DNA Damage and Repair

53BP1 Is Limiting for NHEJ Repair in ATM-deficient Model Systems That Are Subjected to Oncogenic Stress or Radiation

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Abstract

The DNA damage response (DDR) factors ataxia telangiectasia mutated (ATM) and p53 binding protein 1 (53BP1) function as tumor suppressors in humans and mice, but the significance of their mutual interaction to the suppression of oncogenic translocations in vivo has not been investigated. To address this question, the phenotypes of compound mutant mice lacking 53BP1 and ATM (Trp53bp1−/−/Atm−/−), relative to single mutants, were examined. These analyses revealed that loss of 53BP1 markedly decreased the latency of T-lineage lymphomas driven by RAG-dependent oncogenic translocations in Atm−/− mice (average survival, 14 and 23 weeks for Trp53bp1−/−/Atm−/− and Atm−/− mice, respectively). Mechanistically, 53BP1 deficiency aggravated the deleterious effect of ATM deficiency on nonhomologous end-joining (NHEJ)-mediated double-strand break repair. Analysis of V(D)J recombinase-mediated coding joints and signal joints in Trp53bp1−/−/Atm−/− primary thymocytes is, however, consistent with canonical NHEJ-mediated repair. Together, these findings indicate that the greater NHEJ defect in the double mutant mouse resulted from decreased efficiency of rejoining rather than switching to an alternative NHEJ-mediated repair mechanism. Complementary analyses of irradiated primary cells indicated that defects in cell-cycle checkpoints subsequently function to amplify the NHEJ defect, resulting in more frequent chromosomal breaks and translocations in double mutant cells throughout the cell cycle. Finally, it was determined that 53BP1 is dispensable for the formation of RAG-mediated hybrid joints in Atm−/− thymocytes but is required to suppress large deletions in a subset of hybrid joints.

Implications: The current study uncovers novel ATM-independent functions for 53BP1 in the suppression of oncogenic translocations and in radioprotection.

Visual Overview: http://mcr.aacrjournals.org/content/11/10/1223/F1.large.jpg.

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Introduction

In response to DNA double-strand breaks (DSB), mammalian cells have evolved the DNA damage response (DDR), a set of ubiquitous factors responsible for their detection, signaling, and repair (1, 2). Upon DSB induction, the ataxia telangiectasia mutated (ATM) kinase phosphorylates hundreds of substrates to promote cell-cycle checkpoint activation and repair via either nonhomologous end-joining (NHEJ) or homologous recombination (HR; ref. 3). In this context, ATM phosphorylates MDC1 to promote chromatin ubiquitination, an essential step for the recruitment of the 53BP1 adaptor to specific histone marks at sites of DSBs (4). 53BP1 in turn facilitates NHEJ-mediated repair via several mechanisms (reviewed in ref. 5), such as protecting broken ends from extensive end-resection (6, 7) and, in some cellular contexts, regulation of higher order chromatin reorganization (8, 9). Activation of the DDR represents a key barrier to cancer development (10) and, accordingly, ATM and 53BP1 are tumor suppressors in humans. Individuals with the hereditary disease ataxia-telangiectasia (A-T; lacking ATM) are markedly cancer-prone (11). Moreover, acquired ATM mutations are frequent in B-cell malignancies, where they correlate with aggressive disease and poor clinical outcome (12). Similarly, diminished 53BP1 expression is observed in lymphomas as well as solid tumors (13–17). Moreover, low levels of 53BP1 often correlate with aggressive disease (13–17) and may represent a main mechanism for acquisition of resistance to therapy in breast cancers (18).

Previous studies indicate that 53BP1 functions to concentrate activated ATM and other repair factors at sites of...
DSBs (19, 20). In turn, ATM-dependent phosphorylation of the 53BP1 amino terminus is essential for recruitment of R NF1 (21–24), the downstream mediator of 53BP1 in the suppression of end-resection (21–25). However, little is known on how these molecular interactions modulate the formation of chromosomal translocations in vivo, particularly in the context of oncogenic stress. Here, we address this question via analysis of newly generated compound mutant mice (Trp53bp1–/–/Atm–/– mice).

Materials and Methods

Mice and cells

Trp53bp1–/– mice (26) and Atm–/– mice (27) were obtained from Drs. Junjie Chen and Fred Alt, respectively. Trp53bp1–/– mice were backcrossed into 129/Sv mice for 6 generations and then bred to 129/Sv Atm–/– mice to generate double mutants and all corresponding controls. All mouse experiments were carried out in accordance with Institutional Animal Care and Use Committee (IACUC)-approved protocols and following guidelines from the US Public Health Policy on Humane Care and Use of Laboratory Animals. Purification and in vitro activation of mature B and T lymphocytes was done as described (28). For irradiation experiments, mice or cells were exposed to 2 Gy of IR using a 137Cs source (GammaCell40), at a rate of 0.5 Gy/min.

