The Germline- and Tissue-Specific Effects of Endogenous Point-Mutant p53

by

Kenneth Paul Olive

B.S. Biology, Bucknell University, 1998

Submitted to the Department of Biology in Partial Fulfillment
of the Requirements for the Degree of

Doctor of Philosophy

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[June 2005]
February 8, 2005

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Abstract

p53 is frequently altered in human tumors through missense mutations that result in accumulation of mutant p53 protein. These mutations may confer dominant-negative or gain-of-function properties to p53. To ascertain the physiological effects of tumor-associated point-mutations in p53, the structural mutant $p53^{R172H}$ and the contact mutant $p53^{R270H}$ (codons 175 and 273 in humans) were engineered into the endogenous *p53* locus in mice. $p53^{R270H/+}$ and $p53^{R172H/+}$ mice are mouse models of Li-Fraumeni Syndrome (LFS). They developed allele-specific tumor spectra that were distinct from $p53^{+/-}$ mice and that better reflect the broad spectrum of tumors found in LFS patients. Dominant effects that varied by allele and function were observed in primary cells derived from these mice. In addition, $p53^{R270H/-}$ and $p53^{R172H/-}$ mice developed novel tumors compared to $p53^{-/-}$ mice, including hemangiosarcomas and variety of carcinomas. These data support a gain-of-function effect by mutant p53 toward the development of epithelial and endothelial tumors.

Furthermore, conditional mutant p53 alleles were used in combination with a conditional activated *K-ras* allele to generate mouse models of advanced lung adenocarcinoma. In this system, the effects of endogenous mutant p53 were found to be both allele-specific and tissue-specific. This work provides insight into the spectrum of *p53* mutations in human cancers and demonstrates that point-mutant *p53* alleles expressed under physiological control have enhanced oncogenic potential beyond the simple loss of *p53* function.

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PUBLICATIONS

Mutant p53 Gain-of-Function in Two Mouse Models of Li-Fraumeni Syndrome. Kenneth P. Olive, David A. Tuveson, Zachary C. Ruhe, Bob Yin, Nicholas A. Willis, Roderick T. Bronson, Denise Crowley, Tyler Jacks. Cell 119(6): 847-860.

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Up-regulation of c-Myb expression in murine osteoblasts transfected with a 1.3 kbp c-myb HindIII fragment and pSV2neo: Induction of a tumorigenic phenotype. Olive, Kenneth P, Martin, James F, Novak, Josef F, Page, Kathleen C, and Chernin, Mitchell I. Abstract. FASEB J. 11(9): A1430. 1997

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Generation And Analysis Of Two Strains Of Li-Fraumeni Mice. Kenneth P. Olive, Roderick T. Bronson, Denise Crowley, Bob Yin, Tyler Jacks, and David A. Tuveson. Lecture. Cold Spring Harbor Meeting, Cancer Genetics & Tumor Suppressor Genes, Cold Spring Harbor, NY, August 2002.

Increased c-myb Proto-oncogene Expression in Mouse Bone Cells Transfected with a Fragment of the Chicken c-myb Gene: Induction of a tumorigenic phenotype. Kenneth P. Olive, James F. Martin, Josef F. Novak, Kathleen C. Page, and Mitchell I. Chernin. Honors Thesis, Bucknell University. 1998

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It is my belief that personal development- physical, emotional and intellectual- do not cease over the course of one's life. The past six years of my life have been a time of rapid change for me. In addition to having accumulated some small measure of knowledge in the field of biology, my eyes have also been opened more broadly to areas such as politics, international development, gender issues, social interaction and management (both of myself and of others). That these changes have been, I believe, in a positive direction is due in no small part to the influence of a large number of friends, colleagues and family members. My personal growth has not been without a few pains along the way and certainly, as my research has made greater demands of my time and energy, I have necessarily had to make greater demands from those around me. Without their understanding and support, I could not have been successful.

In science, it is understood that all progress derives from the foundation of knowledge lain by others. At the end of a scientific publication, authors are afforded the opportunity to acknowledge those individuals and institutions that contributed directly to the technical work of the experiments. However, by convention, scientists do not acknowledge in journal publications those who contributed to our work in a non-technical sense; indeed it is rare that we have the opportunity to express our gratitude to everyone in our lives that have made it possible to achieve our goals. I am therefore immensely pleased at this chance to thank the following people:

Shana S. Warren- As I am writing these words, it occurs to me that five years ago on this date, on a similarly arctic night, Shana and I ate dinner at the Southwest Grill and then drove to the Kendall Square Cinema to watch Cider House Rules on our first date. Tonight, as Shana flies over the Sahara desert, returning to the United States for the first time in four months, I look forward with great anticipation to our wedding and our future lives together. In the past five years, Shana has opened my eyes to the world and fostered my desire to improve it. She has been my constant companion, confidant and friend as well the anchor of my support system. Her patience, love and unique ability to make me see other points of view have helped me to grow as a person.

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Chapter 1

Introduction

MODELING CANCER IN MICE

In the past six years, substantial advances have been made in the accurate modeling of human cancers using genetically engineered mouse strains. As mouse models have improved in their sophistication and faithfulness to the genetic lesions observed in human tumors, their accuracy in recapitulating the phenotypes of these tumors has also improved. Analysis of carefully designed mouse strains has allowed for explorations into the biology of hundreds of different cancer genes, elucidating many of the basic principles of cancer described above. The following is a discussion of the various classes of genetically engineered mouse strains and the challenges that remain in mouse modeling (Figure 1).

The earliest genetically engineered mouse models were *transgenic mice* into which recombinant DNA was randomly inserted (Gordon et al., 1980). Transgenic mice are still frequently utilized as a quick means of assessing the effects of gene overexpression or as a component of a more complicated regulatory system. The former generally involve the expression of a cDNA from an exogenous promoter. The principal drawback to *cDNA trangenics* is that they lack proper temporal and spatial control of gene expression. All of the subtle details of gene regulation inherent in the endogenous gene are lost and the transgene is often expressed at levels vastly greater than physiological. A variation of this approach is to place a cDNA under the control of the promoter from a gene with a desired expression pattern (Chada et al., 1985). When using this strategy, great care must be taken to confirm the expression pattern of the transgene; missing intronic or distal upstream regulatory elements can affect the expression pattern of a gene, as can epigenetic effects arising from the site of genome integration. More recently, bacterial artificial chromosomes (BACs) have been used to overcome the inherent insert size restrictions of

traditional cloning techniques (Yang et al., 1997). Using this technique, *BAC transgenic mice* can be constructed that incorporate large fragments of DNA (up to ~100kb) into the mammalian genome, allowing for an extra copy of a gene to be expressed from its endogenous promoter and with intact non-coding sequences. However, even in this case, epigenetic effects, such as the precise state of the chromatin surrounding the endogenous gene, may not be accurately recapitulated.

In order to assess the effects of loss of gene expression, methods for precisely targeting endogenous loci were developed using homologous recombination in embryonic stem cells followed by blastocyst injection and implantation (Thompson et al., 1989). The results of this approach were *knockout mice*, which harbor null mutations of targeted genes in the endogenous locus. Knockout mice have been highly successful aids to understanding the functions of tumor suppressor genes or for investigating the developmental roles played by oncogenes. One of the major drawbacks of using knockout mice to model human tumors is that many cancer genes have important developmental roles, the loss of which can result in embryonic lethality. The second major drawback is that tumor suppressor genes are not always lost through large deletions. p53, for example, is generally found to harbor single base-pair point-mutations rather than loss-of-function mutations. A variation on the knockout mouse addresses this second point by targeting subtle mutations into the endogenous locus. These *targeted mutants* express altered gene products under the control of the endogenous gene promoter, allowing for physiologically accurate expression of mutant proteins at the appropriate time and place in development. This approach is particularly useful for determining the functional effects of particular mutant alleles found in human tumors.

Targeted mutant mice are to be distinguished from *knockin mice*, in which a foreign gene or cDNA is targeted into an existing gene locus. For example, a cDNA encoding the *β-galactosidase* gene was targeted into the ROSA26 locus, replacing the ROSA26 gene product but keeping the ROSA26 gene promoter (Friedrich and Soriano, 1991; Zambrowicz et al., 1997). This allows the widespread expression β-galactosidase (commonly used in reporter systems) by placing it under the control of a ubiquitously expressed gene that is known to exist in a region of open chromatin.

Targeted mutants are still prone to embryonic lethal phenotypes, especially in the case of activated oncogenes. In order to circumvent this issue, *conditional knockout mice* were developed in which the loss of a gene is achieved through the action of a site-specific recombinase *in vivo (Orban et al., 1992)*. The most common example of this is the use of the bacterial *Cre* recombinase to excise the region situated between two similarly oriented LoxP sites. Alleles targeted in this way are referred to as *floxed* (flanked by LoxP) and are used to target gene loss to a particular place or time through the managed expression of Cre recombinase. This can be achieved by expressing Cre recombinase from a tissue-specific promoter or by delivering Cre ectopically through the use of viral vectors or protein transduction. Several viral vectors for Cre delivery have been developed including adenoviral, retroviral and lentiviral systems. Likewise, purified HIV-TAT-Cre fusion proteins have also been successfully used to achieve directed recombination (Jo et al., 2001).

In order to investigate the effects of re-activation of a gene, targeted alleles have been developed that incorporate a floxed gene-silencing cassette to prevent expression of the allele until after Cre-mediated excision. A Lox-STOP-Lox (LSL) cassette developed in this laboratory effectively prevents gene expression by using four tandem polyadenylation sequences to block transcription paired with a splice acceptor and mutant splice donor to block translation (Tuveson et al., 2004). Such *conditional activatable* alleles are useful for investigations into the effects of re-expression of a wild-type tumor suppressor gene in a tumor caused by its absence. Alternatively, *conditional mutant* alleles can be constructed to express activated oncogenes from the endogenous promoter in a tissue-specific manner. In this case, heterozygous conditional mutant animals can be used to examine the effects of a dominant oncogene while avoiding the embryonic lethal phenotypes of widespread mutation or homozygous deletion of the targeted locus.

Site-directed recombination technology has been refined in several areas. Multiple LoxP sites have been described that are all effectively recombined by Cre but are mutually incompatible with one another, allowing for the simultaneous directed recombination of multiple Cre-mediated events within a single cell (Sauer, 1996). Furthermore, other bacterial recombinase systems, such as Flp/Frt, have been described and, in some cases, are being utilized in mammalian systems (Vooijs et al., 1998). The combination of multiple recombinase systems targeted to different gene loci within a single animal will allow of the sequential inactivation of several genes in a controlled manner. This will facilitate the ordered dissection of gene function with regards to tumor initiation and progression.

Another approach to targeted gene expression is through the *RCAS/TVA* system (Federspiel et al., 1994). In this system, an avian retroviral vector is used to express a cDNA in infected cells. What makes this system useful is the fact that the particular avian leukosis virus utilized does not infect mammalian cells. By targeting the expression of the avian retroviral receptor TVA, infection by RCAS can be directed specifically to cells of interest. This system has the advantage that once the TVA transgenic mice are established, many different genes can be swapped into the viral vector for testing.

One of the principal drawbacks to conditional control of gene expression using tissue-specific recombinase expression is that the cells harboring the recombined mutant genes are frequently surrounded by other mutant cells. In contrast, it is thought that most human tumors begin as a single mutant cell surrounded by a wild-type cellular context. In order to mimic this effect, a *latent* mouse model was designed that relies on the stochastic homologous recombination of a gene segment duplication to express a mutant gene product (Johnson et al., 2001a). In this case, the allele is null prior to the spontaneous rearrangement of the gene locus in somatic cells, resulting in mutant gene expression in an individual cell surrounded by other cells that are heterozygous knockouts. This strategy could be useful for investigations into tumor surveillance but it is currently hindered by a lack of simple techniques for assessing which cells have undergone rearrangement. This is necessary because it is likely that different cell types will recombine and activate the mutant allele at different rates. It has also been suggested that this model could be useful as an *in vivo* assay for genes that affect genomic stability since any mutation that affected the rate of homologous recombination should influence that rate accumulation of cells with the targeted rearrangement.

Another approach to controlling gene expression *in vivo* is through the use of a drug-sensitive regulatory system. An example of this is the tetracycline regulatory system in which a cDNA of interest is expressed from an inducible promoter that is sensitive to a tetracycline-regulated fusion protein (*rtTA-VP16*)(Kistner et al., 1996). Variants of this system allow for tetracycline-regulated activation or repression of gene expression (rtTA or tTA). Expression of the rtTA-VP16 or tTA-VP16 fusion proteins from a tissue-specific promoter allow for control of gene expression both spatially and temporally within an animal. The one drawback to this approach is that the gene of interest is expressed from an exogenous promoter. Attempts to combine the Cre/Lox system with the Tet responsive system into a Tet/Cre approach have been confounded by the "leakiness" of tetracycline response elements. Since Cre-mediated recombination is an irreversible binary event, even a very low level of Cre expression can result in full activation of the targeted locus.

In another drug-inducible system, a fusion is created between the gene of interest and the ligand-binding domain of the estrogen receptor. This results in the sequestration of the protein of interest in the cytoplasm until binding of 4-hydroxy-tamoxifen (OHT) induces its translocation into the nucleus. This system is very clean with regards to background activity and was therefore successfully utilized as a method of activating Cre using OHT, allowing for drug inducible expression of targeted conditional loci from their endogenous promoters (Indra et al., 1999).

One of the most recent developments in mouse modeling has been the use of short hairpin RNAs (shRNAs) to knock down the expression of a target gene. shRNAs allow for stable expression of small interfering RNAs (siRNAs) which target transcripts for destruction by the RNA-induced silencing complex (RISC). shRNA transgenes can be used to stably knock down a targeted gene in mice. Furthermore, since some siRNA sequences are more effective than others, an allelic series can be produced showing the effects of various levels of gene expression from the endogenous promoter (Hemann et al., 2003).

Another recently developed tool has been the use of lentiviruses to transduce mouse embryonic stem cells (ES cells). By arranging a multiple cloning site within a lentiviral expression vector, shRNA cassettes can be rapidly cloned into the viral transduction system and integrated into ES cells, allowing for the rapid generation of transgenic mouse lines. In elegant work, conditional versions of the lentiviral promoter were created by altering the LoxP site to include a TATA box element. This "lentilox" site was then combined with an shRNA expression cassette to allow for Cre-mediated activation or inactivation of shRNA expression *in vivo* (Ventura et al., 2004). This approach allows for the rapid generation of conditional gene knockdown mice.

Summary

The recent explosion in gene targeting strategies has resulted in some truly impressive mouse models that almost fully recapitulate the biology and pathology of human tumors. Nonetheless, several hurdles remain. For example, the ability to conditionally introduce subtle mutations into the endogenous locus while retaining a wild-type allele prior to induction would allow one to assess the effects of mutations in a fully wild-type cellular context. Furthermore, such a system

would make it possible to homozygose alleles that are embryonic lethal when deleted, making the breeding schemes to produce double- and triple- compound mutant mice vastly more efficient. Another frontier is that of combining imaging modalities with mouse models of cancer. Currently, fluorescence and luminescence technologies are being used at a very basic level to observe tumor growth and to act as a reporter for the activation of inducible systems. However, at this time these systems lack finesse and are plagued by technical glitches (for example, GFP is inactive in tissues processed for histology by most standard techniques). The refinement of these technologies and the adaptation of other imaging systems for use with mice, including magnetic resonance (MR), computed tomography (CT), positron emission spectroscopy (PET) and single photon emission computed tomography (SPECT), will allow for the observation of neoplastic processes as they occur in a living animal. Finally, the role of epigenetics in tumor development has not yet been thoroughly explored using mouse models. As noted previously, many tumor cells exhibit widespread changes in chromatin configuration, and epigenetic silencing is a common mechanism of tumor suppressor gene inactivation. Models that allow the precise control of epigenetic regulatory systems could be extremely useful in generating accurate mouse models of human cancers.

With the great variety of genetic tools either at their disposal or in development, the onus is now on researchers to put them to use in clever and meaningful ways. In creating mouse models of cancer, one should at all times be guided by the human disease. Dozens of mouse models have been generated that fail to adhere to this rule. These models rarely produce pathology even remotely related to that seen in human cancers and the lessons to be derived from them are dubious at best and misleading at worst. The early failure of such models to respond to

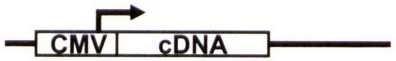
therapeutics in a predictive manner set back the entire field of mouse modeling by fostering the belief among clinical investigators and drug companies that mouse models of cancer are not useful in preclinical drug development. Although genetically engineered mice have been extraordinarily successful in providing information about the basic biology of tumorigenesis, it is the additional responsibility of mouse modelers to correct this misconception by creating truly predictive tools for drug discovery and development.

Figure 1. A summary of mouse modeling strategies. Abbreviations: TSP, tissue specific promoter; CMV, cytomegalovirus promoter (a strong constitutive promoter); Pro, an endogenous gene promoter; Neo^R, neomycin resistance cassette.

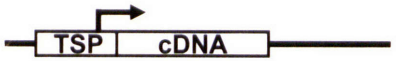
Mouse Modeling Stragegies

Random Integration

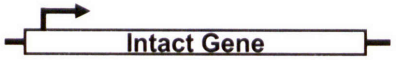
cDNA transgenic



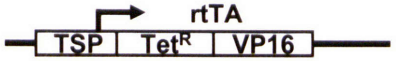
tissues-specific transgenic

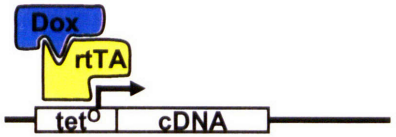


BAC transgenic



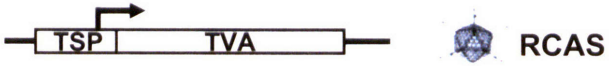
tetracycline inducible



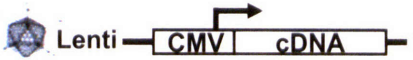


ER^{T2} fusion

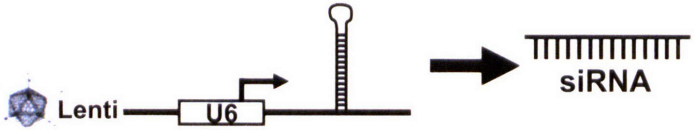
RCAS/TVA



lentiviral transgenic

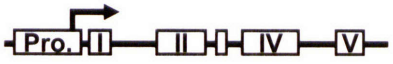


shRNA knockdown

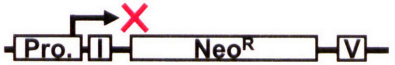


Targeted

genomic locus

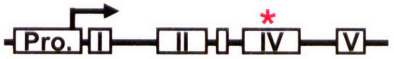


knockout



knockin

targeted mutant



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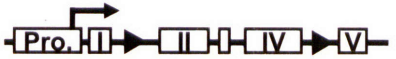
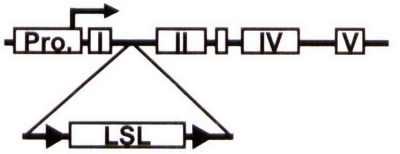
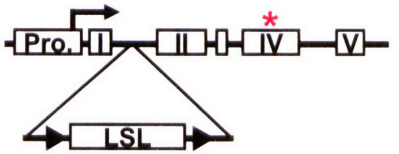


conditional activatable

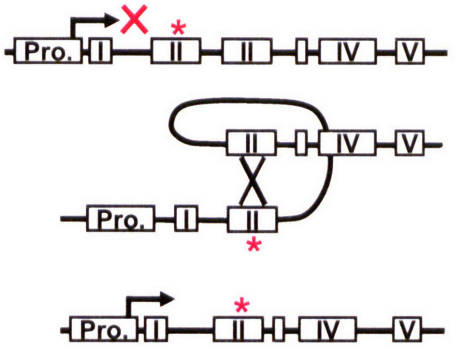


▶ = LoxP site

conditional mutant



stochastic



THE P53 TUMOR SUPPRESSOR GENE

The *p53* tumor suppressor gene is the most extensively studied gene in the human genome. Referenced in over 33,000 papers, our understanding of this critical gene is both extensive and incomplete. The most compelling reason for this intense focus is that *p53* is mutated in over 50% of all human tumors, making it one of the most frequently altered genes in cancer. Indeed, it appears likely that alteration of the *p53* pathway, through any of a multitude of mechanisms, may be a requisite event in the development or progression of nearly all forms of cancer. Furthermore, p53 mutations occur in distinct locations within the gene and the mutational spectra of p53 vary with different classes of tumors. Mutant p53 alleles also yield a mutant protein that is stabilized relative to wild-type p53 leading to the frequent observation of p53 overexpression in human tumors. These alleles appear to be something more than null alleles yet less than true dominant-negative alleles; cells heterozygous for mutant p53 still frequently select for loss of the remaining wild-type allele. Some mutant p53 alleles may even actively promote tumorigenesis, independent of wild-type p53 function. For this reason, a thorough understanding of the structure and function of *p53*, both wild-type and mutant, is essential. Through such an understanding, rational strategies for the treatment of cancer can be pursued that account for and counter-act the alteration of p53 pathway function.

A Brief History

p53 was described 25 years ago as a cellular protein bound by the SV40 large T-antigen and as a tumor antigen expressed in carcinogen-induced mouse tumors (DeLeo et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979). p53 expression was soon found in human tumors (Crawford et al., 1981; Dippold et al., 1981) and the presence of anti-p53 antibodies was

discovered in sera from breast cancer patients (Crawford et al., 1981). Following this, several observations led logically to the hypothesis that p53 was a cellular oncogene, similar in nature to known retroviral transforming proteins such as $p60^{src}$. It was also found that p53 levels fell dramatically during the retinoic acid-induced differentiation of F9 embryonal carcinoma cells (Oren et al., 1982); similar treatment of mice injected with F9 cells resulted in prolonged survival, supporting a correlation between p53 expression and tumorigenicity (Strickland and Sawey, 1980). In a separate experiment, microinjection of anti-p53 antibodies into Swiss 3T3 cells resulted in a decrease in DNA synthesis; this was interpreted as a correlation between the inactivation of p53 through antibody-binding and a decrease in cellular proliferation. Finally, p53 was found to cooperate with H-ras in the transformation of primary rat embryo fibroblasts (Eliyahu et al., 1984; Parada et al., 1984b).

Eventually, a series of observations changed the perception of p53 function in tumorigenesis, suggesting it acted instead as a tumor suppressor gene. First, p53 cDNAs that had been used in oncogene cooperation assays were found to be mutant, and wild-type p53 cDNAs were shown to inhibit cellular transformation (Finlay et al., 1989; Hinds et al., 1989). Second, p53 was determined to be mutated frequently and at numerous codons in a wide range of human tumors and was seen to undergo loss-of-heterozygosity with high frequency (Baker et al., 1990; Hollstein et al., 1991). Finally, loss-of-function mutations in p53 in the mouse led to profound cancer predispositions (Harvey et al., 1993a; Jacks et al., 1994; Purdie et al., 1994).

In this light, p53 came to be viewed a tumor suppressor gene and tumor-associated point-mutations in p53 were generally viewed to be acting in a dominant-negative manner to inhibit wild-type p53. It is now understood that the decrease in wild-type p53 expression observed

during F9 cell differentiation mirrors a similar decrease in p53 mRNA levels that occurs during the normal embryonic development. Prior to differentiation (and in the absence of DNA damage), F9 cells behave similarly to ES cells which express high levels of functionally inactive p53. Furthermore, microinjection of anti-p53 antibodies induced cell cycle arrest in Swiss 3T3 cells because antibodies directed to the C-terminus of wild-type p53 activate its DNA-binding function rather than inhibit its function (Hupp et al., 1995). Therefore, the results of both experiments can be related to the activation of wild-type p53 tumor suppressor functions.

An Overview of p53 Structure and Function

A general understanding of the structure and functions of p53 has developed over the past decade. p53 encodes a transcription factor that responds to cellular stresses by directly binding to DNA and transcriptionally regulating genes involved in cell cycle arrest, apoptosis, and genome maintenance. The p53 gene is comprised of eleven exons spanning approximately 20 kilobases and is translated into a 393 amino acid polypeptide. The p53 protein has five primary functional domains: an N-terminal transactivation domain (TA, residues 1-63), a proline-rich domain (PR, residues 64-94), a core sequence-specific DNA binding domain (DBD, residues 110-286), an oligomerization domain (OD, residues 326-355) and a regulatory domain (CTD, residues 363-393)(Figure 2).

p53 Oligomerization

The regulation of target genes by p53 is dependent on its assembly into a homotetramer, which is mediated by the oligomerization domain and is necessary for efficient binding of DNA (Hainaut et al., 1994; Iwabuchi et al., 1993; Pavletich et al., 1993; Sturzbecher et al., 1992; Wang et al.,

1993). Tetrameric p53 is organized as a dimer of dimers; monomeric p53 cannot bind DNA and dimeric p53 does so only weakly. Although thermodynamic calculations suggest that p53 exists in a monomeric state in undamaged cells (Sakaguchi et al., 1997), this model is based on an analysis of a p53 OD fragment and does not take into account interactions between the OD and other portions of the protein such as the DBD. Such interactions may act cooperatively to stabilize the formation of p53 tetramers. More recently, an elegant set of experiments showed that that the biogenesis of p53 dimers occurs through the association of elongating p53 monomers within a polysome (Nicholls et al., 2002). Mixing experiments suggested that this cotranslational dimerization was irreversible. In contrast, the association of p53 dimers into a tetramer was shown to be both post-translational and reversible. These observations have important implications for the mixing of mutant and wild-type p53 in heterozygous tumor cells (see Chapter 4).

DNA Binding by p53

Crystal structures of the human (Cho et al., 1994) or murine (Zhao et al., 2001) p53 DBD bound to DNA depict a β-sandwich that coordinates the position of a large loop (L3) to make contact with the minor groove and a loop-sheet-helix motif to make contact with the major groove. This structure incorporates one Zn^{2+} ion per monomer which helps to coordinate the L3 loop and is necessary for binding to DNA (Pavletich et al., 1993). An analysis of the p53 DBD by solution NMR found that binding of DNA by the core domain resulted in a substantial chemical shift of the residues that form the DNA binding surface (Rippin et al., 2002). This work also found cooperativity of binding by the DBD in the absence of an OD; two small surface regions that were shifted during DNA binding may participate in DBD-DBD interactions within a tetramer.

Another analysis of the DBD, based on phasing experiments using full length p53, details the bending and twisting of the DNA bound by p53, leading to the supposition that p53 may bind nucelosomal DNA (Nagaich et al., 1999). Finally, work from Carol Prives' laboratory found that binding of several different antibodies against the N-terminal region of p53 greatly reduced the dissociation of the DBD from DNA. Such antibodies may mimic the effects of protein-protein interactions or post-translational modifications at the N-terminus, implying that intramolecular interactions may occur between the N-terminus and the DBD (Cain et al., 2000).

Binding of the p53 DBD to DNA is sequence-specific and the architecture of the p53 binding site reflects the quaternary structure of active p53. The p53 binding site is composed of two half-sites separated by a 0-13 nucleotide linker. Each half-site contains two copies of the sequence PuPuPuC(A/T) arranged in a head-to-head orientation (el-Deiry et al., 1992). This consensus site is fairly degenerate and a large number of different specific binding sites have been found in p53 target genes. Furthermore, p53 binding sites vary in their affinity, conformation, orientation and relative location within target gene promoters or introns; multiple different p53 binding sites are often located within a single p53 target gene. The variation in p53 binding sites may allow for the subtle regulation of target gene choice by p53 under different conditions or in different cell types. However, no clear correlation between gene function and the nature of p53 binding sites has yet been described.

p53 Transcriptional Activation

The p53 transactivation domain is composed of two subdomains that act independently to promote transcription. Activation subdomain 1 (ASD1) extends from residues 1-42 and contains

a highly conserved region (BOX-I) that is necessary for transcription (Fields and Jang, 1990; Raycroft et al., 1990). Transactivation subdomain 2 (ASD2) extends from residues 43-63 and is less well conserved than ASD1 (Candau et al., 1997; Venot et al., 1999; Zhu et al., 1998). Although the precise contribution of each domain to global p53 function is poorly understood, it is clear that following DBD-mediated binding to target gene promoters, both transactivation subdomains are capable of interacting with components of the transcriptional apparatus, including TATA-binding protein (TBP), several TBP-associated factors (TAFs), transcription factors IIB and IIF (TFIIB, TFIIF) and RNA polymerase II (Chang et al., 1995; Farmer et al., 1996; Lu and Levine, 1995). Furthermore, both transactivation domains were found to be necessary for interaction of p53 with the histone acetyltransferase p300 (Liu et al., 2003). p300 is one of several transcriptional coactivators that are recruited by p53 to modify histones near the proximal promoters of p53 target genes. In recent work, chromatin reconstitution experiments and chromatin immunoprecipitation (ChIP) were used to show that protein arginine N-methyltransferase (PRMT1), p300 and coactivator-associated arginine methyltransferase (CARM1) each bind to p53 and act cooperatively, through a temporally ordered mechanism, to promote the transcription of p53 target genes (An et al., 2004). ChIP was also utilized, on a stunningly large scale, to dissect the contribution of specific transcription initiation components to p53-mediated transactivation (Espinosa et al., 2003). This work found that the composition and mechanics of p53-induced transcriptional initiation varied dramatically based on the promoter analyzed as well as the type of stress used to induce p53 function.

Although histone modification of p53 target genes correlates well with their level of transcriptional upregulation following DNA damage, the exact mechanism by which histone

modification contributes to transcription of p53 target genes is unclear. However, studies of other systems suggest that modified histones may preferentially act as substrates for the recruitment of other transcription factors to the initiation complex and, similarly, they may prevent the association of transcriptional repressors (Strahl and Allis, 2000).

p53 Transcriptional Repression

While its most well known role is in the transactivation of target genes, p53 also has an important role in the transcriptional repression. p53 has been shown to inhibit transcription through three different potential mechanisms. First, p53 may have generalized effects on the basal transcription machinery as studies of the cyclin B gene show that p53-mediated transcriptional repression remains intact through successive deletions of all but the basal promoter (Innocente et al., 1999; Krause et al., 2000). This mechanism is the least well understood and may in fact reflect a lack of insight into other mechanisms. In recent years, p53 binding sites have been found in the intronic regions of several genes. The presence of an intronic p53 binding site in cyclin B could provide an alternative explanation for the effects of p53. Second, p53 may affect chromatin remodeling through the recruitment of histone deacetylases (HDACs). For example, p53 was shown to recruit HDACs to the *Map4* and *stathmin* promoters by directly associating with the co-repressor mSin3a (Hoffman et al., 2002; Murphy et al., 1999). This mechanism is reminiscent of the recruitment of the histone acetyltransferase p300 during transcriptional activation. Finally, p53 may interfere with the function of other transcriptional activators. For example, p53 inhibits the anti-apoptotic *bcl-2* gene by binding to a p53 site within the *bcl-2* promoter and displacing the transcriptional activator Brn-3a (Budhram-Mahadeo et al., 1999). In another important example, p53 inhibits

the transactivation of a number of genes including *cdc2*, topoisomerase II, *chk2* and securin by interfering with the function of NF-Y (Joshi et al., 2003; Matsui et al., 2004; Yun et al., 1999; Zhou et al., 2003).

Given that p53 can either activate or repress different sets of genes following DNA binding, there must be determinants that distinguish these two sets. Indeed, at least one group reported the presence of a variant p53 binding site arranged in a head-to-tail orientation in the *MDR1* gene that conferred responsiveness to transcriptional repression by p53. Substitution of this site with a canonical head-to-head version of the same sequence reversed the effect of p53, resulting in transactivation of *MDR1* following induction of p53 (Johnson et al., 2001b). Incidentally, a nearly identical variant p53 binding site was also found in the *cylcin B1*, gene, although its relevance to the transcriptional repression of *cyclin B1* by p53 remains untested.

The Proline-Rich Domain of p53

The 30 residue PR domain contains five different PXXP motifs and was originally described as a domain necessary for the efficient induction of apoptosis (Venot et al., 1998; Zhu et al., 1999). More recently, the PR domain has been shown to act as a second binding site for the transcriptional cofactors mSin3a (Zilfou et al., 2001) and p300 (Dornan et al., 2003; Liu et al., 2003). Interestingly, both mSin3a and p300 appear to bind to p53 in roughly the same place implying their binding may be mutually exclusive. Given their respective roles as co-repressor and co-activator, this could imply that the PR domain acts as a switch to determine whether p53 functions to activate or repress target genes.

The C-Terminal Regulatory Domain

The p53 CTD has never been very clearly understood, but early work suggested that it functions as a negative regulatory domain that interferes with sequence-specific binding by the core DBD. This was thought to be the result of steric inhibition caused by the CTD folding back and occluding the DBD (Gu and Roeder, 1997)(see below for discussion of allosteric regulation of p53). However, recent work has focused primarily on the ability of the CTD to bind non-specifically to various forms of DNA. The p53 CTD has variously been described as capable of associating with RNA (Yoshida et al., 2004), single-stranded DNA overhangs (Bakalkin et al., 1994), short single-stranded DNA (Jayaraman and Prives, 1995), DNA aggregates (Yakovleva et al., 2001), stem-loops (Kim et al., 1997), supercoiled DNA (Fojta et al., 2004), complex junctions and recombination intermediates (Dudenhoffer et al., 1998; Lee et al., 1997), as well as to damaged DNA, including DNA mismatches (Lee et al., 1995), gaps (Zotchev et al., 2000), and by gamma irradiated DNA (Reed et al., 1995). In each case, the p53 CTD recognizes a molecule that in some way exhibits structural constraints that deviate from the conformation of B-DNA. In a particularly elegant report, McKinney and Prives explored the binding of p53 to linear or circularized 66-mers containing either a wild-type or mutated binding site for p53. They found that the p53 DBD and CTD acted cooperatively to promote the high affinity recognition of p53 binding sites that were constrained by forced circularization (McKinney and Prives, 2002). These and other data (discussed below) suggest that rather than negatively regulating DNA binding by p53, the CTD in fact promotes DNA binding of constrained DNA.

Regulation of p53 Activity

p53 activity is regulated through a vast array of different mechanisms. These can be grouped broadly into protein-protein interactions, post-translational modifications, protein localization, and variation in target sites. The aspects of p53 biochemistry that are regulated include the stability of the p53 protein, its affinity for (and rate of dissociation from) DNA, the availability of substrate and the choice of which target gene p53 will bind. The complexity of the regulatory networks affecting p53 is staggering and the mechanisms by which these different regulatory mechanisms are integrated are poorly understood.

Early work on p53 regulation led to an allosteric model of p53 function that included two primary conformation states of p53 protein, "latent" and "active". This view of p53 regulation was based on functional readouts rather than analysis of its physical state and several recent works have challenged this view. An overview of the allosteric model and its shortcomings is presented below, followed by a review of known mechanisms of p53 post-translational regulation. This section will conclude with a discussion of the upstream signaling pathways that induce these modifications.

The Allosteric Model of p53 Function

The allosteric model was proposed following observations that deletion of the CTD resulted in activation of sequence-specific DNA binding by the core domain as measured by electrophoretic mobility shift assay (EMSA)(Hupp and Lane, 1994; Hupp et al., 1992). It was also found that binding of an antibody to the CTD, acetylation of lysine residues in the CTD by p300/CBP or phosphorylation of serine residues in the CTD by protein kinase C (PKC) or casein kinase 2

(CK2), increased DNA binding by p53. Furthermore, p53 expressed from bacteria was found to be completely unable to bind to DNA in the absence of post-translational modifications such as acetylation. This led to the idea that the p53 CTD acts as a negative regulatory domain that inhibits the function of the DBD, either through direct association or by positioning the DBD away from DNA. Competition experiments in which a peptide derived from the CTD was also shown to activate DNA binding were argued to support this model.

However, several recent observations challenge the allosteric model of p53 latency. For example, PAb 421 was found not to affect DNA binding by p53 when measured by DNAse I protection assay rather than EMSA (Cain et al., 2000). Also, though bacterially expressed p53 cannot bind to oligonucleotides containing a p53 binding-site, it is fully competent for binding to p53 response elements in chromatin, even in the absence of CTD acetylation (Espinosa et al., 2003). Furthermore, experiments utilizing ChIP found that the amount p53 binding to DNA following genotoxic stress correlated well with changes in total p53 protein levels, indicating that a conformational change in pre-existing p53 tetramers did not play a substantial role in the regulation of p53 (Kaeser and Iggo, 2002). Finally, latent p53 was found already associated with target gene promoters in unstressed cells by ChIP allowing for the rapid assembly of transcription machinery at certain promoters in response to certain genotoxic signals (Espinosa et al., 2003). These data suggest that the activation of latent p53 is not dependent on an allosteric regulation of sequence-specific DNA binding.

Regulation of p53 Protein Degradation

The most prominent mechanism by which p53 is regulated is through 26S proteasomal degradation. The 26S proteasome is a large, multimeric, barrel-shaped complex that can actively catalyze protein degradation (Crews, 2003; Voges et al., 1999). It consists of three primary subunits: a central proteolytic chamber, called the 20S proteolytic complex, and two 19S regulatory complexes. Proteins are targeted for proteasomal degradation through the covalent ligation of a small protein moiety called ubiquitin. Ubiquitin acts as a molecular tag that is recognized by the 19S complexes. A class of proteins called E3-ubiquitin ligases catalyzes the monoubiquitylation of various target proteins. The ubiquitin tag is then itself ubiquitylated and it is this polyubiquitylated form that is targeted to and degraded by the 26S proteasome.

The principal molecular determinant of p53 protein levels is a protein called mouse double-minute 2 (MDM2, or HDM2 in humans)(Haupt et al., 1997). MDM2 acts as an E3-ubiquitin ligase for p53 (Honda et al., 1997). The MDM2 protein contains a deep hydrophobic cleft that specifically binds to a segment of the N-terminus of p53 extending from residue 17 to residue 27 (Kussie et al., 1996). In unstressed cells, MDM2 binds to p53 and catalyzes the monoubiquitylation of p53 at six known lysine residues near its C-terminus: lysines 370, 372, 373, 381, 382 and 386 (Nakamura et al., 2000; Rodriguez et al., 2000). The monoubiquitin conjugates are polymerized into polyubiquitin through the concerted action of MDM2 with p300/CBP. Following activation by cellular stress, the interaction between MDM2 and p53 is inhibited and p53 rapidly accumulates to higher levels.

MDM2 itself is transcriptionally activated by p53 resulting in a negative feedback loop that regulates the activity of p53 (Barak et al., 1993); a p53 DNA-binding site exists within the first intron of the *MDM2* gene that strongly regulates the expression of MDM2 (Wu et al., 1993). The importance of this interaction was dramatically demonstrated through the analysis of *MDM2* knockout mice. Mice lacking both copies of *MDM2* die early in embryogenesis, shortly after implantation (Jones et al., 1995). However, the further deletion of both copies of p53 fully rescues the developmental defects of *MDM2* loss, resulting in normal mice with an adult-onset tumor predisposition (Montes de Oca Luna et al., 1995). Furthermore, heterozygosity for *MDM2* in the context of wild-type p53 dramatically extends the survival of *Eμ-myc* transgenic mice (Alt et al., 2003). Both of these observations are consistent with a model in which MDM2 deletion or haploinsufficiency results in the activation of p53 function.

