCG-3 and 5-
AAAAATGAAGGCTGCTGGGC-3. *H2Eα:* 5-CCAGAGACCAGGATGCCGC-3 and 5-
TGGGCACCTTAGCACCGTAGTTAC-3. A20: 5-GGACCATGGCTGAACAACTT-3 and
5-ATCTGGCCGTTTGAGACAAC-3. *miR-142*: 5-CGTTGGATTCAAGACTGTGGGTC-3
and 5-AATGAGGGCGTGTGAGAGATGCTC-3. *Anxa4*: 5-
ACGGCACCATCTTCTGCTGTC-3 and 5-TCCTCCACACCTTGTTCTCTTGAG-3.
Wdfy3: 5-AGAGGAGCCTGGTTTATGTAGCAG-3 and 5-
TGGGAGGCTTATTGATTAGGCTG-3. Activated B cells were stimulated for 4 days with
LPS and IL-4. Single B cell preparations from matched mice were compared. For additional
details see Supplemental Table S4. P-values were determined with the Student's-t test using a
two-tailed distribution.

Southern Blot

Rearrangements at the *c-myc* and *miR-142* loci were determined by digesting DNA with SspI
(*c-myc*) and PstI (*miR-142*) and probing with PCR products of 5-
CATTCTGACTCCTTTTGCCC-3 and 5-TCAGAGGTGGCTATTCAGTTGC-3 (*c-myc*); or
5-CCCAGGCATTTTTTCCACG-3 and 5-TTGAATCCAACGGAGGCAGC-3 (*miR-142*).

qPCR

Reverse-transcribed RNA from tumors and controls were amplified for *c-myc* (5-
GCCCCTAGTGCTGCATGAG-3 and 5-CCACAGACACCACATCAATTTCTT-3) and
normalized with *Gapdh* (5-TGAAGCAGGCATCTGAGG-3 and 5-
CGAAGGTGGAAGAGTGGGAG-3) using SYBR Green (Applied Biosystems).

RT-PCR and PCR for T(11;15) breakpoints

Der11 *c-myc/miR-142* fusion transcripts were amplified with 5-
AGTCGGCAAGAAAAGCAGGTG-3 and 5-TCGTCGCAGATGAAATAGGGC-3 and
verified by direct sequencing. Der11 breakpoints were amplified with 5-
TCGCTCTGCTGTTGCTGGTGATAG-3 and 5-TTGTCGCTGGTTTCCTGTCAG-3. Der15
breakpoints were amplified with 5-GTGAAAACCGACTGTGGCCCTGGAA-3 and 5-
CACAACCCCAATAACAGAGTCAGAC-3. The breakpoint was determined by direct
sequencing of the gel-extracted PCR products.

Array painting

Tumor cells growing in culture were arrested by overnight treatment with colcemid. The cells
were treated with hypotonic KCl solution and 0.25% TX-100, before incubation with
Chromomycin A3 (final concentration 40 pg/ml) and Hoechst 33258 (final concentration 2 pg/
ml). Sorting was performed on a FACStar Plus flow sorter (Becton Dickinson) equipped with
two argon ion lasers. 5000 chromosomes were sorted. Amplification of sorted chromosomes
was achieved using WGA4 (Sigma). Oligonucleotide array CGH (aCGH) using mouse 244K
tiling arrays was performed according to the manufacturer's protocol (Agilent Oligonucleotide
Array-Based CGH for Genomic DNA Analysis, version 4.0, June 2006). Unsorted liver
genomic DNA from sex-matched littermates was used as reference DNA. Slides were scanned
using a G2565BA scanner, version 9.1. Feature Extraction software was applied and the data
were visualized using CGH Analytics 3.4.40 (Agilent).

