

## DNA Damage and Cellular Stress Responses

## Phosphorylation of p53 Serine 18 Upregulates Apoptosis to Suppress Myc-Induced Tumorigenesis

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## Abstract

ATM and p53 are critical regulators of the cellular DNA damage response and function as potent tumor suppressors. In cells undergoing ionizing radiation, ATM is activated by double-strand DNA breaks and phosphorylates the NH<sub>2</sub> terminus of p53 at serine residue 18. We have previously generated mice bearing an amino acid substitution at this position (p53S18A) and documented a role for p53 phosphorylation in DNA damage-induced apoptosis. In this present study, we have crossed *Eμmyc* transgenic mice with our p53S18A mice to explore a role for ATM-p53 signaling in response to oncogene-induced tumorigenesis. Similar to DNA damage induced by ionizing radiation, expression of c-Myc in pre-B cells induces p53 serine18 phosphorylation and Puma expression to promote apoptosis. *Eμmyc* transgenic mice develop B-cell lymphoma more rapidly when heterozygous or homozygous for p53S18A alleles. However, *Eμmyc*-induced tumorigenesis in p53S18A mice is slower than that observed in *Eμmyc* mice deficient for either p53 or ATM, indicating that both p53-induced apoptosis and p53-induced growth arrest contribute to the suppression of B-cell lymphoma formation in *Eμmyc* mice. These findings further reveal that oncogene expression and DNA damage activate the same ATM-p53 signaling cascade *in vivo* to regulate apoptosis and tumorigenesis. *Mol Cancer Res*; 8(2); 216–22. ©2010 AACR.

## Introduction

The p53 transcription factor activates the expression of genes encoding negative regulators of growth in cells exposed to various types of stress, including DNA damage, hypoxia, or inappropriate growth stimulation due to oncogene activation (1). These p53 target genes include Cdkn1a (p21), 14-3-3s, cyclin G, and other genes whose products inhibit cell proliferation, as well as Bax, Puma, Noxa, and other p53 target genes that encode proapoptotic proteins (2). Activation of these genes by p53 blocks proliferation or induces apoptosis in damaged cells, thereby preventing the accumulation of oncogenic mutations and subsequent tumorigenesis. The importance of p53 in controlling cell growth and in tumor suppression is underscored by the fact that mutations in *p53* or in other genes encoding proteins that govern p53 function is the most common mechanistic step in the development of human cancer (3).

The activity of p53 is tightly regulated during normal cell growth by Mdm2, an E3 ligase that binds and ubiquitinates the p53 protein to regulate p53 cellular localization

and stability (4). Regulation of p53 activity is also mediated by other, numerous posttranslational modifications of the p53 protein, including acetylation, neddylation, sumoylation, and phosphorylation, perhaps the best understood of these p53 modifications (5, 6). In response to DNA damage, the ATM (mutated in ataxia telangiectasia) kinase becomes activated and phosphorylates p53 on serine residue 15 (p53 Ser18 in mice). This phosphorylation is reduced in ATM-mutant mice and is proposed to upregulate the p53 DNA damage response, as p53 activity is reduced in ATM-deficient mouse and human cells treated with ionizing radiation (IR; refs. 7–12). To explore the significance of this p53 phosphorylation *in vivo*, we generated a p53 knockin mouse model in which the serine residue at p53 position 18 is replaced by alanine (13). Previous analysis of primary fibroblasts isolated from this p53S18A model (p53<sup>S18A/S18A</sup>) revealed that phosphorylation of serine18 did not alter p53 protein stabilization or inhibit the ability of p53 to induce cell cycle arrest in fibroblasts following DNA damage. However, apoptosis was compromised in p53S18A thymocytes treated with IR, indicating that this phosphorylation regulates p53-induced apoptosis in response to DNA damage. Further analysis of this model revealed that p53S18A homozygous mice developed spontaneous tumors, albeit with a very delayed onset of cancer relative to p53-null mice (14). In addition, B-cell lymphomas were the predominant tumor type observed in the p53S18A mice, whereas T-cell lymphomas are most frequently seen in mice deleted for either p53 (15–17) or ATM (18–20). Collectively, these studies reveal that phosphorylation of p53 serine 18 upregulates DNA damage-induced apoptosis in thymocytes and can contribute to p53-mediated tumor suppression.

