The Ataxia Telangiectasia–Mutated Target Site Ser[18] Is Required for p53-Mediated Tumor Suppression

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Abstract

The p53 tumor suppressor is phosphorylated at multiple sites within its NH_2-terminal region. One of these phosphorylation sites (mouse Ser[18] and human Ser[15]) is a substrate for the ataxia telangiectasia–mutated (ATM) and ATM-related (ATR) protein kinases. Studies of *p53*^{S18A} mice (with a germ-line mutation that replaces Ser[18] with Ala) have indicated that ATM/ATR phosphorylation of p53 Ser[18] is required for normal DNA damage–induced PUMA expression and apoptosis but not for DNA damage–induced cell cycle arrest. Unlike *p53*-null mice, *p53*^{S18A} mice did not succumb to early-onset tumors. This finding suggested that phosphorylation of p53 Ser[18] was not required for p53-dependent tumor suppression. Here we report that the survival of *p53*^{S18A} mice was compromised and that they spontaneously developed late-onset lymphomas (between ages 1 and 2 years). These mice also developed several malignancies, including fibrosarcoma, leukemia, leiomyosarcoma, and myxosarcoma, which are unusual in p53 mutant mice. Furthermore, we found that lymphoma development was linked with apoptotic defects. In addition, *p53*^{S18A} animals exhibited several aging-associated phenotypes early, and murine embryonic fibroblasts from these animals underwent early senescence in culture. Together, these data indicate that the ATM/ATR phosphorylation site Ser[18] on p53 contributes to tumor suppression *in vivo*. [Cancer Res 2007;67(24):11696–703]

Introduction

Patients with ataxia telangiectasia exhibit pleiotropic symptoms, including ataxia (motor skill impairment), telangiectasia (dilated superficial blood vessels), early aging, and susceptibility to cancer (1). The gene deleted in ataxia telangiectasia patients encodes the ataxia telangiectasia–mutated (ATM) protein kinase (ataxia telangiectasia mutant), and *ATM*-null mice are a model for the human ataxia telangiectasia disease. *ATM*-null mice develop lymphomas (2–5). Cells from *ATM*-null mice have been shown to senesce, and animals undergo early aging under certain conditions (5, 6). The defects, such as aging and tumor susceptibility, associated with *ATM*-null mice and ataxia telangiectasia patients are associated with loss of DNA repair activity or DNA-damage checkpoints.

One target for the ATM protein kinase is the tumor suppressor p53. In response to genotoxic and cellular stresses, such as DNA damage, the p53 protein undergoes posttranslational modification, including acetylation and phosphorylation (7, 8). Phosphorylation is part of a dynamic process of regulating p53 tumor suppressor function. In DNA-damaged human cells, p53 protein levels are stabilized and ATM and ATM-related (ATR) kinases phosphorylate the p53 tumor suppressor directly at Ser[15] (mouse Ser[18]; refs. 9–11) and indirectly at Ser[20] (mouse Ser[23]) via the Chk2 protein kinase. After recovery from damage, p53 protein levels return to a prestress state and p53 is dephosphorylated (12, 13).

To examine the role of Ser[18] in these processes of p53 and potential mediator of ATM and ATR function, we generated an animal model in which Ser[18] (human Ser[15]) has been changed to an Alanine, an amino acid that cannot undergo phosphorylation. Studies of *p53*^{S18A} mice have indicated that phosphorylation of p53 Ser[18] is required for normal DNA damage–induced PUMA expression and apoptosis but not for DNA damage–induced cell cycle arrest (7). PUMA, a proapoptotic BH3-only protein, is the major mediator of p53-dependent apoptosis (14, 15). Consistent with normal cell cycle arrest, p21 protein levels are induced in response to DNA damage (16). Therefore, studies from these animals showed Ser[18] is able to "fine tune" the induction of particular genes in response to DNA damage. Unlike *p53*-null mice, *p53*^{S18A} mice did not succumb to early tumors (7). In addition, cells from *p53*^{S18A} mice grew poorly and were unable to undergo a modified 3T3 immortalization assay.

Although the roles of p53 in mediating the DNA-damage response and in tumor suppression were proposed to be causally linked, this link has recently been questioned (17, 18). To reexamine the role of the DNA-damage response in p53 tumor suppression, we studied tumor formation in aged *p53*^{S18A} animals. We found that the *p53*^{S18A} mice died between ages 1 to 2 years. Analysis of these animals showed that the mice developed spontaneous tumors, predominantly lymphomas of B-cell lineage. The mice also developed cancers that are unusual in p53 mutant mice, such as leukemia, fibrosarcoma, leiomyosarcoma, and myxosarcoma. A portion of animals that died between 1 to 2 years had no tumors, yet their survival was compromised. These animals presented phenotypes associated with accelerated aging. In addition, we showed that cells from *p53*^{S18A} mice underwent early senescence, similar to *ATM*-null cells (5). Here we present our findings that Ser[18] regulates p53 tumor suppression *in vivo*. Together, these data indicate that the ATM/ATR phosphorylation site Ser[18] on p53 contributes to tumor suppression.

Materials and Methods

Mouse strains and tumor analysis. The generation and genotyping of the *p53*^{S18A} mice (16) and *p53*$^{-/-}$ mice (19) have been previously described. *p53*$^{S18A/+}$ mice were intercrossed to obtain *p53*$^{S18A/S18A}$, *p53*$^{S18A/+}$, and *p53*$^{+/+}$ mice, and similar age-matched mice were aged. Single allele p53^{S18A} mice were generated by mating *p53*$^{-/-}$ and *p53*$^{S18A/+}$ mice to obtain *p53*$^{+/-}$ and *p53*$^{S18A/-}$ mice. All mice were on a mixed 129/Sv × C57BL/6 background. The mice were observed twice a week for any signs of tumors

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or distress. Mice were sacrificed when a tumor was apparent or when the mice became unhealthy (severe weight loss, severe dermatitis, or pronounced lordosis). Mice were examined by necropsy to detect tumors or disease. Tumors or abnormal organs were embedded in paraffin and sectioned, mounted on slides, and stained with H&E. Slides were examined by a board-certified veterinary pathologist. The lineage of some tumor sections was examined by immunostaining with the B-cell–specific antibody B220 (Becton Dickinson).

