Cell Reports
Report

Cell PRESS
Open ACCESS

p53 Efficiently Suppresses Tumor Development in the Complete Absence of Its Cell-Cycle Inhibitory and Proapoptotic Effectors p21, Puma, and Noxa

Liz J. Valente,[1,2] Daniel H.D. Gray,[1,2] Ewa M. Michalak,[1,2] Josefina Pinon-Hofbauer,[3] Alex Egle,[3] Clare L. Scott,[1,2] Ana Janic,[1,2,4] and Andreas Strasser[1,2,4,*]
[1]The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3052, Australia
[2]Department of Medical Biology, University of Melbourne, Parkville, Victoria 3052, Australia
[3]Laboratory for Immunological and Molecular Cancer Research (LIMCR), Third Medical University Department for Hematology and Medical Oncology, Paracelsus Private Medical University Salzburg, Salzburg 5020, Austria
[4]These authors contributed equally to this work and are co-senior authors
*Correspondence: strasser@wehi.edu.au
http://dx.doi.org/10.1016/j.celrep.2013.04.012

SUMMARY

Activation of apoptosis through transcriptional induction of Puma and Noxa has long been considered to constitute the critical (if not sole) process by which p53 suppresses tumor development, although G1/S boundary cell-cycle arrest via induction of the CDK inhibitor p21 has also been thought to contribute. Recent analyses of mice bearing mutations that impair p53-mediated induction of select target genes have indicated that activation of apoptosis and G1/S cell-cycle arrest may, in fact, be dispensable for p53-mediated tumor suppression. However, the expression of Puma, Noxa, and p21 was not abrogated in these mutants, only reduced; therefore, the possibility that the reduced levels of these critical effectors of p53-mediated apoptosis and G1/S-cell-cycle arrest sufficed to prevent tumorigenesis could not be excluded. To resolve this important issue, we have generated mice deficient for p21, Puma, and Noxa ($p21^{-/-}puma^{-/-}noxa^{-/-}$ mice). Cells from these mice were deficient in their ability to undergo p53-mediated apoptosis, G1/S cell-cycle arrest, and senescence. Nonetheless, these animals remained tumor free until at least 500 days, in contrast to p53-deficient mice, which had all succumbed to lymphoma or sarcoma by 250 days. Interestingly, DNA lesions induced by γ-irradiation persisted longer in p53-deficient cells compared to wild-type or $p21^{-/-}puma^{-/-}noxa^{-/-}$ cells, and the former failed to transcriptionally activate several p53 target genes implicated in DNA repair. These results demonstrate beyond a doubt that the induction of apoptosis, cell-cycle arrest, and possibly senescence is dispensable for p53-mediated suppression of spontaneous tumor development and indicate that coordination of genomic stability and possibly other processes, such as metabolic adaptation, may instead be critical.

INTRODUCTION

p53 imposes a critical barrier against the development of cancer (Vogelstein et al., 2000; Vousden and Lane, 2007). Approximately 50% of human cancers have mutations in this tumor-suppressor gene, and many of the remainder harbor mutations or epigenetic changes that impair activation of p53 or some of its effector functions. Inherited heterozygous loss-of-function mutations in p53 cause Li-Fraumeni syndrome in which affected individuals are highly predisposed to developing various types of cancers (e.g., breast cancer, sarcoma, and lymphoid malignancies), often at a young age. Accordingly, mice lacking p53 (Donehower et al., 1992; Jacks et al., 1994) or bearing loss-of-function point mutations in p53 (corresponding to amino acid residues in p53 that are altered in Li Fraumeni syndrome patients or in sporadic human cancers; Olive et al., 2004) develop thymic lymphoma or sarcoma with 100% incidence between 150 and 250 days of age.

The p53 protein mostly resides in the nucleus where, upon activation, it can bind as a homotetramer to specific DNA sequences and thereby regulate the transcription of select target genes (Cho et al., 1994). The p53 protein can be modified in response to diverse stress stimuli, including DNA damage, hypoxia, and, importantly for tumor suppression, the activation of certain oncoproteins, such as deregulated expression of c-Myc. In healthy (unstressed) cells, p53 levels and activity are low, largely due to Mdm2-mediated ubiquitination, which primes p53 for proteasomal degradation (Vousden and Lane, 2007). Cellular stressors activate p53 via posttranslational modifications, including phosphorylation and acetylation of conserved amino acid (aa) residues, or inhibition of Mdm2, for example, via stimulation of p14/ARF by activated oncoproteins (Riley et al., 2008; Vousden and Lane, 2007). p53 can then transcriptionally activate or repress many target genes (~200) to initiate diverse cellular responses, including G1/S boundary cell-cycle arrest, cellular senescence, apoptotic cell death, and

coordination of genomic repair (Riley et al., 2008; Vousden and Lane, 2007). Studies using gene-targeted mice demonstrated critical roles for certain p53 target genes in p53-mediated effector processes. For example, the cyclin-dependent kinase inhibitor (CDKI) p21 is essential for G1/S boundary cell-cycle arrest (Deng et al., 1995), whereas the BH3-only proteins Puma and (to a lesser extent) Noxa are required for induction of apoptosis (Erlacher et al., 2005; Jeffers et al., 2003; Michalak et al., 2008; Shibue et al., 2003; Villunger et al., 2003).