Immunophenotyping and cell-cycle analysis

Thymocytes and splenocytes of 8- to 12-week-old mice were stained with antibodies to T- or B-cell markers, as described (28). For quantification of the mitotic population, cells were co-stained with an α-phospho-histone H3 (Ser10) antibody (Millipore) and propidium iodide (PI). Data were acquired using a FACSCalibur and analyzed with FlowJo software.

Cytogenetic assays

Metaphase preparation and telomere FISH were previously described (28). For TCRα/β locus-specific FISH, we detected the 5′ end of the locus using BAC RP23-304L21 (purchased from Children’s Hospital Oakland Research Institute) and the 3′ end using BAC TCR-Çα (kind gift of Dr. Carol Greider). BAC labeling, hybridization and detection were conducted as described (28). Paints to mouse chromosomes 12 and 14 were purchased from Cambio and Dr. Carol Greider). BAC labeling, hybridization and detection were conducted as described (28). All images were acquired using a Zeiss Axioplan Imager Z.1 microscope equipped with a Zeiss AxioCam and an HXP120 mercury lamp (Jena GmbH) and dedicated software (Zeiss Axiovision Rel 4.6; ref. 28). Spectral karyotyping analysis was done as described (29).

Histopathology, immunohistochemistry, and TUNEL assay on mouse tissues

Five-micrometer sections from formalin-fixed, paraffin-embedded mouse organs were stained with hematoxylin and eosin (H&E) and evaluated by a veterinary pathologist (D.L. Huso). Immunostaining and detection of TUNEL+ cells were done as previously described (29).

Immunoblotting

Cells were resuspended in radioimmunoprecipitation assay (RIPA) buffer and protein transferred to polyvinylidene fluoride (PVDF) membranes as described (29). Antibodies used were: p53 (clone 1C12, Cell Signaling; 1:1,000); phospho-p53 (Ser15; Cell Signaling; 1:1,000); KAP-1 (Abcam, 1:1,000); phospho-KAP1 (Bethyl Laboratories, 1:5,000), or α-tubulin (Millipore, 1:5,000).

Real-time quantitative PCR

Thymocytes were resuspended in TRIzol and RNA extracted following manufacturer’s protocols. Two micrograms of RNA was reverse-transcribed using RT-III (Invitrogen), and cDNA was amplified using Power Sybr Green PCR Master Mix in a 7900HT Fast Real-Time PCR System with SDS v2.3 software. Data were analyzed using RQ Manager v1.2, all from Applied Biosystems. Primers were: p21-F: 5′-TCCACACGGATATCCAGACATT-3′; p21-R: 5′-ACGGCCCTCCCCAGAAGTTG-3′; Bax-F: 5′-CAAGATGCGTCCACCAAGA-3′; Bax-R: 5′-CGTGTC-CACTGCAAGATCT-3′, GAPDH:F: 5′-CATGGCCTTCCGTTGCATCA-3′, GAPDH-R: 5′-TGCTGCTTCACACCTCTTCT-3′.

Indirect immunofluorescence on cells and tissue cryosections

Splenocyte cytopsins or 5-μm thymus cryosections were fixed in 4% paraformaldehyde (PFA) and immunofluorescence for γ-H2AX was done as described (28).

Sequencing of coding joints and signal joints

All analyses were done on thymus gDNA from 7-day-old mice. PCR, cloning, and sequencing of the Vβ5–Dβ2 signal joints (SJ) were conducted as described (30). PCR, cloning, and sequencing of the Vβ2–Jβ1 coding joints (CJ) were done as for the SJ analysis, using published primers (8).

Hybrid joint analysis

For DJβ2–Vβ14 hybrid joint (HJ) analysis, the joint was first amplified using previously described primers (31) or newly designed primers equidistant from the junction. For the latter experiments, the primary reaction primers were: TCR5′β-J: Mus: GTGCACTCCAGAGTGCTCATGC and TCR 3′β Mus: CTAGACAAAGACCATCGTGACTATGC, with an annealing temperature of 56.8°C for 20 cycles. The secondary reaction primers were: TCR 3′β J: Mus inside: GCACAGACAAAGACGATGC and TCR 3′β Mus inside: CCTTCTCCTGGCGTCTGTT, with an annealing temperature of 57.4°C for 30 cycles. A portion of the Rag1 locus was amplified from the genomic templates as a loading control, as described (30). DJβ2–Vβ14 HJ PCR products were transferred to a nylon membrane and hybridized overnight with a 32P-labeled probe that recognizes sequences in the 5′ portion of Vβ14. Specific amplicons representing HJs harboring deletions were cut from the gel, purified, cloned into TOPO-pCR2.1 (Invitrogen), and sequenced.
Statistical analysis
We evaluated significance using the Student t test on 3 to 5 data points per genotype. For analysis of survival, we used the log-rank test.

