p53 Nuclear Localization Control, and p53-dependent Regulation of DNA Repair Gene Transcripts

By

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ABSTRACT

The experiments presented in this thesis use mutation analysis, and study of the cells of mice with a deletion allele for the *Trp53* gene, to explore both the regulation of p53, and its downstream functions mediated by specific activation of target genes. Chapter 2 addresses the regulation of nuclear localization of the p53 protein. Previous reports in the literature had suggested that the p53 negative regulator HDM2 was a nucleocytoplasmic shuttling protein that binds and carries p53 from the nucleus of the cell to the cytoplasm where it is destroyed by the proteasome. We determined that HDM2 with a mutated nuclear export sequence was still able to able to alter p53's cellular localization to a cytoplasmic pattern. The nuclear export sequence in the p53 C-terminus was required for this activity, as was the ability of HDM2 to ubiquitinate p53. Further studies indicated that ubiquitination of the p53 C-terminus was the basis for HDM2's ability to remove it from the nucleus and cause its efficient degradation. C-terminal ubiquitination causes the p53 nuclear export sequence to be activated or made more accessible to the nuclear export machinery of the cell. Chapter 3 summarizes cDNA microarray experiments in which *Trp53*-/- and *Trp53*+/+ fibroblasts were treated with a panel of genotoxic agents, and assayed for p53-dependent upregulation or downregulation of the approximately 15,000 gene sequences represented on the microarray. New candidate p53 target genes were revealed, among them the DNA repair gene *Ercc5*, which encodes the xeroderma pigmentosum disease gene homolog Xpg, a participant in nucleotide excision repair and a mediator of base excision repair of oxidative DNA damage. Further analysis of most of the DNA repair genes in the mouse genome using real-time PCR indicated that a second gene, *Polk*, encoding the translesion DNA polymerase kappa, is also a p53-induced gene. Chapter 4 describes further characterizes the p53-dependent regulation of *Ercc5*, and shows that it is a directly-regulated p53 target gene with a p53-responsive site in its first intron. *Trp53*-null cells show a modest reduction in the ability to repair an oxidatively-damaged DNA construct, and this defect is rescued by exogenous expression of retrovirally transduced XPG, indicating that the lower levels of this gene are likely responsible for the defect.

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Biographical Note

Scott Boyd was born in Winnipeg, Manitoba, Canada. He attended the University of Manitoba, earning the degree of B.Sc. in Biochemistry in 1992, and receiving the Governor General's Medal for Undergraduate Science, the University Gold Medal, and a Rhodes Scholarship. He pursued literary studies at the University of Oxford, where he was granted the degree of B.A. in English Language and Literature in 1994, and was recognized with the Thomas Jefferson award from St.Catherine's College. His travels next took him to Harvard University and the Massachusetts Institute of Technology, where he entered the M.D./Ph.D. degree program in the Health Sciences and Technology society. After initial research work in the lab of Arlene Sharpe at the Brigham and Women's Hospital, studying the CTLA-4 T cell inhibitory receptor, he joined the lab of Tyler Jacks at MIT for his Ph.D., and began studies of the p53 tumor suppressor protein.

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Chapter 1: Table of Contents

1. Historical Overview: Human Cancer, Tumor Suppressor Genes, and p53

a) Understanding Cancer as a Disease of Gene Mutations

The set of diseases referred to as cancer have been known to beleaguered physicians since ancient times. The story of mankind's growing understanding of the causes of cancer closely parallels the development of biological science, for as new knowledge, methods, and tools have been developed, investigators have been quick to subject cancer to fresh scrutiny. Never has this been so apparent as in the past 50 years, following the discovery of the structure of DNA, and the extremely useful technologies afforded by its base-pairing mechanism. In these few initial decades of molecular discovery, the cellular gene *TP53* has emerged as perhaps the most essential of the human genes tasked with preventing normal cells of the body from becoming cancerous. A survey of the roughly 28,000 scientific papers about p53 in the scientific literature shows that although much has been learned about this protein's roles in the cells of the body, and the main themes of its activities are beginning to be agreed upon, many aspects of its functions are still subject to debate. Key points are not yet resolved due to the limitations of current tools, and possibly because of the diversity of cellular contexts in which p53's function may be required.

Variation is the hallmark of biological entities: the different species that successfully populate the earth; the sub-populations of organisms of a given species; the distinct

individual cells in the tissues of multicellular organisms, each with its own unique life-history; and, at the root of all these other differences, the nucleotide sequence variations within the strands of DNA, inflicted by radiation and chemicals from the environment, or generated by rare failures of the cellular DNA replicating machinery. This diversity, in the form of gene mutations, is the key to understanding the causes of cancer, in which a mutant cell can depart from the regulated behavior of its kind and embark on a Darwinian rampage ending in the death of its host.

With an eye to themes that have emerged in the study of p53, a brief recounting of earlier discoveries in cancer biology may be informative. Classical physicians and their intellectual descendants through the medieval period recognized that the lesions known as cancers behaved differently from other swellings and lumps that we now know to be caused by infectious diseases, and they did not dictate the same sort of societal avoidance of cancer sufferers as they did for victims of contagious diseases (Shimkin, 1977). The word cancer, derived from the Greek *karkinos*, the crab, was used to designate lesions with a hard swelling center and prominent veins, most clearly described in the breasts of women (Demaitre, 1998). In the Hippocratic tradition, the cause of cancer was said to be an excess of the black bile humor. The recommended therapy was cutting out the cancer, if it wasn't too large or deeply integrated into the surrounding tissues. Otherwise, there was little hope for the patient, and palliative treatment was administered. Modern understanding of the causes of cancer has advanced tremendously; sadly, with some exceptions, the current treatment options are closer to those of medieval times, although

there are many more sophisticated ways of cutting a cancer out and a short list of drug treatments that show efficacy.

To move rapidly over other important observations about cancer in early times, we will note the rise of anatomical studies and dissection which revealed characteristic tumors in many organs of the body in the 16th century, and the association of tumors with vessels of the lymphatic system discovered by Aselli in the 17th century, foreshadowing metastatic processes whose molecular bases remain largely mysterious (Shimkin, 1977). The beginnings of cancer epidemiology appear in the 18th century in observations of characteristic cancers of the breast in celibate nuns, and Percival Pott's description of British chimney sweeps, who unlike their continental European counterparts, were rarely allowed to wash the carcinogenic soot and tars from their bodies and developed scrotal cancers (Shimkin, 1977). The 19th century brought the first clear recognition of metastases, the application of the microscope to the study of pathological specimens, and new theories about the embryonic cell layer origins of cancer cells. Further epidemiological connections were established between cancers and the new molecules such as aniline dyes generated by organic synthesis (Lee and Wright, 2000). In this same period, experimental oncology can be said to have begun, with demonstrations of the ability to transplant tumors from one animal to another (Shimkin, 1977). This experimental method still finds much use today, although its conceptual descendant, the transfer of a disease trait by transferring the putative genetic cause of the trait, is the current state of the art in mouse transgenic experiments. Finally, the studies of Gregor

Mendel, when rediscovered in the 20th century, would provide the starting point for modern understanding of heredity in diploid organisms.

In the early 20th century, the main ideas that bear on the story of p53 are Theodor Boveri's 1902 hypothesis that cancer might be due to abnormal chromosomes; a countervailing view was put forward by Borrel in 1907, suggesting that cancer was caused by the effects of filterable viruses infecting cells (Balmain, 2001; Shimkin, 1977). In retrospect, both were correct: we are now aware that a subset of cancers are probably triggered by oncogenic viruses, but that the majority of cancers are the result of somatic mutations in cellular genes in the chromosomes. Consistent with its seemingly central role in preventing unwanted cellular proliferation, p53 is the target of both of these mechanisms of cancer causation, being inactivated by proteins of several oncogenic viruses, as well as being altered by gene mutations caused by a variety of chemical agents.

Other key findings in the 20th century leading up to the era of molecular biology in cancer studies were the evidence from inbred mouse strains and human genetic analysis that gene mutations could confer cancer susceptibility; clearer characterization of the chemical agents that could contribute to cancer causation (later connected to their ability to cause mutations in the DNA of cellular genes); and isolation of oncogenic viruses that would provide the first clues, via the altered or truncated pieces of cellular genes that they had accumulated in their genomes, of the genes that are mutated in sporadic human cancers (Lee and Wright, 2000; Shimkin, 1977).

b) Tumor Suppressor Genes

The idea that mutations in endogenous genes could give rise to the transformed phenotype of cancer cells suggested several possible categories for such gene mutations, by analogy with classic genetic analysis of other traits in diploid organisms. There could be "gain of function" mutations, where a cellular gene's activity is enhanced or altered by the mutation, so as to give the novel phenotype. Such mutations could, in theory, act in a dominant fashion, and this possibility was explored in experimental strategies to clone these "oncogenes" by introducing them into other cells (Shih and Weinberg, 1982).

The hypothetical counterpart to oncogenes were designated "tumor suppressor genes". These would be genes whose normal function prevents cells from assuming a transformed phenotype, and whose mutations would act in a recessive manner since both copies of the gene would have to be mutated to ablate wild-type function. Interestingly, these basic concepts of gain of growth-enhancing, and loss of growth-inhibiting genetic elements in cancer development were proposed to explain chromosomal changes by the far-seeing Theodor Boveri at the very beginning of the 20^{th} century, prior to any real understanding of the nature of genes (Balmain, 2001). Early evidence for such genes came from somatic cell hybridization experiments, in which tumorigenic cancer cells were physically fused with parental non-tumorigenic cells. The resultant fusion product cells exhibited the phenotype of the non-tumorigenic fusion partner, indicating that genes in this cell type were able to suppress loss-of-function mutations in the other fusion

partner (Sager and Kovac, 1978; Smith and Sager, 1985; Stanbridge, 1989). Propagation of these genomically unstable fusion product cells resulted in a variety of different hybrid progeny due to errors in mitotic chromosome segregation, thus enabling investigators to narrow down the genetic region responsible for suppressing the tumorigenic phenotype to the level of the chromosome. These ideas and results were fully validated with the molecular cloning of the *Rb* tumor suppressor gene, whose loss results in the formation of retinoblastoma tumors in the affected individual (Friend et al., 1986). As was predicted by the Knudson and DeMars hypothesis, individuals carrying one germline mutation in the *Rb* gene were highly disposed to developing retinoblastoma tumors, as the somatic cells in their retinas had only to suffer mutation of the remaining wild-type allele in order to become nullizygous (Knudson et al., 1975). The loss of the second *Rb* allele on chromosome 13 frequently occurred by deletion, resulting in loss of heterozygosity in restriction-fragment polymorphism analysis; similar LOH patterns in other cancers had been noticed by this time in the short arm of chromosome 17, but their significance was as yet unclear (Mackay et al., 1988).

c) The p53 Tumor Suppressor— Inklings from Early Studies

Between 1979 and 1981, five independent reports described a cellular phosphoprotein of 53 kDa apparent molecular weight expressed at unusually high levels in transformed cells of humans and other mammals (Crawford et al., 1981; DeLeo et al., 1979; Dippold et al., 1981; Jay et al., 1981; Linzer et al., 1979). With the benefit of hindsight, one can discern the germs of much subsequent research on p53 in these initial reports. While most of the

cell lines examined showed high levels of p53 expression, the HeLa line showed no detectable expression; this observation would later point to mechanisms of degradation of the p53 protein in cells expressing oncogenic human papillomavirus gene products (Crawford et al., 1981). The serine and threonine phosphorylation of p53 described in these initial reports would later be revealed to be a key to the protein's activation for normal functions in the cell (Jay et al., 1981). p53 was noted to be a nuclear protein, giving a hint of its role as a transcription factor for gene regulation.

The increased expression of p53 in tumor cells led to the initial hypothesis that p53 might be an oncogene possessing biological activities that could contribute to the successful growth of such transformed cells (Jay et al., 1981). Partial proteolysis of p53 species from different transformed cell sources showed that there were several distinct but related varieties of the protein, which later discoveries would reveal to be different mutated p53 variants (Crawford et al., 1981). However, it was the odd cell line out, the HeLa cells expressing diminished levels of p53, that gave the initial clue that loss of p53 function might play an important role in cancer development, and that *TP53* was an example of a tumor suppressor gene. In time it would be revealed that high levels of p53 in tumor cells are typically the hallmark of mutated p53 that is inactive for its normal functions, including regulation of its own degradation (Crawford et al., 1981; Momand et al., 2000). Much of the p53 research contained in the roughly 28,000 journal articles in the 22 years that followed the reports of 1981 can be regarded as an elaborate exposition of the details accounting for these initial observations. At present, it is clear that many aspects of the normal function of p53 in our cells, and the ways in which derangement of p53

contributes to the development of malignancy, have yet to be revealed. Furthermore, our current understanding of p53 has barely begun have a meaningful impact on the diagnosis of human cancers and does not yet guide the treatment of these disorders. It is to be hoped that the more powerful and comprehensive tools now available in biology will alter this situation in the future, to the benefit of individual patients (Knowles, 2001).

d) TP53 *Gene Sequences: The Eventual Recognition of a New Tumor Suppressor*

Cloning and sequencing of a cDNA encoding the mouse p53 protein was first reported in 1983 (Oren et al., 1983; Oren and Levine, 1983). The human sequence was isolated and sequenced in the following year (Matlashewski et al., 1984). A period of some confusion in the field followed, with several studies indicating that p53 exhibited the behavior of an oncogene, and could immortalize cells and cooperate with other oncogenes in cellular transformation (Eliyahu et al., 1984; Jenkins et al., 1984; Lane, 1984; Parada et al., 1984). Although investigators were alert to the possibility that genetic deletions or rearrangements in the vicinity of the gene could be the cause of the high levels of p53 expression in transformed cells, no such alterations were initially detected, and more subtle point mutations were not initially sought (Crawford et al., 1984).

The first clue to the predominant form of p53 alteration in human tumors was detected in 1986. Noticing that there were two electrophoretically distinct forms of the p53 protein in a transformed human cell line, Harris *et al.* sequenced the cDNAs for the gene, and found that there were two alleles present, which differed only by a point mutation from arginine

to proline (Harris et al., 1986). Ironically, this sequence variation proved to be a widely-distributed polymorphism at codon 72 in genes encoding functional wild-type protein, although there is currently a lively debate in the literature about possible functional differences between the two alleles (Bergamaschi et al., 2003; Buchman et al., 1988; Dokianakis and Spandidos, 2000; Marin et al., 2000; Rosenthal et al., 1998). In the following year, other mutations were reported, and the distinctive epitopes and heat-shock protein binding properties of mutant p53 protein compared to wild-type p53 were described, with the implication that the structural integrity of the mutant protein might be compromised (Chumakov, 1987; Sturzbecher et al., 1987).

By 1989, the current understanding of p53 as a protein with growth-inhibitory properties was coming into focus. The presence of point-mutated *TP53* genes in a variety of human cancer cells was recognized, along with the tendency for tumors to lose portions of the short arm of chromosome 17 in the vicinity of locus 17p13.1, where the *TP53* gene is found (Baker et al., 1989; Eliyahu et al., 1989; Finlay et al., 1989; Nigro et al., 1989; Takahashi et al., 1989). The beginnings of understanding of the functional domains of p53 were revealed by mutational "hot-spots" that mapped onto the regions of the gene sequence that were best conserved between mammalian species (Nigro et al., 1989).

2. Clinical Data Supporting a Role for p53 in Human Cancer

a) Overview

Sparked by the revelation that p53 was a possible tumor suppressor gene, a flood of clinical studies of correlations between p53 gene status and tumor development began in the 1990s. A key finding was the identification of germline mutations in *TP53* in families with the rare autosomal dominant Li-Fraumeni syndrome, where affected members develop mesenchymal and epithelial neoplasms at a high frequency, and with early onset (Friend et al., 1986). Sequencing of the *TP53* gene in sporadic human cancers revealed frequent mutation of the gene, with a number of "hot-spots" within exons 5-8 that appeared especially often, suggesting that their alteration was particularly common, or particularly advantageous to a developing tumor cell. The data for a detailed molecular epidemiology of the *TP53* gene is now available in several databases on the internet containing the accumulated data of over 6,000 publications describing nearly 15,000 sporadic *TP53* mutations, and 201 germline mutations (Beroud and Soussi, 2003). It has become possible to discern patterns of particular p53 mutations that are found in different kinds of tumors, and to relate these mutations to environmental exposures to chemicals or radiation suffered by the tissue of origin. Over 70% of reported mutations are not at "hot-spot" positions in the gene; of these, between 4 and 5% of mutations in the UMD-p53 database have only been reported once, possibly giving an indication of the level of the contribution of sequencing errors to the total number (Beroud and Soussi, 2003).