It has been widely believed that p53's primary (possibly even sole) tumor-suppressive function is the activation of apoptosis. Additional roles for G1/S boundary cell-cycle arrest and cellular senescence have also been proposed (Liu et al., 2004; Riley et al., 2008; Vousden and Lane, 2007). The overall importance of these three cellular responses for p53-mediated tumor suppression has, however, been questioned. First, it was reported that mice deficient for endogenous (mouse) p53 but carrying a human p53 transgene were protected from rapid tumor development, although their cells were unable to undergo DNA-damage-induced apoptosis, which is p53 mediated (Dudgeon et al., 2006). Moreover, it was found that specific aa residues within p53 that are either involved in transactivation (Brady et al., 2011) or modified by acetylation and thereby affect p53 binding to DNA (Li et al., 2012) were differentially required for transcriptional induction of distinct p53 target genes, and hence the activation of certain p53 effector processes. Diverse cell types from mice with mutations in p53's first transactivation domain (L25Q, W26S in TAD1) were markedly resistant to cell-cycle arrest and apoptosis triggered by DNA damage, because this p53 mutant was defective in the transcriptional induction of *p21*, *Puma*, and *Noxa* (Brady et al., 2011). Remarkably, in contrast to p53-deficient mice, the homozygous $p53^{25,26}$ mutant animals did not show accelerated tumor formation in a mutant Ras-driven transgenic model of non-small cell lung cancer (NSCLC) (Brady et al., 2011). Likewise, tumorigenesis induced by other means, such as induction of medullablastoma caused by loss of one allele of *Ptch*, was considerably reduced in homozygous $p53^{25,26}$ mutants compared to $p53^{-/-}$ mice (Jiang et al., 2011). Since the homozygous $p53^{25,26}$ mutant cells could still undergo p53-induced senescence, the authors proposed that this process (triggered by currently "underappreciated" p53 target genes) may be critical for the tumor-suppressive action of p53. However, data from another *p53* mutant mouse strain appear to rule out a critical role for senescence in tumor suppression as well. The $p53^{3KR}$ mutant lacks three conserved aa residues that are acetylated in response to DNA damage (and certain other cytotoxic insults) and is consequently not only unable to induce apoptosis and cell-cycle arrest but also unable to trigger cellular senescence (due to a defect in transcriptional induction of *p21*, *Puma*, and *Noxa* and other classical p53 target genes) (Li et al., 2012). Nevertheless, only a low incidence of spontaneous tumor formation with late onset was seen in $p53^{3KR}$ mice (Li et al., 2012). These data, in conjunction with the finding that $p53^{3KR/3KR}$ mutant cells could still induce *Tigar*, a p53 target gene implicated in the control of metabolism (Li et al., 2012), led the authors to the conclusion that this effector process might be critical for p53's tumor-suppressive action.

An important caveat of both of these *p53* mutants is that the expression of the critical effectors Puma, Noxa, and p21 was reduced, but not completely abrogated. Therefore, it remains unclear whether apoptosis, G1/S boundary cell-cycle arrest, and/or cellular senescence are truly dispensable for p53-mediated tumor suppression. To answer these questions, we have generated mice that lack all of the critical effectors of p53-mediated apoptosis (Puma and Noxa) and G1/S cell-cycle arrest, as well as cellular senescence (p21), and investigated their predisposition to cancer.

RESULTS AND DISCUSSION

Although the aforementioned two studies (Brady et al., 2011; Li et al., 2012) constitute a substantial advance in the understanding of how p53 suppresses tumorigenesis, there is one important caveat. Although the transcriptional induction of the *p21*, *Puma*, and *Noxa* genes was severely compromised in the cells expressing either of these two p53 mutants, their expression was not completely abrogated (Brady et al., 2011; Li et al., 2012). It is therefore possible that in $p53^{25,26/25,26}$ and $p53^{3KR/3KR}$ mutant mice, the residual expression of these effectors, induced in response to oncogene activation, was sufficient to cause growth arrest and/or apoptosis in incipient neoplastic cells to efficiently suppress tumorigenesis. To unambiguously resolve the important issue of whether any expression of these proteins is required for p53-mediated tumor suppression, we generated mice lacking p21 (critical for p53-induced G1/S cell-cycle arrest and cellular senescence; Deng et al., 1995; Riley et al., 2008) as well as Puma and Noxa (essential for p53-mediated induction of apoptosis; Jeffers et al., 2003; Shibue et al., 2003; Villunger et al., 2003).

Cells from $p21^{-/-}puma^{-/-}noxa^{-/-}$ Mice Are Resistant to p53-Mediated Apoptosis Triggered by DNA Damage but Susceptible to p53-Independent Apoptotic Stimuli

The BH3-only proteins Puma, and to a lesser extent Noxa, were previously shown to be critical for apoptosis induced by p53-dependent stress stimuli, such as DNA damage (Erlacher et al., 2005; Jeffers et al., 2003; Michalak et al., 2008; Shibue et al., 2003; Villunger et al., 2003). We therefore wanted to verify that cells from $p21^{-/-}puma^{-/-}noxa^{-/-}$ mice were also resistant to p53-mediated apoptosis. Thymocytes from $p21^{-/-}puma^{-/-}noxa^{-/-}$ mice or control animals (wild-type [WT] and $p53^{-/-}$) were either γ-irradiated or treated with etoposide (two cytotoxic insults that kill these cells entirely via a p53-dependent pathway; Clarke et al., 1993; Lowe et al., 1993; Strasser et al., 1994), and their survival was then measured by flow cytometric analysis. Thymocytes from $p21^{-/-}puma^{-/-}noxa^{-/-}$ mice were profoundly resistant to DNA-damage-induced apoptosis (Figure 1A). For example, after 24 hr of treatment with etoposide (1 μg/ml), there was less than 30% specific survival of WT thymocytes but >75% specific survival of the $p21^{-/-}puma^{-/-}noxa^{-/-}$ thymocytes, which was comparable to the survival of $p53^{-/-}$ thymocytes (Figure 1A). In contrast, $p21^{-/-}puma^{-/-}noxa^{-/-}$, $p53^{-/-}$, and WT thymocytes displayed comparable sensitivity to deregulated calcium flux (Figure 1A), a cytotoxic stimulus that is

