Redundant and Nonredundant Functions of ATM and H2AX in \(\alpha\beta\) T-Lineage Lymphocytes

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Redundant and Nonredundant Functions of ATM and H2AX in αβ T-Lineage Lymphocytes

Bu Yin,*†‡ Baeck-Seung Lee,§ Katherine S. Yang-Iott,†‡ Barry P. Sleckman,§ and Craig H. Bassing*†‡

The ataxia telangiectasia mutated (ATM) kinase and H2AX histone tumor suppressor proteins are each critical for maintenance of cellular genomic stability and suppression of lymphomas harboring clonal translocations. ATM is the predominant kinase that phosphorylates H2AX in chromatin around DNA double-strand breaks, including along lymphocyte Ag receptor loci cleaved during V(D)J recombination. However, combined germline inactivation of Atm and H2ax in mice causes early embryonic lethality associated with substantial cellular genomic instability, indicating that ATM and H2AX exhibit nonredundant functions in embryonic cells. To evaluate potential nonredundant roles of ATM and H2AX in somatic cells, we generated and analyzed Atm-deficient mice with conditional deletion of H2ax in αβ T-lineage lymphocytes. Combined Atm/H2ax inactivation starting in early-stage CD4+/CD8+ thymocytes resulted in lower numbers of later-stage CD4+/CD8+ thymocytes, but led to no discernible V(D)J recombination defect in G1 phase cells beyond that observed in Atm-deficient cells. H2ax deletion in Atm-deficient thymocytes also did not affect the incidence or mortality of mice from thymic lymphomas with clonal chromosome 14 (TCRα/δ) translocations. Yet, in vitro-stimulated Atm/H2ax-deficient splenic αβ T cells exhibited a higher frequency of genomic instability, including radial chromosome translocations and TCRβ translocations, compared with cells lacking Atm or H2ax. Collectively, our data demonstrate that both redundant and nonredundant functions of ATM and H2AX are required for normal recombination of TCR loci, proliferative expansion of developing thymocytes, and maintenance of genomic stability in cycling αβ T-lineage cells.


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In mice and humans, αβ γδ T cells develop in the thymus from common progenitor cells through programs that link the assembly of TCR genes with differentiation (20). TCRβ, TCRγ, and TCRδ genes are assembled from variable (V), diversity (D), and joining (J) gene segments in CD4+/CD8− “double-negative” (DN) thymocytes (20). V(DJ) recombination is initiated by the RAG1/RAG2 (RAG) endonuclease, which induces DSBs adjacent to participating segments, and is completed by NHEJ proteins, which repair RAG DSBs to form V(DJ) coding exons upstream of constant (C) exons (21). Assembly and expression of functional TCRγ and TCRδ genes generates γδ TCRs that promote differentiation into mature γδ T cells (22). In contrast, assembly and expression of functional TCRβ genes leads to TCRβ-pre-Tα (pre-TCR) complexes and β-selection signals that drive proliferative expansion as cells differentiate into CD4+/CD8− “double-positive” (DP) thymocytes (20). This TCRβ-dependent proliferation is associated with spontaneous replication-associated DSBs that must be repaired by HR for normal DN-to-DP thymocyte expansion (23). Assembly and expression of functional TCRα genes in DP cells leads to formation of αβ TCRs, which upon their selection signal differentiation into CD4+ or CD8+ “single-positive” (SP) thymocytes that exit the thymus as CD4+ or CD8+ mature αβ T cells (20).

In developing αβ T cells, ATM and H2AX each suppress oncogenic translocations arising from aberrant repair of DSBs during NHEJ and HR. Germline Atm-deficient (Atm−/−) and H2AX-deficient (H2ax−/−) mice each exhibit fewer numbers of thymocytes and αβ T cells due to impaired proliferation of cells lacking Atm or H2ax (6, 9, 10, 12–14, 24). Atm−/− mice, but not H2ax−/− mice, exhibit a severe defect in DP-to-SP development due to H2AX-independent functions of ATM during coding join formation in G1 phase cells (6, 9, 10, 13, 14, 24–26). Almost all Atm−/− mice succumb to thymic lymphomas with clonal chromosome 14 translocations created through aberrant repair of DSBs induced by RAG during TCRβ recombination and by DNA replication errors during thymocyte expansion (7, 8). In contrast, despite H2AX phosphorylation by ATM and DNA-PKcs along RAG-cleaved Ag receptor loci (27), H2ax−/− mice rarely succumb to these or other tumors due to ATM in p53-dependent activation of checkpoints and apoptosis (12–14).