As a result of this negative feedback circuit, the levels of p53 and MDM2 oscillate over time following activation of p53 by DNA damage. Initially, a careful kinetic analysis of MDM2 and p53 protein levels by immunoblotting suggested an analog model of damped oscillations after high levels of DNA damage (Lev Bar-Or et al., 2000). More recently, an elegant report analyzed fluorescently tagged p53 and MDM2 proteins by time-lapse video microscopy in single cells (Lahav et al., 2004). This work revealed different cells within a single culture undergo varying numbers of oscillations following DNA damage. Furthermore, the amplitude and duration of each pulse in different cells was unchanged following treatment with escalating doses of ionizing radiation. Rather, the number of consecutive pulses within a cell increased with higher levels of DNA damage. This quantized or 'digital' response to DNA damage could imply that p53 and MDM2 act as a circuit that continues to monitor the cell following DNA damage, activating

fresh rounds of stress response pathway signaling until all DNA damage events have been resolved. It remains to be seen if the variation in the numbers of oscillations occurring in different cells reflects varying levels of actual DNA damage experienced by those individual cells or whether successive rounds of p53 activation regulate different subsets of p53 target genes.

The interaction between MDM2 and p53 forms a nexus of integration for a variety of different upstream signaling pathways. Several DNA damage response pathways modulate this interaction by post-translationally modifying p53 (see *Post-Translational Modifications of p53*, below). Phosphorylation of MDM2 (such as by cyclin A/CDK) can also inhibit the p53/MDM2 interaction (Zhang and Prives, 2001). MDM2 is also regulated through changes in its subcellular localization. $p19^{ARF}$ ($p14^{ARF}$ in humans), a tumor suppressor protein produced from the alternative reading frame of the *CDKN2* gene locus, functions primarily by binding to MDM2 and sequestering it away from p53 (Honda and Yasuda, 1999; Weber et al., 1999). This connects p53 to core components of the cell cycle pathway and positions p53 as an important regulator of oncogenic stress.

In addition to ubquitylating p53, MDM2 was recently found to mediate the conjugation of p53 with NEDD8, another small protein moiety similar to ubiquitin (Xirodimas et al., 2004). Following DNA damage, NEDD8 was ligated to p53 at lysines 370, 372 and 373. These are three of the six lysine residues that have been reported to be ubiquitylated by MDM2. Interestingly, neddylation was found to inhibit the ability of p53 to transactivate target genes rather than to affect the ubiquitylation and turnover of p53. The precise mechanism by which

this occurs is poorly understood but this initial work adds yet another layer of complexity to the regulation of p53 function.

A third ubiquitin-like protein, SUMO1, has also been found to be covalently ligated to p53. Sumoylation of p53 occurs at Lys386, a site that is also ubiquitylated by mdm2. It is unclear what effect the sumoylation of p53 has on its function.

Other Post-Translational Modifications of p53

The p53 protein is the subject of a variety of post-translational modifications at numerous different sites. In addition to being ubiquitylated, neddylated and sumoylated, p53 has also been reported to be phosphorylated (Jay et al., 1981), acetylated (Gu and Roeder, 1997), methylated (Chuikov et al., 2004), glycosylated (Shaw et al., 1996) and ribosylated (Wesierska-Gadek et al., 1996). Although the role of glycosylation and ribosylation of p53 have not been further explored, it is clear that acetylation and methylation play an important role in the regulation of p53 function. The kinases, methyltransferases, acetyltransferases and ligases that effect these modifications form the basis of a vast upstream regulatory network feeding into p53. More recently the roles of p53 phosphatases and deacetylases in p53 regulation have also been investigated.

Phosphorylation of p53

p53 is phosphorylated on multiple serine and threonine residues throughout the protein; there is no evidence of phosphorylation of p53 on tyrosine residues (Figure 2). In several cases, a single phosphorylation site is the target of several different known kinases and some kinases are known

to phosphorylate more than one residue on p53. For many of these sites, kinases have been identified that catalyze their phosphorylation in response to stimuli. All of the phosphorylation sites in p53 are found within known functional domains and discrete effects on p53 function have been determined for several of them. The phosphorylation of several sites in the N-terminus of p53 can interrupt or diminish the association of p53 with its primary negative regulator MDM2 (see section of the role of MDM2 in p53 regulation). The phosphorylation of some sites in the C-terminus can directly influence the ubiquitylation of p53 and its subsequent degradation while other sites may directly influence the transactivation activity of p53.

Casein Kinase I (CK1) is the only known kinase for Ser6, Ser9 and Thr18 (Higashimoto et al., 2000; Knippschild et al., 1997; Sakaguchi et al., 2000); the phosphorylation of Thr18 by CK1 is dependent on prior phosphorylation of Ser15 (Dumaz et al., 1999). CK1 is actually a family of serine/threonine protein kinases, of which CK1δ and CK1ε have been shown to phosphorylate p53. Little is known about the direct role of CK1 in cancer but recently, CK1ε was shown to be activated by the *wnt* signaling pathway, tying it to a major pathway involved in development and cancer (Liu et al., 2002). The observation that Ser6, Ser9 and Thr18 are each phosphorylated in response to DNA damage implies either that CK1 kinase activity is also DNA-damage responsive or other kinases for these sites remain to be identified. No specific effect has been ascribed to phosphorylation of Ser6 and Ser9. However, Thr18 is part of the binding site of the MDM2 and phosphorylation of this residue inhibits the binding of MDM2 to p53 (Kussie et al., 1996; Sakaguchi et al., 2000).

p53 is one of over a dozen different substrates of ATM (mutated in ataxia telangiectasia). ATM, and two related proteins, ATR and ATX, are members of the phosphoinositol-3-kinase (PI3K) family and are all capable of phosphorylating p53 at Ser15, resulting in its accumulation and activation (Canman et al., 1994; Shiloh, 2003; Tibbetts et al., 1999). ATM, ATR and ATX are key players in the cellular response to a variety of different forms of DNA damage. ATM is inactive in undamaged cells and rapidly responds to certain forms of DNA damage by relocalizing to the site of damage and by phosphorylating a variety of proteins involved in cell cycle arrest and DNA damage repair pathways (Bakkenist and Kastan, 2003). ATM and ATR are known to respond to different types of DNA damage and there is some indication that this information is communicated to p53 through differential phosphorylation as ATM can additionally phosphorylate Ser9 and Ser46 (Saito et al., 2002) while ATR has been shown to phosphorylate Ser37 (Tibbetts et al., 1999).

ATM also phosphorylates checkpoint kinase 2 (CHK2), which in turn phosphorylates p53 at Ser20. Ser20, like Ser15 and Thr18, makes up part of the binding interface between MDM2 and p53. Phosphorylation of either Thr18 or Ser20 has been shown to interfere with this interaction resulting in the stabilization of p53 protein. In contrast, phosphorylation of Ser15 was shown not to directly affect the binding of a p53 polypeptide to MDM2. Rather, phospho-Ser15 appears to form a component of CK1 binding site, thereby encouraging the phosphorylation of p53 on Thr18 (Chehab et al., 1999; Sakaguchi et al., 2000).

Another member of the PI3K family, DNA-dependent protein kinase (DNA-PK), is also capable of phosphorylating p53 at Ser15 as well as at Ser37 (Shieh et al., 1997). The kinase function of

DNA-PK is dependent on its association with double-stranded DNA breaks. DNA-PK functions in the non-homologous end joining (NHEJ) pathway where it cooperates with Ku70 and Ku86 in synapse formation (the positioning of two DNA ends for ligation) (Lieber et al., 2003). Recently, these three proteins, and specifically the kinase activity of DNA-PK, were shown also to be required for normal telomere end-capping (Bailey et al., 2004). Therefore, DNA-PK has three critical and inter-related functions: sensor of DNA damage, participant in the NHEJ DNA damage response and the initiator of the p53-dependent DNA damage responses.

Several mitogen-activated protein kinases (MAPKs), including ERK1/2 and p38 kinase, have been shown to physically associate with and phosphorylate p53 in response to DNA damage, particularly UV irradiation. For example, p38 has been shown to phosphorylate p53 at Ser15, Ser33, Ser46 and Ser392 while ERK2 was shown to phosphorylate p53 at Thr55 in response to doxorubicin (She et al., 2000; Yeh et al., 2001). Phosphorylation of p53 at these residues was shown to correlate with increased transactivation by p53. While the immediate effects of Ser33 phosphorylation are unclear, one might speculate that phosphorylation of Thr55 may directly influence the transactivation activity of p53 as Thr55 is located directly adjacent to two residues that are critical for the function of activation subdomain 2; the mutation of Trp53/Phe54 to Gln/Ser (53/54QS) results in complete inactivation of ASD2 (Candau et al., 1997).

p53 Ser46 was also shown to be phosphorylated by homeodomain-interacting protein kinase 2 (HIPK2) in response to cisplatin treatment or UV irradiation (D'Orazi et al., 2002; Hofmann et al., 2002). HIPK2 was also found to associate with creb-binding protein (CBP) and was shown to be necessary for CBP-mediated acetylation of the p53 CTD. Given the clear effects of CTD

acetylation of p53 function (see below) the Ser46 phosphorylation should have a substantial effect on p53 function. Indeed, recent follow-up work has found that the inhibition of HIPK2 by RNAi resulted in decreased induction of p53-dependent apoptosis following cisplatin treatment (Di Stefano et al., 2004).

In contrast to the phosphorylation of N-terminal sites, which generally activate p53, the phosphorylation of p53 at Ser376 and Ser378 negatively regulates p53 activity. Phosphorylation of these sites in unstressed cells is constitutive and the advent of DNA damage (in the form of ionizing radiation) results in the dephosphorylation of p53 at these sites. The identity of the phosphatase(s) responsible for this dephosphorylation is unknown. The constitutive phosphorylation of Ser376 and 378 is mediated by protein kinase C (PKC) and stimulates the MDM2-mediated ubiquitylation of p53, resulting in increased p53 degradation (Chernov et al., 2001).

Finally, phosphorylation of p53 can affect its activity through changes in its subcellular localization. The phosphorylation of nuclear p53 at Ser315 or Ser376 by glycogen synthase kinase-3β (GSK3β) results in its relocalization to the cytoplasm and correlates with decreased p53-mediated apoptosis (Qu et al., 2004). Ser315 and Ser376 phosphorylation may interfere with the normal import of p53 as both residues are situated within nuclear localization sequences (NLS's). Aurora kinase A (AURKA) has also been shown to phosphorylate p53 at Ser315; however, in this case, the authors found that Ser315 phosphorylation resulted in the increased ubiquitylation and degradation of p53 rather than a relocalization.

A number of other kinases have also been reported to phosphorylate p53 at some of the same residues described above. These include CDK2 and CDC2 (Blaydes et al., 2001), TAF1 (Li et al., 2004), PKR (Cuddihy and Bristow, 2004), and FACT (Keller et al., 2001). The cop9 signallosome-associated kinase complex (CSN kinase) was reported to phosphorylate a cluster of three residues in the DBD (Ser149, Thr150, Thr155) resulting in increased 26S proteasome-mediated degradation of p53 (Bech-Otschir et al., 2001). Finally, c-jun N-terminal kinase (JNK) has a complicated role in regulating p53 protein levels that includes phosphorylating p53 at Ser20 and Thr81 in addition to regulating its levels through ubiquitylation (Buschmann et al., 2000; Fuchs et al., 1998).

Acetylation

The acetyltransferases p300/CBP and PCAF figure largely in the p53 pathway. In additional to acting as coactivators for p53-mediated transcriptional regulation by acetylating histone residues in target gene promoters (described above), they also can help to regulate p53 function and stability by directly acetylating p53 itself (Gu and Roeder, 1997; Lill et al., 1997). The acetylation of p53 in the C-terminus by p300/CBP helps to regulate the stability of the p53 protein by directly interfering with its ubiquitylation by MDM2. Of the six lysine residues at which p53 has been found to be ubiquitylated, Lys372, Lys373 and Lys382 have been each found to be acetylated by p300/CBP. Ubiquitylation and acetylation of the same residue are mutually exclusive.

In addition to these three residues, p53 may also be acetylated at Lys 305 by p300/CBP and at Lys320 by PCAF. The acetylation of these residues appears to augment the sequence-specific

binding of DNA by p53, thereby positively regulating its function (Luo et al., 2004; Wang et al., 2003). However, the mechanism by which this occurs is unclear. The acetylation status of p53 is also regulated by SIR2, a protein deacetylase (Vaziri et al., 2001). SIR2 has been shown to directly deacetylate p53 at Lys382 in an NAD-dependant manner and evidence suggests that other deacetylases also actively regulate p53 function.

Methylation

Finally, during the preparation of this thesis, p53 was reported to be methylated at Lys373 by Set9 methyltransferase (Chuikov et al., 2004). The effect of methylation of p53 appears to be somewhat similar to that of acetylation in that methylated p53 has increased stability and elevated transactivation activity. It is unclear by what mechanism this is achieved. However, it is striking that at a single residue, p53 can be ubiquitylated, neddylated, acetylated or methylated. The observation that a single amino acid lies at the intersection of four different upstream signaling pathways begs for further analysis, although effectively dissecting the distinct effects of each of these modifications will be technically challenging.

Downstream Effectors and p53 Function

The classic function of p53 is as a transcription factor that regulates target genes involved in cell cycle arrest and apoptosis following cellular stress. However, the complete story of p53 function is substantially broader. Expression analysis experiments using oligonucleotide or cDNA arrays to assay the responses of thousands of genes to p53 activation suggest that between 1-5% of the mammalian genome responds to p53 activation (Mirza et al., 2003; Wang et al., 2001; Zhao et al., 2000). Assuredly, a large portion of these genes are altered indirectly due to the effects of

p53 on cell proliferation and viability. Nonetheless, hundreds of different genes have been reported to harbor p53 response elements and to respond directly to p53 activity. Many of these genes participate in functional pathways distinct from those traditionally associated with p53 function, including cytoskeletal proteins, cell matrix and adhesion molecules, and growth factors and their inhibitors. Functional studies have also implicated p53 in maintaining genome stability by affecting DNA repair pathways and centrosome regulation. A comprehensive picture of p53 function is not yet available, but is slowly coming into focus.

Cell Cycle Arrest

p53 contributes to the regulation of cell cycle progression by initiating cell cycle arrest in response to DNA damage as well as monitoring the cell for aberrant proliferative signals. Following activation by cellular stresses, p53 rapidly induces both G_1 and G_2 arrest through several different pathways. The primary mediator of the DNA damage dependent G_1 arrest is the cyclin-dependent kinase inhibitor p21 (CIP1/WAF1). p21 is a generalized cyclin-dependent kinase inhibitor that is capable of targeting both the G_1-specific cyclin E/Cdk2 complexes as well as the G_2-specific cyclin B/Cdc2 complexes. p21 expression is strongly dependent on p53-mediated transactivation both under basal conditions and following DNA damage. The significance of this pathway is evident from the observations that both $p53^{-/-}$ and $p21^{-/-}$ mouse embryo fibroblasts fail to undergo a G_1 arrest following DNA damage (Brugarolas et al., 1995; Kastan et al., 1992). In contrast, untreated $p21^{-/-}$ MEFs proliferate at the same rate as wild-type MEFs while $p53^{-/-}$ MEFs proliferate for more rapidly. Therefore, p21 is not involved the p53-mediated surveillance of basal proliferation. Although there is a general understanding that p53

monitors proliferative signals via E2F-mediated transactivation of $p19^{ARF}$, the mechanism by which p53 restrains basal proliferation remains to be determined.

p53 also exerts some influence on the G_2 checkpoint, although there are also important p53-independent pathways that affect G_2 progression. In addition to p21, two prominent p53 target genes GADD45 and 14-3-3σ, have been implicated in control of the G_2/M checkpoint. Both GADD45 and 14-3-3σ cause the inhibition of cyclin B/cdc2 complexes. GADD45 directly binds to cyclin B resulting in its translocation from the nucleus while 14-3-3σ binds to and sequesters the cdc25c phosphatase, which is required for activation of cyclin B/cdc2 complexes (Chan et al., 1999; Jin et al., 2002; Yang et al., 2000).

Apoptosis

p53 is one of the primary regulators of the apoptotic response in many mammalian cells. This is particularly apparent in primary murine thymocytes which are fully protected from apoptosis induced by ionizing radiation in cells lacking p53 (Lowe et al., 1993b). p53 regulates apoptosis by transcriptionally regulating a number of different apoptotic genes including important mediators of both the intrinsic and extrinsic apoptotic pathways. p53 may also directly participate in the intrinsic apoptotic pathway in a transcription-independent manner.

p53 can directly transactivate three different cell surface receptors involved in apoptosis. The death-domain containing receptor DR5/KILLER contains a p53 response element and is activated by p53 in response to DNA damage (Burns et al., 2001). Similarly, FAS, a member of the TNF receptor family, is also a p53 target gene. The third receptor, PERP, is a tetraspan

transmembrane receptor related to PMP-22. Although PERP is required for p53-mediated apoptosis in several different cell types, the mechanism by which PERP functions in apoptosis is unclear (Attardi et al., 2000; Ihrie et al., 2003).

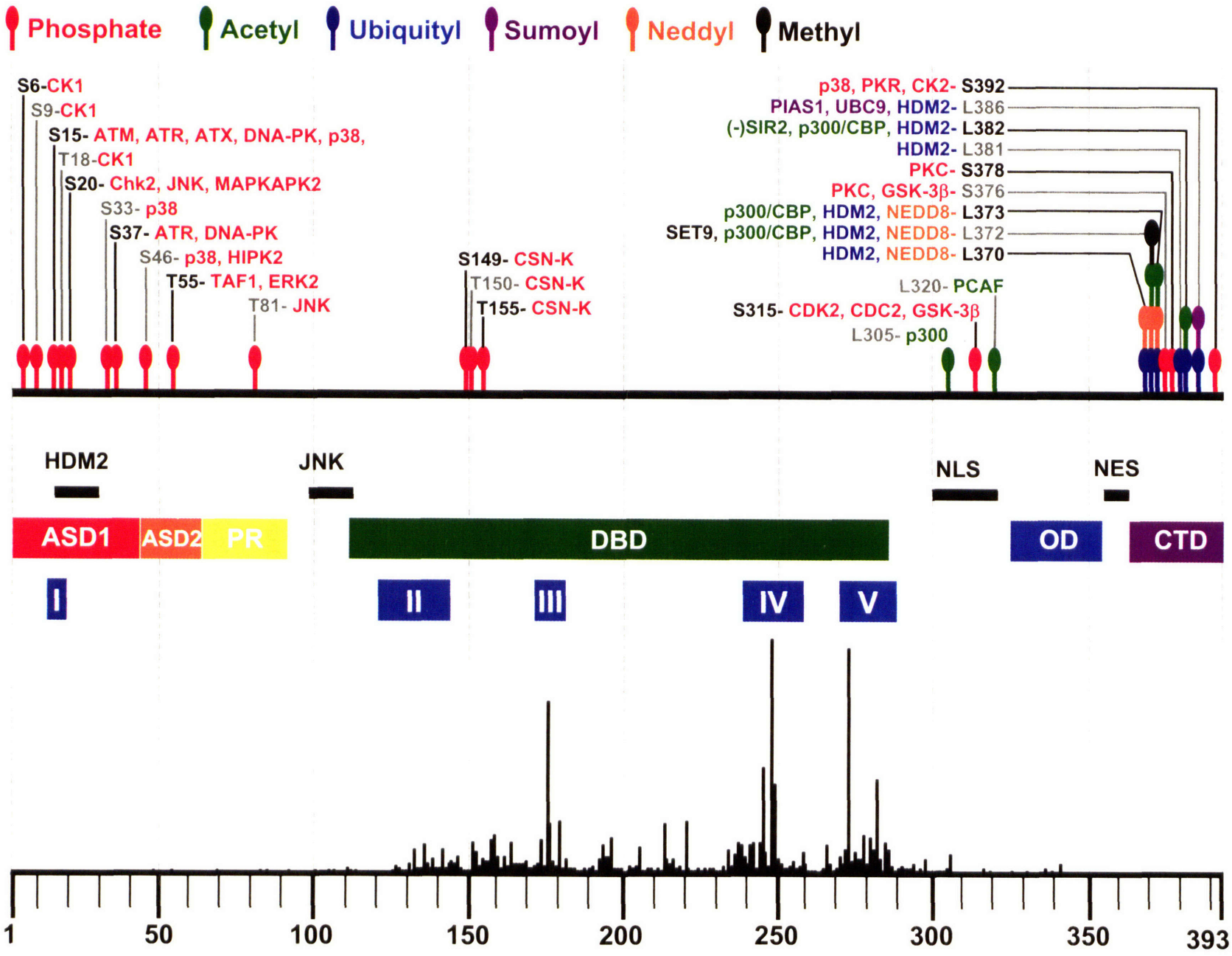
p53 also directly activates several genes involved in the intrinsic apoptotic pathway including Bax (Thornborrow et al., 2002), Bid (Sax et al., 2002), Puma (Nakano and Vousden, 2001), and Noxa (Oda et al., 2000). Each of these proteins localizes to and promotes the permeabilization of the outer mitochondrial membrane, resulting in the release of cytochrome c and Apaf 1 from the mitochondria. p53 can also repress the activation of Bcl-2, an inhibitor of this process (Budhram-Mahadeo et al., 1999). Once released from the mitochondria, cytochrome c binds to APAF1, which is itself a p53 target gene, and causes the activation of two initiator caspases (caspase-9 and caspase-3), followed by downstream activation of the effecter caspases, caspase-6 and caspase-7 (Kannan et al., 2001; Moroni et al., 2001; Robles et al., 2001). Caspase-6 is also a transcriptional target of p53.

Finally, recent work has found that p53 itself participates in the intrinsic apoptotic pathway by translocating from the nucleus to the outer mitochondrial membrane (Chipuk et al., 2003). Although controversial at first, the work of several different groups has begun to establish this activity of p53. For example, γ-irradiation of mice was found to induce a rapid wave of p53-dependent apoptosis that preceded the transactivation of target genes by p53 while the translocation of p53 to mitochondria occurs as early as 30 minutes after irradiation (Erster et al., 2004). Careful cell fractionation experiments have recently established the presence of an endogenous complex in the mitochondria containing p53, Bcl-2 and Bcl-X_L and gold-tagged

antibodies were used to observe p53 localization to the outer mitochondrial membrane by electron microscopy (Ute Moll, unpublished results presented at the 12th International p53 Workshop, Dunedin, New Zealand). Furthermore, p53 can directly induce Bak oligomerization following cell stress, an important step in the pathway leading to cytochrome c release (Leu et al., 2004). Importantly, tumor-associated mutants of p53 are defective in these activities.

In a related story, human p53 contains a polymorphism between arginine and proline at codon 72. The frequency of this polymorphism varies by latitude, with the proline allele being substantially more common in equatorial populations than in those found at higher latitudes (Beckman et al., 1994). The arginine allele has an up to 15-fold greater ability to induce apoptosis compared to the proline allele despite the fact that there is no difference in the ability of the two variants to bind to DNA and transactivate target genes (Dumont et al., 2003; Thomas et al., 1999). Interestingly, the Arg72 variant is translocated from the nucleus to mitochondria more readily than the Pro72 variant. Bak oligomerization is also increased in cells with the Arg72 variant (Maureen Murphy, unpublished results presented at the 12th International p53 Workshop, Dunedin, New Zealand). These data strongly suggest that the effect of the codon 72 polymorphism of p53 is to fine-tune the induction of apoptosis by altering the fraction of p53 protein at the outer mitochondrial membrane.

Figure 2- Mutations, modifications and functional domains of the p53 tumor suppressor gene. The frequency of p53 mutations in human tumors by amino acid is depicted at bottom with black bars. Above this, the positions of evolutionarily conserved regions in the p53 protein are shown in blue boxes. In the middle, the functional domains are shown in colored boxes: ASD, activation subdomains; PR, proline-rich region; DBD; sequence-specific DNA binding domain; OD, oligomerization domain; RD, regulatory domain. Above this, black bars indicate the sites of binding by HDM2 and JNK as well as the positions of nuclear export and nuclear localization signals. The diagram at top demonstrates the positions of various post-translational modifications of p53 as well as the proteins that regulate the modifications.


Phosphate
Acetyl
Ubiquityl
Sumoyl
Neddyl
Methyl
S6-CK1
S9-CK1
S15- ATM, ATR, ATX, DNA-PK, p38,
T18-CK1
S20- Chk2, JNK, MAPKAPK2
S33- p38
S37- ATR, DNA-PK
S46- p38, HIPK2
T55- TAF1, ERK2
T81- JNK
S149- CSN-K
T150- CSN-K
T155- CSN-K
p38, PKR, CK2- S392
PIAS1, UBC9, HDM2- L386
(-)SIR2, p300/CBP, HDM2- L382
HDM2- L381
PKC- S378
PKC, GSK-3β- S376
p300/CBP, HDM2, NEDD8- L373
SET9, p300/CBP, HDM2, NEDD8- L372
HDM2, NEDD8- L370
L320- PCAF
S315- CDK2, CDC2, GSK-3β
L305- p300
HDM2
JNK
NLS
NES
ASD1
ASD2
PR
DBD
OD
CTD
I
II
III
IV
V
1
50
100
150
200
250
300
350
393

p53 FAMILY-MEMBERS

In 1997, two genes with substantial homology to p53 were discovered. Called p63 and p73, they share the general structure and function of the primary p53 functional domains and are particularly well conserved in the region corresponding to the p53 DBD. Both p63 and p73 also contain an extreme C-terminal region with an SAM protein-protein interaction domain. They are capable of binding to p53 response elements in DNA and transactivating p53 target genes in response to DNA damage. Yet for all of these similarities, p63 and p73 were not found to be mutated in human tumors as frequently as p53.

Both p63 and p73 encode multiple variants due to the use of alternative promoters and alternative splicing. In particular, certain variants lack a large region of their N-terminus including most of the transactivation domains of these proteins. These ΔN-p63 and ΔNA p73 variants may act as dominant negative variants by hetero-oligomerizing with the full-length TAp63 and TAp73 variants. *In vitro* experiments suggest that p63 and p73 polypeptides can weakly hetero-oligomerize with one another but not with p53.

Following the reports of multiple p63 and p73 variants, speculation arose concerning whether p53 might also express multiple variants. Shockingly, an analysis of p53 transcripts recently found that p53 also has a number of different variants, including three different N-terminal variants and three different C-terminal alternative splice variants (Jean-Christophe Bourdon, 12th International p53 Workshop, Dunedin, New Zealand). Many of the other variants are poorly recognized by the common p53 antibodies because in the earliest days of the p53 research, antibodies that produced multiple bands for p53 were regarded as low-quality. It is embarrassing

that the p53 field has pretty much ignored the scattered reports of p53 alternative splice variants over the years despite the presence of extra bands appearing on our gels the whole time (Arai et al., 1986; Han and Kulesz-Martin, 1992; Matlashewski et al., 1987; Wu et al., 1994).

Knockout mice have been constructed for both p63 and p73. p63 null mice die perinatally from desiccation resulting from a developmental defect in their skin. They also have craniofacial abnormalities and limb truncations. p73 null mice are viable at birth, but are susceptible to infections making it difficult to raise them through adulthood. They also have hippocampal dysgenesis and defects in the vomeronasal organs. Furthermore, the initial publications describing $p63^{+/-}$, $p73^{+/-}$ or $p73^{-/-}$ mice reported that they do not develop cancer. These observations threw into doubt the status of p63 and p73 as tumor suppressor genes.

A collection of related familial disorders was recently found to result from the germline mutation of p63. The symptoms of these disorders bear a striking resemblance to those of the p63 knockout mouse. These include ectodermal dysplasia-cleft lip/palate (EEC) syndrome (Celli et al., 1999), limb-mammary syndrome (LMS), Hay-Wells syndrome (McGrath et al., 2001), acro-dermato-ungual-lacrimal-tooth (ADULT) syndrome (Chan et al., 2004), Rapp-Hodgkin syndrome (RHS)(Kantaputra et al., 2003) and split-hand/split-foot malformation (SHFM)(Ianakiev et al., 2000). Although these syndromes are not generally reported to be associated with a cancer predisposition, they are so extremely rare that an association may not have been noted. There are several individual case reports of patients with these disorders developing tumors, including malignant lymphoma, malignant melanoma, bilateral nephroblastoma, esophageal carcinoma, non-Hodgkin's lymphoma and perioral papillomatosis

(Akahoshi et al., 2003; Drut et al., 2002; Kaelmann and Ruprecht, 1997; Ogutcen-Toller et al., 2000). No familial syndrome has yet been associated with mutations in p73.

Furthermore, alterations in p63 and p73 in spontaneous tumors have begun to be reported more widely in the literature. For example, overexpression of ΔNp63 (see below) is a relatively frequent event in squamous cell carcinomas while ΔNp73 overexpression correlates with poor prognosis in lung cancer (Choi et al., 2002; Uramoto et al., 2004). Also, a more thorough analysis of p73 knockout mice revealed an elevated tumor incidence compared to wild type and compound heterozygous mutation of any two members of the p53 family in mice resulted in a substantial increase in tumorigenesis compared to the single mutants (Elsa Flores, submitted to Cancer Cell). Taken together, these data support classification of p63 and p73 as tumor suppressor genes.

MOUSE MODELS OF P53

Over a dozen different alleles of p53 have been engineered into mice in the past fifteen years, leading to dramatic progress in the understanding of both mutant and wild-type p53 function. Many of the principles described above were derived from studies genetically engineered strains of mice or cells derived from them. The various p53 mouse strains can be classified into four general groups: transgenic strains, knockout/knockdown strains, reporter strains and those with non-tumor-associated mutations.

Transgenic Mice

The first two genetically engineered p53 mouse strains carried genomic fragments of p53 derived either from a mouse liver tumor ($p53^{A135V}$) or from the Friend cell line CB7 ($p53^{R193P}$, although the presence of this mutation in CB7 cells was not discovered until a year later)(Lavigueur et al., 1989; Munroe et al., 1990). At the time these mice were described, p53 was thought to be a cellular oncogene rather than a tumor suppressor gene. The fact that these mice that overexpressed various fragments of p53 developed tumors actually supported this notion. It was not until about a year later, when wild-type p53 was found suppress transformation, that it was realized that the mutations present in these mice and in many tumors and cell lines were actually dominantly interfering with the function of wild-type p53. In 1995, the $p53^{A135V}$ mouse was crossed onto $p53^{+/-}$ and $p53^{-/-}$ backgrounds (see below) to address the possibility of dominant-negative or gain-of-function effects by mutant p53 (Harvey et al., 1995). In this case, it was found that mutant p53 accelerated tumorigenesis and changed the tumor spectrum of $p53^{+/-}$ mice, but did not affect the phenotype of p53 knockout mice. This was interpreted as supporting the dominant-negative hypothesis and refuting the p53 gain-of-function hypothesis.

However, it is difficult to accept these interpretations. The mutant p53 transgene in these mice was expressed at extraordinarily high levels. Northern analysis of p53 levels in the tissues of these animals found approximately 40- to 50-fold more p53 mRNA in the transgenic mice than in wild-type mice. Furthermore, p53 protein levels were elevated even in normal tissues. In contrast, Li-Fraumeni Syndrome patients show no accumulation of p53 protein in their normal tissues. Therefore, rather than acting as a dominant-negative allele, the mutant p53 protein might be interpreted as simply overwhelming the small fraction of p53 protein that is wild-type in these animals. The chance of there being any fully wild-type p53 tetramers in the cells of these mice is very low.

The lack of an effect by the mutant p53 transgene on the p53 null mouse phenotype does not disprove the mutant p53 gain-of-function hypothesis. Absence of evidence is not evidence of absence. The rapid development of lymphomas and sarcomas in p53 null mice could mask a gain-of-function effect on the development of other tumor types. Furthermore, the $p53^{A135V}$ mutation is not a particularly potent mutation. Indeed, it has been shown to be a temperature sensitive allele and a subsequent mouse model targeting expression of $p53^{A135V}$ to the kidney found that it actually protected the animal from an activated K-ras transgene targeted to the same tissue (Schaffner et al., 1996). Both of these caveats were noted by the authors in the 1995 paper. Nonetheless, this work fostered a general suspicion within the p53 field of the mutant p53 gain-of-function hypothesis.

In addition to the kidney-specific transgene described above, mutant p53 transgenes have also been targeted to the mammary epithelium using the whey acidic protein promoter (WAP-$p53^{R172H}$), the epidermis using the human keratin 1 promoter (HK1-$p53^{R172H}$) and the lung using the surfactant protein C promoter (SPC-$p53^{R270H}$)(Duan et al., 2002; Li et al., 1998; Murphy and Rosen, 2000; Takahashi et al., 1989; Wang et al., 1998a). Due to the non-physiological levels of protein expression (and in one case, the lack of even remotely relevant controls), these strains are primarily useful as models of particular tumor types rather than for investigating the functional properties of mutant p53.

The final transgenic mouse strain harbors extra copies of wild-type p53 in the form of large genomic transgenes (Garcia-Cao et al., 2002). These "super p53" mice were resistant to two types of carcinogen induced cancers and had enhanced cellular responses to DNA damage. A small number of mice had been aged for up to two years with no dramatic loss of viability or other phenotypes. However, a large-scale aging study has not yet been reported for these mice.

p53 Knockout Mice and Knockdown Mice

p53 knockout mice have been generated by several different groups, each with similar phenotypes (Donehower et al., 1992; Jacks et al., 1994; Purdie et al., 1994). $p53^{-/-}$ mice are born at slightly sub-Mendelian ratios due the occurrence of female-specific exencephaly in a subset of p53 null embryos (Sah et al., 1995). p53 null mice that survive to birth develop normally but develop tumors with a mean latency of roughly 4.5 months. In most studies, ~95% of p53 null mice succumb to cancer by six months of age. p53 knockout mice develop primarily lymphomas and sarcomas, although there is some influence of strain background (Harvey et al., 1993b).

$p53^{+/-}$ mice survive an average of ~18 months and primarily develop sarcomas and lymphomas although their tumor spectrum is broader than that of $p53^{-/-}$ mice. Furthermore, when crossed onto the BALB/c genetic background, 100% of $p53^{+/-}$ female mice developed either mammary adenocarcinomas (55%) or mammary hyperplasias (45%), suggesting that a powerful genetic modifier may be present in the BALB/c strain (Kuperwasser et al., 2000). Analysis of $p53^{+/-}$ mice also found that the wild-type p53 allele is frequently, but not inevitably, inactivated in tumors. In fact, tumors that arise in $p53^{+/-}$ mice that survive past 18 months usually retain wild-type p53 function, arguing that a reduced gene dosage of p53 is sufficient to sensitize mice to tumorigenesis (Venkatachalam et al., 1998).

Finally, an epi-allelic series of lentiviral shRNA expressing mice was described in which different p53-directed siRNAs were used to generate mice with intermediate levels of p53 expression (Hemann et al., 2003). Strikingly, the different strains of mice (crossed with the Eμ-Myc transgenic strain) developed a range of tumor phenotypes that directly correlated with the expression levels of the p53 protein. The use of such mice allows for a careful assessment of the effects of different levels of p53 on *in vivo* tumorigenic processes.

Reporter Mice

Two strains of mice have been developed to facilitate p53 mouse research. The first features a targeted allele of p53 that has been tagged with a coat color marker (Zheng et al., 2002). This allows for visual distinction of wild-type, heterozygous and homozygous knockout mice rather

than using PCR analysis. The second strain harbors a p53-responsive lacZ transgene. This strain is useful for analyzing p53 transactivation activity by histology (Komarova et al., 1997).

Non-Tumor-Associated Targeted Mutations

Four strains of mice have been generated with targeted mutations for the purpose of exploring specific biochemical properties of p53. In the first of these strains, exons 4-9 of the murine p53 gene were replaced with exons 4-9 of the human p53 gene. These human p53 knock-in (hupki) mice were generated because mice and humans do not develop identical mutational spectra in p53 following exposure to carcinogens. Indeed, when small sections of the skin of homozygous hupki mice were chronically exposed with UVB radiation, they developed a spectrum of mutations in p53 that matched that seen in humans rather than in mice. These differences can be attributed to the lack of transcribed-strand dipyrimidines at some the sites where they are found in human p53 (Luo et al., 2001a; Luo et al., 2001b).

The other three targeted strains harbor single base-pair mutations in the p53 coding sequence. These include the phosphorylation site mutants $p53^{S23A}$ (analogous the human $p53^{S20A}$), $p53^{S389A}$ (analogous to human $p53^{S392A}$) and the apoptosis-defective $p53^{R172P}$ (analogous human $p53^{R175P}$). $p53^{S23A/S23A}$ mice have a mean survival of ~15 months and develop primarily B-cell lineage tumors rather than the T-cell lineage lymphomas commonly found in p53 knockout mice (MacPherson et al., 2004). Primary cells and tissues from these mice exhibit partial defects in p53 mediated apoptosis but appear to have an intact cell cycle arrest in response to ionizing radiation. This is similar to the phenotype of $p53^{R172P/R172P}$ mice, which are also fully defective for apoptosis induced by ionizing radiation but retain a partial ability to induce cell cycle arrest

(Liu et al., 2004). In contrast, $p53^{S389A/S389A}$ primary cells have an intact ionizing radiation response but are partially compromised in their response to UV irradiation. $p53^{S389A/S389A}$ also have an increased sensitivity to UV-induced skin tumor development (Bruins et al., 2004).

Other p53 Mouse Strains

Finally, three lines of mice have been described that harbor p53 alleles with unintended mutations. The first strain was reported as a transactivation-deficient mouse model of p53. The double amino acid mutation substitution ($p53^{L25Q/W26S}$) has been shown to abrogate the function of the transactivation subdomain 1 of p53. This mutation was targeted into murine embryonic stem cells and reported to cause constitutive binding of p53 to DNA (Jimenez et al., 2000). However, at a recent meeting the primary investigator of this work disclosed that his laboratory had discovered an extraneous mutation in the p53 coding sequence (12th International p53 Workshop, Dunedin, New Zealand). At the same meeting, another investigator reported unpublished data on an independently targeted mouse with the $p53^{L25Q/W26S}$ mutation (Laura Attardi, 12th International p53 Workshop, Dunedin, New Zealand). In this case, the mutant p53 protein was found to be both stabilized, due to a disruption of its interaction with MDM2, and compromised in its ability to activate many target genes. The effect on p53 transactivation was selective as some p53 target genes were found to be regulated in a manner similar to wild-type mice. Further work on this and other transactivation mutant mice should provide insight into the complex differential regulation of p53 target genes.

