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The p53 protein acts a tumor suppressor by inducing cell cycle arrest and apoptosis in response to DNA damage or oncogene activation. Recently, it has been proposed that phosphorylation of serine 15 in human p53 by ATM (mutated in ataxia telangiectasia) kinase induces p53 activity by interfering with the Mdm2-p53 complex formation and inhibiting Mdm2-mediated destabilization of p53. Serine 18 in murine p53 has been implicated in mediating an ATM- and ataxia telangiectasia-related kinase-dependent growth arrest. To explore further the physiological significance of phosphorylation of p53 on Ser18, we generated mice bearing a serine-to-alanine mutation in p53. Analysis of apoptosis in thymocytes and splenocytes following DNA damage revealed that phosphorylation of serine 18 was required for robust p53-mediated apoptosis. Surprisingly, p53Ser18 phosphorylation did not alter the proliferation rate of embryonic fibroblasts or the p53-mediated G_1 arrest induced by DNA damage. In addition, endogenous basal levels and DNA damage-induced levels of p53 were not affected by p53Ser18 phosphorylation. p53Ala18 mice developed normally and were not susceptible to spontaneous tumorigenesis, and the reduced apoptotic function of p53Ala18 did not rescue the embryo-lethal phenotype of Mdm2-null mice. These results indicate that phosphorylation of the ATM target site on p53 specifically regulates p53 apoptotic function and further reveal that phosphorylation of p53 serine 18 is not required for p53-mediated tumor suppression.

The p53 tumor suppressor is a transcription factor that functions to regulate cell proliferation and apoptosis. The importance of p53 in suppressing cancer is highlighted by the very high incidence of spontaneous tumor formation in p53-deficient mice and in mice bearing genetic alterations in other members of the p53 pathway (14, 27, 30, 42). The critical role of p53 in tumor suppression is further underscored by the high frequency of *p53* mutations in human cancer, and malignancies that retain a wild-type *p53* gene often have acquired other mutations that affect one or more of the pathways known to govern p53 function (53). Therefore, it is likely that inactivation of p53 is a common mechanistic step in the development of most human cancers.

Cellular p53 activity is greatly increased following treatment of cells with DNA-damaging agents such as ionizing radiation and adriamycin (62). Induction of p53 activity can also occur in response to inappropriate growth-stimulatory signals, such as expression of an activated oncogene or exposure of the cells to other forms of stress (37, 38, 50, 69). Increased p53 activity resulting from DNA damage blocks the proliferation of cells in the G_1 phase of the cell cycle by inducing the expression of genes such as the cyclin-dependent kinase inhibitor p21 (6, 13, 16, 20). In addition, increased p53 activity in the cell can initiate apoptosis by inducting the expression of proapoptotic BCL-2 family members involved in mitochondrion-associated cell death and by transactivating the expression of death receptor-associated genes (7, 41, 52, 66). Both p53-mediated inhibition of cell cycling and induction of apoptosis appear to be highly dependent upon the cell context. Induction of p53 has also been proposed to regulate passage of cells through the G_2 phase of the cell cycle, chromosomal stability, and mitosis, although the precise molecular mechanisms used by p53 to perform these regulatory functions are less clear.

Given the multiple roles of p53 in controlling cell cycling and cell death, proper regulation of p53 activity is obviously crucial to permit cells to undergo normal growth and to respond appropriately to stress. Regulation of cellular p53 activity has been reported to occur at the level of p53 transcription and translation (19, 49, 63). However, it is clear that Mdm2-mediated alteration of p53 protein stability is a major pathway used by many cells to govern p53 function. The Mdm2 oncoprotein binds to the amino-terminal portion of the p53 protein (11, 48), induces p53 monoubiquitination and nuclear export (35, 39), and targets p53 for proteosomal degradation (23, 34). The importance of Mdm2 as a negative regulator of p53 activity in cell growth and development has been demonstrated in mice by rescue of the embryo-lethal phenotype of Mdm2-deficient mice by deletion of p53 (28, 45).

Numerous in vitro studies have determined that p53 is subject to posttranslational modification. Phosphorylation occurs in response to DNA damage on human p53 serine residues 15 and 20 (mouse Ser18 and Ser23, respectively) and has been found in several in vitro studies to upregulate p53 activity (15, 18, 36, 43, 60) and interfere with Mdm2-mediated degradation of p53 (55).

The ATM (mutated in ataxia telangiectasia) kinase has been demonstrated to phosphorylate human p53 serine 15 (3, 8, 33, 51). Furthermore, both human p53Ser15 phosphorylation (8, 46, 56) and total p53 protein levels (31, 32, 46) are reduced in ATM-null cells following ionizing radiation treatment. These observations suggest that ATM-mediated phosphorylation of this residue increases the stability of p53. Expression of inactive forms of the ataxia telangiectasia-related kinase ATR also interferes with p53Ser15 phosphorylation (47, 58, 65). In addition, mouse embryonic fibroblasts (MEFs) deficient in both

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ATM and ATR fail to undergo G_1 arrest in response to DNA damage and display greatly reduced phosphorylation of p53Ser18, the mouse homologue of human p53Ser15 (5). These data suggest that ATM and ATR functions may depend, in part, upon the ability of these related kinases to phosphorylate p53 at this serine residue.

