Tumor predisposition in mice mutant for *p63* and *p73*: Evidence for broader tumor suppressor functions for the *p53* family

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Summary

***p63* and *p73* are functionally and structurally related to the tumor suppressor *p53*. However, their own role in tumor suppression is unclear. Given the *p53*-like properties of *p63* and *p73*, we tested whether they are involved in tumor suppression by aging mice heterozygous for mutations in all *p53* family genes and scored for spontaneous tumors. We show here that $p63^{+/-};p73^{+/-}$ mice develop spontaneous tumors. Loss of *p63* and *p73* can also cooperate with loss of *p53* in tumor development. Mice heterozygous for mutations in both *p53* and *p63* or *p53* and *p73* displayed higher tumor burden and metastasis compared to $p53^{+/-}$ mice. These findings provide evidence for a broader role for the *p53* family than has been previously reported.**

Introduction

p63 and *p73* were cloned due to their structural similarity to *p53*. However, they have been found to be more complex than *p53* due to the existence of several variant isoforms. Both *p63* and *p73* contain carboxy-terminal spliced variants known as the TA isoforms. So-called ΔN variants also exist that lack the transactivation domain and are transcribed from an internal promoter within exon 3 of the full-length genes (Irwin and Kaelin, 2001; Yang et al., 1998). These different isoforms have been shown to have vastly different activities. The TA isoforms act similarly to *p53*. They have the ability to transactivate *p53* target genes and induce apoptosis. In contrast, the ΔN isoforms have little transactivation activity and are thought to play a role in blocking transactivation of target genes of both *p53* and their respective TA isoforms (Yang et al., 2002). Therefore, the TA isoforms might be expected to have a role in tumor suppression, while increased expression of the ΔN isoforms might be oncogenic.

Gene targeting studies in the mouse have revealed that both *p63* and *p73* have roles in normal development (Mills et al., 1999; Yang et al., 1999; Yang et al., 2000). The mutations delete the central DNA binding domain of *p63* and *p73* and, thus, affect all isoforms of the genes. *p63*-deficient mice are born with craniofacial abnormalities, limb truncations, and a thin layer of undifferentiated skin. They die within hours after birth due to desiccation and maternal neglect (Mills et al., 1999; Yang et al., 1999). *p73* mutant mice also survive to birth but are runted and have hydrocephalus as well as hippocampal dysgenesis. The majority of *p73*-deficient mice live to be only 4 to 6 weeks old and die due to chronic infections (Yang et al., 2000). In our colony, approximately 25% survive to adulthood. Because of the complexity of the *p63* and *p73* loci and the lack of a detailed characterization of the molecular effects of the targeted mutations, it is possible that the mutant alleles might still produce truncated mRNAs and proteins that could complicate the interpretation of the mutant phenotypes.

The literature on *p63* and *p73* is complex and controversial, and their role in tumor suppression has been much debated. In studies performed to date, only a small percentage of human tumors have been shown to harbor *p63* and *p73* mutations (Irwin and Kaelin, 2001). However, recent studies have shown

SIGNIFICANCE

The discovery of the *p53*-related genes *p63* and *p73* raised the possibility that they may be cancer-associated genes. However, initial reports indicated that mutations in *p63* and *p73* are rare in human cancers. We have found that mice that are $p63^{+/-};p73^{+/-}$ develop malignant tumors at high frequency. In addition, $p53^{+/-};p63^{+/-}$ and $p53^{+/-};p73^{+/-}$ developed a more severe phenotype (higher tumor burden and metastases). Using mouse models, we have shown that inactivation of *p63* and *p73* leads to tumor types in the mouse frequently found to lose expression or have mutations in *p63* or *p73* in human tumors. This study identifies a previously unrecognized function of these genes and supports a broader role for the *p53* family in tumor suppression.

that p63 and p73 have tumor-suppressive activities in human tumors. While some studies have shown overexpression of some isoforms of *p63* or *p73* in human tumors (Hibi et al., 2000; Nozaki et al., 2001), certain tumor types (transitional cell carcinomas, mammary adenocarcinomas, squamous cell carcinomas, and osteosarcomas) exhibit loss or reduced expression of p63 and/or p73 (Ahomadegbe et al., 2000; Koga et al., 2003; Park et al., 2000; Park et al., 2004; Puig et al., 2003; Urist et al., 2002). However, these studies have been complicated by the lack of antibodies that distinguish between the TA and ΔN isoforms. More recent studies using antibodies or RT-PCR for specific isoforms of *p63* or *p73* have shown increased or decreased expression of the TA or ΔN isoforms of *p63* and *p73.* The few studies on human tumors that have been performed to date to determine the significance of the TA versus ΔN isoforms have shown that some tumors do specifically lose TA isoform expression (Park et al., 2000), while others have increased expression of the ΔN isoforms (Concin et al., 2004; Moll, 2003; Zaika et al., 2002). In addition to loss of expression of p63 in some human tumors, 12% of chronic myelogenous leukemias examined were found to have mutations in *p63* (Yamaguchi et al., 2001) contributing to the evidence that p63 has tumor-suppressive activities. Many more studies using antibodies or RT-PCR for specific isoforms must be performed in additional human tumors to determine the significance of the loss or gain of each isoform.

To date, a systematic analysis of tumor development in mice that are mutant for *p63* and *p73* has not been performed, and the synergistic effects of the *p53* family in tumor suppression have not been examined. The present study examines the long-term tumorigenic effects of *p63* and *p73* mutation, alone or in combination with *p53*. The data support a model whereby *p63* and *p73* play an important role in tumor suppression in specific tissues in the mouse.

Results

p63 or *p73* mutation leads to tumor predisposition in the mouse

The *p63* and *p73* mutant mice used in this study carry mutations that remove a large segment of the central DNA binding domain that are thought to inactivate all of the isoforms of these genes (Mills et al., 1999; Yang et al., 1999; Yang et al., 2000). Due to the complexity of these loci, it remains possible the mutant alleles produce truncated products; however, for simplicity, we have referred to the mutant alleles here as $p63^{-/-}$ and $p73^{-/-}$. To determine if *p63* or *p73* mutation alone could cause tumor predisposition, a large cohort of *p63* and *p73* heterozygous mice ($p63^{+/-}$ and $p73^{+/-}$) were aged for two years or until moribund. Both groups (consisting of 40 mice) for each genotype had a shortened life span compared to their wild-type littermates (Figure 1). By 14 to 15 months of age, half of the $p63^{+/-}$ and $p73^{+/-}$ mice were moribund and had to be euthanized. Statistical analysis was performed using a log-rank test. The survival time of $p63^{+/-}$ and $p73^{+/-}$ mice was found to be statistically significant compared to the time of survival of wild-type mice, with a p value of 0.0001 for both. After two years, 32 out of 40 $p63^{+/-}$ mice and 36 out of 40 $p73^{+/-}$ mice had been sacrificed or died, compared to 8 out of 40 in the wild-type group. All animals in this study were subjected to full necropsy to determine the frequency and spectrum of spontaneous tumor formation.

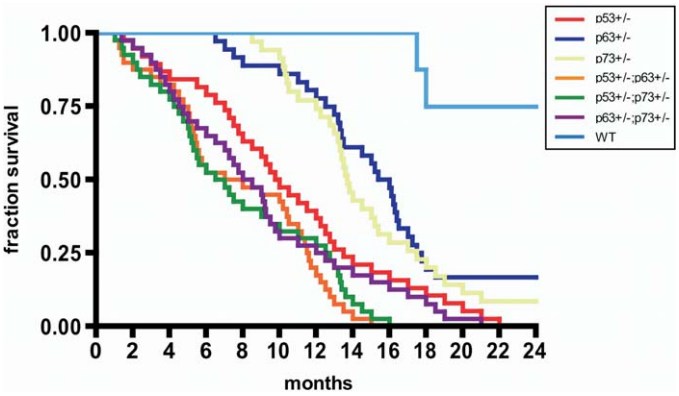

Figure 1. Kaplan-Meier survival curves of wild-type, $p53^{+/-}$, $p63^{+/-}$, $p73^{+/-}$, $p53^{+/-};p63^{+/-}$, $p53^{+/-};p73^{+/-}$, and $p63^{+/-};p73^{+/-}$ mice showing the fraction of mice alive up to 24 months

Genotypes are color-coded. The median time of survival was 10 months for $p53^{+/-}$, 15 months for $p63^{+/-}$, 14 months for $p73^{+/-}$, 7 months for $p53^{+/-};p63^{+/-}$, 6 months for $p53^{+/-};p73^{+/-}$, and 8 months for $p63^{+/-};p73^{+/-}$ mice. The log-rank test was performed to determine the statistical significance between the survival time of wild-type and $p63^{+/-}$ or $p73^{+/-}$ mice (p = 0.0001 for both), the difference between the survival time of $p63^{+/-}$ or $p73^{+/-}$ and $p63^{+/-};p73^{+/-}$ mice (p = 0.0001 for both), and the difference between the survival time of $p53^{+/-}$ and $p53^{+/-};p63^{+/-}$ or $p53^{+/-};p73^{+/-}$ mice (p = 0.001 and p = 0.0004, respectively).