All mice were housed in a pathogen-free facility accredited by the American Association for Laboratory Animal Care. The Institutional Animal Care and Use Committee of the University of Massachusetts approved all studies using animals.

Cell culture and senescence assays. Murine embryonic fibroblasts (MEF) were generated from day 13.5 embryos. The MEFs were maintained in DMEM supplement with 10% fetal bovine serum, penicillin, and streptomycin (Invitrogen). The MEFs were cultured at subconfluence and were passaged no more than four times, unless otherwise indicated. A 3T3 assay was performed by plating 1×10^6/100-mm dish, and trypsinizing and replating every 3 days. Cell counts were obtained during each passage. Retroviral transduction studies were performed with recombinant viruses generated using Bosc cells and the vectors pBabe-Puro and pBabe-*HaRas61L*-Puro. In brief, cells at 80% confluence were transfected with 10 μg plasmid using Lipofectamine (Invitrogen). Retroviral particles were collected at 48 h posttransfection and used for viral transduction of primary MEFs. The cells were selected after 12 h by adding 3 μg/mL puromyocin. Two days postselection, the cells were trypsinized and plated at 25,000 cells per well in a 12-well dish. Viability was determined at days 2, 4, and 6 postplating by trypan blue staining. The number of senescent cells was determined by staining for the senescence marker acidic β-galactosidase (β-gal; 20).

Apoptosis assay and Western analysis. Animals were whole-body irradiated with 8 Gray (*Gy*) using a Gammacel 40. Spleens were harvested 18 h posttreatment. Splenoctyes were released and red blood cells were lysed in red blood cell lysis buffer (0.0009% EDTA, 155 mmol/L NH4Cl, and 9 mmol/L KHCO3) for 10 min on ice. Cells (1×10^6) were analyzed by fluorescence-activated cell sorting (FACS) and B220-FITC–positive cells were identified (1:200; Becton Dickinson). Western blot analysis was done as described (16).

Data analysis. Survival of mice was determined by Kaplan-Meier analysis using JMP software from SAS. Pair-wise comparison was done using the log-rank test. Statistical significance was set at $P < 0.01$.

Results

***$p53^{S18A}$* cells undergo premature senescence.** Interestingly, while performing a 3T9 immortilization assay, we observed that the cells grew poorly (16). We also observed that cells undergoing subconfluent passaging also grew poorly and contained a portion of cells with flattened phenotype, reminiscent of cells that have exited the cell cycle. Because cells are plated at 3×10^6 cells/100-mm dish in the 3T9 assay, the early-demise phenotype of the $p53^{S18A}$ MEFs might have been due to high density plating. To exclude this possibility, we performed the immortalization assay using the 3T3 protocol, in which cells are plated at 1×10^6/100-mm dish. The $p53^{S18A}$ cells again grew poorly and died by passage 5 (Fig. 1*A*). We characterized the different cell passages by staining the cells for acidic β-gal to examine senescence (20). The number of positive blue cells increased with each passage of $p53^{S18A}$ cells, compared with wild-type cells. By passage 3, 30% of $p53^{S18A}$ cells stained positive for β-gal, whereas none of the wild-type cells stained positive (data not shown). Also, the cells seemed to be mostly large flat cells, which is consistent with cells having exited the cell cycle (data not shown). Therefore, the early defects in growth of $p53^{S18A}$ cells in a 3T3 or 3T9 assay were due to increased senescence.

***$p53^{S18A/S18A}$* cells undergo increased senescence in response to oncogenic *Ras*.** *H-Ras*–infected wild-type cells undergo senescence, which is p53 mediated (21). To determine if MEFs from $p53^{S18A/S18A}$ mice responded to oncogenic *Ras*, we infected $p53^{+/+}$, $p53^{-/-}$, and $p53^{S18A/S18A}$ cells with oncogenic *H-Ras* (Fig. 1*B*). Cells were treated with puromycin to kill uninfected cells, replated, and stained at various passages for β-gal (20). Wild-type MEFs did not grow in the presence of oncogenic *Ras* (Fig. 1*B, left*). $p53^{-/-}$ MEFs grew robustly in the presence of oncogenic *Ras* (Fig. 1*B, right*), consistent with published data (21). However, the $p53^{S18A}$ cells did not escape the inhibitory affects of oncogenic *Ras* (Fig. 1*B, middle*). Instead, *Ras* had a greater negative effect on cell growth in $p53^{S18A}$ cells than in wild-type cells.

To examine if the decrease in cell growth was due to senescence, day 6 cells were stained for acidic β-gal. No positive staining was found in 300 *Ras*-infected $p53^{-/-}$ cells (Fig. 1*C*). Almost all Ras-infected wild-type cells stained positive for β-gal, indicating senescence was induced. More *Ras*-infected $p53^{S18A}$ MEFs stained positive for β-gal than *Ras*-infected wild-type MEFs, confirming the negative growth effect of *Ras*. Wild-type and $p53^{S18A}$ cells grown in the presence of *Ras* presented with flat cells (Fig. 1*D*), a phenotype characteristic of cells that have exited the cell cycle. Taken together, these findings suggest a role of Ser^{18} in p53-mediated control of senescence.

p53 Ser^{18} plays a role in mouse survival. To examine the role of p53 Ser^{18} in aging, we tested the effect of loss of Ser^{18} on the life span of mice. A cohort of $p53^{S18A}$ animals under age 1 year were previously shown to be viable and have no tumors (16, 22). In this study, the survival of wild-type, $p53^{+/-}$, $p53^{S18A/+}$, $p53^{S18A/-}$, and $p53^{S18A/S18A}$ mice was examined further, to ages 2 years (Fig. 2*A*). The Kaplan-Meier analysis indicated that the survival of $p53^{S18A/S18A}$ mice was significantly shorter compared with wild-type survival ($P = 0.004$). The median survival of $p53^{S18A/+}$ mice (94 weeks) was similar to that of wild-type $p53^{+/+}$ mice (98 weeks). In contrast, the median survival of p-$53^{S18A/S18A}$ mice (81 weeks) and $p53^{S18A/-}$ mice (66 weeks) were less than wild-type. Interestingly, the survival age of $p53^{S18A/-}$ mice was less than the median survival of $p53^{+/-}$ mice (71 weeks). Together, these data indicate that replacing Ser^{18} on p53 with Ala^{18} decreased mouse survival. Nevertheless, the decrease in survival caused by mutating Ser^{18} was not as severe as that seen in $p53^{-/-}$ mice, which had a median survival age of 18 weeks (23).