Recently mouse p53Ser18 was mutated in embryonic stem (ES) cells, and the p53 apoptotic response and cell cycle arrest following DNA damage of these cells were delayed (10). However, p53-mediated pathways of cell cycle inhibition and apoptosis are not fully functional in ES cells (1). Thus, the relationship between p53Ser18 phosphorylation and p53 stability and function remains unclear.

In order to directly address the role of p53Ser15 phosphorylation in regulating p53 levels and p53 function in a genetically defined in vivo system, we used gene targeting in mouse ES cells to generate mice bearing p53 alleles that contain a p53Ser18 to Ala18 substitution. Our results indicate that the p53-dependent apoptosis induced by ionizing radiation is significantly reduced in the thymocytes and splenocytes of p53Ala18/Ala18 mice. Therefore, phosphorylation of p53Ser18 regulates p53-dependent apoptosis. However, phosphorylation of this residue does not alter the stability of the p53 protein. Wild-type mice and p53Ala18 mice display similar basal p53 levels and similar induced levels of p53 following DNA damage. Surprisingly, phosphorylation of p53Ser18 does not affect the rate of cell proliferation of embryonic fibroblasts. In addition, loss of Ser18 does not alter the p53-dependent G_1 arrest of cell growth following DNA damage. Thus, the G_1 arrest induced by ATM and ATR following DNA damage is not mediated by p53Ser18 phosphorylation. Furthermore, the reduced apoptotic activity of p53Ala18 does not rescue the embryonic lethality of Mdm2-null mice and does not alter p53-mediated tumor suppression. These results indicate that phosphorylation of the ATM/ATR target serine 18 regulates p53 apoptotic functions and not p53-mediated cell growth arrest and that phosphorylation of this site is not required for p53 suppression of spontaneous tumorigenesis.

MATERIALS AND METHODS

Generation of targeted ES cells. The targeting vector was generated with a portion of the p53 gene (129SVBrd-strain) spanning a *Bam*HI fragment 9.9 kb from intron 1 and intron 6 of the p53 gene. Serine 18 in exon 2 was mutated by oligonucleotide site-directed mutagenesis (QuickChange kit; Stratagene) of a subfragment of the targeting vector. The oligonucleotides used were HKS1Ala18 (5′-GGATATCAGCCTCGAACTCCCTCTGGCCCAGGAGACATTTTCAG G-3′) and HKS2BAla (5′-CCTGAAAATGTCTCCTGGGCCAGAGGGAGTT CGAGGCTGATATCC-3′). The subfragment was DNA sequenced to confirm the mutation and subcloned back into the targeting vector. A *neo*r cassette gene flanked by *loxP* sites was introduced in an *Avr*II site, resulting in loss of the *Avr*II site. An MC1-thymidine kinase gene was added to the 3′ end of the targeting vector.

Homologous recombination and generation of p53Ala18 mice. PC3 ES cells (26) were electroporated with the targeting vector and drug selected with G418 and 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl-5-iodouracil (FIAU). A total of 600 ES clones were screened, and two correctly targeted clones were identified by Southern analysis of *Eco*RI-digested genomic DNA with 5′ and 3′ probes external to the targeting vector. The presence of the alanine mutation in the ES clones was confirmed by PCR with oligonucleotide primers to intron1 forward (1F; 5′-GACAAGTTATGCATCATAC-3′) and intron 3 reverse (3R; 5′-CAAA GCCCAAGTCCCTTTC-3′) and subsequent digestion of the PCR fragments with *Xho*I. The wild-type PCR product was digested to yield two fragments, 365 bp and 70 bp. Cells were microinjected into embryonic day 3.5 blastocysts (C57BL/6 strain), and the embryos were surgically implanted into pseudopreg-

nant foster mice by standard procedures. Excision of the *neo*r cassette in F_1 offspring of male chimeric mice was confirmed by digestion with *Avr*II and Southern analysis with a p53 exon 7 probe.

Research involving mice complied with all relevant federal and institutional policies, as well as guidelines established by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School.

Cell culture, proliferation, and immortalization assays. Murine embryonic fibroblasts (MEFs) were generated from day 13.5 embryos from the various genotypes and maintained in Dulbecco's modified Eagle's medium supplement with 10% fetal bovine serum, penicillin, and streptomycin. Cells were maintained at subconfluency, and low-passage MEFs (passages 2 to 4) were used for all subsequent assays. Growth curves were performed with triplicate plating of wild-type, $p53^{-/-}$, p53Ala18/+ and p53Ala18/Ala18 homozygous MEFs. Cells were plated (5×10^5 cells/10-cm dish) and counted every 24 h with a Beckman Coulter counter. Proliferation assays were performed with two independent lines of each genotype. 3T3 assays were performed with multiple plates of each genotype to examine rates of spontaneous immortalization as described previously (22).