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However, some p53 functions may not be dependent on ATM phosphorylation of p53 serine 18, and it is unclear from these studies if phosphorylation of p53 serine 18 is critical in regulating the p53 response in cells exposed to stress other than DNA damage.

The *Eumyc* transgenic mouse is a well-established model of oncogene-induced tumorigenesis. *Eumyc* transgenic mice overexpress c-Myc in progenitor B cells and rapidly develop non-Hodgkin's B-cell lymphoma. (21). Inappropriate levels of Myc in the premalignant B cells of *Eumyc* mice results in the activation of the ARF-Mdm2-p53 signaling pathway and induces p53-dependent apoptosis, and B-cell tumors that arise in this mouse model display either alterations in p19Arf expression or Mdm2 expression, mutation of the p53 gene, or loss of *Puma*, the proapoptotic p53-upregulated modulator of apoptosis (22-24).

To determine if the phosphorylation of p53 serine residue 18 also plays a role in regulating p53 functions in the cellular response to activated oncogenes, we placed the *Eumyc* transgene on a p53S18A background and examined B-cell proliferation, apoptosis, and tumorigenesis. The results indicate that ATM phosphorylation of p53 serine 18 is a critical regulatory step in promoting B-cell apoptosis and in suppressing B-cell lymphomagenesis induced by Myc expression.

## Materials and Methods

### Mice and Tumor Assays

The generation of p53S18A mice and of *Eumyc* transgenic mice has been previously described (13, 21). All mice were backcrossed to C57Bl/6 strain for a minimum of 10 generations. The p53S18A mice and *Eumyc* transgenic mice were intercrossed to generate *Eumyc* transgenic mice that were p53-WT, p53<sup>S18A/+</sup>, or p53<sup>S18A/S18A</sup>. Cohorts of each line of mice were collected and aged to perform a tumor assay. Mice displaying obvious tumors or signs of reduced vitality were euthanized, necropsied, and tissues were harvested and fixed in 10% formalin. All mouse tumors were classified by morphology to be B-cell lymphomas, regardless of p53 status. Mice were maintained and used in accordance with both federal guidelines and those established by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School.

### Western Analysis, Immunohistochemistry, and Antibodies

Protein extracts were generated as previously described (13), using antibodies against p53 (1:1 mix of AB-1 and AB-3, Calbiochem), Puma (4976, Cell Signaling), or  $\alpha$ -tubulin (T5168, Sigma). Spleens (preneoplastic) were isolated from 6- to 8-week-old mice, and a portion of each tissue was fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, and stained with an antibody that recognizes mouse p53 phospho-serine 18 (Cell Signaling). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining and Ki-67 staining of spleen sections was done as previously described

