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Tumorigenesis in *p27/p53*- and *p18/p53-Double Null* Mice: Functional Collaboration Between the pRb and p53 Pathways

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Mice lacking both $p18^{Ink4c}$ and $p27^{Kip1}$ develop a tumor spectrum similar to $pRb^{+/-}$ mice, and loss of p53 function accelerates tumorigenesis in $pRb^{+/-}$ mice. We hypothesized that codeletion of either *p18* or *p27* in conjunction with *p53* deletion will also accelerate tumorigenesis. Mice lacking both *p18* and *p53* develop several tumors not reported in either *single null* genotype, including hepatocellular carcinoma, testicular choriocarcinoma, hemangiosarcoma, leiomyosarcoma, fibrosarcoma, and osteosarcoma. Mice lacking both *p27* and *p53* exhibit a decreased lifespan and develop unique tumors, including papillary carcinoma of the colon, hemangiosarcoma, and leiomyosarcoma. In both *p18/p53* and *p27/p53 double null* genotypes, the incidence and spectra of tissues that develop lymphoma are also increased, as compared to the *single null* genotypes. The development of *p27/p53 double null* colon tumors correlates with secondary changes in cell-cycle protein expression and CDK (cyclin-*d*ependent *k*inase) activity, perhaps contributing to the progression of colorectal cancer. We concluded that p18 and p27 can, not only functionally collaborate with one another, but also can independently collaborate with p53 to modulate the cell cycle and suppress tumorigenesis in a tissue-specific manner.

Key words: Ink4c; Kip1; p53; tumorigenesis; collaboration

INTRODUCTION

Progression through the G_1 cell-cycle checkpoints is mediated by a family of serine/threonine protein kinase complexes consisting of a catalytic *c*yclin-*d*ependent *k*inase (CDK) subunit and a positive regulatory cyclin subunit [reviewed in Refs. 1,2]. The catalytic CDK subunit must be associated with its cyclin subunit for the complex to have activity. The G_1 checkpoints are regulated by three specific CDK complexes: CDK4-cyclin D, CDK6-cyclin D, and CDK2-cyclin E. If growth conditions are met, the CDK complexes become active, allowing progression to the next checkpoint. In contrast to cyclins, CDK inhibitors (CDKIs) repress CDK activity. The seven mammalian CDKIs are divided into two families: the INK4 family ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$) and the CIP/KIP family ($p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$). Ectopic expression of INK4 proteins suppress growth with a correlate dependence on *wild type* (*WT*) pRb function, suggesting that INK4 proteins positively regulate pRb growth suppression by inhibiting CDK4 and CDK6 kinase activities [3–5]. The CIP/KIP proteins inhibit a wide range of CDKs by forming complexes that include both CDKs and cyclins [6–9]. In addition to their inhibitory effect on CDK2-cyclin E complexes, CIP/KIP proteins are thought to promote the assembly and the subsequent activation of CDK4/6-cyclin D complexes [10,11]. Therefore, depending on the growth conditions, CIP/KIP proteins can play either a positive or negative role in determining progression from one cell-cycle checkpoint to the next.

As proteins that maintain the active growth arrest function of pRb, CDKIs should be candidate tumor suppressors. To test this in vivo, $p16^{-/-}$ mice were shown to spontaneously develop lymphomas and sarcomas [12–14], while $p18^{-/-}$ or $p27^{-/-}$ mice developed slow-growing intermediate lobe pituitary adenomas [15–18]. Mice deleted for other CDKIs do not develop significant tumor phenotypes [19–23]. Mice codeleted for both *p18* and *p27* invariably die at 3 mo due to an accelerated rate and incidence of pituitary tumorigenesis [24]. These mice also develop multiple endocrine and gastrointestinal tumors that are not seen in the *single null* (*SN*) genotypes. The tumor spectrum that develops in *p18/p27 double null* (*DN*) mice is very similar to that reported in $pRb^{+/-}$ mice, including pituitary and

Abbreviations: bp, base pairs; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; DN, double null; SN, single null; WT, wild type.

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adrenal tumors [25,26]. These results provided strong genetic evidence that p18 and p27 functionally collaborated as tumor suppressors in a tissue-specific manner with a tumor spectra similar to their downstream target, pRb.

p53$^{-/-}$ mice are predisposed to develop lymphomas and sarcomas [27,28]. In rare cases, teratoma, schwannomas, leydig cell tumors, and carcinomas of the ovary, lung, and mammary gland also occur. As major tumor suppressor pathways, mice deleted for both *Rb* and *p53* were examined [29,30]. In addition to the lymphomas and sarcomas characteristic of the *p53*$^{-/-}$ genotype, and pituitary and adrenal tumors associated with the *pRb*$^{+/-}$ genotype, *p53*$^{-/-}$-*pRb*$^{+/-}$ mice develop pinealblastomas, thyroid tumors, pancreatic islet cell tumors, bronchial hyperplasia, and retinal dysplasia at an increased incidence. Significantly, there was only an acceleration of Rb-, but not p53-specific tumors phenotypes. It is also interesting to note that all of the *p53*$^{-/-}$-*pRb*$^{+/-}$ endocrine tumors are detected in *p18*/*p27 DN* mice [24]. Therefore, p53 and pRb functionally collaborate in tumor suppression, perhaps with an emphasis in endocrine organs.

Because of the overlap in the developing tumor spectra between *p18*/*p27 DN* and *pRb*$^{+/-}$ mice, and the collaboration between pRb and p53 in tumorigenesis, we hypothesized that p18 and/or p27 may each collaborate with the p53 pathway to accelerate tumorigenesis similar to *p53*$^{-/-}$-*pRb*$^{+/-}$ and *p18*/*p27 DN* mice. To address this, we generated *p18*/*p53 DN* and *p27*/*p53 DN* mice. As anticipated, *DN* genotypes possessed increased unique tumor formation. Many of the *DN* tumors, which also develop in the *SN* mice, exhibited acceleration of important tumor parameters including tumor incidence, progression, and age of onset. Finally, we detected alterations in the expression and activity of several important cell-cycle proteins in *p27*/*p53 DN* tumors. Combined, our results clearly indicated collaboration between p18 and p27 with p53 in tumor suppression in a tissue-specific manner.