DNA damage and cell cycle analysis. Replicative DNA synthesis was quantified by bivariate flow cytometry. In brief, cells were irradiated with 2, 5, or 8 Gy. Cells were irradiated with a cesium 137 source (Gammacell 40). Cells were pulsed with bromodeoxyuridine (60 μM) from15 to 18 h postirradiation and harvested by trypsinization and fixation in 70% ethanol. Fixed cells were washed and incubated in 4 N HCl for 30 min at room temperature. The HCl was neutralized by addition of 0.1 M borax. Cells were incubated with a fluorescein isothiocyanate-conjugated antibromodeoxyuridine monoclonal antibody (Becton Dickinson), washed, and resuspended in phosphate-buffered saline containing 50 μg of RNase per ml and 20 μg of propidium iodide per ml. Fluorescence from propidium iodide complexes and fluorescein isothiocyanate-conjugated bromodeoxyuridine was measured by flow cytometry. Similar numbers of cells were analyzed for each sample, and cell cycle distribution was determined with FlowJo software.

Protein analysis and antibodies. Following treatment, cells were lysed in EBC buffer (120 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1.0% NP-40, 0.1% sodium dodecyl sulfate, 1 mM EDTA) with a cocktail of protease and phosphatase inhibitors; 100 μg of cell extract were run on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. The p53 antibody (Ab-7 kit [Oncogene Research Products], diluted 1:1,000), the phospho-p53 (Ser 15) (Cell Signaling 9284S, diluted 1:1,000), the Mdm2 antibody (Santa Cruz sc-812, diluted 1:500), the p21 (Santa Cruz sc-397, diluted 1:500), the α-tubulin antibody (Sigma, diluted 1:10,000), and the Puma antibody (Abcham Limited, diluted 1:1,000) were obtained from the indicated suppliers. Immune complexes were detected by enhanced chemiluminescence (ECL kit, Amersham).

Thymocyte and splenocyte studies. Thymocytes and splenic cells were removed from 4- to 6-week-old mice. Thymuses were removed and ground up with a syringe tip. Cells were washed with phosphate-buffered saline–1% serum solution, and resuspended at 10^6 cells/ml in phosphate-buffered saline–1% serum. For T-cell population profiling, whole animals were irradiated with 8 Gy. Thymocytes were recovered from untreated and irradiated animals and stained with CD4-phycoerythrin and CD8-fluorescein isothiocyanate antibodies (Pharmingen). Thymocytes were then washed and fixed in 1% paraformaldehyde and subjected to fluorescence-activated cell sorting analysis. For sub-G_1 DNA analysis, whole animals were irradiated with 8 Gy. Thymocytes were removed 8 h postirradiation from irradiated and control unirradiated animals. Thymocytes were fixed in 70% ethanol. Sub-G_1 DNA content was determined by fluorescence-activated cell sorting analysis.

Viability analysis was performed on in vitro-cultured thymocytes and thymocytes derived from animals irradiated in vivo. For viability analysis, thymocytes were stained with the annexin V-fluorescein isothiocyanate apoptosis kit (Oncogene Research Products). Viability was determined as the population negative for annexin V/7-amino actinomycin D (7AAD) relative to unirradiated control animals. Spleens were dissected and recovered as described (57). Splenocytes were plated (2×10^5) in RPMI supplemented with 10% serum. Cells were irradiated with 8 Gy. Viable cells were counted by trypan blue exclusion 7 days post-ionizing radiation treatment.

