Functional Interplay of p53 and Mus81 in DNA Damage Responses and Cancer

Ashwin Pamidi,1,2,3 Renato Cardoso,1,2,3 Anne Hakem,1,2,3 Elzbieta Matysiak-Zablocki,1,2,3 Anuradha Poonepalli,1,2,3 Laura Tamblyn,1,2,3 Bayardo Perez-Ordonez,4 M. Prakash Hande,5 Otto Sanchez,6 and Razqallah Hakem1,2,3

1The Advanced Medical Discovery Institute, Ontario Cancer Institute, and 2Department of Medical Biophysics, University of Toronto; 3Department of Pathology, University Health Network, Toronto, Ontario, Canada; 4Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore; and 3Department of Medical Biophysics, University of Toronto, 620 University Avenue, Suite 706, Toronto, Ontario, Canada M5G 1L4.

Abstract
Mus81 plays an integral role in the maintenance of genome stability and DNA repair in mammalian cells. Deficiency of Mus81 in human and mouse cells results in hypersensitivity to interstrand cross-linking (ICL) agents and elevated levels of genomic instability. Furthermore, Mus81-mutant mice are susceptible to spontaneous lymphomas. The role of cellular checkpoints in mediating the phenotypes observed in Mus81-deficient cells and mice is currently unknown. In this study, we have observed increased activation of p53 in Mus81−/− cells in response to ICL-induced DNA damage. In addition, p53 inactivation completely rescued the ICL hypersensitivity of Mus81−/− cells, signifying p53 is essential for the elimination of ICL-damaged cells in the absence of Mus81. Confirming that p53 acts as a critical checkpoint for the Mus81 repair pathway, a synergistic increase of spontaneous and ICL-induced genomic instability was observed in Mus81−/−/p53−/− cells. To clarify the genetic interactions of Mus81 and p53 in tumor suppression, we monitored Mus81−/−/p53−/− and control mice for the development of spontaneous tumors. Significantly, we show that loss of even a single allele of Mus81 drastically modifies the tumor spectrum of p53-mutant mice and increases their predisposition to developing sarcomas. Our results reveal a key role for p53 in mediating the response to spontaneous and ICL-induced DNA damage that occurs in the absence of Mus81. Furthermore, our data show that loss of Mus81, in addition to p53, is a key step in sarcoma development. [Cancer Res 2007;67(18):8527–35]

Introduction
DNA interstrand cross-linking (ICL) agents, such as cisplatin and mitomycin C (MMC), are commonly used as antitumor agents in the treatment of a broad spectrum of cancers (1). The therapeutic use of these drugs has generated increased interest in understanding the mechanisms by which ICLs are repaired in mammalian cells. ICL agents disrupt replication of the genome by inducing the formation of stalled replication forks, which, in turn, can collapse into broken forks that can lead to double-strand breaks (DSB) and chromosomal rearrangements (2). ICL agents also interfere with critical cellular processes, such as transcription and recombination (3). Yeast and bacterial studies have shown that both nucleotide excision repair and homologous recombination pathways are involved in ICL repair, but the mechanisms of mammalian ICL and stalled replication fork repair remain poorly characterized (4–7).

Mus81 (methyl methanesulfonate, UV sensitive, clone 81) was first identified in yeast as a member of the XPF family of endonucleases that physically interacts with the cell cycle checkpoint protein Cds1 (yeast homologue of mammalian Chk2) and the DNA repair protein Rad54 (8, 9). Studies in yeast identified that Mus81 and its binding partner Eme1 formed a DNA structure-specific endonuclease (10–13). Yeast mutants of Mus81 were found to be sensitive to DNA-damaging agents that induce stalled replication forks but not DSBs (8, 9). Meiotic defects in Schizosaccharomyces pombe mutants for Mus81 suggested that the Mus81-Eme1 endonuclease plays a key role in Holliday junction resolution (9). Subsequent in vitro studies indicated that the yeast Mus81-Eme1 endonuclease is involved in the resolution of nicked Holliday junctions that may arise during recombination and repair processes. However, recent studies have indicated that the mammalian Mus81-Eme1 endonuclease preferentially cleaves stalled replication fork intermediates and has weak Holliday junction resolution activity (14–17). Furthermore, Mus81−/− mice were found to be fertile, indicating that mammalian Mus81 is dispensable for the processing of Holliday junctions that may arise during meiosis (18, 19). Cellular phenotypes of Mus81-deficient murine and human cells include exquisite sensitivity to ICL agents and elevated levels of spontaneous and MMC-induced genomic instability (18–20). Importantly, our previous study showed that Mus81−/− and Mus81−/− mice spontaneously develop tumors, thus establishing Mus81 as a novel haploinsufficient tumor suppressor gene (18).