To investigate potential redundant and nonredundant DDR functions of ATM and H2AX in somatic cells, we generated and analyzed Atm−/− mice with conditional deletion of H2ax in developing αβ T lymphocytes. We show that combined Atm/H2ax deletion starting in DN thymocytes results in lower numbers of DP thymocytes without causing a V(DJ) recombination defect beyond that observed in Atm-deficient cells. We also show that H2ax deletion in Atm-deficient thymocytes does not affect the incidence or mortality of mice from thymic lymphomas with clonal chromosome 14 translocations. Yet, we find that proliferating Atm−/− H2ax−/−-deficient αβ T cells exhibit a higher frequency of genomic instability, including radial chromosome translocations and TCRβ translocations, relative to cells lacking Atm or H2ax. Collectively, our data demonstrate that both redundant and nonredundant functions of ATM and H2AX are required for normal TCR recombination, proliferative expansion of developing thymocytes, and maintenance of genomic stability in cycling αβ T-lineage cells.

Materials and Methods

Mice

Lck-cre transgenic (28), Atm−/− (24), and H2ax−/− (12) mice were bred to generate the animals in this study. The background strain of these mice was mixed 129SvEv and C57BL/6, with the 129SvEv background predominant. PCR analyses of H2ax deletion were performed as described (12), demonstrating complete deletion of H2ax in Lck-creAtm−/− and Lck-cre-Atm−/−H2axF/F αβ T-lineage cells. Experiments were conducted on 4- to 6-wk-old mice of each genotype, performed in accordance with national guidelines, and approved by the Institutional Animal Care and Use Committee of the Children’s Hospital of Philadelphia.

Flow cytometry

Single-cell suspensions from thymuses and spleens of 4- to 6-wk-old mice or tumors were stained with Abs in PBS with 2% FBS. For analysis of CD4 and CD8 expression, single-cell suspensions of thymocytes and splenocytes were stained with FITC-conjugated anti-TCRβ, PE-conjugated anti-CD8, and allophycocyanin-conjugated anti-CD4 (BD Pharmingen). Data were collected using a FACSCalibur (BD Biosciences, San Jose, CA) and CellQuest software (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Chromosomal V(DJ) recombination assay

Bone marrow from 3- to 5-wk-old Atm−/− H2ax−/− mice harboring the Eμ-Bcl-2 transgene were cultured and infected with the pMSCV v-abl retrovirus to generate Abelson transformed (abl) pre-B cells as previously described (25). Generation of the H2ax−/− abl pre-B cells was previously described (29). H2ax−/− and Atm−/− H2ax−/− abl pre-B cells containing pMX-DEL3 retroviral recombination substrates were made as described (25). Atm−/− H2ax−/− abl pre-B cells containing pMX-DEL3 were created through Tat-Cre induction of Atm−/− H2ax−/−:pMX-DEL3 abl pre-B cells as previously described (29). Southern blot analysis was performed upon abl pre-B cells after treatment with 3 μM STI571 for the indicated times at a density of 104 cells/ml, as previously described (25).

Southern blotting

Genomic DNA (20–30 μg) of thymocytes, tumors, or kidneys was digested with 100 U of the indicated enzymes (New England Biolabs), separated on a 1.0% TAE agarose gel, transferred onto λ probe membrane (Bio-Rad), and hybridized with 32P-labeled DNA probes as described (12).

Cytogenetics

Metaphase spreads were prepared as described. Spectral karyotyping and chromosome painting were performed according to the manufacturer’s instructions (Applied Spectral Imaging). BAC fluorescence in situ hybridization (FISH) probes were labeled with biotin (Biotin-Nick Translation Mix; Roche). The TCR-β164GJ1 BAC used for FISH has been described (30). Images were captured and analyzed using Case Data Manager version 5.5 configured by Applied Spectral Imaging.

Statistical analysis

The Kaplan–Meier curves were generated in SAS version 9 (SAS Institute). Statistical analyses were performed with two-tailed unpaired Student t tests in Excel (Microsoft) or Prism (AMPL Software). We considered p < 0.05 to be statistically significant.