RESULTS

Generation of p53Ala18 mice. To analyze the effect of p53 serine 18 phosphorylation on p53 functions, a gene targeting experiment was performed in PC3 ES cells (129Sv-Brd strain). A gene replacement vector containing strain 129 mouse p53
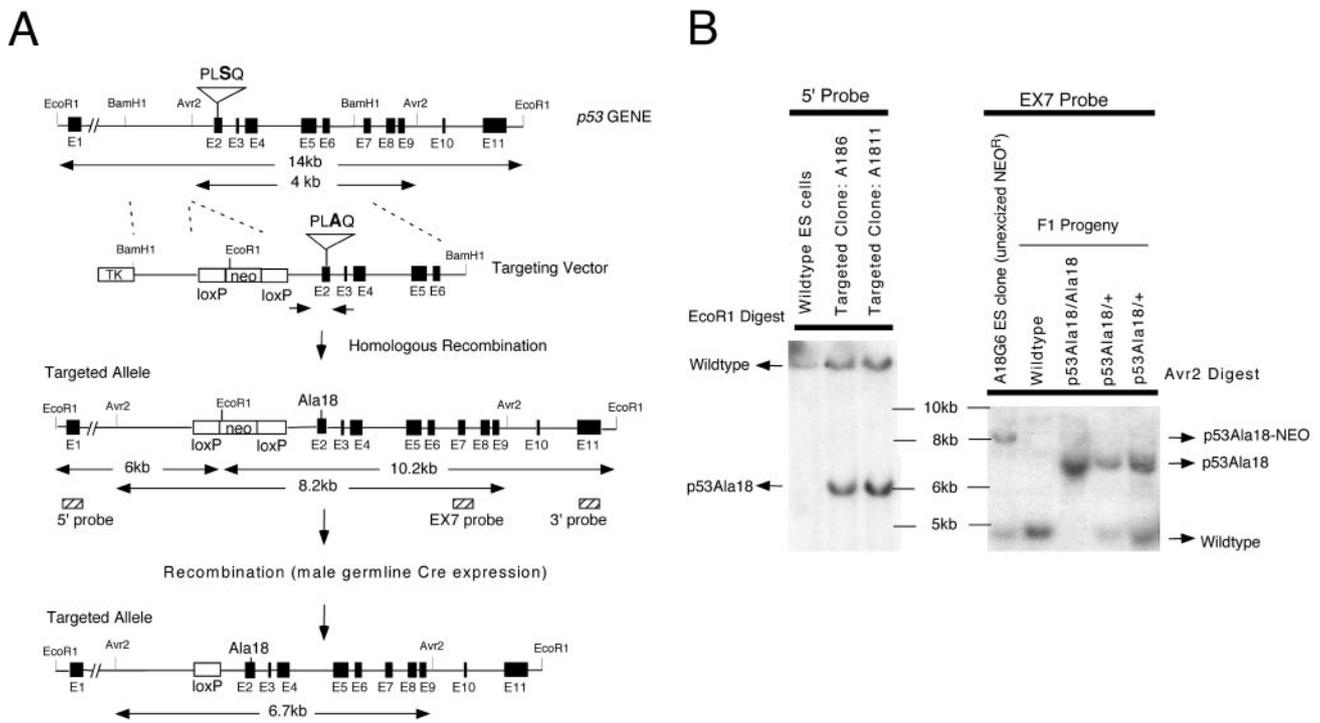
FIG. 1. Mutation of serine 18 in the p53 gene. (A) The targeting vector encodes a serine to alanine missense mutation in exon 2. An *Xho*I restriction site was also deleted in this exon as a diagnostic marker. A floxed *neo*^{r} gene was introduced in intron 1 to permit positive selection of ES cells. Homologous recombination of the targeting vector in ES cells is illustrated. The *neo* gene will be excised in the sperm of chimeric mice due to expression of a protamine-Cre transgene present in the PC3 ES cells. The p53Ala18 allele is shown at the bottom. Probes used for Southern blot analysis are shown (hatched boxes). (B) Southern blot analysis of targeted ES cell DNA with a 5′ external probe (left panel). *Eco*RI digestion of ES cell DNA indicates the presence of the mutant allele (unexcised *neo*^{r} gene, 6 kb) and the wild-type allele (14 kb). Southern blot analysis of F$_1$ progeny with EX7 probe (right panel). An *Avr*II digest distinguishes the wild-type allele (4 kb), unexcised mutant allele (8.2 kb), and excised mutant allele (6.7 kb). (C) The presence of the mutation was determined by PCR amplification of exon 2 and loss of the *Xho*I restriction site. (D) Diagram of the mutation generated in exon 2 of p53. Serine18 was mutated to an alanine. An adjacent *Xho*I restriction site was deleted for diagnostic purposes.

genomic DNA was generated that encoded a serine to alanine substitution at amino acid position 18 (Fig. 1A, D) in exon 2 of the coding sequences. This substitution also deleted an *Xho*I restriction endonuclease site in exon 2 DNA sequences without altering the other amino acids encoded in exon 2. This vector also contained a neomycin drug resistance marker flanked by *loxP* sites inserted in intron 4 of the p53 gene and a herpes simplex virus thymidine kinase gene downstream of the 3′ region of homology. Following positive (G418) and negative (FIAU) drug selection of the ES cells, 600 surviving clones were screened by Southern analysis, and 3 clones were identified that had undergone targeting of the p53 allele.

Detailed analysis of these three clones with 5′ and 3′ probes external to the targeting vector confirmed proper targeting of the locus (Fig. 1B). Two of these clones (A18-6 and A18-11) were used to perform blastocyst injection experiments, and both clones gave rise to multiple high-degree chimeras. Male chimeras were crossed with C57BL/6 mice to obtain agouti F$_1$ offspring. Analysis of genomic DNA isolated from tail biopsies of these mice confirmed that a subset of the mice had inherited the targeted allele and had excised the neomycin drug selection marker due to expression of the protamine-Cre transgene (endogenous in PC3 ES cells, Fig. 1B). The presence of the mutation encoding the Ala18 substitution was confirmed in a

subset of F_1 mice by DNA sequencing and PCR analysis (Fig. 1C). Inheritance of the p53Ala18 allele was subsequently followed in the colony by use of a PCR strategy that used the newly deleted XhoI site in exon 2 adjacent to the Ala18 mutation. Mice heterozygous for the p53Ala18 allele were intercrossed, and mice homozygous for the mutant allele were recovered at the expected frequency of 25%. Mice heterozygous or homozygous for the p53Ala18 allele developed normally. Large cohorts of p53Ala18/+ and p53Ala18/Ala18 mice were generated for further analysis.

In vivo analysis of apoptosis in p53Ala18/Ala18 cells. Mouse thymocytes are induced to undergo p53-dependent apoptosis following ionizing radiation treatment (12, 40). To determine the contribution of p53 ser18 phosphorylation to p53 apoptosis, we compared apoptosis in thymocytes of p53Ala18/Ala18 mice and in wild-type mice and p53-null mice following exposure to DNA damage. Thymocytes were recovered from nontreated mice and from treated mice at 8 h after whole-body exposure of the mice to 8 Gy of ionizing radiation. The thymocytes were analyzed for apoptosis by analyzing sub-G_1 DNA content (Fig. 2A). Following DNA damage, a similar increase in the percentage of apoptotic cells was observed in wild-type thymocytes and in thymocytes bearing one copy of the p53Ala18 allele, suggesting that the presence of the p53Ala18 allele was not exerting a dominant negative effect on wild-type p53 (Fig. 2A). However, mice homozygous for p53Ala18 displayed significant reductions in the percentage of thymocytes undergoing apoptosis following DNA damage (Fig. 2B), indicating that phosphorylation of p53 serine 18 does alter p53-mediated apoptosis in vivo.

