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RAG-Mediated V(D)J Recombination Is Not Essential for Tumorigenesis in *Atm*-Deficient Mice

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***Atm*-deficient mice die of malignant thymic lymphomas characterized by translocations within the *Tcr*α/δ locus, suggesting that tumorigenesis is secondary to aberrant responses to double-stranded DNA (dsDNA) breaks that occur during RAG-dependent V(D)J recombination. We recently demonstrated that development of thymic lymphoma in $Atm^{-/-}$ mice was not prevented by loss of RAG-2. Thymic lymphomas that developed in $Rag2^{-/-}$ $Atm^{-/-}$ mice contained multiple chromosomal abnormalities, but none of these involved the *Tcr*α/δ locus. These findings indicated that tumorigenesis in $Atm^{-/-}$ mice is mediated by chromosomal translocations secondary to aberrant responses to dsDNA breaks and that V(D)J recombination is an important, but not essential, event in susceptibility. In contrast to these findings, it was recently reported that $Rag1^{-/-}$ $Atm^{-/-}$ mice do not develop thymic lymphomas, a finding that was interpreted as demonstrating a requirement for RAG-dependent recombination in the susceptibility to tumors in *Atm*-deficient mice. To test the possibility that RAG-1 and RAG-2 differ in their roles in tumorigenesis, we studied $Rag1^{-/-}$ $Atm^{-/-}$ mice in parallel to our previous $Rag2^{-/-}$ $Atm^{-/-}$ study. We found that thymic lymphomas occur at high frequency in $Rag1^{-/-}$ $Atm^{-/-}$ mice and resemble those that occur in $Rag2^{-/-}$ $Atm^{-/-}$ mice. These results indicate that both RAG-1 and RAG-2 are necessary for tumorigenesis involving translocation in the *Tcr*α/δ locus but that *Atm* deficiency leads to tumors through a broader RAG-independent predisposition to translocation, related to a generalized defect in dsDNA break repair.**

Ataxia telangiectasia (AT) is an autosomal recessive disease caused by mutation of the ataxia telangiectasia mutated (*ATM*) gene (16). Absence of the ATM protein, which plays an important role in cellular responses to the presence of double-stranded DNA (dsDNA) breaks and induction of cell cycle checkpoints (6, 18), results in a pleiotrophic phenotype characterized by progressive cerebellar ataxia, ocular telangiectasias, immunodeficiencies, increased sensitivity to ionizing radiation, and increased incidence of lymphoid cancers (2, 17, 20). While the exact pathophysiological mechanism relating the genetic defect to the clinical manifestations of the disease has not been elucidated, it is plausible that several components of the AT phenotype, including the predisposition to development of lymphoid malignancies, result from aberrant recognition and repair of dsDNA breaks such as those that normally occur during V(D)J recombination in lymphoid cells (15).

Support for this hypothesis comes from studies of the murine model of AT. Karyotypic analysis of the thymic lymphomas that characteristically develop in *Atm*-deficient mice (1, 3, 22) has identified consistent translocations within the *Tcr*α/δ locus, suggesting the involvement of V(D)J recombination in the process of lymphomagenesis (11). Because V(D)J recombination is dependent upon the recombinase-activating genes (*Rag1* and *Rag2*) (13, 19), Liao and Van Dyke generated $Rag1^{-/-}$ $Atm^{-/-}$ mice to assess the requirement for V(D)J recombination in tumorigenesis (8). Consistent with the hypothesis that V(D)J recombination is involved in tumorigenesis in *Atm*-deficient mice, Liao and Van Dyke reported that no thymic lymphomas developed in $Rag1^{-/-}$ $Atm^{-/-}$ mice, whereas all $Rag1^{+/-}$ $Atm^{-/-}$ mice died of thymic lymphoma. In contrast, an analogous study conducted by our group observed that $Rag2^{-/-}$ $Atm^{-/-}$ mice did develop thymic lymphomas and did so through a mechanism that did not involve *Tcr* α/δ translocation (14). Given that absence of either RAG-1 or RAG-2 eliminates V(D)J recombination (13, 19), the disparity in the results of these two tumor incidence studies was surprising. A difference in lymphoma susceptibility between $Rag1^{-/-}$ $Atm^{-/-}$ mice and $Rag2^{-/-}$ $Atm^{-/-}$ mice could signify previously unidentified differences in RAG-1 and RAG-2 function. Alternatively the difference in results of previously reported studies of $Rag1^{-/-}$ $Atm^{-/-}$ and $Rag2^{-/-}$ $Atm^{-/-}$ mice could be attributable to experimental variables such as differences in genetic background and/or environmental differences in mouse colonies used in these studies. To more directly compare the roles of RAG-1 and RAG-2 in the generation of thymic lymphomas, we have studied $Rag1^{-/-}$ $Atm^{-/-}$ mice under the