The mechanisms underlying the ICL sensitivity of Mus81-deficient cells have not yet been elucidated but may involve the activation of p53-dependent or p53-independent cellular checkpoints. Moreover, the role that p53 plays in suppressing the genome instability and tumors associated with Mus81 deficiency still remains to be addressed. In this study, we have generated double-knockout Mus81−/−/p53−/− mice and addressed the effect of dual loss of p53 and Mus81 on development, DNA damage responses, genomic instability, and cancer. Our data show that p53 inactivation rescues the ICL sensitivity of Mus81-deficient cells at the expense of increased genomic instability. Dual inactivation of p53 and Mus81 in mice resulted in accelerated tumorigenesis, showing cooperativity of these tumor suppressors in cancer. Furthermore, Mus81 mutation altered the tumor spectrum of p53−/− mice. p53−/− mice predominantly developed...
thymic lymphomas, whereas Mus81-mutant mice developed mainly T-cell or B-cell lymphomas; however, Mus81+/p53−/− and Mus81−/−p53−/− mice were drastically predisposed to sarcomas and multiple tumors. Our study indicates that p53 plays a critical role in DNA damage responses in Mus81-deficient cells and shows that Mus81 plays an essential role in suppressing sarcoma formation in p53−/− mice.

Materials and Methods

Mice. Mus81−/− mice (18) were crossed with p53−/− mice (Taconic) to obtain Mus81−/−p53−/− mice. Double heterozygote mice were then crossed to obtain Mus81−/−p53−/− and Mus81+/−p53−/− mice. All mice studied were in a mixed 129/×C57BL/6 genetic background and were genotyped by PCR (primer sequences and PCR conditions available on request). All experiments were done in compliance with the Ontario Cancer Institute animal care committee guidelines.

Flow cytometry. Thymocytes, spleen, lymph nodes, and bone marrow cells were harvested from 6- to 12-week-old mice and stained with the following monoclonal antibodies (PharMingen): anti-CD4, anti-CD8, anti-Thy-1, anti-B220, anti-TCRα, anti-CD43, and anti-IgM. Fluorescence-activated cell sorting (FACS) analyses were done using a FACS Calibur (Becton Dickinson).

Proliferation assay. For proliferation in response to T-cell stimuli, lymph node cells were isolated and 5 × 105 cells were placed into round-bottomed 96-well plates in culture medium (RPMI 1640, 10% FCS, and 1% β-mercaptoethanol) and stimulated in triplicate with soluble anti-CD3 (5 μg/mL) or with or without anti-CD28 (1 μg/mL) or interleukin-2 (IL-2; 50 units/mL). Cells were pulsed for the last 18 h with 1 μCi [3H]thymidine (Amersham) per well and harvested at 48 and 72 h after treatment. Similarly, proliferation of B cells was assessed using 5 × 105 splenocytes stimulated with anti-IgM (20 μg/mL; Jackson Immunoresearch) with or without anti-CD40 (5 μg/mL; PharMingen) and lipopolysaccharide (LPS; 10 μg/mL; Sigma).

Cell cycle analysis. Peripheral T lymphocytes were grown on plate-bound anti-CD3 and culture medium for 24 h followed by a 1-day culture in the presence of murine IL-2 (50 units/mL). Untreated and 0.1 μg/mL MMC-treated lymphocytes were grown for an additional 72 h. Cells were analyzed by flow cytometry to determine CFSE levels, and population values were determined using Flowjo analysis software (Tree Star).

Western blot analysis. Mouse embryonic fibroblasts (MEF) were plated on 10-cm plates at 30% to 40% confluency and cultured in the presence of 0 to 25 μg/mL of MMC for 0 to 24 h. Protein cell extracts were prepared using 3% NP40 lysis buffer, and Western blots were done using rabbit polyclonal anti-p53 antibody (FL-393; 1:200 dilution; Santa Cruz Biotechnology), rabbit polyclonal anti-phosphorylated p53 (Ser15; 1:200 dilution; Cell Signaling), an affinity-purified rabbit polyclonal antibody against amino acids 190 to 211 of murine Mus81 (1:500 dilution), antitubulin (1:1,000 dilution; Sigma), and antiactin (1:1,000 dilution; Sigma).