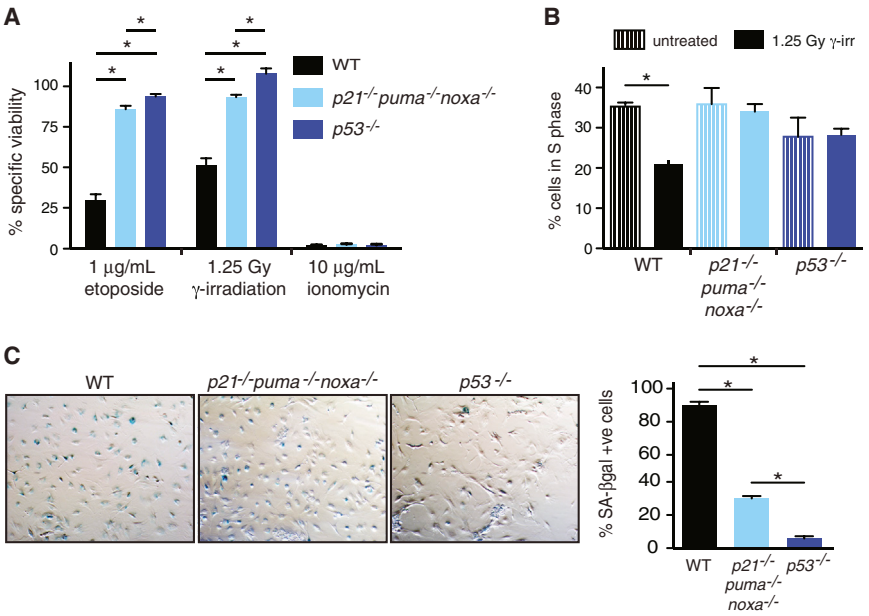

Figure 1. Cells from *p21*$^{-/-}$*puma*$^{-/-}$*noxa*$^{-/-}$ Mice Are Resistant to p53-Mediated Induction of Apoptosis, G1/S Cell-Cycle Arrest, and Senescence

(A) Thymocytes from WT, *p21*$^{-/-}$*puma*$^{-/-}$*noxa*$^{-/-}$, and *p53*$^{-/-}$ mice were left untreated, exposed to 1.25 Gy γ-irradiation, or treated with 1 μg/ml etoposide or 10 μg/ml ionomycin in vitro. Cell viability was assessed after 24 hr by Annexin-V/PI staining followed by flow cytometric analysis. Data represent mean percentage Annexin-V^{neg} PI^{neg} cells relative to medium controls.

(B) Activated (proliferating) T lymphoblasts from WT, *p21*$^{-/-}$*puma*$^{-/-}$*noxa*$^{-/-}$, and *p53*$^{-/-}$ mice were either left untreated or exposed to 1.25 Gy γ-irradiation and then cultured for an additional 8 hr. Distribution of cells within the different stages of the cell cycle (G0/G1, S, G2/M) was determined by PI staining followed by flow cytometric analysis. Data represent mean percentage of S phase cells ± SEM.

(C) Dermal fibroblasts from WT, *p21*$^{-/-}$*puma*$^{-/-}$*noxa*$^{-/-}$, and *p53*$^{-/-}$ mice were either left untreated or treated with 2 μg/ml etoposide and cultured for 96 hr. Induction of cellular senescence was determined by SA-β-galactosidase staining. Representative images of SA-β-galactosidase-stained cells. Quantitative analysis of % SA-β-galactosidase^{+} cells is shown; n=5 images/mouse (10× objective) were counted. For (A)–(C), n = 3–4 mice/genotype. Data represent mean ± SEM. *p < 0.001.

independent of p53 (Strasser et al., 1994) as well as Puma and Noxa (Villunger et al., 2003), but is instead mediated by the BH3-only protein Bim (Bouillet et al., 1999). These data show that cells from *p21*$^{-/-}$*puma*$^{-/-}$*noxa*$^{-/-}$ mice are profoundly resistant to p53-mediated apoptosis.

Cells from *p21*$^{-/-}$*puma*$^{-/-}$*noxa*$^{-/-}$ Mice Are Resistant to p53-Mediated G1/S Cell-Cycle Arrest and Senescence

The cyclin-dependent kinase inhibitor, p21, has previously been shown to be essential for p53-mediated G1/S boundary cell-cycle arrest and cellular senescence triggered by DNA damage (Deng et al., 1995; Riley et al., 2008). Therefore, we examined whether cells from the *p21*$^{-/-}$*puma*$^{-/-}$*noxa*$^{-/-}$ mice were also resistant to p53-mediated G1/S cell-cycle arrest and senescence. First, mitogenically activated splenic T lymphocytes were exposed to low dose (1.25 Gy) γ-irradiation, and after 8 hr their distribution among the different stages of the cell cycle was examined by flow cytometric analysis. A substantial reduction (drop from ~35% to ~20%) in cells in the S phase was seen in WT T lymphoblasts, but no such reduction was observed in activated, proliferating T cells from the *p21*$^{-/-}$*puma*$^{-/-}$*noxa*$^{-/-}$ mice, which behaved like those from the *p53*$^{-/-}$ animals (Figure 1B).

Induction of cellular senescence was examined by culturing nontransformed, primary dermal fibroblasts for 96 hr in the presence of 2 μg/ml etoposide or, as a control, in simple medium. Cells were then stained for the senescence-associated marker, SA-β-galactosidase. Treatment with etoposide caused a substantial (>80%) increase in SA-β-galactosidase^{+} cells from WT mice (Figure 1C). In contrast, considerably fewer cells (~30%) from *p21*$^{-/-}$*puma*$^{-/-}$*noxa*$^{-/-}$ mice displayed this marker of senescence after such treatment (Figure 1C). It is noteworthy that this reduction was not as profound as the reduction observed in etoposide treated cells from *p53*$^{-/-}$ mice (only ~5% SA-β-galactosidase^{+} cells) (Figure 1C). These data are consistent with the notion that transcriptional induction of the CDK inhibitor p21 is a substantial, but not exclusive, mediator of p53-activated cellular senescence (Riley et al., 2008; Vousden and Lane, 2007).