The most striking feature of *TP53* mutation patterns is the preference for G:C to A:T transitions, which accounts for 51% of currently reported mutations, and within these, the preference for mutation at CpG nucleotide sites in the gene (making up 59% of the G:C to A:T transitions) (Soussi and Beroud, 2003). The Cytosines in CpG nucleotide

sequences are frequently methylated in mammalian cells, and it has been shown that this is the case for the 42 CpG sites within *TP53* in nomal tissues. 5-methylcytosine is susceptible to deamination, which converts the nucleotide to a thymidine, resulting in a T:G mismatch mutation that can be "fixed" if it persists long enough for cell division to synthesize the complement to the erroneous DNA strand. This deamination hypothesis is currently favored as the overall mechanism for many *TP53* mutations, but it has not been proved conclusively, particularly with regard to whether or not the participation of other reactive chemical species within the cell, or from exogenous sources, plays an important role in the rate of deamination events; indeed, some alkylating agents may preferentially promote mutations at CpG base sequences (Pfeifer, 2000). Also, the signatures of environmental mutagens can be found in the tumors of different tissues that have differing exposures to irradiation or reactive chemicals (Pfeifer and Denissenko, 1998). Naturally, the susceptibility of individual bases within *TP53* to mutation is not revealed by the tumor mutation databases, since the appearance of a given mutation is very likely the consequence of its ability to promote the transformed phenotype of the cells of the tumor.

Although deletions are frequently found in the region of *TP53* on chromosome 17, it appears from the sequence databases that there is a strong selective advantage for tumor cells expressing missense point mutants of the gene (Soussi and Beroud, 2001). This observation is consistent with dominant gain of function behavior of the mutated gene products. In light of the known tetrameric quaternary structure of the wild-type p53 protein, and a number of *in vitro* studies indicating that point mutant p53 proteins can

interfere with the behavior of the wild-type protein, there is reason to credit the gain-of-function or dominant negative hypotheses, although it is possible that p53 may exhibit haploinsufficiency for some phenotypes that are important for preventing tumor cell development. Also, the true rates of deletional events and point-mutational events at the *TP53* locus in normal cells subjected to normal environmental exposures are not clearly known, and it may well be the case that point mutations are a more frequent sort of genetic alteration in the gene. The loss of the wild-type allele by deletion in a tumor cell containing a point-mutated allele would indicate that there is additional advantage to losing the remaining wild-type protein, either because the dominant negative properties of the point-mutated p53 are only partially effective, or perhaps because the point-mutant p53 has additional favorable gain-of-function behavior that is suppressed by the wild-type protein. Alterations in the rate of unrepaired mutagenic events, or cell survival in the context of mutagenic events, may also contribute to loss of the second allele once one copy of p53 is inactivated or becomes dominant-negative in function (Beroud and Soussi, 2003). Alternatively, the rate of deletions versus point mutations could simply be higher under all circumstances, with the phenotypic growth or survival advantage of point-mutant heterozygosity compared to null allele heterozygosity accounting for the difference in tumor production.

A minor topic of p53 sequence variation that has been the subject of recent dispute is the polymorphism of codon 72, which has two major variants, one coding for an argine, the other a proline (Soussi and Beroud, 2001). A variety of cancers from cervical carcinomas to breast cancers are reported to have skewed representation of these alleles, indicating

preferential interaction of one species with oncogenic viral proteins, or interactions of other point mutant p53 proteins preferentially with the wild-type product of one allelic form of the polymorphism within the *TP53* gene. Studies of other cancers, for example, a series of colon tumors, have found no such connection.

It should be noted that the *TP53* mutant sequence databases contain a significant bias as a result of many investigators having chosen to sequence only exons 5-8 of the gene sequence once it became known that these regions of the gene frequently contained mutations. About 40% of the papers describing mutant *TP53* genes sequenced only these exons (Soussi and Beroud, 2001). Within the entire database, 15% of the mutations fall outside of exons 5-8, with examples from most kinds of cancer, indicating that a substantial number of other mutants were likely missed in the exon 5-8 sequencing studies. A higher frequency of frameshift mutations compared to missense mutations is seen in exons 4, 9 and 10 than is the case for exons 5 to 8, for example. A second under-appreciated source of p53 inactivation is intronic splice-site mutation, which were recently shown to account for 17.5% of the mutations in a series of Li-Fraumeni families. A further source of reporter bias in analysis of p53 gene sequences is the practice of relying on immunohistochemical detection of high levels of p53 protein before deciding to sequence the gene: only about 80% of mutated p53 alleles give rise to high protein levels, and therefore there is likely to have been under-reporting of frame-shift, truncating, and other less customary mutations (Soussi and Beroud, 2001).

These difficulties or inadequate measurements of *TP53* status in clinical studies have probably contributed to the minor role that *TP53* status plays in current clinical decision making. Disagreement in the literature about the prognostic value of knowing *TP53* status has meant that this information is not commonly taken into account for therapeutic decisions. There is some indication now that clinical studies where the entire *TP53* gene is sequenced show better correlations between *TP53* status and clinical outcomes, suggesting that further thorough investigation may allow p53 to be more useful to the oncologist in dealing with the real-world crisis that confronts an individual who learns that they have cancer (Soussi and Beroud, 2001). A final issue in considering the correlations between *TP53* mutation status and human cancers is that several non-mutational mechanisms of p53 inactivation have been discovered and partially elucidated. Many cervical cancers arise from cells infected with papillomaviruses expressing p53-inactivating gene products (Tommasino et al., 2003). Neuroblastomas and some other cancers have been found to exclude wild-type p53 from the nucleus of the cell, apparently via several different mechanisms (Stommel et al., 1999). Amplification of the gene for the p53 negative regulator MDM2, is found in a variety of cancers, most notably sarcomas, while other cancers inactivate the upstream potentiator of p53 activity, $p14^{ARF}$, rather than the gene for p53 itself (Leach et al., 1993; Zhang and Xiong, 1999).

Another major aspect of tumor biology that *TP53* mutation studies have tried to illuminate is the stepwise progression of less-malignant to more malignant lesions. The earliest, and most influential example of this extension of the somatic mutation model of cancer development was the result of a series of studies of progressively more advanced

lesions of colon cancer, in which a fairly well-ordered series of gene mutations and chromosomal abnormalities was found to correlate with the progression and biological behavior of the tumors (Goldberg and Diamandis, 1993). It may be fairly said that for the majority of human cancers, particularly those in less accessible tissues of the body, the life-history and characteristics of progression of different malignant lesions have yet to be puzzled out, and it is probably overly optimistic to suppose that there are not a number of variant pathways of ordered gene mutations that can lead to most kinds of cancer. For example, a form of colonic adenoma has been detected with the use of magnifying endoscopy that typically possesses *TP53* mutations prior to *APC* or *K-ras* gene mutations, reversing the order of the paradigmatic progression series (Baba, 1997). However, current data do support the general idea that for some human cancers such as soft tissue sarcomas, early *TP53* mutations are essentially required, while for others, p53 functional loss contributes to later steps in the accumulation of more malignant behavior.

b) Prevalence and variety of p53 mutations in human cancers

Li-Fraumeni Syndrome

A series of cancer-prone families plagued with soft-tissue sarcomas, breast cancers, and other neoplasms was reported by Li and Fraumeni in 1969, with the suggestion that this might be an example of a dominant genetic disease trait (Li and Fraumeni, 1969). In comparison to other familial cancer syndromes such as von Recklinghausen's

neurofibromatosis, or familial melanoma predisposition, the Li-Fraumeni syndrome was unusual for the wide range of tissues it affected. The current definition of a Li-Fraumeni-like (LFL) case is one family member proband having a childhood tumor or sarcoma, adrenocortical tumor, or brain tumor under age 45, with a first or second-degree relative having a Li-Fraumeni-associated tumor, and an additional first or second-degree relative having any cancer under age 60 (Birch et al., 1994). In a recent compilation of studies where much of the *TP53* gene, including splice junctions, was sequenced, 77% of Li-Fraumeni syndrome patients (23 of 30 total) had a germline mutation of some sort in their *TP53* gene (Varley, 2003). The same study found that 27% of the mutations were outside the traditional exon 5 to 8 region, and a deletion, an insertion, and 7 different splice site mutations were detected. Overall, the most common mutations in Li-Fraumeni patient germlines follow the same patterns as those found in sporadic tumors, with codon 248, 273, 245, 175, and 282 missense mutations being most common, albeit in a different order of ranking in the two cases (Varley, 2003). Families with core DNA-binding domain mutations appear to have a more severe form of the disease, with earlier and more numerous tumor development, compared to families with null alleles, or those where the *TP53* gene has a wild-type sequence. An overview of cancers in families known to possess *TP53* mutations found that the tumors with the highest incidence in these individuals were breast carcinomas (24%), osteosarcomas (12.6%), brain tumors (12%), soft tissue sarcomas (11.6%), followed by ALL and Hodgkin's lymphomas (4.2%) and adrenocortical carcinomas (3.6%) (Kleihues et al., 1997). Li-Fraumeni patients also show some tendency toward earlier than usual development of a wider range of other cancers, for example, in the lung and gastrointestinal tract (Nichols et al., 2001).

The closer to normal risk that Li-Fraumeni syndrome patients will develop lung, bowel, bladder, ovary, or head and neck cancers compared to the general population, despite carrying germline mutations that are the same as the most common *TP53* mutations found in sporadic tumors at these sites, is quite interesting. This mystery may relate to other rare mutational events that must take place first before a nascent tumor in these sites can begin to grow—clearly, the *TP53* mutation is not a decisive first step, as it appears to be in breast cancer and sarcomas. An additional intriguing insight has come from children with adrenocortical carcinoma. It is reported that 80% of children developing this disease have germline *TP53* mutations, in the otherwise rarely mutated codons 152, 158, or 337 (Latronico et al., 2001; Varley et al., 1999). The codon 337 mutations were found in a study of Brazilian children, but there appeared to be no founder effect that could account for the prevalence of this mutation. The protein product of the mutated codon 152 gene appears to be a fairly typical transactivation-deficient mutant, but the product of the codon 337 mutation has an unusually pH-sensitive tetramerization domain which may be the source of its phenotype (DiGiammarino et al., 2002).

Sporadic tumors

Breast Cancer

TP53 mutations are frequently found in breast cancers; the percentage of cancers showing such mutations ranges from 8% to 71% in different studies, with the positive percentage increasing with lymph-node positive tumors, larger tumors, more advanced progression, and recurrence of tumors (Borresen-Dale, 2003). The prevalence of breast cancers in Li-Fraumeni patients gives a clue that p53 inactivation can function as an efficient initiating mutation for these cancers, but it is evident that a variety of paths can lead to breast cancer. Notably, patients carrying germline mutations in the *BRCA1* or *BRCA2* genes appear to have a different spectrum of mutations in their *TP53* genes than do patients with sporadic breast tumors, indicating that the role of these two gene products in the maintenance of genome stability can contribute to the means of inactivating p53 (Gasco et al., 2003). There is some positive evidence from mouse studies now to suggest that increased levels of wild-type p53 protein can be caused by the rise in estrogen and progesterone levels in pregnancy, and that this effect might at least partially explain the protective effect of pregnancy with regard to this kind of cancer.

A recent survey of the prognostic value of measuring p53 overexpression by immunohistochemistry in breast cancer cases gave marginal results. A subset of studies reported so far give a stronger indication of the possible benefit of measuring p53 in these cases, but it is not clear whether differences in various studies are attributable to measurement methods, patient populations, particular *TP53* mutations, or other factors. mRNA expression profiling studies of breast tumors indicated that mutant p53 tended to be found in tumor subtypes that had poorer clinical prognosis (Borresen-Dale, 2003). In addition, there is reason to hope that *TP53* status may contribute useful information in the

future about responses of breast cancers to therapy, since a series of studies have now shown that point mutants in the DNA binding domain of p53 are associated with poorer responses to several different therapeutic regimens.

Cervical Cancer

Cervical carcinomas present one of the clearest examples of the interaction between a very specific environmental factor and p53 functional inactivation. The cells of more than 90% of cervical cancers are found to be infected with human papillomaviruses. The so-called "high risk" viral variants, HPV16 and HPV18, express a version of the viral E6 protein that efficiently targets p53 for proteasomal degradation by coupling the small protein ubiquitin to it with the help of the cellular E6-AP protein (Tommasino et al., 2003). As with many p53-binding proteins, E6 has also been shown to directly interfere with transactivation by p53, which may additionally impair p53 function. Low-risk HPV strains express E6 proteins that have minimal ability to incite p53 degradation in cells. As expected from the efficiency of this epigenetic viral mechanism, there is a low incidence of *TP53* mutations in cervical cancers (between 1% and 6.5%, depending on the study), and only in the setting of HPV variants of intermediate virulence does *TP53* mutation appear to play an important role in carcinogenesis (Tommasino et al., 2003).

Ovarian Cancer

Ovarian cancer is one of the most poorly understood of cancers causing significant mortality. *TP53* mutations have been reported in roughly 50% of cases studied, with the usual caveats about incomplete sequencing of the gene. Mutations have not been found in benign cystadenoma tumors of the epithelium, and are rare in the cystadenomas of borderline malignancy category of lesions. Regrettably, because of the anatomical location of the ovaries and the lack of prominent symptoms early in disease progression, many women present with malignant tumors that have spread throughout the peritoneum. More than half of these cases of stage III or stage IV disease show *TP53* mutations. There is as yet no agreement in the literature about the prognostic value of information about p53 for ovarian cancer patient outcomes (Schuijer and Berns, 2003).

Lung Cancer

TP53 is one of the most frequently mutated genes in lung cancer. Together, lung cancers are the leading cause of cancer deaths worldwide, and perhaps the most preventable variety. The influence of tobacco smoke leaves a unique mutagenic signature in the *TP53* gene, with an unusually high rate of G:C to T:A transversions, and with prevalent mutations of bases that form adducts with polycyclic aromatic hydrocarbons such as benzo-*a*-pyrene. Mutations at codons 157 and 158 are a hallmark of smoking-associated lung cancers. Interestingly, it appears from the current p53 mutation database literature that the overall statistical increase in G:C to T:A transversions in *TP53* of smokers' lung tumors is mainly attributable to differences between female smokers and female non-smokers—for males, there was no significant difference in rates of these mutations

between smokers and non-smokers. These data correlate well with other clinical findings showing that women who smoke are at higher risk for developing all of the different classes of lung cancer than are men who smoke a similar amount. Exposures to Radon-222 gas and asbestos particles are also shown to increase lung cancer rates, but there is little data as yet about p53 mutation spectra in these cases (Toyooka et al., 2003).

A recent study of the DNA repair abilities of p53 mutant and wild-type human fibroblasts in response to damage caused by the polycyclic aromatic hydrocarbon Benzo(g)chrysene, one of the major mutagens in tobacco smoke, showed that p53 was essential for normal global genomic repair of the DNA adducts (Lloyd and Hanawalt, 2002). The concentrations of the mutagen used in these experiments was within the range experienced by the cells in the lungs of a smoker, and the authors suggest that the ultrasensitive assay system that they used in this study may have revealed the appropriate context in which p53 plays a key role in protecting cells from environmental mutagens.

Prognostic value of *TP53* mutation is the subject of controversy at the moment, with different groups claiming negative, positive, or no association between patient outcomes and p53 status in lung tumors of patients. This range of results probably has its roots in the methodological concerns mentioned previously (Soussi and Beroud, 2001). Clinical trials are currently testing the efficacy of delivering p53 to lung tumor cells by adenoviral gene therapy vectors (Swisher and Roth, 2002).

Gastric Cancer

Both the intestinal and diffuse subtypes of gastric cancer are associated with *Helicobacter pylori* infection, which has been proposed to contribute to carcinogenesis by attracting leukocytes that damage the gastric cells with free radical species directed toward the pathogen. A number of foods containing carcinogens such as N-nitroso compounds give rise to increased rates of gastric cancer. At present, there is evidence in the p53 mutation databases that mutational spectra for *TP53* in gastric cancer differ with geographical location, perhaps pointing toward the role played by different foodstuffs, contaminants such as aflatoxin, or other environmental factors. As the lesions of gastric cancer progress from dysplasia to carcinoma, the frequency of cells with p53 immunopositivity and gene mutations increases, although there is a wide variation in reported figures in the literature. Similarly, prognostic use of *TP53* status has not been valuable to date.