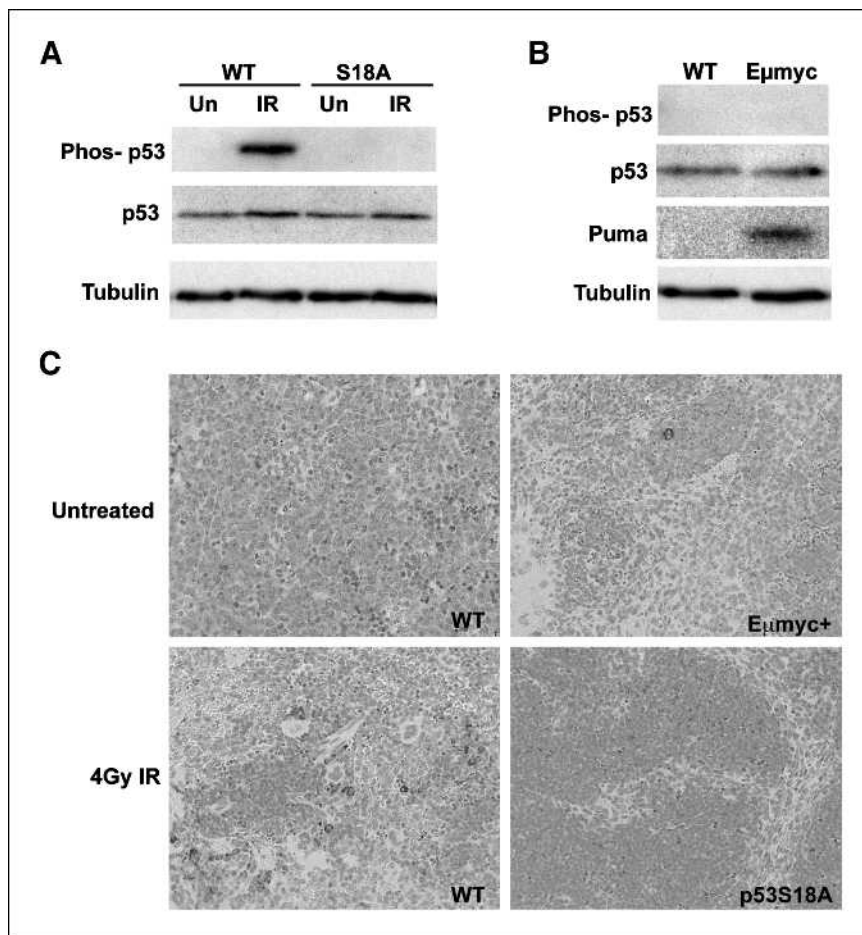
(25). Tumors were isolated from the cohorts of mice as they developed, fixed in 10% phosphate-buffered formalin, and paraffin-embedded sections were stained with H&E or with an antibody to CD45R/B220 (BD Pharmingen, diluted 1/50) before being analyzed by microscopy. Images of stained spleens or tumor sections were obtained using an Axioskop (Zeiss) with a  $\times 40$  optic lens and a 3008 Prog/Res digital camera coupled to a MacIntosh G4 computer, using Photoshop 4 software (Adobe).

### Proliferation and Apoptosis Assays

Proliferation of B220+ cells in wild-type (WT), *Eumyc* transgenic (Myc+), p53<sup>S18A/S18A</sup> (p53S18A), and *Eumyc* transgenic, p53<sup>S18A/S18A</sup> mice (p53S18A, Myc+) was determined by injecting 6- to 8-week-old mice with 50  $\mu$ g BrdUrd/g body weight. After 4 h, the spleens were harvested, made into single-cell suspensions, stained with anti-BrdUrd-PE and B220-APC, and analyzed by fluorescence-activated cell sorting. Proliferating cells were scored as those double positive for B220 and BrdUrd. Apoptosis of B220+ cells in WT, *Eumyc* transgenic (Myc+), p53<sup>S18A/S18A</sup> (p53S18A), or *Eumyc* transgenic, p53<sup>S18A/S18A</sup> mice (p53S18A, Myc+) was determined by harvesting spleens from 6- to 8-wk-old mice, which were made into single-cell suspensions, stained with Annexin V-FITC and B220-APC, and analyzed by fluorescence-activated cell sorting. Cells undergoing apoptosis were scored as those staining double positive for Annexin V and B220+. Flow cytometry was done on a three-laser 12-color LSR II (BD Biosciences) and analyzed with the FlowJo software (Tree Star).

## Results and Discussion

To examine ATM phosphorylation of p53 serine residue 18 in mouse spleen, we whole-body irradiated p53-WT or p53S18A mice (C57Bl/6 inbred background) with 4 Gy IR and recovered the spleens at 4 hour post-IR treatment (Fig. 1A). A slight increase in p53 levels was observed at this early time point in IR-treated mice relative to non-treated mice, but no difference was detected in total p53 levels between WT and p53S18A mice regardless of IR treatment. This result indicates that the phosphorylation of p53 serine 18 by ATM does not alter p53 levels in spleen, in agreement with our earlier finding in fibroblasts that the modification of this p53 residue does not affect p53 stability (13). Western analysis of splenic extracts was also done using an antibody that specifically recognizes p53 when phosphorylated at serine residue 18. As expected, DNA damage induced by IR treatment results in the phosphorylation of p53 serine 18 in WT spleen, whereas this p53 phosphorylation event cannot occur in the spleen of IR-treated p53S18A mice. Phosphorylation of p53 serine 18 cannot be detected in the spleen of *Eumyc* transgenic mice (C57Bl/6 strain) by Western analysis (Fig. 1B). However, these mice do display an increase in Puma levels in the spleen, consistent with a model for Myc-induction of p53 transcriptional activity and p53-mediated apoptosis. Spleen