M-FISH and analysis of genomic instability

For analysis of chromosome and chromatid breaks, metaphase spreads were prepared from tumor cells and hybridized with telomere repeat-specific peptide nucleic acid (PNA) labeled with Cy3 (Applied Biosystems; (Callen et al., 2007)). Metaphases were counterstained with DAPI. For M-FISH, metaphases were hybridized with the 21x mouse probe cocktail (Metasystems). FISH-labeled metaphases were imaged using a Zeiss AxioImager M1, equipped with a motorized scanning stage and analyzed using Isis software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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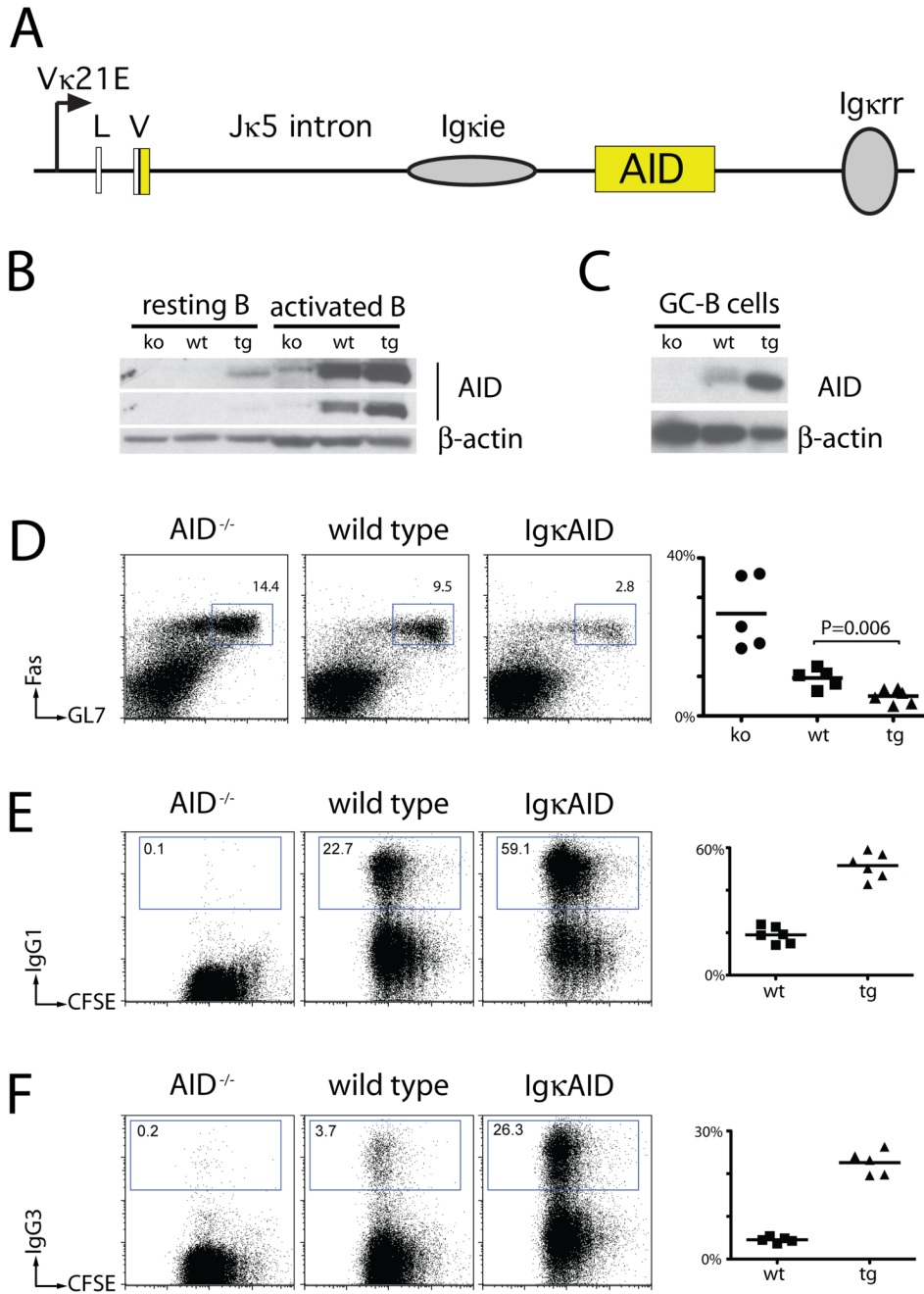
Figure 1. Increased class switching in *Igκ* AID B cells

