Short Telomeres and Ataxia-Telangiectasia Mutated Deficiency Cooperatively Increase Telomere Dysfunction and Suppress Tumorigenesis

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ABSTRACT

To examine the role of ataxia-telangiectasia mutated (Atm) in telomere function, we generated Atm and telomerase null mice (Atm \(^{-/-}\) mTR \(^{-/-}\) iG6 mice). These mice exhibited increased germ cell death and chromosome fusions compared with either Atm \(^{-/-}\) or mTR \(^{-/-}\) iG6 mice. Furthermore, the Atm \(^{-/-}\) mTR \(^{-/-}\) iG6 mice had a delayed onset and reduced incidence of thymic lymphoma compared with Atm \(^{-/-}\) mice. The tumors in the Atm \(^{-/-}\) mTR \(^{-/-}\) iG6 mice showed increased apoptosis and anaphase bridges. Finally, lymphomas from Atm \(^{-/-}\) mTR \(^{-/-}\) iG6 mice were derived from CD8 immature, single-positive T cells, whereas Atm \(^{-/-}\) lymphomas were from CD4\(^+\)/CD8\(^+\) double-positive T cells. We propose that Atm protects short telomeres and that Atm deficiency cooperates with short telomeres, leading to increased cell death, decreased tumorigenesis, and increased overall survival.

INTRODUCTION

Chromosomal rearrangement and instability can initiate tumor formation. Cancer cells accumulate genetic changes because of loss of checkpoint controls that normally ensure genomic integrity. Telomeres protect chromosome ends from rearrangement, and loss of telomere function may play an important role in fueling genomic instability in cancer (1–5). In addition to promoting carcinogenesis, telomere dysfunction can also limit tumor growth (4, 6, 7). Dysfunctional telomeres activate cellular DNA damage responses and induce cell cycle arrest, apoptosis, or senescence (3, 8, 9). The telomerase null mouse, mTR \(^{-/-}\), shows progressive telomere shortening from the first null generation, mTR \(^{-/-}\) G1, through the sixth generation of interbreeding, mTR \(^{-/-}\) G6. Although the early generations, mTR \(^{-/-}\) G1–G3, show few abnormal phenotypes, the later-generation mice, mTR \(^{-/-}\) G4–G6, have critically short telomeres and exhibit germ cell apoptosis and infertility (10, 11), increased genomic instability, and cancer predisposition (1–5). However, the underlying mechanisms of response to dysfunctional telomeres are not yet understood but are under active investigation.

A-T\(^{-}\) is a pleiotropic genetic disorder attributable to recessive mutations in ATM (12, 13). It is characterized by cerebellar degeneration, immunodeficiency, radiosensitivity, and cancer predisposition, particularly lymphoid malignancies (14). The ATM family of protein kinases, which includes ATR and their yeast homologs Tel1 and Mec1/Rad3, plays a critical role in the cellular response to DNA damage (15, 16). In the presence of DNA damage, ATM and ATR phosphorylate downstream targets including NBS, BRCA1, and p53 (15–19). In addition, ATM plays a direct role in telomere maintenance. Loss of the ATM homolog Tel1 in yeast leads to short telomeres (20–24). Similarly, human A-T cells have shorter telomeres than normal cells (25). Finally, ATM may play a role in the cellular response to dysfunctional telomeres. Overexpression of a dominant-negative form of the telomere-binding protein TRF2 induces apoptosis in normal lymphocytes but not in lymphocytes from A-T patients (8).

To study the interplay between Atm and telomeres in telomere function and tumorigenesis, we generated double-knockout mice with short telomeres lacking Atm (Atm \(^{-/-}\) mTR \(^{-/-}\) iG6). Several lines of Atm mutant mice have been described. Although these Atm mutant mouse models recapitulate some features of the A-T patients, the phenotypes of these mouse models vary (26–30), likely because of a different genetic background and/or the different regions of the Atm protein that were targeted. For example, the Atm \(^{-/-}\) mice generated by Barlow et al. (26) on a 129S6/SvEvTac background develop thymic lymphoma at 12–16 weeks of age. In contrast, the Atm mutant mice Atm\(^{+/y}\) generated by Borghesani et al. (29) on a 129Sv/C57BL6 background develop thymic lymphoma at much lower frequency with a longer latency and have a median survival time of over 52 weeks. The increased tumor latency in the latter case may be attributable to the expression of a low level of mutant Atm mRNA with an intact kinase domain in the Atm\(^{+/y}\) mutant mice (29). Recently, Wong et al. (31) crossed mTR \(^{-/-}\) mice onto Atm\(^{+/y}\) mice and showed increased cell death in bone marrow cell culture and gastrointestinal crypts in the double-mutant mice. The incidence of thymic lymphoma was decreased, but the survival of these mice was not changed.