Results

Both ATM and H2AX are required for normal TCRβ-mediated thymocyte expansion

Because the Lck-cre transgene promotes deletion of “floxed” H2ax (H2axf) genes in DN cells prior to V(DJ) recombination (29), we sought to generate and analyze Lck-cre+/− Atm−/−, Lck-cre+/− H2axff, and Lck-cre+/− Atm−/− H2axff mice. Atm and H2ax are closely linked on chromosome 9, therefore we first bred together Atm−/− and H2axff males to generate Atm−/− H2axff males containing the Atm− and H2axf alleles on different chromosomes. These Atm−/− H2axff males were crossed with wild-type females to select for meiotic crossover events that created Atm−/− H2axff mice with the Atm− and H2axf alleles linked on the same chromosome. We frequently observe deletion of H2axf genes in nonlymphoid cells when Lck-cre is transmitted maternally, but not when transmitted paternally (data not shown). Thus, to avoid nonspecific H2ax deletion, as well as potential complications due to homozygous Lck-cre transgene integration, we bred heterozygous Lck-cre males with Atm−/− H2axff females to generate Lck-cre+/− Atm−/− H2axff mice. Because Atm−/− mice are infertile (6,
9, 10, 24), we bred Lck-cre<sup>−/−</sup>Atm<sup>−/−</sup>H2ax<sup>F/F</sup> males with Atm<sup>−/−</sup>-H2ax<sup>F/F</sup> females to generate experimental Lck-cre<sup>−/−</sup>Atm<sup>−/−</sup>H2ax<sup>F/F</sup> mice, hereafter referred to as LAH mice. We used a similar breeding strategy to generate control Lck-cre<sup>−/−</sup>Atm<sup>−/−</sup> and Lck-cre<sup>−/−</sup>H2ax<sup>F/F</sup> mice, hereafter referred to as LA and LH mice, respectively.

To assess potential redundant and nonredundant functions of ATM and H2AX in αβ T-lineage cells, we first analyzed the thymuses and spleens of LA, LH, and LAH mice by cell counting and flow cytometry (FACS) analysis with anti-CD4 and anti-CD8 Abs. We found that LH and wild-type mice exhibited comparable numbers of total thymocytes, cells within each thymocyte developmental stage, and splenic αβ T cells (data not shown). We detected ∼2-fold lower numbers of total thymocytes and splenic αβ T cells in LA mice compared with LH mice (Fig. 1). The fewer numbers of LA thymocytes reflected an ∼2-fold reduction in DP cells and ∼5-fold reductions in CD4<sup>+</sup> SP and CD8<sup>+</sup> SP cells (Fig. 1A, 1B). These data are consistent with the phenotypes of Atm<sup>−/−</sup> and H2ax<sup>−/−</sup> mice (6, 9, 10, 12–14, 24), indicating that Lck-cre expression has negligible effects upon the development of αβ T cells lacking Atm or H2ax. We found that the average numbers of thymocytes and splenic αβ T cells in LAH mice were each reduced ∼2-fold compared with LA mice and ∼4-fold compared with LH mice (Fig. 1), although the difference in numbers of LA and LAH splenic αβ T cells was not significant from the numbers of mice analyzed. Notably, as compared with LA mice, LAH mice contained an ∼2-fold reduction in DP cell numbers, yet showed no significant differences in DN, CD4<sup>+</sup> SP, or CD8<sup>+</sup> SP cell numbers (Fig. 1A, 1B). These observations indicate that TCRβ-dependent DN-to-DP thymocyte expansion is more impaired in Atm/H2ax-deficient cells than in Atm<sup>−/−</sup> or H2ax<sup>−/−</sup> cells. This difference may reflect nonredundant ATM and H2AX functions in coding join formation during TCRβ recombination and/or repair of replication-associated DSBs during TCRβ-driven cellular proliferation.

The similar defect in the DP-to-SP thymocyte differentiation step in LAH and LA mice suggests that combined Atm/H2ax deletion does not impair coding join formation during TCRα recombination beyond that observed in Atm<sup>−/−</sup> thymocytes. Analysis of chromosomal V(D)J recombination in abl pre-B cells enables distinction between NHEJ defects in G1 phase cells and impaired cellular proliferation after the assembly, expression, and selection of functional Ag receptor genes (25). Therefore, to obtain direct evidence that H2ax deletion does not impair coding join formation beyond that observed in Atm<sup>−/−</sup> cells, we sought to analyze chromosomal V(D)J recombination among H2ax<sup>−/−</sup>, Atm<sup>−/−</sup>, and Atm<sup>−/−</sup>H2ax<sup>−/−</sup> abl pre-B cells. For this purpose, we established abl pre-B cell lines from Atm<sup>−/−</sup>H2ax<sup>F/F</sup> mice and incubated these with TAT-Cre protein to delete H2ax<sup>F</sup> genes, generating otherwise identical Atm<sup>−/−</sup>H2ax<sup>−/−</sup> abl pre-B cells. We infected the parental