Collectively, these results demonstrate that cells from *p21*$^{-/-}$*puma*$^{-/-}$*noxa*$^{-/-}$ mice are profoundly resistant to p53-mediated cell-cycle arrest and substantially (albeit not completely) resistant to p53-mediated induction of senescence.

Unlike *p53*$^{-/-}$ Mice, *p21*$^{-/-}$*puma*$^{-/-}$*noxa*$^{-/-}$ Mice Are Not Prone to Spontaneous Tumor Development

The *p53*$^{-/-}$ mice spontaneously develop tumors (mostly thymic lymphomas and, to a lesser extent sarcomas) with high incidence at a relatively young age, with nearly 100% of animals succumbing to malignant disease before 250 days of age (Donehower et al., 1992; Jacks et al., 1994). Since p53-mediated induction of apoptosis, via upregulation of Puma and to a lesser extent Noxa, and induction of G1/S cell-cycle arrest, via upregulation of p21, have been hypothesized to be critical for p53-mediated tumor suppression, it follows that *p21*$^{-/-}$*puma*$^{-/-}$*noxa*$^{-/-}$ mice lacking all of these effectors should also be tumor prone. We therefore monitored *p21*$^{-/-}$*puma*$^{-/-}$*noxa*$^{-/-}$ mice for tumor development (n = 28). Remarkably, none of the *p21*$^{-/-}$*puma*$^{-/-}$*noxa*$^{-/-}$ mice developed a tumor (or any other disease) within the 500 day observation period (Figure 2). In contrast, all (n = 125) *p53*$^{-/-}$ mice had succumbed to lymphoma or more rarely, sarcoma, within 250 days. As expected, none of the WT mice (n = 167) developed malignant disease by 300 days of age (Figure 2). These findings prove beyond doubt that the combined loss of p53-mediated induction (or any induction, for that matter) of the cell-cycle inhibitor p21 plus the apoptosis mediators Puma

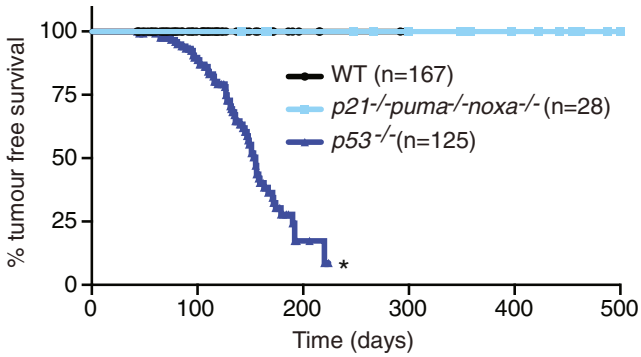

Figure 2. $p21^{-/-}puma^{-/-}noxa^{-/-}$ Mice Are Not Predisposed to Spontaneous Development of Cancer

WT, $p21^{-/-}puma^{-/-}noxa^{-/-}$, and $p53^{-/-}$ mice (all on a C57BL/6 background; see Experimental Procedures for how they were generated) were aged and monitored for tumor development (n = mice/genotype *p < 0.0001).

and Noxa, does not predispose mice to spontaneous tumor development. These data do not exclude that p53-mediated apoptosis, cell-cycle arrest, and/or senescence can exert a tumor-suppressive action in the context of certain oncogenic driver mutations. Indeed, loss of Puma or the combined loss of Puma and Noxa accelerated Myc-induced lymphoma development (Garrison et al., 2008; Michalak et al., 2009). However, lymphoma development occurred considerably more rapidly in $E\mu$-$myc;p53^{+/-}$ mice (note loss of only one p53 allele) compared to $E\mu$-$myc;puma^{-/-}$ or $E\mu$-$myc;puma^{-/-}noxa^{-/-}$ mice (Michalak et al., 2009). These studies show that, while p53-mediated apoptosis can contribute to tumor suppression in the context of deregulated c-Myc expression, additional p53-mediated processes must suppress lymphomagenesis.

Resolution of DNA Damage-Associated γ-H2AX Foci and Induction of Genes Implicated in DNA Repair Are Normal in $p21^{-/-}puma^{-/-}noxa^{-/-}$ Cells but Impaired in Those from $p53^{-/-}$ Mice

Given that p21-induced cell-cycle arrest and Puma/Noxa-mediated apoptosis are dispensable for p53-mediated suppression of spontaneous tumor development, which p53 effector process(es) might be critical? Interestingly, we observed that, after DNA damage (elicited by 10 Gy γ-irradiation), non-transformed dermal fibroblasts from $p53^{-/-}$ mice appeared to exhibit abnormally delayed resolution of γ-H2AX foci (a marker of double-strand DNA breaks and initiation of DNA repair) compared to WT cells (Figure 3A). Quantitative analysis revealed a trend toward persistence of increased numbers of p53-deficient cells with high γ-H2AX foci content (>40 foci/nuclei) 6 hr after γ-irradiation compared to WT cells (Figure 3B; differences are likely to be statistically significant if quantification of images from larger numbers of $p53^{-/-}$ and control mice could be performed). In contrast, this process occurred normally in dermal fibroblasts from $p21^{-/-}puma^{-/-}noxa^{-/-}$ mice (Figures 3A and 3B). Consistent with the notion that p53 is critical for the coordination of the DNA damage response, studies in zebrafish have shown that p53 is critical for the normal kinetics of DNA repair after exposure to UV (Zeng et al., 2009) or γ-irradiation (Guo et al., 2012). Moreover, consistent with our observation that p53-deficient but not $p21^{-/-}puma^{-/-}noxa^{-/-}$ cells are impaired in their response to DNA damage, several p53 target genes implicated in DNA repair (e.g., *Ercc5*, *Mgmt*, and *Polk*) were normally induced in response to γ-irradiation (5 Gy) in thymocytes from $p21^{-/-}puma^{-/-}noxa^{-/-}$ mice, but were not induced in the thymocytes from $p53^{-/-}$ animals (Figure 3C). We therefore favor the idea that coordination of DNA repair is a critical effector process for p53-mediated tumor suppression (Figure 4). After all, p53 and its homologs in lower organisms are considered the "guardians of the genome" (Lane, 1992). Regulation of metabolism and perhaps additional, presently unappreciated effector processes, may also contribute to p53-mediated tumor suppression (Figure 4). Regardless, the hunt for the critical tumor-suppressive p53 target genes is relaunched.

