

ATM Prevents the Persistence and Propagation of Chromosome Breaks in Lymphocytes

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SUMMARY

DNA double-strand breaks (DSBs) induce a signal transmitted by the ataxia-telangiectasia mutated (ATM) kinase, which suppresses illegitimate joining of DSBs and activates cell-cycle checkpoints. Here we show that a significant fraction of mature ATM-deficient lymphocytes contain telomere-deleted ends produced by failed end joining during V(D)J recombination. These RAG-1/2 endonuclease-dependent, terminally deleted chromosomes persist in peripheral lymphocytes for at least 2 weeks in vivo and are stable over several generations in vitro. Restoration of ATM kinase activity in mature lymphocytes that have transiently lost ATM function leads to loss of cells with terminally deleted chromosomes. Thus, maintenance of genomic stability in lymphocytes requires faithful end joining as well a checkpoint that prevents the long-term persistence and transmission of DSBs. Silencing this checkpoint permits DNA ends produced by V(D)J recombination in a lymphoid precursor to serve as substrates for translocations with chromosomes subsequently damaged by other means in mature cells.

INTRODUCTION

Developing lymphocytes initiate antigen-receptor diversification by expression of the RAG-1/2 endonuclease, which introduces DSBs in recombination-signal sequences flanking immunoglobulin (Ig) and T cell receptor-coding gene segments (V, D, and J segments) (Jung et al., 2006). The subsequent ligation of ends to form functional

antigen-receptor genes is carried out by components of the nonhomologous end-joining (NHEJ) DNA-repair pathway. V(D)J recombination-associated DSBs are formed and repaired in the G0/G1 phase of the cell cycle (Schlissel et al., 1993), where NHEJ predominates. Factors involved in DSB-damage surveillance, Nbs1, ATM, 53BP1, and phosphorylated H2AX, are found at sites of antigen-receptor gene DSBs and also appear to suppress V(D)J-associated oncogenic chromosomal translocations (Bassing et al., 2003; Celeste et al., 2003; Chen et al., 2000; Morales et al., 2006; Perkins et al., 2002; Ward et al., 2005). ATM facilitates DSB repair by stabilizing V(D)J recombination-associated DSB intermediates (Bredemeyer et al., 2006).

B and T cell receptors are assembled in the bone marrow and thymus, respectively, in hierarchical maturation processes that take several days to complete (Jung et al., 2006). The B cell receptor (BCR) consists of a pair of heavy chains (IgH) and light (Igλ or Igκ) chains, whereas the T cell receptor (TCRβ/TCRα) is formed by pairing TCR beta and alpha chains. Assembly of the Ig heavy chain is initiated before light-chain gene rearrangements, and similarly TCRβ rearrangements precede TCRα recombination. Productive rearrangement of Ig heavy and TCRβ genes promotes a signaling cascade that extinguishes expression of RAG-1/2 genes and induces proliferation of pre-B and pre-T cells, respectively (Meffre et al., 2000). The developing lymphocytes undergo two to seven rounds of cell division before they re-express RAG-1/2 and initiate Ig light-chain or TCRα gene rearrangements (Meffre et al., 2000; Rolink et al., 2000; Sprent and Tough, 1994). Immature bone marrow B cells and thymic T cells that express a complete antigen receptor emigrate to the peripheral immune system, where they complete maturation and populate secondary lymphoid organs such as spleen and lymph node.

Mature B cells undergo an additional programmed DNA cleavage reaction that is associated with proliferation, class-switch recombination. This recombination reaction

replaces one Ig constant region with another and positions a new constant region next to the rearranged VDJ exon. Switching is initiated by activation-induced cytidine deaminase (AID), an enzyme expressed exclusively in mature B cells (Muramatsu et al., 1999, 2000; Revy et al., 2000).

AID-dependent DSBs in the Ig switch regions are joined together through NHEJ resulting in excision of up to 200 kb of intervening DNA. Like DSBs induced by V(D)J recombination, AID-dependent DSBs form in the G0/G1 phase of the cell cycle and are monitored by the DSB-damage surveillance proteins ATM, H2AX, 53BP1, and Nbs1 (Petersen et al., 2001; Reina-San-Martin et al., 2004). These proteins are required for normal class switching, possibly because they promote synapsis of switch regions (Celeste et al., 2002; Kracker et al., 2005; Lumsden et al., 2004; Manis et al., 2004; Petersen et al., 2001; Reina-San-Martin et al., 2004, 2005, 2007; Ward et al., 2004). In addition, they suppress Ig heavy-chain locus instability during class switching and lymphoma-associated chromosome translocations (Franco et al., 2006; Ramiro et al., 2006a).

Genomic instability associated specifically with the Ig heavy-chain locus has been visualized using a combination of fluorescence in situ hybridization (FISH) probes for chromosome 12 (chr12), IgH, and telomere repeats (Franco et al., 2006; Ramiro et al., 2006a). IgH-associated chromosome breaks and translocations were found in stimulated B cells deficient in the DNA-damage response proteins ATM, 53BP1, Nbs1, and H2AX (Franco et al., 2006; Ramiro et al., 2006a), but such lesions were undetectable in AID^{-/-}H2AX^{-/-} and AID^{-/-}53BP1^{-/-} cells, consistent with the requirement for AID to initiate DSBs (Petersen et al., 2001; Ramiro et al., 2006a; Franco et al., 2006).

IgH-associated breaks and translocations on Chr12 accumulated at high levels in *Atm*^{-/-} B cells (Franco et al., 2006; Ramiro et al., 2006a) despite a relatively mild switching defect (Lumsden et al., 2004; Reina-San-Martin et al., 2004). This suggests the possibility that besides AID, an additional source of IgH-specific DNA damage contributes to Chr12 aberrations in mature ATM-deficient lymphocytes. Here we show that a significant fraction of long-lived peripheral *Atm*^{-/-} lymphocytes harbor unresolved recombination activating gene (RAG)-dependent DSBs that were generated in progenitor cells. These findings uncover that an ATM checkpoint blocks transmission of V(D)J breaks and could thereby prevent translocations between proto-oncogenes and antigen-receptor genes damaged in precursor cells.

RESULTS

DSBs and Translocations in *Atm*^{-/-} B Cells

To determine the etiology of unrepaired chromosome breaks in *Atm*^{-/-} B cells, we generated mice deficient in ATM and AID. Mature (CD43⁻) splenic B cells from *Atm*^{+/+}*Aid*^{+/+} (WT), *Atm*^{-/-}*Aid*^{+/+} (*Atm*^{-/-}), *Atm*^{+/+}*Aid*^{-/-} (*Aid*^{-/-}), and *Atm*^{-/-}*Aid*^{-/-} mice were activated in vitro by stimulation with LPS+IL4. Class switching was assessed

by measuring surface IgG1 expression at day 4, and, as expected, switching was reduced in ATM-deficient mice (Lumsden et al., 2004; Reina-San-Martin et al., 2004) and was undetectable in *Atm*^{-/-}*Aid*^{-/-} mice (Figure 1A). Metaphase spreads analyzed on day 3 cultures using chr12-, telomere-, and IgHC α -specific probes (Ramiro et al., 2006a) showed a small increase in total (IgH specific + other) abnormalities in *Atm*^{-/-} (43%) versus *Atm*^{-/-}*Aid*^{-/-} (34%) (Figure 1B and Table S1). These aberrations included chromosome breaks, chromatid breaks, dicentric, and translocations (Table S1); however, the vast majority of aberrations in *Atm*^{-/-} (76%) and *Atm*^{-/-}*Aid*^{-/-} (68%) B cells were chromosome breaks (Table S1) (Franco et al., 2006; Takao et al., 1999), suggesting that ATM regulates the repair of DSBs generated in the prereplicative phase of the cell cycle.

In contrast to genome-wide aberrations, breaks and translocations specifically associated with the Ig heavy-chain locus were statistically different between the three groups. Ig-specific lesions were found in 23% of *Atm*^{-/-} metaphases (n = 3 independent stimulations), 11% of *Atm*^{-/-}*Aid*^{-/-} metaphases (n = 3), and 0.4% of WT metaphases (n = 3) (Figures 1B and 1C and Table S1). Chr12-specific breaks that accumulated in *Atm*^{-/-} (total 0.2 per cell) and *Atm*^{-/-}*Aid*^{-/-} (total 0.08 per cell) B cells were distributed among those that were centromeric (downstream) and telomeric (upstream) of IgHC α , with the majority localized downstream (Figure 1C and Table S1). Distinct breakpoints in the Ig heavy-chain locus could either reflect different types of initiating lesions (e.g., recombination+ common fragile sites, see below) or could be indicative of a single lesion combined with varying degrees of chromosomal erosion. Altogether, only half of the Chr12 aberrations in *Atm*^{-/-} B cells stimulated with LPS and IL-4 are AID dependent (Figure 1B), which contrasts with 53BP1 and H2AX deficiency, where IgH-associated lesions are entirely dependent on AID (Franco et al., 2006; Ramiro et al., 2006a).

In addition to chromosome breaks, 4% of *Atm*^{-/-} metaphases and 2.5% of *Atm*^{-/-}*Aid*^{-/-} metaphases contained translocations associated with the IgH locus (Figure 1C and Table S1). However, when we assayed for specific translocations between *c-myc* and Ig switch regions by PCR (Figure 1D), these fusions were undetectable in *Atm*^{-/-}*Aid*^{-/-} mice while accumulating at a high frequency (1.6×10^{-6}) in *Atm*^{-/-} mice. This is consistent with the requirement for AID in canonical *c-myc-IgH* switch translocations (Ramiro et al., 2006a). We conclude that only a subset of the Ig heavy chain-associated translocations originate from AID-dependent DNA damage in *Atm*^{-/-} B cells stimulated to undergo CSR.