We used the Atm mutant mouse model generated by Barlow et al. (26) and adopted a different crossing scheme than Wong et al. (31). Consistent with Wong et al. (31), we also found increased cell death and reduced tumorigenesis in the double-mutant mice. Furthermore, we observed the cooperative effect of Atm deficiency and short telomeres on telomere dysfunction without extensive telomere shortening and an increased survival rate of double-mutant mice. We examined possible mechanisms for the increased survival and found that increased apoptosis and altered T-cell development contribute to decreased tumor growth. Finally, our data suggest a role for Atm in protecting short telomeres.

MATERIALS AND METHODS

Mice. mTR \(^{-/-}\) mice on a C57BL6 background were generated as described (10). Atm \(^{-/-}\) mice (The Jackson Laboratory) on a 129S6/SvEvTac background (26) were mated to mTR \(^{-/-}\) mice to generate double-heterozygous Atm \(^{-/-}\) mTR \(^{-/-}\) mice. The Atm \(^{+/y}\) mTR \(^{-/-}\) mice were intercrossed to generate Atm\(^{+/y}\) mTR \(^{-/-}\) and Atm \(^{-/-}\) mTR \(^{+/y}\) mice. Atm \(^{-/-}\) mTR \(^{-/-}\) iG6 mice were generated by intergenerational crosses: Atm \(^{-/-}\) mTR \(^{-/-}\) mice were crossed with mTR \(^{-/-}\) G4 mice to first generate Atm \(^{-/-}\) mTR \(^{-/-}\) iG6 mice. These mice were then intercrossed to generate Atm \(^{-/-}\) mTR \(^{-/-}\) iG6 mice. The Atm \(^{-/-}\) mTR \(^{-/-}\) iG6 and Atm \(^{-/-}\) mTR \(^{-/-}\) control mice are on the same genetic background. Genotyping was done by PCR as described previously for Atm (26) and mTR (32). All of the animals were maintained in a pathogen-free facility, weighed every week, and checked every other day for signs of...
sickness. All mouse protocols were approved by the Institutional Care and Use Committee at the Johns Hopkins University School of Medicine.

Histopathology Examination. When a mouse was sacrificed, its internal organs were fixed in 10% formalin, and the rest of the mouse (skeleton, testis, etc.) was fixed in Bouin’s solution. After being embedded in paraffin, 5-μm tissue sections were stained with H&E. The testis sections from 7- to 10-week-old mice were examined and photographed using a Nikon inverted microscope ECLIPSE TE300 with SPOT RT software (Diagnostic Instruments, Inc.). Over 100 tubules for each mouse were counted and classified into four categories (normal, hypospermatogenesisia, germinal arrest, and Sertoli cell only) by two independent investigators. For the metastasis analysis, pathological diagnoses were determined by two independent pathologists as a blind study.

Immunofluorescence and TUNEL Assay. Sections were deparaffinized and hydrated as described previously (11). For GCNA1 and PCNA staining, sections were stained after antigen retrieval with 1 mM EDTA with GCNA1 (undiluted; from G. C. Enders, University of Kansas, Kansas City, KS (38)), followed by anti-mouse IgG Cy3 (1:400; Jackson ImmunoResearch), PCNA C-20 (1:100; Santa Cruz Biotechnology), followed by donkey anti-goat IgG Cy3 (1:300; Jackson ImmunoResearch). TUNEL assay was performed using the TUNEL kit (Roche) per the manufacturer’s procedure. Four low-power fields (×200) were taken for each thymic lymphoma sample, and TUNEL-positive cells were counted as a blind study.

Metaphase Preparation. Metaphases from spleen, thymus, and bone marrow were generated as described previously (33). In brief, splenocytes and thymocytes were cultured in the presence of 5 μg/ml ConA + 20 μg/ml lipopolysaccharide, 750 ng/ml ionomycin + 20 μg/ml phorbol myristate acetate + 10 units/ml of IL-2, respectively, for 48 h before being harvested for metaphase preparation. To obtain bone marrow metaphases, mice received injections of 0.1 ml of 0.5% colchicine (Sigma) for 45 min before being sacrificed. Bone marrow cells were obtained by flushing femurs and tibias with fresh 75 mM KC1 and incubated at 37°C for 15 min, followed by fixation four times in fresh 3:1 methanol/glacial acetic acid.

Q-FISH and SKY. Q-FISH and SKY were performed on the same sets of metaphases as described previously (34, 35). The analysis of chromosome abnormalities (signal-free ends, translocations, fusions, and fragments) was carried out as a blind study. To avoid counting chromosome fragments as signal-free ends, the size of each chromosome lacking a telomere signal was closely compared with its homolog using SKY. If the chromosome was shorter than its homolog, the event was scored as a fragmentation and not as a signal-free end. Each chromosome fusion was identified by SKY, and the identity of all chromosome fusions was counted directly. In addition, to avoid overestimating the clonal events, each specific chromosome fusion in a given mouse was counted as one independent fusion event.

Kaplan-Meier Survival Curve. If the mouse dropped 20–40% of its body weight in a 3-day interval and was motionless with dramatic trembling or other signs of ill health, it was pronounced dead and evaluated by a complete pathological examination.