EXPERIMENTAL PROCEDURES

Mice

All experiments with mice followed the guidelines of the Melbourne Directorate Animal Ethics Committee. Generation and genotyping of mice deficient for p21 (Brugarolas et al., 1995), Noxa (Villunger et al., 2003), Puma (Villunger et al., 2003), or p53 (Jacks et al., 1994) have been described. The $puma^{-/-}$ and $noxa^{-/-}$ mice were generated on a C57BL/6 background using C57BL/6-derived ES cells. The $p21^{-/-}$ and $p53^{-/-}$ mice were generated on a mixed C57BL/6x129SV background using 129SV-derived ES cells but had been backcrossed with C57BL/6 mice for more than ten generations. To generate mice deficient for p21, Puma, and Noxa, $puma^{+/-}noxa^{-/-}$ mice were first crossed to $p21^{-/-}puma^{+/-}$ to produce mice heterozygous at all alleles ($p21^{+/-}puma^{+/-}noxa^{+/-}$). These triple heterozygous mice were intercrossed to produce $p21^{-/-}puma^{+/-}noxa^{-/-}$ offspring, which were then further intercrossed to produce the triple-knockout ($p21^{-/-}puma^{-/-}noxa^{-/-}$) animals. All mice used for cell-viability, cell-cycle, senescence, and DNA-damage repair experiments were 6–10 weeks of age. For long-term analysis of tumor development, cohorts of WT (C57BL/6), $p21^{-/-}puma^{-/-}noxa^{-/-}$, and $p53^{-/-}$ mice were monitored for >300 days for signs of illness.

Cell-Viability Assays

Thymi were harvested from mice of the indicated genotypes and thymic cell suspensions prepared. Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Bovogen Biologicals), 50 μM 2-mercaptoethanol, 100 μM asparagine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were plated into 96-well flat-bottom plates at a starting density of 2.5×10^5 cells/ml (200 μl/well) and treated, as described (Strasser et al., 1991), with 1 μg/ml etoposide (InterPharma), 1.25 Gy γ-irradiation, or 10 μg/ml ionomycin (Sigma). After 24 hr, cells were harvested and stained with fluorescein-isothiocyanate (FITC)-conjugated Annexin-V (to reveal surface exposed phosphatidyl-serine, a marker of apoptotic cells) plus 2 μg/ml propidium iodide (PI; Sigma). Cell viability was determined by flow cytometric analysis on a BD-Biosciences LSRI flow cytometer with 10,000 events recorded per sample. Data were analyzed using FlowJo (version 8.8.7) analysis software (Treestar). Data are presented as percentage of Annexin-$V^{negative}PI^{negative}$ (viable) cells relative to medium treated controls and calculated using the following equation [(% viable $cells^{treatment}$/% viable $cells^{untreated}$)*100], n = 3 mice/genotype.

Cell-Cycle Analysis

Spleens were harvested from mice of the indicated genotypes and single-cell suspensions were prepared. Cells were then washed and resuspended at a final density of 3×10^6 cells/ml in high-glucose DMEM supplemented with 10% fetal bovine serum (Bovogen Biologicals), 50 μM 2-mercaptoethanol,

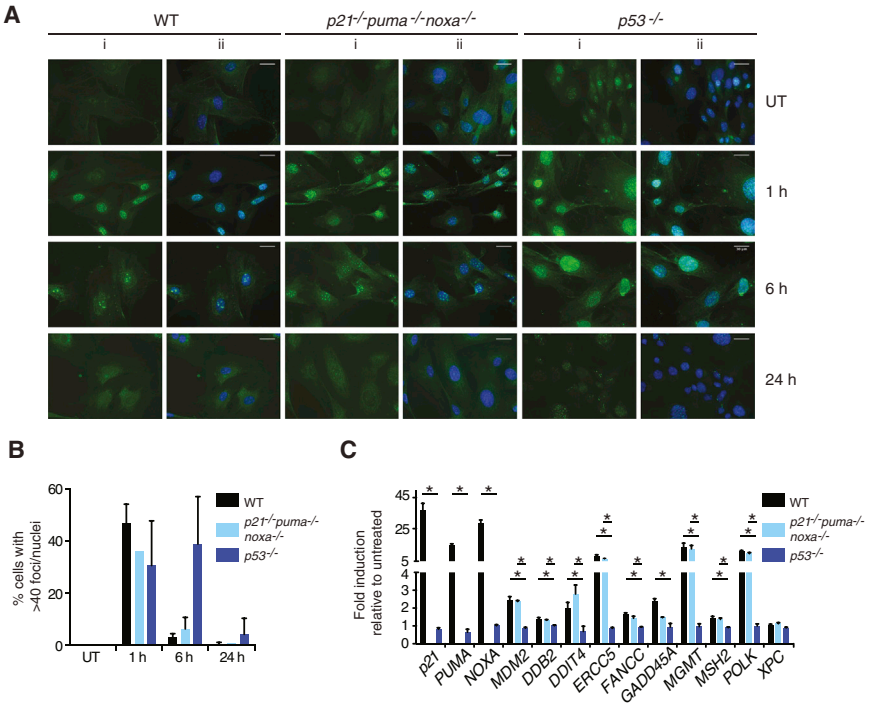

Figure 3. Loss of p53 but Not Combined Loss of p21, Puma, and Noxa Delays Resolution of DNA Damage Foci and Impairs Induction of Genes Implicated