CD4/CD8 thymocytes undergo p53-dependent apoptosis following ionizing radiation damage (40). Mice were irradiated in vivo with 8 Gy, and a time course of CD4/CD8 staining was performed post-ionizing radiation with multiple p53Ala18/Ala18, wild-type, and p53-deficient mice. A significant depletion of CD4/CD8 thymocytes was observed in p53Ala18/Ala18 mice (Fig. 2C). p53Ala18/Ala18 mice most closely resembled the phenotype of p53-heterozygous mice. The fraction of surviving thymocytes was also measured at multiple time points postirradiation by staining cells for annexin V and 7AAD (Fig. 2D). Annexin V (an early marker for apoptosis)-negative and 7AAD (a DNA intercalator)-negative populations were determined and compared to untreated thymocytes. The reduced viability of thymocytes in p53Ala18/ala18 mice confirmed that these cells were compromised in p53-dependent apoptosis.

To investigate if p53 apoptosis is altered in a different cell context, splenocytes were also harvested and analyzed for the percentage of cells undergoing p53-mediated apoptosis following whole-body ionizing radiation treatment (Fig. 2F). Similar to the results obtained with thymocytes, the p53Ala18/Ala18 splenocytes showed increased viability relative to wild-type splenocytes, indicating that p53-mediated apoptosis was also compromised in these cells.

Analysis of the role of p53 serine 18 phosphorylation in p53 stability. Phosphorylation of serine 18 has been proposed to inhibit Mdm2-p53 complex formation (55), leading to the stabilization of p53 levels by preventing Mdm2 from targeting p53 for ubiquitination and proteosomal degradation. Therefore, reduction in p53Ala18/Ala18 thymocyte apoptosis might reflect differences in the stabilization of p53 levels by phosphor-

ylation. To explore this possibility, we examined the basal level of p53 and DNA damage-induced levels of p53 in wild-type and p53Ala18/Ala18 thymocytes. As expected, p53 was strongly induced in these cells 8 h after whole-body irradiation of mice (Fig. 3). However, no difference was detected between the basal p53 levels or the p53 levels induced by ionizing radiation in wild-type and p53Ala18/Ala18 thymocytes. In addition, while expression of p53-responsive genes such as *p21* is upregulated in thymocytes following ionizing radiation, no defect was observed in the *p21* induction in p53Ala18/Ala18 thymocytes. In contrast, induction of the proapoptotic gene *PUMA* was decreased in p53Ala18/Ala18 cells relative to p53 wild-type cells. PUMA has recently been shown to be the principal mediator of p53-dependent death in thymocytes (25).

Analysis of the growth characteristics of p53Ala18/Ala18 embryonic fibroblasts. The rate of embryonic fibroblast proliferation (22, 29) and cell cycle arrest in response to ionizing radiation damage (31) is p53 dependent. To explore a role for p53 serine 18 phosphorylation in p53-mediated control of cell growth, we generated murine embryonic fibroblasts (MEFs) from wild-type, p53Ala18/Ala18, and p53-deficient embryos (day 13.5). Studies on bromodeoxyuridine uptake by these cells revealed no difference between wild-type and p53Ala18/Ala18 MEFs (data not shown). The rate of cell proliferation was indistinguishable between these two cell populations (Fig. 4A).

To determine if p53Ser18 phosphorylation alters p53-mediated cell cycling, we analyzed the p53-dependent G_1 arrest post-ionizing radiation. We treated cells with 8 Gy of ionizing radiation and analyzed cell cycle profiles at 18 h postirradiation by fluorescence-activated cell sorting analysis. Three lines of p53Ala18/Ala18 MEFs generated from different mice were analyzed. Similar to wild-type MEFs, p53Ala18/Ala18 MEFs displayed a large loss of S phase following ionizing radiation treatment, with values indistinguishable from wild-type cells (Fig. 4B).

Analysis of protein levels revealed no difference between the basal and ionizing radiation-induced amounts of p53 in wild-type and p53Ala18/Ala18 MEFs, and while Mdm2 and p21 levels were both increased by treatment of the cells with ionizing radiation, the levels of Mdm2 and p21 were similar in wild-type and p53Ala18/Ala18 MEFs (Fig. 4C). These data indicate that phosphorylation does not alter the level of p53 protein in MEFs and is not required for induction of p21.