Ig and TCR Locus Instability in Mature *Atm*^{-/-} Lymphocytes

Ig-associated DNA breaks in *Atm*^{-/-} B cells might arise as a result of DNA-replication stress incurred at fragile sites during the two to seven rounds of division in B cells stimulated with LPS+IL4, or they might be present in resting

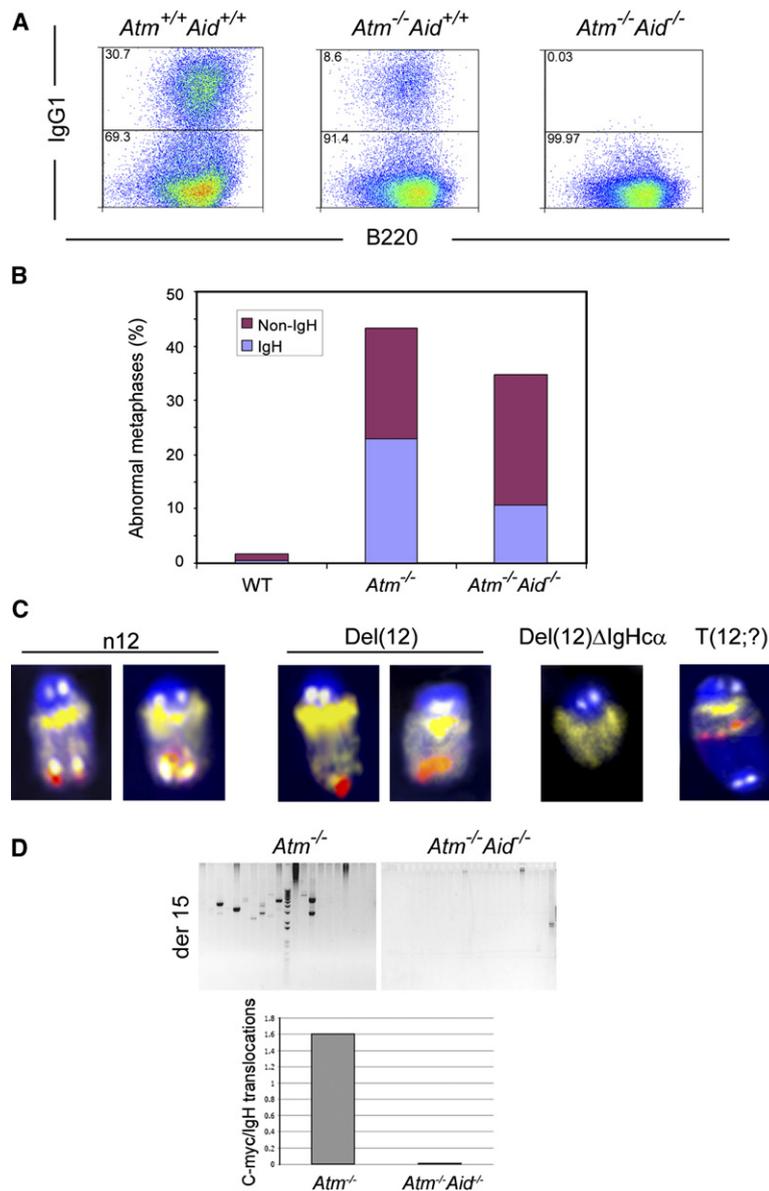


Figure 1. IgH Locus Breaks and Translocations in *Atm*^{-/-}*Aid*^{-/-} B Cells

(A) Flow cytometric analysis of class-switch recombination to IgG1 in *Atm*^{+/+}*Aid*^{+/+}, *Atm*^{-/-}*Aid*^{+/+} and *Atm*^{-/-}*Aid*^{-/-} B cells stimulated with LPS/IL4 for 4 days. Class-switch recombination is decreased in *Atm*^{-/-}*Aid*^{+/+} mice (relative to the normal *Atm*^{+/+}*Aid*^{+/+} littermate) and is abrogated in *Atm*^{-/-}*Aid*^{-/-} mice.

(B) Percentage of metaphases with abnormalities specifically associated with the IgH locus (blue bar) compared to the frequency of general non-IgH instability (red bar). Metaphases from three independent *Atm*^{+/+}*Aid*^{+/+} (WT), *Atm*^{-/-}*Aid*^{+/+} (*Atm*^{-/-}) and *Atm*^{-/-}*Aid*^{-/-} mice (see Table S1) were prepared 72 hr after B cell culture with LPS/IL4 and analyzed using the IgH/telomere FISH assay.

(C) Examples of IgH-associated aberrations in *Atm*^{-/-}*Aid*^{-/-} B cells. Chromosomes from B cells stimulated for 72 hr with LPS/IL4 were hybridized with a chr12 painting probe (yellow; which carries the IgH locus in a telomere-proximal position), IgH Cα probe (red; centromeric of Cγ1), a telomere sequence-specific probe (white), and counterstained with DAPI (blue). Normal 12 (n12), deleted 12 (either positive for IgH Cα and negative for telomeric sequences: Del[12], or negative for both IgH Cα and telomeric sequences: Del[12]ΔIgH Cα) and a IgH-associated translocation (T[12;?]) are indicated.

(D) C-myc/IgH-switch translocations in *Atm*^{-/-} and *Atm*^{-/-}*Aid*^{-/-} mice. Representative agarose gels (top) and frequency of c-myc/IgH translocations per 10⁶ cells (below) is shown. Each lane represents an individual PCR reaction.

mature B cells before stimulation. To assay for Ig-associated fragile sites, we cultured activated WT and *Atm*^{-/-} B cells with low doses of aphidicolin (0.05–0.3 μg/ml), an inhibitor of DNA replication that is a potent inducer of fragile site expression (Arlt et al., 2006). WT B cells cultured with 0.1 μg/ml aphidicolin showed the expected increase in chromatid gaps and breaks in metaphase chromosomes. Chromosomal aberrations were found in 51% of aphidicolin-treated WT cells (Figure 2A) compared to 0.9% in untreated cultures (Figure 1B); however, aphidicolin treatment did not produce chromosomal breaks in the vicinity of the Ig heavy-chain locus (Figure 2A). Moreover, although abrogation of the S and G2/M checkpoint generally enhances fragile-site expression (Casper et al., 2002), treatment of *Atm*^{-/-} B cells with aphidicolin did not increase the percentage of cells with Ig heavy-chain locus

breaks (Figures 1B and 2A). Thus, replication stress does not appear to specifically target the Ig heavy-chain locus in WT or *Atm*^{-/-} B cells stimulated with LPS and IL-4.

To determine whether lesions in the Ig heavy-chain locus are present in unstimulated mature B cells, we assayed purified morphologically preserved splenic B cells using three-dimensional FISH with telomeric V_H and centromeric Cα probes (Figure 2B). Both probes were detectable in >98% of WT (n = 511) and *Aid*^{-/-} cells (n = 402, not shown) as two pairs of closely apposed signals. In contrast, 6.7% of *Atm*^{-/-} (n = 342) and 7.8% of *Atm*^{-/-}*Aid*^{-/-} (n = 422) B cells had lost either V_H or both V_H and Cα signals from one allele (Figure 2B; *Atm*^{-/-} versus WT, p = 0.00085; *Atm*^{-/-}*Aid*^{-/-} versus WT, p = 0.000024; two-tailed p values). Consistent with the fact that AID is not expressed in resting B cells, the loss of integrity of the Ig heavy-chain locus was

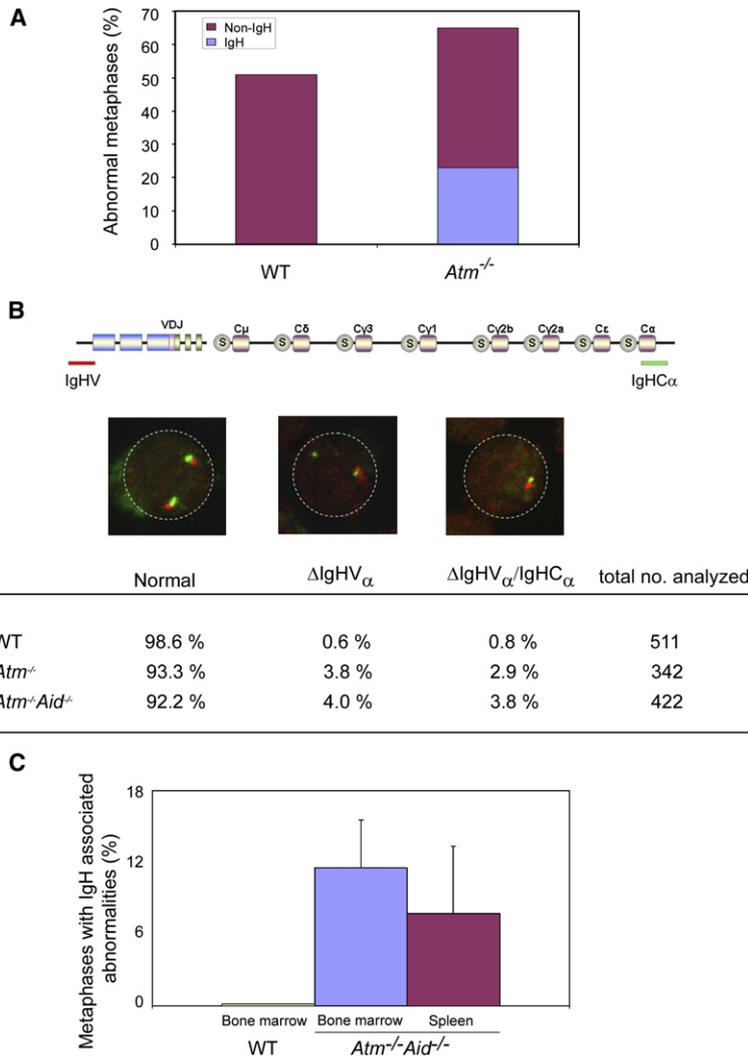


Figure 2. IgH-Associated Aberrations in Resting Mature B Cells and Immature Bone Marrow Cells

(A) WT and *Atm*^{-/-} B cells were cultured for 48 hr with LPS/IL4 and then an additional 24 hr in the presence of 0.1 μ g/ml of aphidicolin. Replication stress increased the frequency of non-IgH (red) but not the IgH-specific aberrations (blue), as determined by the IgH/telomere FISH assay.