***p53S18A* mice develop spontaneous tumors.** To determine whether $p53^{S18A}$ mice older than age 1 year developed late-onset tumors, we examined the animals from the survival study (7) for tumor formation. The tumor burden at time of death of all mice was determined by necropsy and histologic analysis of tissue sections. We found that $p53^{S18A}$ mice developed spontaneous tumors between ages 1 and 2 years.

The predominant tumor detected in mice with p53 mutation at Ser^{18} was lymphoma (Table 1). Among all tumors, the incidence of lymphoma was 72% in $p53^{S18A/S18A}$ animals and 47% in $p53^{S18A/+}$ animals. The presentation of lymphomas in $p53^{S18A}$ mice had a multicentric distribution, with involvement mostly in the spleen and presentation in the liver. Only one lymphoma in a $p53^{S18A/S18A}$ animal exhibited thymic involvement, which was likely a tumor disseminated from the spleen and lymph node. The incidence of lymphoma was similar in $p53^{S18A/+}$ and $p53^{+/-}$ mice. Although $p53^{+/-}$ mice also develop lymphomas, these are primarily of the T-cell lineage.

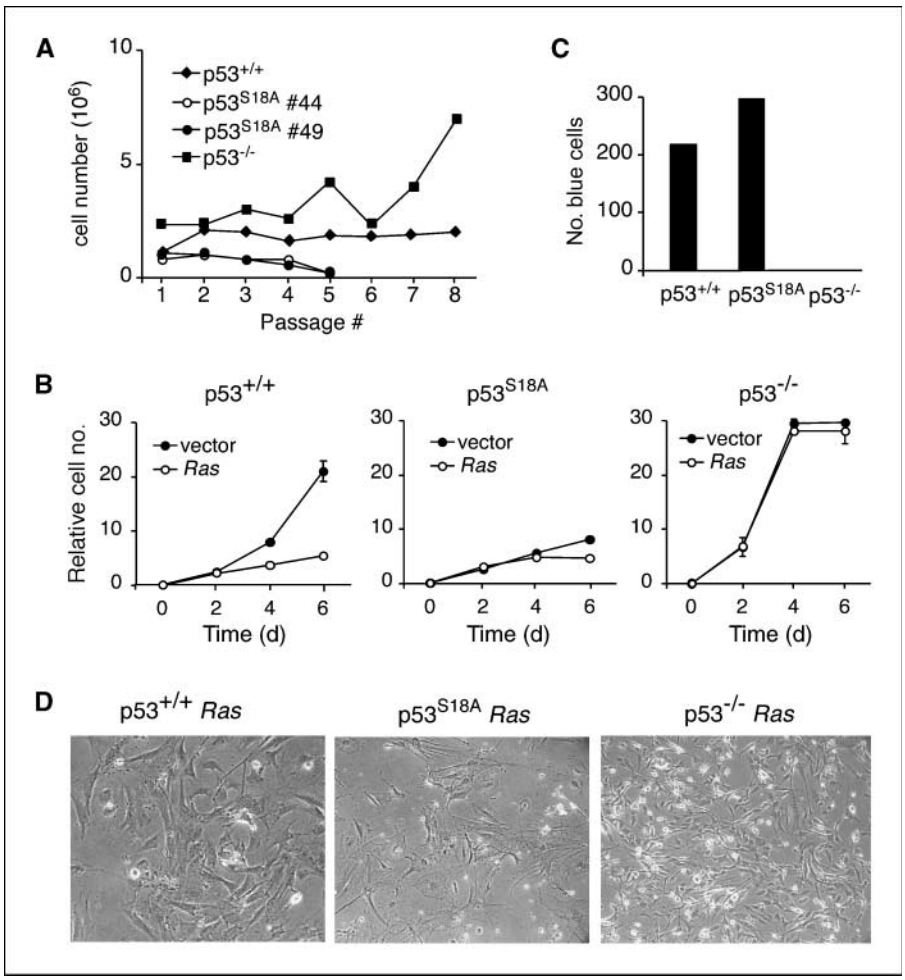

Figure 1. $p53^{S18A}$ cells undergo premature senescence. *A,* $p53^{S18A}$ cells have a shorter life span than other mouse embryo fibroblast cells. Cells were passaged under standard 3T3 protocol and their cell number were determined up to 8 passages. *B,* Ras infection reduces viability of $p53^{S18A}$ cells. Cells were infected with pBabe (vector) or pBabe-*HaRas* and were plated. Cell viability was determined at 2, 4, and 6 d postplating. *C,* $p53^{S18A}$ cells exhibit flat cell phenotype. Phase contrast micrograph of cells infected with *HaRas*. *D,* $p53^{S18A}$ induces increased senescence in response to oncogenic Ras. Number of cells staining positive for acidic β-gal in cells response to oncogenic Ras.

The majority of lymphomas in $p53^{S18A}$ mice were B-cell lineage based on histologic criteria (24). Histopathology of representative tumors from $p53^{S18A}$ mice are shown in Fig. 3. A diffuse lymphoma in one $p53^{S18A/S18A}$ mouse was concentrated in the white pulp and extended into red pulp (Fig. 3*A, left*). Infiltrating cells had a diffuse pattern composed of mostly large centroblastic cells (>80%). The same animal had dissemination of the lymphoma to the liver (data not shown). Almost complete effacement of the splenic architecture was also observed in some diffuse large-cell lymphomas. Follicular lymphomas were less common and remained in the white pulp with a nodular pattern and had a mixture of centroblasts and centrocytes. An example of a follicular lymphoma staining positive for B220, a B-cell marker, is depicted in Fig. 3*A* (*middle*). Interestingly, small focal incipient lymphomas were detected in $p53^{S18A/S18A}$ mice in the spleen and lymph nodes (data not shown). The majority of animals did not present with palpable tumors (hepatosplenomegaly) at the time of sacrifice.