(A) Schematic representation of the *Igκ*AID transgene. AID cDNA (yellow) is embedded in *Igκ* regulatory elements (Vκ21E promoter, non-coding leader (L) and V gene exons, intronic sequence with enhancer (ie), and 3′ regulatory region (rr)). (B) and (C) Western Blot for AID protein in *Igκ*AID B cells. (B) 1 million cells of the indicated genotypes were assayed in each lane. Top and middle are different exposures of the same blot. (C) 0.5 million sorted germinal center (GC) B cells were assayed in each lane. (D) Flow cytometric analysis of Peyer's patch germinal center B cells from matched 6–10 week old mice. Graph (right) shows a summary of two independent experiments with 2–3 mice each, the bar indicates the mean value for each group (25.9% ko, 9.6% wt, 5.0% tg), P value was calculated by unpaired T-test. (E) and (F)

Flow cytometric analysis for class switching to IgG1 and IgG3 and cell division. Graph (right) shows a summary of two independent experiments with 2–3 mice each, the bar indicates the mean value for each group (IgG1: 18.9% wt, 51.6% tg; IgG3: 3.8% wt, 22.6% tg).
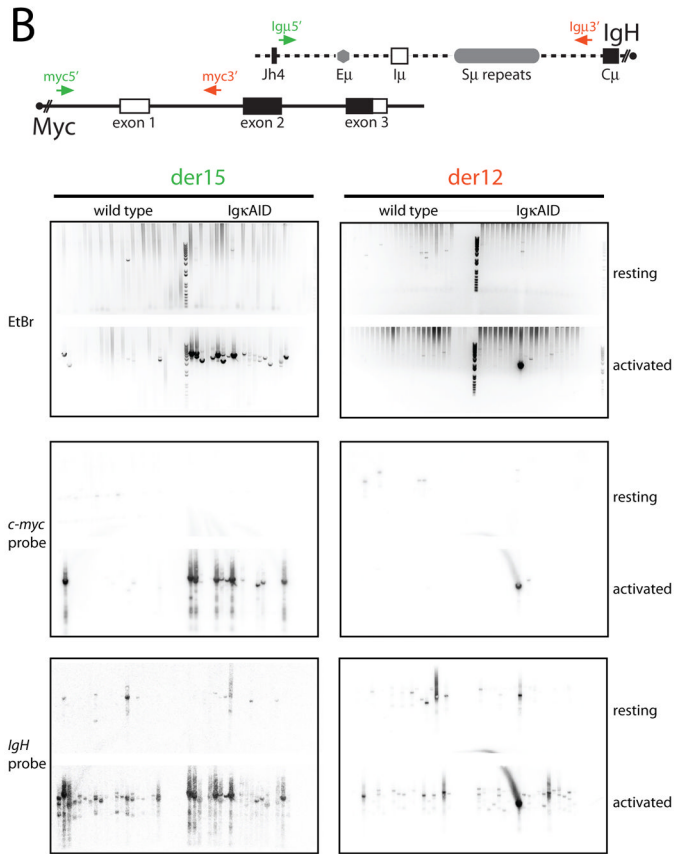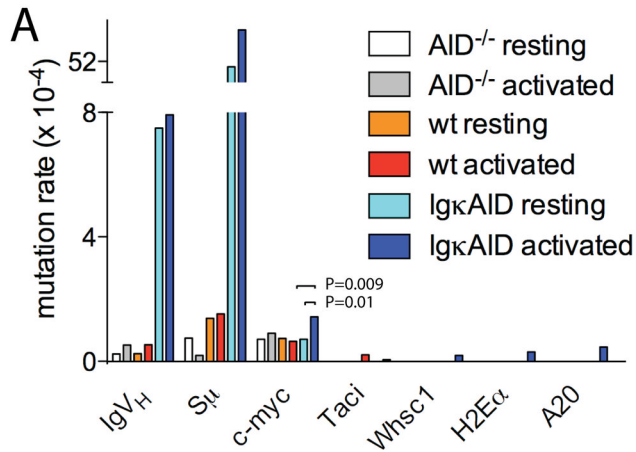
Figure 2. Somatic mutations and *c-myc/IgH* translocations
(A) Frequency of somatic mutations in indicated loci of resting and LPS and IL-4 stimulated
B cells. (B) *C-myc/IgH* translocations in resting B cells or B cells activated with LPS and IL-4
for four days. Top: Schematic representation of the *Myc* and *IgH* alleles with the PCR primers
for detecting der15 and der12 *c-myc/IgH* translocations. Bottom: ethidium bromide (EtBr)
stained gel with PCR bands indicative of translocations was blotted and probed for *c-myc* and
IgH, as indicated, to verify translocations. Translocation frequency: wild type 0.4×10^{-6} (der15)