**FIGURE 1.** ATM and H2AX exhibit nonredundant functions during DN-to-DP thymocyte expansion. (A) Representative CD4/CD8 FACS data of thymocytes from LH, LA, and LAH mice. The average numbers of total thymocytes, the DN, DP, CD4<sup>+</sup> SP, and CD8<sup>+</sup> SP cell quadrants, and the percentages of splenocytes within each of these quadrants are indicated. (B) Graphs showing the average numbers of total thymocytes or DN, DP, CD4<sup>+</sup> SP, and CD8<sup>+</sup> SP cells. Error bars are SE. *p < 0.05. (C) Representative CD4/CD8 FACS data of splenocytes from LH, LA, and LAH mice. The average numbers of total αβ T cells, CD4<sup>+</sup> and CD8<sup>+</sup> quadrants, and the percentages of splenocytes within each of these quadrants are indicated. Each experiment in this figure was done at least five independent times on at least one mouse of each genotype.

**FIGURE 2.** Atm<sup>−/−</sup> and Atm<sup>−/−</sup>H2ax<sup>−/−</sup> cells exhibit similar defects in CJ formation in G1 phase cells. (A) Schematic diagrams of the pMX-DEL<sup>Cr</sup> retroviral substrate in the unarranged (UR), recombined (CJ), and unrepaired (CE) configurations. Coding sequences are shown as open boxes and recombination signal sequences as triangles. Arrows represent the LTR sequences. Indicated are the relative positions of the EcoRV sites (RV) and C4 probe used for Southern blotting and the sizes of the C4-hybridizing EcoRV fragments from pMX-DEL<sup>Cr</sup> in the UR, CE, and CJ configurations. (B) Southern analysis of pMX-DEL<sup>Cr</sup> recombination in H2ax<sup>−/−</sup>, Atm<sup>−/−</sup>, and Atm<sup>−/−</sup>H2ax<sup>−/−</sup> abl pre-B cells untreated or treated with STIS17 for 48 or 96 h. Bands corresponding to fragments obtained from pMX-DEL<sup>Cr</sup> substrates of the UR, CE, and CJ configurations are indicated. This experiment was performed once on pools of infected H2ax<sup>−/−</sup>, Atm<sup>−/−</sup>, and Atm<sup>−/−</sup>H2ax<sup>−/−</sup> abl pre-B cells.
translocations. (14, 4, 16) Atm​/− H2ax​/− and derivative Atm​/− H2ax​/− abl pre-B cells, as well as previously established H2ax​/− abl pre-B cells (29), with the pMX-DELC​/J retroviral V(D)J recombination substrate (Fig. 2A) (25). Rearrangement of pMX-DELC​/J in wild-type cells results in formation of a chromosomal coding join (CJ) through coding end (CE) intermediates (Fig. 2A). We treated populations of H2ax​/−, Atm​/− H2ax​/−, and Atm​/− H2ax​/− abl pre-B cells containing chromosomally integrated pMX-DELC​/J with STI571 for 48 or 96 h. We then conducted Southern blot analysis to identify substrates that rearranged to form CJs, accumulated in G1 phase cells substantially beyond that detected in unrepaired CEs (Fig. 2B). Notably, we detected no obvious differences in the ratio of unrepaired CEs to accumulated unrepaired CEs (data not shown). We found that LA and LAH mice survived tumor-free between 75 and 145 d with both genotypes exhibiting a median age of mortality around 85 d (Fig. 3A). All 20 LA cohort mice succumbed to thymic lymphomas that showed no dissemination to peripheral lymphoid organs (Supplemental Table I), similar to the tumor phenotype of Atm​/− mice (6, 9, 10, 24). Of the 27 LAH cohort mice, 25 succumbed to thymic lymphomas, one succumbed to a peripheral lymphoma, and another succumbed to a sarcoma (Supplemental Table II). These observations demonstrate that Atm​/− mice and Atm​/− mice with H2ax​ deletion initiating in DN thymocytes exhibit similar predisposition to spontaneous thymic lymphomas.