Colon Cancer

The Vogelstein lab's classic surveys of mutations in tumor suppressor gene and oncogene loci in lesions of various stages in the development of colon adenocarcinomas indicated that the development of *TP53* mutations or chromosome 17p loss was rather a late event in the life history of these malignancies (Cho and Vogelstein, 1992; Vogelstein, 1990). Perhaps as an indicator of diagnostic methods to come, one study by Dong et al, showed that it is possible to isolate tumor-derived DNA from the stool of patients with primary colon tumors, and to detect the mutations in the genes for RAS, TP53, and BAT26 in a specific parallel assay (Dong et al., 2001).

Liver Cancer

Although uncommon in Western countries, hepatocellular carcinoma (HCC) is a major international killer, and is an endemic disease in the Far East and Sub-saharan Africa. A variety of chemicals and viruses contribute to the development of HCC, and help to explain its geographical distribution (Staib et al., 2003). The most important of these factors is chronic infection of the liver by hepatitis B and C viruses, but ingestion of Aflatoxin B1 from fungal contamination of grains, heavy ethanol use, and smoking also increase risk, as do rarer causes such as iron or copper overload diseases. Aflatoxin B1 has been clearly shown to cause codon 249 mutations in *TP53*; and vinyl chloride, when activated by microsomal oxidases to form the epoxide chloroethylene oxide, is highly mutagenic. The metal overload diseases hemochromatosis and Wilson's disease are suspected to initiate *TP53* mutations in HCC by generating Fenton oxidants such as the hydroxyl radical, which can then damage the DNA. A large body of evidence now suggests that the protein product of the HBX gene of HBV can bind to the p53 protein and prevent it from transactivating target genes. The mechanism for HCV viral transformation of hepatocytes is not as clear, but may involve the HCV core protein. *TP53* status is not a useful indicator for patient prognosis at this time (Staib et al., 2003).

Skin Cancer

About 50% of skin cancers that arise in the general population possess mutations in *TP53*, suggesting that for the cells of these malignancies there can be an advantage to losing the normal monitoring functions of p53 (Giglia-Mari and Sarasin, 2003). The situation is more extreme for individuals with the xeroderma pigmentosum diseases who possess germline mutations in one of a number of genes that encode key members of DNA repair pathways. These patients develop skin cancers at a greatly elevated rate, and *TP53* is mutated in roughly 90% of these lesions. With a few exceptions, the mutational "signature" in *TP53* reveals that the primary environmental cause of sporadic and XP patient skin cancers is UVA and UVB ultraviolet radiation from the sun, which promotes photocyclization of adjacent pyrimidine nucleotides to form cyclobutane pyrimidine dimers (CPD) or 6:4 photoproducts (6-4PP), depending on which portions of the pyrimidine rings are linked together in the reaction. When these crosslinked photoproducts are in the strand of DNA that is being used as the template in DNA replication, an adenine nucleotide will often be paired with a base in the lesion, leading to the characteristic C to T transition mutations at the dipyrimidine site when the erroneously synthesized strand is used as template in subsequent rounds of cell division. Interestingly, in xeroderma pigmentosum patients, there is an elevated rate of tandem CC to TT double transitions in *TP53*, which have been hypothesized to result from the long half-life of pyrimidine dimers in such repair-deficient cells, which gives a greater opportunity for spontaneous deamination of the cytosines to uracil (or thymine, if the cytosine was methylated, which is common at CpG sites in the *TP53* gene). When the DNA strand containing the uracil or thymine residues is replicated, adenine will be paired with the deaminated bases, thus fixing the dual mutation. Preferential selection due to a

growth advantage conferred by the CC to TT mutant alleles is very unlikely, as most of these result in the same missense amino acid incorporation as would the single mutation (Giglia-Mari and Sarasin, 2003).

The most common human skin cancers can be divided into malignant melanoma and non-melanoma skin cancers. The bulk of the latter category is made up of squamous cell carcinomas and basal cell carcinomas. *TP53* mutation status varies significantly between these lesions. Typically, the dangerously metastatic malignant melanomas do not appear at increased rates in sun-exposed areas of the skin, except in the elderly people, and only about 1 in 10 of malignant melanomas carries mutated *TP53*. It should be noted, however, that melanomas frequently carry mutations in the *INK4a* locus, which encodes both the p16 cell cycle inhibitor, and the p14 ARF upstream regulator of p53, which may indicate that ablation of the p53 pathway is partially accomplished by this alternative mechanism. Further data have suggested that melanomas can carry mutations of the p53 transcriptional target gene *APAF1* , an effector of the apoptosis program whose loss may remove an important component of p53 activity in these cells. In contrast, xeroderma pigmentosum patients do develop more melanomas on sun-exposed skin, and show about 60% rate of *TP53* mutation, often with the UV-induced dipyrimidine mutation signature. Melanoma *TP53* mutations in non-XP patients often occur in unusual regions of the gene, with hot-spots at codons 104, 213, 286, 290, and 296. Of these, only the codon 213 mutation is common in other kinds of cancers. Compared to the basal cell and squamous kinds of skin cancer, the mutations in melanomas are more frequently A:T to G:C

transitions, which indicates a different etiology from crosslinking of nucleotides by UV irradiation.

Within non-melanoma skin cancers, basal cell carcinomas show frequent mutation of *TP53*, with mutational hot-spots reported at codons 177, 196, and 245. The codon 177 mutation is not often found in other malignancies, but the reasons for its preferential appearance in basal cell carcinomas, whether because of selective growth advantage provided by this allele in the basal cell type, or unique susceptibility for developing this lesion in these cells, remain unclear. Squamous cell carcinomas are another sun-exposed skin lesion, less common than basal cell carcinoma, but more prone to metastasis. They show mutations with the UV-damage signature, and appear to have a characteristic hot-spot at codon 278. As with the basal cell carcinomas, the reasons for the hot-spot are still unresolved.

Brain Cancer

The vast majority of tumors in the central nervous system are derived from the glial cell lineages that support the functions of neurons in normal brain tissue, and which, in contrast to neurons, maintain some proliferative potential throughout the life of the individual. Of these gliomas, about 60% are astrocytomas that result from mutations in astrocyte cells or astrocyte developmental precursor cells. Studies of the *TP53* mutation status of astrocytomas in each of the four World Health Organization malignancy grade categories has shown that almost two-thirds of these lesions of all grades carry *TP53*

mutations (Zhu and Parada, 2002). In addition, astrocytomas are one of the tumor types that Li-Fraumeni patients are prone to develop. These data indicate that loss of p53 functions can provide a selective advantage for the growth of an astrocytoma; however, it would appear that other mutations, which do not occur at a high enough frequency to generate astrocytomas in all Li-Fraumeni patients, must be necessary for the formation of these tumors. The most ominous astrocytoma lineage tumors, given the WHO grade IV, are also known as glioblastoma multiforme (GBM), and are thought to either progress from lower-grade lesions (in which case they are known as secondary GBM) or else to arise rapidly without passing through lower-grade phenotype, in which case they are termed primary GBM. Both primary and secondary GBM show loss of p53 function, but via distinct pathways. Secondary GBM show a greater than 60% incidence of *TP53* gene mutation, whereas primary GBM have been found to have a much lower incidence of such mutations, on the order of 10% (Zhu and Parada, 2002). Instead, the primary GBM developmental pathway appears to select for amplification of the p53 negative regulator *MDM2* gene, or inactivation of the p53 upstream pathway regulatory locus *INK4a* which encodes $p14^{ARF}$ as well as the Rb pathway p16 proteins. These mechanisms, although inactivating or at least decreasing the activity of p53, are likely to be somewhat different in their cellular consequences from mutation of the *TP53* gene: for example, the combined p53 and Rb regulatory pathway loss that results from *INK4a* mutation may partially account for the rapid progression of the primary glioblastoma multiforme lesions; in addition, mouse studies indicate that overexpression of MDM2 has effects on cells beyond inactivation of p53.

Hematological Cancers

Mutations in *TP53* are less common in leukemias and lymphomas than in solid tumors, appearing in 10-20% of these lesions (Peller and Rotter, 2003). Acute lymphocytic leukemia in infants exhibits only about a 5% incidence of *TP53* mutations. In patients with acute myeloid leukemia or myelodysplastic syndrome, *TP53* mutations are found in less than 10% of cases, and correlate with abnormal karyotype and poor patient prognosis. When lesions in ALL or MDS patients who have been previously treated with alkylating chemotherapeutic agents were examined, the *TP53* mutation rate jumped to 27% in one study. In contrast to the acute leukemias, the chronic leukemias such as chronic myelogenous leukemia, chronic lymphocytic leukemia, and hairy cell leukemia show higher rates of mutation in *TP53*. For example, *TP53*-mutant CLL has been shown to correlate with aggressive disease course, and the normally treatment-responsive, slowly progressing hairy cell leukemias show poorer patient outcomes if *TP53* is mutated. In lymphomas, the overall picture is similar, with poorer prognosis for patients with *TP53* mutations in both Hodgkin's and non-Hodgkin's lymphomas (Peller and Rotter, 2003).

3. p53 Mutants, and Mouse Models of Human Cancer

a) Cancer-related phenotypes

Detailed experimental manipulation of the p53 pathway in animals and their cells has largely depended on studies in transgenic and knockout mice. An initial attempt to model the biological effects of mutated *TP53* genes in the mouse was reported in 1989, when the tumor suppressor nature of p53 had just been recognized. In these experiments, a transgene expressing the temperature-sensitive V135A mutant of the murine *Trp53* gene was introduced into mice, and roughly 20% of them developed tumors including lung adenocarcinomas, osteosarcomas, and lymphomas. This study, although bedeviled by the possibility that the multiple copies of the integrated transgene in the genome might behave differently than a single point-mutated copy, gave the first *in vivo* evidence for dominant interfering effects of a mutated p53 species on the wild-type protein (Lavigueur et al., 1989).

A more recent transgenic mouse overexpressing wild-type p53 in the cells of the ureteric bud was shown to cause increased levels of cellular apoptosis in the developing kidney, and alter the differentiation of cells to tubular epithelia, resulting in final organs with decreased numbers of nephrons and smaller size (Godley et al., 1996). Similarly, a p53 transgenic mouse expressing the protein in the optic lens develops micropthalmia due to a defect in lens fiber formation (Nakamura et al., 1995). Finally, a series of transgenic mice expressing p53 in postmeiotic cells of the male germline exhibit spermatogenesis defects ranging from apoptosis to altered differentiation of spermatids, depending on the level of p53 expressed (Allemand et al., 1999).

A number of early transgenic mouse studies relating to p53 involved expression of the SV40 viral large T antigen as a transgene under the control of regulatory sequences that are active in different tissues. Expression of large T antigen in pancreatic beta cells, choroid plexus, hepatocytes, enterocytes of the intestine, and mammary epithelium, to name a few examples, resulted in a variety of dysplasias and eventual tumors in the affected tissues, although usually with a latency period indicating that other genetic changes were likely required to manifest the full tumor phenotype (Butel et al., 1990; Efrat et al., 1987; Kim et al., 1994; Marks et al., 1988; Sepulveda et al., 1989). Importantly, the most frequently mutated *Trp53* gene exons were sequenced in tumors in several of these mouse strains, and were found to be unmutated, indicating that the presence of SV40 large T antigen promoted tumor formation by inactivating p53 function (Moore et al., 1992). However, a difficulty in the overall interpretation of these large T antigen-expressing mice is that the large T antigen also binds to, and alters the function of, a number of other important cellular proteins, for example, Rb, p300, PP2A, and the roles of loss or alteration of these other proteins' functions may contribute to tumor formation in the mice. As proof of this, some strains made with mutant variants of the large T antigen that are unable to bind p53 develop tumors nonetheless (Bennoun et al., 1998).

The introduction of methods for making germline modifications to the mouse genome gave rise to opportunities for more subtle investigations of the phenotype of mice carrying mutant *Trp53* alleles. The first reports of this kind described mice with homozygous or heterozygous disruption of the *Trp53* gene by recombination with

constructs containing drug-resistance marker genes (Donehower et al., 1992; Jacks et al., 1994). Although these mutant strains, with their complete inactivation of one or both alleles of the *Trp53* gene, differ from the typical p53 status of human cancers, where missense mutations are the most common abnormality, they nonetheless recapitulate several important features of known human tumor biology. Similar to Li-Fraumeni syndrome patients, *Trp53* heterozygous or null mice are prone to developing lymphomas, sarcomas, and, in some genetic backgrounds, other cancers. The influence of the genetic background of the inbred mouse strain carrying the inactive *Trp53* allele on the development of particular kinds of tumor is striking: for example, on the BALB/c background, *Trp53*-/- mice develop hemangiosarcomas at a significant rate, and also develop mammary tumors when irradiated, while the 129/Sv background predisposes male mice to malignant germ cell tumor formation, for which a genetic modifier locus has been mapped to the murine chromosome 13 (Backlund et al., 2001; Donehower et al., 1995; Muller et al., 2000a). It is interesting to note that the cancers to which *Trp53*-null mice are prone, lymphomas and sarcomas, are similar to a subset of those that develop most commonly in human children; this might be interpreted as evidence for the kinds of tumor suppression that p53 has been actively selected for in evolution (Leroi et al., 2003). The other high-frequency cancers in children, brain tumors, have been suggested to be a possible result of the rapid change of genes affecting the brain in human evolution, with the underlying notion that cellular growth controls might take some time to catch up to other genetic changes that have been selected for different selected advantages in the organism (Leroi et al., 2003). In this case, the failure of p53-mutant

mice to develop brain tumors at a high frequency would be consistent with the absence of the human-specific gene activity or regulation changes.

If modifier genes play an important role in the tumor spectrum of inbred p53-mutant mouse strains, we might expect that the far greater number of genetic variations between mice and humans could result in very substantial differences of biological outcomes in response to impaired p53 gene product activity. However, the similarities between *Trp53* mutant mice and human Li-Fraumeni patients are more striking than the differences, given the dissimilar development time, life-span, and other biological distinctions between the two species. Perhaps the most notable difference between human and mouse biology in this regard is the high incidence of lymphomas (mainly of thymic origin, but with some B-cell derived lesions as well) in *Trp53*+/- and *Trp53*-/- mice. The reasons for this difference, which may relate to the relative rate of other mutational events, or the comparative growth and survival advantages obtained by human and mouse lymphocytes with impaired p53 pathways, remain unclear, but further studies of the other mutations in these tumors may shed light on the matter.

The outstanding question of the true *in vivo* behavior of missense p53 mutants was initially approached by crossing a *Trp53* null strain with the Lavigeur et al. V135A transgene mutant p53 strain (Harvey et al., 1995). Consistent with the idea that missense mutants of p53 could suppress the activities of the wild-type p53 protein, mice heterozygous for *Trp53* inactivation and also carrying the missense transgene displayed an accelerated rate of tumor development, and novel tumor types compared to the

heterozygous inactivated p53 mice. In contrast, the presence of the missense transgene array in a *Trp53* null background showed no change in tumor development, indicating that for this missense allele at least, there was no additional gain of function that manifested itself at the level of tumor formation rate or tumor spectrum (Harvey et al., 1995). Similar results were obtained with *Trp53*+/- or null primary neuroectodermal cells retrovirally-transduced with a brain tumor-associated human codon 236 deletion *TP53* gene and transplanted into recipient mouse brains; the *Trp53* heterozygote cells were augmented in their tumorigenic potential by the putative dominant negative allele expression, while the *Trp53* null cells were unaffected (Hegi et al., 2000).

Attempts to generate germline single-copy missense *Trp53* mutant mice have been underway for several years now, and initial results have begun to emerge. Heterozygotes of one strain carrying one wild-type *Trp53* allele and one allele with an R172H mutation plus an unintended splice site mutation developed more carcinomas and somewhat fewer lymphomas than mice heterozygous for wild-type and null *Trp53* alleles. The tumors of these mice were also more prone to metastasize, indicating a potential gain of function phenotype above and beyond the interference with wild-type p53 function resulting from this allele (Liu et al., 2000). A second study looked at the effects of the missense alleles $Trp53^{R270H}$ and $Trp53^{P275S}$, corresponding to the mutations at human codons 273 and 278 respectively, and found that ES cells and thymocytes heterozygous for these alleles suppressed the wild-type p53 allele-dependent apoptosis in response to doxorubicin treatment, again supporting the idea that these p53 missense allele proteins possess dominant-negative activity (de Vries et al., 2002).