MATERIALS AND METHODS

Mouse Breeding and Genotyping

Genetic breeding of *p18*$^{-/-}$ [18], *p27*$^{-/-}$ [16], and *p53*$^{-/-}$ [25] mice has been reported. *Double heterozygous p18*/*p53* and *p27*/*p53* strains were generated by mating *p18*$^{-/-}$ or *p27*$^{-/-}$ males with *p53*$^{-/-}$ females. *Double heterozygotes* were then mated to generate *p18*/*p53 DN* and *p27*$^{+/-}$-*p53*$^{-/-}$ mice. The *p27*/*p53 DN* mice were generated by mating *p27*$^{+/-}$-*p53*$^{-/-}$ males and females. All of the genotypes were a mix of *C57BL*/*6* and *129sv* genetic backgrounds. For each genotype, we intercrossed for eight to ten generations without any alterations in detected phenotypes resulting from the drifting of genetic backgrounds.

To verify the different genotypes, genomic tail DNA was used as template for PCR using primers specific for the *WT* or *null* alleles. The primer used included (A) p18WT-F (5′-AGCCATCAAATTTATTCATGTTGCAGG-3′), p18WT-R (5′-ACAGCGTGGTGGTACCTTAT-3′), p18Null-R (5′-CCAGCCTCTGAGCCCAGAAAGCGAAGG-3′), (B) p27WT-F (5′-TCAAACGTGAGAGTGTCTAACGG-3′), p27WT-R (5′-AGGGGCTTATGATTCTGAAAGTCG-3′), and p27Null-R (5′-ATTTTGCTGAAGAGCTTGGCGG-3′), (C) p53WT-F (5′-TATACTCAGAGCCGGCCT-3′), p53WT-R (5′-ACAGCGTGGTGGTACCTTAT-3′), and p53Null-R (5′-TCCTCGTGCTTTACGGTATC-3′). Annealing temperatures were 57°C (p18 and p27) or 65°C (p53). PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The *p18 WT* and *null* PCR fragments were 600 and 400 base pairs (bp), respectively. For *p27 WT* and *null* PCR, 200 and 300 bp fragments were expected, respectively. The anticipated PCR fragments for *p53 WT* and *null* alleles were 400 and 550 bp, respectively.

Phenotypic Analysis

Animals were sacrificed using CO_2 inhalation and dissected upon any indication of morbidity, obvious tumor production, and/or failure to thrive. Age-matched *WT* and *SN* animals were compared with *p18*/*p53 DN* or *p27*/*p53 DN* animals. Tumors and organs were either fixed and stained for pathology or processed for biochemical assays, as previously reported [18,24]. Student's *t*-test was applied for statistical analysis of the different lifespans.

Antibodies and Immunochemistry

Antisera for p16, p18, p21, cyclin D1, cyclin D3, CDK2, CDK4, CDK6, α-tubulin (Caltag Laboratories, Burlingame, CA) and procedures for immunoprecipitations, immunoblotting, and kinase assays have been previously described [18,24,31,32]. The protein concentrations were determined using Bradford reagent and standardized for Western analysis and kinase assays. Kinase assays were performed four times with independent sets of normal and tumor lysates. Western experiments were performed from three of the same independent lysate sets.

RESULTS

Viability and Lifespans of *p18/p53* and *p27/p53 DN* Genotypes

Mice deficient for *p18* [18], *p27* [15–17], or *p53* [27] were bred to generate the necessary *DN* genotypes, and the status of each locus was confirmed by PCR genotyping (Figure 1A). The production of *p18*/*p53 DN* and *p27*/*p53 DN* males and females at the expected Mendelian ratios indicated that these genotypes were viable and that neither p18/p53 nor p27/p53 is rate-limiting during embryogenesis.