(B) (Top) Scheme of the mouse IgH locus and probes used for three-dimensional interphase FISH. BAC 224M14 (Vh) hybridizes to the distal 5' (telomeric end) of the VJ588 cluster and the IgH C α BAC extends from C γ 1 to 3' of C α . (Middle) Representative confocal sections through the nuclei of unstimulated CD43⁻ purified *Atm*^{-/-} B cells, analyzed by three-dimensional FISH with IgH Vh (red) and IgH C α (green) probes. Examples of cells with normal, Vh-deleted and Vh/C α -deleted chromosomes are indicated. (Bottom) Percentage of WT, *Atm*^{-/-}, and *Atm*^{-/-}*Aid*^{-/-} B cells with IgH-associated chromosome deletions. A two-tailed Fisher's test revealed statistical differences between WT and ATM mutant genotypes (WT versus *Atm*^{-/-}, $p = 0.00085$; WT versus *Atm*^{-/-}*Aid*^{-/-}, $p = 0.000024$).

(C) Frequency of IgH-associated abnormalities in *Atm*^{-/-}*Aid*^{-/-} bone marrow and spleen was analyzed using the IgH/telomere FISH assay. Metaphase spreads were prepared from purified B220⁺ bone marrow cells after culture on irradiated S17 stromal cells for 5 days and splenic CD43⁻ B cells after culture with LPS/IL4 for 3 days. Data represent mean + standard deviation.

not statistically different between *Atm*^{-/-} and *Atm*^{-/-}*Aid*^{-/-} B cells ($p = 0.58$; two-tailed p values) (Figure 2B). We conclude that a subset of resting *Atm*^{-/-} and *Atm*^{-/-}*Aid*^{-/-} B cells harbor deletions in the Ig heavy-chain locus.

The observed disruption of the Ig locus in resting peripheral *Atm*^{-/-} B cells may have originated earlier in B cell development. To determine whether telomere-deleted chr12 ends were detectable in B cell precursors, we cultured *Atm*^{-/-}*Aid*^{-/-} and WT pre-B cells (CD19⁺IgM⁻) from bone marrow and examined metaphases using the Ig heavy chain locus-specific FISH assay. Whereas Ig breaks were undetectable in WT pre-B cells ($n = 70$), these lesions were found in 11% of *Atm*^{-/-}*Aid*^{-/-} pre-B cells ($n = 80$ metaphases) (Figure 2C). Similar levels of Ig-associated DSBs were detected in the mature splenic B cell compartment from the same mouse (Figure 2C). The finding that a significant fraction of *Atm*^{-/-}*Aid*^{-/-} pre-B cells contain copies of chr12 that are broken at the Ig heavy-chain locus is consistent with the observation that after V(D)J recombination unrepaired coding ends accumulate

and dissociate from the postcleavage complex in *Atm*^{-/-} pre B cells (Bredemeyer et al., 2006; Huang et al., 2007).

Recent studies have demonstrated that the integrity of the TCR α locus is frequently disrupted in immature thymocytes of *Atm*^{-/-} mice (Huang et al., 2007; Matei et al., 2007; Vacchio et al., 2007), and TCR α -specific translocations have been documented in mature T cells (Liyanaage et al., 2000). Analogous to the persistence of AID-independent Ig heavy-chain DSBs in B cells, we hypothesized that telomere-deleted chromosome ends might also accumulate in mature *Atm*^{-/-} T cells. To determine whether terminally deleted chromosomes are present in mature ATM-deficient T cells, we stimulated lymph node T cells for 48 hr with anti-CD28 and -TCR antibodies and examined metaphase spreads using a chromosome 14 (chr14)-painting probe in conjunction with a BAC probe spanning the TCR α locus as well as a telomere-specific probe (Figures S1A and S1B). Consistent with a previous report (Liyanaage et al., 2000), we found that 6.2% of *Atm*^{-/-} T cells ($n = 143$) harbored TCR α translocations. In addition

to TCR α -specific translocations, 5.6% of mature *Atm*^{-/-} T cells harbored telomere-deleted chromosome ends near the TCR α locus (Figures S1A and S1B). As observed for the Ig heavy-chain locus (Table S1), some of the chromosomes were broken between V α and C α (Figure S1A; del[14]), whereas others were deleted beyond the TCR α locus toward the centromere of chr14 (Figure S1A; del[14] Δ TCR α). We conclude that chromosome breaks near the antigen-receptor loci accumulate in mature B and T cells in ATM-deficient mice.

ATM^{-/-} Antigen-Receptor Locus Abnormalities Require RAG

To determine whether TCR α -specific chromosome abnormalities in mature T cells are products of V(D)J recombination, we performed the TCR α FISH assay on metaphase spreads from *Atm*^{-/-}*Rag2*^{-/-}TCRAND T cells (Kaye et al., 1989). Absence of RAG expression interferes with T cell development, but development is reconstituted by expression of a transgenic T cell receptor (Kaye et al., 1989). In contrast to lymph node T cells from *Atm*^{-/-} mice, no chromosome breaks or translocations involving the TCR α locus were observed in *Atm*^{-/-}*Rag2*^{-/-}TCRAND T cells (n = 80) (Figure S1B). Thus, TCR α -associated chromosomal damage in mature T cells is dependent on RAG-mediated DNA cleavage.

To determine whether Ig-specific lesions in *Atm*^{-/-} B cells are also RAG dependent, we generated *Atm*^{-/-}*Rag2*^{-/-}MD4 mice expressing an Ig transgene (Goodnow et al., 1988). Metaphase spreads were analyzed from B cells stimulated with anti-RP105, which induces B cell proliferation (Miyake et al., 1995) but not AID expression (Figure 3A). None of the *Atm*^{-/-}*Rag2*^{-/-}MD4 B cells stimulated with RP105 underwent switching (not shown) or contained chr12-specific lesions (n = 74) (Figure 3B). In contrast, Ig heavy chain-associated lesions were found in 7.5% of RP105-treated *Atm*^{-/-}*Rag2*^{+/+}MD4 B cells (Figure 3B). Taken together, these results demonstrate that in the absence of ATM, broken chromosome ends produced during V(D)J recombination in immature T and B cells can persist in peripheral lymphocytes. These findings in primary lymphocytes are consistent with the requirement for RAG proteins in the generation of lymphomas with TCR α or Ig translocations (Difilippantonio et al., 2002; Gladdy et al., 2003; Petiniot et al., 2002; Zhu et al., 2002).

If RAG proteins were expressed in mature T and B cells, continual V(D)J recombination activity might contribute to antigen-receptor breaks. RAG expression is normally shut off in immature B and T cells (Monroe et al., 1999; Yu et al., 1999) but might be deregulated in the absence of ATM. To test this possibility, we measured RAG messenger RNA in CD43⁻ splenic B cells and lymph node T cells on days 1–3 after stimulation. We found that RAG1 and RAG2 mRNA levels in mature *Atm*^{-/-}*Aid*^{-/-} lymphocytes were at least 10,000-fold lower than in purified pre-B cells (Figure 3C). Furthermore, RAG protein was undetectable in mature T cells (Figure S2).

The B cell population in the spleen is divided between short-lived transitional cells (CD43⁻ HSA^{hi}B220^{lo}) that express low levels of RAG and mature B cells (CD43⁻ HSA^{lo} B220^{hi}) that do not express any RAG (Monroe et al., 1999; Yu et al., 1999). To determine whether IgH-associated breaks were present in long-lived mature B cells, we compared the level of damage in purified transitional and long-lived mature *Atm*^{-/-}*Aid*^{-/-} B cells. We found that similar to the unseparated B cells, 11.3% of the transitional cell population (n = 62) and 9% of the mature B cell population (n = 77) had Ig heavy chain-associated aberrations (Figure 3D). We conclude that neither continued RAG expression nor contaminating transitional cells account for antigen-receptor DNA breaks observed in the peripheral lymphocyte compartment. Rather, our experiments suggest that unresolved chromosome breaks can persist throughout lymphocyte development in the absence of ATM.

Stability of Terminally Deleted Chromosomes In Vivo

To determine the minimum lifespan of a mature lymphocyte that carries an unresolved broken chromosome end, we used reporter mice transgenic for GFP driven by the *Rag2* promoter (RAG2p-GFP transgenic mice) (Yu et al., 1999). In these mice, GFP is highly expressed in the thymus and bone marrow, lower levels are detectable in recent emigrants in peripheral compartments, and GFP is absent in mature lymphocytes (Yu et al., 1999). For example, mature T cells that are GFP⁺ are recent (1 week-old) thymic emigrants, while GFP⁻ cells have been in the peripheral compartment for at least 2 to 3 weeks (Boursalian et al., 2004). GFP⁻ and GFP⁺ T cells were sorted from *Atm*^{-/-} RAG2p-GFP transgenic mice, stimulated for 48 hr with anti-TCR/CD28 antibodies, and metaphase spreads were hybridized with TCR α /Chr14 FISH probes (Figure 4A). We found similar levels of DNA breaks associated with the TCR α locus in both populations. Similarly the percentage of B cells harboring telomere-deleted ends was comparable in *Atm*^{-/-} RAG2p-GFP⁻ (n = 73) and *Atm*^{-/-} RAG2p-GFP⁺ (n = 83) sorted subpopulations that had been stimulated with RP105 for 24 hours, as well as unsorted *Atm*^{-/-}*Aid*^{-/-} splenic B cells (Figures 1B and 4A). Since GFP⁻ cells have been in the peripheral compartment for at least 2 weeks, these results indicate that abnormal antigen-receptor loci bearing chromosome breaks are remarkably stable in vivo in the absence of ATM. Finally, because GFP⁻ cells that harbor such aberrations do not express or re-express RAG (Figure 4A) (Monroe et al., 1999; Yu et al., 1999), this analysis at the single-cell level demonstrates once again that chromosome breaks are not caused by de novo cleavage of antigen-receptor genes in mature lymphocytes.