(A) Dermal fibroblasts from WT, $p21^{-/-}puma^{-/-}noxa^{-/-}$, or $p53^{-/-}$ mice were either left untreated (UT) or exposed to 10 Gy γ-irradiation and subjected to analysis after 1, 6, and 24 hr. Cells were analyzed by immunofluorescent staining for the presence of γ-H2AX foci (green; a marker for detection of double-strand DNA breaks and initiation of repair). Counterstaining with DAPI (blue) was used to label all nuclei. (i) γ-H2AX staining alone; (ii) γ-H2AX staining and DAPI staining. Data are representative of analysis of n = 3 mice/genotype.

(B) Quantification of γ-H2AX foci in dermal fibroblasts from WT, $p21^{-/-}puma^{-/-}noxa^{-/-}$, or $p53^{-/-}$ mice that had been either left untreated (UT) or treated with 10 Gy γ-irradiation (analysis was performed after 1, 6, and 24 hr). Data represent percentage of cells with >40 foci/nuclei. Confocal images from UT, n = 1 mouse/genotype; 1 hr, n = 1–3 mice/genotype; 6 hr, n = 2 mice/genotype; 24 hr, n = 1–3 mice/genotype, were analyzed with 50–100 cells/mouse analyzed per time point.

(C) Thymocytes from WT, $p21^{-/-}puma^{-/-}noxa^{-/-}$, or $p53^{-/-}$ mice were either left untreated or exposed to 5 Gy γ-irradiation and harvested after 6 hr in culture. Quantitative RT-PCR analysis of mRNA levels of p53 target genes implicated in the control of DNA repair was performed; mRNA levels were standardized to the control gene *Hmbs* and data are expressed relative to untreated thymocytes (cultured for 6 hr).

n = 3 mice/genotype and data represent mean ± SEM. *$p < 0.05$.

100 μM asparagine, 100 U/ml penicillin, and 100 mg/ml streptomycin ~100 IU/ml mouse interleukin-2 (IL-2) and ~100 IU/ml mouse IL-7. Cells were plated into 6-well plates (3 ml/well), coated with antibodies to mouse CD3 and CD28, and incubated at 37°C 10% CO_2 for 48 hr (after 24 hr, cells were given 2 ml of fresh medium). At 48 hr, an aliquot of cells was harvested and stained with antibodies to CD25 (phycoerythrin conjugated) and CD44 (FITC conjugated) to determine the purity of activated T cells ($CD25^+CD44^+$) within the culture (routinely >85% $CD25^+CD44^+$ cells). The remaining cells were either left untreated or treated with a single dose of 1.25 Gy γ-irradiation. Cells were then plated into 96-well flat-bottom plates at a starting density of 2.5×10^5 cells/ml (200 μl/well) and incubated at 37°C 10% CO_2 for 8 hr. After 8 hr, cells were harvested, fixed, permeabilized, and stained with propidium iodide for flow cytometric cell-cycle analysis using a BD FACScan flow cytometer with 20,000 events recorded for each sample. Data were analyzed using FlowJo (version 8.8.7) analysis software (Tree Star) and distribution of cells within the distinct stages of the cell cycle determined by applying the Dean-Jett-Fox cell-cycle modeling algorithm to the PI fluorescence intensity profile of the cells. Data are presented as means of average percentage of cells in S phase ± SEM, n = 4 mice/genotype.

Senescence-Associated β-Galactosidase Activity Assay

Tails were harvested from mice of the indicated genotypes and mouse dermal fibroblasts generated. Briefly, a longitudinal incision was made down the length of each tail and skin removed. Skin was incubated for 24 hr at 4°C in 2.5 ml Hanks Balanced Salt Solution (HBSS) (with Ca^{2+} and Mg^{2+}) with 2.1 U/ml Dispase II (Roche). After 24 hr, the dermis was separated from the epidermis and then incubated in 3 ml HBSS (with Ca^{2+} and Mg^{2+}) with 0.4 mg/ml Collagenase II (Sigma) at 4°C, rolling for 48 hr. The dermis was then mashed through a 100 μm mesh sieve and the resulting cell suspensions washed in DMEM supplemented with 8% fetal bovine serum (Bovogen Biologicals). Cells were resuspended at a density of 7.5×10^5 cells/ml and 1 ml plated into 24-well flat-bottom plates. Cells were incubated overnight at 37°C 10% CO_2 to allow cells to adhere. Cells were then either left untreated or were treated with 2 μg/ml etoposide (InterPharma) for 96 hr. Cells were then fixed in 2% formaldehyde 0.2% glutaraldehyde and incubated with X-gal (Invitrogen) at pH 6.0 for 14 hr to determine senescence associated β-galactosidase activity, as previously described (Dimri et al., 1995). Stained cells were imaged using an Olympus IX70 inverted bright-field and fluorescence microscope using the CellSens Standard (Olympus) image-capturing software. Five representative images/genotype at 10× objective were counted. Data are presented as the mean percentage of β-galactosidase-positive cells as a proportion of total cells. Error bars represent SEM, n = 3 mice/genotype.