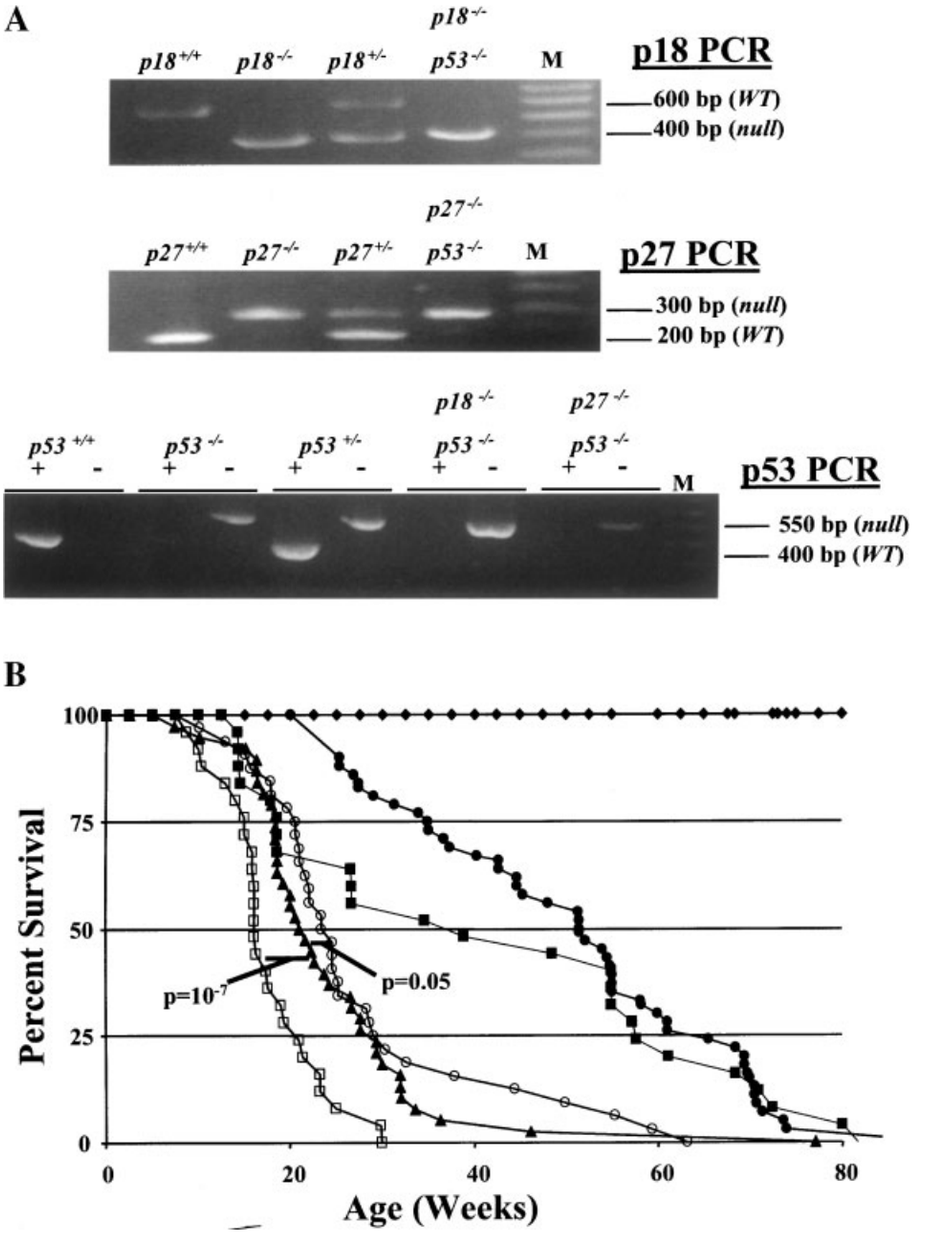

Figure 1. Genotyping and mortality of *p18/p53 DN* and *p27/p53 double null* (*DN*) mice. (A) The genotype of each mouse was confirmed by PCR for the status of the *p18*, *p27*, and *p53* loci, as indicated. The size (bp) of the PCR products for the *wild type* (*WT* or +) and *null* (*null* or −) alleles are indicated for each PCR reaction. Molecular weight DNA marker (M) is indicated. (B) Mortality of mice of different genotypes. Mice of different genotypes were sacrificed upon evidence of tumor production and/or failure to thrive. Genotypes include *WT* (◆), $p18^{-/-}$ (●), $p27^{-/-}$ (■), $p53^{-/-}$ (▲), $p18^{-/-}$-$p53^{-/-}$ (○), and $p27^{-/-}$-$p53^{-/-}$ (□). The statistical analysis of mortality rate of $p53^{-/-}$ versus $p18^{-/-}$-$p53^{-/-}$ ($P = 0.05$) mice or $p53^{-/-}$ versus $p27^{-/-}$-$p53^{-/-}$ ($P = 10^{-7}$) mice was determined by Student's *t*-test.

We investigated the effects of *p18/p53* or *p27/p53* deletions on lifespan (Figure 1B). For *p18/p53 DN* mice, mean survival was 23 weeks, maximum lifespan was 63 wk. In comparison, *p53 null* mice survived an average of 21, 77 weeks maximum. The mean lifespan of *p18 null* mice was 51, 83 weeks maximum. *WT* mice lived past 80 weeks with 100% survival. The mean survival for *p18/p53 DN* mice was about the same as *p53 null* mice ($P = 0.05$), suggesting that the combined p18/p53 loss of functions does not collaborate to decrease the lifespan beyond that of *p53 null* mice. Alternately, the mean and maximum lifespan of *p27/p53 DN* mice was significantly shorter, as compared to *p53 null* genotypes ($P = 10^{-7}$), as previously reported [33]. The mean age of survival of *p27/p53 DN* mice was 16, 30 weeks

maximum, while *p27 null* mice possessed a mean lifespan of 39, 80 weeks maximum. This indicated that the combined loss of p27/p53 functions was rate limiting, collaborating to increase mortality. We proposed that decreased survival in *p27/p53 DN* mice is due to increased tumorigenesis.

Accelerated Tumorigenesis in *p27/p53 DN* Mice

We examined *WT*, *p27 null*, *p53 null*, and *p27/p53 DN* tissues at comparable ages for tumor production. Of 25 *p27/p53 DN* mice, papillary hyperplasias, adenomas, and adenocarcinomas (Figure 2A) of the colon were identified at an incidence of 44% (Table 1). Papillary adenomas and adenocarcinomas were observed as early as 3.8 months, with 4.1 months mean detectable age. Colon tumors were not observed in *WT*, *p27 null*, or *p53 null* genotypes, and were not reported by Philipp et al. [33]. This indicated that p27 and p53 collaborated to suppress tumor growth in the mouse colon.

The *p27/p53* deletion also affected several *p27 null* or *p53 null* tumors. Pituitary adenomas and carcinomas developed in *p27/p53 DN* mice at an incidence of 17.6% (Table 1). In one *p27/p53 DN* mouse possessing a pituitary adenocarcinoma, we also detected metastasis to salivary gland lymph nodes. In comparison, *p27 null* mice developed pituitary adenomas at an incidence of 25%, yet adenocarcinomas did not occur. *WT* and *p53 null* mice did not develop any pituitary phenotypes. Although the incidence of