To determine whether there is selection against cells harboring chromosome breaks, we examined dividing lymph node T and splenic B cells from *Atm*^{-/-}*Aid*^{-/-} mice after labeling with the vital dye CFSE (5- [and 6-] carboxy-fluorescein diacetate succinyl ester) (Figures 4B and 4C). After stimulation for 3 days in culture with either

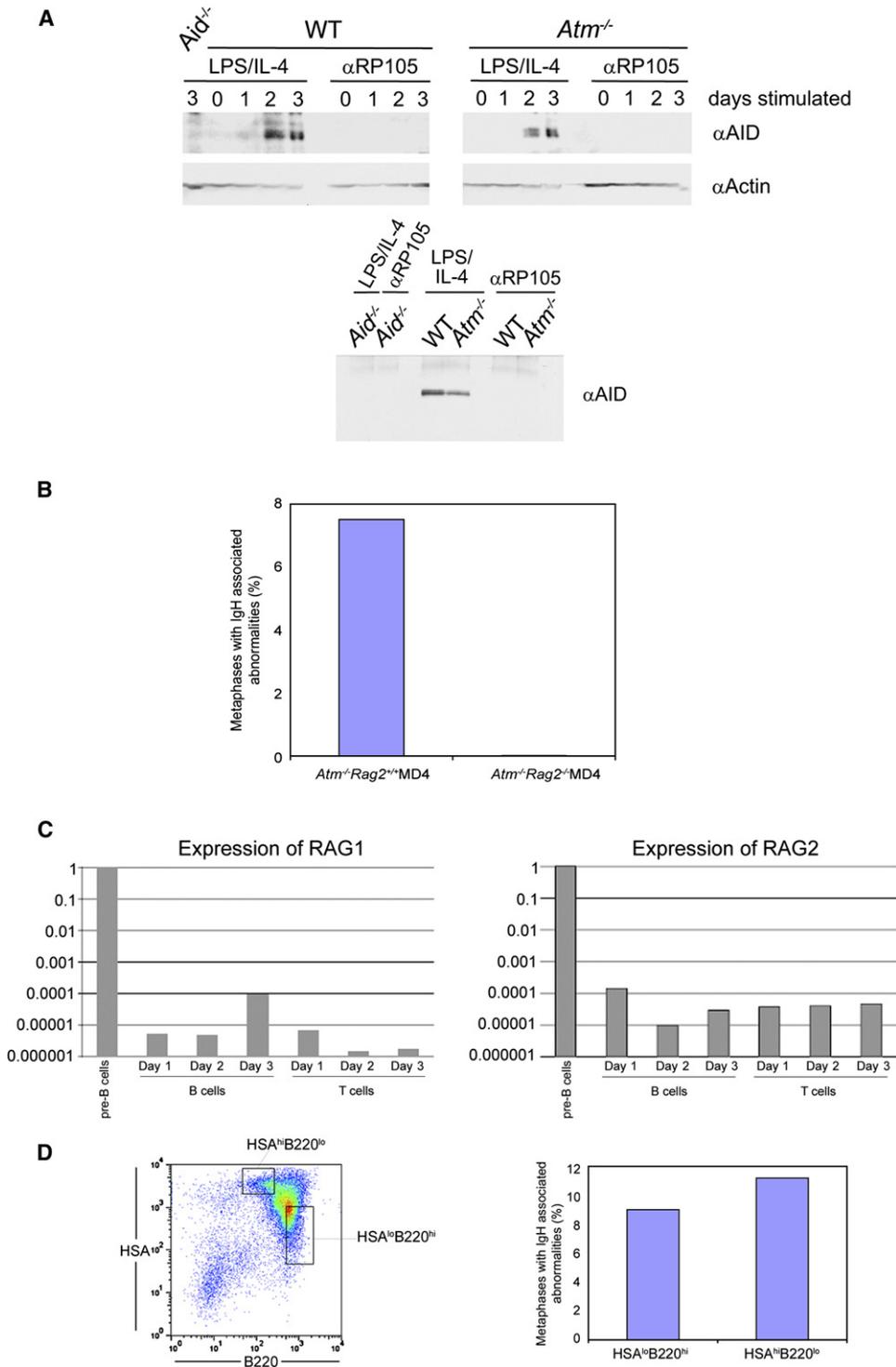
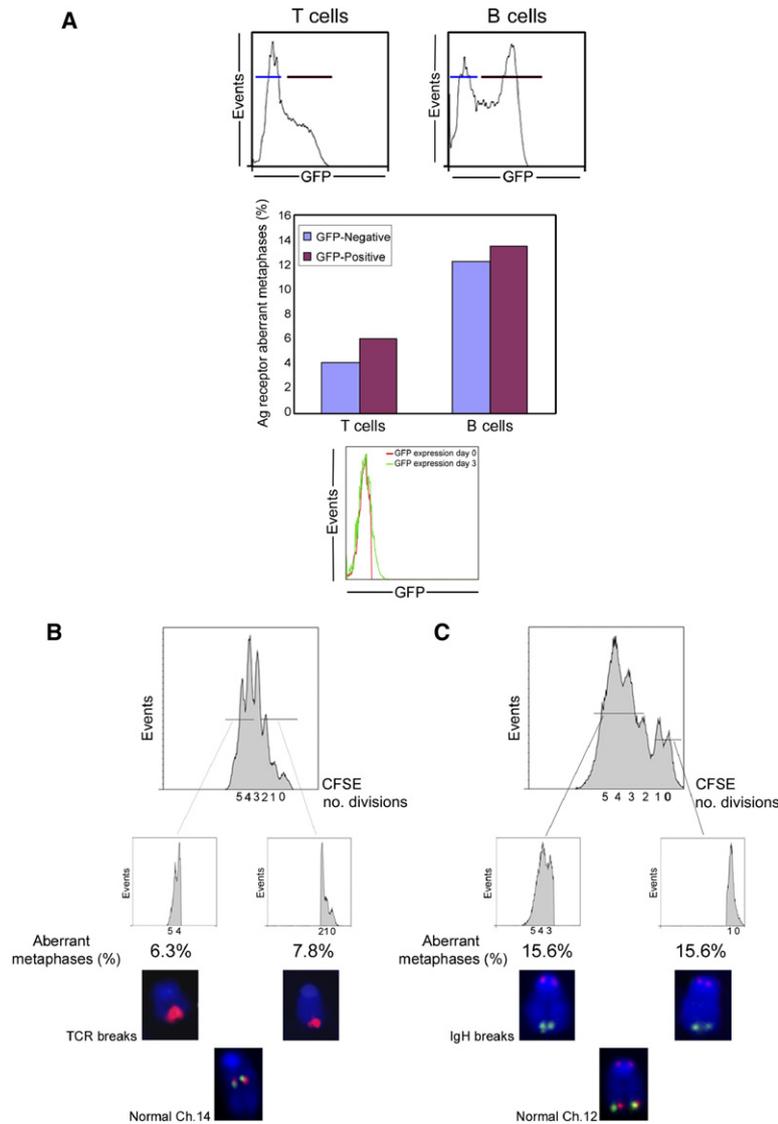


Figure 3. RAG Dependence of IgH-Associated Breaks and Translocations in *Atm*^{-/-} B Cells

(A) (Top) Time course of AID expression in B cells. Anti-AID immunoblots of lysates from *Aid*^{-/-}, WT, or *Atm*^{-/-} B cells stimulated with either LPS/IL-4 or anti-RP105. The number of days cultured is indicated. AID expression is detected in lysates after 2 days of LPS/IL-4 expression. Anti-actin was used as a loading control. (Bottom) AID expression in B cells. Anti-AID immunoblots of anti-AID immunoprecipitates from lysates of 20 million *Aid*^{-/-}, WT, or *Atm*^{-/-} B cells stimulated with either LPS/IL-4 or anti-RP105 for 72 hr.

(B) Quantification of IgH-associated aberrations in *Atm*^{-/-}*Rag2*^{+/+}MD4 (n = 78) and *Atm*^{-/-}*Rag2*^{-/-}MD4 (n = 74) B cells demonstrate that these lesions originate from RAG-mediated cleavage. Metaphase spreads from CD43⁻ splenic B cells stimulated for 24 hr with RP105 were analyzed by IgH/telomere FISH.



anti-TCR/CD28 antibodies (T cells) or LPS/IL4 (B cells), we sorted cells based on the fluorescence intensity of CFSE, which halves at each cell division, and analyzed metaphase spreads for antigen-receptor specific breaks. We found that 6.3% of T cells that had undergone four to five divisions had TCR α breaks, compared to 7.8% of cells that had undergone zero to two divisions (Figure 4B). Moreover, 15% of the *Atm*^{-/-}*Aid*^{-/-} B cells had IgH-associated aberrations whether they had divided only once or three to five times (Figure 4C). Thus, in *Atm*^{-/-} lymphocytes, there does not appear to be selection against the transmission of V(D)J-associated DSBs through DNA replication and cell division.