Genomic instability analysis. Splenocytes were harvested from mice of 8 to 12 weeks of age and cultured in medium containing 10 μg/mL LPS for 48 h in the presence or absence of MMC (40 ng/mL). Cells were then incubated with colcemid (0.1 μg/mL) for 4 h, harvested, and treated with hypotonic buffer (0.075 mol/L KCl) at 37°C for 15 min. After fixation in ice-cold methanol/acetic acid (3:1) buffer, cells were dropped onto glass slides and stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma) and chromosomes were determined for a minimum of 60 metaphase spreads per cell type as described previously (18).

Histology. Tumor and normal tissue paraffin sections were evaluated by H&E staining in conjunction with anti-Ki-67, anti-CD3, anti-CD3, and anti-B220 staining of adjacent sections as described previously (21).

Bone marrow colony forming assay. Bone marrow cells were derived from femurs of 6-week-old mice. Bones were isolated and flushed with culture medium. Cells were seeded on 35-mm culture dishes at a density of 1 × 10^6 cells/mL in MethoCult GF M3434 medium (Stemcell Technologies, Inc.). MMC (0–40 ng/mL) was added to the cell suspensions just before plating. Colonies were counted 10 days after plating.

γ-Irradiation sensitivity. T lymphocytes were irradiated with 0, 2, or 4 Gy. Viability was assessed 12 h after irradiation by doing 7-aminoactinomycin D (7-AAD) staining (Sigma).

Results

Mus81−/− cells express elevated levels of p53 in response to DNA damage. Although studies have shown that Mus81 plays an important role in the response of cells to DNA-damaging agents,
evidence describing the molecular mechanisms that underlie Mus81-mediated DNA repair is lacking. As such, we first assessed if p53 expression is altered in response to DNA damage by examining the effect of the ICL agent MMC on MEFs (Fig. 1A). Western blot analysis showed that Mus81 expression levels remained unchanged in cells treated with 10 μg/mL MMC for 4, 8, or 24 h. To confirm that the MMC did induce genotoxic stress in the treated MEFs, we examined the expression of p53 as it is upregulated in response to MMC-induced damage (22). Elevated levels of p53 were observed 24 h after treatment with 10 μg/mL MMC, confirming that the MMC was inducing a damage response.

To assess the effect of Mus81 mutation on p53 activation, we generated Mus81−/− MEFs. Similar to their wild-type (WT) controls, Mus81−/− MEFs did not show elevated basal levels of p53 expression (Fig. 1B) and their proliferation was unaffected (Supplementary Fig. S1). However, Western blot analysis indicated increased levels of p53 induction in Mus81−/− MEFs compared with WT MEFs treated with 10 and 25 μg/mL of MMC (Fig. 1B).

In response to DNA damage, p53 is phosphorylated at Ser15 by ataxia telangiectasia mutated, resulting in its activation. To evaluate if MMC-induced damage results not only in increased expression but also in activation of p53 in Mus81−/− cells, we examined phosphorylation of p53 at Ser15 by Western blot analysis (Fig. 1C). We observed increased Ser15 phosphorylation of p53 in Mus81−/− MEFs compared with WT MEFs as early as 4 h after treatment with 10 μg/mL MMC, showing that Mus81−/− cells exhibit elevated levels of p53 activation in response to ICL-induced damage.

In accordance with our previous study (18), Mus81−/− MEFs displayed increased cellular sensitivity to MMC (P = 0.03 for 10 μg/mL; Fig. 1D). Our data suggest that the increased activation of p53 observed in Mus81−/− cells following MMC treatment plays an important role in the ICL hypersensitivity of Mus81−/− cells.

**Combined p53 and Mus81 deficiency leads to female embryonic lethality.** Mus81-deficient mice are predisposed to developing lymphomas presumably due to elevated levels of genomic instability, a hallmark feature of carcinogenesis. However, cancer involves multiple genetic alterations, including loss of cellular checkpoints (23). We hypothesized that the p53 tumor suppressor pathway plays an important role in suppressing the genomic instability and tumorigenesis in Mus81-deficient mice and in the hypersensitivity of Mus81-deficient cells to ICL-induced DNA damage.