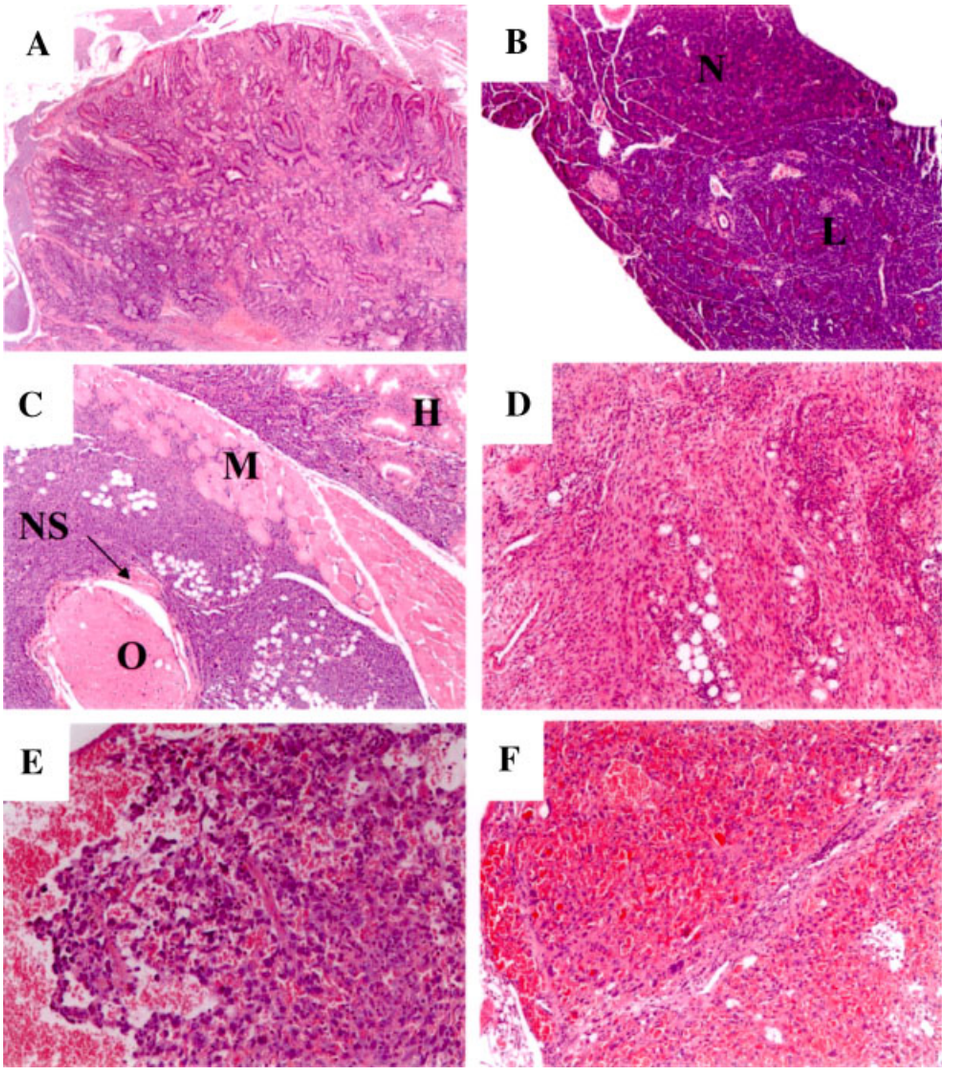

Figure 2. Tumorigenesis in *p27/p53 DN* mice. Hematoxylin and eosin stains of sample *p27/p53 DN* tumors. (A) Colon papillary adenocarcinoma; 400× magnification. (B) Lymphoma (L) infiltrating exocrine pancreas. Normal (N) pancreatic tissue is indicated; 200× magnification. (C) Lymphoma affecting multiple layers of the eye including the harderian gland (H), optic nerve (O), nerve sheath (NS), interstitium of the ocular muscle (M), corneal epithelium (data not shown) and perioccular globe (data not shown); 100× magnification. (D) Leiomyosarcoma affecting the colon; 100× magnification. (E) Spleen hemangiosarcoma; 200× magnification. (F) Liver hemangiosarcoma; 100× magnification. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 1. Tumorigenesis in *Wild Type*, $p27^{-/-}$, $p53^{-/-}$, and $p27^{-/-}$-$p53^{-/-}$ Mice

Organ	Wild type (N = 63)	$p27^{-/-}$ (N = 24)	$p53^{-/-}$ (N = 38)	$p27^{-/-}$-$p53^{-/-}$ (N = 25)
Normal colon	63/63	24/24	38/38	14/25
Papillary hyperplasia				2/25
Papillary adenoma				7/25
Papillary carcinoma				2/25
Normal pituitary	58/58	7/20	18/18	14/17
Hyperplasia		8/20		
Adenoma		5/20		2/17
Carcinoma				1/17
Normal adrenal	58/58	12/21	24/24	11/16
Medullary hyperplasia		4/21		2/16
Pheochromocytoma		5/21		3/16
Normal stomach	63/63	19/24	30/30	15/17
Villous adenoma		7/24		1/17
Squamous papilloma				1/17
Normal testis	27/27	12/12	9/11	5/8
Interstitial hyperplasia			2/11	2/8
Interstitial adenoma				1/8
Lymphoma	0/63	1/24	14/32	15/19
Salivary gland[a]			1/14	5/15
Ovary[a]			1/14	3/15
Kidney[a]			4/14	11/15
Uterus[a]			1/14	2/15
Heart[a]			4/14	8/15
Liver[a]			5/14	9/15
Pancreas[b]				5/15
Pituitary[b]				2/15
Epididymis[b]				1/15
Eye[b]				1/15
Gall bladder[b]				1/15
Parathyroid[b]				1/15
Prostate[b]				1/15
Small intestine[b]				1/15
Testis[b]				1/15
Thyriod[b]				1/15
Sarcoma	0/63	0/24	12/32	11/19
Soft-tissue sarcoma			12/12	5/9
Hemangiosarcoma[b]		1/1[c]		2/9
Leimyosarcoma[b]				4/9

Wild type, WT.
[a]Indicates tissues with lymphomas where the incidence in *p27/p53 DN* mice is greater than that detected in either *p27 null* mice or *p53 null* mice.
[b]Indicates tissues where lymphomas or sarcomas are unique in *p27/p53 DN* mice.
[c]Hemangiosarcoma was detected in a single 16-month-old *p27 null* animal.

p27/p53 DN pituitary tumors was less than that found in *p27 null* mice, this may be explained by the early mortality and age at which this tumor was detected (4 months), as compared to *p27 null* mice (9 months). If *p27/p53 DN* mice lived longer, perhaps the incidence for this tumor would increase. Therefore, p27 and p53 functionally collaborated to suppress pituitary tumorigenesis, and simultaneous *p27/p53* deletions affected the incidence, age of onset, and progression of this tumor.

31.3% of *p27/p53 DN* mice also developed adrenal medullary hyperplasias and pheochromocytomas. Pheochromocytomas developed as early as 4.1 months, with a mean age of 5.2 months (Table 1). In comparison, *p27 null* mice developed medullary hyperplasia and pheochromocytomas at a combined incidence of 43%, respectively, and pheochromocytomas developed by 9 months. *WT* and *p53 null* mice did not develop this tumor. The earlier onset of pheochromocytomas in *p27/p53 DN* mice suggested that the combined loss of p27 and p53 accelerated the progression of this tumor.

p27/p53 DN mice also developed stomach squamous cell papillomas and villous adenomas (both at 6% incidence) by 4 months (Table 1). Only villous adenomas occurred in our *p27 null* mice (29% incidence) by 14 months. *WT* and *p53 null* mice did not develop either gastrointestinal tumor. Although the incidences decreased in the *p27/p53 DN* mice, the age of onset was significantly earlier. Perhaps the

accelerated mortality in *p27/p53 DN* animals decreased the incidence. Combined, these results suggest that p27 and p53 collaborate to suppress gastrointestinal tumors in the mouse stomach, as well as colon.

Testicular leydig cell hyperplasia was detected in both *p53 null* and *p27/p53 DN* mice (18% and 25% incidences, respectively). Leydig cell adenomas also occurred in *p27/p53 DN* males (12.5% incidence), but not *p53 null* males. Leydig cells were normal in *WT* and *p27 null* mice. The age of detection for hyperplasia was also accelerated in the *p27/p53 DN* (4 months), as compared to the *p53 null* animal (6 months). These results indicate that p27/p53 collaborate to accelerate incidence, progression, and age of onset in testicular tumors.