Other tumors were detected in $p53^{S18A}$ animals (Table 1). A myeloid leukemia was found in one $p53^{S18A/-}$ animal (Fig. 3*A, right*). The white pulp of the animal no longer contained the correct architecture, and leukemic infiltrate of myeloid differentiation were apparent. $p53^{S18A/S18A}$ mice developed few cancers other than lymphoma. An oligodendroma, a poorly differentiated sarcoma, and one case of bronchoalveolar carcinoma were seen in homozygous $p53^{S18A}$ animals. The oligodendroma invaded the cerebral cortex (*arrow*, Fig. 3*B, middle*).The neoplastic infiltrate was uniform, and mitotic figures were observed in the infiltrate and trapped neurons (*arrow*, Fig. 3*B, right*). A normal brain section is provided for comparison (Fig. 3*B, left*).

The $p53^{S18A/+}$ and $p53^{S18A/-}$ mice also developed other cancers such as fibrosarcoma, leiomyosarcoma, and myxosarcoma, as well as more common sarcomas and carcinomas (Table 1). Sarcomas were observed in 31.5% of tumors from the $p53^{S18A/+}$ mice. Of these sarcomas, the highest incidence was of histiocytic sarcomas (15.7%) and next highest was hemangiosarcomas (5.2%). Only one bronchoalveolar carcinoma was found in $p53^{S18A/+}$ animals (Fig. 3*C, left*). In addition, one $p53^{S18A/+}$ mouse developed a leiomyosarcoma and an undifferentiated sarcoma. The leiomyosarcoma, a malignancy of smooth muscle origin, is represented in Fig. 3*C* (*middle*). The tumor is seen pushing and extending into the normal muscle wall (*bracketed region*, Fig. 3*C, middle*). The cells are spindle shape in appearance and many mitotic figures are apparent. The $p53^{+/-}$ mice developed mostly osteosarcomas (41.6%) and histiocytic sarcomas (41.6%). Unlike $p53^{+/-}$ mice, the $p53^{S18A/+}$ and $p53^{S18A/S18A}$ mice exhibited no osteosarcomas.

$p53^{S18A/-}$ animals developed one case of histiocytic sarcoma in the liver (Fig. 3*D, left*) and two cases of osteosarcoma (data not shown). The histiocytic infiltrate is primarily around the portal veins and bile ducts. Also detected were two rare cancers: a fibrosarcoma and a myxosarcoma. A myxosarcoma is a cancer of connective tissue. An expanded tumorous area (*bracketed region*, Fig. 3*D, right*) can be observed compared with adjacent to normal

connective tissue (Fig. 3*D, middle*). In the expanded neoplastic area, cells are further apart and cellularity is increased. Control wild-type mice were also necropsied and analyzed for tumors; only one hemangiosarcoma was observed. Thus, the appearance of spontaneous tumors in mice bearing a mutation at p53 Ser^{18} suggests that the Ser^{18} phosphorylation site is critical for tumor suppression by p53.

***$p53^{S18A}$* mice exhibit nonmalignant alterations.** Other changes were detected in the cellular composition of the of *$p53^{S18A}$* animals. Nonmalignant adenomas were detected in *$p53^{S18A/S18A}$* and *$p53^{S18A/+}$* mice. Hyperplasia was observed more often in the spleens of *$p53^{S18A/S18A}$* animals than in wild-type animals. In *$p53^{S18A/S18A}$* animals, lymphoid follicular hyperplasia was detected in 21% of spleens. In *$p53^{S18A/+}$* animals, lymphoid hyperplasia was detected in 45% of spleens. In our older wild-type mice, although hyperplasia was observed in 25% of spleens, the hyperplasia was very minimal.

To determine the tumor burden of each mouse, we analyzed mice for multiple primary neoplasms of different cell types at origin. The percentages of animals with multiple primary tumors for *$p53^{S18A/S18A}$* and *$p53^{S18A/-}$* mice were 13% (2 of 15) and 9% (1 of 11), respectively. The proportion of *$p53^{+/-}$* mice with more than one tumor was 26.3% (5 of 19). Interestingly, the highest percentage of animals [36% (4 of 11)] with multiple tumors of different origin was found in *$p53^{S18A/+}$* mice. In contrast, the percentage of tumor metastases was low in all mice. This increased tumor burden in $p53^{S18A}$ mice supports a role for Ser^{18} in tumorigenesis.

Accelerated aging-associated phenotype in $p53^{S18A}$ animals. Several animals in the survival analysis did not succumb to tumors. Although the *$p53^{S18A}$* animals developed tumors, the tumor penetrance was 35.7% and 36% for *$p53^{S18A/S18A}$* animals, respectively. In contrast, the tumor penetrance for *$p53^{+/-}$* animals was 70%. The survival of nontumor-bearing *$p53^{S18A/S18A}$* animals was significantly shorter compared with nontumor-bearing wild-type animals (P = 0.01). The shorter survival of nontumor-bearing *$p53^{S18A}$* mice suggests that their aging process could be accelerated due to the Ser^{18} mutation. In fact, these mice exhibited heightened expression of several factors associated with aging, such as inability to heal, premature graying, chronic alopecia, and lordokyphosis (Table 1). Many animals had to be sacrificed due to chronic severe dermatitis. The animals also had organ problems suggesting increased loss of function. For example, extramedullary hematopoiesis (EMH) was found in many more aged *$p53^{S18A}$* than in wild-type animals (Table 1). In *$p53^{S18A/S18A}$* animals, EMH was detected in 38.7% of spleens. In *$p53^{S18A/+}$* animals, EMH was detected in 54% of spleens. Splenic EMH was detected in 16% of older *$p53^{S18A}$* animals. Although caution must be taken in interpreting the individual appearance of these phenotypes, taken together, these observations support a model in which early death is due to accelerated aging. This model is supported by the observation of early senescence observed in MEFs (Fig. 1).

Defective apoptosis in B cells. The role of p53 in tumor suppression has been proposed to be through apoptosis. We previously that showed apoptosis is defective in thymocytes derived from *$p53^{S18A}$* animals (16). To determine if loss of apoptosis could contribute to tumor formation, we determined if apoptosis was defective B cells from *$p53^{S18A}$* animals. Thus, we examined the loss of B cells in response to DNA damage. Wild-type B cells are reduced in response to IR to 10.2% (Fig. 4*A*). In contrast, B cells form *$p53^{S18A}$* animals are reduced to 21.3% (Fig. 4*A*). This suggests that apoptosis is defective in $p53^{S18A}$ B cells. We analyzed PUMA induction and found that similar to $p53^{S18A}$ thymocytes (16), induction of the protein was compromised in response to IR (Fig. 4*B*).