To identify the functional interactions between p53 and Mus81, double-knockout mice deficient for Mus81 and p53 were generated. Surprisingly, our crosses to generate Mus81−/−p53−/− and Mus81−/−p53−/− mice did not yield the expected Mendelian ratios (Supplementary Fig. S2A). The number of Mus81−/−p53−/− offspring (47 mice) was much lower than expected (86 mice). Of the 47 offspring, only 3 were females, indicating that the observed decreased viability of Mus81−/−p53−/− mice was female specific (P < 0.001; Supplementary Table S1). Similarly, we observed a
significantly reduced number of female Mus81+/−p53−/− mice (only 1 female of 20 Mus81+/−p53−/− mice). Analysis of embryos at day 9.5 of gestation indicated that 24% of Mus81+/−p53−/− females displayed exencephaly, a defect in neural tube closure (Supplementary Fig. S2B). Previous studies have shown that loss of p53 results in a reduction of female progeny (10–30% depending on background) due to a variety of developmental abnormalities (24, 25). From our double heterozygote crossings (Mus81+/− × Mus81+/−p53+/−), we noticed a slight but not significant deficiency in p53−/− females (P > 0.1; Supplementary Table S1). Our findings show that Mus81 mutation drastically enhances the embryonic lethality associated with p53−/− females.

Deficiency of p53 and Mus81 does not affect immune cell differentiation and proliferation. Male Mus81+/−p53−/− mice are fertile and seem indistinguishable from their WT, Mus81+/+, or p53−/− littermates. Cell counts and determination of the various immune cell populations by FACs analysis of thymus, lymph node, spleen, and bone marrow indicated that the development of the immune system of Mus81+/−p53−/− males was not compromised (Supplementary Fig. S3). The effect of dual loss of p53 and Mus81 on the in vitro proliferative capacity of T and B cells derived from adult mice was also assessed. No significant differences were observed in the activation-induced proliferation of T and B cells from Mus81+/−p53−/− mice when compared with single mutant and WT cells (Fig. 2A and B).

These data indicate that combined loss of Mus81 and p53 in male mice does not affect their embryonic development or the development of their immune system. In addition, combined loss of Mus81 and p53 does not alter the proliferative capacities of T and B cells derived from adult males.

Mus81 is dispensable for the response to γ-irradiation-induced DNA damage. p53 is critical for initiating cell cycle arrest and apoptosis in response to ionizing radiation (26). We previously reported that Mus81 mutation does not impair the cellular response to γ-irradiation–induced DNA damage (18). To investigate the effect of combined loss of Mus81 and p53 in γ-irradiation sensitivity, we assessed the viability of lymphocytes 12 h after irradiation using 7-AAD staining (Fig. 2C). As expected, p53−/− lymphocytes were highly resistant to γ-irradiation compared with WT cells. Mus81-null cells displayed a modest but not significant sensitivity to γ-irradiation compared with WT cells (P > 0.1). Importantly, Mus81−/−p53−/− cells displayed equivalent γ-irradiation resistance when compared with p53−/− cells. These findings show that Mus81 does not play a significant role in cellular responses to γ-irradiation and its inactivation does not impair the resistance of p53−/− cells to γ-irradiation–induced DNA damage.

Loss of p53 rescues the MMC sensitivity of Mus81-deficient cells. Mus81 deficiency increases sensitivity to DNA-damaging agents in yeast and mammals (8, 9, 18–20). In particular, loss of mammalian Mus81 results in hypersensitivity to MMC. We have shown that MMC-induced DNA damage results in elevated levels and activation of p53 in Mus81−/− cells (Fig. 1A). To elucidate the requirement of p53 for the DNA damage sensitivity of Mus81−/− cells, we examined the response of Mus81−/−p53−/− cells to MMC. Because MEFs can lose cellular checkpoints and become immortalized during in vitro culturing, we did colony forming assays in the presence or absence of MMC using primary bone marrow cells isolated from 6-week-old WT, Mus81−/−, p53−/−, and Mus81−/−p53−/− mice. Colony formation was assessed at day 10 of culture and colony numbers were normalized to untreated controls to determine the sensitivity to MMC (Fig. 2D). Mus81-deficient bone marrow cells were found to be 6-fold more sensitive to MMC than WT cells (P < 0.01; colony survival: 13 ± 1% for Mus81−/− and 79 ± 4% for WT cells). p53-null cells displayed a modest but not significant resistance to MMC compared with WT cells (colony survival: 91 ± 11% for p53−/− and 79 ± 4% for WT cells). Remarkably, loss of p53 completely rescued the MMC sensitivity of Mus81−/− cells, as Mus81−/−p53−/− cells displayed similar sensitivity as WT cells (colony survival: 78 ± 5% for Mus81−/−p53−/− and 79 ± 4% for WT cells). These data confirm the MMC hypersensitivity of Mus81−/− cells and show that p53 plays a critical role in mediating this sensitivity.