Three aspects of lymphomas and sarcoma development were accelerated in the *p27/p53 DN* mice. First, many lymphomas developed in tissues that did not develop such tumors in *p53 null* mice, including the pancreas (Figure 2B and Table 1), pituitary, and the eye (Figure 2C). Similarly, unique *p27/p53 DN* leiomyosarcomas developed in the colon (Figure 2D) and unique hemangiosarcomas developed in the spleen and liver (Figures 2E and F, respectively). Thus, there was a widening of the tissue spectrum in which lymphoma and sarcomas developed in *p27/p53 DN* mice. Second, the incidence at which lymphomas and sarcomas formed in *p27/p53 DN* tissues increased, as compared to the *p53 null* animals (Table 1). *p27/p53 DN* mice developed lymphomas at an incidence of 79%, while *p53 null* mice developed lymphomas at an incidence of 44%. Sarcomas developed in *p27/p53 DN* and *p53 null* mice at incidences of 58% and 38%, respectively. Third, there was an accelerated age of onset in *p27/p53 DN* sarcomas, detected as early as 2.4 months, mean of 4.3 mo. Sarcomas were detected in *p53 null* mice as early as 3.8 mo, mean of 6.3 months. Combined, these results indicate that the loss of p27 function accelerates *p53 null* tumor phenotypes, affecting lymphoma, and sarcoma characteristics such as tissue spectra, tumor incidence, and age of tumor onset.

Accelerated Tumorigenesis in *p18/p53 DN* Mice

We next determined if collaboration in tumor suppression between p18 and p53 exists by examining *p18 null*, *p53 null*, and *p18/p53 DN* genotypes. Recently, Zindy et al. reported that *p18/p53 DN* mice develop hemagiosarcomas, medulloblastomas, and additional tumors in an accelerated manner [34]. Our results verified their findings and supplied additional data regarding p18/p53 collaborative tumor suppression in mice. *p18/p53 DN* mice developed unique tumors at low incidence not seen in *WT*, *p18 null*, or *p53 null* mice (Table 2). Hepatocellular carcinoma developed at an incidence of 3.1% (Figure 3A), and this carcinoma metastasized to the lung (data not shown). A choriocarcinoma developed in the testis of one 6-month-old *p18/p53 DN* mouse (6.7% incidence, Figure 3B), and stomach squamous cell papillomas developed in two *p18/p53 DN* mice (6.5% incidence). A leydig cell adenoma developed in one 6-month-old *p18/p53 DN* mouse (6.7% incidence). While the incidences of these tumors are low, none were detected in *WT*, *p18 null*, or *p53 null* mice, nor were they reported by Zindy et al. Interestingly, the Leydig adenoma and stomach papilloma are tumor phenotypes detected in *p18/p27 DN* mice [24], suggesting that the loss of p53 function can exacerbate *p18 null* tumor phenotypes, mimicking the adenomas found in *p18/p27 DN* mice, even when p27 was functionally active. These results also indicated that p18/p53 loss of function can cause unique tumors to develop in the liver, testis, and stomach.

Deletion of *p18/p53* also affected the incidence, age of detection, and progression of several p18 *null* tumor phenotypes (Table 2 and [24]). Pituitary adenocarcinomas were detected in *p18/p53 DN* mice at an increased incidence (11%), as compared to *p18 null* mice (2.7%). Adrenal pheochromocytomas were detected in *p18/p53 DN* mice with a mean of 4.9 mo, while it took 12 mo in *p18 null* mice. A thyroid C cell adenoma developed in a *p18/p53 DN* mouse (5% incidence) at 8 mo, while this tumor developed in one *p18 null* mouse (2.4% incidence) after 14 mo. This suggested that *p53* deletion accelerates the characteristics of several *p18 null* tumor phenotypes by affecting the incidence, age of onset, and progression of these tumors.

Like *p53 null* and *p27/p53 DN* mice, *p18/p53 DN* mice developed lymphomas in numerous tissue types. Lymphomas were detected in *p18/p53 DN* mice at an increased incidence of 59% (Table 2 and [34]), while our *p18 null* and *p53 null* mice developed lymphoma at 5.6% and 44% incidences, respectively. The increased incidence of *p18/p53 DN* lymphoma occurred in the salivary gland, liver, kidney, adrenal, heart, lung, and thyroid, and may be due to the loss of p18 function in the *p53 null* background, especially in tissues where the fold-increase in lymphoma-genesis was more pronounced (e.g., salivary gland). Additionally, unique *p18/p53 DN* lymphomas were detected in the pancreas (Figure 3C), pituitary, testis, and seminal vesicle, which were not affected in *p53 null* mice. Therefore, there was both an increased incidence in lymphomas in certain tissues, and a widening of the spectrum of affected tissues. We concluded that p18 and p53 collaborate to suppress the development of lymphoma by mediating both tissue-specificity and lymphoma incidence.

Similarly, deletion of *p18/p53* also affects characteristics of sarcoma development. Sarcomas developed in our *p18/p53 DN* mice at an incidence of 28% (Table 2). While the overall incidence of sarcoma development was not affected, there was a shift in

Table 2. Tumorigenesis in *Wild Type*, $p18^{-/-}$, $p53^{-/-}$, and $p18^{-/-}$-$p53^{-/-}$ Mice

Organ	*WT* (N = 63)	$p18^{-/-}$ (N = 53)	$p53^{-/-}$ (N = 38)	$p18^{-/-}$-$p53^{-/-}$ (N = 32)
Normal liver	63/63	24/24	32/32	31/32
Hepatocellular carcinoma				1/32
Metastasis to lung				1/32
Normal pituitary	58/58	4/37	18/18	24/27
Hyperplasia		22/37		
Adenoma		10/37		
Carcinoma		1/37		3/27
Normal adrenal	58/58	14/29	24/24	20/26
Medullary hyperplasia		10/29		4/26
Pheochromocytoma		5/29		2/26
Normal thyroid	63/63	36/42	29/29	19/20
C cell hyperplasia		5/42		
C cell adenoma		1/42		1/20
Normal testis	27/27	1/41	9/11	8/15
Interstitial hyperplasia		39/41	2/11	5/15
Interstitial adenoma		1/41		1/15
Choriocarcinoma				1/15
Normal stomach	63/63	38/38	30/30	29/31
Squamous papilloma				2/31
Lymphoma	0/63	3/53[c]	14/32	19/32
Salivary gland[a]			1/14	6/19
Liver[a]		2/3[c]	5/14	14/19
Kidney[a]		1/3[c]	4/14	11/19
Adrenal[a]			3/14	8/19
Heart[a]			4/14	9/19
Lungs[a]			7/14	13/19
Thyroid[a]		1/3[c]		1/19
Pancreas[b]				2/19
Pituitary[b]				2/19
Testis[b]				2/19
Seminal vesicle[b]				1/19
Sarcoma	0/63	1/53[c]	12/32	9/32
Soft-tissue sarcoma		1/1[c]	12/12	4/9
Hemangiosarcoma[b]				2/9
Leiomyosarcoma[b]				1/9
Fibrosarcoma[b]				1/9
Osteosarcoma[b]				1/9