Persistent DNA Damage Induced by Irradiation

Chromosome breaks in *Atm* knockout lymphocytes might persist because of the unusual way the DNA ends are generated by RAG cleavage. To determine whether chromosome aberrations are stable when DNA damage is generated by a different means, we irradiated WT and *Atm*^{-/-}*Aid*^{-/-} B cells with low-dose γ irradiation (IR, 1 Gy), stimulated them with LPS+IL4, and examined the integrity of the chromosomes 1 hr and 72 hr later. At 1 hr postirradiation, chromatid breaks, chromosome breaks, or dicentric chromosomes were found in 77% (n = 40) and 73% (n = 42) of WT and *Atm*^{-/-}*Aid*^{-/-} B cells, respectively (Figure S3). At 72 hr postirradiation, *Atm*^{-/-}*Aid*^{-/-}

Figure 4. Stability of V(D)J-Associated Breaks in the Absence of ATM

(A) (Top) GFP⁻ (blue mark) and GFP⁺ (red mark) lymph node T cells and CD43⁻ splenic B cells from *Atm*^{-/-}RAG2p-GFP transgenic mice were sorted on day 0. (Middle) Sorted T cells were stimulated with anti-TCR/CD28 antibodies for 48 hr, and sorted B cells were stimulated with RP105 for 24 hr. Antigen-receptor chromosome abnormalities in GFP⁻ (blue) and GFP⁺ cells (red) were quantified using the IgH- and TCR α -telomere/FISH assays. (Bottom) RAG-GFP expression does not change during 72 hr of in vitro culture of sorted GFP⁻ cells.

(B) Lymph node T cells from *Atm*^{-/-}*Aid*^{-/-} mice were labeled with CFSE. After stimulation with anti-TCR/CD28 antibodies for 72 hr, cells that had undergone zero to two and four to five divisions were sorted and metaphase spreads were generated. The percentage of metaphases with TCR α abnormalities was quantified. An example of a TCR α -associated break (detected using TCRV α - [red] and TCR α - [green] probes) on both populations, and a normal chr14 is shown below.

(C) Splenic CD43⁻ B cells from *Atm*^{-/-}*Aid*^{-/-} mice were labeled with CFSE to track cellular division. After stimulation with LPS/IL4 for 72 hr, cells that had undergone zero to one and three to five divisions were sorted and metaphase spreads were generated. The percentage of metaphases with IgH-associated abnormalities was quantified. Example of an IgH-associated break (detected using IgHC α - [green], telomere-specific [red] probes) from both populations and a normal chr12 is shown below.

(C) RAG1 and RAG2 messenger RNA measured in *Atm*^{-/-}*Aid*^{-/-} pre-B cells, lymph node T cells, and splenic B cells (days 1–3 after stimulation). (D) (Left) Marked populations from *Atm*^{-/-}*Aid*^{-/-} B cells stained with anti-HSA and -B220 antibodies were sterile sorted on day 0. (Right) Sorted HSA^{lo}B220^{hi} and HSA^{hi}B220^{lo} B cells were stimulated with LPS/IL4 for 72 hr, and chr12 abnormalities were scored using the IgH FISH.

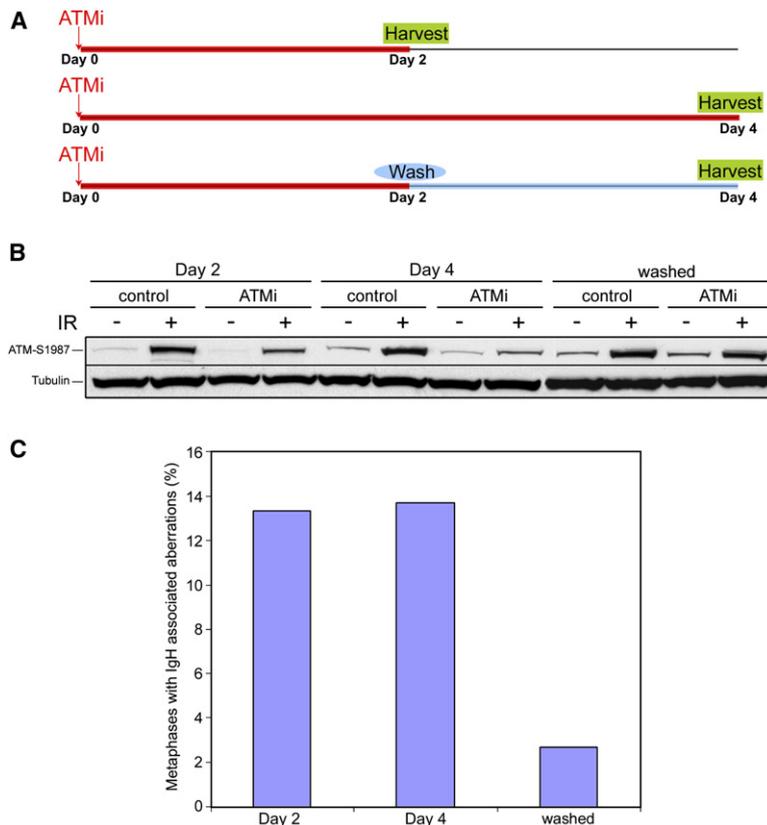


Figure 5. Elimination of IgH-Associated Breaks after Restoration of ATM Kinase Activity

(A) Scheme of the experimental procedure performed with the ATM kinase inhibitor (Ku55933).

(B) Western blot analysis of ATM-S1987 phosphorylation in drug-treated or control WT B cell cultures with or without irradiation (10 Gy).

(C) Percentage of cells with IgH-associated abnormalities. Metaphases were harvested at the indicated time points and analyzed by FISH.

cells continued to show a significant amount of damage (58% of cells, $n = 43$), whereas the frequency of aberrations in WT B cells dropped precipitously (6.8% of cells, $n = 40$). CFSE-labeling experiments revealed that 61% ($n = 52$) and 54% ($n = 62$) of *Atm*^{-/-}*Aid*^{-/-} cells that had undergone zero to two divisions and four to six divisions, respectively, harbored asymmetric chromosome aberrations. We conclude that IR-induced chromosome aberrations persist for several days in ATM-deficient lymphocytes, similar to V(D)J recombination-associated breaks.

To determine whether DNA damage persists in other cell types, we irradiated WT and *Atm*^{-/-} embryonic stem (ES) cells. Four hours after treatment with 1 Gy IR, 100% of the *Atm*^{-/-} ES cells ($n = 40$) harbored structural aberrations. At 72 hr postirradiation, the frequency of chromosome aberrations decreased only to 62% ($n = 42$) (Figure S3). In WT ES cells, the frequency of aberrations already decreased to 60% ($n = 40$) by 4 hr and then to 11% ($n = 40$) at 72 hr. Thus, similar to lymphocytes, chromosome aberrations in *Atm*^{-/-} ES cells do not rapidly disappear after low-dose IR.

Checkpoint Function in Response to Antigen-Receptor Breaks

We next addressed the effect of transiently restoring ATM after accumulation of IgH-associated breaks. To ascertain the impact of restoring ATM function, we took advantage of the highly specific small molecule inhibitor of ATM

kinase activity (KU55933) (Figure 5A). Continuous incubation of LPS+IL4-treated WT splenic B cells with KU55933 for 2 or 4 days lead to a reduction in ATM activity, evidenced by the decrease in ATM-1987 autophosphorylation (Figure 5B). Strikingly, just like *Atm*^{-/-} cells, 13%–14% of drug-treated WT cells harbored IgH-associated aberrations on days 2 and 4, indicating that ATM kinase activity is required for faithful end joining during class switching (Figure 5C). However, when KU55933 was washed out on day 2 and the cells were allowed to progress to day 4 in the absence of the drug, the number of aberrations dropped to 2%, concomitant with the increase in ATM activity (Figure 5C). Thus, it appears that some cells harboring IgH-associated breaks are eliminated from the overall population upon restoration of ATM activity, suggesting the existence of a checkpoint function mediated by ATM in response to antigen-receptor breaks.

In contrast to ATM deficiency, there is a selection against persistent DNA damage in developing *53BP1*^{-/-} lymphocytes. This is evidenced by comparing the integrity of TCR α locus in *53BP1*^{-/-} thymocytes versus mature lymph node T cells (Figure S4A) and levels of IgH locus-associated aberrations in B cells from bone marrow versus spleen (Figure S4B). Like *Atm*^{-/-} thymocytes (Matei et al., 2007), a significant fraction of *53BP1*^{-/-} developing thymocytes (16.9%) had either fewer than or greater than two TCR α C region signals per nucleus compared to 0.9% aberrant cells in WT when assayed using three-dimensional

FISH with $V\alpha$ and $C\alpha$ probes (Figure S4A). However, in contrast to ATM deficiency (Figure S1), TCR α loci were largely intact in mature $53BP1^{-/-}$ lymph node T cells with only 1% (2 out of 180, $n = 3$ mice) of the cells carrying TCR α -associated translocations. In $53BP1^{-/-}$ bone marrow, the fraction of metaphases harboring IgH-associated breaks (2% of the cells) was intermediate between $Atm^{-/-}$ (10%) and WT (0%). However, DSBs did not persist in the periphery since no aberrations were found in RP105-stimulated $53BP1^{-/-}$ B cells (Figure S4B). Similarly, chromosome aberrations were undetectable in RP105-stimulated $DNA-PKcs^{-/-}$ HL B cells, which are deficient for NHEJ but proficient in the DSB checkpoint response. Altogether, these data suggest that abrogation of both faithful end joining and DSB checkpoints in $Atm^{-/-}$ lymphocytes allow damaged antigen-receptor genes in precursor cells to persist.

The finding that restoration of ATM activity reduces the level of IgH instability suggests that IgH-associated breaks in splenic B cells harbor DNA damage response-activating signals. Normally, ATM activation requires prior loading of DNA-damage sensors such as the Mre11/Rad50/Nbs1 complex and 53BP1 to sites of DSBs (Mochan et al., 2004). To determine whether persistent 53BP1 foci form at the IgH locus, we used an immunofluorescence in situ hybridization approach to visualize protein and DNA simultaneously (Chen et al., 2000; Petersen et al., 2001). WT, $Aid^{-/-}$, $Atm^{-/-}$, and $Atm^{-/-}Aid^{-/-}$ B cells were stimulated with LPS+IL4 for 2 days and then were stained with anti-53BP1 antibodies, followed by FISH detection of the IgH locus. The percentage of cells containing 53BP1/IgH foci that showed colocalization was similar in $Atm^{-/-}$ B cells (61%) and WT controls (64%), lower in $Atm^{-/-}Aid^{-/-}$ B cells (36%), and at background frequencies in $AID^{-/-}$ cultures (5.4%) (Figure S5). The finding that a significant fraction of $Atm^{-/-}Aid^{-/-}$ B cells harbor 53BP1 foci at the IgH locus indicates that even persistent DSBs generated in the absence of ATM are detected by components of the DNA damage-response machinery.