DNA Damage Repair Analysis Using γ-H2AX Staining

Mouse dermal fibroblasts were derived as described above and 1×10^4 cells in DMEM supplemented with 8% fetal bovine serum (Bovogen Biologicals) were plated into 8-chamber polystyrene vessel tissue culture treated glass slides (BD Falcon) (500 μl/well). Cells were then incubated at 37°C 10% CO_2 for 2 days before being used for experiments. Cells were exposed to 10 Gy γ-irradiation (or left untreated) and then incubated at 37°C 10% CO_2. Cells were harvested at 1, 6, and 24 hr post-γ-irradiation, fixed, and permeabilized in eBioscience Fix/Perm solution for 30 min according to the manufacturer's instructions. Slides were washed with an excess of eBioscience Perm Wash solution and then stained with FITC-conjugated antibodies to phospho-histoneH2A.X (Ser139; Millipore) in wash solution for 30 min. Slides were then washed, incubated with DAPI for 5 min, washed again, and coverslipped for imaging on a Zeiss LSM5 Live microscope. Images were analyzed with Axiovision v4.8 multidimensional acquisition (n = 3 mice/genotype). γ-H2AX foci were quantified by blinded scoring of images. Data represent percentage of cells with >40 foci/nuclei. Confocal images from one to three mice/genotype were analyzed with 50–100 cells/mouse analyzed per time point.

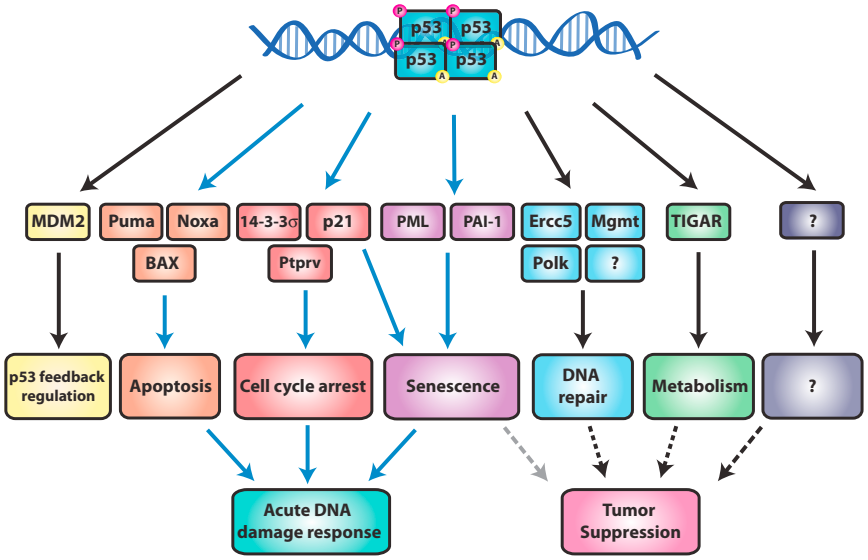

Figure 4. Model for How p53 Suppresses Tumorigenesis

p53's transcriptional control of apoptosis (via Puma and Noxa), cell-cycle arrest, and senescence (via p21) appears largely dispensable for p53's ability to suppress tumorigenesis. Note that a role for cell senescence in p53-mediated tumor suppression cannot be fully excluded on the basis of our results (reflected by a faint dotted line) since induction of senescence was only impaired in cells from the $p21^{-/-}puma^{-/-}noxa^{-/-}$ mice but abrogated in cells from the $p53^{-/-}$ mice (Figure 1C). These processes do, however, exert critical roles in p53-mediated cellular responses to acute DNA damage, but this acute DNA damage response is not essential for p53-mediated suppression of spontaneous tumor development (Christophorou et al., 2006). Instead, p53's underappreciated ability to act as a "guardian of the genome," facilitating coordination of DNA repair and/or control of metabolism (or control of currently unknown processes) is hypothesized to be essential for p53's ability to suppress development of cancer.

Quantitative RT-PCR Analysis

Total RNA was isolated from thymocytes using TRIzol (Invitrogen) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) and Oligo-d(T) primers. Quantitative RT-PCR was performed in triplicate using Taqman Gene Expression assays (Applied Biosystems) and an ABI 7900 Real-Time PCR machine (Applied Biosystems). The mRNA expression levels of p53 target genes of interest were standardized by the transcript levels of the reference gene, Hmbs, based on the comparative threshold method ($\Delta\Delta C_t$). n = 3 mice/genotype.

Statistical Analysis

Prism (version 5; GraphPad Software) software was used for all statistical analyses. Two-group comparisons were made using two-tailed t tests assuming equal variances. Tumor free survival data were plotted using Kaplan-Meier curves. Differences in survival time between cohorts of mice were tested using log-rank tests. p values less than 0.05 were considered to indicate statistical significance.

LICENSING INFORMATION

ACKNOWLEDGMENTS

Work in the authors' laboratories was supported by the National Health and Medical Research Council of Australia (Program Grant 461221, Australia Fellowship), the Leukemia and Lymphoma Society (SCOR grant #7413), the Career Development Fellowship #637353 to D.H.D.G.), the Cancer Council Victoria (to L.J.V.), the Beatriu de Pinos Foundation and the Lady Tata Memorial Trust (to A.J.), and operational infrastructure grants through the Australian Government (IRISS) and the Victorian State Government (OIS). We thank Drs. Y. Haupt and K. Wolyniec (Peter MacCallum Cancer Center, Melbourne, Australia) for reagents and advice.

Received: February 12, 2013
Revised: April 3, 2013
Accepted: April 12, 2013
Published: May 9, 2013

REFERENCES

Bouillet, P., Metcalf, D., Huang, D.C.S., Tarlinton, D.M., Kay, T.W.H., Köntgen, F., Adams, J.M., and Strasser, A. (1999). Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science *286*, 1735–1738.

Brady, C.A., Jiang, D., Mello, S.S., Johnson, T.M., Jarvis, L.A., Kozak, M.M., Kenzelmann Broz, D., Basak, S., Park, E.J., McLaughlin, M.E., et al. (2011). Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. Cell *145*, 571–583.

Brugarolas, J., Chandrasekaran, C., Gordon, J.I., Beach, D., Jacks, T., and Hannon, G.J. (1995). Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature *377*, 552–557.

Cho, Y., Gorina, S., Jeffrey, P.D., and Pavletich, N.P. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science *265*, 346–355.