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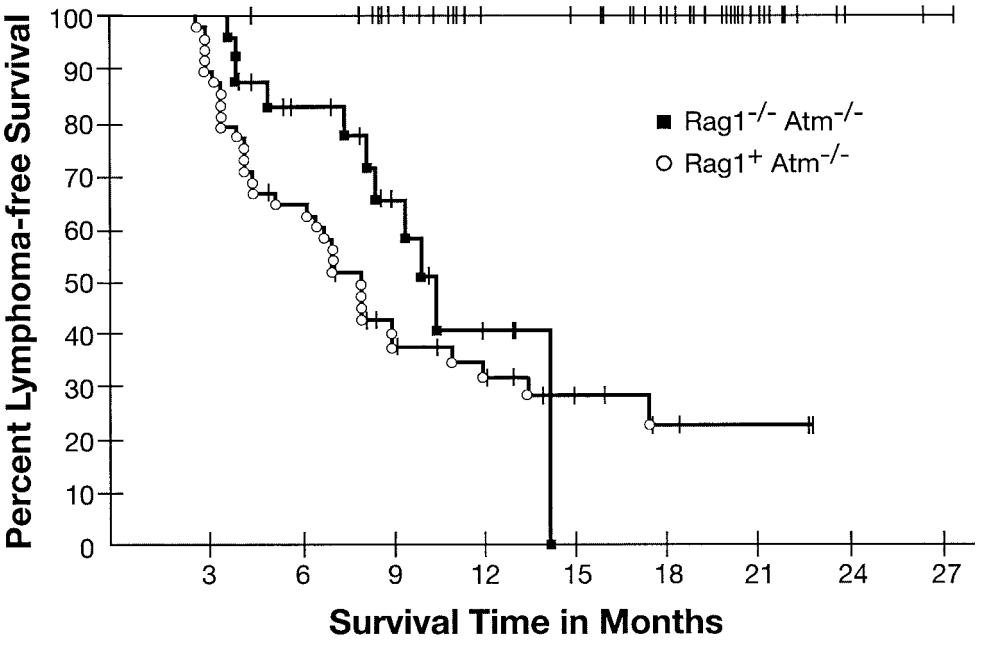

FIG. 1. Kaplan-Meier analysis of tumor incidence. Thymic lymphoma-free survival is plotted versus time in months for $Rag1^{-/-}$ $Atm^{-/-}$ mice and littermate controls. A drop in the curve represents the sacrifice or death of an animal diagnosed at necropsy with malignant thymic lymphoma. Tick marks represent the sacrifice or death of an animal that was not subsequently diagnosed with malignant thymic lymphoma. As no $Rag1^{-/-}$ $Atm^{+/+}$ or $Rag1^{+}$ $Atm^{+/+}$ animals were diagnosed with malignant thymic lymphomas, the curves for these two groups are at 100% event-free survival.

conditions previously used in characterizing susceptibility to lymphomas in $Rag2^{-/-}$ $Atm^{-/-}$ mice.

MATERIALS AND METHODS

Mice. The creation of *Atm*-deficient mice (allele designation $Atm^{ins5790neo}$) has been described previously (1). Progeny of heterozygote matings were genotyped by PCR (9). *Rag1*-deficient mice (C57BL/6J-sv/129; Jackson Labs) were identified phenotypically by flow cytometric analysis of peripheral blood lymphocytes as described previously (14). To generate mice deficient for both *Rag1* and *Atm*, double heterozygotes were crossed and progeny were typed as described above.

Tumor incidence and histopathological analyses. As described previously (14), mice were examined daily for evidence of morbidity and mortality. Mice were sacrificed when death was judged to be imminent and prepared for necropsy. Organs from mice that were found dead were similarly preserved. Tissues were fixed, sectioned, stained, and examined by light microscopy (L. G. Luna, Histopathological methods and color atlas of special stains and tissue, American Histolabs, Inc., Publications Division, Gaithersburg, Md.).