Analysis of survival of tumor-bearing mice. To verify a possible role of apoptosis in p53-mediated tumor suppression, we

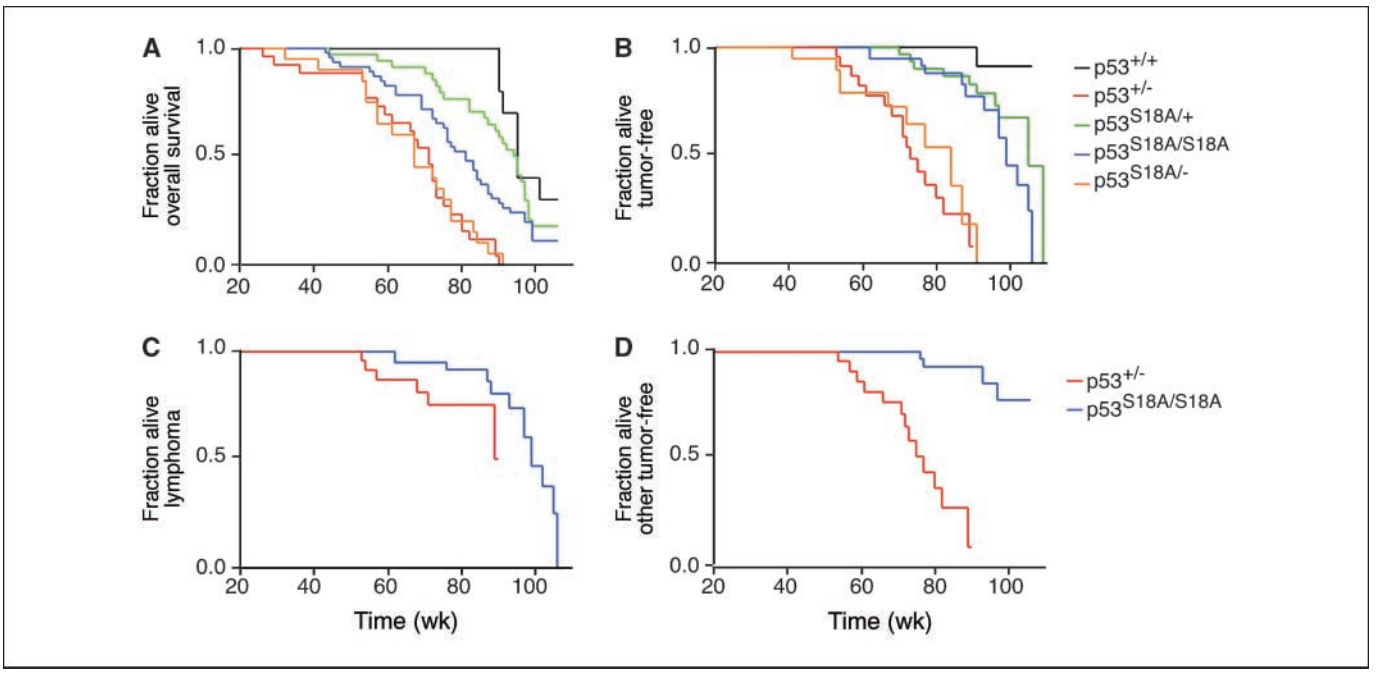

Figure 2. Overall survival and survival of tumor-bearing $p53^{S18A}$ mice. *A,* overall survival. Kaplan-Meier distribution of overall survival of $p53^{S18A/+}$, $p53^{S18A/S18A}$, and $p53^{+/-}$ mice over the course of 2 y. Percent survival is on the *Y* axis and age of death (in weeks) is on the *X* axis. Death was by either presence of tumor, illness, or case unknown (see Table 1). *B,* survival of tumor-bearing animals. Kaplan-Meier analysis of animals that died from tumors from survival curve. *C,* lymphomas develop with the same kinetics in $p53^{S18A}$ and $p53^{+/-}$ animals. Survival of lymphoma-only bearing $p53^{+/-}$ and $p53^{S18A}$ animals is not significantly different ($P > 0.01$). *D,* nonlymphoma tumors do not have the same kinetics in $p53^{S18A}$ and $p53^{+/-}$ animals. Breakdown of survival in animals bearing tumors other than lymphomas.

examined the survival of tumor-bearing $p53^{S18A}$ mice. In a previous study (16), we showed that $p53^{S18A/S18A}$ and $p53^{+/-}$ thymocytes have the same levels of apoptosis in response to DNA damage in thymocytes (16). In addition, B cells from $p53^{S18A}$ animals are defective in apoptosis (Fig. 4). If defects in the DNA-damage response are implicated in p53-mediated tumor suppression, then the tumor latency or survival should be similar. Surprisingly, we found that survival of animals bearing all tumors types differed significantly in the $p53^{+/-}$ and $p53^{S18A/S18A}$ animals (Fig. 2*B*; $P < 0.001$), suggesting that apoptosis was not rate limiting for tumor development. However, because lymphomas arise from either T or B cells, we examined the survival of $p53^{+/-}$ and $p53^{S18A/S18A}$ mice bearing only lymphomas. Kaplan-Meier analysis revealed the survival of lymphoma-bearing animals with one mutant p53 allele were not significantly different than $p53^{S18A/S18A}$ lymphoma-bearing animals (Fig. 2*C*; $P > 0.01$, significance set at $P < 0.01$). This result suggests that the apoptosis may participate in the p53-mediated suppression of lymphomas. Analysis of survival curves for animals bearing nonlymphoma tumors showed that tumors other than lymphomas arising in $p53^{+/-}$ and $p53^{S18A}$ mice have significantly different tumor/survival curves ($P < 0.001$; Fig. 2*D*). This does not rule out a potential role for apoptosis. However, it does show that these tumors develop with different kinetics in $p53^{S18A}$ and $p53^{+/-}$ animals. The development of spontaneous tumors shows a role of p53 Ser^{18} in regulating p53-mediated tumor suppression.

Discussion

The cycle of p53 phosphorylation and dephosphorylation helps to provide the cell with a mechanism to respond to the stress. The p53-mediated cellular response to stress can be either growth arrest, apoptosis, or senescence. Earlier reports of animals with Ser^{18} mutation indicated no early tumors, suggesting no role for Ser^{18} in the tumor suppressor function of p53. We hypothesized that because of the role of Ser^{18} in apoptosis, the animals should develop tumors. We report that Ser^{18}-defective animals develop late-onset tumors (between 1 and 2 years of life).