Christophorou, M.A., Ringshausen, I., Finch, A.J., Swigart, L.B., and Evan, G.I. (2006). The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. Nature *443*, 214–217.

Clarke, A.R., Purdie, C.A., Harrison, D.J., Morris, R.G., Bird, C.C., Hooper, M.L., and Wyllie, A.H. (1993). Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature *362*, 849–852.

Deng, C., Zhang, P., Harper, J.W., Elledge, S.J., and Leder, P. (1995). Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell *82*, 675–684.

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., et al. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. USA *92*, 9363–9367.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A.J., Jr., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215–221.

Dudgeon, C., Kek, C., Demidov, O.N., Saito, S., Fernandes, K., Diot, A., Bourdon, J.C., Lane, D.P., Appella, E., Fornace, A.J., Jr., and Bulavin, D.V. (2006). Tumor susceptibility and apoptosis defect in a mouse strain expressing a human p53 transgene. Cancer Res. *66*, 2928–2936.

Erlacher, M., Michalak, E.M., Kelly, P.N., Labi, V., Niederegger, H., Coultas, L., Adams, J.M., Strasser, A., and Villunger, A. (2005). BH3-only proteins Puma

and Bim are rate-limiting for gamma-radiation- and glucocorticoid-induced apoptosis of lymphoid cells in vivo. Blood *106*, 4131–4138.

Garrison, S.P., Jeffers, J.R., Yang, C., Nilsson, J.A., Hall, M.A., Rehg, J.E., Yue, W., Yu, J., Zhang, L., Onciu, M., et al. (2008). Selection against PUMA gene expression in Myc-driven B-cell lymphomagenesis. Mol. Cell. Biol. *28*, 5391–5402.

Guo, L., Liew, H.P., Camus, S., Goh, A.M., Chee, L.L., Lunny, D.P., Lane, E.B., and Lane, D.P. (2012). Ionizing radiation induces a dramatic persistence of p53 protein accumulation and DNA damage signaling in mutant p53 zebrafish. Oncogene. Published online: October 15, 2012. http://dx.doi.org/10.1038/onc.2012.409.

Jacks, T., Remington, L., Williams, B.O., Schmitt, E.M., Halachmi, S., Bronson, R.T., and Weinberg, R.A. (1994). Tumor spectrum analysis in *p53*-mutant mice. Curr. Biol. *4*, 1–7.

Jeffers, J.R., Parganas, E., Lee, Y., Yang, C., Wang, J., Brennan, J., MacLean, K.H., Han, J., Chittenden, T., Ihle, J.N., et al. (2003). Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell *4*, 321–328.

Jiang, D., Brady, C.A., Johnson, T.M., Lee, E.Y., Park, E.J., Scott, M.P., and Attardi, L.D. (2011). Full p53 transcriptional activation potential is dispensable for tumor suppression in diverse lineages. Proc. Natl. Acad. Sci. USA *108*, 17123–17128.

Lane, D.P. (1992). Cancer. p53, guardian of the genome. Nature *358*, 15–16.

Li, T., Kon, N., Jiang, L., Tan, M., Ludwig, T., Zhao, Y., Baer, R., and Gu, W. (2012). Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence. Cell *149*, 1269–1283.

Liu, G., Parant, J.M., Lang, G., Chau, P., Chavez-Reyes, A., El-Naggar, A.K., Multani, A., Chang, S., and Lozano, G. (2004). Chromosome stability, in the absence of apoptosis, is critical for suppression of tumorigenesis in Trp53 mutant mice. Nat. Genet. *36*, 63–68.

Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A., and Jacks, T. (1993). p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature *362*, 847–849.

Michalak, E.M., Villunger, A., Adams, J.M., and Strasser, A. (2008). In several cell types tumour suppressor p53 induces apoptosis largely via Puma but Noxa can contribute. Cell Death Differ. *15*, 1019–1029.

Michalak, E.M., Jansen, E.S., Happo, L., Cragg, M.S., Tai, L., Smyth, G.K., Strasser, A., Adams, J.M., and Scott, C.L. (2009). Puma and to a lesser extent Noxa are suppressors of Myc-induced lymphomagenesis. Cell Death Differ. *16*, 684–696.

Olive, K.P., Tuveson, D.A., Ruhe, Z.C., Yin, B., Willis, N.A., Bronson, R.T., Crowley, D., and Jacks, T. (2004). Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. Cell *119*, 847–860.

Riley, T., Sontag, E., Chen, P., and Levine, A. (2008). Transcriptional control of human p53-regulated genes. Nat. Rev. Mol. Cell Biol. *9*, 402–412.

Shibue, T., Takeda, K., Oda, E., Tanaka, H., Murasawa, H., Takaoka, A., Morishita, Y., Akira, S., Taniguchi, T., and Tanaka, N. (2003). Integral role of Noxa in p53-mediated apoptotic response. Genes Dev. *17*, 2233–2238.

Strasser, A., Harris, A.W., and Cory, S. (1991). *bcl-2* transgene inhibits T cell death and perturbs thymic self-censorship. Cell *67*, 889–899.

Strasser, A., Harris, A.W., Jacks, T., and Cory, S. (1994). DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. Cell *79*, 329–339.

Villunger, A., Michalak, E.M., Coultas, L., Müllauer, F., Böck, G., Ausserlechner, M.J., Adams, J.M., and Strasser, A. (2003). p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. Science *302*, 1036–1038.

Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307–310.

Vousden, K.H., and Lane, D.P. (2007). p53 in health and disease. Nat. Rev. Mol. Cell Biol. *8*, 275–283.

Zeng, Z., Richardson, J., Verduzco, D., Mitchell, D.L., and Patton, E.E. (2009). Zebrafish have a competent p53-dependent nucleotide excision repair pathway to resolve ultraviolet B-induced DNA damage in the skin. Zebrafish *6*, 405–415.