Strikingly, $p63^{+/-}$ and $p73^{+/-}$ mice developed malignant lesions, demonstrating that these genes can act as tumor suppressors. Ten percent of $p63^{+/-}$ mice (n = 40 total mice examined) developed squamous cell carcinomas, and 20% developed histiocytic sarcomas by 12 months of age (Table 1). Ten percent of $p73^{+/-}$ mice developed lung adenocarcinomas, while 12.5% percent developed thymic lymphoma and 12.5% had hemangiosarcomas (Table 1). In addition to these malignant lesions, heterozygous mutant mice showed an increase in benign premalignant lesions, such as squamous cell hyperplasia and multiple lung adenomas. The frequency of lung adenomas in $p63^{+/-}$ and $p73^{+/-}$ was 2.5 and 4 times that seen in wild-type mice, respectively (Table 1). While not all of the mice developed malignant lesions, the remaining mice died from complications due to the benign lesions. For example, in $p63^{+/-}$ and $p73^{+/-}$ mice, many died due to obstructed airways from hyperplastic or premalignant lesions in the pharynx, larynx, mouth, or tongue. In addition to analyzing heterozygous mice for *p73*, a group of 40 $p73^{-/-}$ mice were aged until moribund. As reported previously, many of them died due to chronic infections at approximately 4 to 5 weeks of age (Yang et al., 2000). We were able to monitor 10 out of 40 to the age of 10 months. Six out of those 10 developed lung adenocarcinomas, indicating that loss of *p73* in this tissue leads to tumor formation (Table 1).

Tumors from $p63^{+/-}$ and $p73^{+/-}$ mice undergo loss of heterozygosity (LOH)

LOH is one of the hallmarks of tumor suppressor gene inactivation in cancer (Knudson, 1975; Knudson, 1986). To determine if the tumors in the $p63^{+/-}$ or $p73^{+/-}$ mice lose the wild-type

Table 1. Tumor spectrum of mice mutant for the p53 family members

Tumor type	Genotype	% mice (n = 40)	Tumor type	Genotype	% mice (n = 40)
Mammary adenocarcinoma	wild-type	0%	Osteosarcoma	$p73^{+/-}$	0%
	$p53^{+/-}$	0%		$p53^{+/-}p63^{+/-}$	20%
	$p63^{+/-}$	0%		$p53^{+/-};p73^{+/-}$	20%
	$p73^{+/-}$	0%		$p63^{+/-};p73^{+/-}$	20%
	$p53^{+/-};p63^{+/-}$	10%	Hepatocellular carcinoma	wild-type	0%
	$p53^{+/-};p73^{+/-}$	0%		$p53^{+/-}$	0%
	$p63^{+/-};p73^{+/-}$	20%		$p63^{+/-}$	0%
Lung adenoma	wild-type	10%		$p73^{+/-}$	0%
	$p53^{+/-}$	0%		$p53^{+/-};p63^{+/-}$	0%
	$p63^{+/-}$	25%		$p53^{+/-};p73^{+/-}$	15%
	$p73^{+/-}$	40%		$p63^{+/-};p73^{+/-}$	0%
	$p53^{+/-};p63^{+/-}$	0%	Transitional cell carcinoma	wild-type	0%
	$p53^{+/-};p73^{+/-}$	0%		$p53^{+/-}$	0%
	$p63^{+/-};p73^{+/-}$	0%		$p63^{+/-}$	0%
Lung adenocarcinoma	wild-type	0%		$p73^{+/-}$	0%
	$p53^{+/-}$	0%		$p53^{+/-};p63^{+/-}$	20%
	$p63^{+/-}$	0%		$p53^{+/-};p73^{+/-}$	0%
	$p73^{+/-}$	10%		$p63^{+/-};p73^{+/-}$	0%
	$p53^{+/-};p63^{+/-}$	0%	Myelogenous leukemia	wild-type	0%
	$p53^{+/-};p73^{+/-}$	10%		$p53^{+/-}$	0%
	$p63^{+/-};p73^{+/-}$	15%		$p63^{+/-}$	0%
	$^{*}p73^{-/-}$	60% (n = 10 mice)		$p73^{+/-}$	0%
Salivary adenoma	wild-type	0%		$p53^{+/-};p63^{+/-}$	15%
	$p53^{+/-}$	0%		$p53^{+/-};p73^{+/-}$	0%
	$p63^{+/-}$	0%		$p63^{+/-};p73^{+/-}$	10%
	$p73^{+/-}$	0%	Thymic lymphoma	wild-type	0%
	$p53^{+/-};p63^{+/-}$	0%		$p53^{+/-}$	20%
	$p53^{+/-};p73^{+/-}$	0%		$p63^{+/-}$	0%
	$p63^{+/-};p73^{+/-}$	25%		$p73^{+/-}$	12.5%
Squamous cell hyperplasia	wild-type	5%		$p53^{+/-};p63^{+/-}$	10%
	$p53^{+/-}$	0%		$p53^{+/-};p73^{+/-}$	22.5%
	$p63^{+/-}$	50%		$p63^{+/-};p73^{+/-}$	10%
	$p73^{+/-}$	30%	Histiocytic sarcoma	wild-type	0%
	$p53^{+/-};p63^{+/-}$	0%		$p53^{+/-}$	22.5%
	$p53^{+/-};p73^{+/-}$	0%		$p63^{+/-}$	20%
	$p63^{+/-};p73^{+/-}$	20%		$p73^{+/-}$	0%
Squamous cell carcinoma	wild-type	0%		$p53^{+/-};p63^{+/-}$	0%
	$p53^{+/-}$	0%		$p53^{+/-};p73^{+/-}$	0%
	$p63^{+/-}$	10%		$p63^{+/-};p73^{+/-}$	0%
	$p73^{+/-}$	0%	Hemangiosarcoma	wild-type	0%
	$p53^{+/-};p63^{+/-}$	50%		$p53^{+/-}$	15%
	$p53^{+/-};p73^{+/-}$	0%		$p63^{+/-}$	0%
	$p63^{+/-};p73^{+/-}$	30%		$p73^{+/-}$	12.5%
Acinar pancreatic carcinoma	wild-type	0%		$p53^{+/-};p63^{+/-}$	0%
	$p53^{+/-}$	0%		$p53^{+/-};p73^{+/-}$	10%
	$p63^{+/-}$	0%		$p63^{+/-};p73^{+/-}$	5%
	$p73^{+/-}$	0%	Rhabdomyosarcoma	wild-type	0%
	$p53^{+/-};p63^{+/-}$	0%		$p53^{+/-}$	20%
	$p53^{+/-};p73^{+/-}$	15%		$p63^{+/-}$	0%
	$p63^{+/-};p73^{+/-}$	0%		$p73^{+/-}$	0%
Osteosarcoma	wild-type	0%		$p53^{+/-};p63^{+/-}$	20%
	$p53^{+/-}$	22.5%		$p53^{+/-};p73^{+/-}$	20%
	$p63^{+/-}$	0%		$p63^{+/-};p73^{+/-}$	20%

allele of the respective genes, DNA was extracted from tumors that were macroscopically visible, and analyzed by Southern blotting. Microscopic tumors were analyzed using laser capture microdissection followed by PCR. As shown in Table 2, a high percentage of tumors in the $p63^{+/-}$ and $p73^{+/-}$ mice exhibited loss of the remaining wild-type allele (see Table 2 for full summary of LOH data). For example, in all 5 of the squamous cell carcinomas (SCC) (Figure 2B [T7]) and 7 of the 10 histiocytic sarcomas from $p63^{+/-}$ mice, loss of the wild-type copy of *p63* was observed (Table 2). Likewise, 8 of 10 thymic lymphomas and 4 of 5 lung adenocarcinomas and hemangiosarcomas in $p73^{+/-}$ tumors exhibited LOH (Table 2 and Figure 2D [T10]). These data strongly support the tumor suppressive functions of *p63* and *p73* and establish a tumor cell autonomous effect for their mutation in tumor development.