and 0.1×10^{-6} (der12); IgκAID 4.6×10^{-6} (der15) and 1.3×10^{-6} (der12). One of two independent
experiments is shown.
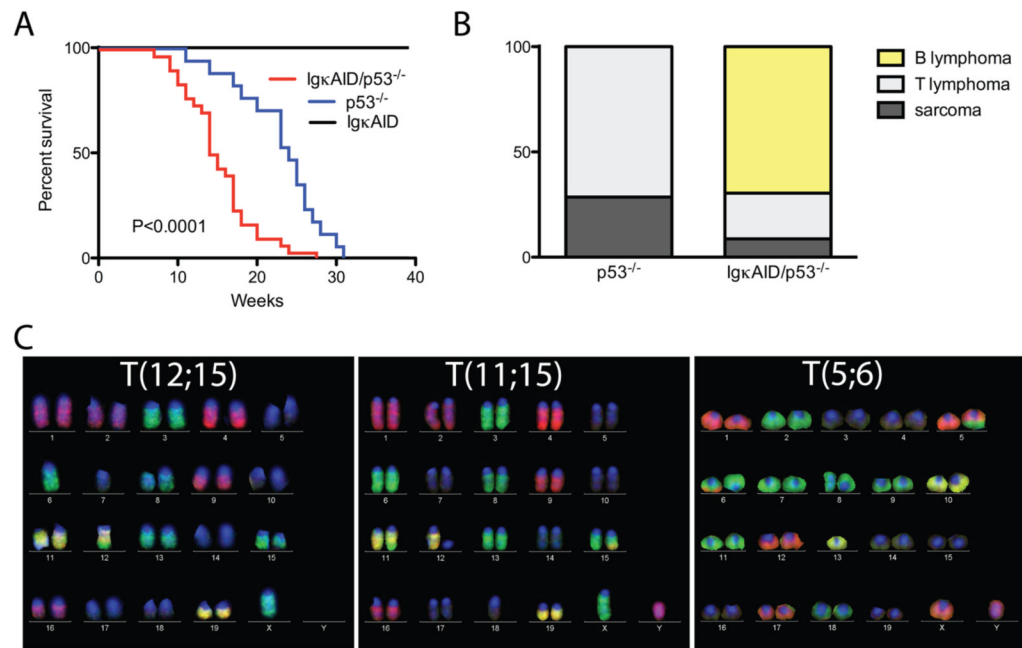
Figure 3. *IgκAID/p53⁻/⁻* mice succumb to B cell lymphomas with reciprocal translocations
(A) Survival curve for n=8 *Igκ*AID, n=17 p53$^{-/-}$ and n=30 *Igκ*AID/p53$^{-/-}$ mice. The P value refers to the difference between *Igκ*AID/p53$^{-/-}$ and p53$^{-/-}$ (log-Rank test). The median survival for *Igκ*AID/p53$^{-/-}$ is 14.5 weeks, for p53$^{-/-}$ it is 24 weeks. (B) Phenotype of tumors at necropsy (n=7 p53$^{-/-}$ and n=23 *Igκ*AID/p53$^{-/-}$). (C) Representative M-FISH images of reciprocal translocations found in B cell lymphomas of *Igκ*AID/p53$^{-/-}$ mice.
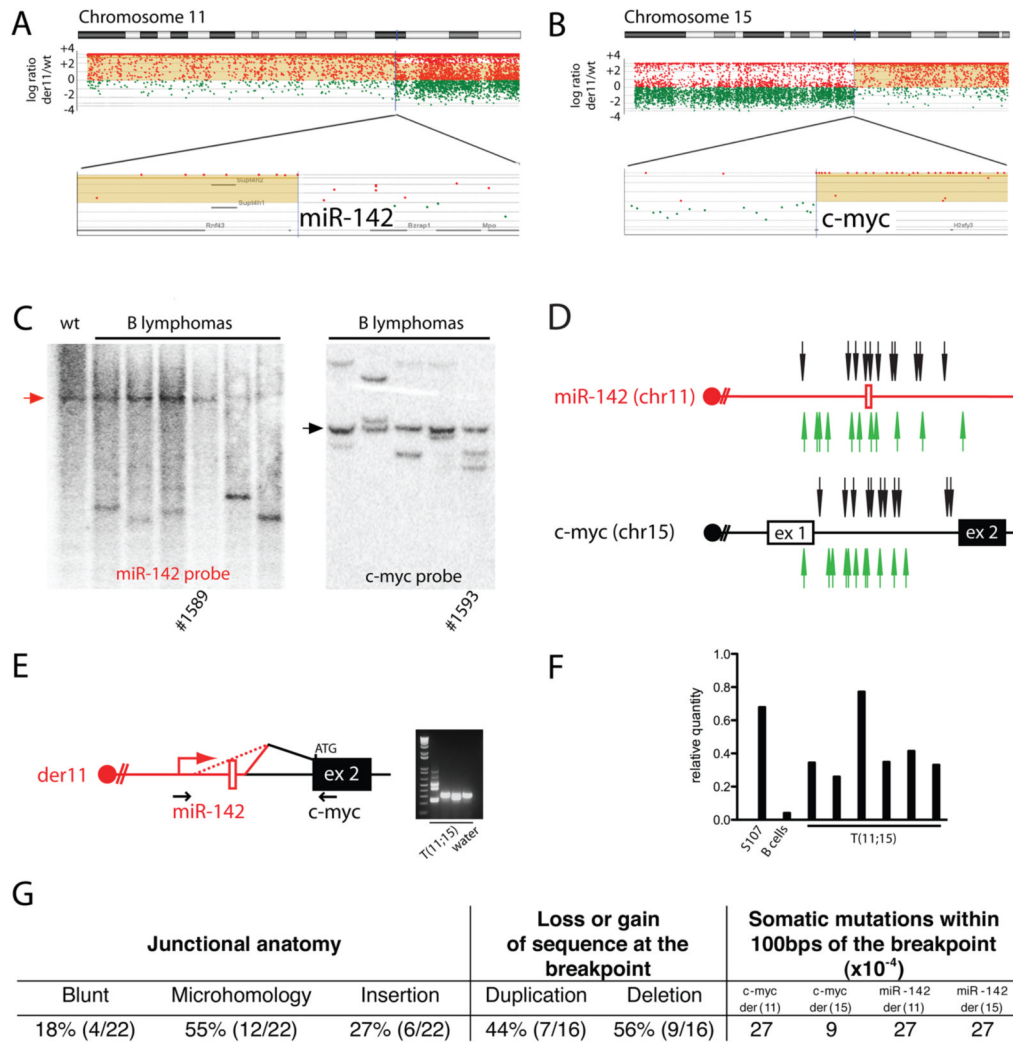
Figure 4. Identification of the T(11;15) translocation partners