Mitotic Figures and Anaphase Bridges. The measurement of mitotic figures and the anaphase bridge index in the thymic lymphoma sections was counted as a blind study by two independent investigators. A total of 10 high-power fields (×600) were counted in each sample. Anaphase bridges were defined as one or more lines spanning two separating anaphase poles.

BrdUrd Labeling. Mice at 7–10 weeks with no signs of sickness received injections i.p. of BrdUrd (0.6 mg/10g body weight). One hour later, mice were sacrificed, and bone marrow cells were flushed out from the femurs and tibias using PBS. Cells were then fixed in cold 70% ethanol and stored overnight at −20°C. All of the following steps were done at room temperature. After being treated with 20 units/ml of Dnase (Roche) in 4.2 mM MgCl2/150 mM NaCl for 10 min, cells were incubated with anti-BrdUrd-FTTC (BD PharMingen; no dilution) for 30 min. Cells were then resuspended in 20 μg/ml of 7-ami- noacetoxydine D (Molecular Probes) for 30 min before being analyzed using the CELLQUEST program (BD).

Flow Cytometric Analysis. Single-cell suspensions were prepared from thymus or thymic lymphoma. After being washed once in PBS/2% BSA, cells were stained with antibodies for 15 min at room temperature before the analysis using the CELLQUEST program. Antibodies purchased from BD PharMingen were as follows: CD4-FTTC (RM4-5, 1:200), CD8-PE (53-6.7, 1:200), CD3-Cyochrome (145-2C11, 1:200) or CD8-FTTC (53-6.7, 1:200), TCR β-PE (H57-597, 1:200), CD4-Cyochrome (RM4-5, 1:300).

RESULTS

Generation of Atm−/− Mice with Short Telomeres Using Inter- generational Cross. Because it is the shortest telomere that triggers the cellular response to dysfunctional telomeres (33), we used an intergenerational breeding method to introduce short telomeres into Atm−/− mice. Atm−/− mTR−/− mice were crossed with fourth generation (G4) mTR−/− mice. The resulting progeny, Atm−/− mTR−/− iG6 (iG6 refers to the progeny of intergenerational crosses) were then intercrossed to obtain Atm−/− mTR−/− iG6 and Atm−/− mTR−/− iG6 mice. The litter size resulting from this cross was 2.8 ± 1.4 pups/litter, which is significantly less than the 7.3 ± 2.6 pups/litter for the cross that generated Atm−/− mTR−/− iG5 mice (20 litters; P = 0.0001), indicating that short dysfunctional telomeres lead to a loss of fertility as shown previously in the late-generation mTR−/− mice (10).

Increased Germ Cell Death in the Atm−/− mTR−/− iG6 Mice. To determine whether Atm is required for sensing telomere dysfunction, we examined germ cell death by assaying seminiferous tubule morphology. Late-generation mTR−/− mice show germ cell apoptosis and seminiferous tubule atrophy (10, 11). In Atm−/− mice, germ cells arrest and accumulate at the leptotene-pachytene stage of meiosis (27, 36). In Atm−/− mTR−/− iG6 mice, 6.5% of the tubules were atrophic and showed a Sertoli cell only phenotype (Ref. 37; Fig. 1, A and C). Notably, there was a more severe phenotype in Atm−/− mTR−/− iG6 mice with 78% of the tubules lacking germ cells (Fig. 1, A and C). The tubules that were not empty (−22%) showed the germline arrest characteristic of Atm−/− mTR−/− mice (Fig. 1, A and C).

To further measure the extent of germ cell loss, we stained testis sections for GCNA1, which is abundantly expressed in spermatogenesis and primary spermatocytes (38). There was a significant reduction in GCNA1 staining in the Atm−/− mTR−/− iG6 mice compared with wild-type mice and both single null mutant mice (Fig. 1, B and C). In addition, staining for PCNA, a marker of mitotic and premeiotic precursors (39), also showed extensive cell loss in Atm−/− mTR−/− iG6 (Fig. 1C). This tubule atrophy is not likely attributable to growth defects of the Atm−/− mTR−/− iG6 mice because at 7–10 weeks, these mice were of the same average size and grew at a rate similar to Atm−/− mTR−/− iG6 (Fig. 1, D). Therefore, unlike in p53−/− mTR−/− late-generation mice (3), tubule atrophy was not rescued by loss of Atm. We conclude that Atm is not essential for germ cell death in response to short telomeres. A similar conclusion was reached recently by Wong et al. (31) from their analysis of apoptosis in bone marrow cell culture and intestinal crypts in Atm+/− mTR−/− mice. Furthermore, the significant increase in seminiferous tubule atrophy in Atm−/− mTR−/− iG6 mice compared with either Atm−/− mTR+/+ or Atm+/+ mTR−/− iG6 suggests that Atm deficiency, together with short telomeres, accentuates telomere dysfunction.