**Figure 1.** Myc overexpression induces p53 serine 18 phosphorylation and upregulates Puma expression and p53-dependent apoptosis in B cells. A, Western analysis of spleen samples from WT or p53S18A mice either nontreated (Un) or treated 4 h after whole body irradiation with 4Gy (IR). Phosphorylation of p53 at serine 18 occurs in response to DNA damage in spleen. B, Western analysis of nontreated spleens reveals upregulation of Puma expression in *Eμmyc* transgenic mice. C, histochemical staining of representative sections of premalignant spleens using a p53-serine18 phospho-specific antibody. Samples were isolated from nontreated WT mice and *Eμmyc* transgenic mice, and from IR-treated WT mice or p53S18A homozygous mice. Sporadic p53 phosphorylation is detected in *Eμmyc* transgenic mouse spleen.

samples were isolated from 5- to 6-week-old WT mice or premalignant, *Eμmyc* transgenic mice, and histologic staining of these tissue sections was done using the p53 phospho-serine 18 antibody (Fig. 1C, top). No p53 phosphorylation was seen in the WT tissue, whereas sporadic staining was observed in the *Eμmyc* transgenic spleen. This modest level of p53 phospho-serine 18 staining in the *Eμmyc*+ spleen likely accounts for the undetected (by Western analysis) level of p53 phosphorylation in the *Eμmyc*+ sample (Fig. 1B). In contrast, IR-treatment of WT mice resulted in robust p53 phospho-serine 18 staining, whereas no staining was observed in the spleen of irradiated p53S18A mice (Fig. 1C, bottom). These results reveal that DNA damage results in p53 serine 18 phosphorylation in spleen, and that p53 serine 18 phosphorylation and Puma activation can be detected in the spleen of *Eμmyc* transgenic mice, albeit at a very low level. These *in vivo* results are in keeping with published results documenting increased p53 serine18 phosphorylation levels in B cells transduced with exogenous Myc (26).

To determine if the phosphorylation of p53 serine 18 regulates the response of p53 to inappropriate Myc expression *in vivo*, we placed the *Eμmyc* transgene on a p53S18A background. *Eμmyc* transgenic mice (C57Bl/6 strain) were

mated with congenic p53<sup>S18A/S18A</sup> mice to obtain *Eμmyc* transgenic, p53<sup>S18A/+</sup> male mice, which were crossed with female p53<sup>S18A/S18A</sup> mice to obtain littermates heterozygous or homozygous for the p53S18A allele, and that contained or lacked the *Eμmyc* transgene. All genotypes were recovered from these matings in the expected Mendelian ratios (data not shown). Spleen samples were isolated from 5- to 6-week-old, premalignant, *Eμmyc* transgenic mice that were either p53-WT or homozygous for p53S18A, and tissue sections were analyzed using an antibody against the proliferation marker Ki-67 or by performing TUNEL staining for apoptosis (Fig. 2A). Cell proliferation seemed to increase in the spleens of *Eμmyc* transgenic mice and *Eμmyc* transgenic, p53S18A mice relative to the levels observed in WT mice. Apoptosis was also upregulated in the spleen of *Eμmyc* transgenic mice relative to WT samples. However, less TUNEL staining was seen in the *Eμmyc* transgenic spleen when p53 was not capable of being phosphorylated at serine residue 18 (compare *Eμmyc* staining to *Eμmyc*, S18A staining). These data suggest that the phosphorylation of p53 serine residue 18 in spleen regulates p53 apoptosis induced by Myc expression.