The second strain of mis-targeted mice harbors a >20 kilobase deletion 5' to the p53 gene and extending through exon 6 of the p53 coding sequence. The deleted region includes other gene

loci. The rearrangement produced a transcript with a 55bp leader of unknown origin fused to the 3' end of the p53 gene with the amino acid that would normally be Met243 serving as the translational start site. In addition, the mutant allele also contains a $p53^{R245W}$ mutation that was engineered in the initial targeting construct. Analysis of mice heterozygous for this allele found that although they survived longer than $p53^{+/-}$ mice, the $p53^{+/m}$ mice developed far fewer tumors (6% versus >80% for $p53^{+/m}$ and $p53^{+/-}$ mice respectively). The precise cause of death of the mice was not determined but the authors presented evidence that the mice suffered from a premature aging syndrome. They argued that the C-terminal fragment of p53 that was expressed in these mice activates the function of the wild-type p53 allele similar to the p53 C-terminal peptides that have been described in cell culture assays (Hupp et al., 1995). The authors put forth the hypothesis that tumor suppression is a balancing act between preventing cancer and inflicting organismal aging. This hypothesis seems to be counteracted by the phenotype of the super-p53 mice, which did not show signs of premature aging.

The final mouse model of p53 harbors a targeted tumor-associated mutation but also has an extraneous splice acceptor mutation that arose inadvertently during cloning. Mice heterozygous for this $p53^{R172H\Delta g}$ allele, developed an increased incidence of carcinomas compared to $p53^{+/-}$ mice and had a very high rate of metastases from carcinomas and osteosarcomas. However, interpreting these results is difficult because the mutant p53 protein is expressed only at very low levels (Liu et al., 2000).

Summary- Mouse Models of p53

The tools described above have, are and will continue to provide invaluable tools for the study of p53 function and dysfunction. Future models, including, I hope, those described in this thesis will further contribute to this effort. One tool that would be useful would be a targeted p53 fusion with a purification tag (e.g. FLAG) for use in biochemical studies on endogenous proteins. Similar tagged versions of other endogenous proteins could facilitate characterization of protein-protein interactions under endogenous conditions.

On another note, the occurrence of three independent lines of mice with unexpected mutations in p53 is disconcerting. Two of the three rearranged alleles were attempts to create tumor-associated mutant p53 mice using standard gene targeting techniques. This prompts me to relate an anecdote from our own initial efforts to target mutant p53 into mice. Prior to my arrival in the Jacks Lab, Annemieke deVries generated ES cells with the targeted tumor-associated mutation $p53^{R270H}$. These ES cells were injected into blastocyts and produced high contribution chimeras. However, in repeated attempts, these chimeras never transmitted the mutant allele. The effort to derive mice from these ES cells was eventually abandoned.

When I joined the Jacks Lab in 1999, it was our opinion that mutant p53 was associated either with a developmental defect or a spermatogenic defect in mice that was not found in human Li-Fraumeni Syndrome patients. The conditional mutant p53 mice designed by David Tuveson and described in Chapters 2 and 3 of this work were engineered to circumvent this potential defect. However, my initial project in the lab was to determine *whether* such a defect occurred in mice. To this end, I activated the conditional $p53^{R270H}$ allele in Dave's ES cells to recapitulate the

experiment that Annemieke performed using targeted ES cells that had already been shown to be competent for germline transmission. Once again, we failed to achieve germline transmission with high contribution chimeras. The mystery is even greater now that we know that when the recombination occurs *in vivo* through the action of a germline-specific Cre, the mutant p53 allele is inherited as readily as a null p53 allele. The implication is extremely odd. The only difference between a chimeric animal and a conditional mutant p53 mouse with a germline-specific Cre is that the chimeras have wild-type cells in direct contact with mutant p53-bearing cells during development. It is as if the wild-type cells prevent the mutant cells from contributing to the germ cell compartment.

Around the same time that I made this observation, my cross of the conditional germline mutant p53 mice to the germline-Cre transgenic mice unexpectedly yielded the first germline mutant p53 mice. I never did return to the question of why directly targeted mutant p53 mice don't achieve germline transmission. The experiment that I would propose to anyone interested in this phenomenon is simple: derive ES cells and blastocysts from both wild-type and germline mutant p53 mice and combine them pair-wise in each of the four possible combinations. If the homotypic combinations successfully achieve germline transmission and the heterotypic combinations do not, there is an interesting story at hand that would be worth pursuing.

Approaches to Therapeutic Intervention

From the standpoint of therapeutic potential, oncogenes have always been viewed more favorably than tumor suppressor genes. It stands to reason that it should be easier to find a therapeutic to inhibit the function of an extant protein than to replace the function of a protein

that has been deleted. However, unlike other tumors suppressor genes, p53 is generally altered through mutation rather than deletion. This has lead to a great deal of interest in molecules that might "reactivate" the normal tumor-suppressive functions of mutated p53. Several compounds have been reported to be capable of reactivating p53, although it is far from clear whether they truly accomplish this feat. For example, a compound called PRIMA1 was reported to restore function to mutant p53 proteins (Bykov et al., 2002a; Bykov et al., 2002b). However, in a small series of unpublished experiments performed in collaboration with Andrea Venture in our laboratory, PRIMA1 had no effect on the accumulation of p53 protein, cell cycle progression or transactivation of p53 target genes in $p53^{M/-}$ MEFs. Therefore, it is therefore unclear whether PRIMA1 actually targets mutant p53. Other drugs have been reported to reactivate mutant p53 but require impractically high cellular concentrations for biological activity.

One caveat to the search for a mutant p53 drug is that different mutations in p53 have different effects on the function of the protein. A drug that shifts the conformational equilibrium of structural mutant p53 proteins back to a wild-type conformation may not be effective against a contact mutant p53 protein. Contact mutants often do not assume a "mutant" conformation. Their defect lies in their inability to make important direct contacts with the DNA backbone, an activity that may be difficult to complement *in trans* with a drug. This suggests that mutant p53 reactivating therapies may need to be combined with diagnostic screening techniques in order to match the specific function of the drug with the particular mutation present in a tumor. In a large genomically unstable tumor, this may simply select for mutations of a different class to emerge, similar to the way CML mutates other residues of the BCR-ABL fusion protein to evade the effects of Gleevec. Nonetheless, even a transient reprieve from tumor progression is an

improvement over none and so the search for p53 reactivating drugs remains a promising avenue of research.

For those tumors harboring wild-type p53, several therapeutic approaches have recently been described for activating p53 function in the absence of DNA damage. A transducible p53 peptide derived from the CTD and composed of a D-isomer polypeptide fused to HIV-TAT was found to potently activate p53 activity and extend lifespan in a tumor xenograft model (Snyder et al., 2004). In separate work, a class of small molecule inhibitors of the p53/MDM2 interaction called nutlins was found to potently activate p53 function in tumor cells but to have little or no effect on normal cells (Snyder et al., 2004). Nutlins or related compounds may be extraordinarily useful in treating those tumors that respond well to standard genotoxic agents in a p53-dependent manner. The substitution of nutlins for classical chemotherapeutics may spare patients some of the substantial morbidity associated with genotoxic treatments.

Finally, there may be an important role for the stratification of patient populations based on p53 mutation status. For some types of tumors, p53 mutations are known to impact the efficacy of chemotherapeutic regimens (Lowe et al., 1993a; Peller, 1998). In this case, the rapid determination of p53 mutation status could save patients from being treated with ineffectual drugs. To this end, a DNA oligonucleotide array protocol was developed that can detect p53 mutations with extremely high sensitivity (Fouquet et al., 2004). For samples contaminated with high proportions of normal stromal tissue, this array protocol outperformed direct sequencing protocols in detecting rare p53 mutant cells. Such an approach may have an important place in the clinic although cost of implementation may preclude this particular strategy.

The search for therapeutic approaches targeted to the p53 pathway is somewhat hampered by a lack of understanding of the basic functions of the common tumor-associated mutant alleles of p53. Thousands of clinical publications have examined p53 protein expression in different types of tumors in order to discern a correlation with prognosis or outcome. However the molecular determinants and consequences of mutant p53 overexpression is poorly understood, as are the implications of a lack of mutant p53 overexpression. Furthermore, the different clinical implications of specific mutations have rarely been explored. A more complete understanding of the functional effects of mutant p53 may allow for the development of a panel of drugs targeted to different potential alterations in the p53 pathway. Such a panel of drugs would have broad applications in treating a very large variety of different human tumors.

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Chapter 2

Mutant *p53* Gain-of-Function in Two Mouse Models of Li-Fraumeni Syndrome

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Construction of the conditional point-mutant p53 mice was performed by David A. Tuveson and Nicholas A. Willis. All subsequent experiments were performed by the author, either personally or with the assistance of two talented MIT undergraduates, Zachary C. Ruhe and Bob Yin. Histology preparation was by Denise Crowley of the MIT Center for Cancer Research Histology Core Facility and analysis of pathology was performed with the assistance of Roderick T. Bronson.

ABSTRACT

The *p53* tumor suppressor gene is commonly altered in human tumors, predominantly through missense mutations that result in accumulation of mutant p53 protein. These mutations may confer dominant-negative or gain-of-function properties to *p53*. To ascertain the physiological effects of *p53* point-mutation, the structural mutant $p53^{R172H}$ and the contact mutant $p53^{R270H}$ (codons 175 and 273 in humans) were engineered into the endogenous *p53* locus in mice. $p53^{R270H/+}$ and $p53^{R172H/+}$ mice are models of Li-Fraumeni Syndrome; they developed allele-specific tumor spectra distinct from $p53^{+/-}$ mice. In addition, $p53^{R270H/-}$ and $p53^{R172H/-}$ mice developed novel tumors compared to $p53^{-/-}$ mice, including a variety of carcinomas and more frequent endothelial tumors. Dominant effects that varied by allele and function were observed in primary cells derived from $p53^{R270H/+}$ and $p53^{R172H/+}$ mice. These results demonstrate that point-mutant *p53* alleles expressed under physiological control have enhanced oncogenic potential beyond the simple loss of *p53* function.

INTRODUCTION

The *p53* tumor suppressor gene encodes a transcription factor that responds to cellular stresses by regulating genes involved in cell cycle arrest, apoptosis, genome maintenance and other pathways (Chang and Kornberg, 2000; Lu and Levine, 1995; Seto et al., 1992; Thut et al., 1995; Xiao et al., 1994). The p53 protein contains a core sequence-specific DNA-binding domain (DBD) that directly binds to sequence elements in target gene promoters (Cho et al., 1994; Pavletich et al., 1993; Zambetti et al., 1992). Missense mutations in the *p53* DBD occur in a large fraction of spontaneous human tumors and have been documented in over 50 different types of cancer. Large deletions, though commonly seen in other tumor suppressor genes, occur rarely in *p53* (Olivier et al., 2002).

The detection of specific p53 mutations in human tumor samples has been reported in nearly 2000 publications. In order to aid in their analysis, two separate p53 mutation databases have been constructed that catalogue data on each of reported mutation: the Universal Mutation Database (UMD) and the International Agency for Research on Cancer p53 mutation database (IARC) (Beroud and Soussi, 2003; Olivier et al., 2002). These two databases are invaluable resources for discerning trends in the p53 mutational spectra of different tumors, although there are some caveats that must be taken into account. For instance, because many investigators limit their sequencing efforts to exons 5-9 of p53, the databases tend to be biased towards mutations in the p53 DBD. Mutations outside the DBD are rare, but they do occur. Also, the samples in many experiments are pre-screened by immunostaining or other techniques, perhaps leading to a bias against mutant p53 alleles that do not induce p53 protein accumulation. Nonetheless, the epidemiology of p53 mutations in sporadic tumors reveals some prominent trends. Certain

tumor types have high frequencies of p53 mutations. These include cancers of the esophagus, ovary, colorectum, head/neck, pancreas, lung and skin. Other tumors, such as those arising in soft tissues, bone, hematological lineages, prostate, kidney, testis and thyroid, have relatively low frequencies of p53 mutation. It is interesting to note that all of the tumors with high frequencies of p53 mutation are of epithelial origin while many of those with a low prevalence of mutations are of mesenchymal or germ-cell origin. Another lesson gleaned from p53 epidemiology is that different p53 mutations occur at varying frequencies in different types of cancers. For example, mutations at codon 273 occur in nearly 16% of brain tumors compared to only 6% for codon 175. This may imply that different mutant in p53 confer subtly different functions. Finally, *p53* mutations have been linked to poor prognosis in a variety of different tumor types and have been shown to affect cellular responses to chemotherapeutics (Cho et al., 1994; Levine, 1997; Milner et al., 1991; Peller, 1998).

At a biochemical level, tumor-associated point-mutations in *p53* generally fall into two classes: 'contact mutations' affect p53 residues that make direct contact with DNA, while 'structural mutations' affect the global structure of the p53 DNA binding domain. Both classes yield mutant p53 proteins that have lost wild-type function and that frequently accumulate to high levels in tumor cells. However, structural mutant proteins assume a globally denatured conformation that exposes novel "mutant-specific" epitopes in the DBD; contact mutant proteins are less likely to assume the mutant-specific conformation.

In addition to sporadic tumors, *p53* point-mutations also occur in the germline of patients with Li-Fraumeni syndrome (LFS), a familial cancer predisposition syndrome in which patients develop a broad spectrum of malignancies including breast carcinomas, soft tissue and bone sarcomas, brain tumors, leukemias, gastrointestinal carcinomas and a variety of additional epithelial cancers (Birch et al., 1994; Kleihues et al., 1997; Varley et al., 1997). The mutations that occur in LFS patients are pretty much the same as those that occur in spontaneous human tumors, with some subtle differences in overall frequency. Few connections have been made between geneotype and phenotype in LFS patients. One exception is the R337H allele which is primarily associated with adrenocortical carcinoma. An analysis of 36 cases of childhood adernocortical cancer in Brazil found that 35/36 patients harbored a germline R337H mutation in p53 (Ribeiro et al., 2001). Codon 337 lies within the oligomerization domain of p53 and its mutation to histidine causes the protein to become extremely pH sensitive and lose the ability to tetramerize at elevated pH. Cells in the adrenal cortex encounter elevated pH in the course of normal developmental processes. Therefore, the R337H mutation may inactivate p53 function specifically in the adrenal cortex (DiGiammarino et al., 2002).

Two prominent models have been proposed to explain the effects of *p53* missense mutation in cancer. The first suggests that hetero-oligomerization between mutant and wild-type p53 polypeptides results in dominant-negative effects on wild-type *p53* function. Considerable biochemical and cell-based data support the ability of different point-mutant alleles to inhibit wild-type *p53* function, but the effects can be complex (Kern et al., 1992). For example, some alleles of mutant p53 can inhibit the ability of wild-type p53 to transactivate target genes involved in apoptosis but not those involved in cell cycle arrest (Aurelio et al., 2000).

Furthermore, the extent to which tumor-derived mutations in *p53* confer dominant-negative effects under physiological conditions is unclear.

The second model of mutant p53 function suggests that *p53* mutations actively promote tumorigenesis independent of wild-type *p53* function. This "gain-of-function" model is supported by several studies in which *p53* null cell lines were transformed with mutant *p53* constructs, resulting in increased tumorigenic potential (Dittmer et al., 1993; Hsiao et al., 1994; Pohl et al., 1988; Shaulsky et al., 1991). In addition, many studies have ascribed novel biochemical properties to mutant p53 that are distinct from the function of wild-type p53. These include the ability to regulate distinct sets of target genes and the ability to participate in novel protein-protein interactions (Cadwell and Zambetti, 2001; Chin et al., 1992; Di Como et al., 1999; Frazier et al., 1998; Gaiddon et al., 2001; Lanyi et al., 1998; Marin et al., 2000; Yang et al., 1999). Very little is known about the relevance of these observations *in vivo*.

Substantial efforts have been made to model LFS in mice. We and others engineered mice lacking expression of the p53 protein. *p53* knockout mice are highly cancer-prone and the study of these mice has yielded significant insight into the cellular and biochemical functions of wild-type *p53*. However, in contrast to the broad spectrum of tumors in LFS patients, the tumor spectrum of *p53* knockout mice is fairly restricted; both heterozygous and homozygous knockout mice develop primarily sarcomas and lymphomas. Carcinomas in these mice are rare, particularly in *p53* null mice (Donehower et al., 1992; Jacks et al., 1994; Purdie et al., 1994). At least four groups have produced transgenic strains that overexpress mutant *p53* from exogenous promoters (Harvey et al., 1995; Lavigueur et al., 1989; Li et al., 1998; Wang et al., 1998b). In

each case, mutant *p53* transgene expression resulted in accelerated spontaneous or carcinogen-induced tumor development, supporting a dominant effect for mutant *p53* that is distinct from simple loss-of-function. For example, a $p53^{135V}$ transgene conferred accelerated tumorigenesis and an altered tumor spectrum on $p53^{+/+}$ and $p53^{+/-}$ mice (Harvey et al., 1995). Similarly, Liu et. al. found an increased incidence of carcinomas and metastatic osteosarcomas, compared to $p53^{+/-}$ mice, in mice heterozygous for an endogenous mutant allele of *p53* ($p53^{R172H\Delta g}$); however, analysis of these mice was confounded by the presence of an extraneous splice acceptor mutation (Liu et al., 2000). Also, we previously reported evidence for dominant-negative effects by two endogenous mutant *p53* alleles in mouse embryonic stem cells and in thymocytes produced by $Rag2^{-/-}$ blastocyst complementation (de Vries et al., 2002). None of these studies, however, found clear evidence of gain-of-function effects by mutant *p53*.

In order to more accurately model LFS in mice and to investigate the potential for dominant-negative or gain-of-function effects by mutant *p53*, we constructed and characterized two mouse strains carrying conditional germline point-mutations of *p53*. These strains harbor contact or structural mutations that together comprise two of the three most commonly mutated *p53* codons in human cancer. These strains more closely mimic humans with LFS both genetically and phenotypically. Moreover, data from these mice provide compelling evidence for gain-of-function effects by mutant *p53*.

RESULTS

Generation of Germline Point-Mutant *p53* Mice

Two conditional point-mutant *p53* alleles were engineered into the endogenous murine *p53* locus. They encode the contact mutant $p53^{R270H}$ and the structural mutant $p53^{R172H}$, both of which are commonly found in spontaneous human tumors and in the germline of LFS kindreds. (Murine *p53* codons 270 and 172 correspond to human *p53* codons 273 and 175.) In order to enable tissue- or stage-specific expression of mutant *p53* under physiological conditions, conditional mutant alleles of *p53* were generated by introducing the LoxP-flanked transcriptional STOP cassette into intron 1 of the *p53* gene. This Lox-STOP-Lox (LSL) cassette was used by our group previously to create a conditional oncogenic allele of *K-ras*; it efficiently blocks expression of targeted loci prior to removal of the STOP element by Cre recombinase (Tuveson et al., 2004). Site-directed mutagenesis was used to introduce an arg→his mutation at either codon 270 or codon 172, resulting in the conditional mutant alleles $p53^{LSL\cdot R270H}$ and $p53^{LSL\cdot R172H}$ (Fig. 1A). These were verified by sequencing the entire *p53* open reading frame and all intron/exon boundries in the targeting vectors (data not shown). Both alleles were targeted into J1 embryonic stem cells and germline transmission was achieved in multiple clones. Presence of the STOP cassette in intron 1 of *p53* in these mice was determined by PCR (Fig. 1B), and the presence of each mutation was reconfirmed by sequencing (data not shown).

In order to study the effects of endogenous expression of the mutant *p53* alleles in all tissues of the mouse, $p53^{LSL\cdot R270H/+}$ and $p53^{LSL\cdot R172H/+}$ mice were crossed to Protamine-Cre transgenic mice, which express Cre recombinase in haploid sperm (O'Gorman et al., 1997). Resulting $p53^{LSL\cdot R270H/+}$; *PrmCre*/+ and $p53^{LSL\cdot R172H/+}$; *PrmCre*/+ male mice were then crossed to wild-type

129S4/SvJae females to generate $p53^{R270H/+}$ and $p53^{R172H/+}$ mice (collectively referred to as "$p53^{M/+}$" mice). Tail DNA samples from offspring were analyzed by PCR (Fig. 1C).

Successful generation of conditional and germline point-mutant *p53* mice was verified by several means. *p53* cDNAs were derived from $p53^{M/+}$ and $p53^{LSL\cdot M/+}$ mouse brains and sequenced. As expected, both mutant and wild-type alleles were found to be expressed in $p53^{R270H/+}$ and $p53^{R172H/+}$ samples, while only the wild-type allele was expressed in $p53^{LSL\cdot R270H/+}$ and $p53^{LSL\cdot R172H/+}$ samples (Fig. 1D, data not shown). In addition, cDNAs derived from $p53^{R270H/-}$ and $p53^{R172H/-}$ primary mouse embryo fibroblasts (MEFs; see below) were sequenced, confirming the presence of only the desired mutations (data not shown). Quantitative real-time PCR analysis for *p53* confirmed that the point-mutant alleles are expressed at levels comparable to wild-type *p53* in MEFs (Fig. 1E).

Mutant p53 protein often accumulates to high levels in human tumors and some mutant proteins adopt an altered conformation. In particular, structural mutants assume a partially denatured conformation that results in the exposure of novel epitopes recognized by mutant-specific α-p53 antibodies. In contrast, contact mutants have been described either as 'flexible' (reactive to both wild-type- and mutant-specific antibodies) or as fully wild-type in conformation (Webley et al., 2000; Yewdell et al., 1986). In order to examine the biochemical properties of mutant p53 proteins, mice that express only point-mutant p53 were generated by crossing $p53^{M/+}$ mice to $p53^{+/-}$ mice (Jacks et al., 1994) to generate $p53^{R270H/-}$ and $p53^{R172H/-}$ mice (collectively referred to as $p53^{M/-}$ mice). These mice appeared grossly similar to $p53^{-/-}$ mice at birth and throughout development (see below). They were born in expected Mendelian ratios except for a slight decrease in the number of $p53^{M/-}$ females, a phenotype that is also observed in $p53^{-/-}$ animals

due to female-specific exencephaly (Sah et al., 1995). Figure 1F shows a Western blot for p53 performed on MEF whole cell lysates confirming that mutant p53 accumulated to high levels in these cells. In addition, these experiments also revealed that the contact mutant $p53^{R270H}$ accumulated to higher levels in $p53^{M/-}$ MEFs than the structural mutant $p53^{R172H}$. This effect was reproduced using three different antibodies against p53 (data not shown). As shown in Figure 1G, the structural mutant $p53^{R172H}$ was preferentially immunoprecipitated by the mutant-specific antibody PAb 240 while the contact mutant $p53^{R270H}$ was preferentially immunoprecipitated by the wild-type-specific antibody PAb 1620.

p53$^{M/+}$ Mice Develop Distinct Tumor Spectra

In order to evaluate whether the $p53^{M/+}$ mouse strains are improved models of LFS and to compare the effects of *p53* point-mutation to *p53* null mutation, a cohort of 37 $p53^{R270H/+}$, 41 $p53^{R172H/+}$ and 37 $p53^{+/-}$ mice were aged and monitored for the onset of malignancy. As shown in Figure 2a, the life-spans of $p53^{R270H/+}$, $p53^{R172H/+}$ and $p53^{+/-}$ mice were similar with mean survival times of 15.8, 15.2, and 15.4 months, respectively (Kaplan-Meyer Log-Rank Test). Each mouse was subjected to a complete necropsy and histopathological analysis of all tissues regardless of whether tumors were apparent. The pathology of each mouse in this study is available in Supplementary Materials.

The $p53^{M/+}$ mice demonstrated significant differences in their tumor spectra, both compared to the $p53^{+/-}$ mice as well as to each other (Fig. 2b). The $p53^{R270H/+}$ mice had an increased incidence of carcinomas compared to $p53^{+/-}$ mice (χ^2, p= 0.017). In particular, 7/36 $p53^{R270H/+}$ mice developed lung adenocarcinomas, including several with malignant features commonly

seen in human lung adenocarcinoma such as nuclear atypia (5/7), desmoplasia (4/7) and metastasis (2/7). Other carcinomas found in $p53^{R270H/+}$ mice included five squamous cell carcinomas, two hepatocellular carcinomas, a transitional cell carcinoma of the kidney and an intestinal carcinoma (Sup. Fig. 2A-C). Many of these tumors were invasive or showed evidence of distal metastases (Sup. Fig. 2D-E). Immunohistochemistry (IHC) was performed on some tumors to further support their epithelial origin (Sup. Table 1, Sup. Fig. 3A). In contrast to the $p53^{R270H/+}$ mice, only four low-grade carcinomas were found in 37 $p53^{+/-}$ mice, similar to previous reports (Donehower et al., 1992; Jacks et al., 1994). The single lung carcinoma observed in a $p53^{+/-}$ mouse was well differentiated and lacked severely dysplastic nuclei or stromal deposition. Pre-malignant epithelial lesions such as adenomas and papillomas were also more common in the $p53^{R270H/+}$ mice than in the $p53^{+/-}$ mice (Sup. Fig. 1).

In addition to carcinomas, $p53^{R270H/+}$ mice also had an increased incidence of B-cell lymphomas. 5/36 $p53^{R270H/+}$ mice developed B-cell lineage tumors (χ^2, p= 0.031; Sup. Fig. 3B,C). They generally arose in the spleen or mesenteric lymph nodes and spread through the abdominal cavity, sometimes invading adjacent organs such as the kidneys and intestines (Sup. Fig. 3B). Overall, $p53^{R270H/+}$ mice had an increased tumor burden compared to $p53^{+/-}$ mice; 44% of $p53^{R270H/+}$ mice had developed multiple tumors at the time of necropsy compared to 19% of $p53^{+/-}$ mice (χ^2, p=0.03).

In contrast to the frequent carcinomas in $p53^{R270H/+}$ mice, the most frequent tumors in the $p53^{R172H/+}$ mice were osteosarcomas. Roughly twice as many $p53^{R172H/+}$ mice developed osteosarcomas as $p53^{+/-}$ and $p53^{R270H/+}$ mice (χ^2, p= 0.043 for $p53^{+/-}$ mice, p= 0.039 for

$p53^{R270H/+}$ mice). These tumors developed primarily in trabecular regions of vertebrae or longbones. Diffuse osteopetrosis was commonly observed concurrent with the osteosarcomas. In addition, the $p53^{R172H/+}$ osteosarcomas metastasized more frequently than those in the $p53^{+/-}$ or $p53^{R270H/+}$ mice, generally to the liver, lung or spleen (Sup. Fig. 2E,F). Only four osteosarcomas in $p53^{R172H/+}$ mice did not metastasize, including two early lesions found in mice that died from other causes. The $p53^{R172H/+}$ mice also developed carcinomas slightly more frequently than $p53^{+/-}$ mice (χ^2, p= 0.38), however, they did not display the high frequency of lung adenocarcinomas noted in $p53^{R270H/+}$ mice.

Tumors that develop in humans with hereditary *p53* mutations frequently lose the remaining wild-type *p53* allele. It was therefore of interest to determine the status of the wild-type *p53* allele in tumors from $p53^{M/+}$ mice. The mutant *p53* reaction described earlier (Figure 1c) was modified to be quantitative by terminating the reaction after 29 cycles rather than 35. The sensitivity of this assay was confirmed by running a dilution series of $p53^{+/+}$ and $p53^{M/M}$ samples (Figure 2C). PCR analysis of DNA derived from $p53^{M/+}$ tumors revealed evidence of LOH in 10/19 tumors analyzed (4/10 $p53^{R270H/+}$ and 6/9 $p53^{R172H/+}$), further validating these mice as models for LFS.

Dominant Effects of Mutant p53 in Primary Cells

There are several potential explanations for the differences in tumor spectra between the $p53^{M/+}$ mice and $p53^{+/-}$ mice. Mutant *p53* may act in a dominant-negative manner to interfere with the function of the wild-type *p53* allele. Alternatively, the mutant *p53* alleles may contribute novel pro-tumorigenic functions. These two mechanisms are not mutually exclusive. In order assess

the dominant effects of endogenous mutant *p53*, several well established assays for *p53*-dependent functions were performed on $p53^{M/+}$ MEFs and thymocytes. For example, *p53* can act to restrain cell proliferation as $p53^{-/-}$ MEFs cycle more quickly than wild-type or $p53^{+/-}$ MEFs. Therefore, we measured cell proliferation in $p53^{M/+}$ MEFs by analyzing the fraction of cells in S-phase via FACS analysis of bromodeoxyuridine (BrdU) incorporation and propidium iodide (PI) exclusion. As expected, a large fraction of $p53^{-/-}$ MEFs were in S-phase during exponential growth compared to wild-type or $p53^{+/-}$ MEFs. Interestingly, both the $p53^{R270H/+}$ and $p53^{R172H/+}$ MEFs had a larger S-phase fraction than $p53^{+/-}$ MEFs, with the $p53^{R172H/+}$ MEFs appearing comparable to $p53^{-/-}$ MEFs in this assay (Fig. 3a, solid bars, Sup. Fig. 4a). Evidence of an elevated proliferation rate in $p53^{R172H/+}$ MEFs was also apparent when they were submitted to the classical 3T3 passaging protocol (Todaro and Green, 1963). $p53^{R172H/+}$ MEFs grew as rapidly as $p53^{-/-}$ MEFs for four passages before entering replicative senescence. Immortalized clones quickly escaped senescence and PCR analysis demonstrated loss of the wild-type *p53* allele in these clones (Sup. Figure 4b; data not shown). *p53* is also required for G_1 arrest in MEFs after DNA damage (Kastan et al., 1992). Surprisingly, the DNA damage-induced G_1 arrest response was intact in $p53^{M/+}$ MEFs: after treatment with the DNA damaging agent doxorubicin, $p53^{M/+}$ MEFs arrested in the G_1 phase to a similar extent as wild-type cells (Figure 3a). Thus, physiological expression of point-mutant *p53* in MEFs appears to dominantly interfere with cellular functions related to basal proliferation but does not affect DNA damage-induced G_1 arrest.

As DNA damage-induced G_1 arrest in MEFs is primarily dependent on the ability of p53 to transactivate the cell cycle inhibitor *p21*, this pathway was investigated in $p53^{M/+}$ MEFs. As

shown in Figure 4A, the amount of total p53 in untreated $p53^{M/+}$ MEFs was elevated compared to $p53^{+/+}$ or $p53^{+/-}$ MEFs, and increased further upon treatment with doxorubicin. p21 levels, however, were comparable to those found in $p53^{+/-}$ MEFs, suggesting that mixed mutant and wild-type p53 tetramers are competent to transactivate at least some target genes. An IP-Western for MDM2 supports this notion and also suggests that the accumulation of high levels of mutant p53 in these cells is not due to a loss of MDM2 transactivation by mutant p53.

Finally, in order to analyze the ability of $p53^{M/+}$ cells to undergo *p53*-dependent apoptosis, primary thymocytes were isolated from 6-8 week old mice. The apoptotic response of $p53^{M/+}$ thymocytes to 5Gy of gamma radiation was assayed by FACS analysis for Annexin-V expression and propidium iodide exclusion. The relative fraction of viable cells after doxorubicin treatment is shown in Figure 3B. As expected, $p53^{-/-}$ thymocytes were completely resistant to radiation-induced apoptosis through 24 hours whereas only 20% of wild-type thymocytes survived to this time-point. Both $p53^{R270H/+}$ and $p53^{R172H/+}$ thymocytes were partially resistant to gamma-induced apoptosis. The $p53^{M/+}$ thymocytes were more resistant to apoptosis than $p53^{+/-}$ cells, consistent with a dominant effect by point-mutant proteins in apoptosis. These results are similar to previous experiments on point-mutant *p53* thymocytes generated by $Rag2^{-/-}$ blastocyst complementation (de Vries et al., 2002).

p53 Gain-of-Function: Mutant *p53* Predisposes Mice to Epithelial and Endothelial tumors

To directly evaluate the *p53* "gain-of-function" hypothesis *in vivo*, spontaneous tumor development in mice expressing only mutant p53 was compared to that in mice lacking p53. If the point-mutant *p53* alleles possess inherent oncogenic functions, $p53^{M/-}$ mice could have a more pronounced tumor phenotype than *p53* null mice. 41 $p53^{-/-}$, 40 $p53^{R270H/-}$ and 45

$p53^{R172H/-}$ mice were generated as described above. All of the mice required euthanasia or died by 8.5 months of age, with a mean survival time of 4.4, 4.5 and 4.6 months for the $p53^{-/-}$, $p53^{R270H/-}$ and $p53^{R172H/-}$ mice, respectively. There was no significant difference in the survival of *p53* mutant mice compared to *p53* null mice. (Kaplan-Meyer Log Rank Test, Fig. 5A)

In support of the mutant *p53* "gain-of-function" hypothesis, two significant differences were observed in the tumor spectra of the $p53^{M/-}$ mice compared to $p53^{-/-}$ mice (Fig. 5B). In previous studies, $p53^{-/-}$ mice primarily developed lymphomas and sarcomas and rarely developed carcinomas. Likewise, none of the $p53^{-/-}$ mice in this study developed carcinomas. In contrast, carcinomas were observed in 18% of the $p53^{R270H/-}$ mice and 16% of $p53^{R172H/-}$ mice (χ^2, p= 0.008 for $p53^{R270H/-}$ and p= 0.015 for $p53^{R172H/-}$). Carcinomas found in the point-mutant *p53* mice arose in a variety of different tissues including lung, small intestine, colon, breast, skin, liver and pancreas. These tumors developed in mice ranging from 1.5 to 6.9 months of age and, in one case, two different primary carcinomas were observed in the same mouse. Most of the carcinomas showed evidence of invasion, metastasis or other features commonly seen in advanced human carcinomas such as desmoplasia and stromal invasion (Fig. 6A-E). In most instances, the epithelial origin of these tumors was readily apparent through histopathology, however IHC was also used to support these diagnoses (Sup. Fig. 3E-F, Sup. Table 1).

In addition to epithelial tumors, the point-mutant *p53* mice also demonstrated an increased incidence of hemangiosarcomas (Fig. 5B, Fig. 6F). The $p53^{R172H/-}$ mice, in particular, showed a significant increase to 62% incidence (27/45) compared to 32% (13/41) in the $p53^{-/-}$ mice (χ^2, p = 0.005). These tumors were highly aggressive, demonstrated marked nuclear atypia and stained positively for the endothelial tumor marker Fli-1 (data not shown).

Similar to previous studies, 66% of the $p53^{-/-}$ mice in this study developed hematological malignancies, primarily T-cell lymphomas. A slight decrease in the incidence of hematological malignancies was observed in $p53^{M/-}$ mice (55% of $p53^{R270H/-}$ mice and 50% of $p53^{R172H/-}$ mice), although there was no shift in the survival time of $p53^{M/-}$ mice with lymphomas. The other tumors observed in $p53^{M/-}$ mice were found at frequencies similar to that of $p53^{-/-}$ mice. Overall, 32% of $p53^{-/-}$ mice developed multiple tumors compared to 43% of $p53^{R270H/-}$ mice (χ^2, $p = 0.31$) and 57% of $p53^{R172H/-}$ mice (χ^2, $p = 0.02$).

Mutant p53 Accumulation in Tumors

Tumors cells from LFS patients often show nuclear accumulation of mutant p53 protein. In order to determine whether tumors from $p53^{M/+}$ mice were similar in this respect, IHC for p53 was performed on tumors from the aging studies. (Sup. Fig. 6A-C, Sup. Table 2). Interestingly, 12/16 carcinomas from $p53^{M/+}$ mice exhibited substantial nuclear accumulation of p53 protein. Three of the four tumors that did not accumulate p53 were low-grade lung adenocarcinomas. More advanced lung adenocarcinomas stained positively for p53 accumulation in patches that correlated with more malignant histology (Sup. Fig. 6C). In addition, 11/13 osteosarcomas in $p53^{M/+}$ mice were found to accumulate p53 to high levels in at least a subset of cells.

Several carcinomas that stained positively for p53 also exhibited stromal desmoplasia. In each case, the stromal cells did not express high levels of p53 (Sup. Fig. 6A,B). Likewise, normal tissues from $p53^{M/+}$ mice did not show evidence of p53 accumulation. These data might indicate that residual *p53* function in $p53^{M/+}$ mice is sufficient to restrain p53 accumulation in normal but not neoplastic cells. Alternatively, the tumor cells that accumulated p53 might have lost their

wild-type *p53* allele. Indeed, in determining whether LOH had occurred in $p53^{M/+}$ tumors (see above), it was observed that LOH in a tumor generally correlated with the accumulation of mutant p53 protein. However, there were several exceptions: tumors were found that had undergone LOH but did not accumulate mutant p53 and, conversely, that accumulated mutant p53 but retained heterozygosity. Furthermore, if mutant p53 protein accumulation is dependent on a loss of wild-type p53 function, it would follow that mutant p53 would accumulate to high levels in the normal tissues of $p53^{M/-}$ mice, which lack any wild-type *p53* function. To test this directly, IHC for p53 was performed on tumors and normal tissues from $p53^{M/-}$ mice. As shown in Sup. Table 2, p53 was readily detected in the majority of, but not all, $p53^{M/-}$ tumors. However, in no case was p53 accumulation observed in normal tissues from these mice. Furthermore, as shown in Sup. Fig. 6D-F, mutant p53 does accumulate in normal tissues of $p53^{M/-}$ mice after gamma irradiation, but only in the same cells and tissues observed to accumulate p53 in wild-type animals. Thus, tumor associated increases in p53 staining cannot be ascribed solely to the *p53* genotype of the cell but, rather, would appear to reflect a secondary event that causes mutant p53 stabilization. Interestingly, thymic lymphomas, which occur frequently in $p53^{-/-}$ as well as $p53^{M/-}$ mice, consistently showed a lack of p53 accumulation, perhaps indicating that the initiation of this tumor requires only the loss of *p53* function.

Loss of Wild-Type Function in $p53^{M/-}$ Primary Cells

Point-mutant *p53* alleles have alternatively been reported to be completely or partially defective in transactivation (Forrester et al., 1995). In order to determine whether the $p53^{R270H}$ and $p53^{R172H}$ mutants had lost wild-type *p53* activity, the cellular assays described above were carried out on $p53^{M/-}$ and $p53^{-/-}$ MEFs. No difference was seen between the point-mutant MEFs and

p53$^{-/-}$ MEFs in basal S-phase fraction or in doxorubicin-induced cell cycle arrest (Sup. Fig. 5A). *p53*$^{M/-}$ MEFs were also found to be immortal by 3T3 assay but they were unable to form foci at high density or to form colonies in soft agar, similar to *p53*$^{-/-}$ MEFs (Sup. Fig. 5B, data not shown). Likewise, there was no difference between *p53*$^{-/-}$, *p53*$^{R270H/-}$ and *p53*$^{R172H/-}$ MEFs in their abilities to induce colony formation at low density or their abilities to proliferate in low serum (data not shown). Finally, mutant *p53* alleles were defective in inducing apoptosis in primary thymocytes after gamma irradiation, similar to *p53*$^{-/-}$ thymocytes (data not shown).

In order to assess the ability of mutant p53 to transactivate target genes, Western blotting for p53, p53$^{\phi\text{-ser15}}$, p21 and MDM2 was performed on protein extracts from p53$^{M/-}$ MEFs. Although mutant p53 accumulated and was phosphorylated in response to doxorubicin treatment, p21 and mdm2 were not induced in mutant *p53* MEFs (Fig. 4b).