Mice mutant for both *p63* and *p73* exhibit a complex tumor phenotype

To examine whether *p63* and *p73* might have overlapping functions related to tumor suppression, we intercrossed mice heterozygous for *p63* and *p73* to obtain mice that were heterozygous for mutations in both genes ($p63^{+/-};p73^{+/-}$) (n = 40 mice).

Table 2. Tumors derived from mice mutant for the p53 family of genes exhibit LOH

Genotype	Tumor	p53	p63	p73	Combined
$p53^{+/-}$	thymic lymphoma, n = 10	90%	N/A	N/A	N/A
	histiocytic sarcoma, n = 5	80%	N/A	N/A	N/A
	rhabdomyosarcoma, n = 5	80%	N/A	N/A	N/A
	osteosarcoma, n = 5	80%	N/A	N/A	N/A
	hemangiosarcoma, n = 5	80%	N/A	N/A	N/A
$p63^{+/-}$	histicytic sarcoma, n = 10	N/A	70%	N/A	N/A
	squamous cell carcinoma, n = 5	N/A	100%	N/A	N/A
	lung adenoma, n = 5	N/A	60%	N/A	N/A
$p73^{+/-}$	thymic lymphoma, n = 10	N/A	N/A	80%	N/A
	lung adenoma, n = 5	N/A	N/A	100%	N/A
	lung adenocarcinoma, n = 5	N/A	N/A	80%	N/A
	hemangiosarcoma, n = 5	N/A	N/A	80%	N/A
$p53^{+/-};p63^{+/-}$	transitional cell carcinoma, n = 20	40%	80%	N/A	40%
	mammary adenocarcinoma, n = 20	0%	80%	N/A	0%
	squamous cell carcinoma, n = 10	0%	100%	N/A	0%
	thymic lymphoma, n = 10	90%	70%	N/A	70%
	rhabdomyosarcoma, n = 10	80%	50%	N/A	40%
	osteosarcoma, n = 10	80%	60%	N/A	40%
	myelogenous leukemia, n = 5	80%	100%	N/A	80%
$p53^{+/-};p73^{+/-}$	thymic lymphoma, n = 10	90%	N/A	80%	80%
	hepatocellular carcinoma, n = 10	0%	N/A	90%	0%
	acinar pancreatic carcinoma, n = 10	0%	N/A	90%	0%
	rhabdomyosarcoma, n = 5	80%	N/A	20%	20%
	osteosarcoma, n = 10	80%	N/A	70%	50%
$p63^{+/-};p73^{+/-}$	mammary adenocarcinoma, n = 10	N/A	90%	90%	90%
	lung adenocarcinoma, n = 10	N/A	80%	90%	80%
	thymic lymphoma, n = 10	N/A	70%	80%	70%
	squamous cell carcinoma, n = 15	N/A	100%	0%	0%
	myelogenous leukemia, n = 4	N/A	100%	50%	50%

N/A, not applicable; LOH, loss of heterozygosity; n, number of tumors analyzed; %, tumors with LOH.

In a two-year aging study, these mice were found to have a shorter life span than wild-type, $p63^{+/-}$, or $p73^{+/-}$ mice (Figure 1). The median survival age for these compound heterozygotes was 8 months, which is similar to the median survival age of $p53^{+/-}$ mice (10 months), compared to 14 and 15 months for $p73^{+/-}$ and $p63^{+/-}$ mice, respectively (Figure 1). Statistical analysis using a log-rank test revealed that the difference in the median survival age between the $p63^{+/-};p73^{+/-}$ mice and the *p63* and *p73* single heterozygotes is statistically significant ($p = 0.0001$). $p63^{+/-};p73^{+/-}$ mice that died by 6 months of age had developed thymic lymphoma, hemangiosarcoma, or myelogenous leukemia (Figure 3C). For mice that died between 6 and 12 months of age, carcinomas were most prevalent: mammary adenocarcinomas, lung adenocarcinomas, and squamous cell carcinomas (Table 1 and Figures 3A, 3B, and 3D). Many of these tumor types (including myelogenous leukemia, lung adenocarcinomas, squamous cell carcinomas, and mammary adenocarcinomas) were not detected in the $p53^{+/-}$ mice (Table 1), suggesting different sites of action for *p63* and *p73* in tumor suppression. Strikingly, the tumor types detected in the mice are precisely those that have been shown to lose expression of *p63* and/or *p73* in human tumors. These include transitional cell carcinomas of the bladder (Koga et al., 2003; Park et al., 2000; Puig et al., 2003; Urist et al., 2002), mammary adenocarcinomas (Ahomadegbe et al., 2000; Wang et al., 2002; Yamamoto et al., 2002), osteosarcomas (Park et al., 2004), and myelogenous leukemia (Inokuchi et al., 2001; Yamaguchi et al., 2001). In fact, a mutation in *p63* and abnormalities in *p73* that are associated with myelogenous leukemia have been found (Inokuchi et al., 2001; Yamaguchi et al., 2001). Interestingly, 10% of $p63^{+/-};p73^{+/-}$ mice develop myelogenous leukemia, providing a suitable mouse model in which to study the disease. In addition to malignant lesions, some $p63^{+/-};p73^{+/-}$ mice also developed benign or borderline malignant lesions, including lung adenomas, salivary adenomas, and squamous cell hyperplasia of various grades (Table 1).

Tumors from $p63^{+/-};p73^{+/-}$ mice exhibit LOH

The tumors from $p63^{+/-};p73^{+/-}$ mice exhibited loss of either wild-type *p63* or *p73* or, in many cases, both genes. For example, in 9 of 10 mammary adenocarcinomas from these mice, loss of both *p63* and *p73* was detected (Table 2 and Figures 2B and 2D [T11]). A similar pattern was observed for lung adenocarcinomas and thymic lymphomas (Table 2). Additionally, half of the myelogenous leukemia cases in $p63^{+/-};p73^{+/-}$ mice lost both *p63* and *p73*. In contrast, all 15 of the squamous cell carcinomas assayed from $p63^{+/-};p73^{+/-}$ mice selectively lost the wild-type allele of *p63* (Table 2). Thus, *p63* and *p73* participate in tumor suppression in tissues distinct from that of *p53*, and the combination of the loss of *p63* and *p73* accelerates tumorigenesis. It was striking that some of the prevalent tumor types detected in the double mutant mice were mammary adenocarcinomas and myelogenous leukemia. These are the tumor types in humans that show loss or downregulation of p63 and/or p73 (Ahomadegbe et al., 2000; Inokuchi et al., 2001; Moll, 2003; Park et al., 2000; Park et al., 2004; Puig et al., 2003; Urist et al., 2002; Wang et al., 2002; Yamaguchi et al., 2001; Yamamoto et al., 2002). In fact, the study of human myelogenous leukemias identified a mutation in *p63* associated with the blast crisis of the disease in 12% of cases analyzed, indi-