A final set of point-mutant *Trp53* allele mice address questions of the importance of p53's activities as a transcription factor, by introducing the double codon mutations L25Q and W26S that together render the N-terminal transactivation domain of the protein inactive (Chao et al., 2000; Jimenez et al., 2000). ES cells and mouse embryo fibroblasts homozygous for this double point mutant allele behave identically to the corresponding cells homozygous for null alleles in assays measuring the ability of p53 to arrest the cell cycle or initiate apoptotic cell death, indicating that transactivation activity (or, arguably, another activity of p53 that is ablated by the two amino acid alterations) is essential for these phenotypic outcomes.

The question of the relationship between normal p53 "dose" in the cells of the body and their resistance to malignant transformation was recently addressed by the generation of a mouse strain carrying an extra *Trp53* gene, complete with upstream and downstream regulatory regions, as a single-copy or duplex transgene in a wild-type p53 genetic background. The phenotype of these mice revealed that they were less cancer-prone than wild-type mice of the same genetic background, suggesting that the possession of three or four copies of *Trp53*, perhaps by further reducing the chances of a single cell achieving mutational inactivation of all copies of p53, was a successful halt to cancer development. An alternative possibility for the phenotypic outcome is that the increased expression of p53 target genes caused by the increased p53 protein expression was more effective than the usual dose in responding to cellular stresses. As mentioned below, there have recently

been claims that p53 dose can play a role in mammalian organismal aging, but these mice seemingly disprove this idea, at least within the range of p53 expression that they exhibit.

b) Developmental and aging-related phenotypes

Additional insights into the normal role for p53 in the development of mammalian embryos were derived when it was discovered that a subset of female, and, much more rarely, male *Trp53*-/- embryos exhibit an exencephalic phenotype due to failure of the neural tube to close completely (Sah et al., 1995). The basis for this incompletely penetrant phenotype and its gender bias has not yet been elucidated but the cells at the margins of the closing neural tube are highly sensitive to a variety of environmental insults and may unmask a protective role of p53 in the response to low frequency mutagenic events or other developmental accidents. Notably, irradiation of *Trp53*-/- male mice causes a higher incidence of exencephaly in their female *Trp53*-/- progeny, but not heterozygote progeny, indicating that DNA-damaged p53 null spermatocytes carry an exencephaly-promoting factor that is revealed in a p53-null embryo; the most likely explanations would perhaps be that in the absence of p53, the damage to the male-derived chromosomes is inadequately repaired, or the consequences of mutant gene expression are handled inappropriately (Shimura et al., 2002). Further subtle developmental phenotypic variations in p53-null mice have been described. For example, craniofacial abnormalities such as fused upper incisors, absence of the hyoid bone, retinal dysplasia, adhesion of the ocular lens to the cornea, and, in one reported case, polydactyly of the

hind limbs are manifested in *Trp53*-/- embryos (Armstrong et al., 1995; Ohyama et al., 1997). In addition, the conduct of meiosis in the male germline appears to be partially dependent on p53. *Trp53*-/- male mice show defective spermatogenesis and accumulation of multinucleate giant cells, perhaps originating in the tetraploid pachytene stage when p53 is expressed, and when directed DNA breakage events occur in the normal course of meiotic recombination (Rotter et al., 1993). When specialists in the biology of other tissues have closely examined the *Trp53*-/- mouse, they have found other unusual phenotypes. For example, one group found that although hepatic development in embryogenesis and early life is apparently normal in the absence of p53, adult livers in *Trp53*-/- mice are composed of hepatocytes that have proliferative indices roughly twice those of wild-type mice. The portal regions of *Trp53*-/- livers also possess clusters of hepatic blast cells similar in phenotype to those found early in liver development (Dumble et al., 2001). These investigators attribute these blast cells to arrested maturation of liver stem cell progeny, but they do not assess the possibility that these cells might be premalignant lesions derived from mature or partially differentiated hepatocytes or biliary epithelial cells (Dumble et al., 2002). Close investigation of the spleens of *Trp53*-/- mice indicated that they have increased numbers of immature B cells, and that these cells had altered responses to extracellular signals (Shick et al., 1997). Another group examined renal development in *Trp53*-/- embryos and pups and discovered previously unrecognized abnormalities, including poor separation of the nephrogenic zone from the differentiated zone of the developing kidney, and aberrant morphology, expansion and hyperplasia of the renal tubular epithelium leading to the development of cysts with abnormal apical localization of Na^+/K^+-ATPase pumps (Saifudeen et al., 2002).

A recent provocative connection between p53 and aging has been proposed, based on the phenotype of mice with a germline deletion covering the N-terminal half of p53, as well as an undefined genetic region further upstream; these mice are less tumor-prone than wild-type mice, but, surprisingly, they also show several biological traits such as osteoporosis, thinning of the adipose layer of the skin, atrophy of a variety of organs, and decreased ability to respond to stress, that are reminiscent of normal mammalian aging. In the same paper, these investigators reveal that the V135A p53 transgenic line shows many of the same phenotypes (Tyner et al., 2002). An alternative possibility for explaining these findings, is that these mouse strains may show a phenotype which results from impairment of normal p53 function; such a phenotype would not have been detected in the *Trp53* null strains due to their rapid development of tumors and subsequent early deaths. It must also be said that the underlying causes of organismal aging are still the subject of lively debate and widely divergent mechanistic explanations, ranging from the accumulation of somatic gene mutations in differentiated cells resulting in inadequate new protein synthesis, to the depletion of tissue stem cell lineages based on genetic damage to these cells, to accumulated physical damage to long-lived biomolecules of all kinds by reactive oxygen species produced in cellular metabolism. Consistent with the "p53-impairment" hypothesis above, it has been noted that mice and humans with mutations in the *Atm* upstream activator kinase of the p53 pathway have also been shown to have an early aging component to their phenotype; however, this phenotype could also be the result of the loss of ATM-dependent activities that operate independently of p53 (Boder, 1985; Gage et al., 2001).

4. The p53 protein

a) Domain structure

A great deal of detailed molecular and structural analysis of the p53 protein has now accumulated in the literature, but as with many areas of p53 biology, important questions still remain unanswered. A schematic diagram of key features of the p53 protein is shown in Figure 1. The human and mouse p53 proteins are composed of 393 and 390 amino acid residues respectively, share roughly 80% homology, and have an actual molecular weight of around 44 kDa (Harlow et al., 1985; Oren et al., 1983; Pennica et al., 1984; Zakut-Houri et al., 1985). The first 28 N-terminal amino acid residues contain an acidic transactivation domain two-beta-turn structure, as was illuminated by an NMR solution structure of this region of the protein (Botuyan et al., 1997). Several serine and threonine residues in this region of the protein have been shown to be important target sites for kinases that activate p53 in response to damage to the cell. In addition, useful mutations such as the dual mutation of codons 22 and 23 (L22Q,W23S) in this domain have been shown to destabilize the structure of the domain and permit experiments testing the requirement for transactivation function for various p53-dependent phenotypic outcomes (Botuyan et al., 1997). A proline-rich region, from about residue 60 to 94 is next in the p53 primary sequence. This portion of the molecule has been proposed to influence the efficiency of transactivation, and possibly to maintain the protein in an inactive "latent"

state by binding to the C-terminal-most 30 residues of the protein's tail (Ruaro et al., 1997; Venot et al., 1998; Walker and Levine, 1996).

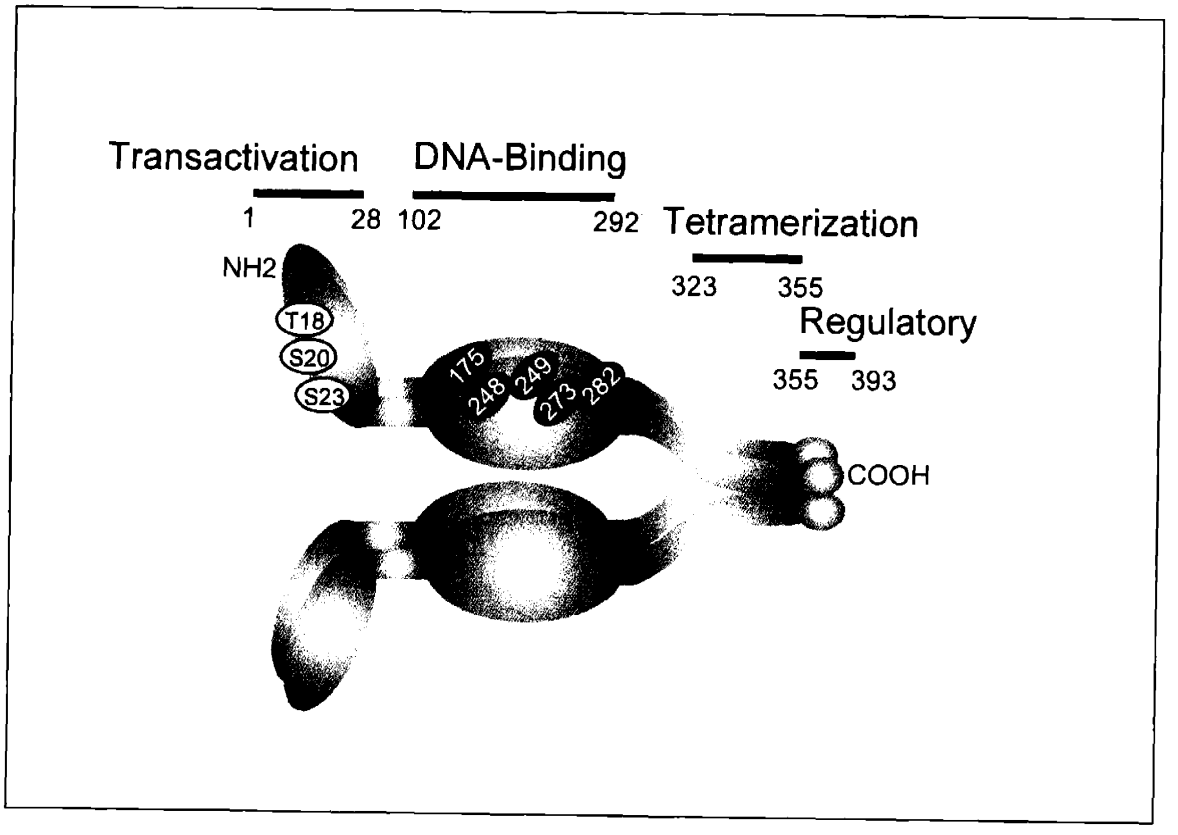

Figure 1 Domain Structure of p53: A schematic diagram of tetramerized p53 is shown. Major domains of the protein are indicated with the black bars, with amino acid numbering from the human p53 sequence. Important targets of protein phosphorylation are indicated in yellow ellipses, while residues frequently mutated in human cancers are indicated in red ellipses.

The central region of the p53 protein, from residues 102 to 292, is the DNA binding domain that confers sequence-specific interaction with the DNA double helix. The Cho *et al.* protein-DNA co-crystal structure of this domain, although it only showed one of three binding domains in the unit cell of the crystal interacting in the predicted way with the known binding site DNA, was extremely informative, for it showed that the known "hot-spot" mutation codons in the p53 DNA-binding domain had a direct relationship to the

structural binding requirements (Cho et al., 1994). The structure of the DNA binding domain was found to be a beta-sandwich supporting a loop-sheet-helix motif and two large loops stabilized by tetrahedral coordination of a divalent Zinc ion (Cho et al., 1994). The loop-sheet-helix, fitting into the major groove, and the L3 loop in the minor groove, form the DNA contacting regions of the domain, and contain the "hot-spot" tumor mutant amino acid residues encoded by codons 249, and 273, and 282 (Cho et al., 1994). Other "hot-spot" codon amino acid residues such as residue 175 were found to be buried in the domain, and their mutation was thus predicted to destabilize the domain structure, a finding that was consistent with known novel antibody epitopes present in these p53 mutant proteins, but not in the DNA-contact base mutants or the wild-type protein. The strong selection bias for point-mutant missense mutations in the DNA binding domain in human tumor development led researchers to suggest that such mutant proteins, by oligomerizing via their intact tetramerization domain with wild-type p53 from the unmutated allele in a mutated cell, could act as dominant-negative proteins, and prevent the wild-type protein from fully exerting its usual tumor-suppressive activity (Srivastava et al., 1993). Recently, small molecule pharmaceutical candidates have been generated that are claimed to stabilize mutationally destabilized p53 DNA binding domains, and thereby restore their function (Bykov et al., 2002; Friedler et al., 2002).

Toward the C-terminus of the protein, the oligomerization domain known to confer tetramerization of the protein is found within residues 323 to 355 (Clore et al., 1994; Jeffrey et al., 1995; Waterman et al., 1995). The NMR and X-ray crystal structures of this isolated domain shows that each p53 monomer contributes a beta-strand and an alpha

helix; each beta-strand and alpha helix interacts with an antiparallel strand and helix from a second monomer, and the tetrameric structure is a perpendicularly opposed dimer of these dimers (Clore et al., 1994; Jeffrey et al., 1995). Interestingly, the lysine amino acid residues known to be involved in several different aspects of p53 regulation appear to cluster around either end of the tetramerization domain, with implications for the regulation of the molecule's stability. A series of detailed physical biochemical studies of the p53 tetramerization domain, and the related oligomerization domains from the p53 family proteins p63 and p73, indicate that the different family members do not form stable hetero-oligomers, suggesting that any interactions between these proteins, to the extent that they occur, must involve additional protein complex members, or rely on interactions between other regions of the molecules (Mateu and Fersht, 1999).

The extreme C-terminal region of p53 has been shown to have some non-sequence specific DNA-binding activity, and to potentially influence the relative positions of the other domains of the protein. This topic is still unresolved, but a number of observations indicate that p53 can exist in more than one conformational orientation, and that this can influence the ability of the protein to bind to DNA. For example, p53 protein produced by overexpression in bacteria has long been known to require some binding interaction with its C-terminus, whether by an antibody specific for this region of the protein, by binding to some other protein, or by covalent modification, in order to be "activated" for sequence-specific DNA binding (Hupp and Lane, 1994; Hupp et al., 1995). However, recent NMR solution structure data generated with a dimeric mutant of p53 are most consistent with a model in which the C-terminal DNA-binding activity competes with the

central DNA binding domain for access to the DNA, rather than inhibiting sequence specific DNA binding by directly interacting with the central domain (Ayed et al., 2001).

It has been difficult to bring the same clarity of analysis to the structure of the intact p53 protein as has been possible with each of its domains in isolation, because there are as yet no crystallographic data available for the entire protein, or any assays that directly measure the spatial relations of all parts of the intact tetrameric protein, although a start has been made with the aforementioned NMR spectrometry studies (Ayed et al., 2001). Most desirable of all would be a structure of the p53 tetramer bound to its complete double-palindromic binding site, with the disposition of tetramerization domains relative to transactivation domains visible, and the influence of C-terminal binding interactions for the overall quaternary structure the complex apparent.The weight of less direct evidence using DNA binding as a readout supports the main theme that binding interactions at the C-terminus of p53 can have important consequences for the ability of the DNA-binding domains to assume the proper positions to bind their specific target DNA sequences. However, recent structural data from NMR studies of a dimeric p53 mutant indicate that the presence or absence of the C-terminal domain does not affect the conformation of the other domains of the protein, arguing that the long-invoked allosteric model of p53 auto-inhibition of DNA binding may be mistaken (Ayed et al., 2001). As for the structural state of wild-type tetrameric p53 in unstressed cells compared to cells where the DNA-damage response pathways have been alerted, further studies, and perhaps new tools, will be required to obtain definitive answers.