Mutant p53 Gain-of-Function in Tumor-Derived Cells

Previous work has demonstrated that overexpression of mutant p53 protein in *p53* null cells results in aggravation of the tumorigenic phenotype of those cells. The reverse experiment, however, has not been performed on contact or structural mutant p53-bearing cells. We therefore utilized a conditional lentiviral shRNA expression construct, pSico-p53 (Ventura et al., 2004), to knock-down mutant p53 expression in an endogenous setting. To allow selection of cells bearing the conditional shRNA, a puromycin resistance cassette was substituted for the GFP cassette of pSico-p53, to give pSico^{P}-p53.

In an effort to find a cellular system sensitive to the expression of mutant *p53,* a cell line, Os1, was derived from an osteosarcoma metastasis that arose in a $p53^{R172H/+}$ mouse. Analysis of DNA from Os1 cells indicated that they had lost their wild-type *p53* allele (data not shown). Os1 cells were infected with pSico^{P}-p53, selected in puromycin, and then superinfected with either a Cre-expressing adenovirus (AdCre) or a control adenovirus (AdEmpty). The percentage of cells infected by AdCre was greater than 90% as assessed by the renewed susceptibility of the cells to puromycin (the puromycin resistance cassette is lost upon activation of shRNA expression by Cre). To control for potential side-effects of expressing Cre recombinase, parental Os1 cells were also treated with AdCre (Fig. 7A). The infected cells were expanded and protein was collected from cells in the presence or absence of the DNA damaging agent (cisplatin). As seen in Fig. 7B, *p53* shRNA expression resulted in substantial knockdown of mutant p53, both before and after treatment with cisplatin.

In the course of expanding the cells, it was noticed that the *p53* shRNA-expressing cells grew more slowly than the control cells. To quantify this, a proliferation assay was performed on *p53* shRNA-expressing and control Os1 cells. As seen in Fig. 7C, Os1 cells proliferate more slowly after knockdown of mutant p53. As this experiment took place several passages subsequent to the initial adenoviral infections, the experiments were repeated just 48 hours after infection with AdCre or AdEmpty. As seen in Figure 7D, the inhibition of proliferation was even more dramatic immediately after infection. This effect was visualized by diluting the cells 1:500 with wild-type MEFs and allowing them to grow to high density (Figure 7E).

It was previously reported that mutant p53 can bind to and inhibit the function of family-member proteins *p63* and *p73*. In order to determine whether this occurs in Os1 cells, p73 immunoprecipitates were probed with an antibody against p53. As shown in Figure 7f, mutant p53 was coimmunoprecipitated with p73, but not by a mouse antibody specific for an unrelated protein. Similar results were seen with an antibody against p63 and a second antibody against p73 (data not shown). Furthermore, qPCR experiments demonstrated an increase in p63 and p73 target gene expression following knockdown of mutant p53 (data not shown). These results support the inhibition of p63 and p73 by protein-protein interaction as a potential mechanism for the observed gain-of-function by mutant p53.

Discussion

Our understanding of the p53 has had a convoluted history. Although it was initially thought to be a cellular oncogene, a series of observations lead eventually to the classification of p53 as a suppressor of transformation. While the tumor suppressive functions of wild-type *p53* are not in question, there have been persistent reports that point-mutant p53 proteins can affect transformation independent of wild-type p53. These studies have led to the suggestion that point-mutant *p53* has gain-of-function oncogenic properties. However, because these experiments have involved ectopic overexpression of the mutant p53 allele, they have been interpreted with caution. Here we describe a definitive test for the existence of oncogenic functions of point-mutant p53.

Several important conclusions can be drawn from the analysis of mice with knock-in alleles of tumor-derived mutations in p53. First, $p53^{M/+}$ mice are models of Li-Fraumeni syndrome that better recapitulate the human familial syndrome than do $p53^{+/-}$ mice. Moreover, $p53^{R270H/+}$ mice develop distinct tumor spectra from $p53^{R172H/+}$ mice, indicating that different *p53* point-mutations confer subtly different functions. Specific functional assays on primary cells derived from $p53^{M/+}$ mice also support this conclusion. $p53^{M/-}$ mice develop tumor spectra distinct from *p53* null mice and tumor cells derived from mutant *p53* mice are sensitive to the shRNA-mediated knock-down of mutant *p53*, strongly supporting a gain-of-function effect by mutant *p53*.

$p53^{M/+}$ mice are models of Li-Fraumeni Syndrome

Of the twenty or so tumor suppresser genes associated with human familial cancer syndromes, *p53* is unusual in that it is commonly altered through missense mutation rather than deletion and

because mutant p53 proteins are upregulated in tumors. A few LFS kindreds have alterations akin to null alleles of p53. An analysis of seven such families concluded that these patients developed fewer cancers overall, and at an older age, than LFS patients with point-mutations in the p53 DBD (Birch et al., 1998). However, a separate study found no difference between point-mutant and truncation-mutant LFS kindreds (Hwang et al., 2003). The small number of these patients combined with their diverse genetic backgrounds complicates this analysis. In contrast, our work shows a clear difference in tumor spectra in $p53^{M/+}$ mice compared to $p53^{+/-}$ mice. Although there was no change in survival time of mice with different genotypes, the $p53^{M/+}$ mice developed more tumors and a different spectrum of tumors compared to $p53^{+/-}$ mice. Moreover, $p53^{M/+}$ mice more accurately recapitulate the phenotype of LFS patients in that they develop a broad spectrum of tumors, including a variety of carcinomas, soft tissue and bone sarcomas, leukemias and even a glioblastoma multiforme (the most common brain tumor observed in LFS patients). The most obvious omission from this tumor spectrum was breast carcinomas, the most common tumor in LFS patients. This could be due to the action of a background modifier not present in the $129S_4$/SvJae genetic background. Indeed, when crossed to the BALB/c background, $p53^{+/-}$ mice develop mammary carcinomas at high frequency (Kuperwasser et al., 2000). This raises the prospect that crossing the point-mutant *p53* mice onto BALB/c or other backgrounds will further refine the accuracy these models.

Differences between contact and structural mutant p53

While numerous studies have reported biochemical differences between contact and structural mutant p53 proteins (Halevy et al., 1990; Hinds et al., 1990), our work represents the first clear evidence of a differential effect on tumorigenesis *in vivo*. $p53^{R270H/+}$ mice develop an increased

incidence of carcinomas and B-cell lymphomas compared to $p53^{+/-}$ mice. $p53^{R172H/+}$ mice develop an increased incidence of metastatic osteosarcomas (and a trend towards more carcinomas). Among LFS patients, few connections between particular mutations and phenotypes have been described.

The molecular basis for the difference in tumor spectra between $p53^{R270H/+}$ and $p53^{R172H/+}$ mice is unclear, but data derived from primary cells isolated from these mice uncovered some functional differences between the contact and structural mutant proteins in the presence of wild-type p53. For example, $p53^{R270H/+}$ MEFs proliferated only modestly faster than $p53^{+/-}$ MEFs while $p53^{R172H/+}$ MEFs doubled nearly as fast as $p53^{-/-}$ cells. In contrast, neither mutant interfered with the induction of G_1 arrest after DNA damage in MEFs.

Dominant effects of mutant p53

In addition to effects on the basal proliferation rate in MEFs, mutant p53 exhibited dominant effects on radiation-induced apoptosis in thymocytes; $p53^{M/+}$ thymocytes had levels of apoptosis intermediate to $p53^{+/-}$ and $p53^{-/-}$ thymocytes. The simplest explanation for this effect is that both $p53^{R270H}$ and $p53^{R172H}$ interfere with the ability of wild-type p53 to transactivate apoptotic target genes through a dominant-negative mechanism. However, these experiments do not rule out dominant gain-of-function effects by mutant p53. That is, the decrease in radiation-induced apoptosis in $p53^{M/+}$ thymocytes may be due to interference with the function of wild-type p53 or interference with other factors involved in radiation-induced apoptosis.

Similarly, the novel tumor phenotypes of $p53^{M/+}$ mice could be due to either dominant-negative effects, dominant gain-of-function effects or a combination of both. For example, one

interpretation of the development of lung carcinomas in $p53^{R270H/+}$ mice is that this allele confers a dominant gain-of-function effect because lung carcinomas are rarely observed in $p53^{-/-}$ mice. However, most $p53^{-/-}$ mice die before six months of age. Were null mice to avoid other tumors for 18 months, perhaps they too would be predisposed to lung adenocarcinoma. In order to assess this properly, *p53* function would need to be examined specifically in the lung without other background tumors. The conditional point-mutant *p53* mice presented in this work will be useful for studying the effects of mutant *p53* in particular tissues or at specific developmental stages.

Mutant p53 gain-of-function

This work presents the first clear *in vivo* evidence for gain-of-function effects by mutant *p53*. Since the initial descriptions of *p53* knockout mice, there have been persistent questions as to why they do not develop more carcinomas when p53 is so often mutated in this class of tumors. As discussed above, one explanation is that background modifiers that promote the development of carcinomas in humans may be absent from certain inbred laboratory mouse strains. However, our data support a model in which epithelial carcinogenesis is enhanced by the production of point-mutant p53, rather than the loss of p53. $p53^{M/-}$ mice spontaneously develop a variety of carcinomas in addition to the normal complement of tumors previously associated with *p53* null mutations in mice. Furthermore, mutant p53 accumulation occurred commonly in carcinomas from $p53^{M/-}$ mice but not in lymphoblastic lymphomas. It is also notable that two of the tumors commonly found in *p53* null mice, lymphoblastic lymphomas and teratocarcinomas, rarely harbor *p53* mutations in humans. Rather, both tumors are associated with functional inactivation of *p53*, such as through HDM2 overexpression (Eid et al., 1999; Zhou et al., 1995). Likewise,

HDM2 overexpression is found in over one third of human sarcomas, the third major tumor type in *p53* null mice (Cordon-Cardo et al., 1994; Oliner et al., 1992). These correlations further support the idea that p53 inactivation (combined with alterations in other genes) is sufficient for tumorigenic progression in a subset of tissues.

In concurrent work, Guillermina Lozano and colleagues have also generated a $p53^{R172H}$ mouse through similar techniques (Lang et. al, this issue). Although they observed a similar spontaneous tumor phenotype in heterozygous mutant p53 mice, they did not observe an increase in carcinomas or other tumors in $p53^{R172H/R172H}$ mice. This may be due to differences in strain background: Lozano's mice were enriched for C57Bl/6 while our mice were enriched for $129S_4$/SvJae.

The data presented here provide strong support for the pro-tumorigenic effects of point-mutant *p53*. At the molecular level, several mechanisms have been proposed to explain mutant p53 gain-of-function effects (reviewed in (Cadwell and Zambetti, 2001). For example, mutant p53 may be able to transactivate novel target genes such as *mdr*, *BAG-1,* or *c-myc*. Alternatively, mutant *p53* may participate in novel protein-protein interactions. Perhaps the most well characterized interaction is with the *p53*-family members, p63 and p73. These two genes share a high degree of homology with p53 and are capable of transactivating several p53 target genes in response to DNA damage. *p63* and *p73* are also activated by DNA damage, similar to *p53* (Irwin et al., 2000; Jost et al., 1997; Yang et al., 1998). Several reports have shown that mutant p53 can bind to and inactivate p63 and p73 (Di Como et al., 1999; Gaiddon et al., 2001; Marin et al., 2000; Strano et al., 2000). We also found mutant p53 capable of binding to its family

members in tumor cells derived from a $p53^{R172H}$ osteosarcoma metastasis. The multiple splice variants of *p63* and *p73* complicate our understanding of their role in tumorigenesis. Although deletions of *p63* or *p73* are rare in human tumors, overexpression of ΔNp63 is common in human squamous cell carcinomas (Hibi et al., 2000) and overexpression of ΔNp73 correlates with poor outcomes in human neuroblastomas (Casciano et al., 2002). Nonetheless, the classification of p63 and p73 as tumor suppresser genes has yet to be commonly accepted. It is therefore striking that compound mutation of p53 with either p63 or p73 results in mice bearing tumor spectra reminiscent of those seen in the mutant p53 mice presented in this study (E. Flores et. al, submitted). Taken together, these data strongly implicate the inhibition of p63 or p73 as a potential mechanism for point-mutant p53 gain-of-function.

MATERIALS AND METHODS

Generation of Conditionally Inactive Mutant *p53* Mice

The LoxP flanked conditional STOP cassette (Tuveson et al., 2004) was cloned into the Xho1 site in intron 1 of the murine *p53* locus. The 270H missense mutation was generated as described (de Vries et al., 2002). To generate the 172H missense mutation, the 1.7 kb Xba1-BamH1 *p53* genomic fragment containing exons 4 and 5 were cloned into pBluescript and site-directed mutagenesis was performed using the Stratagene QuickChange kit with the following oligonucleotides: (172H mutation in bold) F- 5'-cggaggtcgtgagac**a**ctgcccccaccatga-3' R- 5'-tcatggtgggggcag**t**gtctcacgacctccg-3'

Targeting vectors were assembled in pBS and the sequences of all exons and intron-exon boundaries were confirmed by bidirectional sequencing prior to gene targeting in J1 ES cells. Targeting vectors were linearized at a unique 5' Not1 site, and electroporated in to J1 ES cells. ES cells were selected in 2ug/ml puromycin and appropriately targeted clones were identified with a 5' external probe and EcoR1 digested genomic DNA. An internal puromycin cDNA probe confirmed single copy insertion. The presence or absence of the mutation was established in genomic DNA from the ES cell clones. Germline transmission was achieved in 3/3 clones harboring the $p53^{LSL\text{-}R270H}$ allele and 2/2 clones harboring the $p53^{LSL\text{-}R172H}$ allele.

The presence of the 270H mutation was established by PCR amplification of a 565bp genomic fragment encompassing exons 7 and 8: F- 5'-cgcgccggctctgagtatac-3' R- 5'-cgcttgcgctccctgggggc-3'. The reverse primer was used for sequencing. 56/182 clones contained the stop cassette; of these 9/56 contained the novel Msl1 site and the 270H mutation (de Vries et

al., 2002). Two clones negative for the MslI site were sequenced and found to harbor exclusively wild-type *p53*. The 172H mutation was identified after amplification of a 404bp genomic fragment that contained exons 5 and 6: F- 5'-tctcttccagtactctcctcc-3'; R- 5'-aattacagacctcgggtggct-3', with the following primer used for sequencing: 5'-aagctattctgccagctggcg-3'. 27/182 clones contained the stop cassette, and of these, 13/27 unambiguously harbored the 172H mutation.

To determine the presence of the LSL cassette in intron 1 of *p53*, genomic DNA was amplified using the following primers to generate wild-type band of 170bp and a mutant band of 270bp. WT F- 5'-ttacacatccagcctctgtgg-3', Mutant F- 5'-agctagccaccatggcttgagtaagtctgca-3', R- 3'-cttggagacatagccacactg-3'. To determine the presence of the recombined alleles of mutant *p53*, genomic DNA was amplified using the following primers flanking the integration site of the remaining LoxP site in *p53* intron 1: F- 5'-agcctgcctagcttcctcagg-3', R- 5'-ctt ggagacatagccacactg-3'.

Aging Study

Aging cohorts were produced by mating $p53^{R270H/-}$ or $p53^{R172H/-}$ mice to $p53^{+/-}$ mice (19 $p53^{+/-}$ mice and 30 $p53^{-/-}$ mice were derived from the $p53^{R270H}$ cross while 18 $p53^{+/-}$ mice and $14^{-/-}$ mice were from the $p53^{R172H}$ cross). Aging mice were between 93% and 97% enriched for the $129S_4$/SvJae genetic background. Routine testing of sentry mice indicated the colony was pathogen free. Ill or distressed mice were euthanized by CO_2 asphyxiation and all tissues except skin were fixed in 10% formalin/PBS for 24 hours (soft tissues) or Bouin's fixative for 3 weeks (bones). Tissues were embedded in paraffin, sectioned at 5 microns, dewaxed and stained with

hematoxylin and eosin. Tumors were identified with the assistance of a veterinary pathologist (R.B.).

Immunoblotting

Standard techniques were employed for immunoprecipitations and Western Blotting. 1mg samples were immunoprecipitated with 10μL of FL-393 (Santa Cruz), *p53* Ab3 (Oncogene Research Products (ORP)), *p53* Ab5 (ORP), p73 Ab2 (ORP) or mdm2 Ab2 (ORP). Western Blots were probed with the following antibodies: *p53* Ab7 (1:2500, ORP JA1308), SC-1616 (1:2000, Santa Cruz), SC-6426 (1:1000, Santa Cruz), mdm2 Ab-2 (1:1000, ORP).

Immunohistochemistry

Paraffin-embedded tissues were prepared through standard techniques. 5 micron sections for F4/80 staining were unmasked in 0.00025% trypsin/PBS for 20 minutes, 37°C. For all other antibodies, 5 micron sections were unmasked in a pressure cooker for 10-minutes in 0.01M sodium citrate, pH6.0. Endogenous peroxidases were quenched for 10 minutes in 1% hydrogen peroxide solution in PBS. Staining was performed using the Cadenza system according to directions (Thermo Shandon, 407340). Slides were incubated with the following primary antibodies for 12hrs at 4°C: *p53* Ab7, 1:500 (Oncogene Research Products (ORP), JA1308); CK8, 1:250 (Research Diagnostics, RDI-PRO61038); CM5, 1:250 (NCL-*p53*-CM5p); α-Smooth Muscle Actin, 1:100 (Research Diagnostics, RDI-ACTINabm-A4); CK14, 1:500 (Covance, PRB-155P), B220, 1:200 (BD Biosciences, #553090); F4/80, 1:200 (MCAP497). Detection was performed using ABC Peroxidase kits per directions. PAS and Trichrome

staining was performed using kits from Polyscientific R&D, (K047, K037). Whole-mouse irradiation was performed with a GammaCell 40 equipped with a ^{137}Cs source.

Cellular Assays

Thymocyte apoptosis was performed as described previously (de Vries et al., 2002). For cell cycle analysis, $1x10^6$ MEFs were plated in $10cm^2$ plates in DME/HEPES + 10% FBS + 5mM L-glutamine + Pen/Strep. After 24 hours, if indicated, cells were treated with 0.2μg/mL doxorubicin. After 12 hours BrdU was added to 10μM for 4 hours. Cells were collected in trypsin, quenched with medium and washed in PBS; FACS analysis was performed as described (Kastan et al., 1992). Tumor-derived cell lines were grown on plastic in DME/HEPES + 10% FBS + Pen/Strep.

Quantitative Real-Time RT-PCR

Total RNA was extracted from cells and tissue with the RNAeasy kit (Qiagen, 74104). 2ug of RNA was reverse-transcribed using random hexamers and Superscript III (Invitrogen, 18080-044). Quantitative real-time PCR was performed on the ABI 7700 Sequence Detection System using the SYBR Green kit (Applied Biosystems, 4309155) and the following primers: *p53*-5': cccctgtcatcttttgtccctt; *p53*-3': gggaggagagtacgtgcacataa; p21-5': gacaagaggcccagtacttcct; p21-3': caatctgcgcttggagtgata; β-Actin-5': cttcaacaccccagccatgta; β-Actin-3': catcacaatgcctgtggtacg. All reactions were performed in triplicate with β-Actin as the standard.

Lentiviral shRNA Experiments

The GFP cassette was removed from pSico-p53 by EcoRI/Not1 digestion and an EcoR1/Not1 fragment of PGK-Puro was ligated in its place to give pSico^{P}-p53. Lentiviral infections were performed essentially as described (Ventura et al., 2004) except that 293 cells were transfected using the Calphos kit (Clonetech). Recombinant adenoviral stocks were purchased from the Gene Transfer Vector Core facility of the University of Iowa College of Medicine (Iowa City, IA). Adenoviral infections used ~2000 plaque-forming units of virus per cell.

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Competing Interest Statement

The authors declare that they have no competing financial interests.

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Figure 1. Generation, Targeting and Verification of Conditional Point-Mutant *p53* Alleles.

(A) The endogenous *p53* locus is depicted at top, followed by targeting vectors for the $p53^{LSL\cdot R270H}$ and $p53^{LSL\cdot R172H}$ alleles. Asterisks indicate the sights of the point mutations. The integrated mutant *p53* alleles are shown at bottom. Restriction sites are indicated: R, EcoRI; B, BamHI; H, HinDIII; X, Xho; Nd, NdeI, Xb, Xba; S, Sal.

(B) PCR amplification of conditional mutant alleles results in both a 270bp mutant-specific product and a 166bp control product.

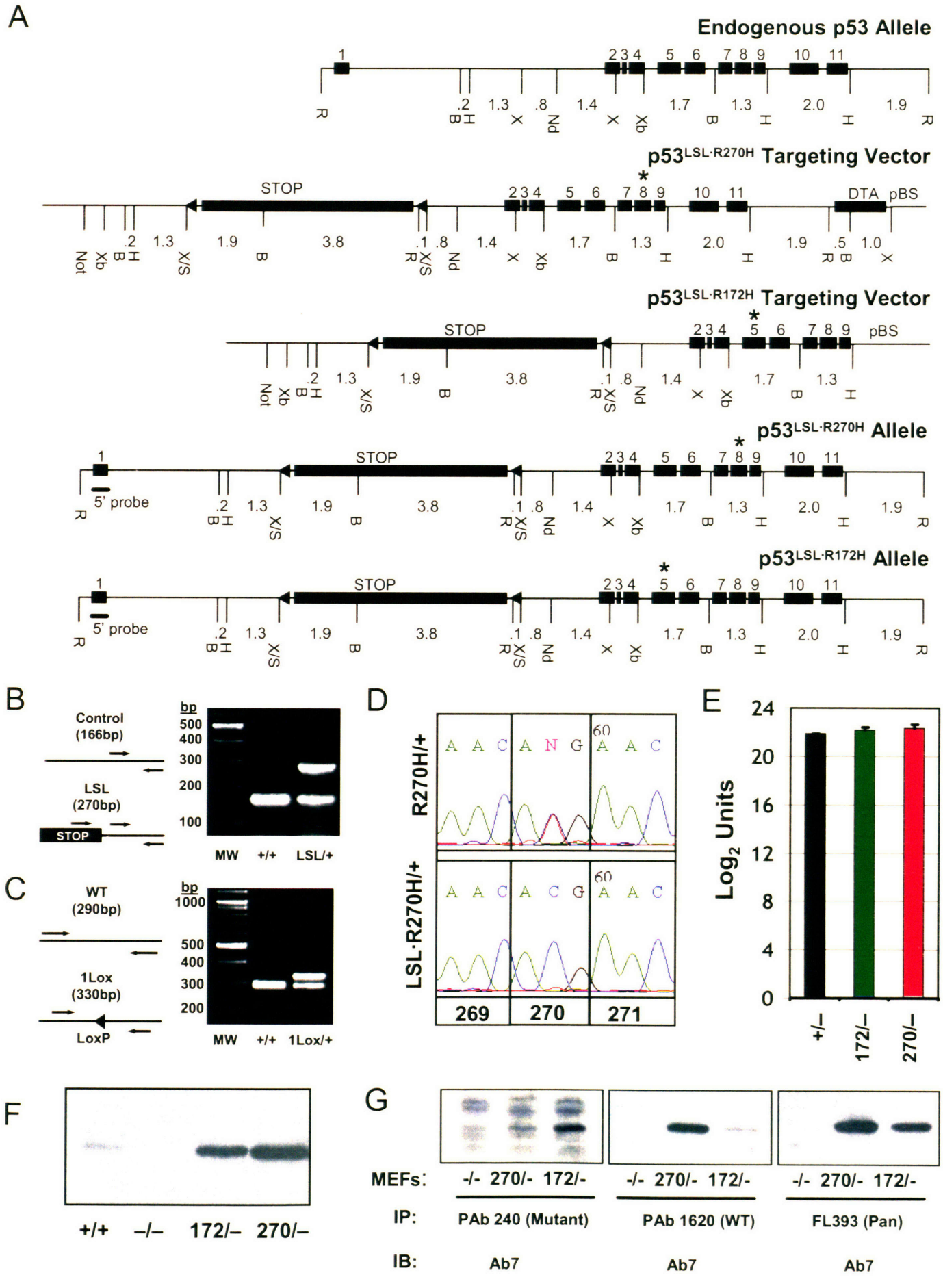
(C) PCR primers flanking the LSL cassette integration site yield a 290bp wild-type band and a 330bp product mutant p53 band.

(D) Sequence-traces from $p53^{LSL\cdot R270H/+}$ (bottom) and $p53^{R270H/+}$ (top) mouse brain cDNAs. N indicates expression of both the wild-type allele (C) and the mutant allele (T).

(E) qRT-PCR analysis of *p53* in WT, $p53^{R172H/-}$ and $p53^{R270H/-}$ MEFs. Histograms depict $\log_2$ induction relative to background in $p53^{-/-}$ MEFs and are the average of three different MEF lines, each assayed in triplicate. Error bars indicate standard error.

(F) Western blot analysis of MEF whole-cell lysates shows accumulation of mutant p53 proteins.

(G) Conformation of mutant p53 proteins was determined by immunoprecipitation of p53 proteins from 1000μg of MEF whole cell lysates with 10μL of either *p53* Ab5 (ORP), *p53* Ab3 (ORP) or FL-393 (Santa Cruz), followed by Western blotting with p53 Ab7 (ORP).


A
Endogenous p53 Allele
p53LSL·R270H Targeting Vector
p53LSL·R172H Targeting Vector
p53LSL·R270H Allele
p53LSL·R172H Allele
STOP
DTA
pBS
5' probe
B
Control (166bp)
LSL (270bp)
STOP
bp
MW
+/+
LSL/+
C
WT (290bp)
1Lox (330bp)
LoxP
1Lox/+
D
R270H/+
LSL·R270H/+
269
270
271
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172/–
270/–
F
+/+
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MEFs:
-/- 270/- 172/-
IP:
PAb 240 (Mutant)
PAb 1620 (WT)
FL393 (Pan)
IB:
Ab7

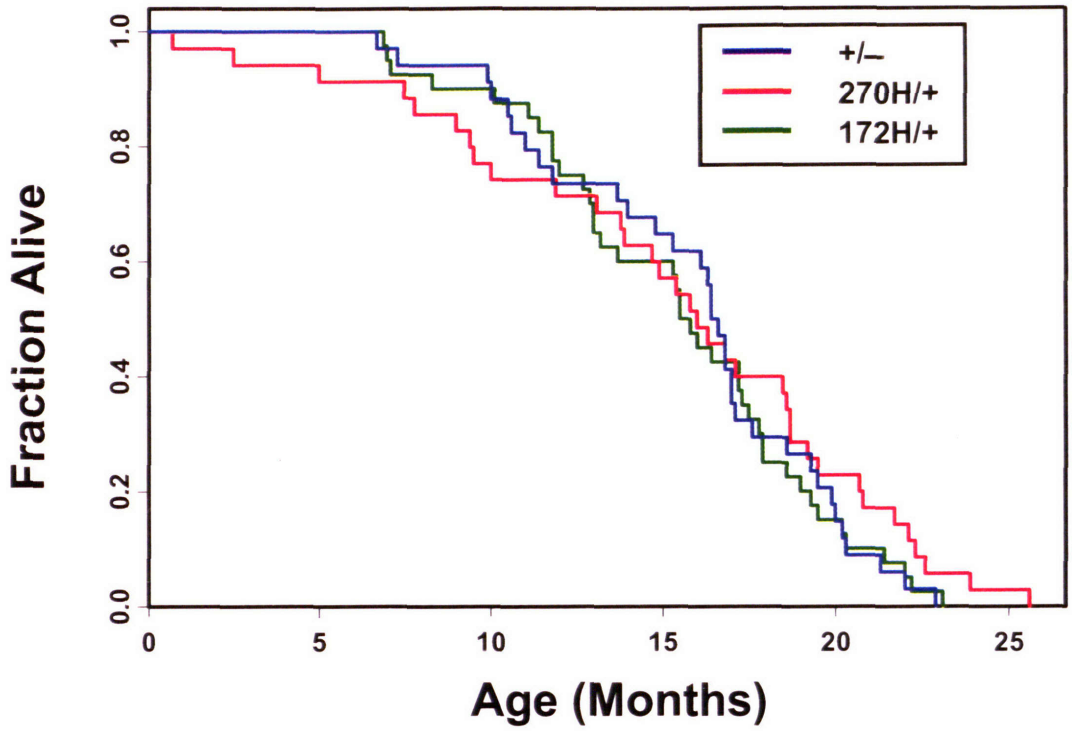
Figure 2. Mice Heterozygous for Mutant p53 Develop Novel Tumors

(A) Kaplan-Meyer plot demonstrates no difference in survival time between $p53^{+/-}$, $p53^{R270H/+}$, and $p53^{R172H/+}$ mice.

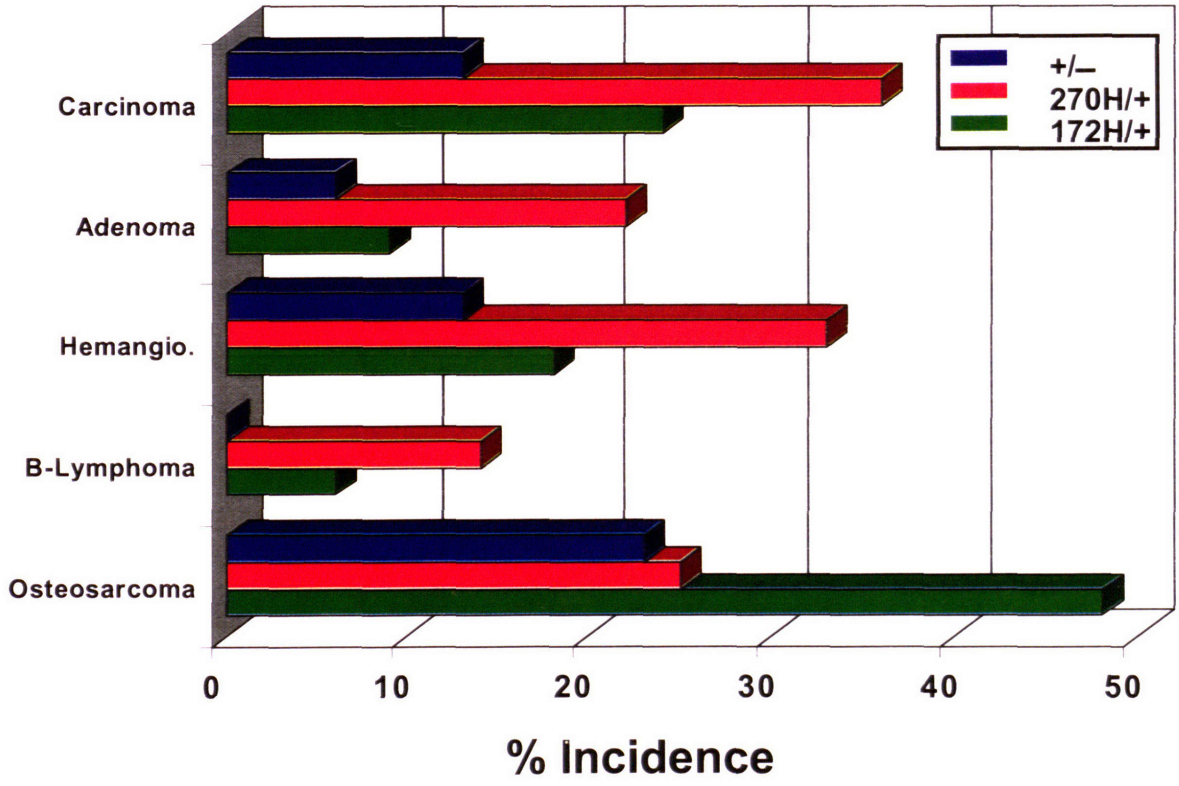
(B) Distinct tumor spectra in $p53^{M/+}$ mice. Histograms show the fraction of $p53^{+/-}$ (blue), $p53^{R270H/+}$ (red) and $p53^{R172H/+}$ mice (green) mice that developed carcinomas, adenomas, hemangiosarcomas, B-cell lymphomas or osteosarcomas. See Sup. Figure 1 for an expanded summary of the pathology of these mice.

(C) PCR analysis was performed on matched tumor (T) and somatic (S) DNA samples to look for LOH. At left is a dilution series of $p53^{M/M}$ and $p53^{+/+}$ DNA demonstrating the sensitivity of this assay.

A


Fraction Alive
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
20
25
Age (Months)
+/–
270H/+
172H/+

B


Carcinoma
Adenoma
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B-Lymphoma
Osteosarcoma
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% Incidence
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270H/+
172H/+

C

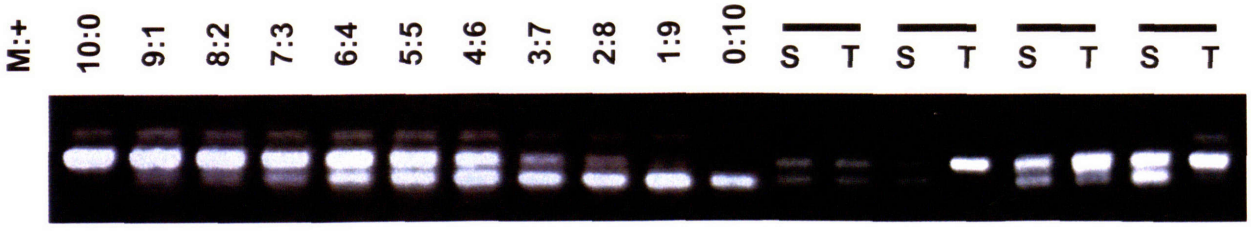
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Figure 3. Dominant effects by mutant p53 in primary cells.

(A) S-phase fraction in $p53^{M/+}$ MEFs. Cell cycle analysis was performed on exponentially growing MEFs. Histograms represent the percent of untreated cells (solid bars) or doxorubicin-treated cells (hashed bars- 0.2μg/mL, 12hrs) in S-phase as measured by FACS analysis of bromo-deoxyuridine and propidium iodide incorporation. Histograms show the average values of four independently derived MEF lines for each genotype. Error bars show standard deviation. Complete cell cycle data are presented in Sup. Figure 3a.

(B) Apoptosis in thymocytes from $p53^{M/+}$ mice. Thymocytes were isolated from 6-9 week old mice and treated with 5Gy of γ-radiation. Apoptosis was measured by FACS analysis of Annexin-V expression and propidium iodide exclusion. Graph depicts the percentage of viable cells (Annexin-V negative, propidium iodide negative) at 12 and 24 hours post treatment, normalized to untreated controls. The average values from at least 3 mice per genotype are shown.