To confirm these findings in the more relevant cell population, we examined the effects of Myc expression on B220+ cells in the p53S18A model. There was BrdUrd uptake in

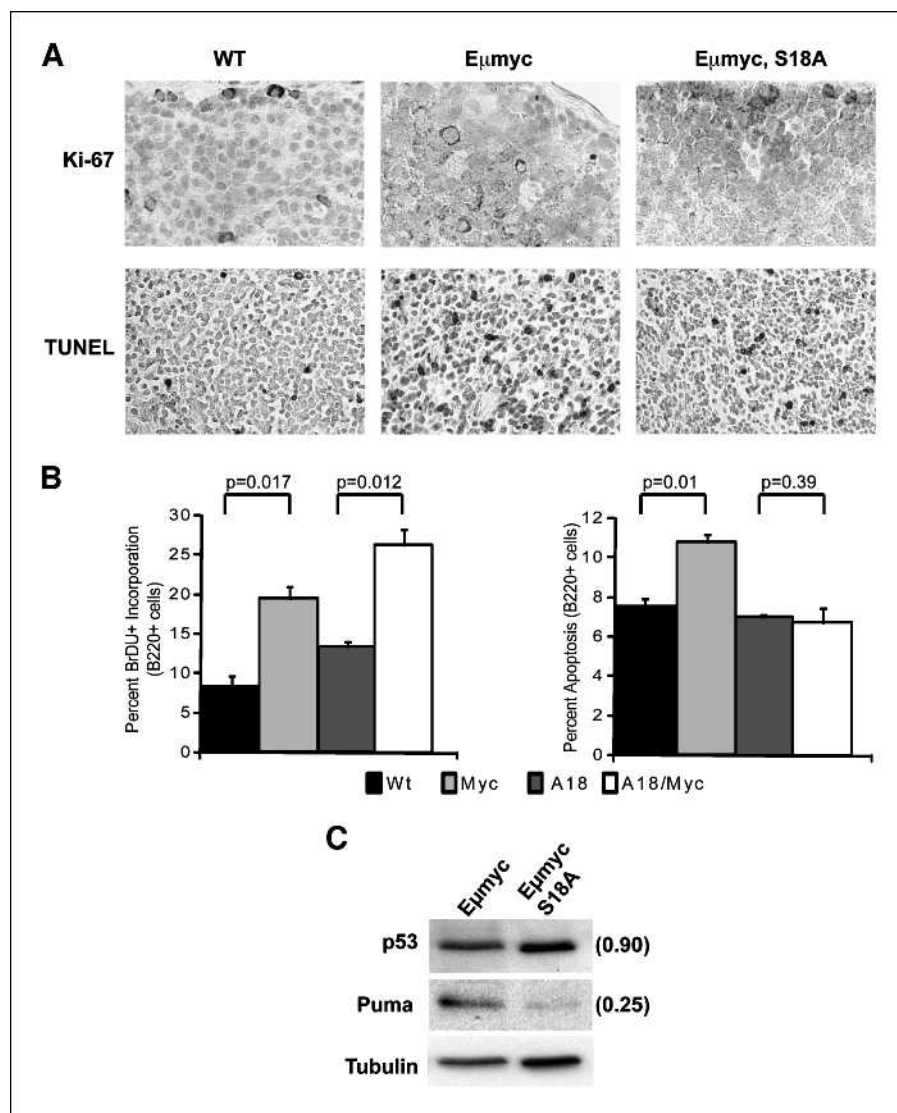
sorted B220+ cells harvested from the spleens of pre-malignant mice. *Eμmyc* transgenic mice displayed a 2-fold increase in the percentage of BrdUrd-positive, B220+ spleen cells relative to WT controls (Fig. 2B, left). As expected, *Eμmyc* expression increased the proliferation of the p53-WT B cells (27). However, the relative increase in the proliferation rate of *Eμmyc* transgenic, p53-WT cells (2.2-fold) was similar to that observed in the *Eμmyc* transgenic, p53S18A cells (2.1-fold). Therefore, although Myc increases B220+ cell proliferation in mice, this increase in B-cell proliferation is not regulated by p53 serine18 phosphorylation.