Having determined that Mus81−/− cells display clonogenic sensitivity to MMC, we questioned if this sensitivity is due to a proliferative defect of Mus81−/− cells in response to ICLs. To assess for this possibility, we examined the proliferation of Mus81−/− cells and their controls by doing CFSE staining on activated lymphocytes (Fig. 3A). In response to MMC, the proliferation of Mus81−/− cells was hampered the greatest, as we observed an 81% reduction in proliferation compared with a reduction of only 66% in WT cells and 20% in p53−/− cells. Strikingly, Mus81−/−p53−/− displayed only a 23% decrease in cellular proliferation in response to MMC. These data show that Mus81−/− cells have impaired proliferation in response to MMC and that this reduction in proliferation is p53 dependent.

Figure 3. MMC-damaged Mus81−/− cells display a p53-dependent G2 arrest. A, cell cycle analysis of WT, Mus81−/−, p53−/−, and Mus81−/−p53−/− peripheral T cells. Cell cycle analysis was done by propidium iodide staining of untreated cells (UT), cells treated with 0.5 μg/mL MMC for 18 h (MMC), and cells that were treated for 18 h with MMC and then subsequently cultured in fresh medium for an additional 18 h (18 h after MMC). Percentage of cells in G0-G1, S, and G2-M phases. Data are representative of three independent experiments. B, representative CFSE staining of WT, Mus81−/−, p53−/−, and Mus81−/−p53−/− activated lymphocytes in response to MMC. FACs analysis was done 120 h after staining of untreated and 100 ng/mL MMC-treated lymphocytes. Percentage of cells achieving two cell divisions.
Role of p53 in the G2 arrest of MMC-treated Mus81<sup>−/−</sup> cells.

DNA damage sensitivity and genomic instability in cells are often accompanied by cell cycle progression defects. Because Mus81-deficient cells are hypersensitive and display impaired proliferation in response to ICLs, we examined the cell cycle progression of WT, Mus81<sup>−/−</sup>, p53<sup>−/−</sup>, and Mus81<sup>−/−</sup>p53<sup>−/−</sup> activated lymphocytes in the absence and presence of MMC-induced damage. WT, Mus81<sup>−/−</sup>, p53<sup>−/−</sup>, and Mus81<sup>−/−</sup>p53<sup>−/−</sup> cells displayed similar cell cycle profiles in untreated conditions (Fig. 3B). Treatment with MMC for 18 h resulted in a significant accumulation of Mus81<sup>−/−</sup> cells in the G2 phase (39%, an increase of 20% compared with untreated cells), whereas only a moderate accumulation of WT cells in the G2 phase was observed (28%, an increase of 11% compared with untreated cells). In contrast to WT and Mus81<sup>−/−</sup> cells, p53<sup>−/−</sup> cells (22%, an increase of 1% compared with untreated cells) did not display a G2 accumulation in response to MMC. Significantly, loss of p53 diminished the arrest of Mus81<sup>−/−</sup> cells in the G2 phase, as Mus81<sup>−/−</sup>p53<sup>−/−</sup> cells (25%, an increase of 8% compared with untreated cells) displayed only a slight G2 accumulation. Taken together, our data show that the ICL repair defect of Mus81-null cells results in an accumulation of cells in the G2 phase and that this accumulation is at least partially dependent on p53.