[a]Tissues with lymphomas where the incidence in *p18/p53 DN* mice is greater than that detected in either *p18 null* or *p53 null* mice.
[b]Tissues where lymphomas or sarcomas are unique in *p18/p53 DN* mice.
[c]Lymphomas and soft tissue sarcoma are detected in *p18 null* animals greater than 13-month-old.

the types of sarcomas that developed in *p18/p53 DN* mice, as compared to *SN* mice. Most *p18/p53 DN* sarcomas are undifferentiated soft-tissue sarcomas (44%), but these mice also developed hemangiosarcomas (22%, Figure 3D), leiomyosarcomas (11%, Figure 3E), osteosarcomas (11%, Figure 3F), and fibrosarcomas (11%). This was compared to our *p53 null* mice, which exclusively developed undifferentiated soft tissue sarcomas. Consistent with our results, Zindy et al. reported an overall increase in vascular tumors, with a specific mention of the increased incidence of hemangiosarcomas [34]. The change in tissue spectra may be due to the rate-limiting effect of combined *p18/p53* deletion, suggesting a functional collaboration between p18 and p53 in suppressing sarcomagenesis in a tissue-specific manner.

Biochemical Analysis of Cell-Cycle Proteins in *p27/p53* Colon Tumors

Changes in the expression of p27 have been correlated with tumor incidence and progression, including in endocrine organs and the colon [35–37]. Additionally, p53 loss of function is a common event in colorectal cancer. We analyzed *p27/p53 DN* colon tumors to determine if changes in other cell-cycle proteins correlated with tumor formation. The colons of *WT*, *p27 null*, *p53 null*, and *p27/p53 DN* mice were used for Western blotting and subsequent kinase experiments. Only *p27/p53 DN* colons that showed grossly visible tumors were used in these experiments. No tumors occurred in the colons of the other genotypes. Normal spleens were also examined to demonstrate any generalized changes

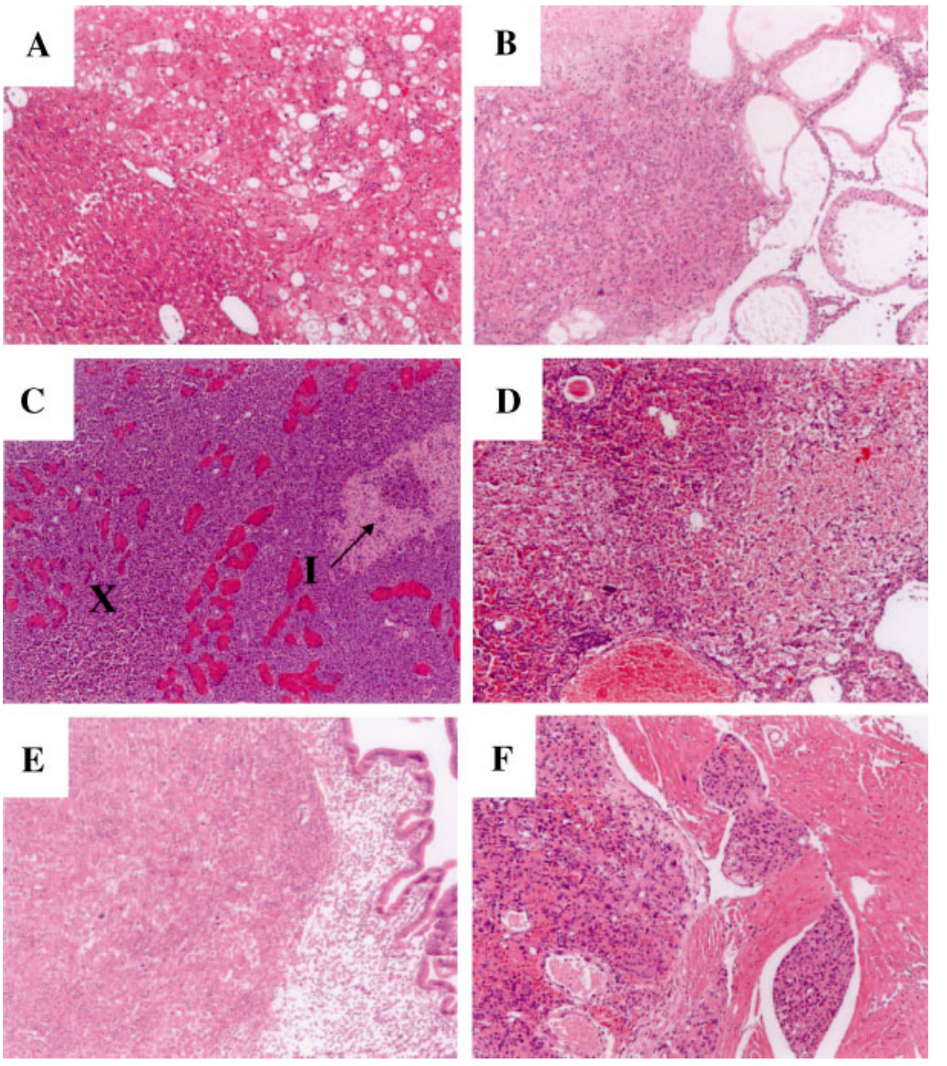

Figure 3. Tumorigenesis in *p18/p53 DN* mice. Hematoxylin and eosin stains of sample *p18/p53 DN* tumors. (A) Hepatocellular carcinoma; 100× magnification. (B) Testis choriocarcinoma; 100× magnification. (C) Lymphoma infiltrating exocrine (X) and endocrine pancreas. An affected endocrine islet (I) is indicated; 100× magnification. (D) Ovarian hemangiosarcoma; 100× magnification. (E) Leiomyosarcoma, also infiltrating the uterine muscle wall; 100× magnification. (F) Metastasis of osteosarcoma into cardiac muscle; 100× magnification. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in cell-cycle profiles that correlated with combined p27/p53 loss of function. Any differences in protein expression or CDK activities between the *p27/p53 DN* colon tumor and spleen samples could indicate events that correlated with tumor formation or progression. Because novel tumors did not develop at a high incidence in *p18/p53 DN* mice, comparable biochemical experiments were not feasible for this genotype.