(A) and (B) aCGH analysis of sorted chromosomes identifies the breakpoint in proximity of *miR-142* (Chr11) and *c-myc* (Chr15). (C) Southern blotting with *c-myc* and *miR142* probes. Unless otherwise noted, tumors have T(11;15) by M-FISH. Arrows point to the unearranged germline bands. (D) Map of translocation breakpoints in T(11;15) B cell lymphomas from IgκAID/p53$^{-/-}$ mice (n=14: in addition to the 9 T(11;15) identified by M-FISH, an additional 5 were identified by PCR). For some tumors the breakpoint could be identified in one only derivative (see Supplemental Table S1). Arrows point to translocation breakpoints for der11 (black) or der15 (green). (E) RT-PCR for hybrid transcripts between *miR-142* and *c-myc*. (F) qPCR for *c-myc* expression in T(11;15) tumors. S107 is a plasmacytoma cell line with *c-myc/ IgH* translocation (positive control). Negative control is activated B cells. (G) Features of the T(11;15) breakpoints.
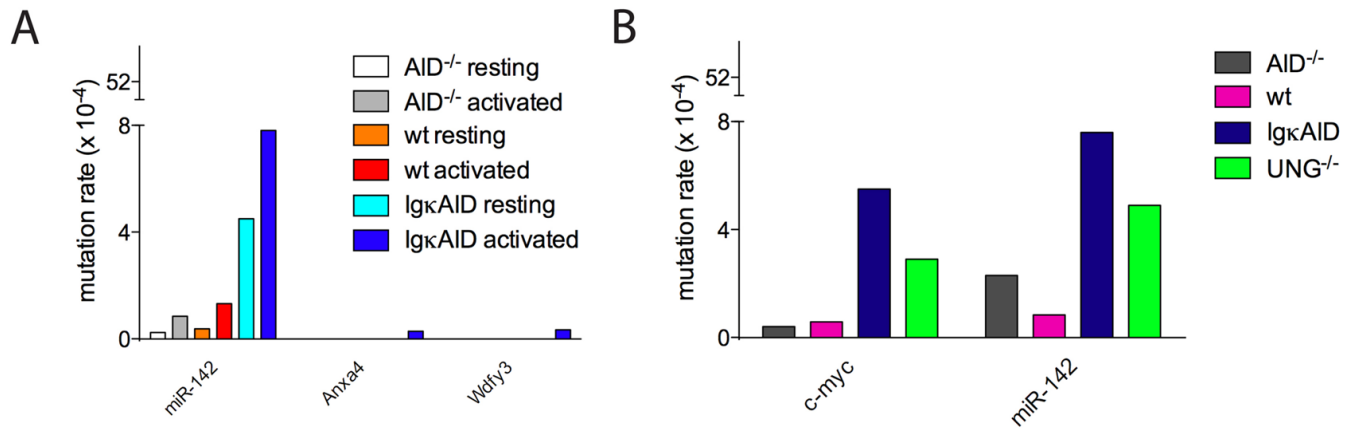
Figure 5. *miR-142* is an AID target
(A) Frequency of somatic mutations in *miR-142, Anxa4* and *Wdfy3* in resting and LPS and IL-4 stimulated B cells. (B) Frequency of somatic mutations in *miR-142* and *c-myc* in purified germinal center B cells from AID$^{-/-}$, wild type, *Igκ*AID, and UNG$^{-/-}$ mice.
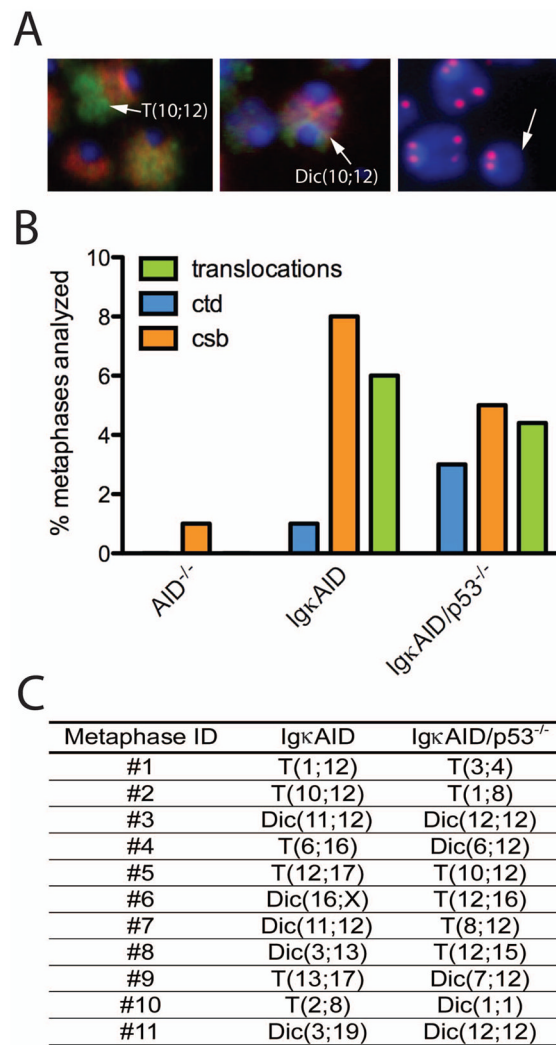
Figure 6. AID-dependent chromosome instability in stimulated primary B cells
(A) Representative M-FISH and telomere FISH images of B cells activated *in vitro* for 4 days
with LPS, IL-4 and RP105. The arrows indicate (left-to-right) a translocation, a dicentric
chromosome and a chromosome break. (B) Genomic instability and translocations in *Igκ*AID
primary B cells. Frequencies of ctd (chromatid breaks) and csb (chromosome breaks) were
determined by analysis of metaphases hybridized with a telomere probe and counterstained
with DAPI (n=100). Frequency of translocations (including dicentrics) was determined by
analysis of M-FISH images (n=250). (C) Table of chromosome aberrations detected in *Igκ*AID
and *Igκ*AID/p53$^{-/-}$ primary B cells. See also Supplemental Table S3.