Immortalization of p53Ala18/Ala18 MEFs. Spontaneous immortalization of embryonic fibroblasts is also dependent upon p53 function, and p53-null MEFs display rapid immortalization in a 3T3 assay, whereas wild-type MEFs do not immortalize or show delayed immortalization in these assays (22). To assess the contribution of p53 serine 18 phosphorylation to immortalization, a modified 3T3 assay was performed with multiple plates of two individual lines of p53Ala18/Ala18 MEFs and one line of p53Ala18/+ MEFs (Fig. 5A). Whereas p53-null MEFs immortalized after passage 4 in the assay and continued to grow rapidly, p53Ala18/Ala18 MEFs stopped dividing by passage 8 and failed to spontaneously immortalize.

p53Ala18 allele fails to rescue Mdm2-null mice. As p53 levels are unchanged by the presence of the p53Ala18 mutation, it is unlikely that phosphorylation alters Mdm2-p53 complex formation. We previously demonstrated the importance of Mdm2-mediated downregulation of p53 activity in early devel-
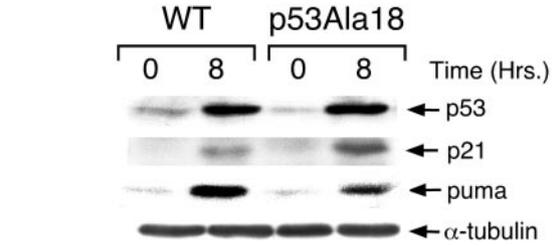
A

B

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D

E

FIG. 2. p53-mediated apoptosis is defective in p53Ala18 mice. (A) Analysis of sub-G_1 content of thymocytes removed from irradiated and unirradiated animals (8 Gy, 8 h posttreatment). The y axis depicts cell number, and the x axis represents DNA content. The percentage of sub-G_1 cells is given for each sample. WT, wild type. (B) Histogram of sub-G_1 DNA from irradiated and nonirradiated mice. Two independent animals were analyzed in triplicate for each genotype. (C) Time course of CD4$^+$ CD8$^+$ profile of mice irradiated (8 Gy) in vivo and nonirradiated mice. The graph depicts double-positive cells. (D) Viability of thymocytes over time in response to ionizing radiation. Thymocytes were removed from mice, irradiated (8 Gy), and harvested at various times (0, 8, 16, and 24 h). Cells were stained with annexin V-fluorescein isothiocyanate, anti-CD4$^+$, and 7AAD. Values given are the averages of the viable population (annexin V-fluorescein isothiocyanate and 7AAD negative) and are normalized to the number of viable cells in the untreated animal for each genotype. (E) Apoptosis in splenocytes is reduced in p53Ala18 mice. Splenocytes were prepared and plated (2×10^5 cells). Cells were irradiated with 8 Gy. Cell viability was determined 7 days post-ionizing radiation. The x axis represents time (days), and the y axis represents viability relative to unirradiated control cells.
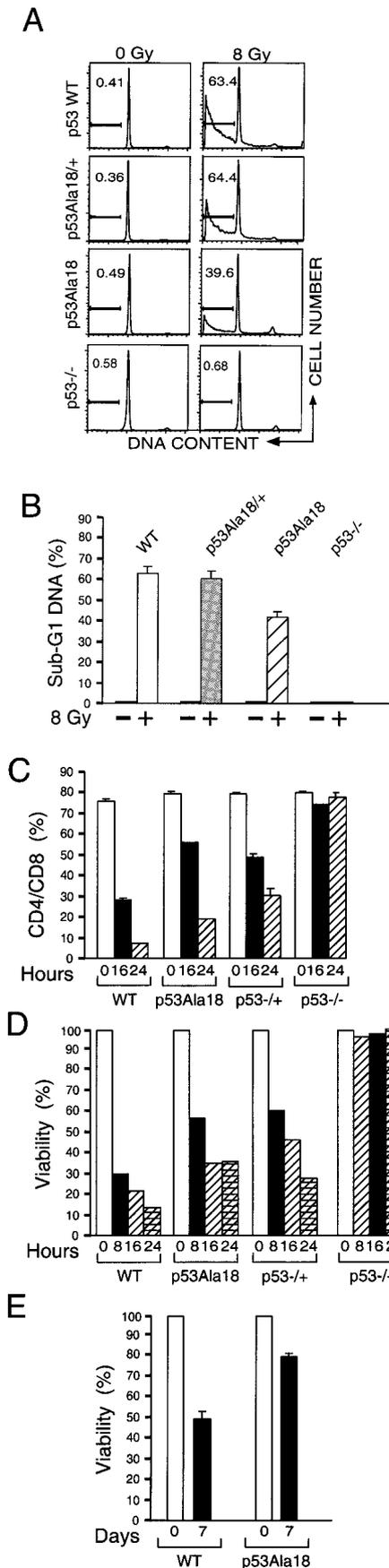

FIG. 3. Mutation of p53Ser18 does not affect basal or ionizing radiation-induced levels of p53 protein. Western analysis of extracts from irradiated (20 Gy) and nonirradiated thymocytes. Extracts were prepared at 0 h (nonirradiated) or 8 h after ionizing radiation treatment. α-Tubulin was used as a control for the Western blot. WT, wild type.

opment: Mdm2-null mice die between day 5 and 6 of development but are rescued by deletion of p53 (28, 45). It is presently unclear which p53 functions are inhibited by Mdm2 during embryogenesis. As loss of p21 fails to rescue Mdm2-nullizygous mice, it has been proposed that unregulated p53 apoptosis is responsible for the early demise of the Mdm2-null mice (44). However, the paucity of tissue in the Mdm2 null embryos makes it difficult to determine the precise p53 functions regulated by Mdm2 in this early stage of development. In order to explore whether reduced apoptotic functions of p53Ala18 would rescue Mdm2-null mice, we crossed Mdm2$^{+/-}$ mice with p53Ala18 mice as well as intercrossed Mdm2$^{+/-}$ p53Ala18/Ala18 mice. Genotyping of the Mdm2 and p53Ala18 status of the resulting pups determined that, unlike p53$^{-/-}$, p53Ala18/Ala18 does not rescue the embryonic lethality of Mdm2-null mice (Table 1).