Statistical analysis. Kaplan-Meier survival curves were generated as previously described (7, 14). Statistical significance was determined by the Mantel-Haenszel method (12). Individual *P* values are provided after adjustment for multiple comparisons according to the method of Hochberg (4). All *P* values are two-tailed.

Thymic lymphoma cell culture. Thymic lymphomas were harvested, and single-cell suspensions were cultured in complete medium supplemented with 100 IU of human recombinant interleukin 2 per ml and 6 ng of recombinant murine interleukin 7 per ml as described previously (14). Tail DNA was isolated from all $Rag1^{-/-}$ $Atm^{-/-}$ mice developing thymic lymphomas and genotyped by PCR for both *Rag1* and *Rag2* to verify genotypes. *Rag2* PCR typing was carried out as previously described (5), and *Rag1* typing was carried out under identical conditions, with substitution of *Rag1*-specific primers as follows: RAG-1 forward, CAA CAT CTG CCT TCA CTT C; RAG-1 reverse, TAC CCT GAG CTT CAG TTC TG; neo, TAT CAG GAC ATA GCG TTG GCG.

SKY and fluorescent in situ hybridization (FISH). Metaphase chromosomes were prepared from tumor cell lines at early-passage numbers (0 to 2) as described previously (14). Spectral karyotyping (SKY) of the tumor cell lines was performed following published procedures (10, 14, 21). Six to ten metaphases were analyzed for each tumor. BAC clones (Genome Systems, St. Louis, Mo.) for the TcrCa and TcrVd were labeled with digoxigenin-dUTP and biotin-dUTP (Roche Biomolecular, Indianapolis, Ind.) and detected with fluorescein- and rhodamine-conjugated avidin and antibody as described previously (11). Images were acquired with a Leica DMRXA microscope (Leica, Wetzlar, Germany) and Q-FISH software (Leica Imaging Systems, Cambridge, United Kingdom).

RESULTS AND DISCUSSION

Incidence of malignant thymic lymphomas in mice deficient in both *Atm* and *Rag1*. As in our previous study, $Rag1^{-/-}$ $Atm^{-/-}$ mice, like $Rag2^{-/-}$ $Atm^{-/-}$ mice, were found to develop malignant thymic lymphomas (Fig. 1). Of 24 $Rag1^{-/-}$ $Atm^{-/-}$ mice monitored, 11 (46%) were diagnosed with malignant thymic lymphoma at necropsy compared with 0 of 33 $Rag1^{+}$ $Atm^{+/+}$ mice ($P < 0.001$ by Mantel-Haenszel test comparing overall probability as a function of time). Median age at death for $Rag1^{-/-}$ $Atm^{-/-}$ mice was 8.3 months (range of times to death, 3.75 to 14.25 months). As expected, $Rag1^{+}$ $Atm^{-/-}$ littermate controls also developed malignant thymic lymphomas, with 33 of 48 $Rag1^{+}$ $Atm^{-/-}$ mice (69%) having this diagnosis at necropsy (Fig 1) and a median age at death of 7.0 months (range, 2.75 to 17.5 months).

In addition to malignant thymic lymphomas, *Atm*-deficient mice also showed an increased incidence of nonthymic tumors compared with control mice. Four of 24 $Rag1^{-/-}$ $Atm^{-/-}$ mice (17%) and 6 of 48 $Rag1^{+}$ $Atm^{-/-}$ mice (13%) developed various nonthymic tumors (carcinomas and sarcomas) compared to 0 of 33 $Rag1^{+}$ $Atm^{+/+}$ mice ($P = 0.0002$ and $P < 0.0001$, respectively, based on actuarial probability of developing nonthymic tumors and after adjustment for both comparisons). The occurrence of tumors other than thymic lymphomas was

TABLE 1. Chromosomal aberrations in *Rag1*$^{-/-}$ *Atm*$^{-/-}$ thymic lymphomas

Rag1$^{-/-}$ Atm$^{-/-}$ tumor no.	SKY-detected structural aberrations	SKY-detected gains and losses
8567	Del(9) T(12D-E;9) T(17;15)	+5, +10, +15 −7
20343	T(9;15;14) T(12D-E;15) T(Dp(14);15;9)	+18
20154	T(4;9) Del(9) T(12D-E;4) Dp(15B-D)	+3, +4 −6

similar to our previously reported observation with *Rag2*$^{-/-}$ *Atm*$^{-/-}$ mice.