$p53^{S18A}$ mice spontaneously develop tumors. The $p53^{S18A}$ mice almost exclusively developed generalized lymphomas, mostly in the spleen and liver, with some involvement of the lymph nodes. The lymphomas were predominantly of B-cell origin. In contrast, $p53^{-/-}$ mice are known to develop T-cell thymic lymphoma (19). This high incidence of lymphomas in $p53^{S18A}$ mice is interesting as a high incidence of lymphomas are found in patients with ataxia telangiectasia, a disease caused by mutation of the ATM protein kinase, which targets Ser^{18} (1). We also detected incipient or low-grade tumors in the mice. These low-grade tumors could account for the difference in observations of Xu and colleagues (22) where no tumors were reported for a group of live animals at 18 months. In addition to lymphomas, $p53^{S18A}$ mice developed sarcomas, carcinomas, and one oligodendroma. The mice also developed less common cancers such as leiomyosarcoma, myxosarcoma, and fibrosarcoma. This development of spontaneous tumors in mice carrying a mutation in p53 at Ser^{18} suggests that the Ser^{18} phosphorylation site is critical for p53-mediated tumor suppression.

An interesting observation is that the $p53^{S18A}$ animals, unlike the $p53^{-/-}$ animals, did not develop early tumors. This result indicates that the $p53^{S18A}$ allele contributes some tumor suppressor function in the $p53^{S18A}$ mice, as they did not behave like *p53-null* mice. $p53^{S18A}$ cells can respond to DNA damage by inducing cell

Table 1. Life span and tumorigenesis in $p53^{S18A}$ mice

Phenotype	$p53^{S18A/+}$	$p53^{S18A/S18A}$	$p53^{S18A/-}$	$p53^{+/-}$	$p53^{+/+}$
No. mice analyzed	31	42	16	27	12
Life span (median; wk)	94	81	66	71	98
Organ phenotypes					
Spleen					
Hyperplasia	14	9	NA	NA	3
EMH	12	23	NA	NA	1
Liver					
Mineralization	9	9	NA	NA	3
Lordokyphosis	4	9	3	0	1
Hair/skin abnormalities	10	25	5	3	1
Animals with tumors	11	15	11	19	1
Total tumors	19	18	12	24	1
Tumor classification					
Lymphoma	9	13	3	8	0
Leukemia	0	0	1	0	0
Osteosarcoma	0	0	2	5	0
Histiocytic sarcoma	3	1	1	5	0
Hemangiosarcoma	1	0	0	2	1
Leiomyosarcoma	1	0	0	0	0
Myxosarcoma	0	0	1	0	0
Fibrosarcoma	0	0	1	0	0
Other sarcoma	1	0	1	2	0
Carcinoma	1	1	2	1	0
Oligodendroma	0	1	0	0	0
Adenoma	3	2	0	1	0