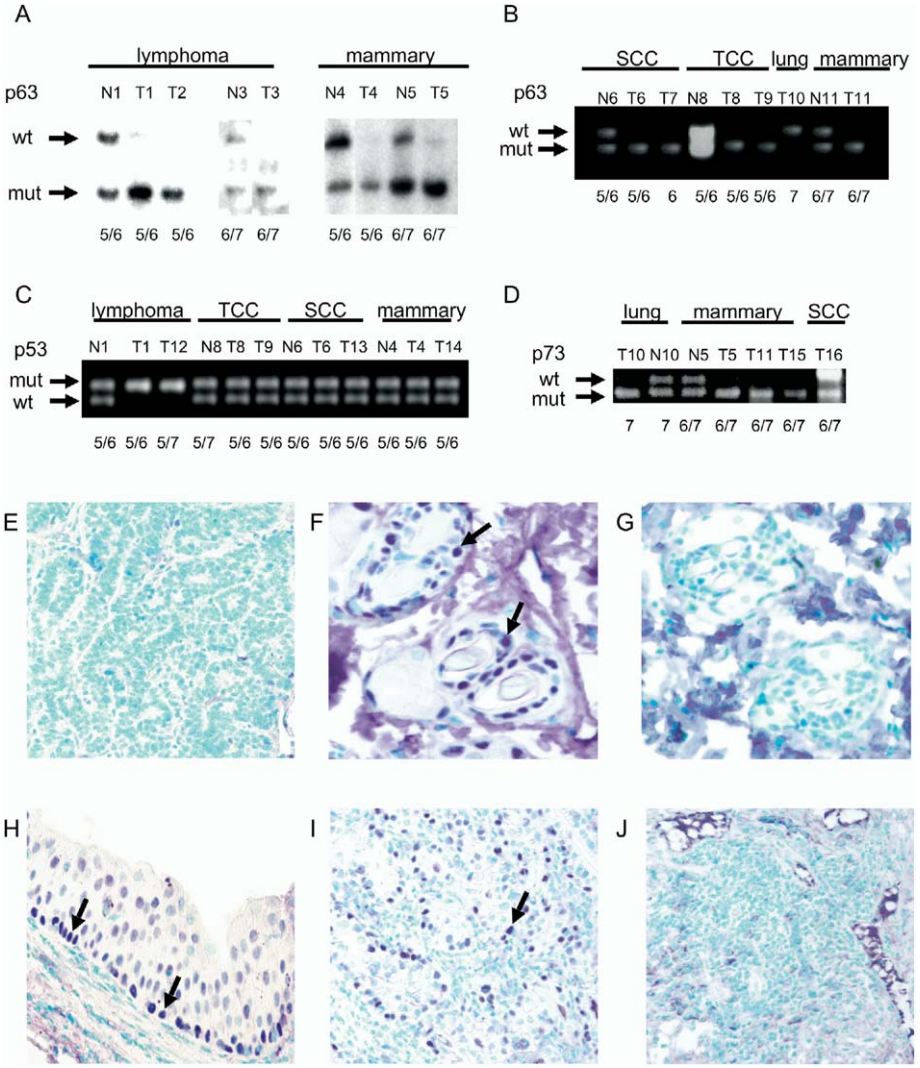

Figure 2. Representative examples of LOH analyses and immunohistochemistry (IHC) on tissue samples from mice mutant for the *p53* family members

LOH of the gene assayed is indicated to the left of each blot or gel. The genotypes of the mice from which the tumors were derived are written below each blot or gel: $p53^{+/-};p63^{+/-}$ (5/6), $p63^{+/-};p73^{+/-}$ (6/7), $p63^{+/-}$ (6), $p53^{+/-};p73^{+/-}$ (5/7), $p73^{+/-}$ (7).

A: Southern blot analysis for LOH of *p63* from macroscopic or laser capture microdissected normal (N) and tumor (T) tissue of thymic lymphomas from $p53^{+/-};p63^{+/-}$ (N1, T1, T2) and $p63^{+/-};p73^{+/-}$ mice (N3, T3) and mammary adenocarcinomas from $p53^{+/-};p63^{+/-}$ (N4, T4) and $p63^{+/-};p73^{+/-}$ mice (N5, T5).

B: PCR for *p63* from squamous cell carcinomas (SCC) from $p53^{+/-};p63^{+/-}$ (N6, T6) and $p63^{+/-}$ mice (T7) and transitional cell carcinomas (TCC) (N8, T8, T9) from $p53^{+/-};p63^{+/-}$ mice. Lung tumor from $p73^{+/-}$ (T10) mouse and mammary adenocarcinoma from $p63^{+/-};p73^{+/-}$ (N11, T11) mouse.

C: PCR for *p53* from thymic lymphoma from $p53^{+/-};p63^{+/-}$ mice (N1, T1) and from a $p53^{+/-};p73^{+/-}$ mouse (T12). TCC (N8, T8, T9), SCC (N6, T6, T13), and mammary adenocarcinomas (N4, T4, T14) from $p53^{+/-};p63^{+/-}$ mice.

D: PCR for *p73* from a lung adenocarcinoma (T10, N10) from a $p73^{+/-}$ mouse and mammary adenocarcinomas (N5, T5, T11, T15), and SCC (T16) from $p63^{+/-};p73^{+/-}$ mice.

E: IHC using an anti-p63 antibody on a mammary adenocarcinoma that has undergone LOH for both *p63* and *p73* from a $p63^{+/-};p73^{+/-}$ mouse.

F: IHC using an anti ΔNp63 antibody on a mammary adenocarcinoma from a $p53^{+/-};p63^{+/-}$ mouse. The genotype of the tumor is $p53^{+/-};p63^{+/-}$.

G: IHC using an anti TAp63 antibody on a mammary adenocarcinoma from a $p53^{+/-};p63^{+/-}$ mouse. The genotype of the tumor is $p53^{+/-};p63^{+/-}$.

H: IHC using an anti-*p63* antibody on the normal epithelium lining the bladder in a $p53^{+/-};p63^{+/-}$ mouse.

I: IHC using an anti-ΔNp63 antibody on a transitional cell carcinoma (TCC) of the bladder from a $p53^{+/-};p63^{+/-}$ mouse. The genotype of the tumor is $p53^{+/-};p63^{+/-}$.

J: IHC using an anti-TAp63 antibody on a transitional cell carcinoma (TCC) of the bladder from a $p53^{+/-};p63^{+/-}$ mouse. The genotype of the tumor is $p53^{+/-};p63^{+/-}$.

Arrows in **F**, **H**, and **I** point to positive nuclei shown in purple. Methyl green was used as a counterstain.

cating again a correlation between the human and mouse data (Yamaguchi et al., 2001).

Mice mutant for *p63* or *p73* in combination with *p53* mutation lead to a more aggressive tumor phenotype

Given that *p63* and *p73* participate in tumor suppression, we next examined whether they might genetically interact with *p53* in tumor formation. Therefore, $p63^{+/-}$ and $p73^{+/-}$ mice were intercrossed with $p53^{+/-}$ mice to obtain mice heterozygous for mutations in both *p53* and *p63* ($p53^{+/-};p63^{+/-}$) and for both *p53* and *p73* ($p53^{+/-};p73^{+/-}$). As shown in Figure 1, $p53^{+/-};p63^{+/-}$ and $p53^{+/-};p73^{+/-}$ cohorts (n = 40 mice each) had a shorter life span than the $p53^{+/-}$ mice. The median age of survival of the $p53^{+/-};p63^{+/-}$ and $p53^{+/-};p73^{+/-}$ mice was 7 and 6 months, respectively, compared to 10 months for $p53^{+/-}$ mice. This difference was found to be statistically significant using a logrank test ($p = 0.001$ and $p = 0.0004$, respectively). The shortened life span in the compound mutants is most likely explained by increased tumor burden and more extensive metastatic disease in these animals. While the $p53^{+/-}$ mice developed primarily thymic lymphomas and sarcomas of various kinds, the $p53^{+/-};p63^{+/-}$ mice developed squamous cell carcinomas with high frequency (50%) in multiple tissues (larynx, pharynx, cervix, and esophagus) (Figure 3E), transitional cell carcinoma of the bladder (20%), and mammary adenocarcinomas (10%) (Table 1, Figure 3F). These mice also developed some of the tumors commonly observed in the $p53^{+/-}$ mice, such as thymic lymphomas (10%), osteosarcomas (20%), and rhabdomysarcomas (20%) (Table 1). Myelogenous leukemia was detected in 15% of $p53^{+/-};p63^{+/-}$ mice, again indicating that *p63* plays an important role in this disease in mice and humans. The $p53^{+/-};p73^{+/-}$ mice also displayed a distinct tumor spectrum