Telomere-length Independent Cooperative Effect of Atm Deficiency and Short Telomeres. To determine whether the increased telomere dysfunction was telomere length dependent, we performed Q-FISH on metaphase chromosome spreads from lymphocytes of 7–10 week old mice to measure the change in telomere length. There was a slight reduction in average telomere length in Atm−/− mTR−/− compared with wild-type mice (Fig. 2B), consistent with the shortening seen in human A-T cells (25). Similarly, Atm−/− mTR−/− iG6 mice showed only a slight decrease in telomere length compared with Atm−/− mTR−/− iG6 mice (Fig. 2D). Because the shortest telomere triggers apoptosis (33), we quantitated the number of signal-free chromosome ends in Atm−/− mTR−/− iG6 and Atm−/− mTR−/− iG6 mice. To distinguish chromosome fragments from signal-free ends, we used FISH along with SKY. When fragments were eliminated, we

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found no significant difference in signal-free ends in Atm−/− mTR−/− iG6 lymphocytes compared with Atm+/+ mTR−/− iG6 (P = 0.46). Although overall telomere length was not measured by Wong et al. (31), they did report an increase in signal-free ends. The different results may be attributable to the methods used to exclude chromosome fragments from the analysis or different Atm mutant mouse models. Our study suggests that telomere shortening is not likely to account for the increased telomere dysfunction in the Atm−/− mTR−/− iG6 mice.

Chromosome Instability Induced by Increased Telomere Dysfunction. Next, we examined the impact of Atm deficiency and short telomeres on chromosomal rearrangements using SKY. A high rate of chromosome fusions was seen in both Atm−/− mTR−/− iG6 and Atm+/+ mTR−/− iG6 animals (Table 1). In contrast, only one fusion was present in the Atm single null mouse (Table 1), suggesting that loss of Atm alone does not lead to telomere dysfunction. Although the total number of fusion events was higher in the Atm+/+ mTR−/− iG6 mice compared with the Atm−/− mTR−/− iG6 littermates (Table 1), this increase was primarily attributable to the clonal expansion of two fusion events, 12p;14p and 9p;14p (Fig. 3C). Although they are likely clonal, these two fusions do not represent germ-line events because they were not present in all metaphases from one given mouse (data not shown). To avoid overrepresenting clonal fusion events, we quantified the number of fusions between specific chromosomes in each mouse as “independent fusion events” (Table 1 and Fig. 3C). The spectrum of chromosomes involved in independent fusion events differed significantly between Atm−/− mTR−/− iG6 and Atm−/− mTR−/− iG6 mice (Fig. 3C). Although all fusions in Atm−/− mTR−/− iG6 mice were p-arm-to-p-arm, the Atm−/− mTR−/− iG6 mice showed both p-arm-to-p-arm and q-arm-to-q-arm fusions (Fig. 3 and Table 1). In addition, many more independent chromosome ends were involved in fusions in Atm−/− mTR−/− iG6 mice than in Atm+/+ mTR−/− iG6 mice (Fig. 3C). Of 162 metaphases examined for both genotypes, 25 different chromosome ends were involved in 25 independent fusion events in Atm−/− mTR−/− iG6 mice, whereas only 8 chromosome ends were involved in 12 independent events in Atm+/+ mTR−/− iG6 mice (Fig. 3C). Finally, there was an expected increase in chromosome fragments and translocations in both Atm−/− mTR−/− and Atm−/− mTR−/− iG6 mice compared with the Atm+/+ mice (Table 1), as has been documented previously in Atm−/− mice (26, 40). Therefore, the increase in q arm fusions and the increased number and change in the repertoire of the chromosomes involved in fusion in Atm−/− mTR−/− iG6 mice suggest that loss of Atm function increases telomere dysfunction of short telomeres.

Delayed and Reduced Incidence of Thymic Lymphoma in Atm−/− mTR−/− iG6 Mice. We next determined how dysfunctional telomeres affect tumorigenesis in the Atm background. The majority of Atm−/− mice from this mouse model typically develop thymic lymphoma and die around 16 weeks of age (26–28, 41). In our colony, the median survival time was 15 weeks for Atm−/− mTR−/− iG6 mice (Fig. 4A). Strikingly, the Atm−/− mTR−/− iG6 mice had a median survival time of 36 weeks (P = 0.0009; Fig. 4A). In total, 47% of the Atm−/− mTR−/− iG6 mice (7 of 15) survived for over 44 weeks (Fig. 4A). Thymic lymphomas were found in 7 of 8 Atm−/− mTR−/− iG6 mice that died. A lymphoma of B-cell lineage was observed in one Atm−/− mTR−/− iG6 mouse with an atrophic thymus (Fig. 4A, asterisk). Thymic lymphoma did not occur in one Atm−/− mTR−/− or Atm+/+ mTR−/− iG6 mouse that died (Fig. 4A). Histopathological examination revealed lymphoma cells invading a variety of organs including liver, kidney, bone marrow, and skeletal
Atm LOSS ACCENTUATES TELOMERE DYSFUNCTION