Results

Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup> mice are viable and show no additional phenotypes in growth and development relative to Atm<sup>−/−</sup> mice

Intercrosses of Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup> mice produced Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup> mice at approximately Mendelian ratios (10 of 102 mice; expected = 6.4; Supplementary Table S1). Like Atm<sup>−/−</sup> and, to a lesser extent, Trp53bp1<sup>−/−</sup> mice, double mutants are growth retarded (Supplementary Fig. S1A and S1B). However, the effect of the combined deficiency on growth was not additive, but comparable to the defect in Atm<sup>−/−</sup> mice (Supplementary Fig. S1A and S1B). Similarly, the decreased cellularity and relative paucity of mature T cells observed in Atm<sup>−/−</sup> thymi and spleens were not further aggravated by concomitant 53BP1 deficiency (Supplementary Fig. S1C–S1H). Pathology of Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup> organs revealed severe ovarian/testicular atrophy (comparable to Atm<sup>−/−</sup> mice; Supplementary Fig. S1I and data not shown) and no significant alterations in the brain (not shown). Altogether, these findings suggest that 53BP1 and ATM function in mostly overlapping pathways during growth and development.

53BP1 deficiency accelerates lymphomagenesis in Atm<sup>−/−</sup> mice

Organismal development is mostly impaired in the context of deficiencies for HR, a pathway that is dependent on ATM but not 53BP1 (28, 32). We therefore sought to investigate nonoverlapping roles for ATM and 53BP1 in an NHEJ-dependent process: V(D)J recombination in developing thymocytes. Here, recombination-activating gene (RAG) introduces DSBs within antigen receptor loci in the G<sub>1</sub> phase of the cell cycle and NHEJ repairs them before DNA synthesis (30). In Atm<sup>−/−</sup> thymocytes, defective NHEJ leads to their aberrant rejoining to DSBs elsewhere to form chromosomal translocations (31, 33). Over time, selection for oncogenic translocations drives lymphomagenesis (33). To assess the effect of 53BP1 deficiency on this process, we first examined lymphoma proneness of Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup> and Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup> mice, relative to Atm<sup>−/−</sup> controls. Consistent with previous findings (27), 18 of 39 (46.1%) Atm<sup>−/−</sup> mice had succumbed at 6 months of age.

Figure 1. 53BP1 deficiency accelerates thymic lymphomagenesis in Atm<sup>−/−</sup> mice. A, survival (Kaplan–Meier) curves of Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup> and control mice. B, moribund Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup> mice were euthanized and organs stained with H&E. Thymus architecture was effaced by lymphoma growth. Black arrows point to lymphoblasts infiltrating the kidney, epicardium, and lung parenchyma. C, immunophenotyping of Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup> tumor cells after staining with antibodies to CD4 and CD8. D, examples of clonal chromosomal rearrangements involving chromosome 14 in Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup> tumor cells using chromosome paints. E, schematic of the TCR<sub>α/β</sub> locus in mouse chromosome 14 and location of BAC probes (not to scale). F, read-out of FISH assay. G, representative example of a Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup> tumor metastasis showing “split” BAC signals and massive amplification of the S probe (red).
Significantly, 35 of 38 (92.1%) Trp53bp1$^{+/–}$/Atm$^{-/–}$ mice had died at the same time point. Overall, average survival was 23 and 14 weeks for Atm$^{-/–}$ and Trp53bp1$^{+/–}$/Atm$^{-/–}$ mice, respectively (Fig. 1A; $P = 0.0001$). In addition, 37 of 38 (100%) Trp53bp1$^{+/–}$/Atm$^{-/–}$ necropsies revealed thymic lymphoma as the cause of death. Frequent tumor infiltrates were present in all major organs of Trp53bp1$^{+/–}$/Atm$^{-/–}$ mice at the time of death (Fig. 1B). We conclude that loss of 53BP1 markedly decreases the latency and increases the penetrance of thymic lymphogenesis in Atm$^{-/–}$ mice. In contrast, the average survival of Trp53bp1$^{+/–}$/Atm$^{-/–}$ was comparable to Atm$^{-/–}$ mutants (25 weeks; Fig. 1A), indicating that one copy of 53BP1 is sufficient to suppress lymphogenesis in this setting.

Atm$^{-/–}$ lymphomas originate from thymic precursors undergoing V(DJ) recombination and invariably harbor clonal translocations with a breakpoint at the TCRα/δ locus in chromosome 14 (33) and amplification of upstream sequences (34). Similarly, Trp53bp1$^{+/–}$/Atm$^{-/–}$ lymphoblasts were typically double positive (CD4$^+$/CD8$^+$) or, less frequently, single positive (CD4$^+$ or CD8$^+$; Table 1; Fig. 1C), lacked significant aneuploidy (Table 1; Supplementary Fig. S2) and 6 of 6 tumors contained a clonal translocation involving chromosome 14, with 4 of 6 harboring a t(12,14) in more than 90% of metaphases (Table 1; Fig. 1D). Moreover, hybridization with BAC probes that recognize sequences over the Cα region or immediately centromeric to the Vα region revealed “split” BAC signals and marked amplification of sequences 5′ to the TCRα/δ locus in most lymphoma cells ($n = 6$ tumors; Fig. 1E–G; Supplementary Fig. S3 for additional examples). More limited analyses by spectral karyotyping (SKY) confirmed these findings and revealed other co-existing clonal translocations (see Supplementary Fig. S4), in line with previous analyses of Atm$^{-/–}$ lymphomas (33). Altogether, these findings suggest that loss of 53BP1 promotes lymphogenesis in Atm$^{-/–}$ mice by accelerating the rate of formation of oncogenic translocations involving the TCRα/δ locus and possibly other loci.