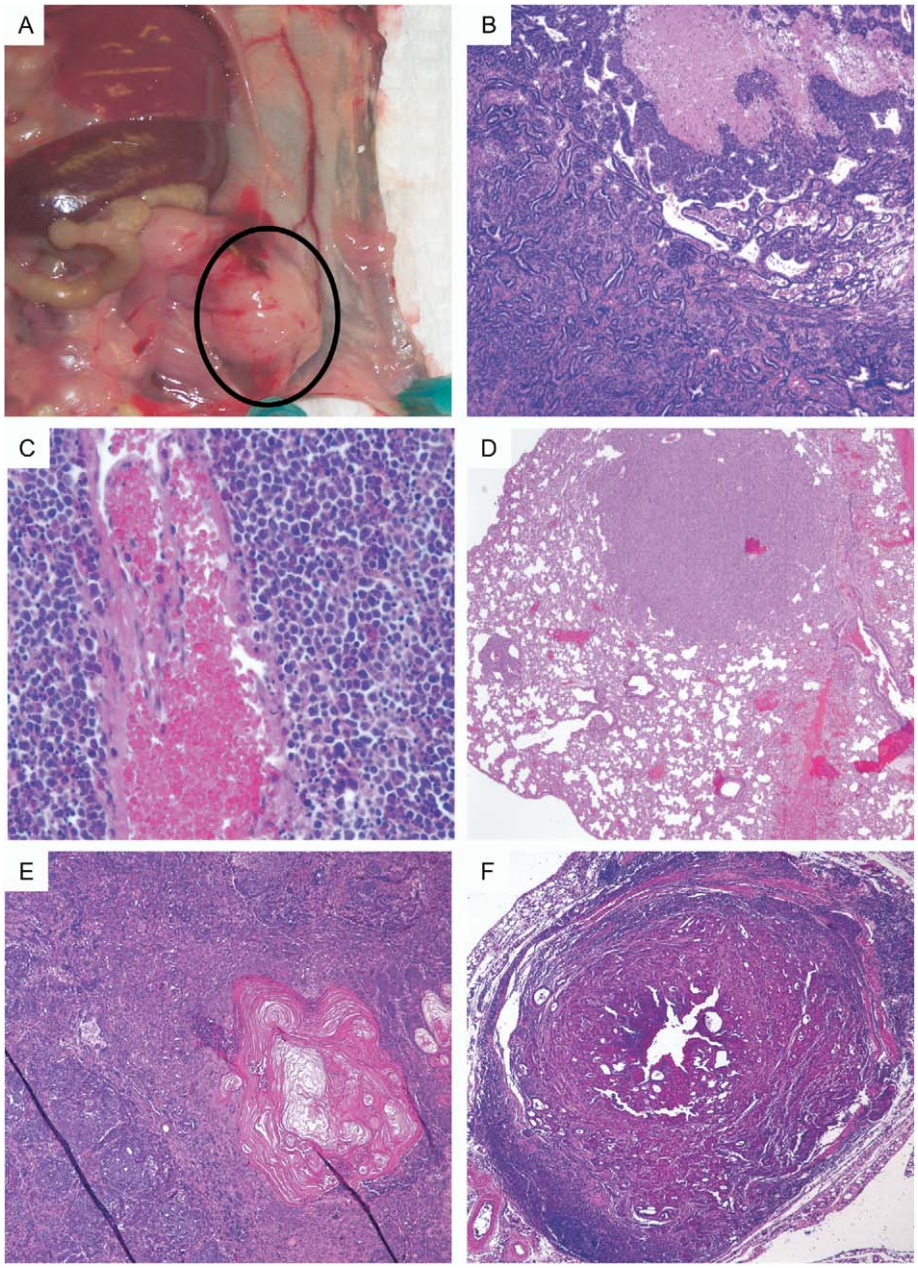

Figure 3. Examples of macroscopic and hematoxylin and eosin (H&E)-stained cross-sections of tumors found in mice mutant for the *p53* family members

A: Mammary adenocarcinoma found on necropsy in a $p63^{+/-};p73^{+/-}$ mouse. Tumor is circled.
B: H&E-stained cross-sections of mammary adenocarcinoma shown in **A**.
C: Thymic lymphoma from a $p63^{+/-};p73^{+/-}$ mouse.
D: Lung adenocarcinoma from a $p63^{+/-};p73^{+/-}$ mouse.
E: Squamous cell carcinoma from a $p53^{+/-}$; $p63^{+/-}$ mouse.
F: Transitional cell carcinoma from a $p53^{+/-}$; $p63^{+/-}$ mouse.

from $p53^{+/-}$ mice. The unique tumors detected in these mice included acinar pancreatic carcinoma (15%), hepatocellular carcinomas (15%), and lung adenocarcinomas (10%) (Table 1). Additionally, $p53^{+/-};p73^{+/-}$ mice developed thymic lymphoma (22.5%) and sarcomas (50%) as detected in the $p53^{+/-}$ mice (Table 1).

$p53^{+/-};p63^{+/-}$ and $p53^{+/-};p73^{+/-}$ mice developed metastatic tumors at a strikingly high frequency and a higher tumor burden than $p53^{+/-}$ mice (Figure 4). Metastatic tumors were distinguished from primary tumors based on histology and tumor location. The compound mutants developed metastatic disease at a frequency of 50% ($p53^{+/-};p63^{+/-}$) and 45% ($p53^{+/-}$; $p73^{+/-}$) compared to 5% of $p53^{+/-}$ mice (Figure 4C). 30% of $p63^{+/-};p73^{+/-}$ developed metastatic disease as well (Figure 4C). The metastatic tumor types included squamous cell carcinomas that metastasized to the heart and lung (Figure 4A) and osteosarcomas and rhabdomyosarcomas that metastasized to the lung and liver (Figure 4B and data not shown). Many of the lung adenocarcinomas in $p53^{+/-};p73^{+/-}$ and $p63^{+/-};p73^{+/-}$ mice metastasized to the lymph nodes. To more clearly quantify and compare the frequency of metastasis in mice from the various genotypes compared to $p53^{+/-}$ mice, two tumor types that were most prevalent and common among the genotypes were analyzed. These were osteosarcomas and rhabdomyosarcomas. For osteosarcomas, 7 of 8 were metastatic in $p53^{+/-}$; $p63^{+/-}$ mice, 7 out of 8 in $p53^{+/-};p73^{+/-}$ mice, and 4 out of 8 in $p63^{+/-};p73^{+/-}$ mice, compared to only 1 out of 8 in $p53^{+/-}$ mice (Figure 4E). For rhabdomyosarcomas, 7 of 8 were metastatic