Thymic lymphomas of Atm​/− mice are clonal malignancies that contain TCRβ rearrangements, lack surface TCRβ expression, and consist of CD4+/CD8−, CD4−/CD8+, and/or CD4−/CD8− cells (6–10, 24). To evaluate whether H2ax-deletion affects the phenotype of Atm​/− thymic lymphomas, we determined the clonality and developmental stage of LAH and LA tumors. Most of the 19 LAH and 19 LA lymphomas analyzed by Southern blotting contained two TCRβ alleles with rearrangements involving DJ1-JB1 segments (Fig. 3B; data not shown). The others contained another rearrangement involving DJ2-JB2 segments and/or deleted TCRβ locus sequences (Fig. 3B; data not shown). Southern blot analysis also confirmed H2ax deletion in all LAH tumors analyzed (data not shown).

Atm​/− H2ax​/− and Atm​/− thymocytes exhibit similar predisposition to transformation

Almost all Atm​/− mice succumb by 6 mo of age to thymic lymphomas (6, 9, 10, 24), whereas H2ax​/− mice rarely succumb to cancer within the first year of life (12–14). Thus, to evaluate potential nonredundant functions of ATM and H2AX in suppression of αβ T-lineage lymphomas, we generated and analyzed parallel cohorts of 20 LA and 27 LAH mice. Although we did not assess a parallel cohort of LH mice, we have never observed any tumors in LH mice (data not shown). LAH and LAH mice were clonal malignancies that contain TCRβ rearrangements, lack surface TCRβ expression, and consist of CD4+/CD8−, CD4−/CD8+, and/or CD4−/CD8− cells (6–10, 24). To evaluate whether H2ax-deletion affects the phenotype of Atm​/− thymic lymphomas, we determined the clonality and developmental stage of LAH and LA tumors. Most of the 19 LAH and 19 LA lymphomas analyzed by Southern blotting contained two TCRβ alleles with rearrangements involving DJ1-JB1 segments (Fig. 3B; data not shown). The others contained another rearrangement involving DJ2-JB2 segments and/or deleted TCRβ locus sequences (Fig. 3B; data not shown). Southern blot analysis also confirmed H2ax deletion in all LAH tumors analyzed (data not shown).
not shown), demonstrating these cells lacked both Atm and H2ax. Of the 17 LA and 21 LAH thymic lymphomas analyzed by flow cytometry, most were TCRβ– and similarly composed of CD4−/CD8+, CD4+/CD8+ cells (Supplemental Tables I, II). Collectively, these data reveal that Atm−/− and Atm+/− mice with H2ax deletion DN cells succumb to clonal αβ T-lineage tumors of similar developmental stages.

Most Atm−/− thymic lymphomas harbor clonal chromosome 14 translocations formed by aberrant repair of RAG DSBs induced during TCRβ recombination in DN thymocytes and additional clonal and nonclonal translocations generated through aberrant repair of replication-associated DSBs (7, 8). To assess whether deletion of H2ax in Atm−/− DN thymocytes affects the translocation pattern of Atm−/− thymic lymphomas, we conducted spectral karyotyping (SKY) on metaphases prepared from four LA and five LAH tumors. Three LA thymic lymphomas contained clonal chromosome 14 translocations, as well as other clonal or nonclonal translocations (Table I); the other harbored clonal t (15, 6), t (12, 7), and t (4, 15) translocations and several nonclonal translocations (Table I). This pattern of translocations is similar to that observed in Atm−/− tumors (7, 8), indicating that Lck-cre expression does not substantially affect the type or frequency of translocations that arise in Atm−/− thymic lymphomas. Four LAH tumors harbored clonal chromosome 14 translocations and many nonclonal translocations (Fig. 3C, Table II). The remaining LAH thymic lymphoma lacked clonal translocations but contained numerous nonclonal translocations (Fig. 3D, Table II). These findings indicate that LA and LAH tumors exhibit similar patterns of translocations compared with each other and with Atm−/− thymic lymphomas. Thus, we conclude that conditional H2ax deletion in Atm−/− thymocytes does not substantially affect the incidence or mortality of mice from thymic lymphomas with clonal chromosome 14 translocations.