Absence of spontaneous tumorigenesis in p53Ala18 mice. To determine if phosphorylation of p53 serine 18 is required for tumor suppression, cohorts of p53Ala18/Ala18 mice were collected and assayed for spontaneous tumor formation. At present, we have 30 p53Ala18/Ala18 mice that are between 40 and 60 weeks of age. Although 100% of p53-null mice and 25% of p53-heterozygous mice (21) succumbed to cancer by 40
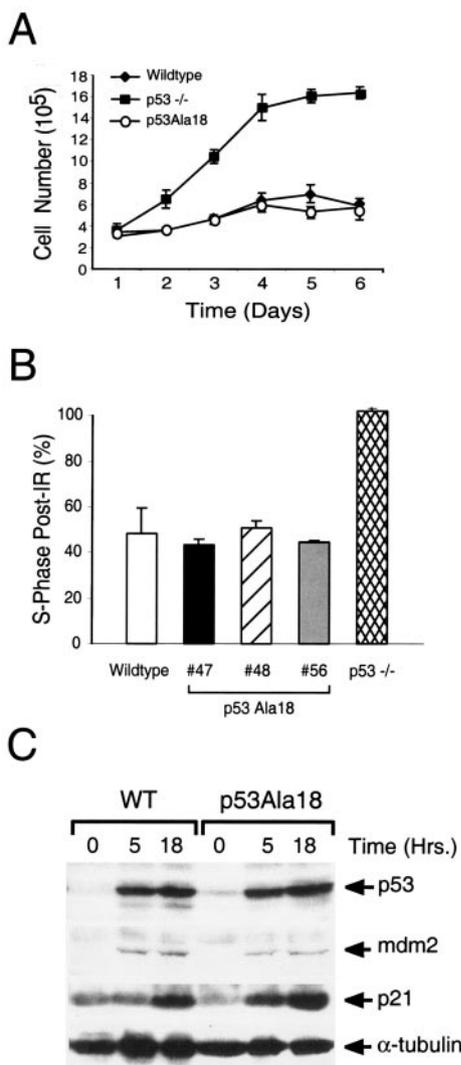
FIG. 4. (A) Proliferation of wild-type, p53Ala18, and p53-null MEFs. (B) Histogram of percent of MEFs in S phase (treated or untreated). MEFs were treated with ionizing radiation and pulsed with bromodeoxyuridine 15 to 18 h postirradiation. Three independent lines of p53Ala18 MEFs were analyzed. (C) Western analysis of extracts from irradiated (8 Gy) and nonirradiated wild-type MEFs and p53Ala18 MEFs harvested at 5 and 18 h post-ionizing radiation treatment. α-Tubulin was used as a control for the Western blot.

weeks of age, we have had no incidence of tumorigenesis in any of our p53Ala18/Ala18 mice, indicating that the reduced apoptotic activity of the mutant p53Ala18 does not alter p53 tumor suppression.

DISCUSSION

It is becoming increasingly apparent from numerous in vitro experiments that p53 is highly posttranslationally modified by phosphorylation. Several studies have demonstrated that p53 serine 18 is phosphorylated in vitro by the ATM and ATR kinases (3, 8, 33, 46, 51, 58). Analysis of p53 protein levels and p53 phosphorylation in ATM-deficient cells has suggested that phosphorylation of this residue by ATM helps regulate the
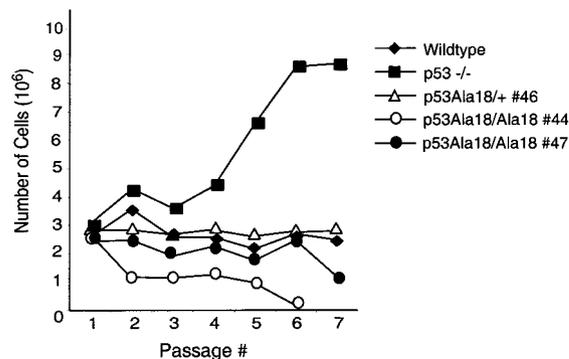

FIG. 5. Immortalization is not induced in p53Ala18 MEFs. 3T3 analysis of two independent p53Ala18/Ala18 MEFs compared to p53Ala18/+, MEFs, and p53-null MEFs.

stability of p53 following DNA damage (3, 8, 32, 56). In addition, it has been suggested that modification of this serine regulates p53 stability by altering Mdm2-p53 interactions (55). However, the role of serine phosphorylation in p53 stability has been placed in question by the results of other transient transfection studies with a mutant p53 that lacks all amino-terminal phosphorylation sites (2).

In order to better understand the role of p53 serine 18 phosphorylation in regulating p53 stability and functions, we generated mice bearing a missense mutation (p53Ser18 to Ala18) and analyzed p53 functions in p53Ala18 mice and in primary cells derived from these mice. Our in vivo analysis of p53Ala18 thymocyte and splenocyte apoptosis revealed a clear reduction in p53 apoptotic activity when p53Ser18 was incapable of being phosphorylated. This result is in agreement with a previous report of diminished p53 apoptosis in p53Ala18 ES cells (10) and suggests that phosphorylation of this residue by ATM and ATR contributes to the p53 apoptotic response.