V(D)J-Associated DSBs Participate in Translocations with DSBs Subsequently Generated in Mature Lymphocytes

We hypothesized that a persistent DSB induced by V(D)J recombination in lymphoid precursors might occasionally participate in a translocation reaction with a partner chromosome that is broken in daughter cells. To determine whether telomere-deleted ends might participate in such translocations, we isolated V(D)J DSB containing $Atm^{-/-}Aid^{-/-}$ B cells, stimulated them to divide in vitro with LPS+IL4 for 48 hr, and then treated the cells with 4 Gy of IR to induce additional DSBs. Metaphase spreads were prepared 6 hr after irradiation and analyzed for Ig heavy chain-associated translocations using the IgH/telomere-specific FISH assay (Figure 6). The number of Ig-specific translocations was consistently higher in the irradiated population compared to the unirradiated samples in three independent experiments (Figure 6; unirradiated

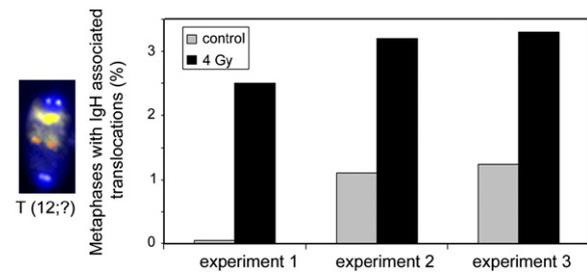


Figure 6. Fusion of Persistent V(D)J Breaks and Irradiation-Induced Breaks

Splenic CD43⁺ B cells from three independent $Atm^{-/-}Aid^{-/-}$ mice were stimulated with LPS/IL4 for 48 hr and then were either untreated (control) or treated with 4 Gy of γ irradiation. Metaphase spreads from the control and irradiated group were assayed for IgH-associated translocations (control versus irradiated, $p = 0.02$). An example of a T(12:?) translocation with IgH $C\alpha$ located near the breakpoint is shown. Chr12 (yellow), IgH $C\alpha$ (red), and telomere (white) probes were utilized.

versus irradiated, $p = 0.02$, two sided Cochran-Mantel-Haenszel test). These results suggest that DSBs generated in daughter cells with γ irradiation can fuse with DNA ends that were produced several days earlier by RAG-mediated cleavage during failed V(D)J recombination in developing B cells.

DISCUSSION

ATM and ATR are members of the phosphatidylinositol 3-kinase (PIKK)-like kinase family, which activate the cell-cycle checkpoint, DNA repair, and apoptotic machineries. ATM is loaded onto DSBs through an interaction with the Mre11/Rad50/Nbs1 complex, whereas ATR recognizes single-stranded DNA (ssDNA) complexed with RPA. Our study provides evidence that ATM is required to select against the propagation of cells with severed chromosomes generated either by RAG or low-dose γ irradiation. This indicates that persistent V(D)J recombination or IR-induced breaks in $Atm^{-/-}$ mice are not recognized by ATR to initiate arrest/apoptosis during replication. One potential explanation for this is that ATR activation is regulated in part by ATM. Indeed, recent studies have demonstrated that the Mre11 nuclease and ATM are required to process IR-induced DSBs into ssDNA and facilitate ATR recruitment (Cuadrado et al., 2006; Jazayeri et al., 2006; Myers and Cortez, 2006). This mechanism would explain how loss of ATM could impair resection of V(D)J recombination-associated breaks to ssDNA and thereby inhibit ATR activation in cells that are in S and G2 phases.

We find that terminally deleted chromosomes in mature $Atm^{-/-}$ lymphocytes arise from antigen-receptor gene-associated DSBs generated in precursor cells. This observation suggests that $Atm^{-/-}$ lymphocytes, which fail primary V(D)J assembly, leaving a DSB on one allele, can achieve a productive rearrangement through the ongoing independent recombination of the second allele. According to this model (Figure 7A), the presence of an Ig-associated DSB in

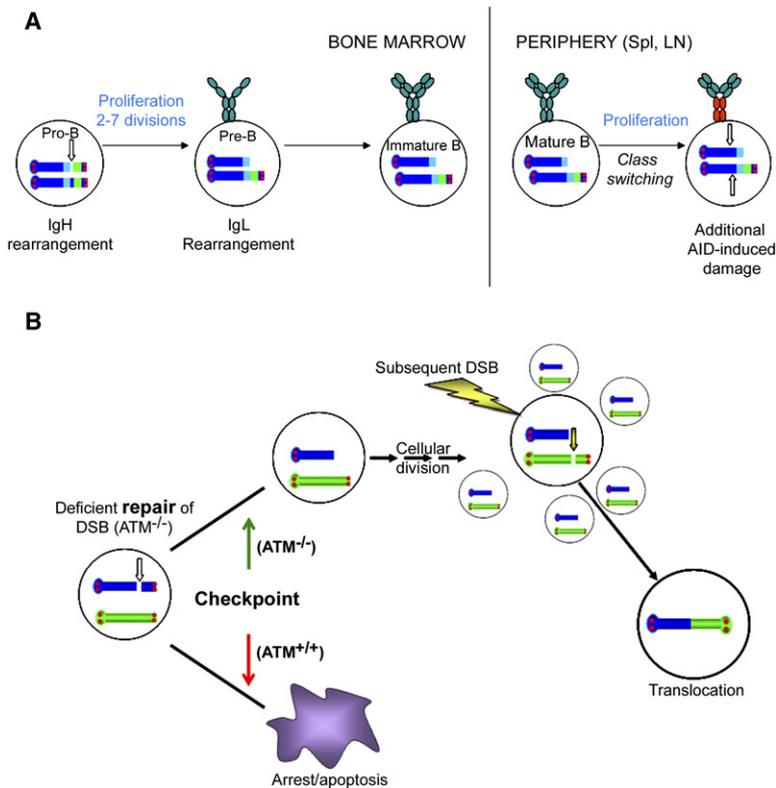


Figure 7. Model for the Etiology of IgH-Associated Breaks and Delayed Genomic Instability in *Atm*^{-/-} B Cells

(A) In the absence of ATM, RAG-dependent IgH-associated breaks may persist throughout B cell development in the bone marrow and during class-switch recombination in the periphery.

(B) Combined deficiency in repair and checkpoints leads to transmission of a DSB (blue chromosome). This DSB can serve as a substrate for translocations with chromosomes damaged in subsequent generations (green chromosome).

Atm^{-/-} pre-B cells would not prevent any of the essential steps in their developmental program, i.e., (1) signaling through the pre-B cell receptor by the productively assembled μ heavy-chain protein, (2) proliferative expansion of pre-B cells, (3) initiation of light-chain rearrangement, (5) production of a B cell receptor, (6) selection of nonself reactive B cells and editing or deletion of self-reactive cells, and (6) immature B cell migration to the periphery. Therefore, DSBs produced in precursor cells would persist in mature B cells in peripheral lymphoid tissues. Upon stimulation with LPS+IL4, B cells undergo class-switch recombination, which leads to further AID-dependent DNA damage. This accounts for the increase in Ig-associated breaks in stimulated *Atm*^{-/-} compared to *Atm*^{-/-}*Aid*^{-/-} B cells (Figure 1B) and the equivalent levels of damage in resting *Atm*^{-/-}*Aid*^{-/-} and *Atm*^{-/-} B cells (Figure 2B). Similarly, loss of ATM permits the survival and proliferation of cells harboring failed V(D)J recombination intermediates produced during T cell development (Figure S1) as well as the propagation of lymphocytes harboring nonantigen receptor-specific DSBs (Figure S3). As discussed below, the finding that precursor cells can divide in the presence of a DSB suggests that a form of delayed genomic instability may contribute to mature B and T cell lymphomagenesis (Figure 7B).

Delayed Genomic Instability

Two theories have been proposed to explain how translocations form between two broken chromosomes. The contact-first model suggests that DSBs fuse together

only if they happen to be in close spatial proximity at the time of DSB induction. The breakage-first model suggests that DNA breaks are mobile and coalesce by an adhesion process mediated by the DSB sensor complex Mre11/Rad50/Nbs1 (Aten et al., 2004). The two models assume that both breaks on the partner chromosomes arise in similar time frames and in the same cell. However, a persistent DSB might move away from its original position, allowing it to sample distinct chromosome territories, and, if transmitted, it could participate in a translocation reaction with a chromosome that was broken in a progeny cell.

We speculate that this delayed form of genomic instability might contribute to the transformation of mature lymphocytes (Figure 7B). Mantle cell lymphoma is a mature B cell lymphoma, whose hallmark feature is a translocation between the IgH-associated recombination signal sequences found adjacent to V, D, or J gene segments and the proto-oncogene cyclin D1. Up to 50% of cases of mantle cell lymphoma have mutations/deletions in ATM, which is likely to be an early event in the transformation (Rosenwald et al., 2003). It is presumed that the cancer promoting IgH/Cyclin D1 translocation occurs in an immature lymphocyte undergoing V(D)J recombination, followed by additional transforming hits in the mature cell. Alternatively, we suggest that early loss of ATM could result in the persistence of an unresolved V(D)J-associated break that recombines with a broken cyclin D1 locus in the mature B cell. Our finding that a radiation-induced break in a mature *Atm*^{-/-}*Aid*^{-/-} B cell can fuse to a V(D)J break generated earlier is consistent with this hypothesis.

In summary, we have documented two complementary genome maintenance functions for ATM (Figure 7): in addition to its role in the repair of chromosomal breaks, an ATM checkpoint prevents the long-term persistence and transmission of DSBs to daughter cells.