### Table I. SKY analysis of translocations in LA thymic lymphomas

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Clonal Translocations</th>
<th>Nonclonal Translocations</th>
</tr>
</thead>
<tbody>
<tr>
<td>728</td>
<td>t (12, 14)</td>
<td>t (12, 7)</td>
</tr>
<tr>
<td>873</td>
<td>t (12, 14), t (8, 6), and t (X;16)</td>
<td>t (4, 5), t (17, 16), t (9, 4), t (1, 15), t (1, 6), t (7, 14), t (2, 11), t (15, 11), t (17, 14), t (4, 7), t (14, 6), t (17, 9), t (8, 15, 16)</td>
</tr>
<tr>
<td>26</td>
<td>t (12, 14) and t (14, 18)</td>
<td>t (12, 2), t (14, 1), t (3, 8), t (2, 12), t (9, 15), t (2, 14), t (16, 15), t(X;15), t (8, 14), t (14, 19), t (5, 16), t (3, 7), t (5, 9), t (15, 9), t(X;5), t (19, 3), reciprocal t (1, 12) and t (12, 1)</td>
</tr>
<tr>
<td>560</td>
<td>t (15, 6), t (4, 15), and t (12, 7)</td>
<td>t (12, 4), t (12, 15), t (17, 15), t (4;X), t (14, 16), t (12, 15), t (15, 8), t (6, 12), t (6, 14), t (7, 15), t (1, 6), t (13, 1), t (15, 15, 6)</td>
</tr>
</tbody>
</table>

Translocations classified as clonal were detected in at least 80% of metaphases.

### Table II. SKY analysis of translocations in LAH thymic lymphomas

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Clonal Translocations</th>
<th>Nonclonal Translocations</th>
</tr>
</thead>
<tbody>
<tr>
<td>798</td>
<td>t (12, 14) and t (14, 15)</td>
<td>t (2, 8), t (2, 15), t (12, 2), t (2, 12), t (2, 15), t (2, 14), reciprocal t (8, 2) and t (2, 8)</td>
</tr>
<tr>
<td>629</td>
<td>t (12, 14) and t (14, 15)</td>
<td>t (17, 14), t (12, 8), t (5, 14), t (1, 2), t (2, 14), t (2, 1), t (17, 13), t (18, 11), t (13, 19), t (19, 11), reciprocal t (1, 2) and t (2, 1)</td>
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<tr>
<td>884</td>
<td>t (12, 14) and t (14, 15)</td>
<td>t (12, 2), t (14, 12), t (1, 2), t (1, 7), t (1, 17), t (8, 7), t (7, 15), reciprocal t (6, 7) and t (7, 6), reciprocal t (4, 10) and t (10, 4)</td>
</tr>
<tr>
<td>590</td>
<td>t (4, 14), t (14, 16), and t (16, 4, 14)</td>
<td>t (14, 4), t (13, 9), t (16, 4, 14), t (13, 2), t (14, 3), t (4, 8), t (2, 17), t (12, 6), t (12, 8), t (16, 12), t (2, 6), t (19;Y), t (8, 16), t (8, 19), t (8, 14), t (12, 2), t (12, 15), t (12, 14), t (2, 16), t (2, 12), t (8;X)</td>
</tr>
<tr>
<td>589</td>
<td>None</td>
<td>t (2, 1), t (4, 5), t (12, 2), t (14, 15), t (15, 4), t (4, 15), t (4, 6), t (12, 14), t (5, 4), t (4, 15, 16), t (8, 19), t (1, 17), t (7;15;14), t (17, 19), t (15, 7), t (2, 12), t (1, 2), t (1, 8), t (1, 2), t (8, 1), t (18, 12), t (14;15;Y), t (5, 1), reciprocal t (4, 15) and t (15, 4), reciprocal t (5, 17) and t (17, 5), reciprocal t (6, 18) and t (18, 6), reciprocal t (3, 4) and t (4, 3)</td>
</tr>
</tbody>
</table>