Fig. 2. ATM deficiency has no significant effect on telomere length. A. Q-FISH data from Atm−/− mTR+/− are shown in blue. B, the data from A are shown overlayed with telomere signals from Atm−/− mTR+/− metaphases shown in magenta. The mice examined are littersmates from a cross of double heterozygotes. C. Q-FISH data from Atm−/− mTR+/− iG6 mice are shown in red. D, the Q-FISH signal from Atm−/− mTR+/− iG6 (green) is overlayed with the data from the Atm−/− mTR+/− iG6 littersmates in red as in C. Overlays are shown to highlight the small difference in telomere length seen in the Atm−/− mice. A total of 18 metaphases (~3,000 telomere signals) were analyzed for each genotype. The data represent three independent experiments with three mice for each genotype. E, signal-free ends (SFE) were counted manually after overexposing the telomere signal. A total of 75 metaphases from 4 independent Atm−/− mTR+/− iG6 mice, 88 metaphases from 5 independent Atm−/− mTR+/− iG6 mice, 73 metaphases from 5 independent Atm−/− mTR+/− iG6 mice, and 100 metaphases from 6 independent Atm−/− mTR+/− iG6 mice were analyzed. Frequency of SFE, the number of SFE per metaphase per mouse. Bars, SD of the means from each mouse of that genotype.

Table 1 Summary of chromosome abnormalities

<table>
<thead>
<tr>
<th>Mouse genotype</th>
<th>No. of metaphases</th>
<th>Independent fusion eventsa</th>
<th>Fragments</th>
<th>Translocations</th>
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<tbody>
<tr>
<td></td>
<td>(n = number of mice)</td>
<td>p&lt;sub&gt;i&lt;/sub&gt;</td>
<td>q&lt;sub&gt;i&lt;/sub&gt;</td>
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</tr>
<tr>
<td>Atm−/− mTR+/− (n = 4)</td>
<td>112</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Atm−/− mTR+/− (n = 5)</td>
<td>101</td>
<td>0</td>
<td>1 (1)</td>
<td>44</td>
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<tr>
<td>Atm−/− mTR+/− iG6 (n = 5)</td>
<td>162</td>
<td>12 (100)</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Atm−/− mTR+/− iG6 (n = 6)</td>
<td>162</td>
<td>17 (35)</td>
<td>8 (14)</td>
<td>36</td>
</tr>
</tbody>
</table>

a One independent fusion event is one specific fusion event in a given mouse. Numbers in parentheses indicate total fusion events scored. p<sub>i</sub>G indicates p-arm-to-p-arm Robertsonian-type fusion. q<sub>i</sub>G indicates q-arm-to-q-arm dicentric fusion.

muscle in both Atm−/− mTR+/− and Atm−/− mTR−/− iG6 mice (Supplementary Table 1). In addition to the lymphoid (T- and B-cell) tumors, we also saw ovarian tubulostromal adenoma and squamous cell carcinoma in the dying Atm−/− mTR−/− iG6 mice (Supplementary Table 1). The development of the non-T-cell tumors may be attributable to the increased life span of these mice. A recent study also found a decrease in thymic lymphoma in Atm−/− mTR−/− mice (31), but because the mouse model they used developed thymic lymphoma at a very advanced age (52 weeks; Ref. 29), no increase in life span was seen in that study (see “Discussion”).

The increase in survival of the mice was not attributable to the change in growth rate because the growth rates of Atm−/− mTR+/− and Atm−/− mTR−/− iG6 mice were similar (Fig. 4B). To further exclude the possibility that extended survival was attributable to the proliferation defects in Atm−/− mTR−/− iG6 cells, we analyzed BrdUrd incorporation in bone marrow cells from healthy, tumor-free mice 7 to 10 weeks of age. No significant difference was found between Atm−/− mTR+/+ and Atm−/− mTR−/− iG6 mice (Fig. 4C). Similar results were obtained for BrdUrd incorporation in thymocytes and splenocytes from the same mice (data not shown). Thus, the delayed incidence of thymic lymphoma in the Atm−/− mTR−/− iG6 mouse was not attributable to general growth or proliferation defects.

Increased Apoptosis in Thymic Lymphoma of Atm−/− mTR−/− iG6 Mice. The delayed incidence of thymic lymphoma in the Atm−/− mTR−/− iG6 mice may result from the cooperative effect of short telomeres and Atm deficiency on apoptosis as we found in the testis. To examine apoptosis directly on thymic lymphoma sections, we used the TUNEL assay. Tumors from Atm−/− mTR−/− iG6 mice (n = 4) exhibited significantly more TUNEL-positive cells than those from Atm−/− mTR+/+ mice (n = 6; P = 0.01; Fig. 5A). Moreover, unlike Atm−/− mTR+/+ thymic lymphomas that were easily cultured in vitro, we were not able to establish cultures from Atm−/− mTR−/− iG6 tumors, even in the presence of IL-2 and other mitogens (data not shown). Thus, increased apoptosis, presumably induced by telomere dysfunction, may in part explain the decreased incidence and/or increased latency of thymic lymphoma.