A number of viral and cellular proteins have been shown to bind to p53. The earliest example of this was the association between p53 and the SV40 large T antigen, which involved the DNA-binding domain (Li and Fields, 1993). Other viral proteins, such as the papillomavirus E6 protein, and the hepatitis B HBX protein bind to the DNA-binding domain and the C-terminus of p53, respectively (Elmore et al., 1997; Mansur et al., 1995).

b) Transcription factor function vs. non-transcriptional functions

As a nuclear protein found in association with the SV40 large T antigen, suspicions were raised early on that p53 might be involved in the regulation of transcription. Initially, fusion proteins of the GAL4 DNA-binding domain with p53 established the presence of a transactivation domain (Fields and Jang, 1990; O'Rourke et al., 1990; Raycroft et al., 1990; Reich and Levine, 1982; Steinmeyer and Deppert, 1988). At the same time, non-specific DNA-binding activity was identified in the C-terminal 47 amino acids of p53, and, more importantly, a sequence-specific DNA-binding activity inactivated by the "hot-spot" mutations in the central regions of the protein was discovered (Bargonetti et al., 1991; Foord et al., 1991; Kern et al., 1991).

Very rapid progress in this topic took place in the 1990s, with the identification of a preferred DNA-binding site for p53 from the analysis of genomic DNA fragments that immunoprecipitated with p53 (el-Deiry et al., 1992). It was found from these initial 18 genomic p53-binding sites that the p53 protein recognized the following sequence motif:

RRRCWWGYYY (0-13 bp spacer) RRRCWWGYYY (el-Deiry et al., 1992). Like many transcription factor binding sites, the p53-binding site displayed palindromicity of each of the two half-sites, and, consistent with the tetrameric oligomerization state of the protein, the complete binding site is made up of two pairs of half-sites. p53 has been found to recruit the basal transcription factors TFIIA, TFIIB, and TFIID to the genes where it is bound, which would seem sufficient explanation for its transactivation ability. However, there is currently some debate in the literature about which components of the transcriptional machinery are bound by p53 (Liu and Berk, 1995; Xing et al., 2001).

A number of different subtractive hybridization studies followed the p53 binding site elucidation, and led to the identification of the first known p53 transcriptional target genes. The first published effort described a gene called $p21^{WAF}$, which was found to be able to prevent the proliferation of a variety of different cultured cancer cells (el-Deiry et al., 1993). The transcript from this gene had been previously found to be upregulated in senescent cells, and its protein product was soon shown to be an inhibitor of the cell cycle protein kinase machinery (Harper et al., 1993; Rubelj and Pereira-Smith, 1994). A DNA binding site sufficient to confer p53-responsiveness to a reporter gene, and fitting the previously defined consensus was found 2.4 kb upstream of the start site of the gene (el-Deiry et al., 1993). A second p53 transcriptional target gene, *MDM2*, was discovered by investigators investigating the regulation of a novel p53-binding protein, then termed p95 (Barak et al., 1993). They found that the levels of the transcript from the gene were dependent on the presence of p53, and that this effect was not prevented by inhibiting protein synthesis, suggesting that a direct transcriptional activation was the most likely

explanation. Indeed, a pair of p53-binding sites separated by 17 base pairs was identified in the first intron of the mouse *Mdm2* gene, and found to be conserved in the human homolog; these sites gave strong p53-dependent activation, although the sequences of the individual sites were not in perfect agreement with the initial consensus site described above (Kaku et al., 2001; Zauberman et al., 1995). Intronic regulatory sites in other p53 target genes have subsequently been found to be quite common. Interestingly, the protein product of the *Mdm2* gene was found to be a negative regulator of p53, consistent with the observation that its genetic locus, 12q13-14, is frequently amplified in sarcomas, and therefore the identification of *Mdm2* as a p53-regulated gene revealed a direct negative feedback loop for controlling p53 activity in the cell (Zauberman et al., 1995).

As various new technologies for examining gene expression differences were developed in the 1990s, a host of other p53-regulated target genes were identified, the functions of some of which are still quite mysterious, while others fitted easily into the paradigms of p53 regulation of the cell cycle, cell death, and genomic stability. These genes are discussed more fully in section 7.

With the availability of the human and mouse genomic sequences in the past two years, it has become possible to try to make use of the known characteristics of the p53 binding site to try to predict new target genes. Two initial reports of such efforts reveal that, in fact, knowledge of the factors that determine whether p53 will make use of a site similar to the el-Deiry *et al.* consensus binding sequence is still too limited to make such searching efforts specific and successful (Hoh et al., 2002). The combination of the

relatively generous degeneracy of the p53 binding site motif, and the large range of separations from the start site of transactivated genes (up to several kb away) ensures that over one-third of all genes in the human genome have a site fitting the p53 binding motif at least as well as some confirmed target genes (S. Boyd, personal observation) (Hoh et al., 2002; Wang et al., 2001). For example, the *MDM2* binding site is sufficiently different from the "consensus" binding motif not to be detected by current methods, unless the stringency of the site search is relaxed to the point where specificity is greatly compromised. Evidently, the cell, its transcription factors, and the basal transcription machinery have other sources of information about the suitability of interacting with a particular region of DNA, perhaps as a result of the local chromatin environment, or the presence of other DNA binding proteins in the vicinity. In this regard, the ASPP1 and ASPP2 proteins have been proposed to increase p53 DNA-binding tendency when they are present at a subset of promoters, and p53 interaction with the transcriptional coactivators CBP and p300 is described as enhancing transcription from target genes (Grossman, 2001; Samuels-Lev et al., 2001).

There have also been reports of transcription-independent activities of p53 in the literature, suggesting that phenotypic effects of p53 expression, particularly the initiation of apoptosis, need not derive solely from the increased levels of expression of transcriptional target genes (Mihara et al., 2003; Schuler and Green, 2001). For example, Mihara et al. present data that a fraction of p53 protein localizes to the mitochondria and directly increases release of cytochrome c, probably via interactions with Bcl-X_L. These results, while intriguing, are likely to reflect a process that occurs in parallel with the

obvious and abundantly documented transcriptional upregulation of pro-apoptotic Bcl-2 family members by p53 for the execution of the apoptotic program (Mihara et al., 2003; Schuler and Green, 2001).

5. Stimuli that induce p53 in mammalian cells

a) DNA-damage inducing stimuli

The responsiveness of wild-type p53 protein levels to DNA-damaging stimuli was noticed shortly after the tumor suppressor identity of p53 became clear (Kastan et al., 1991). Many sorts of irradiation and drugs that cause various different lesions in the DNA have been shown to be efficient inducers of p53, and the cellular detection systems for some of these lesions are exquisitely sensitive. Even a single DNA strand break in the cell can be sufficient for p53 induction (Huang et al., 1996). In the case of the double-stranded DNA breaks produced by the intense oxidative pulses generated by photons of gamma irradiation entering the cell, the pathways responding to the insult have been fairly clearly mapped out, but the true identity of the proteins necessary for recognizing that damage has occurred to the cell has been slower to emerge. An intriguing possibility is that the DNA-damage repair systems are an integral part of the damage-sensing mechanisms of the cell. Some data supportive of this hypothesis have begun to emerge, for example, documenting the failure of cells to respond to cisplatin treatment if a mismatch-repair component of the cell's DNA repair machinery is missing (Brown et al., 2003; Gong et al., 1999).

A second category of p53-inducing agents consists of those drugs or forms of irradiation that cause bulky adducts or crosslinking of the strands of the DNA. Well-known examples of such agents include the crosslinking drugs cisplatin and psoralen, and ultraviolet irradiation, which, in addition to causing some oxidative damage to the DNA, catalyzes the formation of intra- and inter-strand pyrimidine dimers. It has been proposed that these agents cause their effects by establishing a situation where the DNA replicative machinery, stalling at the lesion or cross-link, causes strand breakage by trying to replicate through the covalently joined blockage point. Insofar as this is the case, these agents may give rise to a more indirect form of strand breakage, and are likely to make use of the same induction machinery as the direct strand-breaking mechanisms. A similar argument is made for topoisomerase inhibitors such as etoposide, based on the observation that they cause DNA strand breakage at the replication fork by increasing the stability of topoisomerase-DNA complexes (Ferguson and Baguley, 1996).

The question of what happens to the cell's DNA when a drug- or irradiation-induced lesion is encountered by the RNA polymerase complex is similarly complicated. One might expect that the movement of the RNA polymerase over active gene regions would be a rather efficient sort of damage-sensing system for the most useful portions of the genome, and that it would be surprising if the cell had no provision to make use of these circumstances. There are data that indicate that this scenario is operative in some cell types (Ljungman et al., 2001).

A similar indirect mechanism of action may account for the activity of drugs that activate p53 by depleting cellular pools of nucleotides needed to synthesize new DNA. In the absence of the appropriate building blocks, cells may find themselves with partially completed strands of DNA that alert the strand-breakage detection systems in the cell. However, it has been reported that some nucleotide-depletion protocols induce p53 without causing measurable DNA breakage (Linke et al., 1996).

b) Putative DNA-damage independent stimuli for p53 induction

There are several documented circumstances where p53 levels increase in response to stimuli that apparently have little connection to DNA-damaging mechanisms. One case is the induction of p53 by the hypoxia-induced factor (HIF-1α) protein. In this case, protein-protein interactions are thought to be responsible for the increase in p53 levels by preventing ubiquitylation; however, it has been demonstrated that the presence of high levels of HIF-1α alone are insufficient to induce p53 protein. Therefore, it is likely that other cellular events induced by hypoxic conditions may be required for this pathway of p53 upregulation (An et al., 1998; Wenger et al., 1998).

Other examples are less obvious, or at least have been thrown into some question, for example, the induction of p53 by expression of oncogenes. One pathway of this kind proposes that activated Ras causes the induction of E2F1, which induces the p53 upstream regulator p14 ARF, which induces p53. However, recent data suggest that at least some oncogenes can induce the expression of oxidative species in the cell, via

unknown mechanisms, and that these reactive species could in fact be causing direct damage to the DNA (Vafa et al., 2002).

6. Regulation of p53

a) Ubiquitin-mediated degradation

A key insight into the main mode of p53 protein level regulation was provided by studies of the E6 protein encoded by the oncogenic human papillomavirus types 16 and 18 (Huibregtse et al., 1991). The E6 protein is required for HPV immortalization of keratinocytes. E6 was shown to bind to p53 and to cause it to undergo ubiquitin-mediated degradation (Huibregtse et al., 1991). This oncogenic viral protein was eventually shown to make use of a cellular partner protein, E6-AP, to recruit the same underlying ubiquitin-dependent proteasomal degradation machinery that the MDM2 protein uses to keep p53 protein levels low in unstressed cells (Hubbert et al., 1992; Huibregtse et al., 1993; Scheffner et al., 1993). This mechanism is apparently quite efficient—the half-life of p53 in a resting cell is measured in minutes, but it climbs to over half an hour after irradiation (Maki et al., 1996; Maltzman and Czyzyk, 1984; Reich et al., 1983). The post-translational regulation of p53 has been retained in evolution despite what might appear to be its "wasteful" futile cycling of synthesizing and degrading the p53 protein. This observation indicates that either the metabolic cost of regenerating p53 protein is not much of a burden to the cell, or that the rapid induction of protein levels made possible

by merely having to prevent degradation, has been set at a premium in evolution. This is logical, if p53 activating events have the potential to inactivate the cell's normal mechanisms for maintaining and repairing its structure— there may be no time to waste, if the cell is to survive the immediate damage, or alternately, if the cell is to successfully execute programmed cell death, and thus achieve an outcome that is optimal for the organism whose offspring must derive from a healthy parent to carry their germline genetic material to the next generation.

The ubiquitin protein, true to its name, is one of the most highly conserved proteins in eukaryotes, and has clearly been optimized through evolution for its function of signaling that the protein to which it is linked must be destroyed by the multimeric proteasome enzyme complex. Ubiquitin becomes attached to proteins destined for degradation via an isoamide linkage between the carboxyl group of its C-terminal glycine, and a lysine residue of the modified protein. A complicated series of cellular enzymes specialize in attaching ubiquitin to its substrate proteins, in response to a variety of different circumstances, for example, if the protein is incorrectly folded, or, as in the case of p53, if the protein has not been specially modified to protect it from the degradation system. The ubiquitylation machinery was originally described as consisting of E1, E2, and E3 proteins. The E1 enzyme "charges" the ubiquitin subunit by forming a bond with the C-terminal glycine carboxyl group, and hands it off by forming a thioester bond between the glycine carboxyl group and the cysteine side-chain of an E2 enzyme. This enzyme then catalyzes the formation of an isopeptide bond with the target protein's lysine residue, with or without the help of an additional "specificity factor" E3 enzyme which

can either facilitate the bond formation or can carry the ubiquitin itself in a final transfer step before attachment to the target protein (VanDemark and Hill, 2002).

In the case of p53 in an unstressed cell, the MDM2 protein plays the part of the E3 enzyme; the identity of the E2 enzyme in this case is still unknown. MDM2 binds to the N-terminal transactivation region of the protein and, in addition to preventing this portion of p53 from interacting with the transcriptional machinery of the cell, MDM2 directs the coupling of ubiquitin to the C-terminal lysines of p53 (Honda et al., 1997). Thereafter, exactly what happens is not clear, except that p53 is efficiently degraded in a proteasome-dependent fashion. There are some data from *in vitro* experiments to suggest that MDM2-mediated ubiquitylation of p53 can only attach single ubiquitin moieties to each lysine of p53, and if full polyubiquitin chains consisting of lysine 48-ubiquitinated ubiquitin proteins are to be assembled, that another enzyme must be involved. Surprisingly, the p300 coactivator protein has been shown to be a candidate for this role, as it possesses a ubiqutin ligase activity able to couple ubiquitin to a growing chain, and cellular manipulations that inactivate p300, such as expression of the E1A oncoprotein, result in the generation, and accumulation, of monoubiquitinated p53 (Grossman et al., 2003).

Another recent new twist on the story of p53 protein degradation control was provided by a report of a protein deubiquitinase enzyme, HAUSP, which has the activity of removing ubquitin from cellular proteins (Li et al., 2002). Increased levels of HAUSP were able to prevent MDM2 from decreasing the steady-state amount of p53 present in cells. Whether

this activity is truly p53-specific, or is part of a less-specific cellular system for regulating the levels of a variety of ubiquitinated proteins, remains to be seen.

Finally, a number of additional small ubiquitin-like proteins have recently been discovered and found to alter the localization, activity, and conformation of their targets, One of these, SUMO-1, apparently becomes coupled to different lysine residues of the p53 C-terminus than does ubiquitin, and this modification can increase the transcriptional activity of p53 (Muller et al., 2000b; Rodriguez et al., 1999).

b) Phosphorylation of p53

The rate of ubiquitylation of p53 in the cell is governed by a second set of enzymes that couple p53 regulation to the damage-sensing systems of the cell and catalyze a different covalent modification—phosphorylation. A number of serine and threonine residues of p53 have been shown to be phosphorylated *in vivo* under various circumstances; the most elegant of these studies being Abraham *et al.*'s mass spectrometry analysis of p53 from untreated and irradiated cells (Abraham et al., 2000). Attention has chiefly focused on several amino acid residues in the N-terminal transactivation domain of p53 because it appears that phosphorylation of serine 15, serine 20, and perhaps threonine 18 can disrupt MDM2 binding to this part of p53. There has been considerable debate about the importance of individual residues, however, beginning with claims that DNA-PK phosphorylation of serine 15 of p53 decreased its interaction with MDM2 and prevented p53 degradation (Shieh et al., 1997). Subsequent studies of serine 20 mutants of p53

indicated that this residue was also important for the ability of p53 to be stabilized in response to irradiation (Unger et al., 1999). Results from the simplest experimental system, using purified MDM2 protein and various purified p53 N-terminal peptides with the desired phosphorylations, found a third result: that only threonine 18 phosphorylaton affected the affinity of the p53-N terminal peptide for MDM2 (Schon et al., 2002).

As with the modification, so with the modifiers: a set of different kinases have been implicated as the enzymes that add the phosphates to the p53 N-terminus in times of stress. Foremost among these is the ATM phosphoinositide kinase-related kinase, the homolog of the Mec kinase that responds to similar stresses in the budding yeast. ATM is the product of the gene mutated in Ataxia Telangiectasia, an illness characterized by sensitivity to a variety of environmental stresses like radiation, and susceptibility to developing cancers at a young age. ATM was implicated in the regulation of p53 when it was found that AT patient cells failed to show p53 protein level increases in response to irradiation (Kastan et al., 1992). ATM has proved to be one of the key responders to DNA damage in the cell, and recent data indicate that it may be directly activated by intermolecular autophosphorylation of dimeric inactive ATM following interaction with chromatin containing damaged DNA (Bakkenist and Kastan, 2003). ATM preferentially phosphorylates serine 15 of p53, and appears to be responsible for the first wave of serine 15 phosphorylation in response to gamma irradiation (Brown and Baltimore, 2003). A curious secondary finding has been that p53 in irradiated cells is dephosphorylated at serine 376 in an ATM-dependent manner (Waterman et al., 1998). This is likely to be an indirect effect, but the dephosphorylation of this residue is claimed to reveal a binding

site for a 14-3-3 protein, whose interaction with the C-terminus of p53 activates its DNA binding ability (Waterman et al., 1998).