Table 1

Karyotype and phenotype of B lymphomas in *Igk*AID/p53$^{-/-}$.

Tumor ID	Clonal translocation	Additional changes in at least one metaphase	Tumor phenotype
#1461	T(11;15)	Dic(13;13)	CD19$^+$ IgM$^+$ IgD$^-$
#1535	T(11;15)	−12, +13, −19	CD19$^+$ IgM^{dim} IgD$^{+/-}$
#1536	T(5;6)	−13, T(6;19)	CD19$^+$ IgM$^+$ IgD^{dim}
#1550	T(11;15)	−13, −16, −17, −18, T(16;17), T(16;Y)	CD19$^+$ IgM$^+$ IgD$^{dim/+}$ CD138$^{+/-}$
#1561	T(11;15)	+3,−12, −Y, T(12;16), T(12;17)	CD19$^+$ IgM$^+$ IgD$^-$ CD138^{dim}
#1583	T(11;15)	−12, −13, −14	CD19$^+$ IgM$^{+/++}$ IgD$^{+/-}$ CD138^{dim}
#1584	T(11;15)	−12, −18	CD19$^+$ IgM$^{+/++}$ IgD$^{+/-}$ CD138^{dim}
#1589	none	−5, −14, −18, −X, T(3;4), T(3;11), T(3;19)	CD19$^+$ IgM$^-$ IgD$^-$ Igκ$^-$ CD138$^{+/-}$
#1592	T(11;15)	−7, +16	CD19$^+$ IgM$^+$ IgD$^{+/-}$ CD138$^{+/-}$
#1593	T(12;15)	−7	CD138^{++} CD19$^+$ IgM$^-$ IgD$^-$ Igκ$^-$
#1594	T(11;15) T(12;17)	T(2;11), T(9;15), T(11;16), T(13;5)	CD138^{++} CD19$^+$ IgM$^-$ IgD$^-$ Igκ$^-$
#1595	T(11;15)	−12, −13, −19, T(13;16)	CD19$^+$ IgM$^+$ IgD$^-$ CD138$^{+/-}$