Telomere dysfunction often results in the appearance of anaphase bridges through the formation of dicentric chromosomes (4, 42, 43). To examine telomere dysfunction in tumors, we measured the anaphase bridge index in H&E stained sections of thymic lymphomas from Atm−/− mTR+/+ and Atm−/− mTR−/− iG6 mice (n = 4 for each genotype). There was a significant increase in the number of anaphase bridges in the Atm−/− mTR−/− iG6 tumor sections (Fig. 5C). Over 90% of the anaphases from Atm−/− mTR−/− iG6 mice had at least one bridge in contrast to 37% in the Atm−/− mTR+/+ mice (P = 0.0001; Fig. 5, B and C). Thus, the increase in the number of anaphase bridges in the Atm−/− mTR−/− iG6 tumors is consistent with the increased telomere dysfunction.

To determine whether a decrease in tumor cell proliferation also contributed to increased tumor latency, we measured the mitotic index in H&E sections of thymic lymphomas. No significant difference in the number of mitotic figures was seen between Atm−/− mTR+/+ and Atm−/− mTR−/− iG6 mice (n = 4 for each; P = 0.23; Fig. 5D). Thus, although we saw no evidence of decreased proliferation of Atm−/− mTR−/− iG6 tumor cells, we did find evidence for increased telomere dysfunction in these tumors.

Transformation of a Different T-Cell Type in Atm−/− mTR−/− iG6 Mice. Thymic lymphomas in Atm−/− mice are positive for both CD4 and CD8 immune T-cell lineage markers (26, 27). The double-positive state of these tumors suggests that they arise early in T-cell development before the transition to more mature, CD4+ or CD8+ single-positive cells. Tumors that arise in double-knockout mice Atm−/− p21−/− and Atm−/− p53−/− (44) and the NBS1−/− mouse (45) also are CD4+CD8+ double positive. Consistent with early observations, we found that the thymic lymphomas in Atm−/− mTR−/− mice (n = 5) were CD4+CD8+ with either high or low levels of T-cell maturation markers CD3 (Fig. 6A) and TCR-β chain (data not shown; Ref. 46). Surprisingly, thymic lymphomas derived from the Atm−/− mTR−/− iG6 mice (n = 4) were CD8+CD4− and did not express TCR-β/CD3 complex on their surface (Fig. 6A and data not shown).

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shown). Despite the lack of surface TCR-β/CD3 expression, PCR analysis using the primers specific for V/H9252/J/H9252 5.1 and V/H9252/H9252 8.2 revealed that these cells had undergone V(D)J rearrangement (Supplementary Fig. 1). Thus, these CD4⁻CD8⁻ cells had apparently progressed beyond the CD4⁻CD8⁻ double-negative stage. These cells, however, are not derived from mature CD8⁺ T cells but rather may represent immature single-positive cells, a stage between the double-negative and the double-positive stages (47–49). When cultured in vitro, cells from the Atm⁻/⁻ mTR⁻/⁻ iG6 tumors did not respond to a variety of mitogenic stimuli, including phorbol myristate acetate, ionomycin, and IL-2 (data not shown), consistent with their being intermediate, immature, single-positive cells (47, 48, 50). Furthermore, after 24 h of in vitro culture, tumor cells differentiated into CD8⁺CD4⁻ T cells with increased expression of CD3 (Fig. 6B), suggesting that these CD8⁺ cells from Atm⁻⁻ mTR⁻⁻ iG6 mice are at an earlier developmental stage than the double-positive cells (47, 48, 51, 52).

To exclude the possibility that in the Atm⁻⁻ mTR⁻⁻ iG6 mice T-cell development was blocked at the CD8⁺ immature, single-positive stage, we examined T-cell types in thymuses of healthy mice that were 7–10 weeks of age. The percentages of double-positive, double-negative, and single-positive (CD4⁺ or CD8⁺) thymocytes

<table>
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<tr>
<th>Mouse genotype</th>
<th>Shared events</th>
<th>Non-shared events</th>
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<tr>
<td></td>
<td>Fusion</td>
<td>Numbers</td>
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<tr>
<td>Atm⁻/⁻ mTR⁻/⁻ iG6 (n=5)</td>
<td>9p;9p 1 (3)</td>
<td>2p;15p 1 (1)</td>
</tr>
<tr>
<td></td>
<td>12p;12p 3 (6)</td>
<td>10p;10p 1 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16p;18p 1 (1)</td>
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<td>9p;9p 1 (1)</td>
<td>1p;17p 1 (1)</td>
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<tr>
<td></td>
<td>12p;12p 2 (6)</td>
<td>3p;14p 1 (1)</td>
</tr>
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<td>6p;Xp 1 (1)</td>
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<td></td>
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</tr>
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</table>

Fig. 3. Increased independent fusion events in the Atm⁻⁻ mTR⁻⁻ iG6 mice. A and B, two representative unique fusion events in the Atm⁻⁻ mTR⁻⁻ iG6 mice indicated by arrowheads: A, single q-arm fusion f(1q;Xq); B, p-arm-to-p-arm fusion f(5p;17p). C, summary of independent fusion events. Number indicates independent fusion events scored. Number in the parentheses indicates total events scored for the independent fusion event from each genotype. n is the number of mice for each genotype.