p53 suppresses Mus81-associated genomic instability. Previous reports have shown that Mus81<sup>−/−</sup> cells have increased levels of spontaneous genomic instability (18–20). Unexpectedly, Mus81<sup>−/−</sup> cells have comparable survival and proliferation rates and do not have elevated levels of p53 in the absence of MMC by comparison with WT cells. This may be explained by the fact that only a small fraction of Mus81<sup>−/−</sup> cells have increased genomic instability (<10%); therefore, it may be difficult to detect changes in proliferation and p53 expression at a population level under these conditions. Therefore, we analyzed metaphase spreads of splenocytes from WT, Mus81<sup>−/−</sup>, p53<sup>−/−</sup>, and Mus81<sup>−/−</sup>p53<sup>−/−</sup> mice for chromosomal aberrations to evaluate the role of p53 in suppressing the genomic instability occurring in the absence of Mus81 (Fig. 4A and B). In untreated conditions, Mus81<sup>−/−</sup> (9.8 ± 1% of total metaphases) and p53<sup>−/−</sup> cells (7.3 ± 3% of total metaphases) displayed elevated genomic instability compared with WT cells (0.8 ± 1% of total metaphases). Increased aneuploidy and chromosomal breaks were observed in Mus81<sup>−/−</sup> cells (5.3 ± 0.1% and 6.2 ± 1%, respectively) in comparison with WT cells (0% and 0.8 ± 1%, respectively). p53<sup>−/−</sup> cells also displayed an increase in aneuploidy but only a mild increase in spontaneous chromosomal breaks (7.3 ± 1.5% and 3.6 ± 2%, respectively). Remarkably, combined loss of Mus81 and p53 resulted in a 3-fold increase in spontaneous genomic instability compared with Mus81<sup>−/−</sup> cells and a 4-fold increase compared with p53<sup>−/−</sup> cells, as 28% of Mus81<sup>−/−</sup>p53<sup>−/−</sup> metaphases displayed chromosomal aberrations. Elevated levels of aneuploidy (12.9 ± 3%), chromosomal breaks (12 ± 0.5%), and triradial-like structures (2.4 ± 1%) were observed in Mus81<sup>−/−</sup>p53<sup>−/−</sup> metaphases.

Because Mus81<sup>−/−</sup> cells are particularly sensitive to MMC, we examined the level of genomic instability in WT, Mus81<sup>−/−</sup>, p53<sup>−/−</sup>, and Mus81<sup>−/−</sup>p53<sup>−/−</sup> cells that were treated with 40 ng/mL MMC.

Figure 4. Mus81 and p53 cooperate in maintenance of genomic stability. A, representative metaphases of Mus81<sup>−/−</sup>, p53<sup>−/−</sup>, and Mus81<sup>−/−</sup>p53<sup>−/−</sup> lymphocytes displaying chromosomal aberrations. t, triradial-like structure; b, chromosome break; t:b, triradial- or quadiradial-like structures with chromosome exchanges and breaks/fragments; t/q, triradial- or quadiradial-like structures resulting in chromosome exchanges. B, frequency of aneuploidy, fragments/breaks, triradial-like structure, and total chromosomal aberrations was scored for untreated (black columns) and MMC-treated (white columns) lymphocytes. Activated lymphocytes were cultured in the presence or absence of 40 ng/mL MMC for 48 h. Cells were arrested with colcemid and stained with DAPI. Columns, mean of two independent experiments; bars, SD. A minimum of 50 metaphase spreads was analyzed for each genotype and treatment.
Elevated rates of aneuploidy, chromosomal breaks, and triradial-like structures were observed in all four genotypes in response to MMC. Strikingly, MMC treatment affected Mus81+/−/p53−/− cells the most, as they displayed the largest increase in total chromosomal aberrations in response to MMC (98% compared with 68%, 54%, and 38% increases for p53−/−, Mus81−/−, and WT cells, respectively). Taken together, our data highlight the important role of p53 in the suppression of genomic instability in Mus81−/− cells.