Loss of 53BP1 aggravates the defect in canonical NHEJ in Atm$^{-/–}$ cells

Because NHEJ normally suppresses RAG-dependent translocations, the findings above strongly suggested a greater defect in NHEJ in double mutants. To evaluate this pathway in more detail, we took advantage of the fact that murine splenocytes are mostly noncycling and therefore rely on NHEJ for DSB repair. To assess the kinetics of NHEJ-mediated repair, resting wt, Trp53bp1$^{+/–}$, Atm$^{-/–}$ and Trp53bp1$^{+/–}$/Atm$^{-/–}$ splenocytes were irradiated and the number of γ-H2AX foci, that mark sites of DSBs, quantified over time. At 1 hour after irradiation (IR), the number of foci was comparable across genotypes (Fig. 2A and B and data not shown), validating the use of this assay in our backgrounds. At later time points however, we observed a delay in the resolution of γ-H2AX foci in double mutants relative to Atm$^{-/–}$ controls, suggesting delayed DSB repair (Fig. 2A and B; $n = 2$ independent experiments). These differences did not reflect

Table 1. Analysis of Trp53bp1$^{+/–}$/Atm$^{-/–}$ and control Atm$^{-/–}$ and Trp53bp1$^{+/–}$/Atm$^{-/–}$ thymic lymphomas

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>Genotype</th>
<th>Gender</th>
<th>Age at death, wk</th>
<th>Tumor CD4/CD8 labeling$^a$</th>
<th>Chromosome 14 abnormalities (% metaphases)</th>
<th>Chromosome 12 abnormalities (% metaphases)</th>
<th>No. of chromosomes/cell$^b$ (range)</th>
</tr>
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<tr>
<td>M#4</td>
<td>Atm$^{-/–}$</td>
<td>F</td>
<td>16.5</td>
<td>DP</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>M#36</td>
<td>Atm$^{-/–}$</td>
<td>F</td>
<td>16</td>
<td>CD4 SP</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>M#30</td>
<td>Atm$^{-/–}$</td>
<td>F</td>
<td>23</td>
<td>DP</td>
<td>Yes (98%)</td>
<td>Yes (98%)</td>
<td>41.7 ± 0.5 (41–42)</td>
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<tr>
<td>M#35</td>
<td>Atm$^{-/–}$</td>
<td>M</td>
<td>24</td>
<td>DP</td>
<td>Yes (100%)</td>
<td>Yes (100%)</td>
<td>40.0 ± 0.0 (40–40)</td>
</tr>
<tr>
<td>M#2</td>
<td>Trp53bp1$^{+/–}$/Atm$^{-/–}$</td>
<td>M</td>
<td>12</td>
<td>DP</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>M#5</td>
<td>Trp53bp1$^{+/–}$/Atm$^{-/–}$</td>
<td>F</td>
<td>18</td>
<td>DP/CD4 SP</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>M#49</td>
<td>Trp53bp1$^{+/–}$/Atm$^{-/–}$</td>
<td>M</td>
<td>12</td>
<td>DP/CD4 SP</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
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<td>M#10</td>
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<td>F</td>
<td>27</td>
<td>DP/CD4 SP</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
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<td>M</td>
<td>14</td>
<td>DP</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
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<td>M#11</td>
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<td>M</td>
<td>14.5</td>
<td>DP</td>
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<td>Yes (98%)</td>
<td>39.8 ± 0.4 (39–40)</td>
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<td>M#16</td>
<td>Trp53bp1$^{+/–}$/Atm$^{-/–}$</td>
<td>M</td>
<td>15</td>
<td>CD4 SP</td>
<td>Yes (96%)</td>
<td>No</td>
<td>41.7 ± 103 (38–44)</td>
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<td>M#21</td>
<td>Trp53bp1$^{+/–}$/Atm$^{-/–}$</td>
<td>M</td>
<td>16.5</td>
<td>DP</td>
<td>Yes (100%)</td>
<td>Yes (100%)</td>
<td>40.8 ± 0.6 (40–42)</td>
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<td>M</td>
<td>16</td>
<td>CD4 SP</td>
<td>Yes (98%)</td>
<td>Yes (94%)</td>
<td>40.1 ± 0.6 (39–41)</td>
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<tr>
<td>M#37</td>
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<td>M</td>
<td>14</td>
<td>DP</td>
<td>Yes (100%)</td>
<td>Yes (100%)</td>
<td>40.0 ± 0.5 (39–41)</td>
</tr>
<tr>
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<td>M</td>
<td>13.5</td>
<td>DP/CD8 SP</td>
<td>Yes (92%)</td>
<td>Yes (92%)</td>
<td>40.0 ± 0.2 (40–41)</td>
</tr>
</tbody>
</table>

Abbreviation: n.d., not done.