Fig. 4. Decreased and delayed incidence of thymic lymphoma in Atm⁻⁻ mTR⁻⁻ iG6 mice. A, Kaplan-Meier survival curve of Atm⁻⁻ mTR⁻⁻ iG6, Atm⁻⁻ mTR⁻⁻ iG6, Atm⁻⁻ mTR⁻⁻ iG6, and Atm⁻⁻ mTR⁻⁻ iG6 mice is shown. Tick marks indicate the current age of individual surviving mice. *, the mouse died without thymic lymphoma. n indicates the number of mice. B, similar growth rates were observed between Atm⁻⁻ mTR⁻⁻ iG6 and Atm⁻⁻ mTR⁻⁻ iG6 mice. The body weight of each cohort was followed over a period of 24 weeks. Each data point represents at least 7 individual mice, except for Atm⁻⁻ mTR⁻⁻ iG6 mice from week 20 only represents 1–2 surviving mice. A and B share the same legend. C, BrdUrd analysis of the bone marrow cells from young tumor-free mice shows no difference in cellular proliferation among four different genotypes. The boxed area refers to the BrdUrd⁺ cells that have been through or are still in S-phase. The numbers indicate the percentage of BrdUrd⁺ cells in the population. The data represent two independent experiments.
DISCUSSION

Telomere dysfunction is thought to mimic a DNA double-strand break and activate cell cycle arrest or apoptosis (53, 54). In cultured human cells, the cellular response to telomere dysfunction is both p53 and ATM dependent (8). Our study and the recent one by Wong et al. (31) indicate that ATM deficiency and short telomeres lead to increased apoptosis in mice, indicating that ATM is not required for cell death induced by progressive telomere shortening. The discrepancy in the requirement of ATM for apoptosis triggered by dysfunctional telomeres could be attributable to the differences in human versus mouse cells (55), the use of cultured cell lines versus in vivo models, or a mechanistic difference in how loss of telomere function through progressive telomere shortening versus loss of telomere binding proteins are recognized by the cell. If ATM is not required for apoptosis in response to telomere dysfunction, then other damage sensors such as ATR (56, 57) are likely to be involved. Recent data show that ATM and ATR act in distinct but partially overlapping pathways in response to specific types of DNA damage. ATM is more specialized for response to ionizing radiation, whereas ATR responds to a broad range of damage including ionizing radiation, UV, and stalled replication forks (58). In addition, unlike ATM, ATR binds to asynapsed chromosomes during meiosis (59, 60), suggesting that ATM and ATR may play a different role in recognition of DNA damage. Recent work in yeast suggests that Mec1 (ATR homolog), but not Tel1 (ATM homolog), is required for the cell cycle arrest of telomerase-deficient cells (61, 62).

The cooperative effect of ATM deficiency and short telomeres could be attributable to several causes. Telomere dysfunction sensitizes cells to ionizing radiation (63, 64), suggesting that the total amount of damage determines the cellular response. Thus, the increased apoptosis may result from the sensitizing effect of chromosome breaks that are present in the absence of ATM on the response to short telomeres. Alternatively, ATM loss may directly impair the residual protective function of short telomeres leading to increased telomere dysfunction. Our data support the latter model. In the ATM−/− mTR−/− iG6 and not the ATM−/− mTR−/− iG6 lymphocytes, there was an increase in end-to-end fusions consistent with increased telomere dysfunction but not with an additive effect of DNA breaks. We saw no increase in chromosome breaks in lymphocytes from ATM−/− mTR−/− iG6 mice compared with ATM−/− mTR−/− mice (Table 1). Furthermore, ATM clearly plays a direct role in telomere function in yeast. Absence of the ATM homolog Tel1 causes telomere shortening (20, 21), and loss of both the ATM and ATR homologs inhibits the ability of telomerase to elongate telomeres (23, 24). Recent work in yeast indicates a direct role for Tel1 in telomere protection (65). Thus, ATM may function at telomeres through direct binding to short telomeres, phosphorylation of telomere-binding proteins, or through a less direct regulation of the function of other telomere proteins. We propose that in the absence of ATM, the loss of this protection drives short telomeres to become prematurely dysfunctional without further shortening. The protection provided by ATM is not essential for long telomeres, perhaps because of the ample presence of telomere-binding proteins, such as TRF1, TRF2, or Pot1.