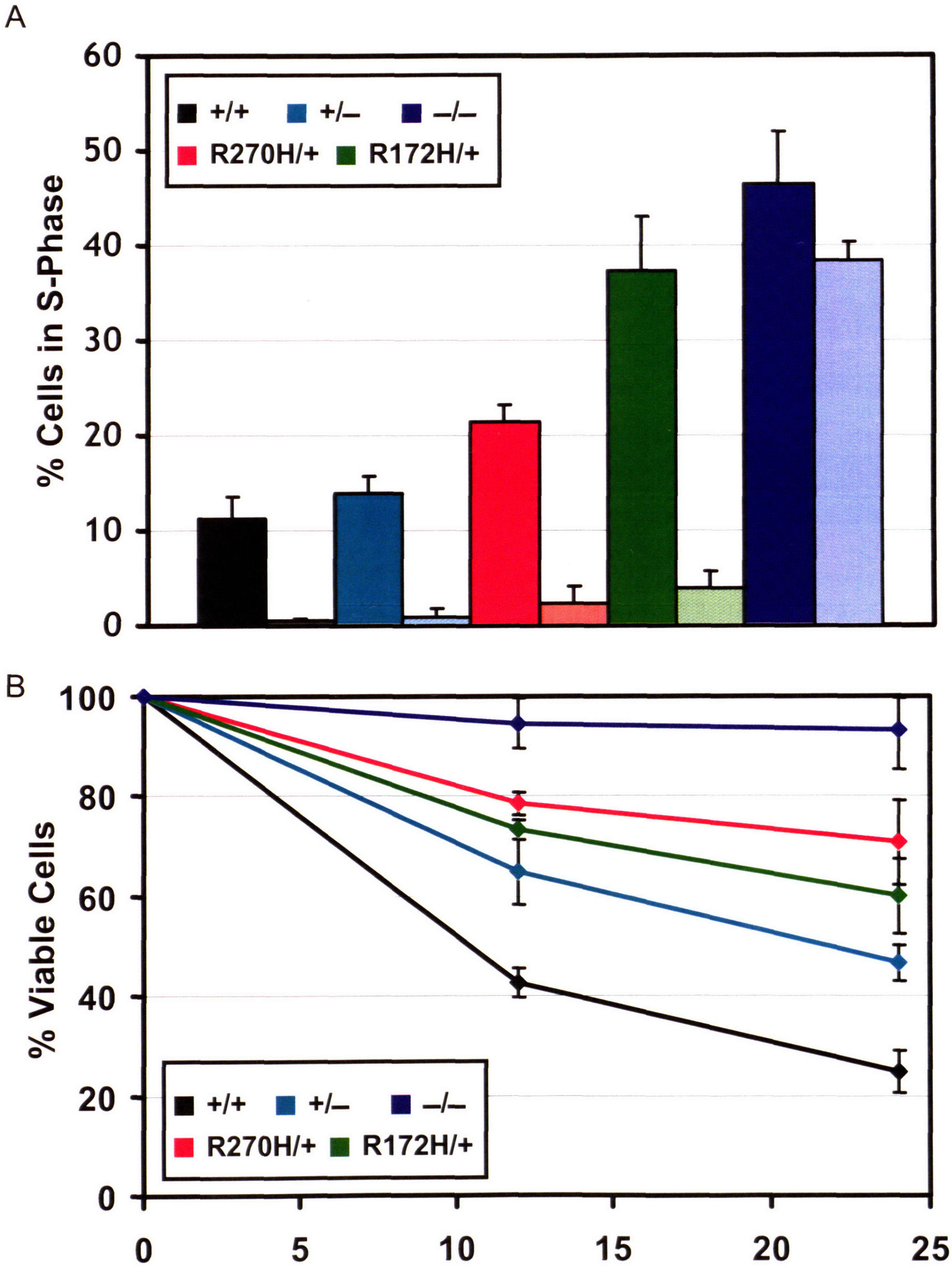
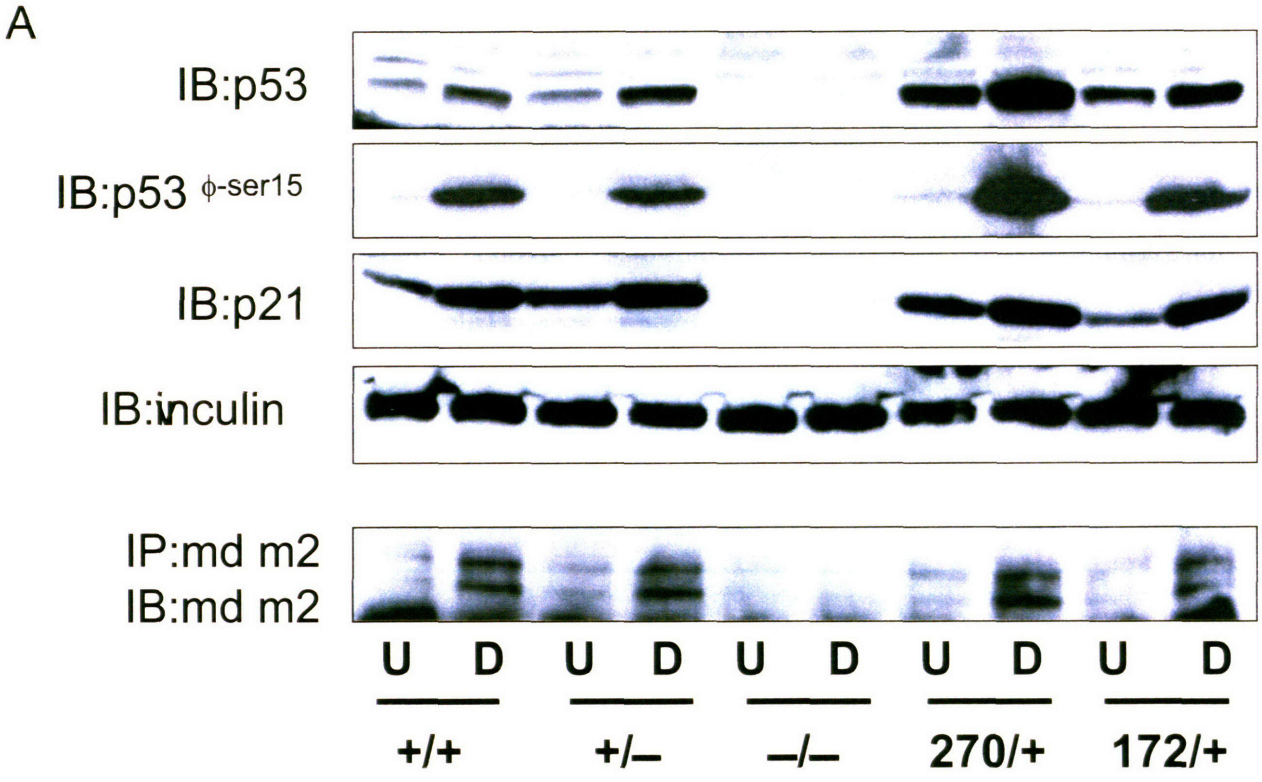
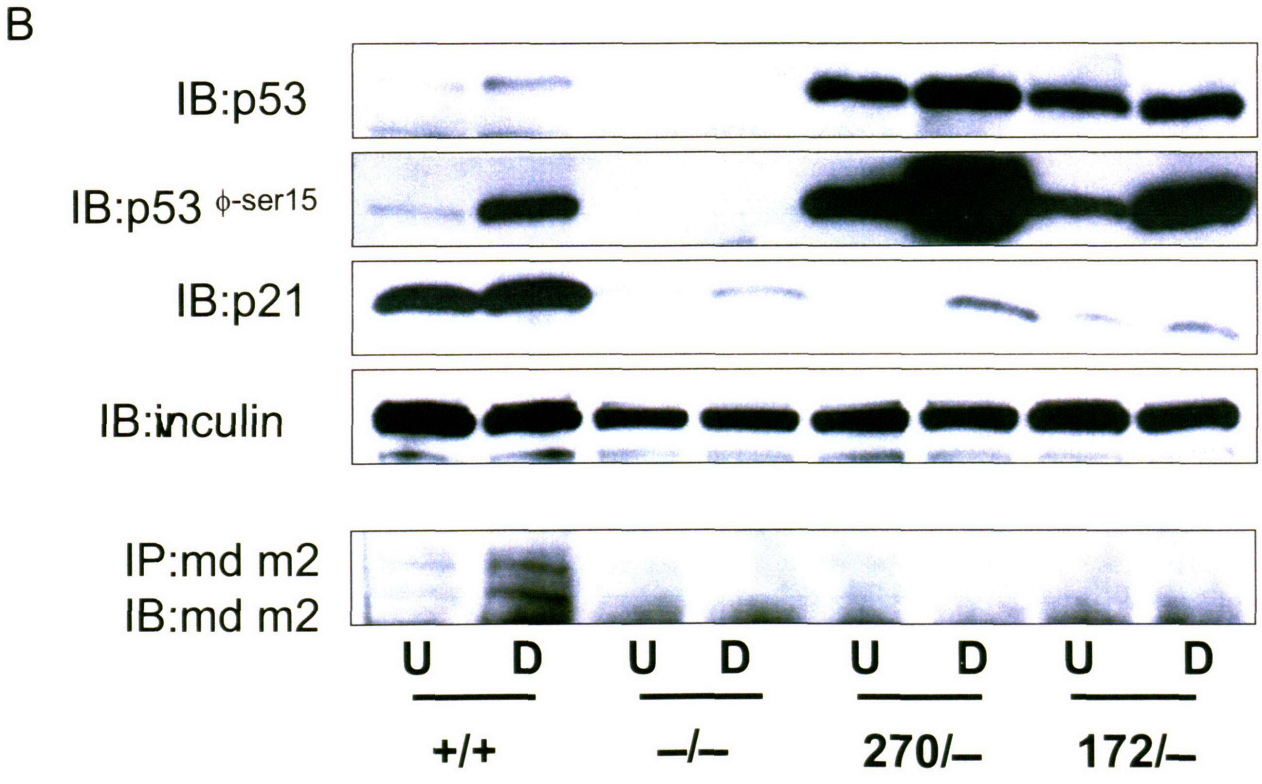

A
60
50
40
30
20
10
0
% Cells in S-Phase
+/+
+/–
–/–
R270H/+
R172H/+
B
100
80
60
40
20
0
% Viable Cells
+/+
+/–
–/–
R270H/+
R172H/+
0
5
10
15
20
25

Figure 4. *p53* Target Gene Expression in Mutant *p53* Cells

(A) Immunoblots for p53, ϕ-ser^{15}-p53 and p21 on MEF whole cell lysates are shown. Mdm2 immunoprecipitates from the same extracts are also shown.

(B) Immunoblots for p53, ϕ-ser^{15}-p53 and p21 on MEF whole cell lysates are shown. Mdm2 immunoprecipitates from the same extracts are also shown.


A
IB:p53
IB:p53 ϕ-ser15
IB:p21
IB:inculin
IP:md m2
IB:md m2
U D U D U D U D U D
+/+ +/– –/– 270/+ 172/+


B
IB:p53
IB:p53 ϕ-ser15
IB:p21
IB:inculin
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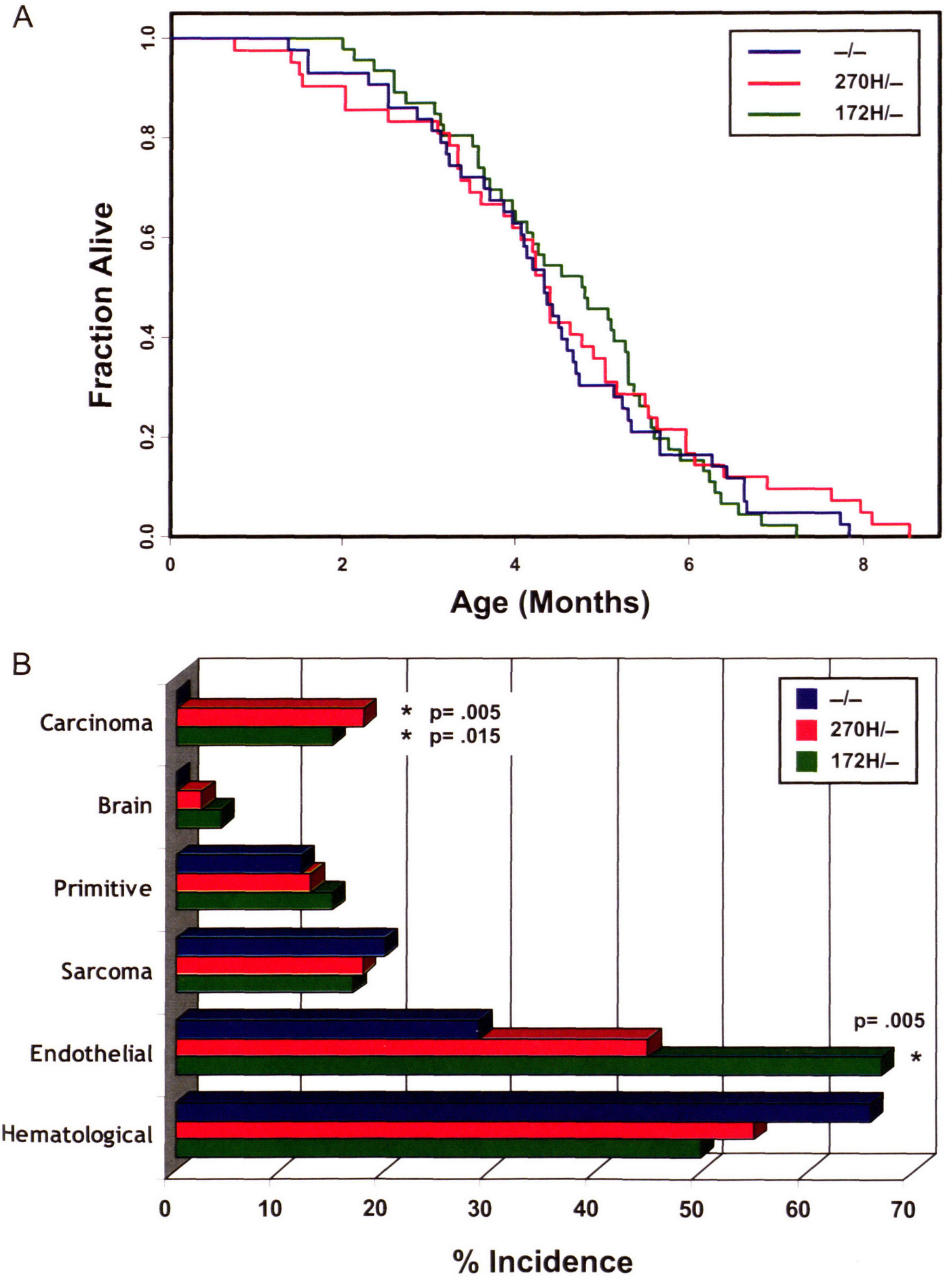
IB:md m2
U D U D U D U D
+/+ –/– 270/– 172/–

Figure 5. Point-Mutant *p53* Mice Develop Novel Tumors Compared to *p53* Knockout Mice

(A) Kaplan-Meyer Plot demonstrates no difference in survival time of $p53^{-/-}$, $p53^{R270H/-}$ and $p53^{R172H/-}$ mice.

(B) Histograms show the fraction of $p53^{-/-}$ (blue), $p53^{R270H/-}$ (red) and $p53^{R172H/-}$ mice (green) mice that developed carcinomas, brain tumors, primitive tumors (mostly teratocarcinomas), soft-tissue or bone sarcomas, endothelial tumors, or hematological tumors.


A
Fraction Alive
1.0
0.8
0.6
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8
Age (Months)
–/–
270H/–
172H/–
B
Carcinoma
Brain
Primitive
Sarcoma
Endothelial
Hematological
* p= .005
* p= .015
p= .005
*
–/–
270H/–
172H/–
0
10
20
30
40
50
60
70
% Incidence

Figure 6. Histopathology of Point-Mutant *p53* Tumors stained with hematoxylin and eosin.

(A) A moderately differentiated $p53^{R172H/-}$ colon adenocarcinoma with a high mitotic index, severe nuclear dysplasia (arrows), desmoplasia and stromal invasion. (400X)

(B) A $p53^{R172H/-}$ squamous cell carcinoma with well differentiated cells forming a keratin pearl (K) and poorly differentiated cells alone or in small clusters (arrows) surrounded by tumor stroma . (400X)

(C) A highly pleomorphic hepatocellular carcinoma (T) from a $p53^{R270H/-}$ mouse. Note the bizarre, oversized nuclei found within the tumor compared to the nuclei of adjacent normal liver cells (N). (400X)

(D) Intra-lymphatic metastasis (M) from a $p53^{R172H/-}$ colon adenocarcinoma. Arrow indicates the lymphendothelium. (400X)

(E) Wright-Giemsa stained peripheral blood smear from a $p53^{R172H/-}$ mouse with a colon adenocarcinoma. Circulating tumor cells are indicated (T) including an actively dividing, polyploid cell with a roughly 8N complement of chromosomes. Nearby are a normal neutrophil (N) and a cluster of platelets (P). (1000X)

(F) A $p53^{R172H/-}$ hemangiosarcoma. (400X)

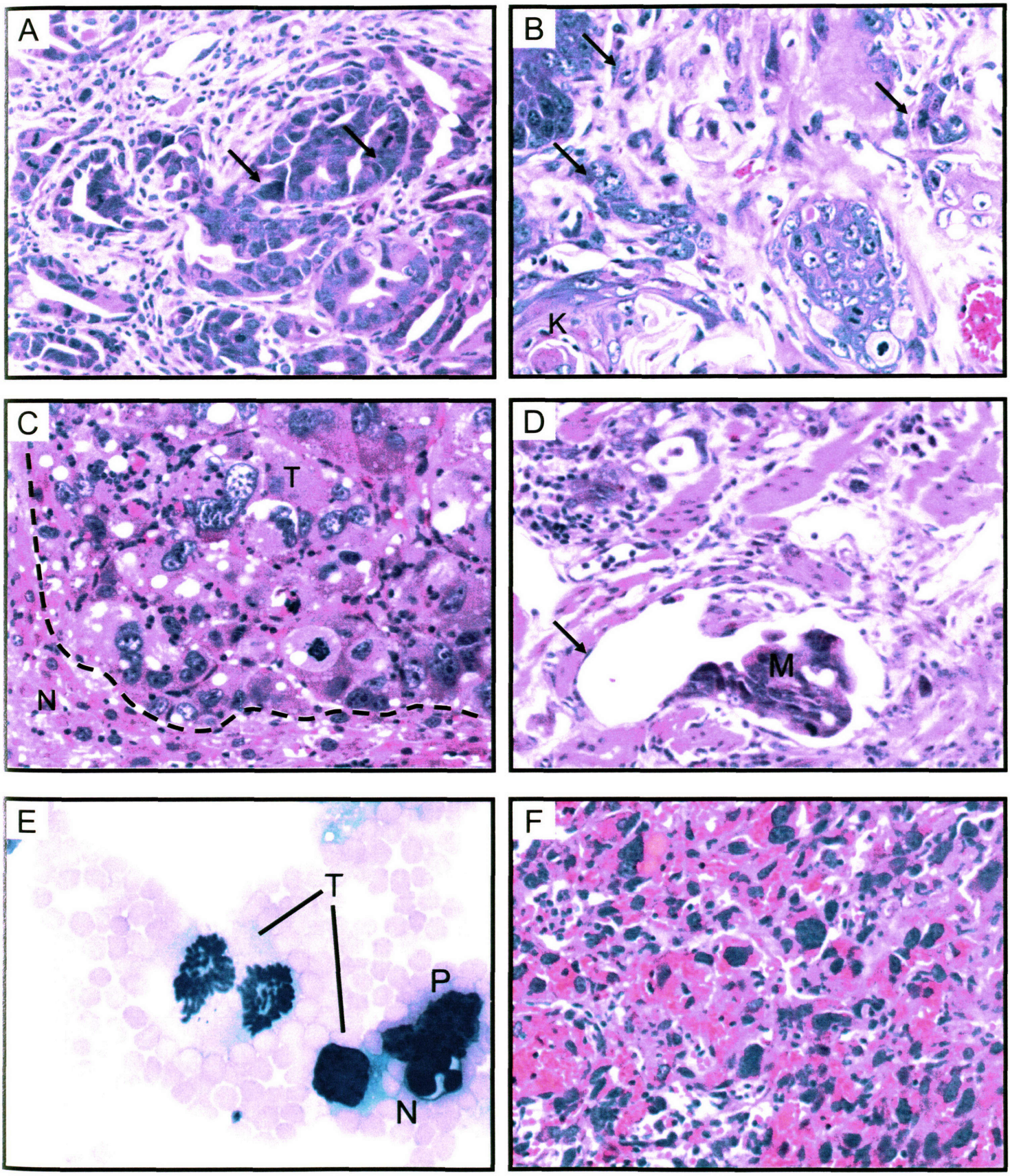
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Figure 7. Mutant p53 Gain-of-Function in Tumor-Derived Cells

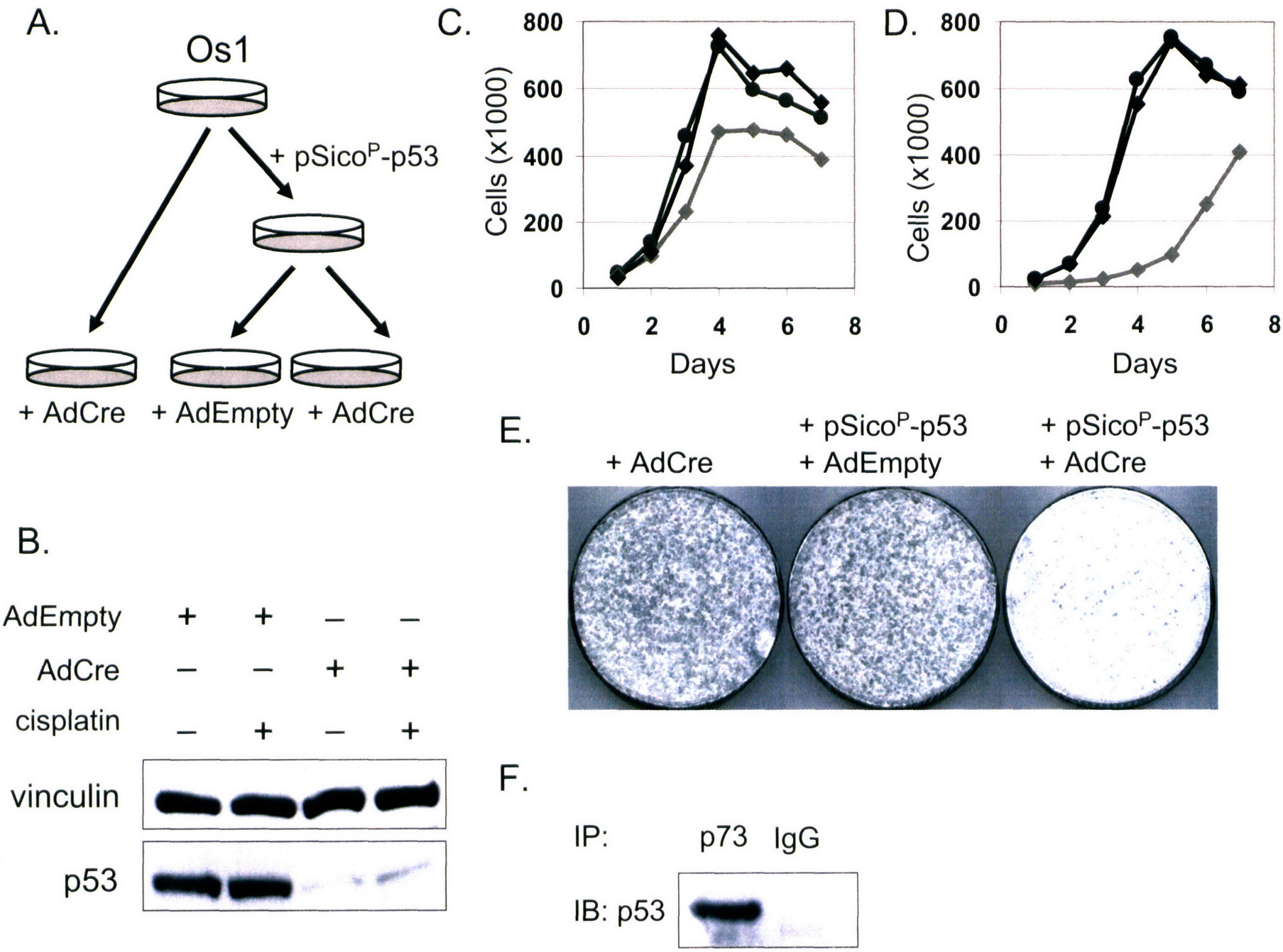
(A) Os1 cells were infected with pSico^{P}-p53 and AdCre in order to knock down endogenous mutant p53 expression. Control cells were infected with AdCre alone or pSico^{P}-p53 + AdEmpty.

(B) Western blot for p53 in Os1 + pSico^{P}-p53 after infection with AdCre or AdEmpty demonstrate a strong shRNA-mediated knock-down of mutant p53. The membrane was also probed with an antibody against vinculin as a loading control.

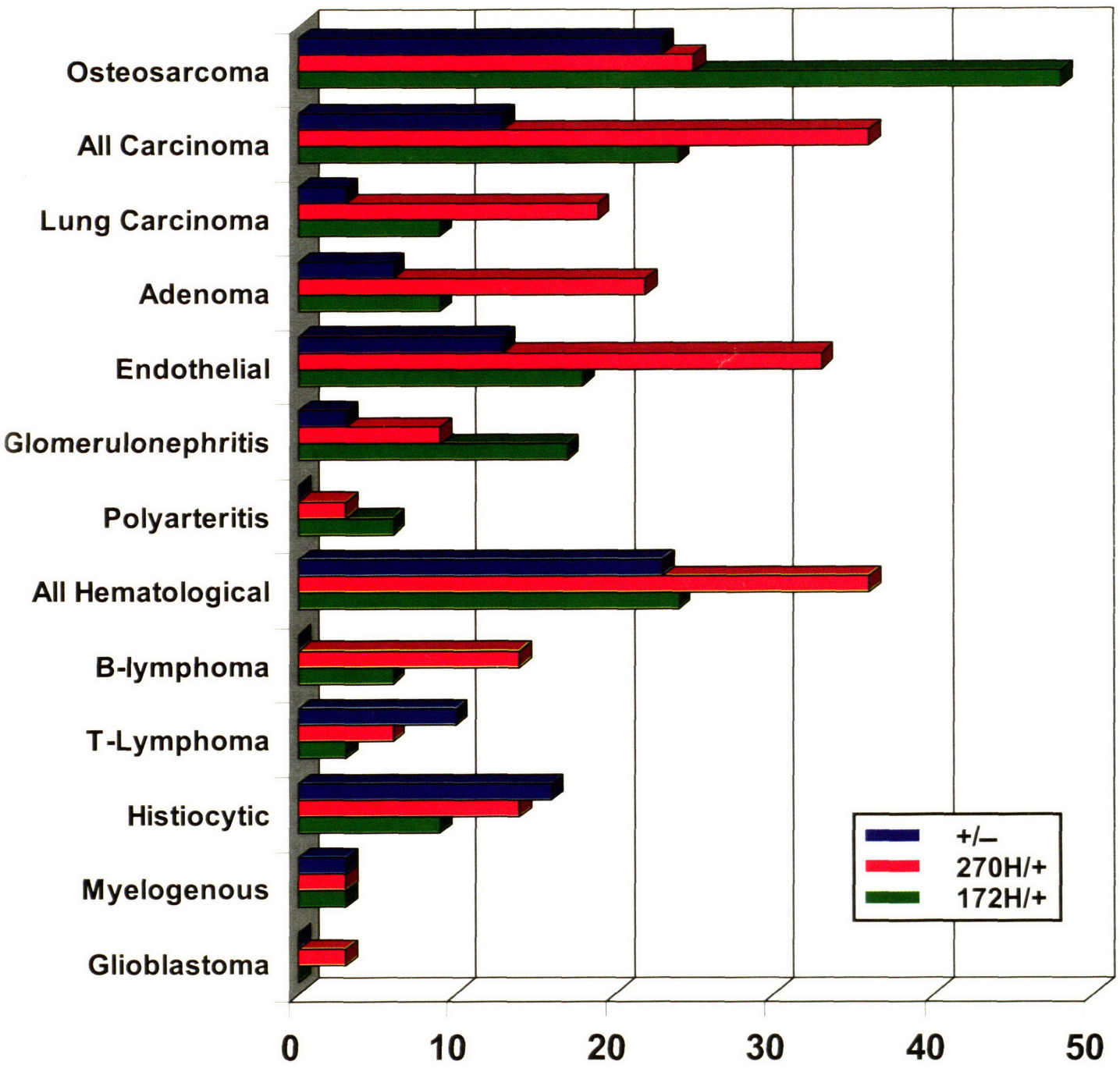
(C-D) Os1 cells proliferate more slowly after mutant p53 knockdown. 3 x 10^4 cells/well were plated in 12 well dishes and cells from two wells were counted daily. Experiments were initiated either three passages after adenoviral infection (C) or immediately following infection (D).

(E) Os1 cells proliferate to higher density prior to mutant p53 knockdown. Immediately following adenoviral infection, 2 x 10^4 Os1 cells were diluted into 1x 10^6 wild-type MEFs and plated in 10cm dishes. After 1 week, cells plates were stained with Wright-Giemsa. Darker staining in plates without mutant p53 KD reflect the ability of Os1 cells to rapidly grow to high density rather than their ability to form classic foci.

(F) Mutant p53 is coimmunoprecipitated by p73 Ab2 (ORP), but not by a control antibody. Immunoblot was performed using p53 Ab7.


A.
Os1
+ pSicoP-p53
+ AdCre
+ AdEmpty
+ AdCre
B.
AdEmpty + + – –
AdCre – – + +
cisplatin – + – +
vinculin
p53
C.
Cells (x1000)
800
600
400
200
0
0 2 4 6 8
Days
D.
Cells (x1000)
800
600
400
200
0
0 2 4 6 8
Days
E.
+ AdCre
+ pSicoP-p53
+ AdEmpty
+ pSicoP-p53
+ AdCre
F.
IP: p73 IgG
IB: p53

Supplemental Figure 1. Full Tumor Spectrum of $p53^{M/+}$ Mice. $p53^{M/+}$ mice develop distinct tumor spectra. Histograms represent the fraction of $p53^{+/-}$ (blue), $p53^{R270H/+}$ (red) and $p53^{R172H/+}$ mice (green) mice that developed the indicated pathologies.


Osteosarcoma
All Carcinoma
Lung Carcinoma
Adenoma
Endothelial
Glomerulonephritis
Polyarteritis
All Hematological
B-lymphoma
T-Lymphoma
Histiocytic
Myelogenous
Glioblastoma
0
10
20
30
40
50
+/–
270H/+
172H/+

Supplemental Figure 2. Histopathology of $p53^{M/+}$ Mouse Tumors Stained with H&E.

(A) Lung adenocarcinoma in a $p53^{R270H/+}$ mouse. **D** indicates the presence of a desmoplastic stroma. Arrow indicates a small cluster of tumor cells embedded in tumor stroma. (400X)

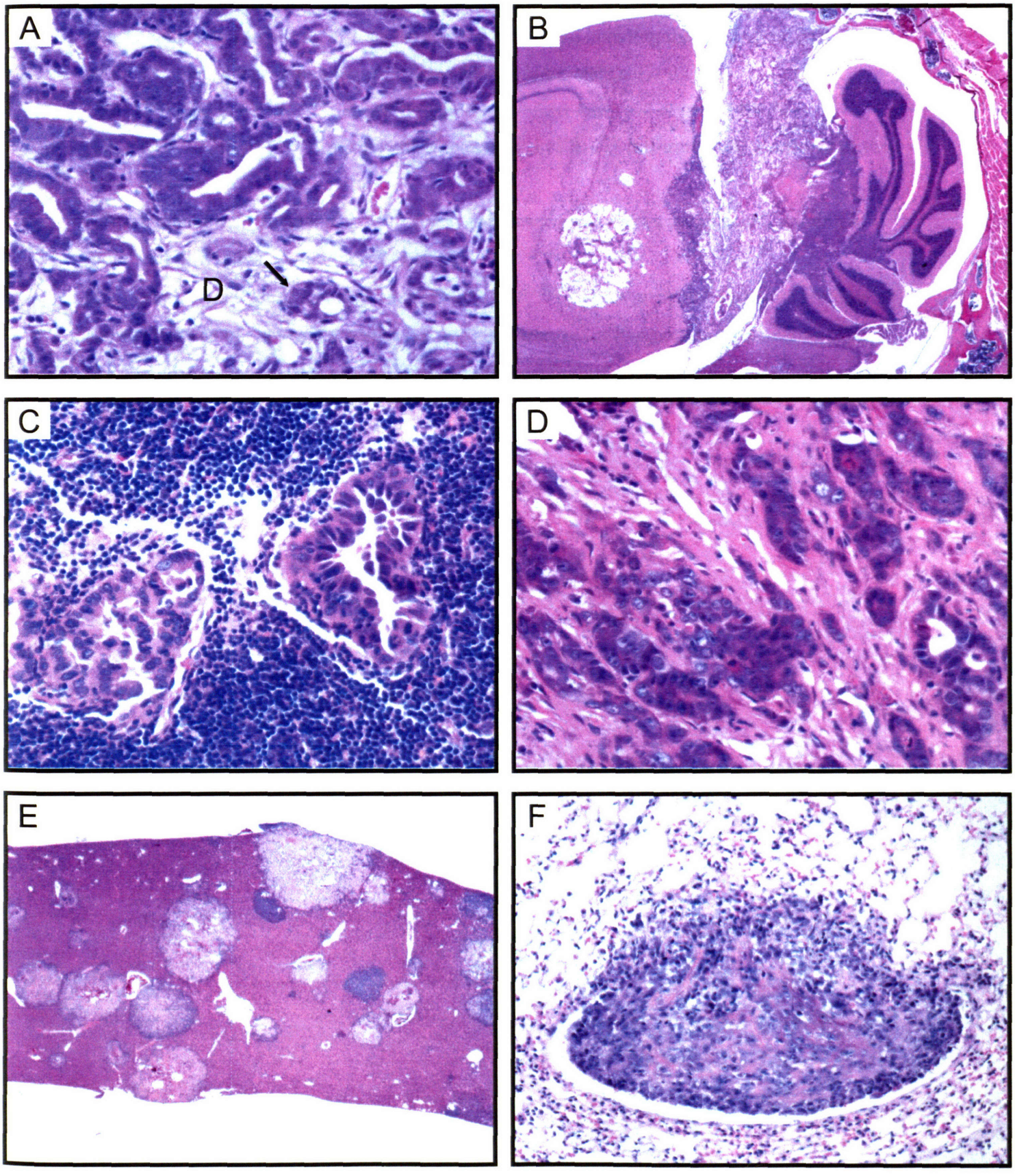
(B) Squamous cell carcinoma in a $p53^{R270H/+}$ mouse. This highly malignant tumor arose in the epidermis of the face and invaded through the skull into the brain, destructively penetrating the cerebellum. (4X)

(C) Metastases from a lung adenocarcinoma in a thoracic lymph node from a $p53^{R172H/+}$ mouse. (400X)

(D) Desmoplasia and stromal invasion in an intestinal adenocarcinoma from a $p53^{R270H/+}$ mouse. Most of these tumors cells are poorly differentiated and have lost their glandular architecture although one small structure can be observed at the left side of the panel. (400X)

(E) Osteosarcoma metastases in the liver of a $p53^{R172H/+}$ mouse. (4X)

(F) Osteosarcoma metastasis in the lung of a $p53^{R172H/+}$ mouse. (400X)


A
D
B
C
D
E
F

Supplemental Figure 3. Immunohistochemistry on $p53^{M/+}$ and Point-Mutant *p53* Tumors

(A) Cytokeratin 14 staining of a squamous cell carcinoma invading the cerebellum of a $p53^{R172H/+}$ mouse (see Supplemental Figure S2b for H&E stain). (200X)

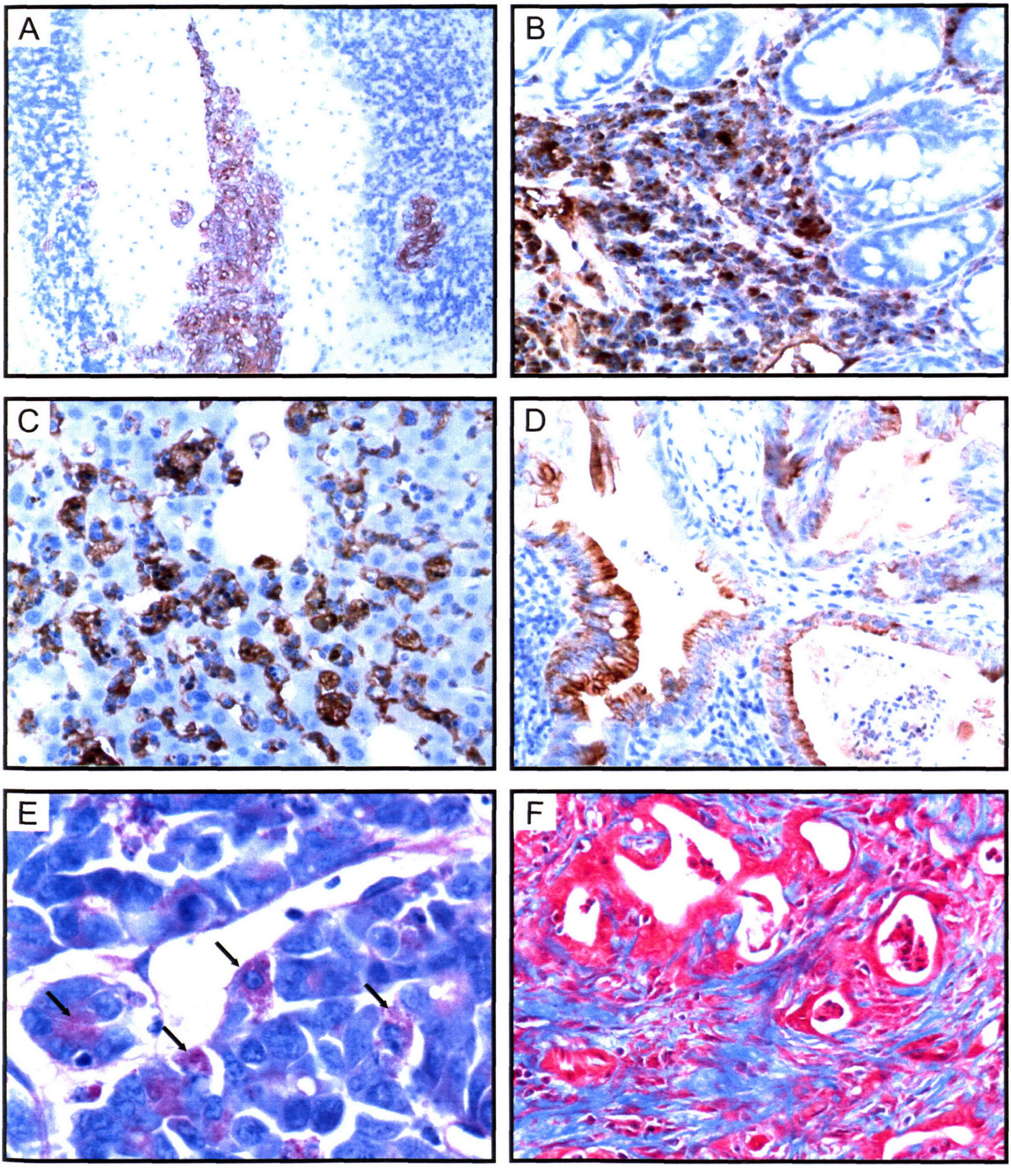
(B) B220 IHC in a $p53^{R270H/+}$ B-cell lymphoma invading intestinal crypts. (400X)

(C) F4/80 IHC in $p53^{R172H/+}$ histiocytic sarcoma cells diffusely infiltrating the sinusoids of the liver. (400X)

(D) Cytokeratin 8 IHC in a $p53^{R270H/-}$ colon adenocarcinoma shows cytoplasmic staining in more well differentiated tumor cells. (200X)

(E) Periodic Acid Schiff staining (in pink) of a $p53^{R270H/-}$ acinar pancreatic adenocarcinoma shows evidence of zymogen granules (arrows) in more well differentiated tumor cells. (1000X)

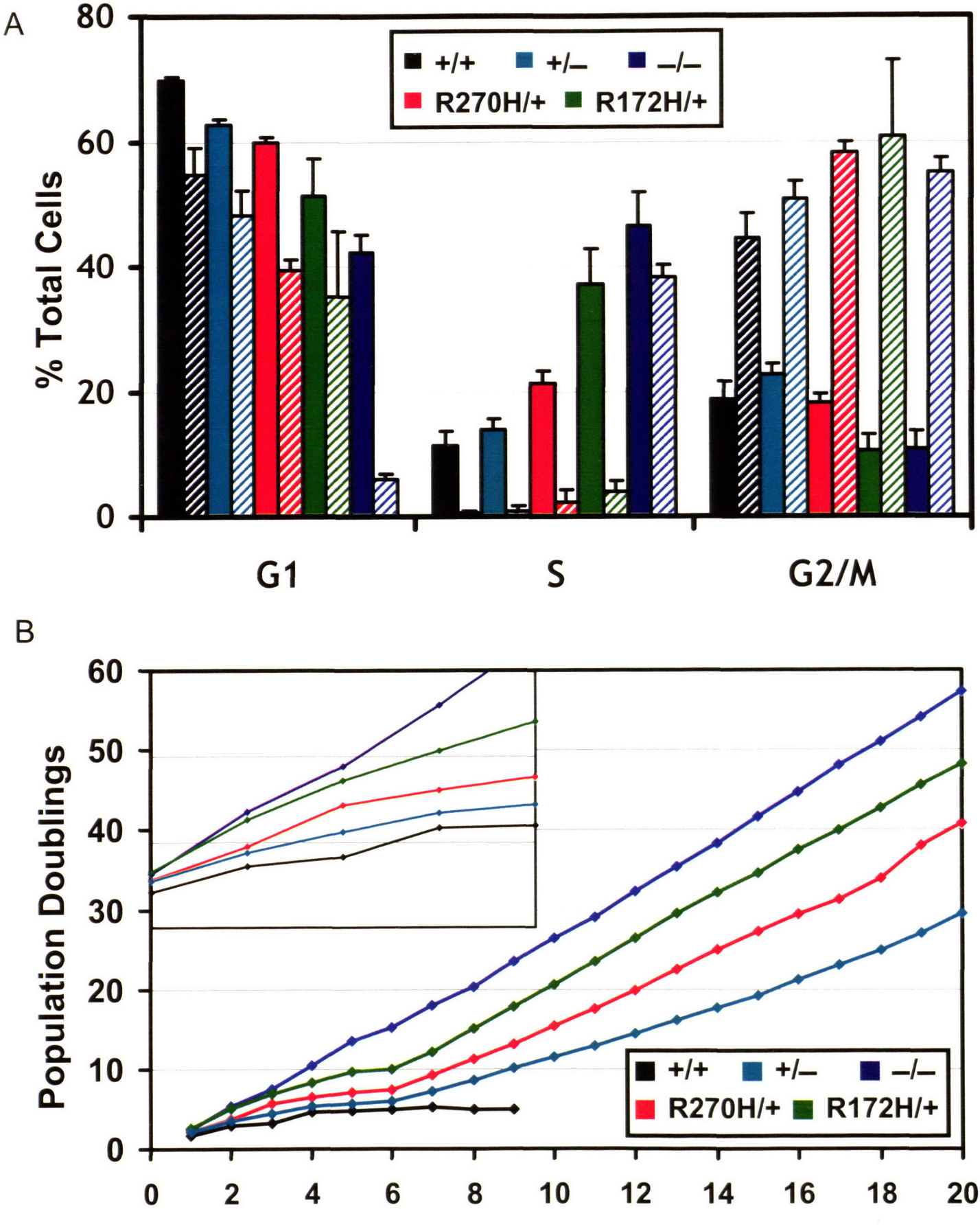
(F) Trichrome staining on a $p53^{R270H/-}$ colon adenocarcinoma showing evidence of desmoplasia (collagen stains light blue). (400X)


A
B
C
D
E
F

Supplemental Figure 4. 3T3 Immortalization and Cell Cycle Analysis of $p53^{M/+}$ MEFs.