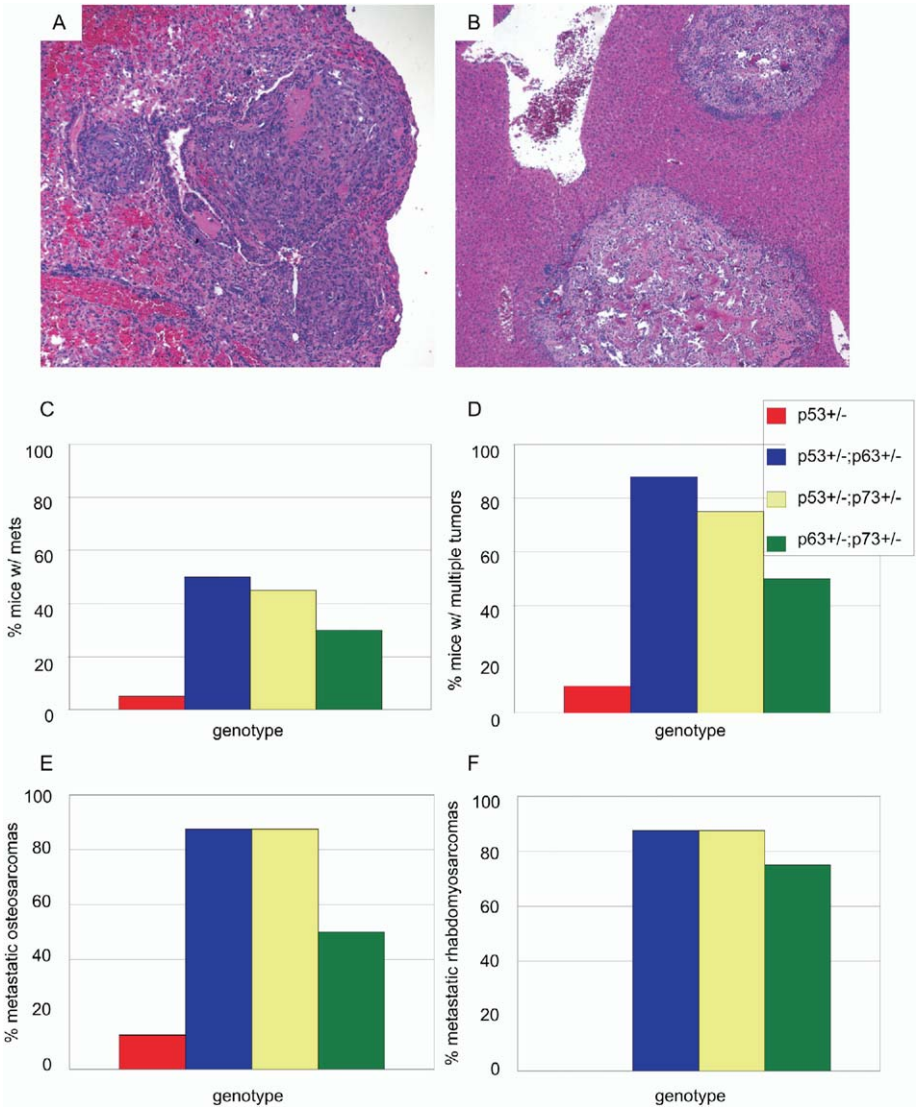

Figure 4. *p53*$^{+/-}$;*p63*$^{+/-}$, *p53*$^{+/-}$;*p73*$^{+/-}$, and *p63*$^{+/-}$;*p73*$^{+/-}$ mice have high tumor burden and tumors that are highly metastatic

A: Squamous cell carcinoma of the esophagus that has metastasized to the lung from a *p63*$^{+/-}$;*p73*$^{+/-}$ mouse.
B: Osteosarcoma from a *p53*$^{+/-}$;*p73*$^{+/-}$ mouse that has metastasized to the liver.
C: Percent mice with metastatic tumors.
D: Percent mice with multiple tumors.
E: Percent mice with metastatic osteosarcomas.
F: Percent mice with metastatic rhabdomyosarcomas.

in *p53*$^{+/-}$;*p63*$^{+/-}$ mice, 7 out of 8 for *p53*$^{+/-}$;*p73*$^{+/-}$ mice, and 6 out of 8 for *p63*$^{+/-}$;*p73*$^{+/-}$ mice, compared to 0 out of 8 for *p53*$^{+/-}$ mice (Figure 4F). These results were found to be statistically significant using a χ^2 test. For osteosarcomas, *p53*$^{+/-}$;*p63*$^{+/-}$ and *p53*$^{+/-}$;*p73*$^{+/-}$ mice had a p value of .01 and *p63*$^{+/-}$;*p73*$^{+/-}$ mice had a p value of 0.025. For rhabdomyosarcomas, *p53*$^{+/-}$;*p63*$^{+/-}$, *p53*$^{+/-}$;*p73*$^{+/-}$, and *p63*$^{+/-}$;*p73*$^{+/-}$ mice had a p value of .01. Interestingly, data from human tumors has indicated that *p63* and *p73* may play a role in progression, as their loss is seen in higher-grade tumors (Koga et al., 2003; Park et al., 2000; Park et al., 2004; Urist et al., 2002; Wang et al., 2002; Yamamoto et al., 2002). Data from the mice shows that loss of *p63* and *p73* leads to more aggressive disease.

These compound mutants also exhibited an increase in tumor burden (Figure 4D). 90% of *p53*$^{+/-}$;*p63*$^{+/-}$, 75% of *p53*$^{+/-}$;*p73*$^{+/-}$, and 50% of *p63*$^{+/-}$;*p73*$^{+/-}$ mice had multiple tumors of the same or distinct types compared to only 10% of *p53*$^{+/-}$ mice (n = 40) (Figure 4D). For example, many of the *p63*$^{+/-}$;*p73*$^{+/-}$ mice with mammary adenocarcinomas had multiple lesions within multiple mammary glands of a single mouse. The *p53*$^{+/-}$;*p63*$^{+/-}$ mice developed collision tumors composed of mammary adenocarcinomas directly adjacent to squamous cell carcinomas and/or rhabdomysarcomas. These data indicate that *p63* and *p73* contribute to tumor suppression, and that loss of these genes increases the severity of disease on the *p53*$^{+/-}$ background.

Mice mutant for *p53* and *p63* or *p53* and *p73* exhibit LOH of one or both genes

LOH in tumors from *p53*$^{+/-}$;*p63*$^{+/-}$ and *p53*$^{+/-}$;*p73*$^{+/-}$ mice was also examined using the techniques described previously (n = 10 or 20 for each tumor type). Certain tumor types in *p53*$^{+/-}$;*p63*$^{+/-}$ mice underwent loss of *p63* at a higher frequency than loss of *p53*, including the transitional cell carcinomas (40% for *p53*, 80% for *p63*, 40% for both) (Figures 2B and 2C [T8 and T9]), mammary adenocarcinomas (0% for *p53* and 80% for *p63*) (Figures 2A and 2C [T4]), squamous cell carcinomas (0% for *p53* and 100% for *p63*) (Figures 2B and 2C [T6]), and my-

elogenous leukemias (80% for *p53*, 100% for *p63*, 80% for both) (Table 2). Other tumor types in these animals showed the opposite pattern. For example, 80% of rhabdomyosarcomas had *p53* LOH, while only 50% showed LOH for *p63*. Examination of tumors from $p53^{+/-};p73^{+/-}$ mice revealed LOH of *p73* in 90% of hepatocellular carcinomas and 90% of acinar pancreatic carcinomas, while LOH of *p53* was not detected in these tumors (Table 2). These data indicate that the tumor-suppressive function of the *p53* family members seems to be tissue-specific, and imply that there is differential selection pressure for their inactivation.

Transitional cell carcinoma (TCC) and mammary adenocarcinoma selectively lose expression of TAp63

Many recent reports have shown the loss of expression of p63 and p73 in various human tumors, including malignant mammary adenocarcinomas (Ahomadegbe et al., 2000; Wang et al., 2002; Yamamoto et al., 2002), bladder adenocarcinomas (Koga et al., 2003; Park et al., 2000; Puig et al., 2003; Urist et al., 2002), osteosarcomas (Park et al., 2004), myelogenous leukemia (Inokuchi et al., 2001; Yamaguchi et al., 2001), and buccal carcinomas (Chen et al., 2004b; Chen et al., 2004c). To determine whether loss of expression of p63 occurs in the tumors from $p53^{+/-};p63^{+/-}$ and $p63^{+/-};p73^{+/-}$ mice, immunohistochemistry was performed using an antibody for p63. p63 was clearly detectable in the basal layer of the epithelium in mammary ducts and in the epithelial lining of the bladder (Figure 2H and data not shown); however, the mammary tumors with LOH not surprisingly lost expression of *p63* (Figure 2E). A subset of mammary adenocarcinomas and transitional cell carcinomas from $p53^{+/-};p63^{+/-}$ mice did not exhibit LOH (Table 2, n = 4 tumors each) of *p63*. These tumors were histologically less aggressive than those that lost the remaining wild-type allele of *p63*. These tumors were analyzed using isoform-specific antibodies for TA versus ΔN p63 to determine whether a similar pattern of *p63* isoform expression is seen in mouse and human tumors. Indeed, these tumors exhibited a loss of expression of specifically the TAp63 isoforms (Figures 2G and 2J), while retaining expression of the ΔN variants (Figures 2F and 2I), as has been reported for these tumor types in human patients (Park et al., 2000). This result indicates that loss of p63 and perhaps more specifically loss of TAp63 is important for the progression of some types of mammary adenocarcinomas and transitional cell carcinomas. These data also indicate that these mouse models mimic the human condition and may be a suitable system to study mechanisms of tumorigenesis and the effects of cancer therapies.