Cooperativity of p53 and Mus81 in tumor suppression. To clarify the in vivo role of p53 in Mus81-associated tumorigenesis, we monitored the health and survival of Mus81+/−, p53+/−, Mus81+/−/p53+/−, Mus81−/−/p53−/−, and WT cohorts for a period of 1 year (Fig. 5A). All moribund mice that were examined were found to have tumors. Only 67% of Mus81+/− mice (26 of 39; mean survival, 44.7 weeks) and 15% of p53−/− mice (3 of 20; mean survival, 27.8 weeks) were viable and healthy at the end of the year. Remarkably, no Mus81−/−/p53−/− mice (0 of 17; mean survival, 25.6 weeks) or Mus81−/−/p53+/− mice (0 of 20; mean survival, 17.5 weeks) survived up to 1 year and all were moribund by 47 and 30 weeks of age, respectively. Log-rank analysis indicated that tumor latency was significantly decreased in Mus81−/−/p53−/− mice compared with p53−/− or Mus81−/−/ mice (P < 0.001), showing that the combined loss of Mus81 and p53 cooperates in tumorigenesis. Characterization of tumors was done by examination of cellular morphology and staining of tissue sections with cell- and proliferation-specific markers. Similar to our previous study (18), the majority of sick Mus81−/− mice that were examined were found to be afflicted with T-cell or B-cell lymphomas (8 of 12, 66%; Supplementary Table S2). As has been described previously (27), p53−/− mice predominantly developed thymic lymphomas (10 of 12, 83%). Interestingly, the combined loss of Mus81 and p53 led to a unique tumor spectrum, as 64% of Mus81−/−/p53−/− mice (9 of 14; Supplementary Table S2; Fig. 5B and C) developed sarcomas. In contrast, only 8% of Mus81−/− mice (1 of 12) and 17% of p53−/− mice (2 of 12) developed sarcomas (Supplementary Table S2; Fig. 5B). In addition, Mus81−/−/p53−/− mice also developed T-cell or B-cell lymphomas, carcinomas, and thymic lymphomas that are associated with inactivation of Mus81 or p53 (Supplementary Table S2; Fig. 5C, D, and H). Loss of one allele of Mus81 in a p53-null background did not decrease tumor latency in comparison with p53−/− mice (P > 0.1; Fig. 5A) but did alter tumor spectrum. Similar to Mus81−/−/ p53+/− mice, Mus81−/−/ p53−/− mice predominately developed sarcomas (7 of 12, 58%; Supplementary Table S2; Fig. 5B and C), indicating that loss of one allele of Mus81 is sufficient for increasing the predisposition of p53−/− mice to sarcomas (P < 0.01). Southern blot analysis of the Mus81 locus was done on three sarcomas isolated from Mus81−/−/p53−/− mice (Supplementary Fig. S4). We did not observe any gross genomic alterations or loss of the Mus81 allele, indicating that loss of heterozygosity (LOH) is not essential for the sarcoma development in Mus81−/−/p53−/−-mutant background.

Combined loss of Mus81 and p53 not only resulted in decreased tumor latency and changes in the tumor spectrum but also promoted the incidence of multiple tumors (P < 0.01; Fig. 6).
Whereas only 8% of Mus81\(^{-/-}\) mice (1 of 12) and no p53\(^{-/-}\) mice (0 of 12) developed multiple independent tumors, 43% of Mus81\(^{-/-}\) p53\(^{-/-}\) mice examined (6 of 14) were characterized with multiple tumors (Supplementary Table S2; Fig. 6A–C). Similarly, Mus81\(^{-/-}\) p53\(^{-/-}\) mice (6 of 12, 50%) also had an increased incidence of multiple tumors, indicating that biallelic expression of Mus81 is required for the suppression of multiple independent tumors in a p53-null background.

**Discussion**

Previous studies have shown that Mus81 plays an important role in the repair of ICL-induced structures, such as stalled replication forks, primarily in the late S phase and G\(_2\) phase of the cell cycle (12, 20, 28). The role of p53 in irradiation-induced cell cycle checkpoints and DSB repair responses has been well documented. However, the role of p53 in responding to stalled replication forks remains poorly characterized. Elucidating the tumor suppressor functions of p53 in the response to stalled replication forks is of significant importance, as defective repair can predispose for cancer and ICL agents, such as MMC, are frequently used for cancer therapy.

To examine the cooperativity of Mus81 and p53 in DNA repair responses and tumorigenesis, we generated Mus81\(^{-/-}\) p53\(^{-/-}\) mice. Unexpectedly, we observed smaller litter sizes and abnormal Mendelian ratios for Mus81\(^{-/-}\) p53\(^{-/-}\) and Mus81\(^{-/-}\) p53\(^{-/-}\) pups. Sex genotyping and embryo analysis indicated that Mus81\(^{-/-}\) p53\(^{-/-}\) and Mus81\(^{-/-}\) p53\(^{-/-}\) female embryos had drastically reduced viability, showing that Mus81 is critical for proper female embryonic development in the absence of p53. Previous studies have identified that loss of p53 results in lethality of a subset of females during embryonic development due to a variety of developmental defects, including excencephaly (24, 25). It has been speculated that the developmental defects in these mice may be due to increased genomic instability in p53-mutant backgrounds. Although the mechanisms behind the decreased viability of Mus81\(^{-/-}\) p53\(^{-/-}\) and Mus81\(^{-/-}\) p53\(^{-/-}\) female embryos require further investigation, it may be linked at least partially to the overall increased genomic instability observed in the absence of Mus81 and p53. Our data establish a requirement for the biallelic expression of Mus81 to suppress female-specific embryonic lethality in the absence of p53.

Studies of embryonic stem cells, transformed MEFs, and mice have shown that Mus81 deficiency leads to ICL sensitivity (18, 19). In this study, we have examined the role that p53 plays in mediating the MMC sensitivity of Mus81-deficient cells and have found that Mus81\(^{-/-}\) cells display elevated levels of p53 in response...
to MMC. Furthermore, inactivation of p53 rescued the MMC sensitivity of Mus81+/− cells to WT levels. Our data show that p53 is critical for the elimination of Mus81+/− cells damaged by MMC.