(A) Cell cycle analysis was performed on exponentially growing $p53^{+/+}$, $p53^{+/-}$, $p53^{-/-}$, $p53^{R270H/+}$, and $p53^{R172H/+}$ MEFs. Histograms represent the percent of untreated cells (solid bars) or doxorubicin-treated cells (hashed bars- 0.2μg/mL, 12hrs) in G_1, S or G_2/M phases as measured by FACS analysis of bromo-deoxyuridine and propidium iodide incorporation. Data represents the average values of four independently derived MEF lines for each genotype.

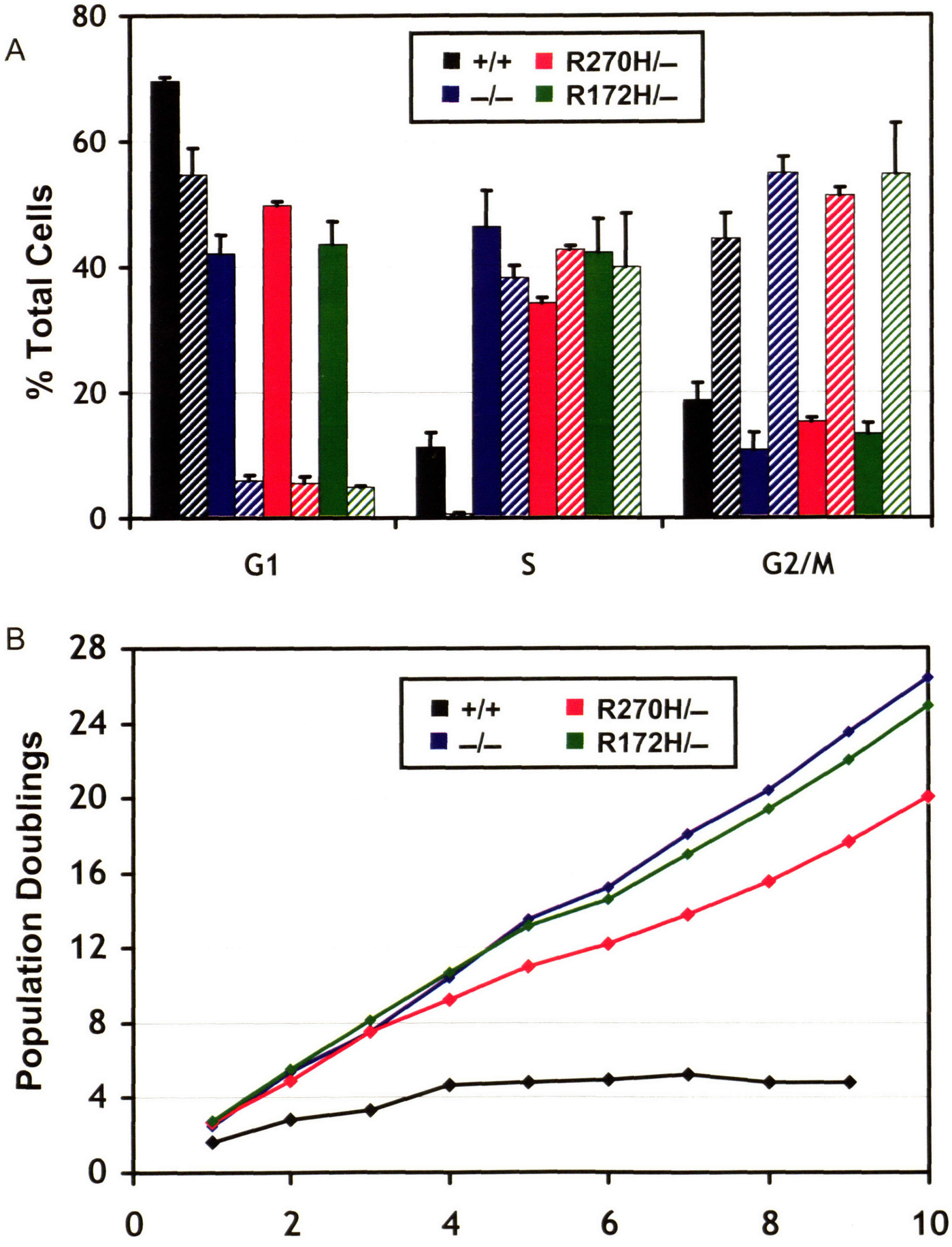
(B) Immortalization assays were performed on $p53^{+/+}$, $p53^{+/-}$, $p53^{-/-}$, $p53^{R270H/+}$, and $p53^{R172H/+}$ MEFs using the 3T3 protocol. Lines represent the average cumulative population doublings for four independently derived MEF lines for each genotype. Inset shows the first four doublings more clearly.


A
80
60
40
20
0
% Total Cells
+/+
+/–
–/–
R270H/+
R172H/+
G1
S
G2/M
B
60
50
40
30
20
10
0
Population Doublings
0
2
4
6
8
10
12
14
16
18
20
+/+
+/–
–/–
R270H/+
R172H/+

Supplemental Figure 5. 3T3 Immortalization and Cell Cycle Analysis of Mutant *p53* MEFs.

(A) Cell cycle analysis performed on exponentially growing $p53^{+/+, p53-/-}$, $p53^{R270H/-}$, and $p53^{R172H/-}$ MEFs. Histograms represent the percent of untreated cells (solid bars) or doxorubicin-treated cells (hashed bars- 0.2μg/mL, 12hrs) in G_1, S or G_2/M phases as measured by FACS analysis of bromo-deoxyuridine and propidium iodide incorporation. Data represents the average values of four independently derived MEF lines for each genotype.

(B) Immortalization assays were performed on $p53^{+/+}$, $p53^{-/-}$, $p53^{R270H/-}$ and $p53^{R172H/-}$MEFs using the 3T3 protocol. Lines represent the average cumulative population doublings for four independently derived MEF lines for each genotype.


A
80
60
40
20
0
% Total Cells
+/+
R270H/–
–/–
R172H/–
G1
S
G2/M
B
28
24
20
16
12
8
4
0
Population Doublings
+/+
R270H/–
–/–
R172H/–
0
2
4
6
8
10

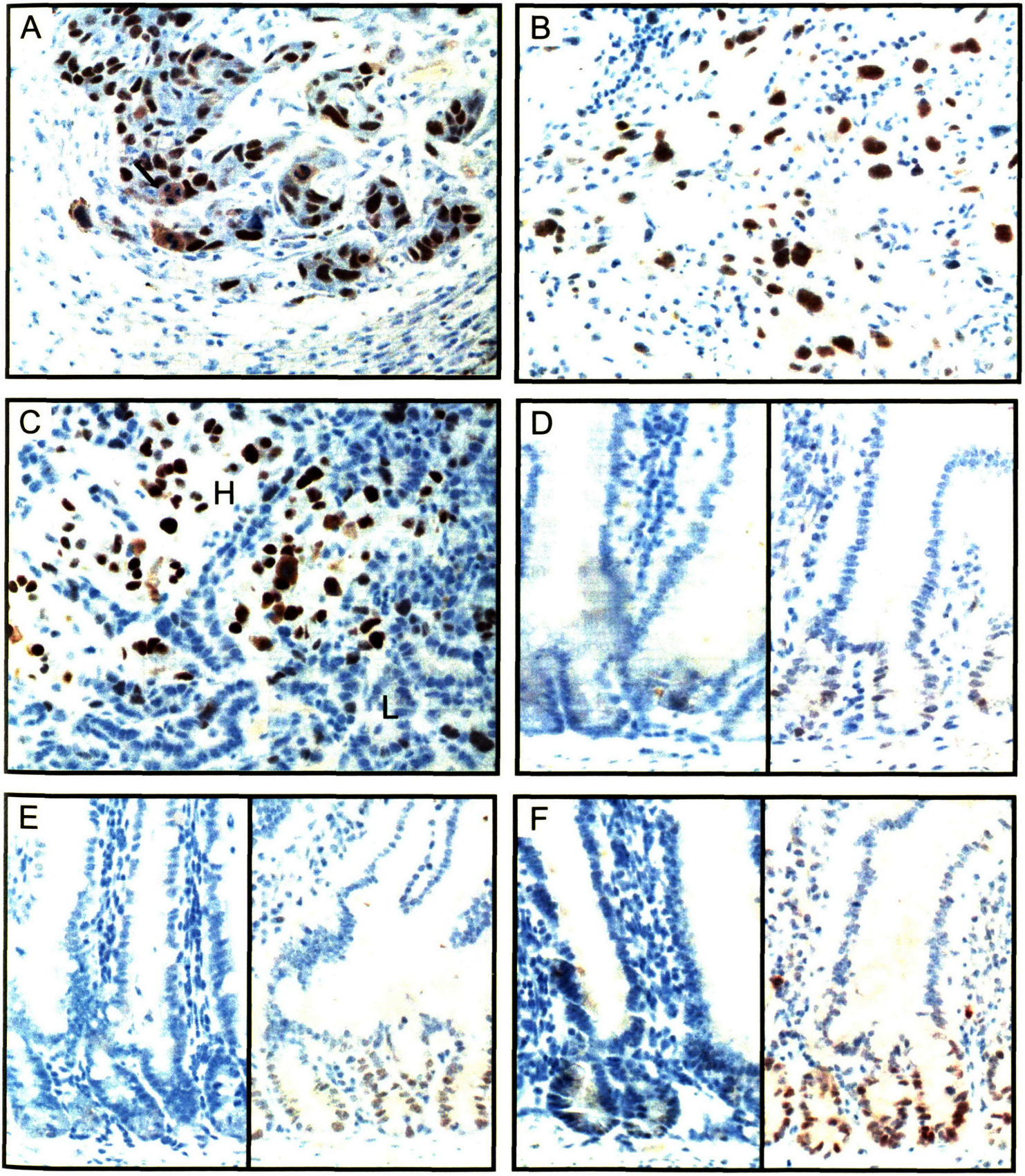
Supplemental Figure 6. Accumulation of Mutant p53 in Tumor Cells.

(A) *p53* IHC on a $p53^{R270H/+}$ squamous cell carcinoma demonstrating strong nuclear accumulation of mutant *p53* in tumor cells but not normal cells. Arrow indicates an aberrant mitosis. (400X)

(B) *p53* IHC on a $p53^{R270H/-}$ hemangiosarcoma. The large, pleomorphic tumor cells accumulate mutant *p53* while surrounding normal cells do not. (400X)

(C) *p53* IHC on a $p53^{R172H/+}$ lung adenocarcinoma. (L) indicates a less aggressive region of the tumor, with organized cells that form glandular structures, and smaller, more regular nuclei. (H) indicates a more aggressive region with poor organization, enlarged and irregular nuclei and desmoplasia (unstained, white regions). Strong p53 accumulation is restricted to the more aggressive regions of the tumor. (400X)

(D-F) *p53* IHC on normal $p53^{+/+}$ (D), $p53^{R172H/-}$ (E) and $p53^{R270H/-}$ (F) intestines. Each panel presents sections from untreated mice (left) and from mice 24 hours after receiving 8Gy of whole-body gamma radiation. All six images were acquired with equal contrast and brightness settings. Mutant p53 accumulates to high levels in many cells of the intestinal crypts after irradiation. (400X)


A
B
C
H
L
D
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Supplemental Table 1- Indicates immunohistochemistry (IHC) performed on tumors to confirm their diagnosis as carcinomas.

ID	Gntp	Tumor	IHC
K2950	R172H/-	Ductal Pancreatic Carcinoma	CK8+
K3080	R172H/-	Colon Adenocarcinoma	CK8+
K3846	R172H/-	Lung Adenocarcinoma	CK8+
K1274	R270H/-	Lung Adenocarcinoma	CK8+
K1275	R270H/-	Pancreatic Acinar Adenocarcinoma	PAS+, Fli1-
K1521	R270H/-	Colon Adenocarcinoma	CK8+
K1765	R270H/-	Colon Adenocarcinoma	CK8+
K3068	R172H/+	Lung Adenocarcinoma	CK8+
K3262	R172H/+	Rectal Carcinoma	CK8+
K3441	R172H/+	Lung Adenocarcinoma	CK8+
K3738	R172H/+	Lung Adenocarcinoma	CK8+
K1074	R270H/+	Lung Adenocarcinoma	CK8+
K1074	R270H/+	Squamous Cell Carcinoma	CK14+
K1276	R270H/+	Intestinal Adenocarcinoma	CK8+
K1280	R270H/+	Lung Adenocarcinoma	CK8+
K1438	R270H/+	Squamous Cell Carcinoma	CK14+
K1440	R270H/+	Lung Adenocarcinoma	CK8+
K1518	R270H/+	Lung Adenocarcinoma	CK8+
K1545	R270H/+	Squamous Cell Carcinoma	CK14+
K1555	R270H/+	Squamous Cell Carcinoma	CK14+
K1674	R270H/+	Squamous Cell Carcinoma	CK14+
K1770	R270H/+	Lung Adenocarcinoma	CK8+
K1549	+/-	Lung Adenocarcinoma	CK8+

Supplemental Table 2- p53 protein accumulation in mutant p53 mouse tumors. 0- no accumulation, 1- weakly positive staining 2- strongly positive staining

ID	Gntp	Tumor	p53	ID	Gntp	Tumor	p53
K3436	+/-	Bladder Carcinoma	++	K1341	-/-	BCL	0
K1532	+/-	Histiocytic Sarcoma	+	K1661	-/-	BCL	0
K1658	+/-	Histiocytic Sarcoma	0	K1313	-/-	Hemangiosarcoma	0
K3424	+/-	Histiocytic Sarcoma	0	K1603	-/-	Hemangiosarcoma	0
K1442	+/-	Leiomyosarcoma	0	K1733	-/-	Hemangiosarcoma	0
K1549	+/-	Lung Adenocarcinoma	0	K1548	-/-	Histiocytic Sarcoma	0
K1549	+/-	Osteosarcoma	0	K3442	-/-	Histiocytic Sarcoma	0
K3482	+/-	Osteosarcoma	0	K1075	-/-	TCL	0
K3659	+/-	Osteosarcoma	0	K1527	-/-	TCL	0
K3743	+/-	Osteosarcoma	0	K1656	R270H/-	Rhabdomyosarcoma	++
K1076	R270H/+	BCL	++	K1519	R270H/-	BCL	+
K1520	R270H/+	BCL	++	K1668	R270H/-	breast carcinoma	++
K1551	R270H/+	BCL	0	K1521	R270H/-	Colon Carcinoma	++
K1623	R270H/+	BCL	++	K1765	R270H/-	Colon Carcinoma	0
K1730	R270H/+	BCL	0	K1149	R270H/-	Hemangiosarcoma	++
K1671	R270H/+	Histiocytic Sarcoma	++	K1620	R270H/-	Hemangiosarcoma	++
K1732	R270H/+	Histiocytic Sarcoma	0	K1654	R270H/-	Hemangiosarcoma	++
K1782	R270H/+	Histiocytic Sarcoma	++	K1762	R270H/-	Hemangiosarcoma	++
K1276	R270H/+	Intestinal Carcinoma	++	K1783	R270H/-	HCC	0
K1074	R270H/+	Lung Adenocarcinoma	++	K1274	R270H/-	Lung Adenocarcinoma	++
K1280	R270H/+	Lung Adenocarcinoma	++	K1274	R270H/-	Myelogenous Leukemia	++
K1440	R270H/+	Lung Adenocarcinoma	0	K1240	R270H/-	Osteosarcoma	++
K1518	R270H/+	Lung Adenocarcinoma	0	K1275	R270H/-	Pancreatic Carcinoma	++
K1555	R270H/+	Lung Adenocarcinoma	++	K1546	R270H/-	Rhabdomyosarcoma	0
K1770	R270H/+	Lung Adenocarcinoma	++	K1668	R270H/-	Rhabdomyosarcoma	0
K1518	R270H/+	Myelogenous Leukemia	0	K1245	R270H/-	TCL	0
K1277	R270H/+	Osteosarcoma	++	K1279	R270H/-	TCL	0
K1551	R270H/+	Osteosarcoma	+	K1526	R270H/-	TCL	+
K1611	R270H/+	Osteosarcoma	++	K1600	R270H/-	TCL	0
K1770	R270H/+	Osteosarcoma	++	K1657	R270H/-	TCL	+
K1074	R270H/+	SCC	++	K1876	R270H/-	TCL	0
K1438	R270H/+	SCC	++	K1886	R270H/-	TCL	0
K1545	R270H/+	SCC	++	K1613	R270H/-	Undiff. Neoplasm	0
K1674	R270H/+	SCC	++	K3662	R172H/-	BCL	0
K1280	R270H/+	Undiff. Neoplasm	++	K4031	R172H/-	BCL	++
K3325	R172H/+	BCL	0	K3662	R172H/-	BCL	0
K3557	R172H/+	BCL	++	K3080	R172H/-	Colon Carcinoma	++
K3653	R172H/+	Pancreatic Carcinoma	0	K2950	R172H/-	Pancreatic Carcinoma	++
K3435	R172H/+	Hematological Tumor	0	K3660	R172H/-	Hemangiosarcoma	++
K3354	R172H/+	Histiocytic Sarcoma	++	K3825	R172H/-	Hemangiosarcoma	++
K3654	R172H/+	Histiocytic Sarcoma	0	K3236	R172H/-	Hemangiosarcoma	++
K3068	R172H/+	Lung Adenocarcinoma	0	K4106	R172H/-	Hemangiosarcoma	++
K3441	R172H/+	Lung Adenocarcinoma	++	K2927	R172H/-	HCC	0
K3738	R172H/+	Lung Adenocarcinoma	++	K3349	R172H/-	Histiocytic Sarcoma	0
K3328	R172H/+	Myelogenous Leukemia	++	K3909	R172H/-	Leiomyosarcoma	0
K2955	R172H/+	Osteosarcoma	++	K4099	R172H/-	Leiomyosarcoma	++
K3265	R172H/+	Osteosarcoma	++	K3846	R172H/-	Lung Adenocarcinoma	++
K3266	R172H/+	Osteosarcoma	++	K3919	R172H/-	Myelogenous Leukemia	++
K3325	R172H/+	Osteosarcoma	++	K3545	R172H/-	Osteosarcoma	0
K3328	R172H/+	Osteosarcoma	++	K4027	R172H/-	Rhabdomyosarcoma	0
K3354	R172H/+	Osteosarcoma	++	K2927	R172H/-	SCC	0
K3422	R172H/+	Osteosarcoma	0	K3946	R172H/-	SCC	++
K3477	R172H/+	Osteosarcoma	++	K3291	R172H/-	TCL	+
K3620	R172H/+	Osteosarcoma	++	K3080	R172H/-	TCL	+
K3262	R172H/+	Rectal carcinoma	++	K3273	R172H/-	TCL	0
				K3497	R172H/-	TCL	0
				K3910	R172H/-	TCL	+
				K3552	R172H/-	TCL	0
				K3850	R172H/-	TCL	0
				K3946	R172H/-	TCL	0

Chapter 3

Differential Effects of Mutant p53 Alleles on the Development of Advanced Murine Lung Cancer and Nasopharyngeal Carcinoma

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* These authors contributed equally to this work

Submitted in similar form to PLOS Biology for consideration.

This work was carried out as an equal collaboration between the author and Erica L. Jackson. Assistance with genotyping was provided by Zachary C. Ruhe and Bob Yin. A substantial volume of histology preparation was performed by Michael Brown and Denise Crowley of the MIT Center for Cancer Research Histology Core Facility and analysis of pathology was performed with the assistance of Roderick T. Bronson.

ABSTRACT

We report the creation of a murine model of spontaneous advanced lung adenocarcinoma through compound mutation of *K-ras* and *p53* which closely recapitulates several aspects of advanced human pulmonary adenocarcinoma. We generated conditional knock-in mice with mutations in *K-ras* combined with one of three *p53* alleles: a contact mutant, a structural mutant or a null allele. *p53* loss strongly promoted the progression of *K-ras*-induced lung adenocarcinomas. Strikingly, the contact mutant $p53^{R270H}$, but not the structural mutant $p53^{R172H}$, acted in dominant-negative fashion in this system. Furthermore, a subset of these mice also developed nasopharyngeal carcinoma (NPC). In contrast to the lung tumors, expression of the point-mutant *p53* alleles strongly promotes the development of NPCs compared to simple loss-of-function.

SIGNIFICANCE

K-ras and *p53* mutations are common in human lung adenocarcinomas; the *K-ras* pathway and *p53* family-members are commonly altered in NPC. Building on these observations, this work yielded the first murine model of nasopharyngeal carcinoma and the first model of lung adenocarcinoma to recapitulate the hallmark features of advanced lung cancer. Furthermore, this work clearly demonstrates the tissue-specific effects of mutant *p53*. Specifically, while expression of mutant p53 in the absence of wild-type p53 promoted the development of NPCs, it did not influence lung tumor development differently than loss of p53 function. The observation of a dominant-negative effect by $p53^{R270H}$, but not $p53^{R172H}$, may explain the over-representation of codon 273 mutations and the under-representation of codon 175 mutations in human lung adenocarcinomas.

INTRODUCTION

Lung cancer is the leading cause of cancer deaths worldwide, with 171,900 new cases and 157,200 deaths predicted for the year 2003 in the United States alone (Jemal et al., 2003). For treatment purposes lung cancer is divided into two classes: small cell lung cancer, which comprises ~20% of cases, and non-small cell lung cancer (NSCLC), which comprises the remainder. NSCLC is further subdivided into 3 histologic subtypes: large cell carcinoma, squamous cell carcinoma and adenocarcinoma. Adenocarcinoma is the single most common form of lung cancer, comprising ~40% of cases (Jemal et al., 2003). The prognosis of patients with NSCLC is determined mainly by the stage of disease at the time of diagnosis. A 5-year survival rate of about 50% can be achieved in patients diagnosed with early stage disease that can be treated surgically. However, in the majority of patients, the cancer has invaded the surrounding tissues and spread to regional lymph nodes or distant organs by the time of diagnosis, making their prognosis far worse.

Activating mutations in the *K-ras* proto-oncogene are found in ~30% of human NSCLCs (Rodenhuis et al., 1988), and are also found in a large percentage of sporadic and carcinogen-induced lung tumors in mice (Herzog et al., 1997). Recently, several mouse lung cancer models have been created using conditionally or spontaneously activatable transgenic and knock-in alleles of oncogenic *K-ras* (Fisher et al., 2001; Jackson et al., 2001; Johnson et al., 2001a; Meuwissen et al., 2001). These models have contributed significantly to our understanding of the role of *K-ras* in tumor initiation, and in maintenance of the tumor phenotype. However, these models do not accurately recapitulate all aspects of the human disease. In particular, the lung tumors that develop in these models resemble early-stage human lung adenocarcinoma.

Features of late stage disease, including invasion of the surrounding pleura, induction of desmoplastic stroma and metastasis are not prominent.

Alterations in the *p53* tumor suppressor gene are also common in NSCLC, occurring in ~50% of cases (Chiba et al., 1990; Takahashi et al., 1989). Similar to other cancers, the majority of these alterations are missense mutations that result in the accumulation of high levels of mutant p53 protein. Studies of *p53* mutations from a variety of tumors show that missense mutations in *p53* occur primarily in the DNA binding domain (DBD). The frequency of mutation at individual codons can vary dramatically between tumor types. The most common *p53* missense mutations in human pulmonary adenocarcinomas occur in descending order at codons 273, 248, 249, 245, and 158 (Olivier et al., 2002). Of note, mutations at codon 175 are significantly less common in lung adenocarcinomas than in other cancers. This may suggest a difference in the tumorigenic potential of individual mutant p53 proteins in different tissues. Indeed, *p53* mutations are commonly grouped into two classes that display different behaviors in several *in vitro* assays (Cadwell and Zambetti, 2001). Class I mutations alter residues that make direct contact with DNA, while Class II mutations alter residues that are critical for maintaining global domain structure (Cho et al., 1994). Structural mutants are frequently described as more potent mutations than contact mutants in promoting cancer. However, in the case of NSCLC, structural mutations at codon 175 are found at a decreased frequency compared to the contact mutations at codons 273 and 248.

An alternative explanation for the variation in mutation frequency between tumor types is that different codons may vary in their exposure and susceptibility to mutagens. For example,

mutations at codon 249 are found in over 34% of spontaneous hepatocellular carcinomas but in only 3% of tumors overall. This is due primarily to a common G→T transversion at codon 249 induced by food contamination with aflatoxin B (Bressac et al., 1991; Hsu et al., 1991). In the case of lung cancer, ~90% of tumors are associated with exposure to carcinogens from tobacco smoke. It has been shown that polycyclic aromatic hydrocarbons found in tobacco smoke, such as benzo[a]pyrene diol epoxide, preferentially bind to and form adducts with several of the p53 codons frequently mutated in lung cancer, including codons 273 and 248. However, this may not completely explain the distribution of p53 mutations in lung cancer since these compounds also efficiently form adducts at codon 175 (Smith et al., 2000). In general, the presence of point-mutant *p53* confers an adverse prognosis in patients with pulmonary adenocarcinoma (Ahrendt et al., 2003; Mitsudomi et al., 2000).

Tumor-derived *p53* mutations can have two broad effects beyond simple loss of function. There is significant evidence that mutant *p53* alleles can act in a dominant-negative manner to inhibit the function of wild-type *p53* through hetero-oligomerization between mutant and wild-type p53 polypeptides. *In vitro* studies have demonstrated that both classes of mutants can exert dominant-negative effects on wild-type p53 activity with the effects being stronger for class II mutants (Sigal and Rotter, 2000). Furthermore, certain point-mutant *p53* alleles have been shown to promote tumorigenesis through gain-of-function effects that are not dependent on wild-type *p53* (Dittmer et al., 1993).

We recently generated two strains of conditional point-mutant *p53* mice that allow for endogenous expression of mutant p53 upon Cre-mediated recombination (Olive et. al, in press,

Cell). The first strain carries the contact mutation $p53^{R270H}$ (homologous to human $p53^{R273H}$) while the second strain carries the structural mutation $p53^{R172H}$ (homologous to human $p53^{R175H}$). Germline point-mutant *p53* mice derived from these strains demonstrated a gain-of-function in the development of epithelial and endothelial tumors. Furthermore, $p53^{R270H/+}$ mice, but not $p53^{R172H/+}$ mice, had a significantly increased incidence of lung adenocarcinomas compared to $p53^{+/-}$ mice. These data support the notion that the $p53^{R270H}$ mutant drives the development of lung adenocarcinoma more strongly than the $p53^{R172H}$ mutant. However, the relatively small number of lung tumors that developed in this study and the high incidence of background tumors prevented a thorough analysis of this effect. Furthermore, it was unclear from this work whether the increase in lung adenocarcinomas was the result of a dominant-negative or a gain-of-function effect by $p53^{R270H}$, since *p53* null mice do not survive for the ~18 months it took the heterozygous mutant *p53* mice to develop lung cancer. This distinction requires an assessment of the influence of mutant and null *p53* on lung cancer at comparable time-points.

We report here the creation of an improved murine lung cancer model through the generation of compound mutant animals with conditional mutations in *K-ras* and *p53*. This model closely recapitulates several aspects of advanced human pulmonary adenocarcinoma not commonly seen in existing models. To gain further insights into putative dominant-negative and gain-of-function effects of *p53* missense mutations *in vivo*, we have used this model to directly compare the oncogenic properties of different *p53* mutations ($p53^{R270H}$, $p53^{R172H}$ and a loss-of-function mutation of p53), in the context of *K-ras*-induced lung tumors. Our comparison of mutant *K-ras*-induced lung tumors with mutant or null alleles of p53 clearly demonstrates a dominant-negative effect by the $p53^{R270H}$ allele that is not conferred by the $p53^{R172H}$ allele. Furthermore,

we describe here a tissue-specific gain of oncogenic potential conferred by both the $p53^{R172H}$ and the $p53^{R270H}$ alleles toward the development of nasopharyngeal carcinoma (NPC).

RESULTS

Generation of Compound Conditional Mice Harboring Various *p53* Alleles

In order to assess the effect of *p53* mutation or loss on *K-ras*-induced lung carcinogenesis, compound conditional mutant mice were generated that harbored the conditional activatable *K-ras*^{G12D} allele (Jackson et al., 2001; Tuveson et al., 2004)(Fig. 1A) and combinations of three different conditional p53 alleles: $p53^{LSL.R270H}$, $p53^{LSL.R172H}$ and $p53^{Flox}$ (Jonkers et al., 2001)(Fig. 1A, Olive et al, in press). Importantly, the use of conditional alleles permits examination of the effects of these mutations in the emerging tumors of interest without the complications of widespread tumorigenesis or other effects of germline mutation. In this way, these models more closely resemble spontaneous tumor development in humans. For clarity, the genotypes of the compound mutant mice will be described in this paper using the abbreviations shown in Table 1 and referred to collectively as K,P mice. The following compound mutant mice were generated: K;Fl/+, K;270/+, K;172/+, K;Fl/Fl, K;270/Fl, K;172/Fl (Fig. 1B). These combinations allow us to examine the effects of point-mutant p53 proteins in the presence and absence of wild-type p53, compared to the complete elimination of p53 function. Crosses were designed to minimize effects caused by mixed genetic backgrounds (Fig. 1B and Materials and Methods). Cohorts of K,P compound conditional mutant mice were infected with $5x10^5$ PFU of a recombinant adenovirus expressing Cre recombinase (AdenoCre) by intranasal instillation and then sacrificed at various time-points (described below). As expected, all of the mice developed multiple primary lung tumors following infection with AdenoCre.

Infection With AdenoCre Induces Recombination of the Conditional Alleles

In order to assess recombination efficiency of each allele when multiple alleles were present, tumors were dissected from the lungs of K;270/Fl mice, and the genomic DNA was isolated. Because the only differences between the $p53^{R270H}$ and the $p53^{R172H}$ alleles are the sites of the single base mutations downstream of the LSL cassette, the recombination efficiency of the LSL cassette in these alleles should be identical. PCR was performed on the tumor DNA samples to detect successful recombination of the conditional K-ras and p53 alleles. In every tumor, PCR amplification of the K-ras allele produced a product 40bp larger than the wild-type allele, due to the single loxP site remaining after Cre-mediated recombination (Fig. 2A). Therefore, the K-rasG12D allele recombined with 100% efficiency. Similar analyses showed that the $p53^{LSL.R270H}$ allele recombined in 90% (9/10) of the tumors analyzed (Fig. 2B). The recombination efficiency of the $p53^{Flox}$ allele was comparable, generating the appropriate PCR product in 80% (8/10) tumors analyzed (Fig. 2C). The effective genotypes that would result from the recombination of only one p53 allele for each mouse are delineated in Table 1.

Loss of p53 Function Promotes Malignant Progression of Lung Tumors.

As mentioned earlier, several mouse models of K-ras-initiated lung adenocarcinomas have been generated that produce low to medium grade lung tumors. The effect of p53 loss was investigated in two of these models (Fisher et al., 2001; Johnson et al., 2001a); in each model, crossing onto a p53 null background resulted in an acceleration of tumorigenesis as well as an increase in cellular pleomorphic and nuclear atypia. However, the mice succumbed to

overwhelming tumor burden and background tumors consistent with homozygous p53 deletion prior to the development of more advanced features such as stromal desmoplasia or metastases.

In order to investigate the effects of p53 loss in the conditional K-ras system, without the induction of background tumors, we evaluated the tumor phenotypes of K;Fl/+ and K;Fl/Fl mice at various time-points after AdenoCre infection. A cohort of K;Fl/+ mice were euthanized at a late time-point (26 weeks) after infection with AdenoCre. The tumors present in the K;Fl/+ mice resembled those seen previously in LSL-K-rasG12D single mutant mice, ranging from adenomas to early stage adenocarcinomas (Jackson et al., 2001). This indicates that the loss of a single copy of p53 does not significantly affect the progression of K-ras-initiated lung adenocarcinomas; these mice were therefore used as controls for the remainder of this study.

A cohort of K;Fl/Fl mice was also generated for analysis at the late time-point. However, it soon became apparent that these mice would not survive for 26 weeks after infection, and they were instead sacrificed after 19 weeks. In contrast to the K;Fl/+ mice, even at the 19 week timepoint, all of the K;Fl/Fl animals developed advanced pulmonary adenocarcinomas. Histological analysis of the tumors found a variety of morphologies that were more diverse than those seen in K-rasG12D single mutant animals. Multiple high-grade tumors were present in each mouse that ranged in appearance from well-differentiated tumors with papillary architecture in which the cells maintained an apical basal orientation and uniform nuclei, to highly dysplastic lesions that had lost their cellular orientation and contained enlarged pleomorphic nuclei. These advanced tumors contained large sheets of dysplastic cells, abnormal mitoses and multinucleate giant cells.

In order to quantify the extent of progression of K;Fl/Fl tumors, we devised a grading system by which to evaluate the stage of every individual tumor in each mouse. Tumors were scored in a blinded manner on a scale of 1-5 with grade 5 indicating the most advanced tumor phenotype. The criteria for each grade are as follows: Grade 1 tumors are lesions with completely uniform nuclei showing no nuclear atypia. Grade 2 tumors contain cells in which the nuclei are uniform but slightly enlarged and exhibit prominent nucleoli. Grade 3 tumors have cells with enlarged, pleomorphic nuclei showing prominent nucleoli and nuclear molding. Grade 4 tumors have very large, highly pleomorphic nuclei exhibiting a high degree of nuclear atypia including abnormal mitoses and hyperchromatism. These tumors also contain multinucleate giant cells. Grade 5 tumors have all the features of Grade 4 tumors and also show stromal desmoplasia surrounding nests of tumor cells. Examples of each grade of tumor are shown in Figure 3.

Using this grading scheme to analyze 944 tumors from 12 K;Fl/+ mice and 1161 tumors from 15 K;Fl/Fl mice, we confirmed that loss of p53 resulted in a markedly more severe tumor phenotype in K-ras-initiated lung adenocarcinomas (Fig. 4A). In addition, K;Fl/Fl tumors demonstrated several characteristics of human lung tumors that had been lacking in previous murine lung cancer models (Tuveson and Jacks, 1999). Perhaps most striking was the presence of tumors containing a large stromal component in which nests of tumor cells could be found growing within a field of desmoplastic stroma (Fig. 4B). Masson's trichrome staining of these tumors demonstrated the abundant production of collagen by the stromal fibroblasts (Fig. 4C) and immunohistochemistry confirmed the expression of smooth muscle actin by the stromal fibroblasts (data not shown).

A subset of K;Fl/Fl tumors were highly invasive, growing into the hilus, the heart and the overlying pleura (Fig 4D). Furthermore, within the tumor mass, blood and lymphatic vessels were found with tumor cells growing within them (data not shown). Therefore, to determine the metastatic potential of the tumors we examined the regional lymph nodes. Lymph node metastases were present over 50% (8/15) of the K;Fl/Fl mice (Fig 4E). A careful histological examination of all of the organs revealed the presence of distant metastases in a small percentage of these mice (Fig. 4F).

Having established that p53 loss affects the progression of K-ras-initiated lung adenocarcinomas at late time-points, we were curious whether this effect was also apparent early in tumor development. Therefore we examined the lungs of K;Fl/+ and K;Fl/Fl mice six weeks after infection with $5x10^5$ PFU of AdenoCre. The lungs of K;Fl/+ mice contained a spectrum of lesions ranging from atypical adenomatous hyperplasia (AAH) to small adenomas (Fig. 5A). AAH is a hyperproliferation of epithelial cells growing along the existing alveolar septae that does not disrupt the underlying lung architecture (Fig. 3A). The cells in these lesions have uniform nuclei with no nuclear atypia. The adenomas present in these mice were very small and, with a few rare exceptions, had regular nuclei (Fig. 5B). AAH and adenomas were also present in the K;Fl/Fl mice, but these lesions often larger and frequently displayed nuclear atypia (Fig. 5C). Even the smallest of lesions were sometimes found to have aberrant nuclei (Fig. 5D). Furthermore, rare tumors could be found that contained multinucleate giant cells (Fig 5C). In order to quantitate these observations, 111 tumors from 12 K;Fl/+ mice and 215 tumors from K;Fl/Fl mice were graded according to the scheme described above (Fig. 5A). This analysis

showed a clear shift towards more malignant lesions in the K;Fl/Fl mice indicating the contribution of p53 loss to lung tumor progression occurs very early in tumor development.

The $p53^{R270H}$ Allele Shows Dominant-Negative Effects in Promoting Malignant Progression

Endogenous expression of mutant p53 can result in a gain-of-function effect on tumor development in some tissues (Olive, et. al, in press). Therefore, we examined whether endogenous expression of point-mutant p53 in K-ras-initiated lung adenocarcinomas would result in a more severe tumor phenotype than homozygous deletion of p53. Cohorts of K;270/Fl and K;172/Fl mice were sacrificed 19 weeks after AdenoCre infection, and their lung tumors were compared to the K;Fl/Fl mice described above. Surprisingly, grading of 841 tumors from 11 K;270/Fl mice and 839 tumors from 10 K;172/Fl mice did not reveal any differences in the distribution of tumor grades compared to K;Fl/Fl mice (Fig. 6A). All three genotypes had substantially fewer low grade, and more high grade tumors than the K;Fl/+ animals sacrificed at 26 weeks post infection. In addition, tumors from the K;Fl/Fl, K;270/Fl and K;172/Fl animals were highly invasive and frequently metastasized to the thoracic lymph nodes. Together, these data support the loss of wild-type p53 function as a factor in the progression of lung adenocarcinoma, but they do not support a gain-of-function effect by point-mutant p53.