Mice mutant for *p63* exhibit signs of advanced aging

In addition to the development of spontaneous tumors, mice mutant for *p63* also showed increased signs of aging (Figure 5). These mice developed severe degenerative disc disease of the spine at ages ranging between 6 and 18 months of age (Figures 5B and 5D). The degenerative disc disease was most consistent with spondylosis and was most frequently detected in the cervical and thoracic regions. All groups of mice heterozygous for the *p63* mutation exhibited this phenotype with high frequency ($p63^{+/-}$ = 40%, $p53^{+/-};p63^{+/-}$ = 40%, and $p63^{+/-};p73^{+/-}$ = 60%). None of the $p53^{+/-}$ or wild-type mice develop these disease symptoms, and only a small percentage of $p73^{+/-}$ or $p53^{+/-};p73^{+/-}$ mice were affected. In 10% of $p63^{+/-};p73^{+/-}$ mice, the degenerative disc disease was so severe that it resulted in partial paralysis.

Discussion

p63 and *p73* were cloned due to their structural similarity to *p53* and have been shown to share some functions with this well-studied tumor suppressor gene, but their own contributions to the inhibition of tumor formation has been uncertain. Indeed, there has been much debate and speculation about the possible roles of *p63* and *p73* in tumor suppression. Many studies have revealed *p53*-like functions of *p63* and *p73*, such as their ability to induce apoptosis, suggesting that they may play a role in tumor suppression, yet initial studies indicated that *p63* and *p73* were not frequently mutated in human cancers (Hagiwara et al., 1999; Irwin and Kaelin, 2001). Many of these studies focused on the DNA binding domain and may have missed mutations in other regions of the genes or on specific isoforms of *p63* or *p73* (Hagiwara et al., 1999; Hibi et al., 2000; Ng et al., 2000). The study of *p63* and *p73* has also been hampered by the existence of multiple isoforms with opposing activities. Some reports have shown that the ΔN isoforms are overexpressed in certain malignancies and may have oncogenic potential (Concin et al., 2004; Zaika et al., 2002). However, recent analyses of human tumors have shown the loss of expression of p63 and/or p73 in many tumors, including transitional cell carcinomas, mammary adenocarcinomas, and squamous cell carcinomas (Ahomadegbe et al., 2000; Chen et al., 2004a; Chen et al., 2003; Koga et al., 2003; Moll, 2003; Park et al., 2000; Park et al., 2004; Puig et al., 2003; Urist et al., 2002), indicating that these genes have tumor-suppressive activities. The present study provides direct evidence that *p63* and *p73* play a role in tumor suppression, and several important conclusions can be drawn from these data. *p63* and *p73* are by themselves tumor suppressor genes, and are more potent when deleted in combination. Additionally, loss of *p63* and *p73* can cooperate with *p53* in tumor suppression. These observations correlate with data from specific human tumors. The most prevalent tumor types in mice mutant for *p63* and *p73* are those that have been found to lose expression of p63 or p73 in human tumors.

Previous reports on the mice mutant for *p63* and *p73* seemingly contradict the data reported here. However, tumors detected in $p63^{+/-}$ and $p73^{+/-}$ mice in this study were evident, starting at approximately 12 months of age. Most of these tumors were small and not grossly apparent. Analysis of these mice required a full necropsy of each mouse at the time of death and careful examination by a mouse pathologist. Additionally, analysis of compound *p53* family crosses had not been performed previously, and the data from these studies show that in combination with each other or with *p53*, *p63* and *p73* are potent tumor suppressor genes.

There is accumulating evidence from human tumors that *p63* and *p73* play a role in tumor suppression. Some human tumor types have been shown to lose expression of *p63* and *p73*, supporting a role for these genes in tumor suppression. Interestingly, multiple studies have shown that loss of *p63* expression is associated with tumor progression and poor prognosis in human bladder cancers (Koga et al., 2003; Park et al., 2000; Urist et al., 2002). Bladder tumors were prevalent in $p53^{+/-};p63^{+/-}$ mice, and these tumors also exhibited loss of *p63* ex-

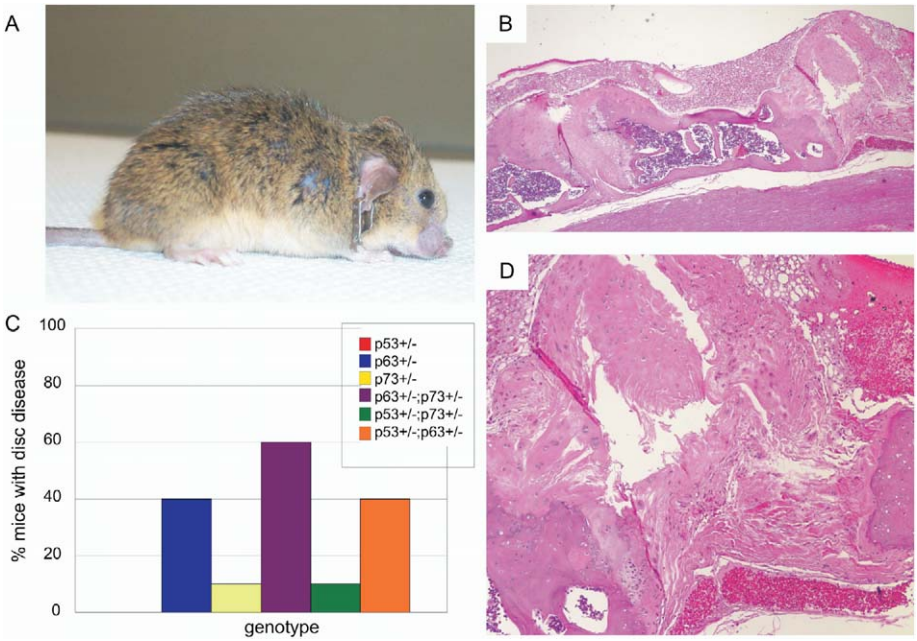

Figure 5. Mice mutant for *p63* exhibit early signs of aging

A: $p63^{+/-};p73^{+/}$ mouse at 6 months of age with a hunched appearance.

B: Hematoxylin and eosin stained cross-section of the spine of a 6-month-old $p63^{+/-};p73^{+/-}$ mouse with degenerative disc disease.

C: Percentage of mice for each genotype with disc disease at 10 months of age.

D: Higher magnification of **B**.

pression through LOH. Moreover, we observed that the few transitional cell carcinomas and mammary adenocarcinomas that did not exhibit LOH of *p63* selectively lost expression of TAp63 and not ΔNp63, indicating that loss of TAp63 may be an important event in the development of transitional cell carcinoma and mammary adenocarcinoma. This pattern of expression has been detected in transitional cell carcinomas in humans (Park et al., 2000). Likewise, LOH, allele silencing, and decreased expression of p73 and p63 have been detected in breast cancers (Ahomadegbe et al., 2000; Wang et al., 2002; Yamamoto et al., 2002). The mammary adenocarcinomas from the $p63^{+/-};p73^{+/-}$ mice also exhibited LOH for both *p63* and *p73*. Another important piece of evidence tying *p63* and *p73* to human tumor suppression is a mutation in *p63* that has been identified in 12% of chronic myelogenous leukemia cases. Two groups of mice that harbor *p63* mutations ($p53^{+/-};p63^{+/-}$ and $p63^{+/-};p73^{+/-}$) developed myelogenous leukemia.