Through its ability to arrest the cell cycle progression of cells with DNA damage, p53 prevents amplification of chromosomal aberrations that can otherwise lead to oncogenic-promoting genetic alterations. By monitoring the proliferation of cells using CSFE staining and cell cycle analysis, we have shown that the increased MMC sensitivity of Mus81+/− cells is due to a proliferative defect in response to ICLs, resulting in an arrest of cells in the G2 phase of the cell cycle.

Furthermore, we have shown that the G2 arrest of MMC-treated Mus81+/− cells is p53 dependent and speculate that the G2 arrest is important in preventing the replication of cells that harbor chromosomal aberrations due to defective ICL repair mechanisms. Supporting this notion are reports describing other ICL-sensitive cells, such as Fanconi anemia and Brcal-deficient cells, which display a G2 arrest in response to MMC (29, 30).

The predisposition of Mus81-mutant mice for T-cell and B-cell lymphomas suggests that cellular checkpoints may play a critical role in the suppression of tumors in other tissues. Our demonstration that the loss of p53 in Mus81+/− mice significantly accelerates tumorigenesis, and alters the tumor spectrum, leading to increased susceptibility to multiple tumors and sarcomas, supports this notion. Importantly, the elevated levels of genomic instability observed in Mus81+/− p53+/− mice are correlated with accelerated tumorigenesis and thus provide further evidence that genomic instability is the driving force behind Mus81-associated tumorigenesis.

Unlike Mus81+/− p53+/− mice, Mus81−/− p53+/− mice did not display significantly decreased tumor latency. However, both Mus81−/− p53−/− and Mus81−/− p53+/− mice displayed a strong shift in tumor spectrum toward sarcomas. Southern blot analysis indicated that LOH of Mus81 is not required for sarcoma development in p53−/− background. We have previously shown that loss of a single allele of Mus81 results in an ~50% reduction in protein levels and spontaneous genomic instability and tumor susceptibility (18). Based on our findings in this study, we speculate that a gene dosage reduction of Mus81 in p53-null mice promotes the genetic alterations required for sarcoma development.

Several recent studies have indicated that p53 plays an important role in the repair of stalled replication forks (31–33). In fact, Subramanian and Griffith (34) have shown that cancer-derived p53 mutants have weaker binding to stalled replication forks. Because Mus81 is able to efficiently resolve stalled replication forks in vitro, it is possible that Mus81 deficiency in vivo results in an increase in the frequency of stalled forks and that the binding of p53 to these structures is critical for repair by alternate pathways. We suggest, in addition to the cell cycle and apoptotic functions of p53, its role in stalled fork repair may play an important role in maintaining genome stability and suppressing tumorigenesis of Mus81−/− mice. Whereas human studies have identified p53 inactivation as the most common genetic alteration leading to sarcoma formation (35), no studies have yet linked mutation of Mus81 or other molecules involved in ICL repair with sarcoma formation. We have identified a novel role for Mus81 as a critical haploinsufficient tumor suppressor that collaborates with p53 in suppressing sarcoma formation. Furthermore, our data warrant investigation of the collaborative roles of human Mus81 and p53 in cancer suppression, which will provide important insight into the molecular mechanisms that underlie sarcoma initiation and progression.

Acknowledgments


Grant support: Academic Research Fund, National University of Singapore and National Medical Research Council, Ministry of Health, Singapore (M.P. Hande); and National Cancer Institute of Canada grant 13071, Canadian Institute of Health Research grant 74509, and Canadian Institute of Health Research salary award (R. Hakem).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank D. Durocher, M. Soenagas, L. Salmena, D. Sealey, and members of the Hakem lab for critically reviewing the manuscript and helpful discussions and J. Squire, J. Karaskova, P. McPherson, and A. Elia for assistance.

References

Functional Interplay of p53 and Mus81 in DNA Damage Responses and Cancer

Ashwin Pamidi, Renato Cardoso, Anne Hakem, et al.


**Updated version**

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/18/8527

**Supplementary Material**

Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2007/09/17/67.18.8527.DC1

**Cited articles**

This article cites 35 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/18/8527.full#ref-list-1

**Citing articles**

This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/67/18/8527.full#related-urls

**E-mail alerts**

Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.