EXPERIMENTAL PROCEDURES

Generation of Mice

Atm^{-/-}, *Aid*^{-/-}, *53BP1*^{-/-}, and *DNA-PKcs*^{-/-} mice were provided by A. Wynshaw-Boris, T. Honjo, J. Chen, and F. Alt, respectively. *Rag2*^{-/-} mice were obtained from Taconic laboratories, transgenic mice expressing a T cell receptor specific for a fragment of pigeon cytochrome c (TCRAND), and MD4 mice expressing a B cell receptor transgene recognizing hen egg lysozyme were obtained from Jackson Laboratory. RAG2p-GFP transgenic mice have been described (Yu et al., 1999).

Lymphocyte Cultures and FISH Detection of Antigen Receptor-Associated Lesions

B cells were isolated from WT and mutant spleens by immunomagnetic depletion with anti-CD43 beads (Miltenyi Biotec) and stimulated with LPS (25 µg/ml; Sigma) and IL4 (5 ng/ml; GIBCO) or RP105 (2.5 µg/ml; PharMingen). Lymph node T cells were stimulated with anti-TCR (H57, 2 µg/ml; PharMingen) and anti-CD28 antibodies (5 µg/ml; PharMingen). B220⁺ bone marrow cells were purified with anti-B220 beads (Miltenyi Biotec) and cultured on irradiated S17 stromal cells for 5 days. PCR analysis of *c-myc*/IgH translocations in splenic B cells was performed as described (Ramiro et al., 2006a). Cultured lymphocytes were arrested at mitosis with Colcemid (GIBCO/BRL) treatment. For FISH analysis, metaphases were hybridized with chromosome-painting probes generated by DOP-PCR using flow-sorted single chromosomes; BAC probes containing the TCR α locus (TCR C α -232F18, TCR V α -RP24-74E19); the IgH locus (IgV-224M14), a generous gift from Roy Riblet; and IgH C α [from C γ 1-3' of C α (Difilippantonio et al., 2002)] and telomere-repeat specific peptide nucleic acid (PNA) probes (Applied Biosystems) as described (Difilippantonio et al., 2002; Ramiro et al., 2006b). Three-dimensional FISH was performed essentially as described (Sayegh et al., 2005). Briefly, B cells and thymocytes were transferred to a glass slide coated with Cell Tak (Becton Dickinson), fixed for 10 min with 4% paraformaldehyde, permeabilized in 0.1% saponin/0.1% Triton X-100 (PB) for 10 min, and immersed in liquid nitrogen for three freeze/thaw cycles. Coverslips were incubated in 0.1 M HCL, blocked in 3% BSA/100 µg/ml Rnase A, and re-permeabilized in PB. Genomic DNA was denatured and hybridized with antigen locus-specific probes as described above. Images of approximately 20 serial optical sections spaced by 0.4 microns were captured with a LSM-510 confocal microscope.

Flow Cytometry and Cell Sorting

To monitor class-switch recombination, B cells were stained with anti-mouse IgG1 and -B220 antibodies (PharMingen) and analyzed on a Becton Dickinson FACSAria cell sorter. GFP⁺ and GFP⁻ cells from Rag2p-GFP transgenic mice and HSA/B220-stained B cells from *Atm*^{-/-} *Aid*^{-/-} mice were sorted sterily before in vitro culture. To examine the propagation of chromosome aberrations in vitro, lymphocytes were labeled with 5 µM CFSE on day 0, stimulated for 72 hr, treated with colcemid for 1 hr, and then sorted based on the fluorescence intensity of CFSE. Mitotic chromosome spreads were prepared immediately after sorting. The purity of the separated fractions was always greater than 98%.

Real-Time PCR Analysis of RAG-1/2 Expression

Total RNA was extracted with TRIzol (Invitrogen) and was reverse transcribed with random hexamers and Superscript II reverse transcriptase (Invitrogen). The first strand was used for SYBR green fluorogenic

dye real-time PCR (Applied Biosystems). The specificity of the RAG1, RAG2, and Gapdh primers was checked with negative controls and by the analysis of dissociation curves (Yu et al., 1999).

Western Blotting and Immunofluorescence FISH

Wild-type, *Atm*^{-/-} or *Aid*^{-/-} B cells were purified from mouse spleens by depletion with anti-CD43 beads and cultured in RPMI with IL-4 plus LPS or anti-RP105. Cells were extracted in RIPA buffer (20 mM Tris pH 8, 200 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 1 mM DTT). For immunoprecipitation, extracts from 20 million cells were incubated with anti-AID antibody (McBride et al., 2006) and protein A sepharose (Amersham), and western blots were performed on the immunoprecipitates or 50 µg extracts with anti-AID antibody and anti- β actin (Sigma-Aldrich). Western blotting for RAG expression was performed as described (Yu et al., 1999). For 53BP1 foci analysis, B cells were spun onto coverslips after 48 hr stimulation and immunofluorescence and FISH detection was performed as described (Peterson et al., 2001). Rabbit polyclonal anti-53BP1 antibodies (Novus, NB100-304) were used at a 1:1,000 dilution.

Supplemental Data

Supplemental Data include five figures and one table and can be found with this article online at <http://www.cell.com/cgi/content/full/130/1/63/DC1/>.

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REFERENCES

- Arlt, M.F., Durkin, S.G., Ragland, R.L., and Glover, T.W. (2006). Common fragile sites as targets for chromosome rearrangements. *DNA Repair (Amst.)* 5, 1126–1135.
- Aten, J.A., Stap, J., Krawczyk, P.M., van Oven, C.H., Hoebe, R.A., Essers, J., and Kanaar, R. (2004). Dynamics of DNA double-strand breaks revealed by clustering of damaged chromosome domains. *Science* 303, 92–95.
- Bassing, C.H., Suh, H., Ferguson, D.O., Chua, K.F., Manis, J., Eckersdorff, M., Gleason, M., Bronson, R., Lee, C., and Alt, F.W. (2003). Histone H2AX: a dosage-dependent suppressor of oncogenic translocations and tumors. *Cell* 114, 359–370.
- Boursalian, T.E., Golob, J., Soper, D.M., Cooper, C.J., and Fink, P.J. (2004). Continued maturation of thymic emigrants in the periphery. *Nat. Immunol.* 5, 418–425.
- Bredemeyer, A.L., Sharma, G.G., Huang, C.Y., Helmink, B.A., Walker, L.M., Khor, K.C., Nuskey, B., Sullivan, K.E., Pandita, T.K., Bassing, C.H., et al. (2006). ATM stabilizes DNA double-strand-break complexes during V(D)J recombination. *Nature* 442, 466–470.