To explore the effects of Myc on B-cell apoptosis in p53S18A mice, we isolated B220+ cells from the spleens of pre-malignant *Eμmyc* mice and performed Annexin V staining (Fig. 2B, right). The expression of Myc increased the rate of spontaneous apoptosis in B220+ cells, in keeping with previous reports indicating that Myc induces ATM/p53-dependent apoptosis in *Eμmyc* transgenic mice

(22, 23, 28). Although no difference was observed in the frequency of cells undergoing apoptosis in nontransgenic p53S18A mice and WT mice, the increase in B-cell apoptosis documented in the *Eμmyc* transgenic, p53-WT model was not observed in *Eμmyc* transgenic, p53S18A mice. Thus, in contrast to our proliferation results in B cells, p53-mediated apoptosis induced by Myc expression in B cells is dependent on phosphorylation of p53 serine18.

To confirm that Myc-induced apoptosis is compromised in p53S18A mice, we examined *Puma* expression levels in pre-malignant, age-matched *Eμmyc*, p53-WT mouse spleen and *Eμmyc*, p53S18A mouse spleen (Fig. 2C). *Puma*, a BH3-only protein that activates Bax and Bak, is a major mediator of p53-induced apoptosis (29), and we have reported previously that p53S18A thymocytes and splenocytes display reduced *Puma* levels after exposure to IR (13, 14), indicating that p53-induction of *Puma* following DNA damage is dependent on p53 serine18 phosphorylation.

**Figure 2.** Myc expression induces B-cell proliferation as well as B-cell apoptosis that is regulated by p53 serine 18 phosphorylation. A, histochemical staining of representative sections of pre-malignant spleens isolated from WT mice, from *Eμmyc* transgenic mice, and from *Eμmyc* transgenic, p53S18A mice. Ki-67 staining indicates that Myc expression upregulates cell proliferation *in vivo* regardless of p53 status, whereas TUNEL staining reveals that Myc expression upregulates splenic apoptosis in a p53 serine 18 phosphorylation-dependent manner. B, B-cell growth in *Eμmyc* transgenic mice. Left, Myc-induced proliferation is not regulated by p53 serine18 phosphorylation in B cells. Columns, mean proliferation derived from four mice per genotype; bars, SD. *P* values are given above brackets. Right, Myc-induced apoptosis is regulated by p53 Serine 18 phosphorylation. Columns, mean apoptosis derived from four mice per genotype; bars, SD. *P* values are given above brackets. C, Western analysis of p53 and *Puma* levels in pre-malignant spleens harvested from *Eμmyc* transgenic mice, or *Eμmyc* transgenic, p53S18A mice. Tubulin was used as a loading control. Values for protein amounts in *Eμmyc* transgenic, p53S18A mice are given in parentheses, as determined by densitometry, with p53 or *Puma* levels in *Eμmyc* transgenic spleens adjusted for Tubulin value and set at 1.