To investigate potential mechanisms of tumorigenesis in *Rag1*-deficient mice, molecular cytogenetic analysis using SKY was employed to identify chromosomal aberrations in early-passage cells cultured from three *Rag1*$^{-/-}$ *Atm*$^{-/-}$ thymic lymphomas. Thymic lymphoma cells from *Rag1*$^{-/-}$ *Atm*$^{-/-}$ mice had multiple translocations in each tumor (Table 1). A representative metaphase from tumor no. 20154 is shown in Fig. 2. This tumor had a translocation [T(12;4)] in addition to other chromosomal aberrations. Remarkably, each of three *Rag1*$^{-/-}$ *Atm*$^{-/-}$ tumors analyzed had a chromosomal translocation involving chromosome 12, bands D and E. This same breakpoint region has been identified in both *Atm*$^{-/-}$ and *Rag2*$^{-/-}$ *Atm*$^{-/-}$ thymic lymphomas (11, 14). Hybridization using a FISH probe containing the Igh-6 gene (at 12F1) confirmed

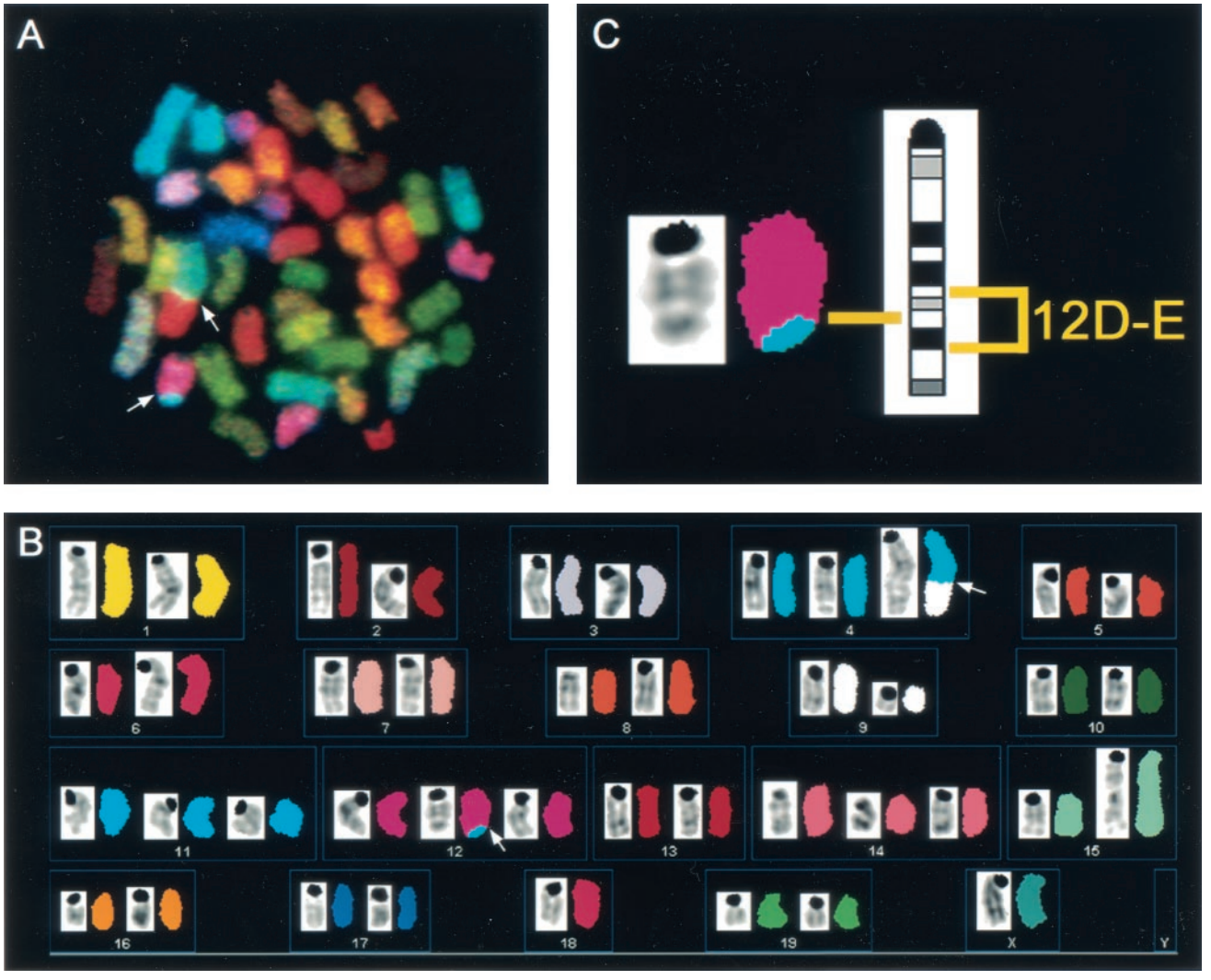

FIG. 2. Spectral karyotyping analysis of a metaphase cell from *Rag1*$^{-/-}$ *Atm*$^{-/-}$ thymic lymphoma no. 20154 displaying chromosome aberrations. The entire metaphase is shown in display colors in panel A, with arrows indicating translocated chromosomes. The spectrum-based classification karyotype is shown in panel B, with each pseudocolored chromosome positioned next to its inverted 4′,6′-diamidino-2-phenylindole-banded image. The T(12;4) is enlarged in panel C next to the G-band ideogram for chromosome 12, to highlight the band region involved in the translocation. The full karyotype of this metaphase is 42, X, T(4D;9A), Del(9B-F), T(12D-E;4D), Dp(15B-D), +4, +11, +12, +14, −18, −Y. Note that this representative metaphase for tumor no. 20154 does not contain every clonal aberration that is described in Table 1.

that the region distal to the breakpoint was lost (data not shown). One tumor (no. 20343) revealed a complex translocation involving chromosome 14. In order to exclude the involvement of the *Tcr* locus in this rearrangement, we used FISH with BAC clones for the constant and variable regions of the *TCR* α/δ locus, respectively. All metaphase spreads and interphase nuclei analyzed ($n = 19$) showed colocalization of the differentially labeled probes. We can therefore exclude the possibility that intrachromosomal rearrangements, such as inversions, disrupt the integrity of the *Tcr* locus.