Translocations classified as clonal were detected in at least 80% of metaphases.
Although most abnormalities were nonrecurrent, we observed higher frequencies of two types of chromosomal lesions in LAH metaphases compared with LA and LH metaphases. We detected radial chromosome translocations involving aberrant fusion and cohesion of heterologous chromatids in 5.5% of LAH metaphases but not in any LA or LH metaphases (5.5% versus 0, $p = 0.0005$) (Fig. 4B, 4C). These radial translocations arise in HR-defective backgrounds and form when chromatid breaks are not repaired via HR but instead aberrantly joined via NHEJ before G2/M (31). Thus, our detection of radial translocations in LAH, but not LA or LH, metaphases reveals that ATM and H2AX have nonredundant functions during HR in proliferating T cells. The Ag receptor loci that recombine in developing $\alpha\beta$ T cells are located on chromosomes 6 (Tcrb), 13 (Tcrry), 14 (Tcrro6), and 12 (Igh). We detected similar frequencies of translocations involving chromosomes 13, 14, and 12 among LH, LA, and LAH metaphases (Supplemental Table IV). Notably, we observed a higher frequency of chromosome 6 translocations in LAH metaphases than in either LA or LH metaphases (Supplemental Table IV). To identify and quantify potential TCRb translocations, we conducted FISH with chromosome 6 paints and a genomic DNA probe that binds downstream of D$\beta$-J$\beta$ segments (3'TCRb). We scored TCRb translocations when 3'TCRb FISH signals were located at breakpoints of chromosome 6 translocations (Fig. 4D). We found a greater frequency of TCRb translocations in LAH metaphases compared with LA (3.5 versus 0.46%, $p = 0.0045$) or LH metaphases (3.5 versus 0.63%, $p = 0.014$) (Fig. 4E). Because TCRb-selection is required for $\alpha\beta$ T cell development, the elevated frequency of TCRb translocations in LAH cells versus LA and LH cells demonstrates that nonredundant functions of ATM and H2AX suppress the aberrant repair of RAG DSBs induced on nonselected TCRb alleles. For reasons explained in the Discussion, we conclude from our data that nonredundant functions of ATM and H2AX protect proliferating $\alpha\beta$ T-lineage cells from genomic instability initiated by spontaneous and programmed DSBs.

**Discussion**

In this study, we generated and analyzed Atm-deficient mice and cells with conditional deletion of H2ax to assess potential redundant and nonredundant functions of ATM and H2AX in developing lymphocytes. We had previously demonstrated that G1 phase Atm$^{-/-}$, but not H2ax$^{-/-}$, abl pre-B cells exhibit defects in chromosomal CJ formation (25, 29). In the current study, we showed that Atm$^{-/-}$/H2ax$^{-/-}$ and Atm$^{-/-}$/abl pre-B cells display similar defects in NHEJ-mediated CJ formation in G1 phase cells. Analogous to the situation for H2AX, inactivation of the XLF DDR has no obvious effect upon CJ formation in G1 phase abl pre-B cells (19). However, combined inactivation of H2ax and Xlf leads to a major defect in CJ formation that is associated with degradation of RAG-generated chromosomal CEs (32). In addi-

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**TABLE III.** SKY analysis of genomic instability in metaphases prepared from in vitro-stimulated splenic $\alpha\beta$ T cells

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Metaphases Analyzed</th>
<th>Metaphases with Genomic Instability</th>
<th>Total Number of Lesions</th>
<th>Lesions per Metaphase</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH</td>
<td>330</td>
<td>75 (23%)</td>
<td>109</td>
<td>0.33</td>
</tr>
<tr>
<td>LA</td>
<td>254</td>
<td>66 (26%)</td>
<td>97</td>
<td>0.38</td>
</tr>
<tr>
<td>LAH</td>
<td>237</td>
<td>98 (41%)</td>
<td>164</td>
<td>0.69</td>
</tr>
</tbody>
</table>

*a* Indicated are the total numbers of abnormalities and the percentage of metaphases with each type of abnormality.

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**FIGURE 4.** ATM and H2AX exhibit nonredundant functions in maintenance of genomic stability in proliferating $\alpha\beta$ T cells. (A) Graph showing the average percentages of metaphases with chromosome abnormalities in metaphases prepared from in vitro-stimulated LH, LA, and LAH splenic $\alpha\beta$ T cells. Error bars are SE. This experiment was conducted three independent times on cells from one mouse of each genotype. (B) DAPI, spectral, and classified SKY images of a tri-radial chromosome translocation observed in metaphases from LAH $\alpha\beta$ T cells. (C) Graph showing the numbers of tri-radial translocations detected in metaphases prepared from in vitro-stimulated LH, LA, and LAH splenic $\alpha\beta$ T cells. Error bars are SE. This experiment was conducted three independent times on cells from one mouse of each genotype. (D) Schematic and cytogenetic images of a normal chromosome 6 and a dicentric translocation involving the TCRb locus on chromosome 6 detected in metaphases from LAH $\alpha\beta$ T cells. Metaphases were stained with DAPI (blue) to label chromosomes, whole chromosome 6 paints (red) to identify chromosome 6 material, and 3’TCRb FISH probes (yellow) to detect these TCRb sequences. (B and D) Original magnification $\times$60. (E) Graph showing the numbers of TCRb translocations detected in metaphases prepared from in vitro-stimulated LH, LA, and LAH splenic $\alpha\beta$ T cells. Error bars are SE. This experiment was conducted three independent times on cells from one mouse of each genotype. *$p < 0.05$. 