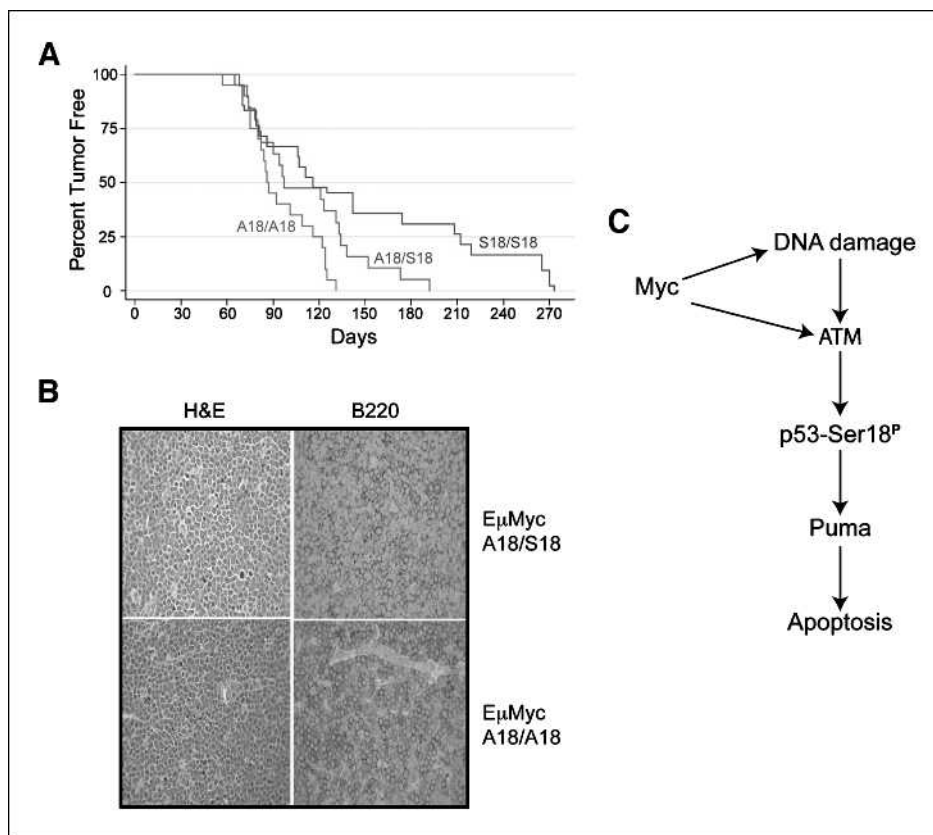


Western blot analysis of spleen extracts harvested from 5-week-old mice revealed little difference in p53 levels between *Eμmyc* transgenic mice, or *Eμmyc* transgenic mice homozygous for the p53S18A allele (as adjusted for control anti-tubulin staining). In contrast, Puma levels are reduced by 75% in *Eμmyc* transgenic, p53S18A premalignant cells. These results indicate that Myc overexpression (in pre-B cells) induces apoptosis that requires p53 serine18 phosphorylation and p53 activation of Puma expression. As loss of Puma is frequently observed in B-cell lymphomas arising in *Eμmyc* mice (24) our data suggest that p53S18A mice would be more susceptible to Myc-driven lymphomagenesis.

To determine if serine18 phosphorylation regulates p53 suppression of Myc-induced tumorigenesis, cohorts of *Eμmyc* transgenic mice that were either p53 WT (S18/S18), heterozygous for the p53S18A allele (A18/S18), or homozygous for the modified serine to alanine p53 allele (A18/A18) were established and surveyed for tumor formation. Tumorigenesis in the *Eμmyc* transgenic, p53- WT mice ( $n = 42$ ) displayed kinetics similar to those previously described for *Eμmyc* transgenic mice (Fig. 3A), with a mean time to tumorigenesis of 116 days (30, 31). *Eμmyc* mice heterozygous for the p53<sup>S18A</sup> mutation ( $n = 19$ ) had an increased rate of tumor onset and a mean time to tumorigenesis of 96 days, whereas *Eμmyc* mice homozygous for the p53<sup>S18A</sup> mutation ( $n = 20$ ) developed tumors even more rapidly, with a mean time to tumorigenesis of 87 days. Log-rank analysis

indicates that *Eμmyc* mice heterozygous for the serine18 mutation (p53S18/A18) or homozygous for the p53Ser18 mutation (p53A18/A18) have reduced survival compared with *Eμmyc*, p53- WT mice ( $P = 0.023$ , and 0.0013, respectively), and *Eμmyc* transgenic, p53A18/A18 are reduced in their survival relative to *Eμmyc* transgenic, p53S18/A18 mice ( $P = 0.033$ ). All mice in the three cohorts developed cancer, and presented with enlarged spleens and lymph nodes. Because mice presenting with hallmarks of disease typically die within a few days due to B-cell lymphomagenesis, and because we did not find any tumors in asymptomatic mouse, the time to tumorigenesis reflects the time of tumor onset and not the rate of disease progression. Tumors were isolated by necropsy, fixed, stained, and were classified by morphology as B-cell lymphomas. To confirm the tumor type, select tumors were fixed and immunostained for B220 antigens (Fig. 3B). All mice developed B220+ lymphomas, as expected, because *Eμmyc* mice rapidly develop B-cell lymphomas (21). Notably, *Eμmyc* transgenic, p53S18A mice develop B-cell lymphomas more rapidly than *Eμmyc* transgenic, p53- WT mice, indicating that phosphorylation of p53 at serine18 regulates p53 suppression of oncogene-induced tumorigenesis.