The ATM and Rad3-related kinase, ATR has also been shown to have a role in p53 phosphorylation (Tibbetts et al., 1999). Experiments using phospho-p53-specific antibodies indicate that ATR can phosphorylate p53 at serine 15 and serine 37 in the N-terminus, and that interfering with ATR activity by gene ablation or expression of dominant-negative constructs prevents a late phase of serine 15 phosphorylation in response to gamma irradiation, and prevents all phosphorylation in response to ultraviolet irradiation (Brown and Baltimore, 2003; Tibbetts et al., 1999). ATR is particularly interesting because it seems to respond to S-phase replication blockage events, such as may be caused by a variety of different DNA-damaging drugs or irradiation treatments that leave bulky adducts in the DNA, or other replication-arresting conditions such as topoisomerase inhibition (Cliby et al., 2002). The kinase is recruited to regions of single-stranded DNA by the single-stranded DNA-binding protein RPA, and once recruited, is activated (Zou and Elledge, 2003).

A third exciting candidate kinase for the p53 N-terminus, CHK2, was identified as the homolog of the budding yeast Rad53, a checkpoint kinase that can halt the cell cycle in times of stress (Chaturvedi et al., 1999; Matsuoka et al., 1998; Tominaga et al., 1999). The CHK2 kinase is activated by ATM, but evidence from *Chk2*-/- mice indicated that Chk2 could also mediate p53-dependent apoptosis of irradiated thymocytes in an ATM-independent manner (Hirao et al., 2002). Further support for the idea that Chk2 and p53

were in the same pathway was provided by genetic data showing that some families with Li-Fraumeni-like cancer predisposition were carriers of germline mutations in the *CHK2* gene (Lee et al., 2001). However, recent studies in human cells where CHK2 levels were decreased by RNA interference, or by homologous recombination disruption of the gene indicate that p53 can be activated by gamma irradiation in the complete absence of CHK2, suggesting that there may be species-specific variations between mice and humans in the importance of this kinase for the p53 pathway (Ahn et al., 2003; Jallepalli et al., 2003). These results were anticipated in part because there are human tumors that carry mutations in both *TP53* and *CHK2*, suggesting that the genes may act in parallel or at least partially redundant pathways (Jallepalli et al., 2003).

A panoply of other cellular kinases are also reported to be able to phosphorylate p53. These include Casein kinases I and II, PKC, MAPK, JNK, and Raf (Lakin and Jackson, 1999). However, convincing *in vivo* evidence for the importance of most of these modifications has yet to be shown. To further complicate the issue, a recent paper describing p53 mutants with alteration of all known phosphorylation residues indicated that p53 was still able to be stabilized in response to cellular stresses (Ashcroft et al., 1999). Perhaps these findings reflect a contribution of the less well known translational control mechanisms mentioned below.

c) Acetylation of p53

Interactions between the p53 N-terminus and p300, CBP or P/CAF histone acetyltransferase proteins correlate with activation of p53 transactivation activity (Avantaggiati et al., 1997; Gu et al., 1997; Scolnick et al., 1997). These proteins have been shown to be able to acetylate p53 at lysines 320, 373, and 382, with the expected enhancement of p53 transactivation activity, and they are also implicated in histone acetylation at p53-regulated promoter sites, thus contributing to a chromatin environment amenable to active p53 target gene transcription.

d) Physical Interactions with other Cellular Proteins

The p19ARF-encoding alternate reading frame transcript of the murine *Ink4a* locus, and the human homolog p14ARF have a significant role to play in p53 regulation in response to stimuli such as overexpression of the MYC oncogene (Zindy et al., 1998). ARF appears to regulate p53 by affecting the function of the MDM2 negative regulator of p53 in several ways: it can inhibit the ubiquitin ligase activity of MDM2, and can, under some conditions, cause MDM2 to become sequestered in the nucleoli (Honda and Yasuda, 1999; Weber et al., 1999). Very recently it has been shown that N-terminal residues 2 to 14 of ARF are able to prevent ribosomal RNA maturation from occurring in the nucleoli, possibly explaining the ability of ARF to arrest the growth of even *Trp53*-/-;*Mdm2*-/- cells (Korgaonkar et al., 2002; Llanos et al., 2001; Sugimoto et al., 2003)

e) Nuclear Import and Export Control

The main functions of p53 take place in the nucleus, and it is no surprise that evolution, both at the level of the healthy organism, and in the pathological development of cancers, has seized upon the fundamental compartmentalization of the eukaryotic cell to add other layers of regulation or, in the case of the cancer cell, misregulation, to the control of p53 activity. Like many transcription factors, p53 is subject to active energy-dependent processes of nuclear import and export; this has been studied in permeabilized cell systems (Middeler et al., 1997). Nuclear import is mediated by three positively-charged nuclear localization signal sequences in the C-terminus of the protein and a pair of positively charged residues upstream of the tetramerization domain; these elements have been shown to bind to the nuclear import receptor importin α (Shaulsky et al., 1990; Shaulsky et al., 1991).

The story of nuclear export control of p53 localization is more complicated. Initially, one group reported that HDM2 (the human Mdm2 homolog) possessed a nuclear export signal similar to that of the HIV Rev protein, an archetypal "nucleo-cytoplasmic shuttling protein" that constantly moves in and out of the nucleus of the cell (Freedman and Levine, 1998; Roth et al., 1998). It was shown that HDM2 also had this shuttling property as a result of its nuclear export sequence (NES), and then it was proposed that HDM2 acted as a shuttle protein to carry p53 to the cytoplasm, where it would be degraded by cytoplasmic proteasomes (Freedman and Levine, 1998). At this time, another study demonstrated that p53 itself had a functional NES sequence in the region of

its tetramerization domain, and could apparently act as a shuttling protein on its own (Stommel et al., 1999).

At this point, the studies outlined in Chapter 2 of this thesis were undertaken; their discussion, and subsequent developments in the field will be discussed there.

f) Minor Modes of p53 Regulation: Transcription and Translation

It has also been reported that in some cell types, for example, lymphoma cells, protein translation rate can play a major role in regulating the levels of p53 protein in the cell in response to stress signals (Fu et al., 1996). Regions within the 5' and 3' ends of the human p53 transcript have been mapped that appear to carry the information for this level of regulation (Fu et al., 1996). Transcriptional regulation of p53 has not been shown to play a prominent role in the regulation of its biological activities.

7. Transcriptional Targets of p53 and cellular consequences of p53 expression

A number of transcriptional profiling studies of cells expressing different levels, or different sequence variants of p53 have been published in the past decade, (Aldaz et al., 2002; Attardi et al., 2000; Madden et al., 1997; Maxwell and Davis, 2000; Polyak et al., 1997; Yu et al., 1999; Zhao et al., 2000). These studies, and lower-throughput experimental approaches, have revealed over 300 transcripts whose level of expression is

affected by, or appears to be affected by, p53. These genes, grouped by functional categories, are listed in Table 1. A brief overview of the known cellular functions of the most prominent p53 direct target genes is presented below and summarized in Figure 2. Some of the reported p53 target genes in the literature are unlikely to be true direct targets, for the many substantial effects of p53 expression on the behavior of cells would be expected to give rise to indirect changes in gene expression, for example, as a result of altering cell cycle behavior.

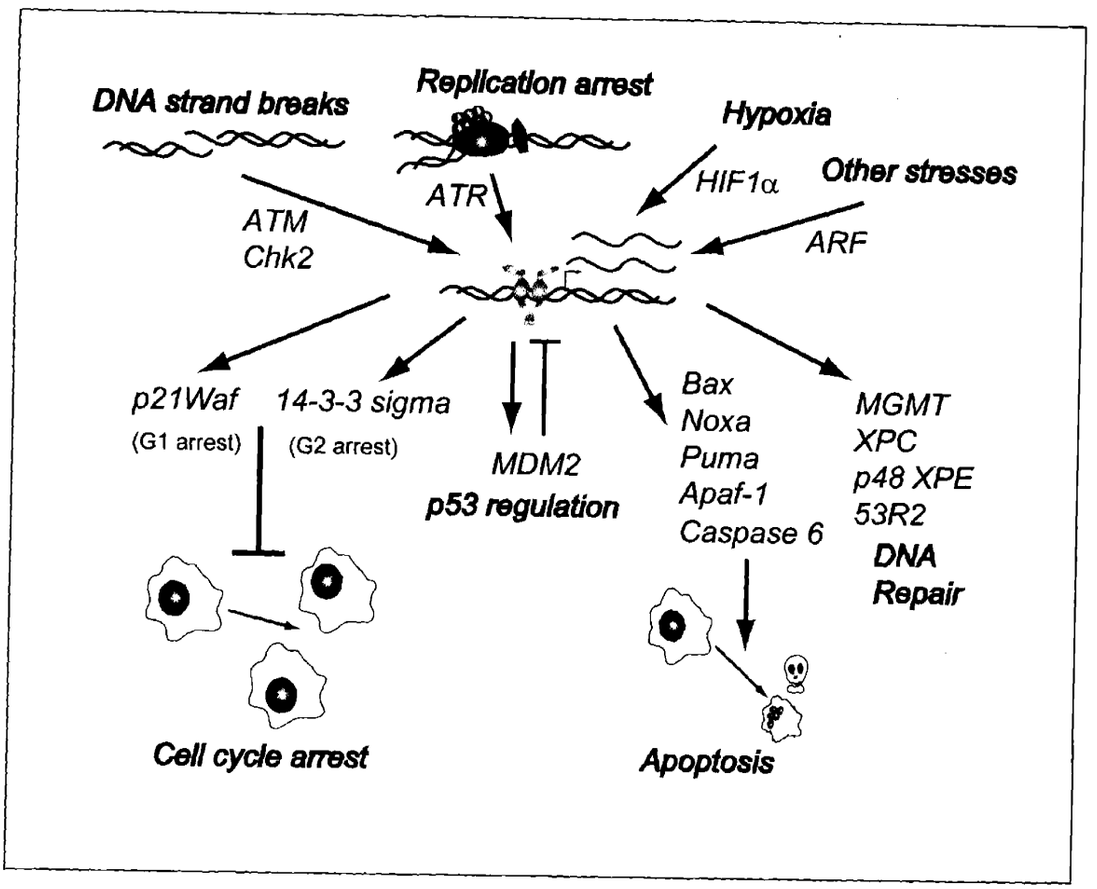

Figure 2 Major Inducers and Transcriptional Targets of p53: Inducing stresses are indicated at the top in purple, with the genes whose products transmit the stress signals to p53 noted beside the arrows. Direct transcriptional targets of p53 are listed in the lower half of the figure, with functional outcomes from p53-induced genes marked in red.

a) Auto-regulatory targets: Mdm2

Early investigations of the Mdm2 gene focused on the fact that it was frequently found to be amplified in double-minute chromosomes in murine transformed cells, indicating that it could have an oncogenic role in the development of tumors. As previously mentioned, Mdm2 was subsequently found to bind to p53 and govern the levels of its target protein in the cell by acting as an E3 enzyme for p53 ubiquitylation, thereby forming a negative feedback loop to control p53 activity. *In vivo* evidence of the importance of this mode of p53 regulation came from mice carrying a disrupted *Mdm2* gene: in the absence of MDM2, embryos die *in utero* around the time of implantation (Jones et al., 1995; Montes de Oca Luna et al., 1995). This lethal phenotype is, however, completely rescued if the *Trp53* gene is also mutated in the mouse, showing that any disruptive consequences of lack of Mdm2 expression are dependent upon p53. The most likely explanation for these findings is that in the absence of Mdm2 p53 levels in the early embryo are unnaturally high, and as a result, the embryonic cells die by apoptosis (Jones et al., 1995; Montes de Oca Luna et al., 1995). Of course, it is also possible that prolonged growth arrest at an inappropriate stage of cell maturation, or other p53-mediated effects could also lead to the same final lethal outcome for the cells.

b) Cell-cycle regulatory targets: p21 and 14-3-3

The discovery of *p21* as a p53-transactivated gene came just as the function of the protein was elucidated, making for a moment of exciting convergence of genetic and biochemical

understanding of the nascent p53 pathway (Dulic et al., 1994; el-Deiry et al., 1993; Harper et al., 1993). p21 is able to bind and inhibit the G1/S cell cycle phase cyclin-dependent kinase CDK2 in association with cyclin A or cyclin E, and by so doing, prevent cells from entering a new round of cell division (Harper et al., 1993). As understanding of the dynamics of the different cell cycle inhibitors has evolved, it has become clear that $p21^{CIP}$ and the similar "broad spectrum" CDK inhibitor $p27^{KIP}$ are actually required for the normal assembly and function of the cyclin D-dependent CDK4 and CDK6 enzymes with their cyclin partners, and that when cyclin D levels rise in the cell in response to mitogenic signals, $p21^{CIP}$ and $p27^{KIP}$ are titrated away from CDK2 complexes, releasing them from inhibition and permitting the initiation of the cell cycle (Sherr and Roberts, 1999). Therefore, p53-mediated induction of p21 can be seen to act in opposition to the effects of increased cyclin D induced by mitogenic stimuli. The phenotype of cells and mice deficient in $p21^{CIP}$ supports the unique importance of this CDK inhibitor for causing the G1/S-phase cell cycle arrest in DNA-damaged cells (Brugarolas et al., 1995). However, the organismal consequences of loss of $p21^{CIP}$ on tumor development are relatively modest: *p21*-null mice show increases in tumor development only very late in life, and fail to show increased tumors following irradiation; in fact, they are protected from irradiation-induced tumors (Martin-Caballero et al., 2001). These findings highlight the complexity of the overall phenotype of tumor development *in vivo*, where, strictly speaking, the initiating mutations, their causes, their detailed effects on cells, and the selection pressures in response to which they give growth or survival advantages, are currently guessed at, but not known with certainty.

The *14-3-3 sigma* gene was cloned on the basis of the protein's ability to bind CDK2; it was shortly thereafter found by the Vogelstein lab to be a p53-induced gene (Hermeking et al., 1997; Laronga et al., 2000). When overexpressed in cells, it causes them to arrest in the G2 phase of the cell cycle via an unknown mechanism. The *Efp* transcriptional target gene of the estrogen receptor has been found to encode an ubiquitin ligase E3 protein for 14-3-3 sigma; overexpression of this Efp protein in breast cancer cells promotes their growth (Urano et al., 2002). As further supporting evidence, although not confirmation, that 14-3-3 sigma may have a growth-suppressive effect in the context of tumor development, mutations in the gene have been found in a subset of lung tumors (Osada et al., 2002). Interestingly, *14-3-3 sigma* appears to be one of the few p53 target genes that is also dependent on BRCA1 for its expression, at least according to a recent paper using microarray methods to study *Brca1*-null embryonic stem cells (Aprelikova et al., 2001).

Some experimental data have been presented that appear to show a role for the p53-induced genes *Reprimo*, *B99*, and *Mcg10* in the regulation of the G2/M checkpoint, however mechanistic details are scant so far (Taylor and Stark, 2001).

c) Pro-apoptotic target genes

A tremendous amount of interest was generated by initial findings that p53 could induce cells to execute the program of apoptotic cell death in response to genotoxic and other potentially damaging stimuli (Lotem and Sachs, 1993; Lowe et al., 1993; Yonish-Rouach et al., 1993). Examination of p53-induced transcripts soon yielded several appealing

candidates for mediating this response, beginning with the gene for the pro-apoptotic Bcl2-family member Bax (Miyashita et al., 1994; Miyashita and Reed, 1995; Zhan et al., 1994). Several other Bcl-2 family proteins that are likely contributors to the mitochondrial cytochrome c-releasing apoptotic cell death pathway have subsequently been found to be encoded by p53 target genes, including *NOXA* and *PUMA* (Oda et al., 2000; Schuler and Green, 2001; Yu et al., 2003; Yu et al., 2001). PUMA has been shown to bind to BCL-XL and to promote Bax multimerization, while NOXA can bind Bcl-2, Bcl-XL. and Mcl-1 (Oda et al., 2000; Yu et al., 2003; Yu et al., 2001). The mitochondrial pathway of cell death is known to make use of cytochrome C release from the mitochondrial membrane to activate Apaf-1, a cytosolic Ced-4 homolog that promotes the cleavage activation of Caspase 9 (Wang, 2001). *APAF-1* was belatedly discovered to be transcriptionally regulated by p53 as well (Fortin et al., 2001; Kannan et al., 2001b; Robles et al., 2001).