The absence of a gain-of-function effect in this system allowed us to cleanly evaluate the role of dominant-negative effects by mutant p53 in the progression of K-ras-initiated lung adenocarcinomas. Cohorts of K;270/+ and K;172/+ mice were sacrificed 26 weeks after AdenoCre infection and compared to the K;Fl/+ mice described above. Grading analysis was

performed on 932 tumors from 11 K;270/+ mice and 773 tumors from 10 K;172/+ mice. Strikingly, while the distribution of tumor grades was similar between K;Fl/+ and K;172/+ mice, the K;270/+ mice displayed a decrease in grade 1 tumors (K;Fl/+ = 30$\pm$ 6% vs. K;270/+ =12$\pm$ 3%, mean $\pm$ SEM, χ^2, p=.019) and an increase in high-grade tumors (grade 3,4 and 5 = 28$\pm$ 4% vs. 46$\pm$4% χ^2, p=.018)(Fig. 6B). The tumor grade distribution of the K;270/+ mice fell between that of the K;Fl/+ and the K;Fl/Fl animals (Fig 6A,B). Therefore, $p53^{R270H}$, but not $p53^{R172H}$, appeared to act as a partial dominant-negative allele toward the development of high-grade lung adenocarcinoma.

In order to determine whether this effect also extended to the growth rate of the tumors, we examined the size of the tumors after 26 weeks of growth. The average number of tumors in the K;Fl/+, K;172/+ and K;270/+ were very similar (79$\pm$21, 77$\pm$24 and 85$\pm$17 respectively). To calculate tumor size we used the Bioquant Image Analysis system to measure the total area of tumor in the lung as well as the total area of lung to determine the percent of lung area involved with tumor. Similar to the tumor grading results, we found that the K;Fl/+ and the K;172/+ mice had similar sized tumors, occupying 24% and 27% of the total lung area respectively. However, tumors in the K;270/+ mice were consistently larger, comprising 36% of the lung area (Fig 4C). The size difference between K;Fl/+ and K;270/+ mice was statistically significant (p=.024). We did not compare the tumor size among the K;Fl/Fl, K;172/Fl, and K;270/Fl animals, as in most cases nearly the entire lung was filled with tumor at sacrifice. These findings further support a dominant-negative effect for the $p53^{R270H}$ allele on lung tumor progression that is not seen with the $p53^{R172H}$ allele.

Upregulation of the Raf/MAPK Pathway Occurs in Advanced Tumors

Immunohistochemical analysis of lung tumors in K;+/+ mice using anti-phospho-MAPK antibodies demonstrated that increased activation of the MAPK pathway is a late event in the progression of K-ras-induced murine lung tumors, occurring in only a small subset (3.5%) of late-stage tumors (data not shown). Studies in primary fibroblasts have linked high levels of Raf/MAPK activity to Ras-induced premature senescence in part through activation of p19ARF, a positive regulator of p53 (Lin et al., 1998). Therefore, we reasoned that mutation of p53 might allow for earlier and more widespread activation of the signaling pathway, which could account for its tumor-promoting effects. To address this question, immunohistochemistry was performed on both early- and late-stage tumors from the K,P mice of all six genotypes using anti-phospho-p42/44MAPK antibodies.

At six weeks post infection no phospho-MAPK positive tumors were present in any of the animals evaluated. This indicates that loss of p53 alone is not sufficient to promote upregulation of the Raf/MAPK pathway. However, immunostaining of late-stage tumors revealed that a subset of tumors contained large foci of phospho-MAPK positive cells. In some cases entire tumors were immunoreactive. The percentage of tumors that were phospho-MAPK positive varied depending on the p53 genotype (Fig. 4D). Roughly half of all the tumors in K;Fl/Fl, K;172/Fl and K;270/Fl animals stained positively for phospho-MAPK (52.2%, 51.2% and 50.6% respectively). Strikingly, more than half (53.1%) of the tumors in K;270/+ mice also stained positively, suggesting that the $p53^{R270H}$ allele acted as a complete dominant-negative allele in this respect. A mild dominant-negative effect was also seen by the $p53^{R172H}$ allele as 32.2% of K;172/+ tumors were phospho-MAPK positive. In contrast, only 13.7% of p53Fl/+ tumors were

phospho-MAPK positive. Therefore, while p53 loss alone is not sufficient to promote upregulation of the Raf/MAPK pathway, mutation or loss of p53 may cooperate with other events that accumulate during the development of a tumor to allow MAPK upregulation. Furthermore, the $p53^{R270H}$ and, to a lesser extent, $p53^{R172H}$ alleles both dominantly interfere with the ability of wild-type p53 to restrain MAPK upregulation.

Wild-type specific LOH in K;270/+ Tumors Despite Dominant-Negative Effects by the $p53^{R270H}$ allele.

To determine whether the dominant-negative effect of the $p53^{R270H}$ allele alleviates the selective pressure to lose the wild-type allele of p53 during tumor progression, we examined the frequency of loss of heterozygosity (LOH) in p53 Fl/+ and p53 ^{R270H}/+ tumors. Quantitative real time PCR analysis was performed on genomic DNA isolated from tumors micro-dissected from the lungs of K;Fl/+ and K;270/+ mice using TaqMan MGB probes. For both genotypes, 100% of the tumors analyzed (n=11 for K;270/+ and n=8 for K;Fl/+) showed loss of the wild-type p53 allele (Supplemental Table 1). Because the microdissection technique required the isolation of larger tumors, the sampling may have been biased towards more advanced tumors. However, these data demonstrate that there is still selective pressure for full loss of p53 function despite the dominant-negative effects of the $p53^{R270H}$ allele, suggesting an incomplete dominance over the wild-type allele. This is consistent with data from human lung tumors and other tumor types (Baker et al., 1990).

Mutant p53 promotes the development of nasopharyngeal carcinomas.

The intranasal instillation method of AdenoCre administration used to generate lung tumors exposes the entire respiratory tract to the virus. Thus, it is possible that infection and subsequent recombination of the conditional alleles would occur in cells outside of the lung. However, analysis of the conditional K-ras^{G12D} single mutant mice did not provide any evidence of tumorigenesis in the upper respiratory tract. It is possible that the epithelial cells of the upper respiratory tract and nasal mucosa are not susceptible to adenoviral infection. Alternatively, the expression of K-ras^{G12D} in these cells may not be sufficient to induce tumorigenesis. In fact, in the course of performing the experiments described above, we observed that K;172/Fl and K;270/Fl compound mutant mice had a higher mortality than the K;Fl/Fl compound mutants, which could not be explained by differences in their lung tumor phenotype. A small number of these mice presented with grossly apparent head tumors. Therefore, we systematically examined the nasal mucosa of these mice for evidence of tumors that might have contributed to their early lethality. Histological analysis of perisagital skull sections revealed the presence of nasopharyngeal carcinomas (NPCs) in a subset of the 19 week K;Fl/Fl, K;270/Fl and K;172/Fl mice. The NPCs arose in the respiratory epithelium of the sinuses. These tumors were extremely poorly differentiated with dramatically pleomorphic nuclei and contained large numbers of multi-nucleate giant cells, giving the tumors a characteristic syncytial appearance. The nuclei also appeared highly vessiculated and had prominent nucleoli (Fig. 5B). Furthermore, the tumors were destructively invasive, commonly penetrating through the cribriform plate and into the olfactory bulb (Fig. 5C).

NPC is a form of head and neck cancer arising from the nasal mucosa (Lo et al., 2004). Although rare in the United States and Europe, NPC is common in Cantonese populations where it occurs in roughly 1:4000 individuals. The World Health Organization describes three histological subtypes of NPC: keratinzing squamous (type I), non-keratinizing squamous (type II) and undifferentiated (type III). The lesions arising in AdenoCre-treated K,P mice are highly reminiscent of undifferentiated NPC. In particular, the striking syncytial growth pattern and bizarre, vessiculated nuclei are both prominent in these tumors.

Consistent with their increased mortality, the incidence of NPC was >4 fold higher in the K;172/Fl (64%) and K;270/Fl (69%) mice than in the p53Fl/Fl mice (14%)(Fig 5D). Given the significantly higher incidence of NPC upon expression of point-mutant p53 compared to loss of p53 expression, these data provide the most dramatic evidence to date of a gain of oncogenic potential by point-mutant p53 *in vivo*.

DISCUSSION

In this work, we present compound mutant mice incorporating somatically manipulatable conditional mutations in the *K-ras* proto-oncogene and the *p53* tumor suppresser gene. Cooperation between oncogenic ras and point-mutant *p53* was one of the earliest examples of genetic interaction between two cancer genes (Parada et al., 1984a). Based on the observation that both *K-ras* and *p53* are frequently mutated in human tumors, we expect that the K,P compound mutant mice described in this work will be useful in accurately modeling a number of different types of cancer, beyond the advanced pulmonary adenocarcinoma and nasopharyngeal carcinoma described here.

K,P Mice Develop Advanced Lung Adenocarcinoma

The K,P compound mutant mice also successfully recapitulate the pathology of advanced human pulmonary adenocarcinoma. These mice combine several modeling techniques to achieve this success. First, the conditional nature of both the *K-ras* and *p53* mutant alleles allows for analysis of mutant cell behavior in the context of an otherwise wild-type animal. This circumvents the developmental effects of mutant *K-ras* expression and the background tumor phenotype of germline *p53* mutation. Second, the delivery of a relatively low titer of AdenoCre prevented the K,P mice from succumbing to the burden of a large number of low-grade tumors, as occurs in the sporadic *K-ras*^{LA} models. Finally, the K,P mice utilize compound mutations of the endogenous *K-ras* and *p53* genes rather than transgenic overexpression of mutant cDNAs. The effect of the endogenous *K-ras*^{G12D} mutation in mouse embryo fibroblasts is substantially different than that of ectopic overexpression, underscoring the importance of physiologically accurate models (Tuveson et al., 2004).

The K,P mutant mice developed lung adenocarcinomas with several feature not commonly observed in previous models. Not only do the tumors in these mice exhibit a high degree of nuclear atypia, they also elicit stromal desmoplasia and are invasive and metastatic. Although it has been shown in other mouse lung cancer models that *K-ras* and *p53* cooperate in the promotion of tumor growth and progression, the induction of a desmoplastic response and the development of metastatic disease was not reported (Fisher et al., 2001; Johnson et al., 2001a).

Human pulmonary adenocarcinomas often contain a large stromal component. The microenvironment in which a tumor resides may play a critical role in tumor growth and progression, and interactions between cells in the tumor and the microenvironment (stroma) have been shown to elicit changes in the other's behavior (Elenbaas and Weinberg, 2001; Liotta and Kohn, 2001). The induction of desmoplastic stroma is commonly seen in NSCLC and may contribute to disease progression. Correlative studies suggest that stromal production of the cell adhesion molecule hyaluronan (HA) (Pirinen et al., 2001) correlates with poor prognosis. The production by stromal fibroblasts of HA and various matrix metalloproteinases (MMPs) involved in reorganization of the extra-cellular matrix are thought to influence the invasive and metastatic properties of the tumor (Liotta and Kohn, 2001). Co-culture experiments have shown that NSCLC cells induce pulmonary fibroblasts to secrete βFGF and IL-8 (Anderson et al., 2000; Mari et al., 1998), suggesting that they may contribute to tumor angiogenesis. Furthermore, stromal production of thymidine phosphorylase, which has angiogenic activity, correlates with poor prognosis in early stage NSCLC (Kojima et al., 2002).

Human NSCLC is highly metastatic. Patients diagnosed with early stage disease that has not spread to lymph nodes or distant organs have an average 5-year survival of about 50%. However, only 15% of lung cancer patients are diagnosed at this stage; most are diagnosed with metastatic disease and have an overall 5-year survival rate only 14%. The current chemotherapeutic regimens have not been effective in the treatment of advanced NSCLC. Numerous murine lung cancer models have been created that have provided valuable insights into the molecular alterations driving lung tumorigenesis. However, because these models do not generally develop advanced disease, their growth properties and response to therapeutics may not accurately reflect those of human tumors. Thus, the model described here will provide a valuable system for the study of lung tumor biology and the pre-clinical testing of novel therapeutics.

The Role of p53 Loss in Lung Tumor Progression

Although it is generally accepted that p53 mutation is an important event in the development of lung cancer, the specific contribution of particular p53 mutations has not been previously analyzed. By comparing the effects of different p53 alleles under identical conditions, we have established that loss of p53 function is sufficient to promote the progression of *K-ras* initiated lung adenocarcinomas in mice. The primary functions of p53 are to respond to cellular stresses by transactivating genes involved in cell cycle arrest, apoptosis and genome maintenance. Although we did not determine the contribution of specific *p53* effecter pathways, it is unlikely that the apoptotic functions of p53 are significant in this setting since we could not detect any differences in the rates of apoptosis within lung tumors of K,P mice by immunostaining for cleaved caspase 3 (data not shown). In fact, the presence of apoptotic cells in *K-ras*-induced

lung tumors was extremely rare even in tumors with wild-type *p53*, implying either that the bronchoalveolar epithelia of the lung is not prone to oncogene-induced apoptosis or that the evasion of apoptosis occurs extremely early in tumor development.

Instead, it appears likely that loss of the genome maintenance and anti-proliferative functions of p53 are critical to the development of advanced lung adenocarcinoma. One of the most prominent features of the tumors in K;Fl/Fl mice is the development of severe nuclear dysplasia. The observation of lesions with prominent nuclear atypia just six weeks after infection with AdenoCre indicates a rapid accumulation of genetic abnormalities following loss of *p53*. Furthermore, loss of *p21*, a primary effector of p53-mediated cell cycle arrest, was shown to significantly accelerate tumorigenesis in a c-raf induced mouse model of lung adenocarcinoma (Fedorov et al., 2003).

In any case, the role of p53 loss appears to be primarily in the progression of lung adenocarcinomas, rather than their initiation. Lung tumors are extremely rare in $p53^{-/-}$ mice, presumably due to the early onset of other tumors such as lymphomas and sarcomas. A small subset of $p53^{+/-}$ mice does develop lung adenocarcinomas but the tumors are generally solitary and have a long latency (>18months). Likewise, $p53^{Fl/Fl}$ mice treated with Adeno-Cre also develop lung adenocarcinomas, but only 1 to 1½ years after treatment (Meuwissen et al., 2003). Therefore, p53 mutation alone is not sufficient for the initiation of lung cancer. Instead, activation of *K-ras* serves to initiate the tumor and p53 loss contributes to the progression. *K-ras* activation is also required for lung adenocarcinoma maintenance as withdrawal of doxycycline-inducable K-ras-$4b^{G12D}$ resulted in regression of existing tumors. Finally, *K-ras* activation may

affect the cell-specificity of tumor development since compound deletion of *p53* and *Rb* in the lung results in the neuroendocrine tumors characteristic of small cell lung cancer rather than pulmonary adenocarcinoma (Meuwissen et al., 2003).

$p53^{R270H}$ Acts as a Partial Dominant-Negative Allele

Although p53 loss of function is sufficient to drive tumor progression in *K-ras* induced lung adenocarcinomas, p53 is rarely deleted in human lung tumors. Rather, p53 is commonly point-mutated at specific hot-spot residues in the DNA binding domain in lung tumors as well as many other cancers. In particular, contact mutations at codons 248 and 273 are very common in human lung adenocarcinomas. In contrast, an analysis of spontaneous tumors from the IARC p53 mutation database (v. R9) confirms that codon 175 mutations are substantially underrepresented in lung adenocarcinomas and non-small-cell lung tumors compared to all tumors (χ^2, p= 0.000026). Previously explanations have focused on the carcinogenic effects of cigarette smoke on different p53 codons as a mechanism for this variation in the codon distribution of p53 mutations, especially in light of *in vitro* evidence that suggested that codon 175 mutations are more potent in oncogenic assays. However, our work found that the $p53^{R270H}$ mutation is more strongly dominant-negative over wild-type p53 than is the $p53^{R172H}$ mutation and therefore more effective in tumor promotion.

There are a variety of possible explanations for this effect. For example, $p53^{R270H}$ may have a stronger intrinsic capacity to interfere with wild-type p53 than $p53^{R172H}$. However, our analysis of mouse embryo fibroblasts heterozygous for endogenous mutant p53 alleles suggested exactly the opposite effect (Olive et. al, in press). $p53^{R172H/+}$ MEFs proliferate significantly more rapidly

than do $p53^{R270H/+}$ MEFs. Alternatively, $p53^{R270H}$ may accumulate to higher levels in the tumor cells than $p53^{R172H}$. Most *in vitro* studies attempt to analyze identical amounts of transfected p53 mutants. However, analysis of endogenous mutant p53 MEFs as well the intestinal crypts of irradiated mutant p53 mice showed that $p53^{R270H}$ consistently accumulates to higher levels than $p53^{R172H}$. One final possibility is that $p53^{R270H/+}$ tumors undergo LOH at an earlier point than $p53^{R172H/+}$ tumors. This could be due to a specific dominant-negative effect by $p53^{R270H}$ on the genome maintenance functions of p53. Alternatively, perhaps $p53^{R270H}$ is less strongly dominant-negative than $p53^{R172H}$ as is suggested by the data on mutant p53 MEFs. In that case, there could be an increased selective pressure for $p53^{R270H/+}$ tumors to undergo LOH early in tumorigenesis. Analysis of p21 and mdm2 levels in $p53^{R270H}$ MEFs found increased levels of these target genes compared to $p53^{+/-}$ MEFs, further supporting this hypothesis.

The Role of *K-ras* and *p53* Mutations in Nasopharyngeal Carcinoma

In addition to NSCLC, some of the K,P mice developed nasopharyngeal carcinomas, thereby establishing the first spontaneous mouse model of NPC. The most prominent etiological factor in human NPC is infection with Epstein-Barr virus (EBV). The episomal EBV genome produces several known viral gene products that contribute to NPC. In addition, several genes are known to be deleted or hypermethylated in NPC cells including $p16^{Ink4a}$, RARβ2 and *RASSF1* (Kwong et al., 2002; Lo et al., 1996). *RASSF1* is particularly interesting because it is located at 3p21, a region commonly deleted in both NPC and lung adenocarcinomas. In contrast to human lung adenocarcinomas, however, mutations in *K-ras* or *p53* are both rare in NPC (Yung et al., 1995). Instead, it appears that these pathways are frequently altered through other means. For example, the epidermal growth factor receptor (*EGFR*), of which the ras pathway is a primary downstream

effector, is overexpressed in ~85% of all NPCs. Likewise, although wild-type p53 accumulates to high levels in most NPC, p53 is clearly blocked from inducing cell cycle arrest or apoptosis. Interestingly, both of these effects may be a consequence of EBV infection as recent reports suggested that the viral protein *LMP1* can both induce the expression of *EGFR* through the activation of NF-κB (Tao et al., 2004) and inhibit the activity of p53, perhaps through upregulation of the anti-apoptotic protein A20 (Fries et al., 1996; Fries et al., 1999) (Sup. Fig. 1A,B).

Finally, ΔNp63 has been reported to be overexpressed in 100% of NPCs (Crook et al., 2000). ΔNp63 is a dominant-negative variant of the *p53* family-member, *p63*. Previously, it was suggested that ΔNp63 may act in a trans-dominant-negative manner to inhibit the function of wild-type *p53* through competition for *p53* binding sites. However, more recently, wild-type p53 has been shown to bind to ΔNp63 through direct interaction of their DNA binding domains, resulting in the targeted destruction of ΔNp63 through caspase-mediated protein degradation (Ratovitski et al., 2001). Therefore, the upregulation of ΔNp63 in NPC may be explained by inhibition of wild-type function p53 in these tumors. This model may also help explain the gain-of-function effects by point-mutant p53 towards NPC development in the K,P mice. Several groups have shown that mutant *p53* can bind to and inhibit the function of *TAp63*, the full-length, transactivation competent form of *p63* (Di Como et al., 1999; Marin et al., 2000). Therefore, the expression of point-mutant p53 may act to inhibit the function of TAp63 in K,P mice, mimicking the effect of ΔNp63 overexpression in human NPC (Sup. Fig. 1C).

These observations suggest that the compound mutation of *K-ras* and *p53* in the respiratory epithelium of K,P mice is substituting for the effects of EBV infection in the development of NPC. It also suggests that rather than playing a minor role, the *ras* and *p53* pathways are, in fact, extremely important in the pathogenesis of NPC.

Tissue-specific Effects of Mutant p53 Function

Endogenous expression of mutant p53 has different effects in different tissues. Previously, we showed that endogenous expression of mutant p53 in the germline of mice resulted in a gain-of-function effect on the development of both endothelial and epithelial tumors (Olive, et. al, in press). In this work, we describe dominant-negative effects by mutant p53 in the pulmonary epithelium and gain-of-function effects in the respiratory epithelium of the nasopharynx. It is unclear why mutant p53 failed to produce a gain-of-function effect in K-ras-induced lung tumors. Lung carcinomas were among those found in germline point-mutant p53 mice, leading to our original expectation of a gain-of-function effect by mutant p53 in lungs of K,P mice. However, since the lung adenocarcinomas arising in the K,P mice all harbored activated *K-ras*, any effect by mutant *p53* on the initiation of lung tumors might be obscured in this system. Alternatively, the novel pathways affected by mutant *p53*, for example *p63* and *p73*, may be relatively less important during the development of lung tumors compared to other sites. Given the prevalence of p53 mutations in human cancer, elucidating the different allele- and tissue-specific effects of mutant p53 is critical to developing a more complete understanding of molecular carcinogenesis.

MATERIALS AND METHODS

Breeding schemes

LSL-K-ras^{G12D} mice were crossed to p53Fl/+ mice to generate K-rasG12D; p53Fl/+ mice. The p53^{R172H} and p53^{R270H} strains were each crossed to the p53Fl/+ strain to p53^{R172H}/Fl and p53^{R270H}/Fl mice. By crossing the K-rasG12D;p53Fl/+ mice to the p53^{R172H}/Fl mice offspring of the following genotypes were generated for use in tumor studies: K-rasG12D;p53Fl/+, K-rasG12D;p53^{R172H}/+, K-rasG12DFl/Fl and K-rasG12D;p53^{R172H}/Fl. The K-rasG12D;p53Fl/+ mice were crossed to the p53^{R270H}/Fl mice to yield: K-rasG12D; p53Fl/+, K-rasG12D;p53^{R270H}/+, K-rasG12D;p53Fl/Fl and K-rasG12D^{R270H}/Fl. Thus comparison of the properties of each point-mutant allele to the floxed (null) allele was performed between littermates.

Molecular Analysis of Recombination Efficiency

To assess the efficiency of Cre mediated recombination of the conditional alleles DNA was prepared from dissected tumors. For the K-ras allele, PCR was performed using the Advantage-GC-cDNA kit from Clontech with primers flanking the lox-stop-lox cassette: forward primer K5'-1 = 5'-GGGTAGGTGTTGGGATAGCTG-3' and reverse primer K3'-3 = 5'-TCCGAATTCAGTGACTACAGATGTACAGAG-3'. The wild-type K-ras allele yields a 265-bp product and the recombined conditional allele containing a single loxP site yields a 305-bp product. Analysis of the conditional p53 point-mutant alleles was performed using standard PCR buffer with primers flanking the lox-stop-lox cassette: forward primer dt020200.1 = 5'-AGCCTGCCTAGCTTCCTCAGG-3' and reverse primer dt011200.3 = 5'-CTTGGAGACATAGCCACACTG-3'. The wild-type and Flox p53 alleles produce a 291bp product and the recombined condtional allele produces a 325bp product. PCR analysis of the

floxed allele was performed using standard PCR buffer with primers flanking the loxP sites in exons 2 and 10: forward 5'CACAAAAACAGGTTAACCCAG-3' and reverse 5'-GAAGACAGAAAAGGGGA GGG-3'. The recombined allele yields a 612-bp product; no product is produced from the wild-type allele.

AdenoCre Infection

Mice were infected with 5x10^5 PFU of AdenoCre virus at 6-8 weeks of age. AdenoCr:CaPi co-precipitates were prepared as described (Fasbender et al., 1998). Mice were anesthetized with avertin. AdenoCr:CaPi co-precipitates were administered intranasally in two 62.5ul instillations. The second instillation was administered when breathing rates had returned to normal.

Tissue Harvesting

Mice were sacrificed by CO2 asphyxiation. The trachea was exposed and the lungs were inflated with formalin. Trachea, heart and lungs were removed en mass and placed in formalin. The remaining organs were then removed and placed in formalin. All tissues were fixed in formalin overnight at room temperature and then placed into 70% ethanol and sent for processing through paraffin. Once processed each lobe of the lung was cut in a set pattern and embedded in paraffin. Remaining organs were embedded according to standard protocols.

Immunohistochemistry and Trichrome Staining

All lungs were sectioned in the same fashion; 5 step sections were taken at 100um apart, with 5 unstained sections taken after section 3. *P53* immunohistochemistry was performed following Trilogy dewaxing/unmasking according to manufacturer's instructions. *p53* CM5 rabbit

polyclonal antibody (Novo Castra) was used at a 1:500 dilution in Tris-Buffered Saline + Tween-20 (.05%). Sections were incubated in primary antibody overnight at 4°C. Staining was completed with an HRP-anti-rabbit kit (Vector Labs) according to manufacturer's instructions. For smooth muscle actin immunohistochemistry unmasking was not required. Sections were incubated overnight at 4°C in Anti-Smooth muscle actin (Reasearch Diagnostics Inc.) diluted 1:100. For phospho-MAPK immunohistochemitsry high temperature citrate buffer unmasking was performed. Sections were incubated overnight at 4°C anti-phosph-p42/44MAPK (Cell Signaling) diluted 1:100. Trichrome staining was performed using a Masson's Trichrome kit from Poly Scientific R&D, following manufacturer's instructions.

Tumor Grading

Tumor grading was performed without knowledge of genotype on the level 3 section of each lung. Each tumor was given a score of 1-5 based on pre-determined criteria regarding nuclear morphology and stromal component. Grade 1 = completely uniform nuclei showing no nuclear atypia. Grade 2 = nuclei are uniform but slightly enlarged and exhibit prominent nucleoli. Grade 3 = enlarged, pleomorphic nuclei showing prominent nucleoli and nuclear molding. Grade 4 = very large highly pleomorphic nuclei exhibiting a high degree of nuclear atypia including abnormal mitosis and contain multi-nucleate giant cells. Grade 5 = highly pleomorphic nuclei and stromal desmoplasia surrounding nests of tumor cells. Grading was performed in the absence of knowledge of p53 genotype.

Tumor size measurement

Lung and tumor areas were determined using Bioquant Image Analysis software in manual measurement mode.

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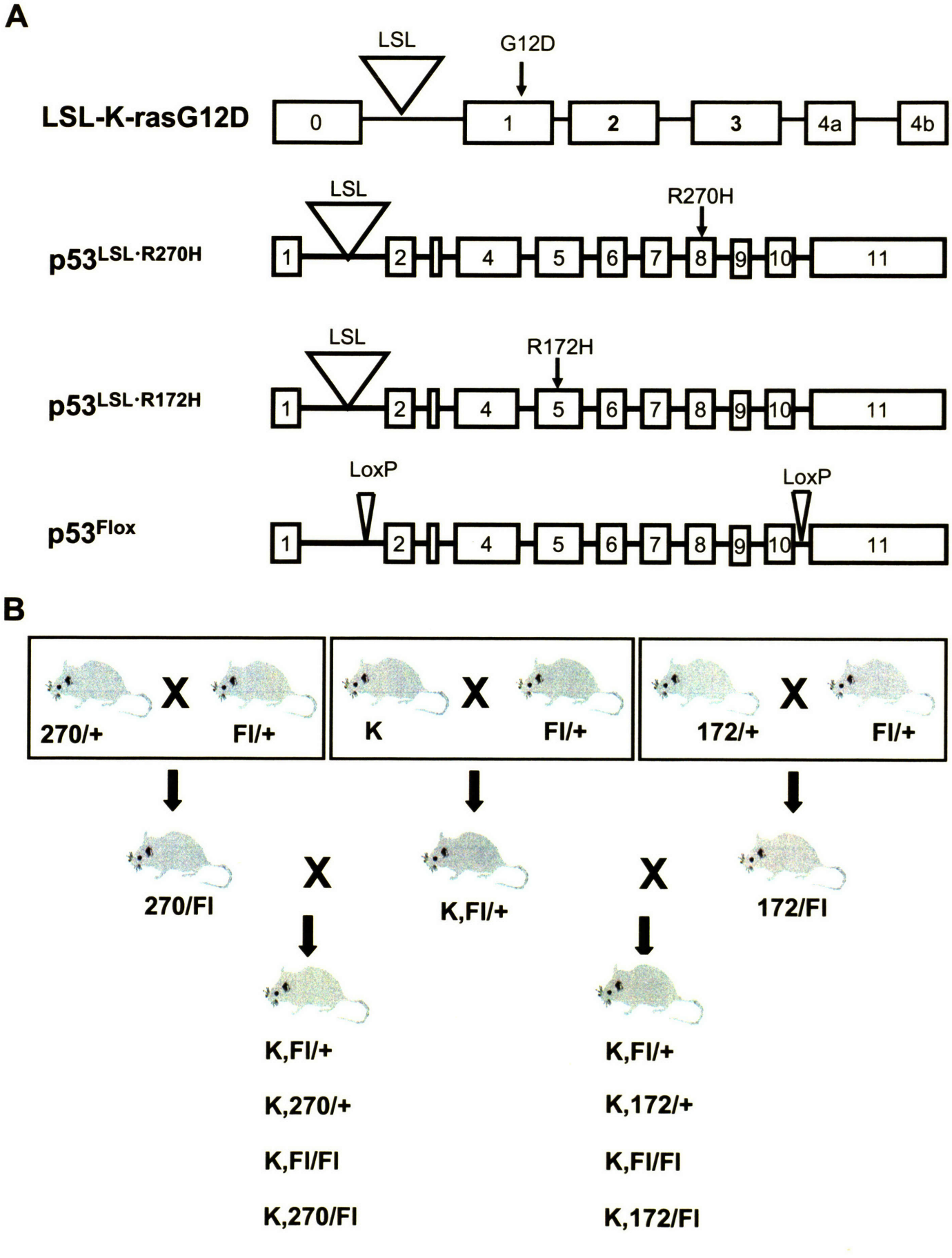
Figure 1. Generation of K,P Compound Mutant Mice

A) Diagram of *K*-ras and *p53* Conditional Alleles. The *LSL-K-ras*^{G12D} allele contains an activating mutation at codon 12 and a Lox-Stop-Lox (LSL) cassette inserted into intron 0 upstream of the transcriptional start site. The *p53*$^{LSL.R172H}$ and *p53*$^{LSL.R270H}$ alleles contain an Arg→His mutation in exons five and eight, respectively, and a LSL cassette inserted into intron 1. The *p53*^{Flox} allele encodes the wild-type p53 protein and contains LoxP sites flanking exons 2 and 10.

B) Breeding Scheme. *LSL-K-ras*^{G12D} mice were crossed to p53$^{Fl/+}$ mice to produce *LSL-K-ras*^{G12D}; *p53*$^{Fl/+}$ mice (K;Fl/+). The p53$^{LSL.R172H}$ and *p53*$^{LSL.R270H}$ strains were each crossed to the *p53*$^{Fl/+}$ strain to produce *p53*$^{R172H/Fl}$ and *p53*$^{R270H/Fl}$ mice. By crossing the K;Fl/+ mice to the *p53*$^{R172H/Fl}$ and to the *p53*$^{R270H/Fl}$ mice, offspring of the following genotypes were generated for use in tumor studies (abbreviated genotypes used): K;Fl/+, K;Fl/Fl, K;172/+, K;172/Fl, K;270/+, K;270/Fl.

Figure 1


A
LSL
G12D
LSL-K-rasG12D
0
1
2
3
4a
4b
LSL
R270H
p53LSL·R270H
1
2
4
5
6
7
8
9
10
11
LSL
R172H
p53LSL·R172H
1
2
4
5
6
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Fl/+
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Fl/+
270/Fl
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172/Fl
K,Fl/+
K,270/+
K,Fl/Fl
K,270/Fl
K,Fl/+
K,172/+
K,Fl/Fl
K,172/Fl

Figure 2. PCR Analysis of recombination efficiency of conditional alleles. Genomic DNA was isolated from 10 different K;270/Fl tumors (lanes 1-10).

A) To assess recombination of the LSL-K-ras^{G12D} allele, PCR was performed using primers flanking the LSL cassette. The recombined K-ras^{G12D} allele and the wild-type allele yield 305 bp (top arrow) and 265 bp (bottom arrow) products respectively.

B) PCR analysis of the p53 locus using primers flanking the LSL cassette in intron 1. The 335 bp product (arrow) is produced from the recombined conditional allele. An asterisk indicates an unrecombined sample.

C) PCR analysis of the p53^{Fl} allele using primers flanking exons 2 and 10. The recombined p53^{Fl} allele produces a 612 bp product. No product is produced from the unrecombined allele, as indicated by the asterisks.

Figure 2

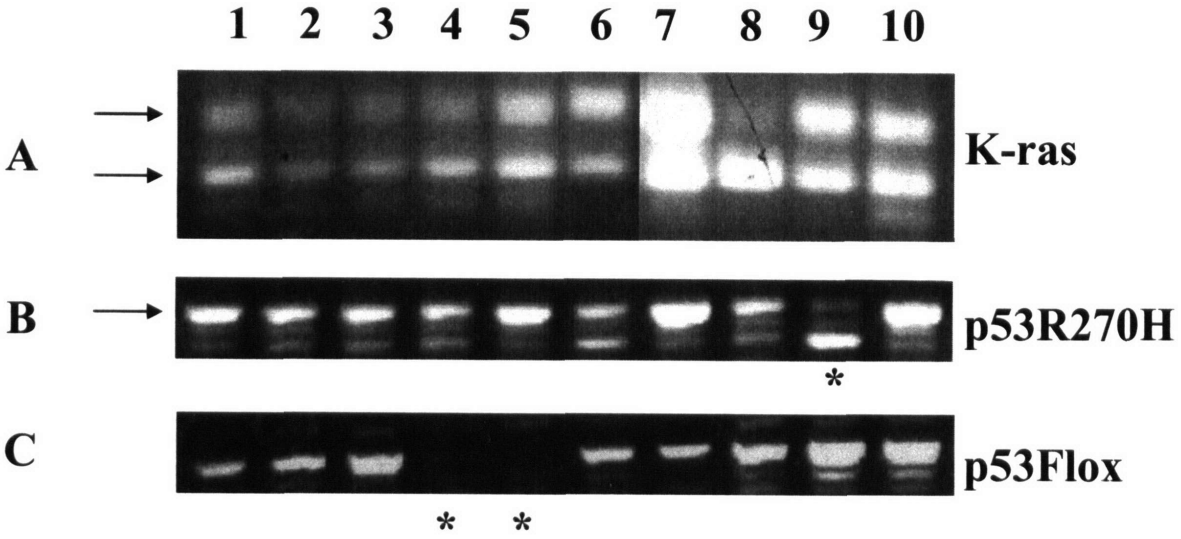


Figure 3. Tumor Grades in K,P Compound Conditional Mutant Mice.

A) At right, a region of advanced adenomatous hyperplasia (AAH), adjacent to normal lung (left). Hyperplastic cells are enlarged and may accumulate slightly, but generally follow the underlying lung architechture.

B) Grade 1 lesions form a solid tumor but have regular nuclei.

C) Grade 2 lesions may have slightly irregular nuclei and prominent nucleoli.

D) Cells in Grade 3 lesions exhibit pleiomorphic nuclei, prominent nucleoli and nuclear molding.

E) Grade 4 lesions have enlarged, pleiomorphic nuclei (single arrows), abberrent mitoses (double arrows), and tumor giant cells (triple arrows).

F) Grade 5 lesions exhibit all the criteria of Grade 4 lesions as well as nests of tumors cells surrounded by a desmoplastic stroma.

igure 3

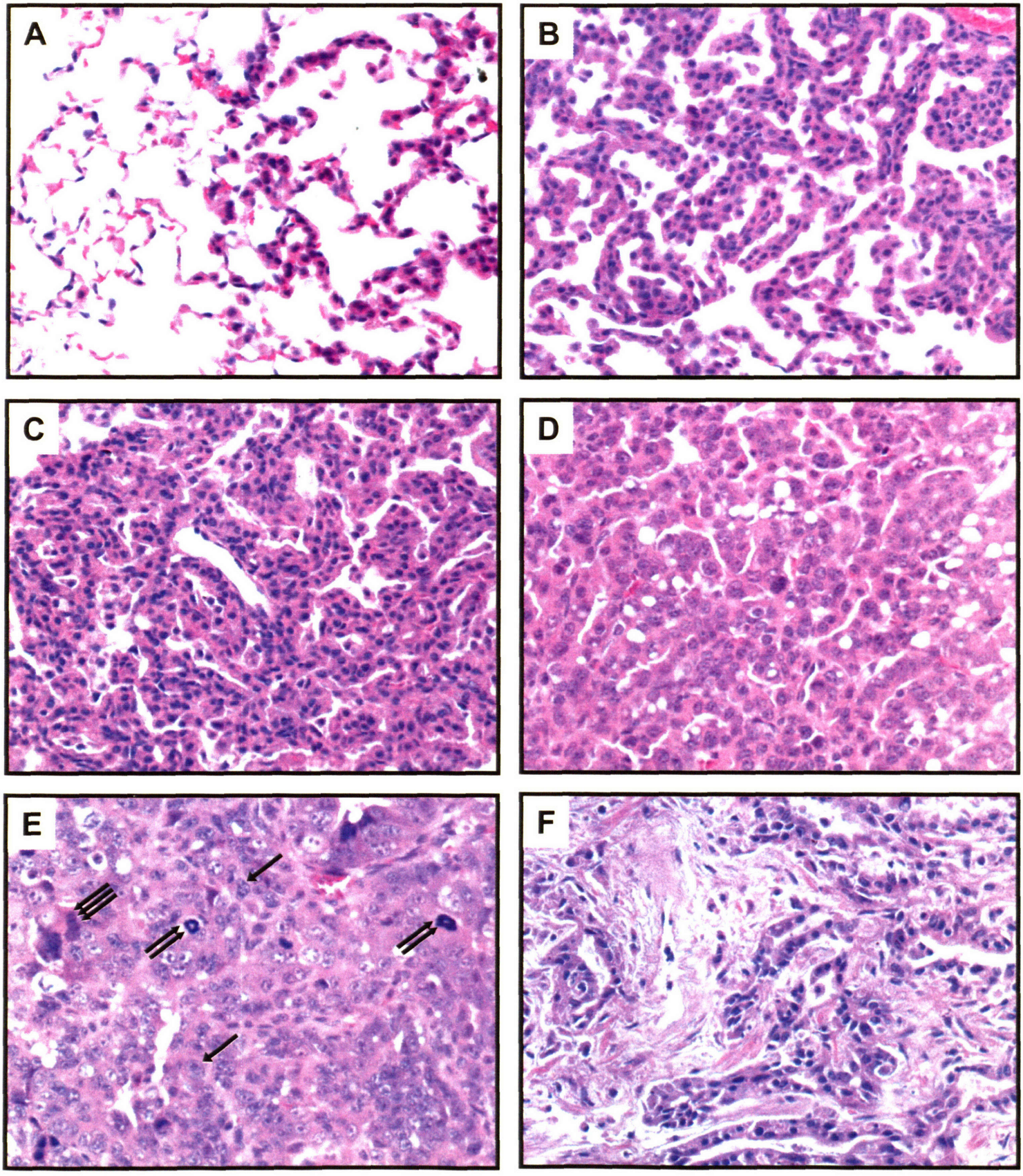


Figure 4. Loss of *p53* results in progression of *K-ras*-induced lung adenocarcinomas. Slides are stained with hematoxylin and eosin unless otherwise indicated.