- Casper, A.M., Nghiem, P., Art, M.F., and Glover, T.W. (2002). ATR regulates fragile site stability. *Cell* **111**, 779–789.
- Celeste, A., Difilippantonio, S., Difilippantonio, M.J., Fernandez-Capetillo, O., Pilch, D.R., Sedelnikova, O.A., Eckhaus, M., Ried, T., Bonner, W.M., and Nussenzweig, A. (2003). H2AX haploinsufficiency modifies genomic stability and tumor susceptibility. *Cell* **114**, 371–383.
- Celeste, A., Petersen, S., Romanienko, P.J., Fernandez-Capetillo, O., Chen, H.T., Sedelnikova, O.A., Reina-San-Martin, B., Coppola, V., Meffre, E., Difilippantonio, M.J., et al. (2002). Genomic instability in mice lacking histone H2AX. *Science* **296**, 922–927.
- Chen, H.T., Bhandoola, A., Difilippantonio, M.J., Zhu, J., Brown, M.J., Tai, X., Rogakou, E.P., Brotz, T.M., Bonner, W.M., Ried, T., et al. (2000). Response to RAG-mediated VDJ cleavage by NBS1 and gamma-H2AX. *Science* **290**, 1962–1965.
- Cuadrado, M., Martinez-Pastor, B., Murga, M., Toledo, L.I., Gutierrez-Martinez, P., Lopez, E., and Fernandez-Capetillo, O. (2006). ATM regulates ATR chromatin loading in response to DNA double-strand breaks. *J. Exp. Med.* **203**, 297–303.
- Difilippantonio, M.J., Petersen, S., Chen, H.T., Johnson, R., Jasin, M., Kanaar, R., Ried, T., and Nussenzweig, A. (2002). Evidence for replicative repair of DNA double-strand breaks leading to oncogenic translocation and gene amplification. *J. Exp. Med.* **196**, 469–480.
- Franco, S., Gostissa, M., Zha, S., Lombard, D.B., Murphy, M.M., Zarrin, A.A., Yan, C., Tepsuporn, S., Morales, J.C., Adams, M.M., et al. (2006). H2AX prevents DNA breaks from progressing to chromosome breaks and translocations. *Mol. Cell* **21**, 201–214.
- Gladdy, R.A., Taylor, M.D., Williams, C.J., Grandal, I., Karaskova, J., Squire, J.A., Rutka, J.T., Guidos, C.J., and Danska, J.S. (2003). The RAG-1/2 endonuclease causes genomic instability and controls CNS complications of lymphoblastic leukemia in p53/Prkdc-deficient mice. *Cancer Cell* **3**, 37–50.
- Goodnow, C.C., Crosbie, J., Adelstein, S., Lavoie, T.B., Smith-Gill, S.J., Brink, R.A., Pritchard-Briscoe, H., Wotherspoon, J.S., Loblay, R.H., Raphael, K., et al. (1988). Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* **334**, 676–682.
- Huang, C.-Y., Sharma, G.G., Walker, L., Bassing, C.H., Pandita, T.K., and Sleckman, B.P. (2007). Defects in coding joint formation *in vivo* in developing ATM-deficient B and T lymphocytes. *J. Exp. Med.* **334**, 676–682.
- Jazayeri, A., Falck, J., Lukas, C., Bartek, J., Smith, G.C., Lukas, J., and Jackson, S.P. (2006). ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat. Cell Biol.* **8**, 37–45.
- Jung, D., Giallourakis, C., Mostoslavsky, R., and Alt, F.W. (2006). Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. *Annu. Rev. Immunol.* **24**, 541–570.
- Kaye, J., Hsu, M.L., Sauron, M.E., Jameson, S.C., Gascoigne, N.R., and Hedrick, S.M. (1989). Selective development of CD4+ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. *Nature* **341**, 746–749.
- Kracker, S., Bergmann, Y., Demuth, I., Frappart, P.O., Hildebrand, G., Christine, R., Wang, Z.Q., Sperling, K., Digweed, M., and Radbruch, A. (2005). Nibrin functions in Ig class-switch recombination. *Proc. Natl. Acad. Sci. USA* **102**, 1584–1589.
- Liyanage, M., Weaver, Z., Barlow, C., Coleman, A., Pankratz, D.G., Anderson, S., Wynshaw-Boris, A., and Ried, T. (2000). Abnormal rearrangement within the alpha/delta T-cell receptor locus in lymphomas from Atm-deficient mice. *Blood* **96**, 1940–1946.
- Lumsden, J.M., McCarty, T., Petiniot, L.K., Shen, R., Barlow, C., Wynn, T.A., Morse, H.C., 3rd, Gearhart, P.J., Wynshaw-Boris, A., Max, E.E., et al. (2004). Immunoglobulin class switch recombination is impaired in Atm-deficient mice. *J. Exp. Med.* **200**, 1111–1121.
- Manis, J.P., Morales, J.C., Xia, Z., Kutok, J.L., Alt, F.W., and Carpenter, P.B. (2004). 53BP1 links DNA damage-response pathways to immunoglobulin heavy chain class-switch recombination. *Nat. Immunol.* **5**, 481–487.
- Matei, I.R., Gladdy, R.A., Nutter, L.M., Canty, A., Guidos, C.J., and Danska, J.S. (2007). ATM deficiency disrupts Tcr α locus integrity and the maturation of CD4+CD8+ thymocytes. *Blood* **109**, 1887–1896.
- McBride, K.M., Gazumyan, A., Woo, E.M., Barreto, V.M., Robbiani, D.F., Chait, B.T., and Nussenzweig, M.C. (2006). Regulation of hypermutation by activation-induced cytidine deaminase phosphorylation. *Proc. Natl. Acad. Sci. USA* **103**, 8798–8803.
- Meffre, E., Casellas, R., and Nussenzweig, M.C. (2000). Antibody regulation of B cell development. *Nat. Immunol.* **1**, 379–385.
- Miyake, K., Yamashita, Y., Ogata, M., Sudo, T., and Kimoto, M. (1995). RP105, a novel B cell surface molecule implicated in B cell activation, is a member of the leucine-rich repeat protein family. *J. Immunol.* **154**, 3333–3340.
- Mochan, T.A., Venere, M., DiTullio, R.A., Jr., and Halazonetis, T.D. (2004). 53BP1, an activator of ATM in response to DNA damage. *DNA Repair (Amst.)* **3**, 945–952.
- Monroe, R.J., Seidl, K.J., Gaertner, F., Han, S., Chen, F., Sekiguchi, J., Wang, J., Ferrini, R., Davidson, L., Kelsoe, G., et al. (1999). RAG2:GFP knockin mice reveal novel aspects of RAG2 expression in primary and peripheral lymphoid tissues. *Immunity* **11**, 201–212.
- Morales, J.C., Franco, S., Murphy, M.M., Bassing, C.H., Mills, K.D., Adams, M.M., Walsh, N.C., Manis, J.P., Rassidakis, G.Z., Alt, F.W., et al. (2006). 53BP1 and p53 synergize to suppress genomic instability and lymphomagenesis. *Proc. Natl. Acad. Sci. USA* **103**, 3310–3315.
- Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y., and Honjo, T. (2000). Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* **102**, 553–563.
- Muramatsu, M., Sankaranand, V.S., Anant, S., Sugai, M., Kinoshita, K., Davidson, N.O., and Honjo, T. (1999). Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J. Biol. Chem.* **274**, 18470–18476.
- Myers, J.S., and Cortez, D. (2006). Rapid activation of ATR by ionizing radiation requires ATM and Mre11. *J. Biol. Chem.* **281**, 9346–9350.
- Perkins, E.J., Nair, A., Cowley, D.O., Van Dyke, T., Chang, Y., and Ramsden, D.A. (2002). Sensing of intermediates in V(D)J recombination by ATM. *Genes Dev.* **16**, 159–164.
- Petersen, S., Casellas, R., Reina-San-Martin, B., Chen, H.T., Difilippantonio, M.J., Wilson, P.C., Hanitsch, L., Celeste, A., Muramatsu, M., Pilch, D.R., et al. (2001). AID is required to initiate Nbs1/gamma-H2AX focus formation and mutations at sites of class switching. *Nature* **414**, 660–665.
- Petiniot, L.K., Weaver, Z., Vacchio, M., Shen, R., Wangsa, D., Barlow, C., Eckhaus, M., Steinberg, S.M., Wynshaw-Boris, A., Ried, T., et al. (2002). RAG-mediated V(D)J recombination is not essential for tumorigenesis in Atm-deficient mice. *Mol. Cell. Biol.* **22**, 3174–3177.
- Ramiro, A.R., Jankovic, M., Callen, E., Difilippantonio, S., Chen, H.T., McBride, K.M., Eisenreich, T.R., Chen, J., Dickins, R.A., Lowe, S.W., et al. (2006a). Role of genomic instability and p53 in AID-induced c-myc-Igh translocations. *Nature* **440**, 105–109.
- Ramiro, A.R., Nussenzweig, M.C., and Nussenzweig, A. (2006b). Switching on chromosomal translocations. *Cancer Res.* **66**, 7837–7839.
- Reina-San-Martin, B., Chen, H.T., Nussenzweig, A., and Nussenzweig, M.C. (2004). ATM is required for efficient recombination between immunoglobulin switch regions. *J. Exp. Med.* **200**, 1103–1110.
- Reina-San-Martin, B., Nussenzweig, M.C., Nussenzweig, A., and Difilippantonio, S. (2005). Genomic instability, endoreduplication, and

- diminished Ig class-switch recombination in B cells lacking Nbs1. *Proc. Natl. Acad. Sci. USA* *102*, 1590–1595.
- Reina-San-Martin, B., Chen, J., Nussenzweig, A., and Nussenzweig, M.C. (2007). Enhanced intra-switch region recombination during immunoglobulin class switch recombination in 53BP1(–/–) B cells. *Eur. J. Immunol.* *37*, 235–239.
- Revy, P., Muto, T., Levy, Y., Geissmann, F., Plebani, A., Sanal, O., Catalan, N., Forveille, M., Dufourcq-Labelouse, R., Gennery, A., et al. (2000). Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* *102*, 565–575.
- Rolink, A.G., Winkler, T., Melchers, F., and Andersson, J. (2000). Precursor B cell receptor-dependent B cell proliferation and differentiation does not require the bone marrow or fetal liver environment. *J. Exp. Med.* *191*, 23–32.
- Rosenwald, A., Wright, G., Wiestner, A., Chan, W.C., Connors, J.M., Campo, E., Gascoyne, R.D., Grogan, T.M., Muller-Hermelink, H.K., Smeland, E.B., et al. (2003). The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. *Cancer Cell* *3*, 185–197.
- Sayegh, C., Jhunjhunwala, S., Riblet, R., and Murre, C. (2005). Visualization of looping involving the immunoglobulin heavy-chain locus in developing B cells. *Genes Dev.* *19*, 322–327.
- Schlissel, M., Constantinescu, A., Morrow, T., Baxter, M., and Peng, A. (1993). Double-strand signal sequence breaks in V(D)J recombination are blunt, 5'-phosphorylated, RAG-dependent, and cell cycle regulated. *Genes Dev.* *7*, 2520–2532.
- Sprent, J., and Tough, D.F. (1994). Lymphocyte life-span and memory. *Science* *265*, 1395–1400.
- Takao, N., Kato, H., Mori, R., Morrison, C., Sonada, E., Sun, X., Shimizu, H., Yoshioka, K., Takeda, S., and Yamamoto, K. (1999). Disruption of ATM in p53-null cells causes multiple functional abnormalities in cellular response to ionizing radiation. *Oncogene* *18*, 7002–7009.
- Vacchio, M.S., Oлару, A., Livak, F., and Hodes, R.J. (2007). ATM deficiency impairs thymocyte maturation because of defective resolution of T cell receptor {alpha} locus coding end breaks. *Proc. Natl. Acad. Sci. USA* *104*, 6323–6328.
- Ward, I.M., Difilippantonio, S., Minn, K., Mueller, M.D., Molina, J.R., Yu, X., Frisk, C.S., Ried, T., Nussenzweig, A., and Chen, J. (2005). 53BP1 cooperates with p53 and functions as a haploinsufficient tumor suppressor in mice. *Mol. Cell. Biol.* *25*, 10079–10086.
- Ward, I.M., Reina-San-Martin, B., Oлару, A., Minn, K., Tamada, K., Lau, J.S., Cascalho, M., Chen, L., Nussenzweig, A., Livak, F., et al. (2004). 53BP1 is required for class switch recombination. *J. Cell Biol.* *165*, 459–464.
- Yu, W., Nagaoka, H., Jankovic, M., Misulovin, Z., Suh, H., Rolink, A., Melchers, F., Meffre, E., and Nussenzweig, M.C. (1999). Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. *Nature* *400*, 682–687.
- Zhu, C., Mills, K.D., Ferguson, D.O., Lee, C., Manis, J., Fleming, J., Gao, Y., Morton, C.C., and Alt, F.W. (2002). Unrepaired DNA breaks in p53-deficient cells lead to oncogenic gene amplification subsequent to translocations. *Cell* *109*, 811–821.