$^a$DP, double positive for CD4 and CD8; SP, single positive; mixed populations were observed in some tumors, as indicated.

$^b$Average and SD; $n = 20–30$ metaphases per tumor.
on increased radioresistance of double mutant cells because the viability of Trp53bp1/−/Atm−/− and Atm−/− irradiated cells was similar and, as expected, markedly increased relative to wt or Trp53bp1/−/− cells (Supplementary Fig. S5).

To further examine this question in a cell type more relevant to our tumor model and bypass potential artifacts associated with cell culture, we also quantified IR-induced γ-H2AX foci in thymus cryosections after allowing for in vivo repair (Fig. 2C). Foci analysis was done on the thymic cortex, which consists mostly of nondividing lymphocytes. Consistent with our observations in B cells, the number of γ-H2AX foci per nucleus was increased in irradiated double-mutant thymocytes relative to Atm−/− controls, particularly 24 hours after IR (Fig. 2C). Altogether, these experiments indicate that loss of 53BP1 delays NHEJ-dependent repair in ATM-deficient cells in prereplicative phases of the cell cycle, suggesting a mechanism for accelerated formation of RAG-dependent translocations.

53BP1 is thought to promote canonical, ligase IV–dependent NHEJ by providing a chromatin context that suppresses inappropriate end-resection, a molecular event that triggers alternative, error-prone end-joining (A-NHEJ; refs. 6, 35). In the context of RAG-mediated DSBs, end-resection results in sequence alterations that can be readily

**Figure 2.** 53BP1 deficiency aggravates the NHEJ defect in primary Atm−/− cells. A, cells were irradiated and the number of γ-H2AX foci per nucleus quantified by indirect immunofluorescence. N = 100 cells per histogram. B, representative examples of cells in (A). C, mice were irradiated, allowed to repair in vivo, and euthanized at the indicated time points. The number of γ-H2AX foci per nucleus was quantified in thymus cryosections by indirect immunofluorescence. N = 150 cells per histogram.
identified by examining specific joints. However, sequences at 2 endogenous recombination junctions (the V62-J81 CJ [Supplementary Fig. S6] and the V65-D62 SJ [Supplementary Fig. S7]), in *Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup> mice were indistinguishable from wt, suggesting that canonical ligase IV–dependent NHEJ remains the predominant pathway in double mutants (see Supplementary Fig. S8 for the distribution of deletion size at coding and signal ends across genotypes). Specifically, we did not observe an increase in the frequency of microhomologies (MH), a footprint of alternative NHEJ (35), nor did we observe templated additions associated with products of mismept V(D)J recombination (30) or with some oncogenic chromosomal translocations (36). We noted only rare deletions, consistent with previous observations in 53BP1-deficient cells (8).

Severe defect in p53 activation in *Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup>* thymocytes

In lymphoid cells, unrepaired DSBs in G<sub>1</sub> elicit p53 activation to regulate the G<sub>1</sub>–S checkpoint and growth arrest/apoptosis. p53 activation is severely compromised in *Atm<sup>−/−</sup>* primary thymocytes, allowing for replication of G<sub>1</sub>-induced DSBs (37). To investigate an effect of 53BP1 deficiency on the ATM/p53 axis, we monitored IR-induced p53 stabilization and effector functions in double mutant and control thymocytes (Fig. 3). As expected, IR-induced p53 stabilization and phospho-p53 (Ser15) formation were robust in irradiated wt and *Trp53bp1<sup>−/−</sup>* cells but markedly attenuated in *Atm<sup>−/−</sup>* thymocytes (Fig. 3A and B). A similar defect was observed in double mutants, regardless of whether radiation was administered in *vivo* (Fig. 3A) or in *vitro* (Fig. 3B). IR-dependent phosphorylation of KAP-1, another posttranslational modification primarily dependent on ATM, was also compromised to a comparable extent in *Atm<sup>−/−</sup>* and *Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup>* cells (Fig. 3A and B). As for *Atm<sup>−/−</sup>* cells (38), residual p53 activation and KAP-1 phosphorylation in double mutants was dependent on DNA-PKcs because it was abrogated by pretreatment with the DNA-PKcs inhibitor NU7026 (Fig. 3C). Finally, IR-induced transcriptional activation of 2 main p53 targets, p21 and Bax, and thymocyte apoptosis were also attenuated to a similar extent in *Atm<sup>−/−</sup>* and *Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup>* cells (Fig. 3D–F). Collectively, these experiments indicate that double mutant cells show a severe defect in p53 activation, providing a mechanism for amplification of their increased NHEJ defect.