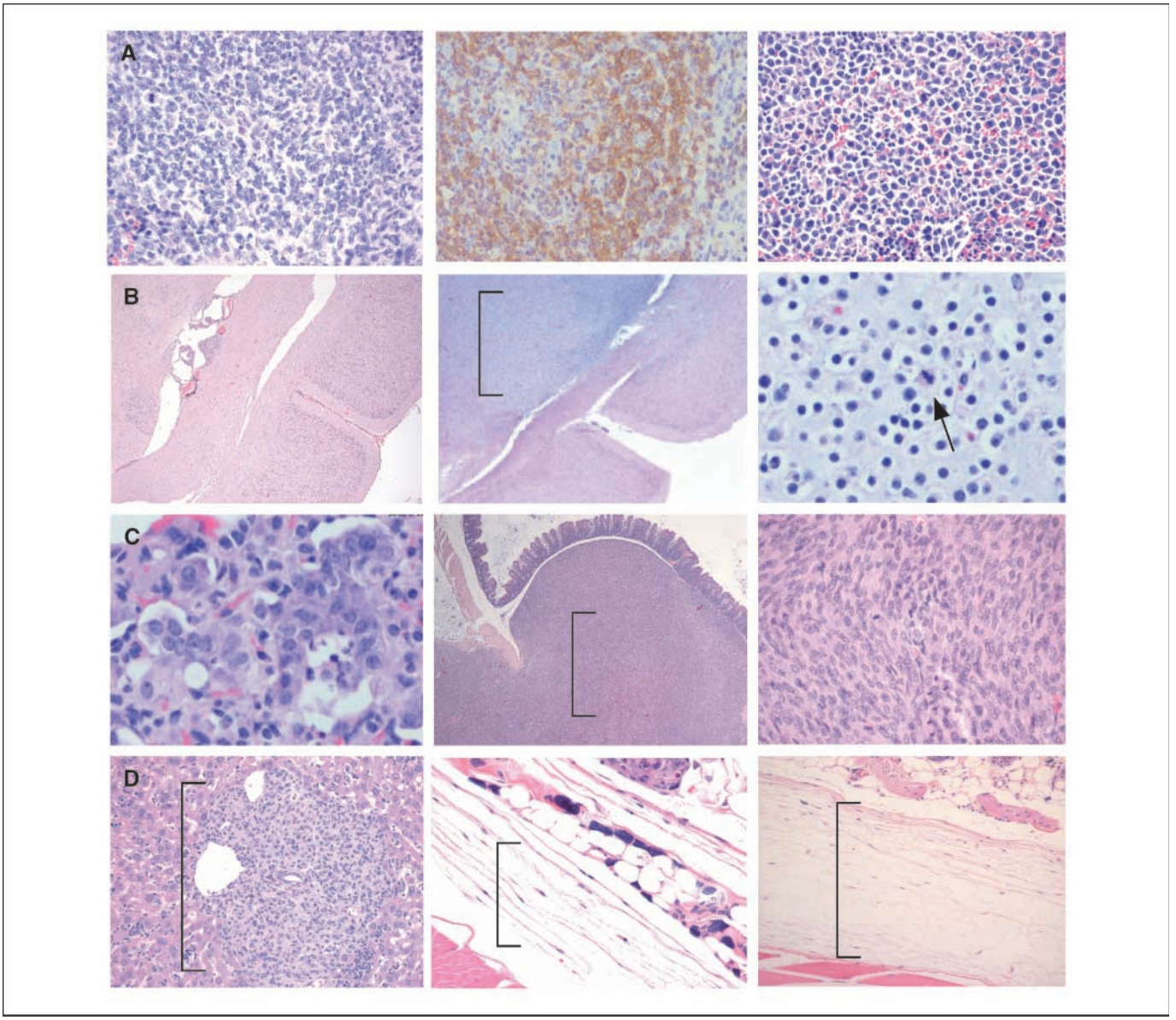

Figure 3. Representative tumors in $p53^{S18A/S18A}$ mice. *A,* lymphomas and myeloid leukemia. Malignant lymphoma in spleen (*left*; ×20). A lymphoma stained with the B-cell–specific antibody, B220 (*middle*; ×20). One $p53^{S18A}$ mouse had a myeloid leukemia (*right*). *B,* oligodendroma. Wild-type normal brain (*left*; ×4) compared with an oligodendroma (*middle*; ×4) and ×40 (*right*). *Bracketed region,* area of neoplastic change; *arrow,* metastasis. *C,* representative carcinoma and leiomyosarcoma. Lung carcinoma from a heterozygous animal (*left*; ×40). A leiomyosarcoma (*middle*; ×4). *Bracketed region,* region of expansion. Spindle cell shape of leiomyosarcoma (*right*; ×40). *D,* representative sarcomas. Histiocytic sarcoma in liver (*left*, ×20); *bracketed region*, region of neoplasm. Normal muscle (*middle*; ×4), and a myxosarcoma in leg muscle (*right*; ×4). Compare expanded tissue (*bracketed region, right*) to normal muscle (*bracketed region, middle*).

cycle arrest (16), suggesting that cell arrest is necessary for suppression of early onset thymic lymphoblastic lymphomas. A role for cell cycle arrest in p53 tumor suppression is supported by the absence of early tumors in mice with a p53 mutation at R172 (25). Although these mutant mice were defective in apoptosis, they were still able to undergo a cell cycle arrest (26). However, older p53 R172 animals developed cancer. Likewise, older $p53^{S18A}$ animals developed tumors, implying that the $p53^{S18A}$ allele lacks tumor suppression capability in other contexts and further supporting a role for p53 Ser^{18} in tumor suppression.

The single allele $p53^{S18A}$ mice in our study succumbed to tumors as rapidly as $p53^{+/-}$ mice. However, the tumor profile was different. Of note, the $p53^{S18A/-}$ mice developed two rare cancers — a myxosarcoma and a fibrosarcoma. In addition, the $p53^{S18A}$ heterozygous animals developed a rare leiomyosarcoma. It is possible that the allele is having a gain-of-function allele, but this is unlikely. Apoptosis assays with heterozygous cells have shown the allele does not act as a dominant negative (16). In addition, the onset of tumors is longer in $p53^{S18A}$ heterozygous and homozygous animals compared with $p53^{+/-}$ animals. Presumably, the decreased levels of p53 and, thus, decreased p53 activity in $p53^{+/-}$ mice contributed to tumor formation. In view of this result, it is interesting that the $p53^{S18A/S18A}$ mice developed tumors. Cells from $p53^{S18A}$ animals do not show concentration defects of p53 (in basal or stimulated states). However, the cells do show different levels of p53-dependent gene induction. The role of this phosphorylation site, Ser^{18}, on gene control is likely to have a rate-limiting role in different cells. Once the animals have

overcome the rapid early-onset of thymomas associated with loss of p53, the critical role of this site in tumor suppression is uncovered.

Although cell cycle may be regulating the early growth suppression (showed by lack of early thymic lymphomas), apoptosis may play a significant role in late-onset tumor suppression. The survival of lymphoma-bearing $p53^{+/-}$ and $p53^{S18A/S18A}$ animals was the same — suggesting the mechanism of tumor induction may be similar. The cells that originate these tumors both display defective p53-dependent apoptosis (Fig. 4; refs. 16, 27). In contrast, the survival of nonlymphoma-bearing animals of the $p53^{S18A/S18A}$ mice was less than the $p53^{+/-}$ mice. The signal for p53 suppression in these $p53^{-/-}$ tumors may be different than that in T- and B-cell lymphomas in older $p53^{S18A}$ animals.

A role for ATM-dependent phosphorylation in p53-mediated tumor suppression is supported by studies of animals with mutant Ser^{23} on p53. p53 Ser^{23} can also be targeted by ATM and ATR by activating Chk1/Chk2 protein kinases (28–30). $P53^{S23A}$ mice show a partial defect in apoptosis, a decreased induction of p53 protein, and an intact cell cycle arrest (27). $p53^{S23A}$ mice also succumb to spontaneous tumors and develop lymphomas, except with a different profile than that of $p53^{S18A}$ mice. Lymphomas in $p53^{S23A}$ mice predominantly involve the lymph nodes, with dissemination to the spleen and rarely to the liver. The $p53^{S23A}$ mice also did not develop unusual sarcomas observed in $p53^{S18A}$ mice. The $p53^{S23A}$ mice had a shorter median survival (64 weeks) than that of our $p53^{S18A}$ mice (81 weeks). These differences in tumor onset and tumor-type distribution suggest that the two phosphorylation sites are not redundant in the role of p53 in tumor suppression.

In fact, these two phosphorylation sites have recently been shown to not act synergistically in the onset of tumors, although they likely cooperate in DNA-damaged death in thymocytes (31). Apoptosis in $p53^{S18A/S23A}$ thymocytes was greatly reduced compared with thymocytes from $p53^{S18A}$ and $p53^{S23A}$ animals. However, $p53^{S18A/S23A}$ thymocytes were not as resistant to apoptosis as those from *p53-null* mice. The double-mutant animals developed tumors that included lymphomas, leukemia, fibrosarcoma, and adenoma. These unusual tumors were observed in the $p53^{S18A}$ animals (Table 1). Thus, these tumors in the $p53^{S18A/S23A}$ mice are most likely attributable to loss of Ser^{18}. Moreover, Kaplan-Meier analysis of survival showed no synergy in the survival of double-mutant mice compared with single-mutant mice. The life span of the double-mutant mice ($p53^{S18/23A}$) was ~87 weeks (31), whereas the life spans of single-mutant mice ($p53^{S23A}$ and $p53^{S18A}$) were 63 weeks (27) and 81 weeks (Fig. 2; Table 1), respectively. This study further supports the theory that these sites are not redundant. We can also rule out that the lack of early tumors (in single-mutant mice) may be due to a compensatory mechanism as the double mutants do not show accelerated tumorigenesis.