The relevance of the p53-regulated expression of these genes to spontaneous or induced cancer development is not yet clear, however. For example, in the case of the most fully substantiated pro-apoptotic p53 target gene, *Bax*, knockout mice for this gene show no increased tendency toward tumor development, and transgenic expression of Bax in T cells of p53-deficient mice actually increases tumorigenesis (Knudson et al., 2001). However, contradictory data come from a lymphoma model where Myc is overexpressed in B cells. In this setting, Bax deficiency accelerates tumor formation, and removes the selection pressure for loss of p53 (Eischen et al., 2001).

A similar story is found with the *Apaf-1* gene: mice homozygous for a null allele show increased rates of exencephaly and craniofacial deformities, along with overproliferation of neurons in the developing central nervous system, but the null mice that survive to adulthood have not been reported to have a tumor-prone phenotype (Honarpour et al., 2000; Yoshida et al., 1998). Clearly, impaired Apaf-1 function in *Trp53-/-* mice is a candidate for the low incidence exencephaly of female embryos. In addition, loss of either Bax or Apaf-1 can cause testicular developmental abnormalities, apparently due to initial overproliferation of spermatocyte progenitor cells (Honarpour et al., 2000; Russell et al., 2002). These findings may partly explain the testicular phenotype of *Trp53-/-* mice.

Other apoptotic pathways appear to be influenced by p53 upregulation of different target genes. The membrane receptor-activated cell death pathway is triggered by the TNF-receptor family member KILLER, which is under p53 transcriptional control (Takimoto and El-Deiry, 2000). So too is the "executioner" Caspase 6, which is a downstream caspase that carries out cleavage of other cellular proteins in the course of the apoptotic program (MacLachlan and El-Deiry, 2002). Downregulation of some anti-apoptotic genes, for example, *BCL-2* and *BCL-X_L* by p53 has been described, however, these studies have not been widely taken up or replicated in the literature (Wu et al., 2001).

d) Redox target genes

With the arrival of methods of measuring cellular transcript levels for thousands of genes at a time came the surprising observation that p53 regulates many genes that are likely to

have roles in adjusting the redox balance of the cell (Polyak et al., 1997). A speculative model proposed at the time was that p53 might induce apoptosis by upregulating transcripts for genes whose products would generate reactive oxygen species, thereby damaging cellular components and triggering cell death. Some data in support of this idea came from measurements of the oxidation state of cells expressing inducible p53, where, indeed, increased levels of oxidative species were observed when high levels of p53 were induced (Polyak et al., 1997). Subsequent studies in other labs, however, have indicated that expressing high levels of a variety of other genes, for example, *p21*, can also cause increased levels of oxidative species, and therefore the validity of the model is uncertain (Macip et al., 2002). At this point, there is little in the way of functional data supporting the role of these genes in preventing tumor development in animals.

e) DNA repair genes

Armed with the foreknowledge that cancer is a disease of acquired somatic mutations, many investigators have been curious to see whether p53 can regulate genes involved in the maintenance of the integrity of cellular DNA. A few DNA repair genes have in fact proved to have p53-inducible transcripts, and there is growing evidence that loss of p53 control of some of these may play significant roles in the development of cancers.

One example is the direct dealkylating enzyme O^6-Methylguanine-DNA-methyltransferase (MGMT), which removes methyl or other short alkyl groups from guanine nucleotide bases before they can induce mutations in the cellular DNA

(Grombacher et al., 1998). Researchers studying this enzyme discovered that it was induced in a p53-dependent manner (Grombacher et al., 1998; Rafferty et al., 1996). This enzyme is of general interest in part because it appears to be a candidate gene for the resistance of tumors to some kinds of chemotherapy, and because transgenic overexpression of the enzyme has proved to be able to protect *Trp53+/-* heterozygous mice from developing lymphomas after treatment with alkylating agents (Reese et al., 2001; Russell et al., 1995). Further, *Trp53-/-* cells have been reported to be more sensitive to the effects of alkylating agents than are wild-type cells (Seo et al., 2002).

Two genes involved in the nucleotide-excision repair (NER) pathway of DNA repair have also been reported to be p53 target genes. The *XPC* gene, which encodes one of the enzymes involved in the early steps of assembly of the NER complex, is induced in a p53-dependent manner, as is p48 XPE (DDB2), an NER complex component important for global genomic repair (Adimoolam and Ford, 2002; Amundson et al., 2002; Kannan et al., 2001a; Tan and Chu, 2002). According to current reports, the DDB2 regulation is species-specific, for no difference was seen in its expression between wild-type and p53-null mice (Tan and Chu, 2002).

Much work has been done to try to figure out the function of the GADD45α and GADD45γ proteins, which are encoded by p53-responsive genes. GADD45α binds to PCNA, and has been shown to increase the rate of excision repair of DNA *in vitro*, and to arrest the cell cycle in G1 phase when it is expressed; but the details of its functions are still unclear (Smith et al., 1994). It appears from the phenotype of *GADD45α* mice,

however, that the gene is needed for maintaining genomic stability, for the cells of these mice develop chromosomal abnormalities at a high rate, and the mice develop cancers when exposed to radiation (Hollander et al., 1999). Recent results *in vitro* show that GADD45α is able to inhibit the mitotic cyclin-dependent kinase CDC2, this activity could account for the G2 arrest caused by expression of the protein (Jin et al., 2000).

Another novel p53 target gene whose function is related to DNA repair is the inducible ribonucleotide reductase 2 gene, which generates deoxyribonucleotides for use in DNA synthesis and is important for the viability of cells faced with genotoxic stress (Nakano et al., 2000; Tanaka et al., 2000).

f) Other target genes

Perhaps the best known of the remaining p53-responsive genes are related to the topic of cellular interactions with the extracellular network. Several target genes, including Matrix Metalloproteinase 2, Thrombospondin, and Cathepsin D may play either positive or negative roles in the potential of primary tumors to grow and to metastasize (Sun et al., 1999). Testing of the effects of loss of Thrombospondin on the tumor phenotype of *Trp53* null and heterozygous mice indicated that the mice died sooner, and also were better hosts for tumor implants, which grew more rapidly and developed denser vasculature (Lawler et al., 2001).

8. Findings in this Thesis

The experiments presented in subsequent chapters use mutation analysis, and study of the cells of mice with a deletion allele for the Trp53 gene, to explore both the regulation of p53, and its downstream functions mediated by specific activation of target genes.

Chapter 2 is a study of the control of nuclear localization of the p53 protein. Previous reports in the literature suggested that the p53 negative regulator HDM2 was a nucleocytoplasmic shuttling protein that was able to bind and carry p53 from the nucleus of the cell to the cytoplasm where it is destroyed by the protasome. I found that the HDM2 nuclear export sequence was not required for it to be able to alter p53's cellular localization to the cytoplasm. Rather, the p53 nuclear export sequence was required for this activity, as was the ability of HDM2 to ubiquitinate p53. Further studies indicated that ubiquitylation of the p53 C-terminus was the basis for HDM2's ability to remove it from the nucleus and cause its efficient degradation. It is suggested that this may be because C-terminal ubiquitylation causes the p53 nuclear export sequence to be activated or made more accessible to the nuclear export machinery of the cell.

Chapter 3 presents the results of cDNA microarray experiments in which *Trp53-/-* and *Trp53+/+* fibroblasts were treated with a panel of genotoxic agents and then assayed for p53-dependent upregulation or downregulation of any of the roughly 15,000 gene sequences on the micrarray. A number of new candidate p53 target genes were revealed,

including the DNA repair gene *Ercc5*, which encodes the DNA repair protein Xpg, a participant in nucleotide excision repair and a mediator of base excision repair of oxidative DNA damage. Further analysis of most of the DNA repair genes in the mouse genome using real-time PCR indicated that a second gene, *Polk*, encoding the translesion DNA polymerase kappa, is also a p53-induced gene.

Chapter 4 further characterizes the p53-dependent regulation of the Xpg-encoding DNA repair gene *Ercc5*, and shows that it is a directly-regulated p53 target gene with a p53-responsive site in its first intron. *Trp53-/-* cells show a modest reduction in the ability to repair damage to an oxidatively-damaged DNA construct, and this defect is rescued by exogenous expression of retrovirally transduced XPG, indicating that the lower levels of this gene are likely responsible for the defect.

Table 1: Reported p53-responsive genes

Function	Accession	Gene	Activated/ Repressed	Direct Target?	Evidence	Abbreviated References
Apoptosis						
	NM_001160	Apaf-1	+	yes		Moroni Nat Cell Biol 3(6):552-8
	U16811	Bak	+			Zhao G&D 14:981-993
	L22473	Bax	+	yes	rp,gs	Miyashita, Cell 80:293-9
	M14745	Bcl-2	-			Budhram-Mahadeo JBC 274:15237-44
	Z23115	Bcl-XL	-			Bartke Oncogene 20(5):571-80
	XM_040142	Bik	+			Bartke Oncogene 20(5):571-80
	NM_001223	Caspase 1	+	yes		Gupta J Biol Chem 276(14):10585-8
	NM_001226	Caspase 6	+	yes	gs,rp	MacLachlan PNAS 99: 9492-97
	XM_006121	Cathepsin D	+	yes	gs,rp	Wu Oncogene 16:2177-83
	AF010127	c-FLIP	+			Bartke Oncogene 20(5):571-80
	NM_001450	DRAL	+	?		Scholl J Cell Biol 151(3):495-506
	XM_004684	ecNOS	-		gs,rp	Mortensen JBC 274:37679-84
	M67454	Fas	+	yes	rp	Zhao G&D 14:981-993
	H04238	FASL	+			Zhao G&D 14:981-993
	U45878	IAP				Kannan Oncogene 20(18):2225-34
	AF016266	KILLER/DR5	+	yes	rp,gs	Yu PNAS 96:14517-22
	AB041230	Noxa	+	yes	rp	Oda Science 288:1053-8
	NM_022112	p53AIP1	+			Matsuda Cancer Res 62(10):2883-9
	AF249870	PERP	+	yes		Attardi G&D 14:704-18
	NM_014417	PUMA	+	yes		Nakano Mol Cell 7(3):683-94
	AF016267	TRID/ TRAIL-R3	+	?		Sheikh Oncogene 18:4153-9
Oxidative stress						
	I14577	Cystathione-beta-synthetase	+			Zhao G&D 14:981-993
	M21304	Glutathione peroxidase	+	yes	gs,rp	Tan JBC 274:12061-6
	NM_002133	Heme oxygenase (decycling) 1	+			Yu PNAS 96:14517-22

	M22538	NADH-Ubiquinone dehydrogenase	-			Zhao G&D 14:981-993
	M32011 ?	neutrophil cytosolic factor 2	+			Zhao G&D 14:981-993
	L07769	Pig1 (Galectin-7)	+			Polyak Nature 389:300-305
	AF010316	Pig12 (glutathione transferase)	+			Polyak Nature 389:300-305
	AF010309	Pig3 (quinone oxidoreductase)	+	yes	rp,chip,gs	Polyak Nature 389:300-305
	M10906, M26152	Pig4 (serum amyloid a protein)	+			Polyak Nature 389:300-305
	AF010310	Pig6 (proline oxidase homolog)	+			Polyak Nature 389:300-305
	AF10312	Pig7	+			Polyak Nature 389:300-305
	AF010313	Pig8 (etoposide-induced mRNA - ei24)	+	yes	rp,gs	Polyak Nature 389:300-305
	J02947	superoxide dismutase 3, extracellular	-			Zhao G&D 14:981-993
Other stress response						
	T51913	Alpha crystallin B chain	+			Zhao G&D 14:981-993
	T67986	Clusterin	+			Zhao G&D 14:981-993
	T48904	Heat shock protein Hsp27	+			Zhao G&D 14:981-993
	T87527	Heat shock protein Hsp 84	-			Zhao G&D 14:981-993
	NM_010431	HIF-1(hypoxia inducible factor 1)	-			Blagosklonny JBC 273:11995-8
	NM_005345	Hsp70	-			Madden Oncogene 15:1079-85
DNA Repair						
	NM_006763	BTG2	+	yes		Kannan Oncogene 20(18):2225-34
	NM_000107	DDB2 (XPE p48)	+			Kannan Oncogene 20(18):2225-34
	NM_000136	Fanconi anemia C	+	yes	gs	Liebetrau Hum Mol Genet 6(2):277-83
	NM_001924	GADD45 alpha	+	yes	rp,gs,fp	Zhao G&D 14:981-993
	U04045	hMSH2	+	yes		Scherer BiochBiophyResCom221:722-8
	AB036063	p53R2	+			Nakano Oncogene 19:4283-9
	D14134	RAD51	"- (P21-dep)"			de Toledo CG&D 9: 887-96
	NM_004628	XPC	+			Amundson Cancer Biol Ther 1(2):145-9
Transcription factors/coactivators						
	BC010998	Four and a half LIM domain (Fhl2)	+	yes		Scholl J Cell Biol 151(3):495-506
	NM_001964	EGR-1 (Zif268/Krox-24)	+			Madden Oncogene 15:1079-85

	R85690	Myelin transcription factor 1	+			Zhao G&D 14:981-993
	X63380	RSRFR2 (SRF-related, B-cell expr.)	+			Zhao G&D 14:981-993
	R52081	GCN5	+			Zhao G&D 14:981-993
	T81919	CREB-binding protein	+			Zhao G&D 14:981-993
	T95318	Transcription initiation factor IIF alpha	+			Zhao G&D 14:981-993
	R55041	SRF	+			Zhao G&D 14:981-993
	H15813	CBP beta	+			Zhao G&D 14:981-993
	R06239	TFIID	-			Zhao G&D 14:981-993
	AJ404688/NM_006497	HIC-1 (hypermethylated in cancer)	+	yes		Wales Nature Med 1:570-7
	XM_086136	Nhlh2	+			Kannan Oncogene 20(18):2225-34
	NM_004024	ATF3	+			Kannan Oncogene 20(18):2225-34
	NM_015925	LISCH7	+			Kannan Oncogene 20(18):2225-34
	NM_002200	IRF5	+			Mori Oncogene 21(18):2914-8
	XM_085179	Cyclin K	+	yes		Mori Neoplasia 4(3):268-74
	X77956	Id1	-			Zhao G&D 14:981-993
RNA Stability/RNA binding						
	M92843	Tristetraprolin	+			Zhao G&D 14:981-993
	AF257770/1/2	MCG10	+	yes	rp,gs	Zhu MCB 20:5602-18
	U28686	RNPL (Putative RNA binding protein)	-			Zhao G&D 14:981-993
Protein Stability						
	XM_083867	MDM2	+	yes		Barak EMBO J 12(2):461-8
	R02151	Proteasome subunit RC10-II	-			Zhao G&D 14:981-993
Metabolic						
	L19956	Aryl sulfotransferase	+			Zhao G&D 14:981-993
	X55740	Placental 5' nucleotidase	+			Zhao G&D 14:981-993
	X72389	4-hydroxyphenylpyruvate dioxygenase	+			Zhao G&D 14:981-993
	R59181	6-phosphofructokinase	+			Zhao G&D 14:981-993
	T54891	Adenylate kinase isozyme 1	+	yes?	none	Zhao G&D 14:981-993
	NM_000693	ALDH6	+			Okamura Oncology Res 11:281-5
	R65697	ATP synthase A chain	+			Zhao G&D 14:981-993