53BP1 is dispensable for ATM-dependent repair of replication-associated DSBs

In addition to regulating the repair of prereplicative DSBs, ATM promotes the signaling and repair of replicative DSBs. To assess an effect of 53BP1 in this context, we next examined the kinetics of DSB repair in mature in *vivo* activated B cells (Fig. 4). We previously showed that B-cell activation with α-CD40/IL-4 counteracts p53 activation in response to spontaneous DSBs (39). Consistent with those findings and a recent report (23), activated wild-type (wt) B cells exposed to IR (2 Gy) did not undergo significant G<sub>1</sub>–S arrest or apoptosis but rather a transient arrest in G<sub>2</sub> (Supplementary Fig. S9). This was determined by the accumulation of cells with 4N DNA content that were also negative for the mitotic marker phospho-H3 (Ser10). In wt cells, the G<sub>2</sub> arrest peaked by 6 hours post-IR and was resolved by 24 hours post-IR (Supplementary Fig. S9). In contrast, all single and double mutants showed persistent arrest at 24 hours (*n* = 5 independent experiments; Fig. 4A and B; Supplementary Fig. S9). Significantly, double mutants consistently showed further delayed cell-cycle kinetics relative to *Atm<sup>−/−</sup>* cells (Fig. 4A and B; Supplementary Fig. S9), correlating with increased number of γ-H2AX foci at the same time points (Fig. 4C and D). Finally, these analyses revealed that, upon irradiation, a subset of *Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup>* and, to a lesser extent, *Atm<sup>−/−</sup>* and *Trp53bp1<sup>−/−</sup>* activated B cells undergo a second round of inappropriate synthesis, resulting in the formation of tetraploid (8N) cells (Fig. 4E and F). The greater frequency of cells with DNA content greater than 4N in double mutants is consistent with their greater load of DSBs (Fig. 4C and D) and suggests an additional mechanism for increased genomic instability (40).

The increased load of DSBs in G<sub>2</sub> likely results from "carry-over" of G<sub>1</sub> breaks as well as deficient repair of newly introduced DSBs during replication. To determine whether 53BP1 may also modulate genomic instability associated with replication in *Atm<sup>−/−</sup>* cells, we examined genomic stability in metaphase spreads of activated B cells treated with the PARP inhibitor olaparib (Supplementary Fig. S10). As expected (41), olaparib induced frequent chromatid breaks in α-CD40/IL-4–activated *Atm<sup>−/−</sup>* cells. 53BP1 status had no measurable effect on this phenotype, suggesting that 53BP1 aggravates DSB repair in ATM-deficient cells specifically in the context of NHEJ. These findings are consistent with our previous observations that mice lacking 53BP1 and PARP1 lack additional phenotypes (28). In further support of this notion, the frequency of hydroxyurea (HU)-induced DSBs was also comparable in *Atm<sup>−/−</sup>* and *Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup>* cells (Supplementary Table S2).