While this article was being prepared for submission, an independent group reported a defect in cell cycle arrest and an apoptotic defect in a p53Ala18 mouse model (9). In contrast, the ability of p53 to regulate cell proliferation and to invoke cell cycle arrest following DNA damage was unaltered in the p53Ala18 MEFs, indicating that modification of this residue does not affect the ability of p53 to regulate cell growth. Thus, apoptosis and cell growth arrest are p53 functions that are distinctly regulated by p53Ser18 phosphorylation.

Interestingly, the amount of p53 protein in wild-type and p53Ala18 thymocytes and MEFs was similar under basal and DNA damaging conditions, indicating that Mdm2 is properly regulating the stability of p53Ala18 in these cells. In addition, p53Ala18 homozygosity failed to rescue Mdm2 deficiency during embryogenesis. Since the absence of Mdm2 is lethal to both wild-type p53 and p53Ala18/Ala18 embryos, Mdm2 must still govern p53Ala18 activity in development. Thus, phosphorylation of serine 18 does not alter Mdm2 regulation of p53 stability in these cells.

The cyclin-dependent kinase inhibitor p21 is a major regulator of the G_1 to S transition of the cell cycle and has been shown previously to be upregulated by p53 (6, 13, 16, 20). Cell growth and ionizing radiation-induced G_1 arrest was not compromised in p53Ala18 cells in vivo or in MEFs. In agreement

TABLE 1. p53Ala18 does not rescue the embryonic lethality of Mdm2-null mice

Cross	Genotype	No. of offspring with genotype:	
		Expected[a]	Obtained
Mdm2$^{+/-}$ p53Ala18/Ala18 × Mdm2$^{+/-}$ p53Ala18/Ala18 ($n = 15$)	Mdm2$^{+/+}$ p53Ala18/Ala18	4	3
	Mdm2$^{+/-}$ p53Ala18/Ala18	8	12
	Mdm2$^{-/-}$ p53Ala18/Ala18	4	0
Mdm2$^{+/-}$ p53Ala18/+ × Mdm2$^{+/-}$ p53Ala18/+ ($n = 96$)	Mdm2$^{+/+}$ p53$^{+/+}$	7	18
	Mdm2$^{+/+}$ p53Ala18/+	15	15
	Mdm2$^{+/+}$ p53Ala18/Ala18	7	4
	Mdm2$^{+/-}$ p53$^{+/+}$	15	25
	Mdm2$^{+/-}$ p53Ala18/+	30	24
	Mdm2$^{+/-}$ p53Ala18/Ala18	15	8
	Mdm2$^{-/-}$ p53$^{+/+}$	0 (lethal)	0
	Mdm2$^{-/-}$ p53Ala18/+	0 (lethal)	0
	Mdm2$^{-/-}$ p53Ala18/Ala18	7	0

[a] Calculated assuming p53Ala18/Ala18 rescues Mdm2 deficiency, rounded to nearest whole number.

with these findings, the levels of p21 were not reduced below wild-type levels in the p53Ala18 MEFs or thymocytes. Apoptosis was compromised in p53Ala18 cells, and the proapoptotic PUMA protein was found to be downregulated in p53Ala18 thymocytes. As p53 protein levels do not differ in p53Ser18 and p53Ala18 cells, these results indicate that phosphorylation of p53Ala18 likely regulates the ability of p53 to transactivate apoptotic genes.

The growth rate of mouse embryonic fibroblasts is significantly increased following deletion of a single p53 allele (22), and p53 haploinsufficiency is sufficient to predispose mice to spontaneous tumorigenesis without loss of heterozygosity for functional p53 in tumors (61). These results indicate that even a partial reduction in p53 activity can alter cell growth in some context and interfere with p53-mediated tumor suppression. However, the reduced levels of p53 apoptotic function in the p53Ala18 mice still induced the embryonic demise of Mdm2-null mice and prevented tumorigenesis in p53Ala18/Ala18 mice. These findings indicate either that cell cycle arrest is an important component of p53-mediated tumor suppression and regulation of development or that p53-induced apoptosis is not rate limiting in these situations.

The role of ATM in cell growth and tumor suppression has been well established (4, 17, 57, 64, 68). The ATM and the related ATR kinase have been demonstrated to regulate p53 activity. In vitro studies have provided evidence suggesting that ATM/ATR-induced phosphorylation of p53 amino-terminal serines regulates p53 function. In addition to phosphorylating p53Ser18, ATM has been proposed to influence Chk2-mediated phosphorylation of p53Ser20 (p53Ser23 in the mouse) following DNA damage (24, 54, 59). However, p53 stability and p53 transactivation of gene expression were found recently to be unaltered in thymocytes and MEFs derived from chimeric mice generated by using p53Ala23 ES cells (67). These findings suggest that p53Ser18 must be the key regulatory target of ATM/ATR phosphorylation. Although p53 apoptosis was partially compromised in our p53Ala18 model, it is clear from our data that ATM and ATR must rely upon targets other than serine 18 to fully regulate p53 activity in response to DNA damage and to suppress tumor formation.

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