In three independent experiments, CDK2 expression (α and β) was consistently increased in *p27 null* and *p27/p53 DN* colons, compared to *WT* or p53 *null* samples (Figure 4A). This increased pattern of CDK2 expression was not detected in the spleen samples. Additionally, we did not detect an increase in CDK2 expression in other normal $p27^{-/-}$ tissues, including the heart, kidney, lung, and testis (data not shown). Because there was no significant difference in CDK2 expression between the colon *p27 null* and *p27/p53 DN* samples, it appeared that the increase in CDK2 expression was p27-dependent. This suggested no collaborative mechanism between p27 and p53 in determining CDK2 expression during colon tumorigenesis. CDK4 expression was elevated in both *p27/p53 DN* colon tumor and spleen samples, as compared to the *WT*, *p27 null*, or *p53 null* control tissues. This may represent a general mechanism that was present in the *p27/p53 DN*, but not the other genotypes. Interestingly, a similar pattern occurred for expression of cyclin D1 (colon only). Perhaps the

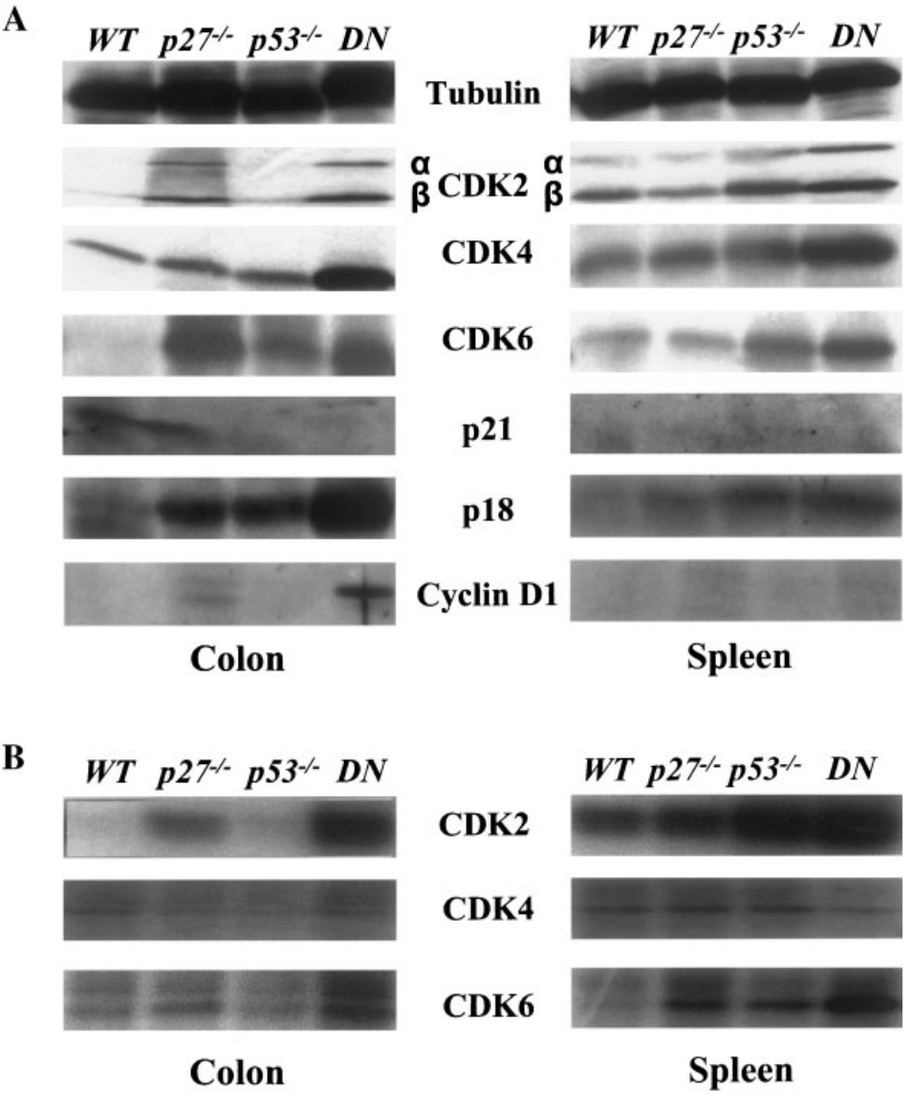

Figure 4. Cell-cycle analysis in *WT*, *p27* null, *p53* null, and *p27/p53 DN* tissues. (A) Western blot cell-cycle analysis. Protein lysates were isolated from *WT*, $p27^{-/-}$, $p53^{-/-}$ and $p27^{-/-}$-$p53^{-/-}$ (*DN*) colon or spleen tissues, resolved by SDS-PAGE and immunoblotted with the indicated antisera. Tubulin was used as a protein loading control. Expression of p16 and cyclin D3 were not detected (data not shown). The result is representative of three sets of independent tissue sample preparations. (B) Kinase assays. The same protein lysates were immunoprecipitated with the indicated antisera and assayed for activity using histone H1 (for CDK2) or GST-pRb (for CDK4 or CDK6) as substrates. Results are representative of four sets of independent tissue sample preparations.

increase of cyclin D1 expression was due to either p27/p53 collaboration, or a secondary amplification of the D1 locus commonly seen in the colorectal cancer. CDK6 levels were also elevated in the *p27/p53 DN* colon tumor and spleen samples. However, CDK6 expression was also elevated in the normal *p27 null* and/or *p53 null* tissues. Combined, these results suggest that p27 and p53 might functionally collaborate to regulate expression of several G_1 CDKs.

We also detected changes in the expression patterns of two CDKIs, p21 and p18 (Figure 4A). p21 was equally expressed in *WT* mice and *p27 null* mice, but was undetectable in *p53 null* and *p27/p53 DN* colon samples. Not surprisingly, p21 was expressed in a p53-dependent manner in the colon, and loss of p53 downregulates p21 expression. p21 was not detected in the spleen. The expression of p18 in both the colon and the spleen was elevated in *p27 null* or *p53 null* genotypes as compared to the *WT* sample, and was even further increased in the *p27/p53 DN* samples. Hence, the loss of both p27 and p53 correlated with increased levels of p18, possibly to compensate for the rate limiting conditions brought about by the loss of p27 and p53

functions (and possibly the correlate loss of p21 expression).