53BP1 suppresses chromosomal translocations in primary *Atm<sup>−/−</sup>* cells

To test the effect of persistent DSBs on translocation formation, we next quantitated breaks and translocations in mitotic chromosomes of activated B cells. As expected (42), we observed an increased frequency of both spontaneous and IR-induced aberrations in all DNA repair–deficient mutants relative to wt controls (*n* = 6 independent experiments; Fig. 4G–I; Supplementary Table S3 for summary, Supplementary Table S4 for individual experiments). Consistent with the lack of additional organismal phenotypes in growth and development (Supplementary Fig. S1), the frequency of spontaneous breaks was similar in *Atm<sup>−/−</sup>* and *Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup>* B cells (Fig. 4G "mock"). In contrast, IR consistently induced an "excess" of chromosomal aberrations in *Trp53bp1<sup>−/−</sup>/Atm<sup>−/−</sup>* cells relative to *Atm<sup>−/−</sup>* controls (*n* = 6 independent experiments; *n* = 0.5–3.2 "excess" breaks per cell [Fig. 4G], "2 Gy"). When normalized...
Figure 3. Effect of 53BP1 deficiency on the ATM/p53 axis function. A, thymocytes were irradiated in vitro and harvested at the indicated time points for immunoblotting. Total KAP1 serves as loading control. Unspecific bands are marked by asterisk and serve as additional loading controls. B, mice were irradiated, allowed to repair in vivo, and euthanized at the indicated time points. Thymocytes were analyzed as in (A). C, Trp53bp1+/-/Atm-/- thymocytes were irradiated in the presence or absence of the DNA-PKcs inhibitor NU7026 and harvested at 3 hours for immunoblotting. D-F, mice were irradiated and euthanized at either 3 or 6 hours post-IR. D, thymus RNA was reverse-transcribed and p21 or Bax cDNA amplified by real-time reverse transcription (RT)-PCR. E and F, apoptosis was quantified in thymic sections using the TUNEL assay. Representative examples are shown in F. Bars represent the average and SD of 3 experiments.
53BP1 deficiency increases the number of chromosomal translocations in irradiated Atm⁻/⁻ primary cells. A and B, α-CD40/IL-4–activated B cells were irradiated, allowed to repair for 24 hours, and stained with propidium iodide (PI) for analysis of cell-cycle distribution. Data for 5 independent experiments are shown in (A) and summarized in (B). The increase in the percentage of cells with 4N DNA content for each irradiated mutant, relative to the irradiated wt control in the same experiment, was calculated and designated delta 4N (%4N). Bars represent the average and standard deviation of the %4N in the 5 experiments in (A). C, distribution of the number of γ-H2AX foci per nucleus. N = 100 cells per histogram. D, representative microphotographs of cells in (C). E, frequency of cells with >4N DNA content 24 hours after IR. Data for the Trp53bp1⁻/-Atm⁻⁻ culture was normalized to the Atm⁻⁻ control in the same experiment. Bars represent the average and SD of 5 mice per genotype in 5 independent experiments. F, representative fluorescence-activated cell-sorting (FACS) plots of (E). G, quantification of the number of chromosomal breaks 24 hours after IR using telomere FISH. H, the frequency of chromosomal breaks per metaphase in Trp53bp1⁻/-Atm⁻⁻ cultures was normalized to Atm⁻⁻ cultures in the same experiment. Bars represent the average and SD of 6 mice per genotype in 6 independent experiments. I, chromosomal breaks in G were classified as “chromosome-type” (centric and acentric chromosomes) or “chromatid-type.” The total number of aberrations analyzed is indicated.
Table 2. Analysis of genomic stability in irradiated (2 Gy) B cells deficient for 53BP1 and/or ATM

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cytokine</th>
<th>No. of metaphases analyzed</th>
<th>No. of broken ends analyzed</th>
<th>No. of translocated ends/no. of nontranslocated ends</th>
<th>% translocated ends/% nontranslocated ends</th>
<th>No. of breaks per cell/no. of translocated breaks per cell</th>
<th>Ratio translocated/nontranslocated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt 1/1</td>
<td>α-CD40/IL-4</td>
<td>90</td>
<td>91</td>
<td>37/54</td>
<td>40.7/59.3</td>
<td>1.0/0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Trp53bp1−/− 1/1</td>
<td>α-CD40/IL-4</td>
<td>90</td>
<td>301</td>
<td>93/208</td>
<td>30.9/69.1</td>
<td>3.3/1.0</td>
<td>0.4</td>
</tr>
<tr>
<td>Atm−/− 1/1</td>
<td>α-CD40/IL-4</td>
<td>90</td>
<td>471</td>
<td>150/321</td>
<td>31.8/68.2</td>
<td>5.2/1.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Trp53bp1−/−/Atm−/−</td>
<td>α-CD40/IL-4</td>
<td>90</td>
<td>741</td>
<td>220/521</td>
<td>29.7/70.3</td>
<td>8.2/2.4</td>
<td>0.4</td>
</tr>
<tr>
<td>wt LPS</td>
<td>LPS</td>
<td>90</td>
<td>82</td>
<td>33/49</td>
<td>40.2/59.8</td>
<td>0.9/0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Trp53bp1−/−</td>
<td>LPS</td>
<td>90</td>
<td>207</td>
<td>44/163</td>
<td>21.3/78.7</td>
<td>2.3/0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Atm−/−</td>
<td>LPS</td>
<td>90</td>
<td>332</td>
<td>99/233</td>
<td>29.8/70.2</td>
<td>3.7/1.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Trp53bp1−/−/Atm−/−</td>
<td>LPS</td>
<td>90</td>
<td>121/271</td>
<td>392/132</td>
<td>30.9/69.1</td>
<td>4.4/1.3</td>
<td>0.4</td>
</tr>
</tbody>
</table>

NOTE: Metaphases were obtained 24 hours after exposure to IR and stained with a telomere probe. N = 3 mice per genotype.
or several additional lower molecular weight bands in Trp53bp1−/−/Atm−/− samples (Fig. 5B; Supplementary S12A). Sequencing of gel-extracted, high-mobility bands confirmed the deletions (Fig. 5C and D, Supplementary Fig. S12B and S12C), consistent with the interpretation that 53BP1 functions to protect a subset of DNA ends from extensive end-resection before HJ formation. Sequence analysis enabled us to distinguish independent events on the basis of differential N-region insertion. Incidentally, deletion endpoints appeared to cluster at 2 sites within the DJβ2 signal flank, residing 267 to 268 and 347 to 348 bp from the signal end (Fig. 5D). Moreover, an apparent bias toward deletion from the signal end was observed when recombinants were amplified with primers placed at similar distances from the coding or signal ends (Supplementary Fig. S12B and S12C). Taken together, these observations indicate that in the context of HJ formation in ATM-deficient cells, removal of 53BP1 does not affect the frequency of incorrect end use but rather markedly increases the extent of end resection that accompanies the joining of a subset of incorrect ends.