Several lines of evidence suggest that telomere dysfunction can limit tumor growth: (a) inhibition of telomerase in human cancer cells in culture leads to cell death (65–70); (b) double mutants of telomerase and Ink4a/Arf−/− resulted in a reduction of tumors in the late-generation mice with short telomeres (6); (c) telomere dysfunction enhances the chemotherapeutic effect on cancer cells (71); and (d) double-mutant ApChimaeric mTR−/− late-generation mice show increased tumor initiation (microscopic adenomas) but decreased progression of the tumors into macroscopic adenomas (4). In support of the role of short telomeres in blocking tumor progression, this study and the work by Wong et al. (31) found a decrease in thymic lymphoma incidence in the ATM−/− mTR−/− double null mice. In addition, our double-mutant mice had a significant survival advantage. This difference in overall survival is likely attributable to the differing genetic background and/or mutagenesis strategies used in generating the ATM single-mutant mice (26, 29).

The observed reduction in thymic lymphoma incidence could be attributable to several different mechanisms: increased apoptosis, decreased tumor cell growth, delayed tumor initiation, or a combination of these factors. Our data suggest that the increased apoptosis attributable to the cooperative effect of short telomeres and ATM loss plays a major role in tumor reduction. Similar to the increased apoptosis seen in the germ cells in ATM−/− mTR−/− iG6 mice, we found increased apoptosis in the ATM−/− mTR−/− iG6 lymphomas compared with ATM−/− mTR−/− lymphomas. The significant increase in...
Fig. 6. Thymic lymphomas from Atm−/− mTR−/− iG6 mice are derived from CD8+ immature, single-positive T cells. A and B, Flow cytometric analysis on thymocytes and thymic lymphoma cells. A, fresh thymocytes from one control Atm+/+ mTR+/+ and Atm+/− mTR−/− iG6 mouse and thymic lymphomas derived from two Atm−/− mTR−/− and Atm−/− mTR−/− iG6 mice were stained for CD4, CD8, and CD3. The CD4+CD8+ double-positive (DP) and CD8+ single-positive (SP) cells were gated for expression of CD3. The percentages of DP, SP, and CD3+ cells are indicated in each panel. B, thymic lymphomas from Atm−/− mTR−/− iG6 mice cultured for 24 h in vitro and stained for CD4, CD8, and CD3. The CD4+CD8+ double-positive (DP) and CD8+ single-positive (SP) cells were gated for expression of CD3. C, T-cell development in the Atm−/− mTR−/− iG6 mice was similar to wild type. Thymocytes from tumor-free mice, 7–10 weeks of age, of four different genotypes were examined for CD4 and CD8 expression. The genotype is indicated on top of each panel. The percentage of each population is shown in each quadrant.

in anaphase bridges in the Atm−/− mTR−/− iG6 lymphomas indicates ongoing telomere dysfunction in the tumors. In contrast, the mitotic index in Atm−/− mTR−/− iG6 lymphomas was similar to Atm−/− mTR+/+ mice, suggesting that decreased proliferation did not play a major role.

A decrease in tumor initiation might also contribute to the decreased latency of death from thymic lymphoma in Atm−/− mTR−/− iG6 mice. The switch in cell type from the CD4+CD8+ double-positive cells in Atm−/− to CD8+ immature, single-positive cells in Atm−/− mTR−/− iG6 lymphomas suggests that tumors arise in a different cell population in Atm−/− mTR−/− iG6 mice. T-cell development progresses from a CD4+CD8+ double-negative stage through a CD8+ immature, single-positive stage to CD4+CD8+ double-positive and further to mature CD4+ or CD8+ single-positive cells (46). The CD8+ immature, single-positive cells represent 1–2% of developing T cells in thymus and are actively cycling (47–49, 72).
The developmental transition from immature, single-positive cells to double-positive cells involves a large clonal expansion (46). If the cycling and the expansion of immature, single-positive cells in Atm$^{-/-}$ mTR$^{-/-}$ iG6 mice are limited by telomere dysfunction, tumor initiation at the double-positive stage may be inhibited. Eventually however, sufficient instability may accumulate in the CD8 immature, single-positive cells and lead to tumor initiation. The delay in initiation may contribute to the increased latency of death attributable to thymic lymphoma in Atm$^{-/-}$ mTR$^{-/-}$ iG6 mice. Further analysis of the TCR rearrangements in these tumors will help pinpoint the specific subpopulation of cells that are transformed.

The increased survival of the Atm$^{-/-}$ mTR$^{-/-}$ iG6 mice provides a powerful model for the further study of A-T disease. In A-T patients, tumors account for only 10–15% of deaths, whereas the majority die from infections attributable to immunodeficiency (73). Although thymic lymphoma is the only tumor type seen in the Atm$^{-/-}$ mouse, A-T patients develop a variety of tumors including B-cell tumors and non-lymphoid tumors, such as ovarian, uterine, and stomach tumors (73, 74). The short life spans of the Atm$^{-/-}$ mice likely contribute to the phenotype difference with human A-T patients. The significant increase in the life span of the Atm$^{-/-}$ mTR$^{-/-}$ iG6 mouse will allow examination of the older Atm$^{-/-}$ mTR$^{-/-}$ iG6 mice and may thus provide insights into other clinical features of A-T patients.

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