Any changes in expression profiles of important cell-cycle proteins could result in alterations in CDK activity. An increase in CDK2, CDK4, CDK6, and/or cyclin D1 expression patterns, or a decrease in CDKIs, could provide a mechanism for increased CDK function, contributing to tumorigenesis. To assess this possibility, we assayed CDK2, CDK4, and CDK6 activity in the same colon and spleen samples (Figure 4B). Particularly in the colon, CDK2 activity parallels CDK2 expression. However, there was more CDK2 activity in the *p27/p53 DN* colon tumor, as compared to the *p27 null* colon sample, despite relatively equal CDK2 protein expression between those two genotypes. This suggested that additional factors were affecting CDK2 activity. Perhaps this was due to the absence of both p27 and p21 in the *p27/p53 DN* samples, leading to enhanced CDK2 activity. CDK4 activity did not appear to change, and was very low in both colon and spleen samples. This was despite elevated CDK4 expression in both colon and spleen *p27/p53 DN* tissue samples. Perhaps increased p18 levels maintain minimal CDK4 activity. CDK6 activity was increased in both *p27/p53 DN* colon tumor and spleen samples, somewhat paralleling CDK6 protein expression. These results suggest that the combined loss of p27 and p53 function has have a profound effect on the activities of G_1 CDK complexes. The loss of expression of p27 and p21 (in *p27/p53 DN* cells) could potentially increase CDK2 activity, and the elevated levels of CDK6 and cyclin D1 could enhance CDK6 activity. Correlate with combined *p27/p53* deletions, the alterations in expression and activities of both G_1 and G_1/S CDK complexes might provide a mechanism for cell-cycle deregulation during colon tumorigenesis.

DISCUSSION

Based on published reports [15–18,24,25,29,30], we hypothesized that: (1) p53 functionally collaborates with either p18 or p27 (or both) to suppress tumorigenesis, (2) the combined loss of functions in the *p18/p53 DN* or *p27/p53 DN* mice would produce accelerated tumor phenotypes, and (3) *p18/p53 DN* or *p27/p53 DN* mice might develop a tumor spectrum similar to that reported for the $p53^{-/-}$-$pRb^{+/-}$ mice. Should novel or accelerated tumors develop in *p18/p53 DN* or *p27/p53 DN* mice, this would demonstrate collaboration between the p53 pathway and the pRb pathway, through the CDKIs, p18 and p27. Moreover, if the tumor spectrum of either p18/p53 DN or p27/p53 DN mice resembles the tumor spectrum of $p53^{-/-}$-$pRb^{+/-}$ mice, this would further support the notion that p18 and p27 are the upstream regulators of pRb function.

The analysis of our *p18/p53 DN* and *p27/p53 DN* mice clearly shows the collaborative nature of p18/p53 or p27/p53 in tumor suppression. Both *p18/p53 DN* and *p27/p53 DN* mice developed unique tumors, including colon and liver adenocarcinomas, and testis choriocarcinomas, none of which were detected in the *SN* control animals. In addition, there was accelerated tumorigenesis in *p18/p53 DN* and *p27/p53 DN* mice with respect to different tumor parameters, including tumor spectrum, age of onset, incidence, and progression, as compared to the *SN* mice. The loss of p53 function enhanced the age of onset and/or progression of tumors that were normally detected in *p18/p27 DN* mice, including adenomas of the pituitary, adrenal gland, thyroid, stomach, and testis. Similarly, the loss of p18 or p27 functions widened the tissue spectrum for lymphomas to include many tissues not affected in the *SN* mice. The concomitant loss of p18/p53 or p27/p53 functions also altered the types of sarcomas that were reported to form in $p53^{-/-}$ mice, especially to include the development of hemangiosarcomas and leiomyosarcomas. Therefore, loss of p53 function enhanced *p18/p27 DN* tumor phenotypes, and the loss of function of either of the CDKIs accelerated the tumor phenotypes of $p53^{-/-}$ animals. This latter observation was somewhat unexpected, as p53 loss of function accelerated pRb-specific tumors, yet pRb loss of function did not affect any p53 tumor phenotypes. As positive upstream regulators of pRb activity, and each with their own additional functions, p18 and p27 were able to modulate tumorigenesis in a p53 null background. Combined, our results demonstrate that p18, p27, and p53 collaborate in tumor suppression of numerous tissues and cell types, and that the combined loss of function of any two can result in accelerated tumor formation [24,34].

The combined loss of p27/p53 function also correlates with alterations in the expression and activity of several important cell-cycle proteins. In colon tumors of *p27/p53 DN* mice, we observed increased expression of CDK2, CDK4, CDK6, cyclin D1, and the CDKI, p18. Loss of p53 function also inhibited expression of the CDKI, p21. These secondary changes in cell-cycle expression correlate with increased CDK2 and CDK6 activity, providing a mechanism for how loss of function of p27 and p53 can result in colorectal tumorigenesis. Indeed, an increase in CDK2 expression and correlate pRb hyperphosphorylation is observed in human colorectal cancers [38]. We postulated that loss of p27 and p53, coupled with a downregulation of p21, correlates with increased CDK2 and CDK6 activity, and that this increase in CDK activity can promote colon tumorigenesis.

To date, we have no clear appreciation for how deletion or inactivation of widely expressed tumor suppressor genes correlates into a narrow or defined tissue-specific tumor spectrum. Such answers should be forthcoming from this and other studies, in which tumor spectra are defined for specific gene deletions

and codeletions. Specifically, we must characterize not only the tumor spectra that develop, but also endeavor to determine the underlying mechanisms that are affected by such deletions and how those combined alterations function in mediating tumor formation. In one recent study, *p18/p53 DN* mice were shown to develop hemangiosarcomas, medulloblastomas, and additional tumors in an accelerated manner [34]. Our results verified the results from this study and added hepatocellular carcinomas, choriocarcinomas, as well as acceleration of $p18^{-/-}$ and $p53^{-/-}$ tumor phenotypes to the reported spectrum of tumors that developed in *p18/p53 DN* tissues. Similarly, another study on *p27/p53 DN* mice showed a shortened rate of survival, and novel tumors including glioblastomas, sertoli cell tumors, as well as poorly undifferentiated carcinomas [33]. Our results added a significant increase in colon tumorigenesis to the tumor spectrum that resulted from this codeletion. Results from their biochemical analysis agree with our results in that they find an increase in CDK2 expression and activity in thymic lymphomas. However, they did not detect any increase in cyclin D1 expression. Combined, these studies provide novel insight into the types of tumors that can result from the deletion of specific tumor suppressors, and the secondary cell-cycle deregulation that can occur in the actual tumors.

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