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tion, combined inactivation of Atm and Xlf leads to a near complete block in chromosomal CJ formation in G1-arrested abl pre-B cells (32). These XLF studies revealed that XLF has essential nonredundant functions with ATM and H2AX during chromosomal CJ formation in G1 phase cells. Because the identical assay was used in these experiments and our current study, our results provide strong evidence that ATM promotes NHEJ-mediated CJ formation in G1 phase lymphocytes through mechanisms that are not critically dependent upon H2AX.

Despite showing that G1 phase Atm−/−H2ax−/− and Atm−/−H2ax−/− lymphocytes exhibit similar defects in CJ formation, we discovered a 7-fold higher frequency of TCRβ translocations in splenic αβ T cells from Lah mice compared with La mice. TCRβ genes are assembled in G1 phase DN cells through an ordered process where δβ-to-ββ recombination is followed by Vβ rearrangement to DjβJ complexes on one allele at a time (33). TCRβ gene expression from one allele selects DN thymocytes for further differentiation and drives G1-to-S progression and multiple cell cycles as cells differentiate into DP thymocytes (33). Although ATM activates the G1/S checkpoint to prevent DN cells with RAG DSBs from entering into S phase (34), a fraction of TCRβ-selected thymocytes enters S phase with unrepaird RAG DSBs induced during Vβ recombination on nonalleles (35). We previously showed that H2AX stabilizes RAG-cleaved DNA strands to prevent DSBs that persist into S phase from progressing into chromosome breaks and translocations during cellular proliferation (29). We also recently found that H2AX phosphorylation blocks resection of CEs to prevent RAG DSBs from entering into S phase (36), a fraction of TCRβ-selected thymocytes enters S phase with unrepaird RAG DSBs induced during Vβ recombination on nonalleles (35).

Our finding that Lah mice exhibit a greater reduction of TCRβ-mediated thymocyte expansion than LA mice indicates that ATM and H2AX serve nonredundant functions in developing αβ T cells. Because ATM and H2AX have redundant functions during CJ formation in G1 phase cells, the impaired proliferative expansion of Lah thymocytes likely reflects nonredundant functions of H2AX and ATM in response to replication-associated DSBs. Our detection of radial translocations in in vitro-stimulated αβ T cells of Lah mice, but neither LA nor LH mice, indicates that ATM and H2AX exhibit nonredundant functions during HR in proliferating lymphocytes. H2AX phosphorylation by ATM and DNA-PK is required for HR repair of DNA replication-associated DSBs and restart of DNA replication (38). Thus, we conclude that the more substantial defect in TCRβ-mediated thymocyte expansion in Lah mice, compared with LA mice, is due to loss of DNA-PK-mediated H2AX functions that suppress translocations from replication-associated DSBs. Such H2AX functions could be the recruitment of HR proteins to promote rapid DNA repair and/or the cohesion of sister chromatids to prevent separation of broken DNA ends.

Our finding that Lah and LA mice exhibit similar mortality from thymic lymphomas with chromosomal 14 translocations implies for treatment of human cancers with ATM inactivation. The clonal t (12, 14) translocations of Atm−/− mice delete one allelic copy of the Bcl11b haploinsufficient tumor suppressor gene that encodes a protein required for activation of the DDR during DNA replication stress (7, 39, 40). Mutation or deletion of ATM and BCL11B often occur in human T cell acute lymphoblastic leukemia (T-ALL) (5, 41–45), whereas inactivation of only ATM is frequently observed in human T cell prolymphocytic leukemia (T-PLL) (5, 46–48). ATM inactivation in T-ALL is associated with therapy resistance and relapse (42), whereas no cure exists for T-PLL with ATM mutation (49). Clearly, the development of more effective therapies for these malignancies is warranted. Targeted inhibition of tumor cell intrinsic nonredundant DDR mechanisms holds great promise for more effective and less toxic cancer treatments (50–52). This approach leads to increased genomic instability that causes apoptosis in tumor cells while minimally affecting normal cells (50–52). Although combined inactivation of Atm and H2ax impairs cellular proliferation and causes substantial genomic instability, our data suggest that targeted inhibition of ATM-independent H2AX functions would not be an effective treatment for T-ALL tumors with ATM and BCL11B inactivation. However, additional studies are warranted to evaluate whether this strategy would be effective for other T-ALL subtypes, T-PLLs, and/or other cancers with current ATM inactivation, such as mantle cell lymphoma (53).

Disclosures

The authors have no financial conflicts of interest.

References