Our observation of increased senescence in MEFs from $p53^{S18A}$ animals may seem to contradict our finding of spontaneous tumors. Senescence has been shown to play a role in tumor suppression (32). It is possible that an early senescence is contributing to the suppression of early tumors. However, this is unlikely, as $p53^{S23A}$ animals, which do not display senescence, develop tumors at the same rate. Senescence can also have deleterious consequences on stem cells and lead to their aging. Replicative senescence is often associated with organismal senescence and rapid aging.

p53 has been shown to have a role in aging. Animals with hyperactivating alleles of p53 have compromised life spans and die with characteristics of early aging, although the animals are tumor resistant (33, 34). These p53-based models of accelerated aging have suggested that this phenomenon is due to a truncated p53 allele, which hyperactivates the wild-type allele in the presence of the mutant protein (33, 34). We determined a potential role for Ser^{18} in aging. The $p53^{S18A/S18A}$ and $p53^{S18A/+}$ animals survived longer than $p53^{+/-}$ mice and had a lower incidence of tumors. Several animals in our $p53^{S18A}$ survival cohort did not die from tumors. An obvious cause of illness was not detected. Based on criteria used for other aging models, we propose that $p53^{S18A}$ animals may be dying at an accelerated rate due to accelerated aging. We found several characteristics of aging-related phenotypes and early senescence, including early lordokyphosis, hair graying and alopecia, decreased hair regrowth, ulcerative dermatitis, and liver pathologies (6, 35, 36). Therefore, although the mice did not present with all the aging-related phenotypes, accelerated aging is consistent with our observations of reduced life span and replicative senescence.

Our observation of accelerated aging in the $p53^{S18A}$ mice was surprising. Based on the p53 models of accelerated aging, one would predict that $p53^{S18A}$ mice would not have a reduced life span but a longer life span due to their reduced p53-dependent apoptosis/activity. Instead, we found that loss of Ser^{18} had a negative effect on survival. The early aging seen in these $p53^{S18A}$ mice might be related to the senescence seen in MEF cultures from

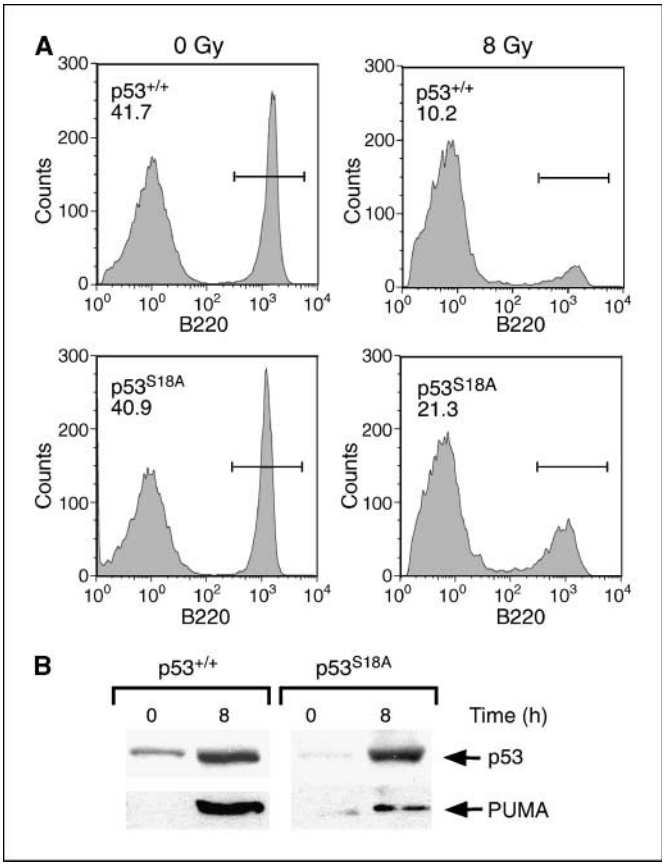

Figure 4. Defective apoptosis in $p53^{S18A}$ B cells and reduced PUMA induction. Mice were irradiated or not treated, and the cells were removed 24 h posttreatment. *A,* FACS analysis of B220-positive cells in response to IR. *B,* Western blot analysis of PUMA expression in spleens treated or not treated with IR.

the mice, whereas the accelerated aging in the hyperactive alleles might occur by a different pathway. Interestingly, an *in vitro* study showed that Ser^{18} phosphorylation is a common event during senescence and DNA damage (37). Our aging model is most similar to the early aging seen in compound $Brca1^{-/-}$ and $p53^{+/-}$ animals (38). Nonetheless, although senescence can be an important means to neutralize unwanted cells, our studies support a model where a negative consequence can be decreased viability of stem cells.

This study has elucidated a role for p53 Ser^{18} in p53-mediated tumor suppression. Models expressing truncated p53 (33, 34) and engineered to produce extra p53 (39, 40) show protection from tumor burden. In the former models expressing mutant p53, lack of tumors has been proposed to come at the expense of life span. Here we present a p53 mutant tumor model where animals can exhibit tumors or accelerated aging. It suggests that Ser^{18} is at a pivotal axis where p53 mediates a cellular response to stress, which influences the long term outcome for the organism. The importance of the finding that Ser^{18} regulates tumor suppression is underscored by observations that not all p53-phosphorylation mutants succumb to cancer. For example, mice deficient in Ser^{389} phosphorylation do not develop spontaneous tumors (41). Here we show that perturbation of phosphorylation of p53, specifically at Ser^{18}, can result in defective p53 function and result in cancer.

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Correction: Tumor Analysis of $p53^{S18A}$ Mice

In the article on tumor analysis of $p53^{S18A}$ mice in the December 15, 2007 issue of *Cancer Research* (1), Dr. Stephen N. Jones should have been included as the third author. The affiliation for Dr. Jones is the Department of Cell Biology and Cancer Biology, University of Massachusetts Medical School, Worcester, Massachusetts. Dr. Jones and Dr. Sluss are co-senior authors of the article. Also, the grant support should have included NIH grant CA77735.

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The Ataxia Telangiectasia–Mutated Target Site Ser^{18} Is Required for p53-Mediated Tumor Suppression

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