A) Distribution of tumor grades in K;Fl/+ mice (blue bars) and K;Fl/Fl mice (red bars). Histograms represent the percent of total tumors for each grade. Error bars indicate standard error.

B) A region of stromal desmoplasia with invasion of tumor cells in a Grade 5 tumor. (200X)

C) Masson's Trichrome stained section of a tumor with tumors cells (red with black nuclei) invading a region of collagen-containing stroma (blue). (400X)

D) Section of a lung adenocarcinoma (T) invading into adjacent cardiac muscle (C). (200X)

E) Section of a lung adenocarcinoma metastasis (M) in a thoracic lymph node. (100X)

F) Section of a distal lung tumor metastasis (M) to the kidney. Arrows indicate normal glomeruli in the kidney. Dashed line indicated the boundary of the metastasis.

Figure 4

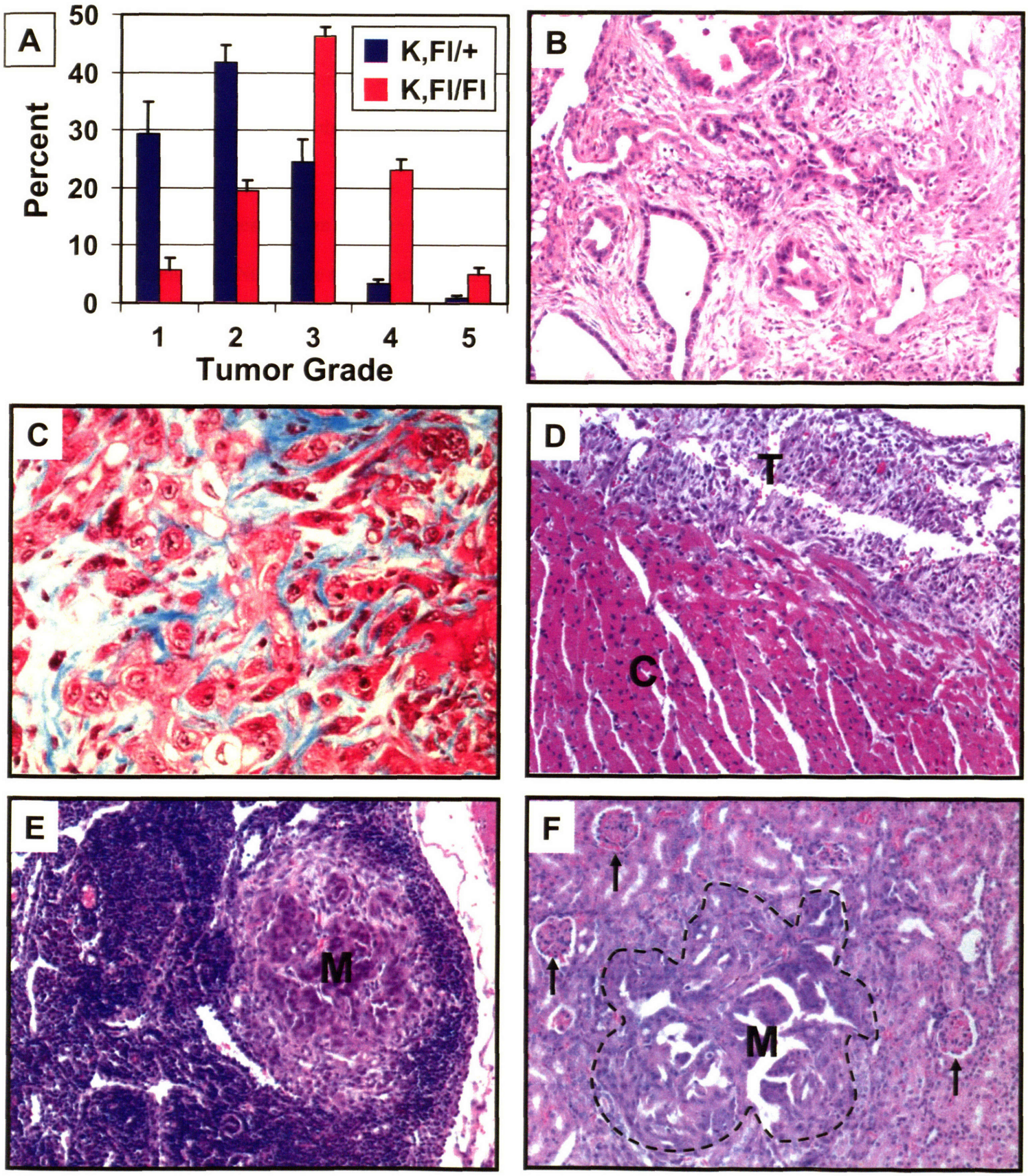

A
50
40
30
20
10
0
Percent
K,Fl/+
K,Fl/Fl
1
2
3
4
5
Tumor Grade
B
C
D
T
C
E
M
F
M

Figure 5. Effects of *p53* Loss are Apparent Six Weeks After AdenoCre Infection. Slides are stained with hematoxylin and eosin.

A) Distribution of tumor grades in K;Fl/+ mice (blue bars) and K;Fl/Fl mice (red bars). Histograms represent the percent of total tumors for each grade. Error bars indicate standard error.

B) Section of a lesion from a K;Fl/+ mouse, six weeks post infection with $5x10^5$ pfu of AdenoCre. Nuclei are regular and retain apical/basal polarity.(400X)

C) Section of a lesion from a K;Fl/Fl mouse, six weeks post infection with $5x10^5$ pfu of AdenoCre. Several enlarged cells with aberrant nuclei are indicated with arrows.(400X)

D) An example of a very small lesion from a K;Fl/Fl mouse, six weeks post infection with $5x10^5$ pfu of AdenoCre. Tumor cells have irregular nuclei, including one enlargerd, aberrant cell (arrow). (400X)

Figure 5

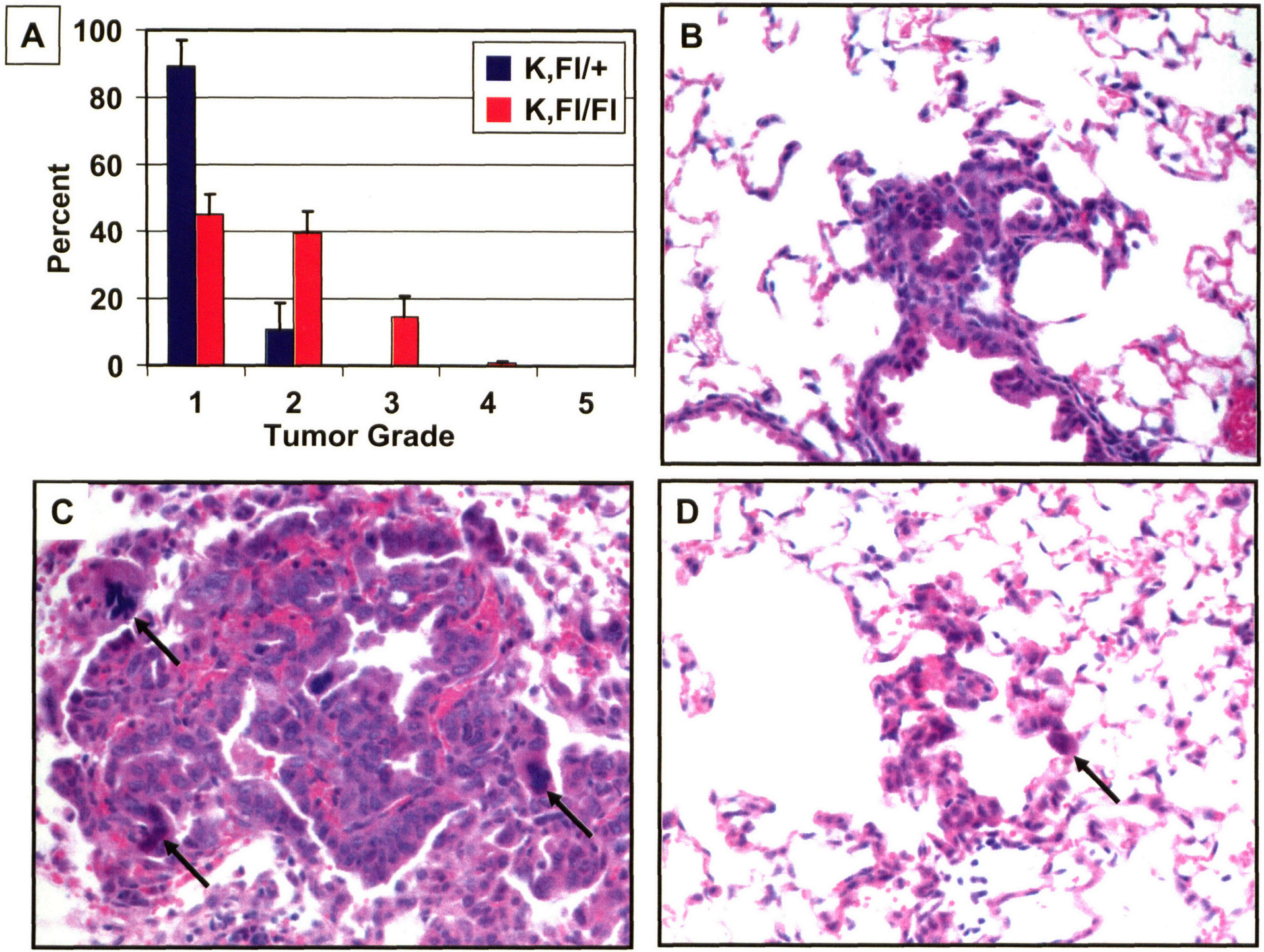


Figure 6. Dominant-Negative Effects by the Contact Mutant 53^{R270H}.

A) Distribution of tumor grades in K;Fl/Fl mice (blue bars), K;172/Fl mice (green bars) and K;172/Fl mice (red bars). Histograms represent the percent of total tumors for each grade. Error bars indicate standard error.

B) Distribution of tumor grades in K;Fl/+ mice (blue bars), K;172/+ mice (green bars) and K;172/+ mice (red bars). Histograms represent the percent of total tumors for each grade. Error bars indicate standard error.

C) Area of tumors in K;Fl/+ mice (blue bars), K;172/+ mice (green bars) and K;172/+ mice (red bars). Histograms represent the percent of lung area occupied by tumors.

D) MAPK activation in K,P mice. Histograms show the percent of tumors with positive staining for ϕ-MAPK in the indicated genotypes.

Figure 6

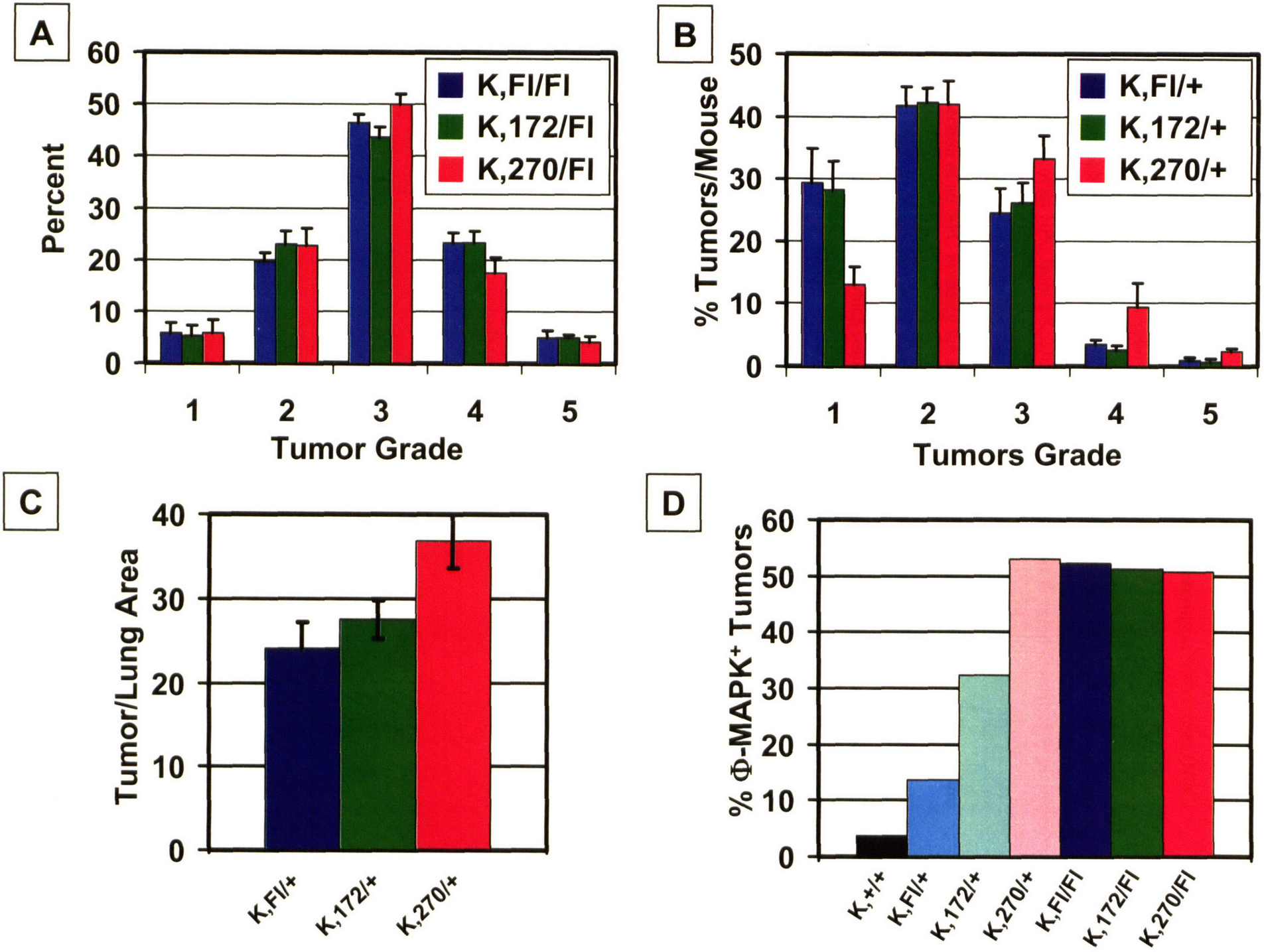

A
Percent
60
50
40
30
20
10
0
K,FI/FI
K,172/FI
K,270/FI
1
2
3
4
5
Tumor Grade
B
% Tumors/Mouse
50
40
30
20
10
0
K,FI/+
K,172/+
K,270/+
1
2
3
4
5
Tumors Grade
C
Tumor/Lung Area
40
30
20
10
0
K,FI/+
K,172/+
K,270/+
D
% Φ-MAPK+ Tumors
60
50
40
30
20
10
0
K,+/+
K,FI/+
K,172/+
K,270/+
K,FI/FI
K,172/FI
K,270/FI

Figure 7. AdenoCre Infection of K,P mice Results in Nasopharyngeal Carcinoma (NPC). Slides are stained with hematoxylin and eosin.

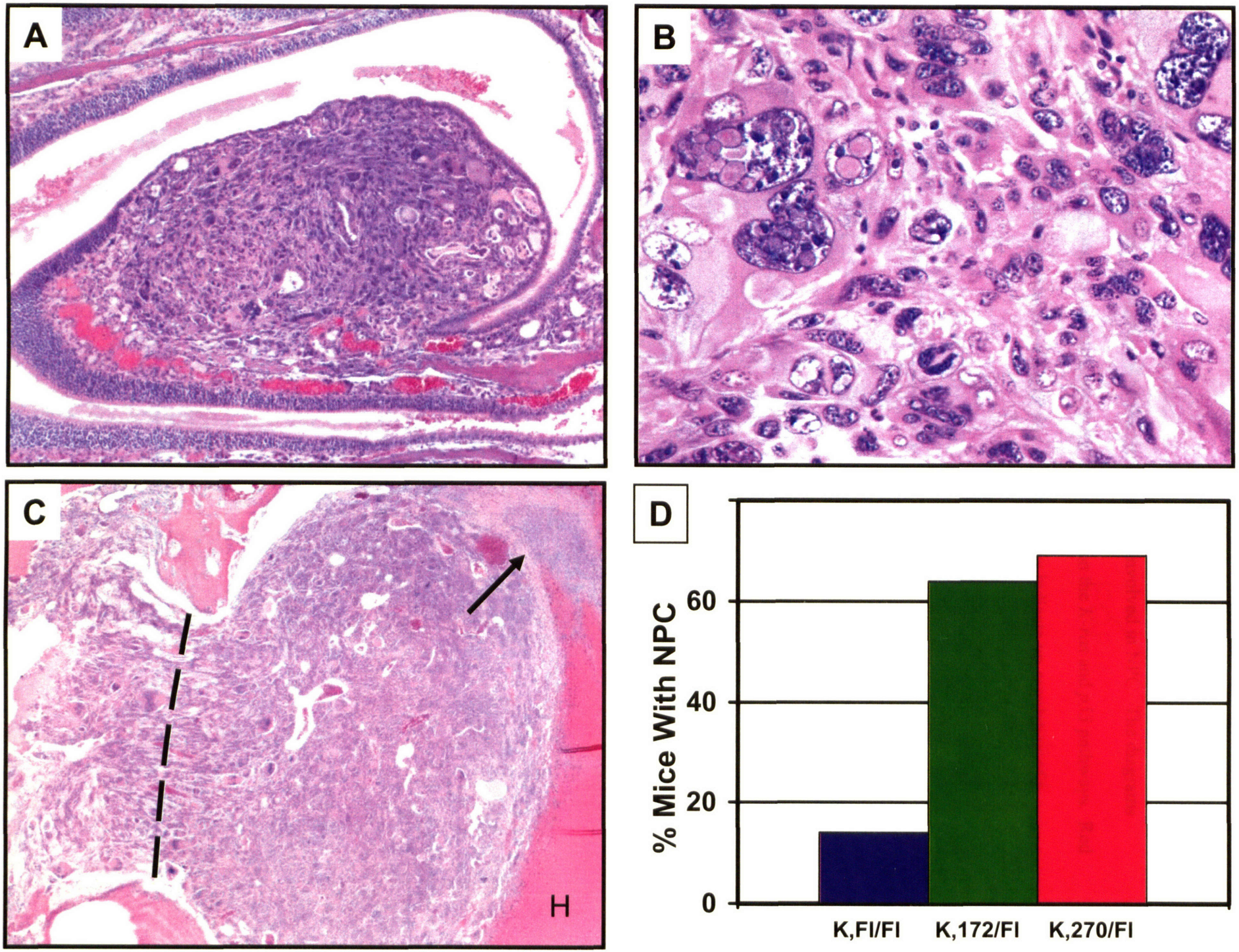
A) A small NPC is shown developing in the nasal mucosa. (100X)

B) Section of an NPC from a K;270/Fl mouse. These tumors develop dramatically pleiomorphic nuclear atypia. (400X)

C) Section of an NPC invading the brain. Dashed line indicates the former location of the cribriform plate. The arrow indicates the leading front of the NPC invading into the remains of the olfactory bulb, which resulted in the accumulation of hemorrhaged blood (H). (40X)

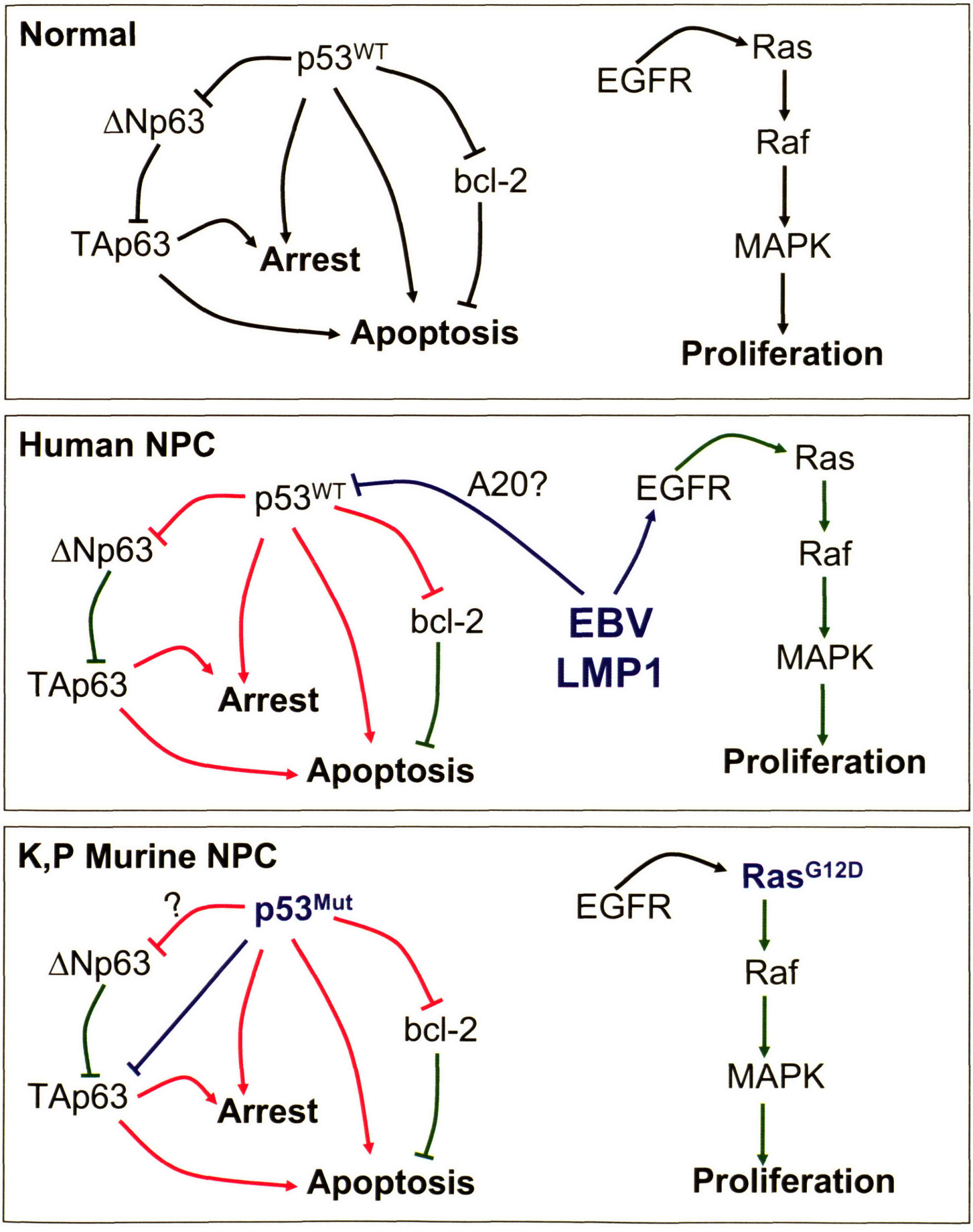
D) Histogram shows the fraction of K;Fl/Fl, K;172/Fl and K;270/Fl mice that developed NPC.

Figure 7



Supplementary Figure 1. Diagram of Molecular Pathways Involved in NPC. The diagrams depict the potential impacts of mutations or EBV infection on the *K-ras* and *p53* pathways. Red arrows indicate down-regulation of a pathway. Green arrows indicate up-regulation of a pathway.

Supplementary Figure 1



Chapter 4

Overview and Conclusions

The focus of this thesis is the function of tumor-associated mutant alleles of p53 and their use in generating accurate mouse models of human cancer. To this end, we engineered two strains of mice with conditional mutant p53 alleles targeted to the endogenous locus. The results of activating these conditional alleles were explored in the context of the entire animal and specifically in a *K-ras*-initiated model of lung cancer. These separate approaches provided complementary information about the basic biology of p53 and its role in tumorigenesis.

MUTANT P53 FUNCTION

Gain of function by endogenous mutant p53

The mutant p53 gain-of-function hypothesis has long been a topic of controversy. It was our intent to rigorously test the hypothesis in a physiologically accurate way. We therefore performed a very simple genetic analysis by comparing mice expressing only mutant p53 to mice lacking p53. The development of novel tumors in $p53^{M/-}$ mice is the first *in vivo* demonstration of a gain of oncogenic potential by mutant p53 and should go a long way towards establishing the mutant p53 gain-of-function hypothesis. The increased incidence of nasopharyngeal tumors is also supportive of the p53 gain-of-function hypothesis. However, the possibility also exists that the increase in NPCs found in K,270/Fl and K,172/Fl mice is the result of non-cell autonomous haplo-insufficiency. That is, the non-infected cells of K,270/Fl and K,172/Fl mice were phenotypically $K\text{-}ras^{+/-}$; $p53^{+/-}$. In contrast, the non-infected cells of K,Fl/Fl mice were $K\text{-}ras^{+/-}$; $p53^{+/+}$. In theory it is possible that NPCs are influenced by non-intrinsic factors that are dose-sensitive to p53. Although such an effect has never before been reported, the only way to rule it out will be to repeat the NPC experiment on K,Fl/Null mice and affirm that they do not have an increased incidence of nasopharyngeal carcinomas.

$p53^{M/-}$ mice develop tumors of epithelial origin in addition to the tumors commonly observed in p53 null mice. This brings to mind the prevalence of p53 point-mutations in different types of sporadic human tumors; the types of tumors that most frequently harbor p53 mutations are all of epithelial origin while most of the tumor types with low rates of p53 mutations are derived from the mesenchyme or from germ cells. This may imply that certain epithelial tissues are specifically susceptible to those aspects of mutant p53 biology that are distinct from p53 loss.

Epithelial tumors are also thought to require higher rates of genomic instability than tumors of hematopoietic lineages (Loeb, 1991; Renan, 1993) and p53 mutations are known to be associated with genomic instability. This may be simply due to a loss of apoptotic surveillance of damaged cells through inactivation of wild-type p53 function or it could be related to a specific function of mutant p53 (Zhivotovsky and Kroemer, 2004). The latter possibility is supported by an experiment in which $p53^{-/-}$ SAOS-2 cells were stably transfected with inducible mutant p53 expression plasmids and then treated with N-(phosphoacetyl)-L-aspartate (PALA) over the course of 16 population doublings. PALA toxicity can be overcome through amplification of the carbamoyl-P-synthetase (CAD) gene, so this assay acts as an indirect measure of genomic instability. Each of three cell lines bearing different inducible alleles of mutant p53 were found to have an increased frequency of gene amplification when mutant p53 protein expression was induced, supporting a gain-of-function effect by mutant p53 on genomic instability (El-Hizawi et al., 2002).

The mechanism by which mutant p53 could induce genomic instability remains speculative. However, wild-type p53 was shown to directly interact with and activate topoisomerase I, a protein that plays a direct role in managing DNA topology, in a transient but tightly regulated manner. In contrast, this interaction was reported to be constitutive in a cell line harboring mutant p53 (Gobert et al., 1999). Since topoisomerase I has been implicated in causing illegitimate recombination (Zhu and Schiestl, 1996), hyperactivation of topoisomerase I could play a role of mutant p53 mediated genomic instability. Alternatively, the inhibition of p63 and p73 by mutant p53 could affect any role in regulating genome stability that the two p53 family-member genes harbor; expression array profiling found that several genes involved in DNA repair were activated following expression TAp73α (Fontemaggi et al., 2002).

We also found evidence for a gain-of-function effect by mutant p53 through experiments performed on a cell line from a mutant p53 osteosarcoma. The induction of cell cycle arrest following shRNA-mediated knockdown of mutant p53 provides an ideal system for mechanistic studies of mutant p53 function. Some of these cells also appear to undergo apoptosis following p53 shRNA activation based on the appearance of highly refractile cells by light microscopy (personal observation).

Several experiments should be performed in order to follow up on these initial observations. To begin, FACS analysis for cleaved caspase-3 expression can be used to determine the presence or absence of apoptotic cells following shRNA induction. Other apoptosis assays could also be performed to look for Annexin-V cell-surface expression, the presence of a subG_1 population by propidium iodide staining or the presence of DNA laddering. Also, the precise nature of the cell

cycle arrest should be worked out by BrdU/PI incorporation or ^{3}H-thymidine incorporation. The accumulation of tumor cells in a particular stage of the cell cycle following mutant p53 knockdown might provide evidence for the activation of a particular cell cycle checkpoint.

At the molecular level, if mutant p53 inhibits the function of p63 and p73, then expression of p63 and p73 target genes should rise following shRNA-mediated knockdown of mutant p53. Preliminary experiments to assess p63 and p73 target gene expression by qRT-PCR supported this prediction, but they require verification (personal observation). An even more stringent experiment would be to perform p63 or p73 chromatin immunoprecipitations to determine whether p63 and p73 binding to target gene promoters increased upon mutant p53 knockdown. It may also be useful to assess the effects of mutant p53 on global gene expression. Having derivatives of the same cell line before and after mutant p53 knockdown should help to reduce the background noise that is common in expression arrays.

The osteosarcoma cell described in this work, as well as other tumor cell lines that were derived from mutant p53 mice, could also be used to identify proteins that interact with mutant p53 using immunoprecipitation and mass spectroscopy. In recent years, p53 has been reported to participate in several direct protein-protein interactions involving the core DBD. These include interactions with TAp63, TAp73, ASPP1, 53BP1, BRCA1 and Bcl-2/Bcl-X_L (Chai et al., 1999; Di Como et al., 1999; Gorina and Pavletich, 1996; Joo et al., 2002)(Ute Moll, unpublished results presented at the 12th International p53 Workshop, Dunedin, New Zealand). One of these interactions, between fragments of p53 and ASPP1, has been visualized using X-ray crystallography (Gorina and Pavletich, 1996). This and other published p53 crystallographic

studies have been invaluable in understanding the basic properties structural properties of p53. It would be a great advantage to have more crystal structures of p53 participating in protein-protein interactions. It would be especially interesting to see a structure of full-length p53 in order to get an idea of how the three independently folding regions of p53 interact with one another. An attempt to crystallize p53 in complex with the small DNA circles described by Carole Prives might have some success as this interaction is far stronger than p53 binding to linear DNA and involves the coordination and binding of multiple regions of p53, including the non-specific DNA binding domain of the CTD. It would also be extremely interesting to observe the protein-protein interactions between mutant p53 and p63 or p73 (this might be accomplished using just the core domain fragments of each protein). This might give an idea as to whether it would be possible to chemically inhibit the interaction between mutant p53 and p63/p73. By inhibiting this interaction, p63 and p73 might then be free to act as tumor suppressors. Their function could then be further augmented either by genotoxic agents or perhaps even the nutlin compounds. Since p63 and p73 do interact with Mdm2, it would be interesting to know whether the nutlin compounds can activate p63 and p73 in addition to p53.

Dominant Effects by Mutant p53

This work also reaffirmed that mutant p53 can act dominantly in the presence of wild-type p53 to promote tumorigenesis. Mice heterozygous for p53 point mutations develop more tumors than heterozygous knockout mice in both the germline and lung adenocarcinoma studies. Although a distinction between dominant-negative effects and dominant gain-of-function effects could not be made in the germline study, a partial dominant-negative effect was established for the $p53^{R270H}$ mutant in the progression of K-ras initiated lung adenocarcinomas. Furthermore,

dominant effects by mutant p53 were apparent in cell cycle experiments on MEFs and apoptosis experiments on primary thymocytes. Whether these cellular assays are indeed indicative of dominant-negative effects by mutant p53 could be established by performing p53 chromatin immunoprecipitations for p53 target genes in the $p53^{M/+}$ cells.

It should be noted that the term "dominant-negative p53" is deceptive. The tumor-associated mutations in p53 do not act as pure dominant-negative alleles as there is substantial evidence from this work and others that mutant p53 cannot fully inhibit the function of wild-type p53 when they are expressed at comparable levels. Indeed, the mere fact the LOH occurs fairly frequently in the tumors of LFS patients proves that there is a selective pressure to lose the remaining wild-type allele of p53. The incomplete dominant inhibition of wild-type p53 by mutant p53 may reflect a partial transcriptional activity by p53 tetramers composed of both mutant and wild-type p53 polypeptides. Alternatively, it may reflect a residual pool of fully wild-type tetramers arising through the random association of four wild-type p53 polypeptides. This latter case becomes substantially more probable if it is true that the initial dimerization of p53 occurs co-translationally (Nicholls et al., 2002). An interesting experiment could be performed that would address this possibility *in vivo* using published mouse strains. The Hupki mice, created by swapping exons 4-9 of human p53 into the murine p53 locus, produce a fully functional chimeric p53 protein that is six residues shorter than the endogenous p53 allele (based on genome data from Ensembl)(described in Chapter 1- mouse models of p53). By crossing Hupki mice to the germline mutant p53 mice to get $p53^{M/Hupki}$ mice, one should be able to distinguish how many differently sized populations of p53 proteins occur by non-denaturing PAGE and immunoblotting. If tetramerization occurs completely randomly, there should be five

different populations of p53 tetramers in a 1:4:6:4:1 ratio, reflecting tetramers containing 0,1,2,3 or 4 mutant subunits. However, if irreversible cotranslational dimerization occurs *in vivo*, then only three populations should be found in a 1:2:1 ratio. Furthermore, the DNA binding activity of each of these populations could also be tested using DNA affinity immunoblotting. Following the addition of biotinylated DNA (containing p53 target sites) to cell lysates from $p53^{M/Hupki}$ cells, a streptavidin immunoprecipitation will pull down only those populations of proteins that successfully bind to DNA. p53 immunoblotting of the immunoprecipitates under non-denaturing conditions would then distinguish which populations of tetramers bound to p53 target sites. This experiment could be performed with a variety of different p53 response sites in order to examine the dominant-negative effects of mutant p53 on different genes.

Mutant p53 protein accumulation

The observation that $p53^{M/-}$ mice do not accumulate mutant p53 protein in their normal tissues, or even in many of their tumors, suggests that there is an *in vivo* regulatory mechanism for p53 protein stability that is not strictly dependent on the Mdm2/p53 autoregulatory loop. In contrast, $p53^{M/-}$ or $p53^{M/+}$ MEFs have elevated levels of p53 protein even in the absence of DNA damage. What is the mechanism by which mutant p53 protein levels are kept in check *in vivo*? One possibility is that p53-independent Mdm2 activation plays an important role *in vivo*. Alternatively, another protein such as JNK may regulate mutant p53 accumulation. One interesting observation comes from the K,P lung cancer models. Immunohistochemical staining for p53 in the lung tumors that develop in K,270/Fl mice and K,172/Fl mice found that only a subset of tumors accumulated mutant p53. This subset tended to include the more advanced tumors from these mice. Interestingly, staining of adjacent serial sections of lung with p53 and

phospho-ERK antibodies found that all of the tumors that accumulated mutant p53 also had expression of phospho-ERK. This might suggest that the MAPK pathway could intersect with the regulators of mutant p53. Preventing the accumulation of mutant p53 could be useful in tumors types where mutant p53 actively promotes tumorigenesis.

MOUSE MODELLING OF HUMAN CANCERS

By targeting the compound mutation of both K-ras and p53 to the lungs, we were able to create mouse models of lung adenocarcinoma that accurately recapitulate the phenotypes of human lung cancer. In particular, tumors arose in the K,P mice that had advanced features of human lung adenocarcinoma, including nuclear atypia, tumor giant cells, stromal desmoplasia, invasion and metastases. These successes reflect the careful targeting of the exact mutations found in human cancers to the appropriate cellular compartment.

Mouse models of cancers have found broad use as an educational tool for discovery. The K,P mouse models of lung cancer have already provided several important lessons regarding the biology of lung adenocarcinomas. As expected, these experiments confirmed that loss of p53 strongly promotes the progression of lung adenocarcinomas. However, the presence of mutant p53 does not confer a gain-of-function effect on the promotion of lung tumors. It should be noted that these experiments were designed in a way that might not detect a gain-of-function effect by mutant p53 on lung adenocarcinoma initiation. For example, if mutant p53 in some way inhibits DNA repair pathways, then its expression might promote the acquisition of K-ras mutations. Such an effect would obviously promote the initiation of lung adenocarcinomas but would not be detected by the experiments performed in this work.

It was also found that the $p53^{R270H}$ allele has a partial dominant-negative effect on the progression of K-ras induced lung adenocarcinomas while the $p53^{R172H}$ allele does not. This may explain why codon 273 of human p53 is more frequently mutated in human lung adenocarcinomas than codon 175.

Finally, it should be noted that even in the K,Fl/Fl mice, only a small subset of tumors possessed the most malignant features of lung cancer such as stromal desmoplasia, invasion and metastasis. This suggests that further genetic alterations must occur for the tumor to fully progress. Future analyses of the genetic abnormalities in these tumors, including expression profiling and CGH, may provide insight into the further events required for progression. One possibility that I find interesting is a comparison of the genetic abnormalities occurring in K,+/+ lung tumors versus K,Fl/Fl lung tumors. If the loss of p53 from a tumor obviates the need for a particular genetic alteration, one might expect that some abnormalities would be found in the K,+/+ tumors that would not be found in the K,Fl/Fl tumors. This could provide insight into what losing p53 truly accomplishes for the tumor.

Use of mouse models in preclinical drug testing

To date, mouse models of cancer have primary been used for learning about the biology of cancer. However, accurate mouse models may also be useful as preclinical models for drug discovery and development. Clinical testing of drugs is an extraordinarily expensive endeavor. In the year 2000, the mean out-of-pocket cost to perform Phase I, II and III trials on a single investigational compound was 15.2, 23.5 and 86.3 million dollars, respectively (DiMasi et al.,

2003). For this reason, a great amount of effort is devoted to trying to predict which compounds will be the most successful. The current standard for preclinical testing is the use of xenograft models. This is a low standard. Xenograft models are extremely ineffective for predicting drug efficacy in humans. Therefore in order to be useful, we need only demonstrate that mouse models of cancer are more predictive of efficacy in humans than xenograft models.

Mouse models have a number of advantages over xenografts. For example, in every tumor that arises in a genetically engineered mouse model, one or more of the genetic alterations in that tumor are identified *a priori*. In contrast, generally very little is known about the genetic abnormalities present in a xenograft tumor. This information could be extremely useful when testing rationally targeted drugs. Small, initial trials could be given more power by selecting the subset of patients most likely to be responsive to a particular drug, i.e. those harboring tumors with particular mutations.

Another benefit is that genetically matched primary cells or tumor cells can be cultured from a mouse for use in high-throughput cellular screens. Relevant reporter molecules can even be engineered directly into the mouse model to facilitate screening. Indeed, several rounds of screening could be used in order to assay different aspects of the tumor biology. Intermediate-throughput screens could conceivably be designed using 3D organ culture or tumor explants. Testing on the organismal level could then commence using a small number of compounds that have been vetted in a series of genetically matched systems. One way to help validate whether any of these screens are predictive will be to test all the drugs that have been used in humans to treat a particular cancer as controls. If cisplatin is an ineffective drug when used in humans

against a particular cancer, one should verify that it is also ineffective against the mouse model of that cancer. As always, when working with mouse models, one should, at all times, be guided by the human disease.

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