The results of this study further support our previous finding that V(D)J recombination is not required for tumorigenesis in *Atm*-deficient mice (14). *Rag1*$^{-/-}$ *Atm*$^{-/-}$ mice, like *Rag2*$^{-/-}$ *Atm*$^{-/-}$ mice, develop malignant thymic lymphomas. The basis for the difference between the findings reported here and those of Liao and Van Dyke (8), who failed to observe thymic lymphomas in *Rag1*$^{-/-}$ *Atm*$^{-/-}$ mice, is not clear but may reflect variables including genetic differences in the strains employed and/or environmental conditions under which animals were housed. Tumors from *Rag1*$^{-/-}$ *Atm*$^{-/-}$ thymic lymphomas resemble those from *Rag2*$^{-/-}$ *Atm*$^{-/-}$ thymic lymphomas in several aspects, including cell surface phenotype (variable levels of CD4 and CD8 expression and absence of TCR α/β expression; data not shown), the presence of multiple translocations in individual tumor cells, and the absence of *Tcr* α/δ locus translocation—a translocation which is consistently seen in *Atm*$^{-/-}$ thymic lymphomas (11, 14). It thus appears that the mechanism of thymic lymphoma development in *Rag1*$^{-/-}$ *Atm*$^{-/-}$ mice, as in *Rag2*$^{-/-}$ *Atm*$^{-/-}$ mice, is related to abnormal repair of dsDNA breaks that occur secondary to processes other than V(D)J recombination. The observance of multiple chromosomal aberrations as well as the lack of translocations within the *Tcr* α/δ locus in *Rag1*$^{-/-}$ *Atm*$^{-/-}$ thymic lymphomas is consistent with this hypothesis. Of note is the frequent translocation site at chromosome 12D-E seen in thymic lymphomas from *Atm*$^{-/-}$ mice, *Rag2*$^{-/-}$ *Atm*$^{-/-}$ mice, and *Rag1*$^{-/-}$ *Atm*$^{-/-}$ mice. Further studies to elucidate the genes involved in this region may help to define the mechanism of tumorigenesis in *Atm*-deficient mice.

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L.K.P., Z.W., and M.V. contributed equally to this work.

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