Discussion

53BP1 is being increasingly recognized as a tumor suppressor in a wide range of human cancers (13–18). Significantly, its functions in NHEJ-mediated repair were previously reported to be epistatic with those of ATM (20), consistent with their mutual regulation (7, 20–24). Here, we find that their functional interaction in NHEJ is context-dependent. In this regard, we observe epistasis during organismal growth and development and in the suppression of spontaneous genomic instability. In contrast, we find that 53BP1 becomes limiting for NHEJ-mediated repair after IR or during oncogenic stress, when the load of DSBs per cell is greater. Importantly, this work shows that, in the pro-oncogenic environment generated by ATM deficiency, even a modest aggravation of the NHEJ defect in the absence of 53BP1 may have a profound clinical effect. This observation is in keeping with a recent report that 53BP1 deficiency similarly cooperates with oncogenic stress associated with AID overexpression to suppress B-cell lymphomagenesis (45).

The clonal RAG-dependent translocations that drive Atm−/− tumors are normally suppressed by NHEJ (31),
We propose that the accelerated acquisition of these translocations in double mutants is a direct result of their greater defect in NHEJ. Although the number of TCRβε8 breaks detected by cytogenetics in *Atm−/−* and *Trp53bp1−/−/Atm−/−* primary cells was too low for a direct comparison (our unpublished observations), we find that NHEJ-mediated repair in irradiated G1 splenocytes and thymocytes is consistently delayed in double mutants. When coupled with the severe checkpoint defects documented here, this deficiency alone could be sufficient to explain our observations in cycling cells, including (i) increased numbers of γ-H2AX foci in G2; (ii) increased polyploidy; and (iii) increased chromosomal breaks and translocations in mitotic chromosomes. Our findings differ from those of a previous study which concluded that 53BP1 and ATM are fully epistatic in the repair of IR-induced DSBs induced in G1 (20). This apparent discrepancy may reflect differences in methodologies, including but not limited to differential radiation dose and our use of ATM null cells rather than a chemical inhibitor of ATM.

Most relevant to our translocation-driven tumor model, we find a net increase in the formation of “random” chromosomal translocations in primary double mutant cells relative to single mutants. Although 53BP1 is required for the fusion of uncapped telomeres (9) and intrachromosomal recombination (8, 46), 53BP1-deficient cells can nevertheless form intra- and interchromosomal rearrangements robustly (8, 45). In agreement with this notion, we find that approximately 30% of IR-induced chromosomal breaks are rearranged in 53BP1-deficient cells, regardless of ATM status. Intriguingly, the percentage of DNA ends undergoing translocation was higher (about 40%) in irradiated wild-type cells, despite the fact that they harbored fewer total breaks per cell and consequently fewer translocation “acceptors.” These observations suggest a model in which 53BP1 and ATM may play dual roles in the context of translocations. In this model, they would primarily function to suppress translocations by promoting appropriate rejoining of DSBs. However, if repair fails and DNA ends are dissociated, 53BP1 and/or ATM would promote “aberrant” repair to form translocations, perhaps by helping bring the ends together and/or promoting the recruitment of classical or alternative NHEJ factors (9, 24). The observed translocation frequency would then reflect the balance between their anti- and protranslocation activities, a notion that will be further examined in future work.

Mechanistically, 53BP1 promotes NHEJ by suppressing inappropriate DNA end-resection (6–8, 23, 45, 47, 48), preventing large deletions and/or use of highly error-prone pathways (35). Previous work has shown that ATM regulates end-resection in 53BP1-deficient cells in a cell-cycle–dependent manner (47). Specifically, ATM suppressed end-resection in 53BP1-deficient cells in G1 but not in S–G2 (6, 7). Consistent with these findings, we report here increased deletions in a subset of RAG-mediated HJs in 53BP1-deficient thymocytes also lacking ATM. Together with previous observations (6, 7), this finding suggests that formation of at least a subset of HJs (and possibly chromosomal translocations) may require cell-cycle progression. However, our finding that sequences at coding, signal, and hybrid joints in double mutants are consistent with ligase IV–dependent canonical NHEJ suggests that this may be nevertheless the main pathway mediating HJs and chromosomal translocations, a notion that will be further examined in future work.

Finally, this work has important implications for our understanding of cancers harboring genetic alterations in DDR factors. Given that mutations in ATM are common in human cancers, it will be interesting to determine whether they are nonmutally exclusive with 53BP1 mutations and to define their interplay in the natural history of disease and the response to therapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Rybanska-Spaeder, T.L. Reynolds, J. Chou, M. Prakash, D.L. Huso, S. Desiderio, S. Franco

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Study supervision: S. Desiderio, S. Franco

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**References**


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