It is interesting to note that the mean time to tumorigenesis for *Eμmyc* transgenic mice on the p53S18A background (87 days) is delayed relative to the mean time to tumorigenesis for *Eμmyc* transgenic mice on an ATM-null background



**Figure 3.** Myc-induced, B-cell tumorigenesis is suppressed by p53 serine18 phosphorylation in mice. **A**, Kaplan-Meier survival curve of *Eμmyc* transgenic mice that are either WT for p53 (S18/S18), heterozygous for the p53S18A allele (S18/A18), or homozygous for the p53S18A allele (A18/A18). **B**, representative tumor sections from *Eμmyc* transgenic mice heterozygous for the p53S18A allele (S18/A18) or homozygous for the p53S18A allele (A18/A18) stained with H&E, or with B220 antibody. **C**, model for Myc activation of p53 tumor suppression. Inappropriate levels of Myc may activate ATM, p53 Serine18 phosphorylation, and p53-mediated apoptosis either by inducing DNA damage within the cell or by directly activating ATM-p53 signaling.

(69 days; ref. 23) or on a p53-heterozygous background (35 days; ref. 26). Furthermore, *Eμmyc* transgenic, p53-null mice cannot be recovered through normal breeding strategies due to the very rapid onset of neoplasia in *Eμmyc* transgenic, p53-heterozygous mice. In contrast, *Eμmyc*, p53S18A mice are easily recovered by breeding. As these mice are viable and also delayed in tumorigenesis relative to p53-haploinsufficient, *Eμmyc* transgenic mice, some p53 tumor-suppressing functions must still exist in p53S18A mice to slow the onset of the disease. That p53S18A retains some ability to suppress Myc-induced tumorigenesis agrees with our previous findings that p53S18A mice form spontaneous tumors more slowly than p53-null mice (13, 14). As p53S18A mice are compromised in Puma induction and apoptosis (Fig. 2A and C), the ability of the p53S18A allele to inhibit the Myc upregulation of B-cell proliferation (Fig. 2B) must also be an important facet of p53 suppression of Myc-induced tumorigenesis.

ATM and p53 are critical regulators of the cellular DNA damage response, and both proteins function as potent tumor suppressors. In cells undergoing DNA damage, ATM activation induces the phosphorylation of p53 at serine residue 18, and we have documented previously that p53 serine18 phosphorylation promotes DNA damage-induced apoptosis in murine thymocytes and splenocytes (13). However, the role of p53 phosphorylation in modulating p53 activity in response to oncogene expression is not well understood. Interestingly, deregulation of oncogene expression has been proposed to induce DNA damage in human cells (32, 33). In keeping with this paradigm, ATM deficiency has been found to impair Myc-induced apoptosis and augment tumorigenesis in mouse epithelial cells (34) and mouse B cells (26, 28). However, oncogene-induced DNA damage was not detected by phospho-H2AX staining in premalignant, *Eμmyc*-expressing B cells (26). Therefore, it is unclear if Myc overexpression activates p53 tumor sup-

pressor functions by inducing DNA damage and ATM-p53 signaling in the *Eμmyc* mouse model, or if Myc induces p53 activity in B cells by some other mechanism. It has been established that Myc can activate p53 levels and activity by upregulating the levels of p19Arf in mice (22, 35), and other oncogenes such as *Ras* have been recently found to induce p53 in an ATM-independent manner (36). Thus, it is possible that inappropriate oncogene expression may induce p53 tumor suppression in mice without invoking a DNA damage-response signaling pathway (Fig. 2C). However, if Myc expression is not causing DNA damage, then the p53 signaling pathway induced by Myc expression shares several mechanistic steps with DNA damage-induced p53 activation, as the results of our study strongly supports a role for ATM-p53 signaling in Myc activation of p53 tumor suppression.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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