The tumor-suppressive activities of *p63* and *p73* seem to play an important role in specific tissues. Based on the tumor spectrum of *p63* and *p73* mutant mice, these genes seem to have unique sites of action. The tumor spectrum detected in mice mutant for *p63* and *p73* is quite different from that of *p53*-deficient mice (Jacks et al., 1994). These *p53* mutant mice primarily develop thymic lymphomas and sarcomas. In contrast, *p63* and *p73* mutant mice develop primarily carcinomas and fewer sarcomas. This is most likely due to the pattern of expression of *p63* and *p73*. While *p53* is ubiquitously expressed, *p63* and *p73* are highly expressed in epithelial tissues, the site of carcinoma development. Additionally, loss of *p63* or *p73* in combination with *p53* led to a more severe and aggressive tumor phenotype. Compound *p53*/*p63* and *p53*/*p73* mutant mice displayed high tumor burden and highly metastatic disease. Earlier work had revealed that cells lacking both *p53* and *p63* ($p53^{-/-};p63^{-/-}$) and *p53* and *p73* ($p53^{-/-};p73^{-/-}$) were more resistant to the induction of apoptosis by DNA damaging agents than cells lacking just *p53* ($p53^{-/-}$) (Flores et al., 2002). These data indicated that *p63* and *p73* have antiapoptotic functions that are independent of *p53*. The tumor data from the *p53*/*p63* and *p53*/*p73* mice correlate with the earlier data and show that *p63* and *p73* have functions independent of *p53* that lead to more aggressive tumor phenotypes in the compound mutant mice. The data from human tumors, such as in transitional cell carcinoma, also indicate that loss of p63 and p73 are indicators of more aggressive tumors and those with poor clinical prognosis (Koga et al., 2003; Park et al., 2000; Urist et al., 2002).

Many possible mechanisms for *p63* and *p73*'s role in tumor suppression exist, including the dependence of *p53* on *p63* and *p73* to induce apoptosis in certain tissues (Flores et al., 2002). This does not hold true in all tissues, because combined mutation of *p63* and *p73* does not affect *p53*-dependent apoptosis in thymocytes (Senoo et al., 2004). However, the *p63*/*p73* compound mutant mice develop thymic lymphomas, indicating that apoptosis may not be the primary mechanism of action for tumor suppression in this tissue. Another mechanism of *p63* and *p73*'s tumor suppressive activities could be through the known inhibition of these genes by point mutant *p53*, a common mutation in human tumors (Di Como et al., 1999; Gaiddon et al., 2001). Numerous cell-based and biochemical studies have shown that point mutant *p53* can associate with and inhibit the transcriptional activation of *p63* and *p73* on various target genes such as *p21*, an important cell cycle regulator (Di Como et al., 1999; Gaiddon et al., 2001; Irwin et al., 2003; Strano and Blandino, 2003; Strano et al., 2002). This transdominant negative regulation could account for the promotion of tumorigenesis by tumor-derived point mutants of *p53*, and may explain the low frequency of mutations detected in *p63* and *p73* in human tumors, providing another possible mechanism of action. Additionally, recent in vivo data indicates that mice carrying point mutant *p53* exhibit a similar tumor spectrum,

tumor burden, and rate of metastasis to the compound *p53/p63* and *p53/p73* mutant mice in this study (Lang et al., 2004; Olive et al., 2004).

In addition to the tumor phenotype detected in mice mutant for *p63* and *p73*, these mice developed signs of premature aging. This phenotype was most severe in the $p63^{+/-};p73^{+/-}$ mice. The $p63^{-/-}$ mice were reported to have bone defects: craniofacial abnormalities and defects in limb development (Mills et al., 1999; Yang et al., 1999). Although the exact mechanism for the disc disease in these mice is not known, it is possible that a gene dosage effect in the $p63^{+/-}$ mice could account for bone defects in the adult mouse.

The study of *p63* and *p73* in tumor suppression is indeed complex. Some tumors have been shown to upregulate the ΔN isoforms while downregulating the TA isoforms (Zaika et al., 2002), while other studies have shown exactly the opposite (Park et al., 2000). The data to date is so complex and puzzling that no clear conclusion can be drawn about the definitive function of each isoform at this time. These opposing data sets most likely reflect tissue specificity of the various isoforms of p63 and p73. The future generation of isoform-specific knockout mice will likely shed much-needed light on the activities of the various *p63* and *p73* isoforms in various tissues and cellular processes. Even so, the data presented here clearly show that loss of function of *p63* or *p73* leads to tumor development. This may have important implications for the treatment of certain cancers, particularly those that retain wild-type *p53* and exhibit loss or mutation of *p63* or *p73* or those that carry point mutant alleles of *p53*. Tumors that arise in these mice (or cells derived from them) should be helpful in determining what therapeutic strategies might be most useful in human cancers that arise through mutation or functional inactivation of the broader *p53* family.

Experimental procedures

Mouse husbandry and tumor analysis

Mice double heterozygous for the *p53* family members ($p53^{+/-};p63^{+/-}$, $p53^{+/-};p73^{+/-}$, and $p63^{+/-};p73^{+/-}$) on a mixed C57BL/6 × 129/SvJae background were backcrossed to C57BL/6 for four generations to enrich for the C57BL/6 background. The published $p63^{+/-}$ and $p73^{+/-}$ mice were on a mixed (C57BL/6 × 129/SvJae) background. Resulting mice were intercrossed to generate the mice (wild-type, $p53^{+/-}$, $p63^{+/-}$, $p73^{+/-}$ $p53^{+/-};p63^{+/-}$, $p53^{+/-};p73^{+/-}$, and $p63^{+/-};p73^{+/-}$) for the study. Mice were aged until moribund for up to 24 months. Ill and distressed mice were euthanized by asphyxiation using carbon dioxide following the guidelines of the IACUC. Necropsies were performed and soft tissue organs were fixed in 10% neutral buffered formalin, and bones were placed in Bouin's fixative for 3 weeks. Fixed tissues were embedded in paraffin and sectioned onto slides. Slides were stained with hematoxylin and eosin. Histopathological analysis for each organ was performed.

Immunohistochemistry

Sections from paraffin-embedded tissue were dewaxed with xylene and rehydrated in a graded series of ethanol following standard protocols. Slides were incubated with primary antibody (clone 4A4, F. McKeon) that detects multiple isoforms of p63, or the TAp63-specific antibody (D20, Santa Cruz) or Δ*Np63*-specific antibody (N16, Santa Cruz) at a dilution of 1:100 for 12 to 15 hr at 4°C. For detection, the Vectastain Kit (Vector Labs) was used, followed by the VIP kit (Vector Labs), and counterstained with methyl green (Vector Labs).

Analysis for LOH of *p63* and *p73*

For macroscopic tumors, samples were taken from fresh tumor tissue and adjacent normal tissue at the time of necropsy. Genomic DNA was extracted from the tissue using DNAzol reagent (GibcoBRL). For detection of *p63*, the genomic DNA was digested with Bgl1 and BamHI, electrophoresed on a 0.8% agarose gel, and transferred to Hybond N+ membrane (Amersham) using standard Southern blotting techniques. Blots were probed with a radiolabeled fragment of genomic *p63* DNA. Probes were prepared by digesting genomic *p63* DNA with BamHI and NheI and by radiolabeling with the Prime-It II kit (Stratagene) and [α-^{32}P]dATP. The 13 kb band corresponds to the wild-type allele and the 7.5 kb band to the mutant allele of *p63*. Microscopic tumors were microdissected by laser capture (Arcturus II) from 10 μM paraffin sections on uncoated glass slides. DNA was prepared from captured samples by overnight digestion at 37°C in 100 μl of LCM buffer (pH 8.0) (0.04% proteinase K, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Tween 20). Proteinase K was inactivated by heating to 95°C for 8 min. PCR was performed for *p53*, *p63*, or *p73* as described previously (Jacks et al., 1994; Yang et al., 1999; Yang et al., 2000).

Quantification of tumor burden and metastases

Histopathology of hematoxylin and eosin stained slides was performed on every soft tissue organ and bone for each mouse. Mice with more than two tumors were scored as containing multiple tumors. When multiple tumors were detected in the same organ, serial sectioning of the organ was done to ensure that the two lesions were not part of one contiguous lesion. Mice with metastases of the primary tumor in a lymph node or distant organ were scored as having metastatic disease. Metastases were distinguished from primary tumors by histopathology.

Statistical analysis

χ^2 test using Microsoft Excel was performed on metastatic osteosarcomas and rhabdomyosarcomas from $p53^{+/-};p63^{+/-}$, $p53^{+/-};p73^{+/-}$, and $p63^{+/-};p73^{+/-}$ mice compared to metastatic osteosarcomas and rhabdomyosarcomas from $p53^{+/-}$ mice. Survival curves were generated using mice that were euthanized when moribund. The logrank (Mantel-Haenszel) test was used to calculate statistical significance (PRISM4, GraphPad).

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