	U04636	Cyclooxygenase 2	-			Subbaramaiah JBC 274:10911-5
		Cytochrome c oxidase subunit 1	+			Okamura Oncology Res 11:281-5
	Z49878	Guanidinoacetate N-methyl transferase	+			Polyak Nature 389:300-305
	L31409	Homo sapiens creatine transporter	+			Zhao G&D 14:981-993
	M34192	Isovaleryl-CoA dehydrogenase	+			Zhao G&D 14:981-993
	Y00711	L-lactate dehydrogenase H chain	-			Zhao G&D 14:981-993
	M14780	Muscle creatine kinase	+	yes		Jackson Oncogene 16:283-92
	J05073	Muscle-specific phosphoglyceromutase	+	yes	rp, gs	Ruiz-Lozano CG&D 10:295-306
	J04173	Phosphoglycerate mutase 1 (brain)	-			Zhao G&D 14:981-993
	T64167	Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase	+			Zhao G&D 14:981-993
	NM_000189	Type 2 hexokinase	+	yes	gs,fp,rp	Mathupala JBC 272:22776-80
	M27160	Tyrosinase	+	yes		Nylander J Path 190:39-46
	AF001295	Tyrosinase-related protein	+	yes		Nylander J Path 190:39-46
Channels/Transporters						
	H65116	Na-K-Cl channel (NKCC1)	-			Zhao G&D 14:981-993
	XM_005543	Aquaporin 3	+ (+++ P73)	yes	rp	Zheng FEBS 489:4-7
	T59627	Brain calcium channel BI-2	+			Zhao G&D 14:981-993
	M14758	MDR1b	+	yes	rp, gs	Zhou JBC 273: 15387-94
	NM_013943	mtCLIC	+		rp	Fernandes-Salas Mol Cell Biol 22(11):3610-20
	U16799	Na/K ATPase beta 1 subunit	-			Zhao G&D 14:981-993
	M99564	P protein (melanocyte-specific)	+			Zhao G&D 14:981-993
	L36069	Potassium channel alpha subunit	+			Zhao G&D 14:981-993
	L16242	Sodium channel type I, beta (SCN1B)	+			Zhao G&D 14:981-993
	L21204	TAP1 peptide transporter	+	yes		Yu PNAS 96:14517-22
	NM_006996	Thiamine transporter 1 (THTR-1)	+	yes	rp,gs	Lo J Biol Chem 276(40):37186-93
Cell Cycle						
	X57348	14-3-3 sigma	+	yes	rp	Zhao G&D 14:981-993
	U02509	APC (adenomatous polyposis coli)	+/-	yes	gs,rp	JBC manuscript M101298200

	X05360	Cdc2	"- (P21-dep)"			de Toledo CG&D 9: 887-96
	M68520	CDK2	-			Zhao G&D 14:981-993
	U66838	Cyclin A	"- (P21-dep)"			de Toledo CG&D 9: 887-96
	M25753	Cyclin B	"- (P21-dep)"			de Toledo CG&D 9: 887-96
	NM_001238	Cyclin E				Kannan Oncogene 20(18):2225-34
	U53328	Cyclin G	+	yes	rp,gs	Okamoto EMBO 13:4816-22
	L25931	Lamin B receptor	-			Zhao G&D 14:981-993
	U31278	Mad2 homolog	-			Zhao G&D 14:981-993
	U03106	p21/WAF1	+	yes	rp,gs	el-Deiry Cell 75:817-25
	XM_004486	PA26 (p53 activated gene 26)	+	yes	rp,gs	Velasco-Miguel Oncogene 18:127-37
	M14630	Prothymosin alpha	-			Zhao G&D 14:981-993
	U35143	Rb-binding protein RbAP46	-			Zhao G&D 14:981-993
	L13463	Regulator of G-protein signaling 2	+			Zhao G&D 14:981-993
	XM_002619	Reprimo	+			Ohki JBC 275:22627-30
	K02581	Thymidine kinase	"- (P21-dep)"			de Toledo CG&D 9: 887-96
	M17733	Thymosin beta-4	-			Madden Oncogene 15:1079-85
	NM_001067	Topoisomerase 2 alpha	"- (P21-dep)"			de Toledo CG&D 9: 887-96
"Growth Regulatory"						
	NM_006569	CGR11	+			Madden Oncogene 15:1079-85
	U66469	CGR19	+			Madden Oncogene 15:1079-85
	U72649	BTG2	+			Rouault Nat.Gen 14:482-6
	AF156598	DDA3	+	yes		Lo Oncogene 18:7765-74
		p202	-	yes		D'Souza J Biol Chem 276(1):298-305
	X96438	p22/PRG1	+/-	yes	gs,rp	Schafer Oncogene 16:2479-87
DNA Replication						
	M12623	Chromosomal protein HMG17	-			Zhao G&D 14:981-993
	X74330	DNA primase polypeptide 1	-			Zhao G&D 14:981-993
	D28480	DNA replication licencing factor cdc47	-			Zhao G&D 14:981-993
	J04088	DNA topoisomerase 2 alpha	-			Zhao G&D 14:981-993
	X62534	HMG2	-			Zhao G&D 14:981-993
	H09351	MCM3 homolog	-			Zhao G&D 14:981-993

Extracellular Matrix						
	X83412	B1 mucin	+			Zhao G&D 14:981-993
	X06268	Collagen type 2 alpha 1	+			Zhao G&D 14:981-993
	X15880	Collagen type 6 alpha1	+			Zhao G&D 14:981-993
	NM_013882	Fibronectin (FN)	-			Iotsova Cell Growth Differ 7:629-34
	XM_006274	hMMP 13 (collagenase 3)	-			Sun JBC 275:11327-32
	NM_002421	MMP 1 (collagenase 1)	-			Sun JBC 274: 11535-40
	J03210	MMP-2	+	yes	gs,rp	Bian MCB 17:6330-8
	M94132	Mucin 2 (MUC2)	+			Zhao G&D 14:981-993
	R55650	POM-ZP3	+			Zhao G&D 14:981-993
	T62932	Procollagen alpha 1(V)	+			Zhao G&D 14:981-993
	D14043	Putative mucin core protein 24	-			Zhao G&D 14:981-993
Cell Migration						
	M74178	Macrophage stimulating protein (msp)	+			Zhao G&D 14:981-993
	M16006	Plasminogen activator inhibitor PAI-1	+	yes		Zhao G&D 14:981-993
Nuclear Matrix						
	AF059611	Nuclear matrix protein NRP/B	+			Zhao G&D 14:981-993
Cytoskeleton						
	R48602/AF064238	Smoothelin	+			Zhao G&D 14:981-993
	J05192	Actin alpha2	+	yes	rp,gs	Zhao G&D 14:981-993
	X53416	Actin-binding protein (filamin)	+			Zhao G&D 14:981-993
	X06956	Alpha tubulin (1)	-			Madden Oncogene 15:1079-85
	L11645	Alpha tubulin (2)	-			Madden Oncogene 15:1079-85
	NM_013882	B99 / Gtse-1 (G2- and S-phase-expressed)	+		rp	Utrera EMBO 17:5015-25
	R80966	Clathrin light chain B	+			Zhao G&D 14:981-993
	M34482	Cytokeratin 8	+	yes	rp,gs	Zhao G&D 14:981-993
	T78096	Dynactin	+			Zhao G&D 14:981-993
	BC005660	Elongation factor 1 alpha	+?	weak?	rp	Kato Blood 90:1373-78

	H72824	EST similar to cytokeratin 8	?			Mukhopadhyay Anticanc Res 16:105-12
	M19283	Gamma actin	-			Madden Oncogene 15:1079-85
	X62571	Keratin 17	+			Zhao G&D 14:981-993
	Y00503	Keratin 19	+			Zhao G&D 14:981-993
	M21389	Keratin 5	+			Zhao G&D 14:981-993
	XM_002993	MAP4 (microtubule associated protein)	-			Murphy G&D 10:2971-80
	AF010314	Pig10 actin-binding protein	+			Polyak Nature 389:300-305
	R37660	Stathmin	-			Zhao G&D 14:981-993
Membrane proteins						
	Z11502	Annexin 8	+			Zhao G&D 14:981-993
	Z18951	Caveolin	+	yes	rp,gs	Zhao G&D 14:981-993
	H64001	CD9	-			Zhao G&D 14:981-993
	L35545	Endothel cell protein C/APC receptor	+			Zhao G&D 14:981-993
	T48950	Erythrocyte memb 50kDa glycoprot	+			Zhao G&D 14:981-993
	AB006780	Galectin-3	-		rp	Raimond FEBS Lett. 363:165-9
	XM_018264	Galectin-7	+			Polyak Nature 389:300-305
	H82820	Glycophorin B	+			Zhao G&D 14:981-993
	D84290	GML (GPI-anchored molecule-like)	+	yes?		Zhao G&D 14:981-993
	Z48042	GPI-anchored protein p137	-			Zhao G&D 14:981-993
	L25941	LBR	-			Zhao G&D 14:981-993
	R69448	Integrin alpha-3	+			Zhao G&D 14:981-993
	H64489	Leukocyte antigen CD37	+			Zhao G&D 14:981-993
	AF065388	NET-1 tetraspan protein	+			Zhao G&D 14:981-993
	NM_004148	Ninjurin				Kannan Oncogene 20(18):2225-34
	D16111	PE binding protein PEBP	-			Zhao G&D 14:981-993
	U28369	Semaphorin 5	+			Zhao G&D 14:981-993
	NM_004636	Semaphorin 3B	+	yes		Ochi Neoplasia 4(1):82-7
	M11507	Transferrin receptor	-			Zhao G&D 14:981-993
Signal Transduction						
	U01147	ABR	+			Zhao G&D 14:981-993
	AB002058	P2XM	+	yes	chip	Zhao G&D 14:981-993

U14603	HU-PP-1	-			Zhao G&D 14:981-993
U35143	RbAp46	-			Zhao G&D 14:981-993
U70426	A28-RGS14p	+			Buckbinder PNAS 94:7868-72
L13738	Ack p21cdc42Hs kinase	+			Zhao G&D 14:981-993
J04809	AK1 cytosolic adenylate kinase beta	+			Collavin Oncogene 18:5879-88
L13687	ARL2 ADP-ribosylation factor-like	-			Zhao G&D 14:981-993
Y11525	C/EBP alpha	-			Zhao G&D 14:981-993
V01512	c-Fos	-	yes	rp, gs	Kley Nucl Acids Res 20:4083-7
	Diacylglycerol kinase				Kannan Oncogene 20(18):2225-34
M59371	Eck tyrosine protein kinase receptor	+			Zhao G&D 14:981-993v
NM_004428	Ephrin A1	+			Yu PNAS 96:14517-22
U07695	Ephrin B4	+			Yu PNAS 96:14517-22
NM_001405	EphrinA2	+	yes		Dohn Oncogene 20(45):6503-15
L32866	EPR-1	-			Zhao G&D 14:981-993
X57206	1D-myo-inositol-trisphosphate 3-kinase B	+			Zhao G&D 14:981-993
X62055	PTP1C	+			Zhao G&D 14:981-993
X74262	RbAp48	-			Zhao G&D 14:981-993
L36719	MAP kinase kinase 3 (MKK3) mRNA.	+			Zhao G&D 14:981-993
	H-ras				Kannan Oncogene 20(18):2225-34
L25665	Hsr1 possible GTP-binding protein	-			Zhao G&D 14:981-993
M26062	IL-2 receptor beta	+			Zhao G&D 14:981-993
U51336	inositol 1,3,4 triphosphate 5/6 kinase	+			Yu PNAS 96:14517-22
X77567	inositol triphosphate 5-phosphatase	-			Zhao G&D 14:981-993
X51345	Jun B	+			Yu PNAS 96:14517-22
NM_005569	LIMK-2	+			Madden Oncogene 15:1079-85
M15024	Myb	-			Zhao G&D 14:981-993
D87953	NDRG1 (N-myc downstream reg)	+			Yu PNAS 96:14517-22
X73478	PP2A regulatory subunit PTPA	-	no	rp, gs	Zhao G&D 14:981-993
R89715	PKC gamma	+			Zhao G&D 14:981-993
Z15108	PKC zeta	+			Zhao G&D 14:981-993
H07860	PKC-like 2	+			Zhao G&D 14:981-993
U14417	Ral GDP dissociation stimulator	+			Zhao G&D 14:981-993

	R56401	RAN-GAP	-			Zhao G&D 14:981-993
	M54968/M38506	RAS	+			Madden Oncogene 15:1079-85
	M28209	RAB-1A	+			Zhao G&D 14:981-993
	NM_000321	Rb	+			Osifchin JBC 269:6383-9
	D85815	RhoHP1	+			Zhao G&D 14:981-993
	L07597	S6 kinase 90kDa pp2	-			Zhao G&D 14:981-993
	H06970	PAK	+			Zhao G&D 14:981-993
	NM_005627	Sgk	+	yes	rp, gs	Maiyar JBC 271:12414-22
	U76247	Siah-1A	+			Matsuzawa EMBOJ 17:2736-47
	NM_003177	Syk	-			Okamura Oncology Res 11:281-5
Growth Factors/ Receptors						
	NM_002006	bFGF	-			Ueba PNAS 91:9009-13
	D30751	BMP 4	+			Zhao G&D 14:981-993
	M22490	BMP 2B	-			Zhao G&D 14:981-993
	NM_000710	Bradykinin B1 receptor	-	no		Yang J Cell Biochem 82(1):38-45
	AF177394	Dickkopf	+			Wang Oncogene 19:1843-48
	NM_005228	EGF receptor	?	yes		Sheikh Oncogene 15:1095-101
	X53655	NGF-2.	+			Zhao G&D 14:981-993
	X54936	PlGF	+			Zhao G&D 14:981-993
	NM_001945	HB-EGF	+			Fang EMBO J 20(8):1931-9
	R94967	HGF-like protein	+			Zhao G&D 14:981-993
	X16323/S80567	HGF	+	yes	rp, gs	Metcalfe Nucl Acids Res 25:983-6
	M31159	IGFBP-3	+			Zhao G&D 14:981-993
	M64347	Novel growth factor receptor	+			Zhao G&D 14:981-993
	NM_000582	Osteopontin (SPP1)	+	yes	rp, chip	Morimoto Gen Chrom Canc 33(3):270-8
	T40653	P01243 lactogen precursor	+			Zhao G&D 14:981-993
	uni 74034	PET-5 EST similar to Hedgehog	+			Yu PNAS 96:14517-22
	AB000584	PTGF-beta TGF-beta superfamily	+	yes		Zhao G&D 14:981-993
	X70340	TGF-alpha	+	yes		Zhao G&D 14:981-993
	R59097	TIE-1	+			Zhao G&D 14:981-993
	T91043	UFO	+			Zhao G&D 14:981-993

Angiogenesis				
	NM_001702	BAI1	+	da K. Cytogenet&Cell Gen 84:75-82
	M65199	Endothelin 2	+	Zhao G&D 14:981-993
	X14787	Thrombospondin 1	+	Zhao G&D 14:981-993
	NM_003247	Thrombospondin 2	?	Adolph Gene 193: 5-11
	R70008	VEGF		Zhao G&D 14:981-993
Immune system-related				
	L33930	CD24	-	Zhao G&D 14:981-993
	K02403	Complement protein 4A	+	Zhao G&D 14:981-993
	R60883	HLA class II DQ(W1.1) beta	+	Zhao G&D 14:981-993
	T62633	HLA class II DR-1 beta	+	Zhao G&D 14:981-993
	T72403	HLA class II DQ(3) alpha	+	Zhao G&D 14:981-993
	T94834	HLA-DR associated protein I		Zhao G&D 14:981-993
	M13755	Interferon-induced 17kDa protein	+	Zhao G&D 14:981-993
	M59807	NK cell protein 4	+	Zhao G&D 14:981-993
Ribosomal/Translation				
	13683	16S rRNA	+	Madden Oncogene 15:1079-85
	M27830	28S rRNA	+	Madden Oncogene 15:1079-85
	R05707	EF-1 beta	+	Zhao G&D 14:981-993
	R60195	EIF-4B	-	Zhao G&D 14:981-993
	L11567	L37	+ (mut p53)	Loging CancEpidemBiomark&Prev 8:1011-6
	J01866/M25598	Ribosomal 5.8S rRNA	+	
	NM_012423	Ribosomal protein L13a	-	Madden Oncogene 15:1079-85
	NM_015920 ?	Ribosomal protein S27	+	Madden Oncogene 15:1079-85
	M17887	RPP-1	+ (mut p53)	Loging CancEpidemBiomark&Prev 8:1011-6
	NM_002952	S2	+ (mut p53)	Loging CancEpidemBiomark&Prev 8:1011-6
RNA Splicing				
		U6 snRNA (likely artifact– no polyA)	+	Madden Oncogene 15:1079-85

Unknown						
(or unknown relevance)	R42736	EST sim to KIAA0835 protein	+			Zhao G&D 14:981-993
	M36263	Estradiol 17 beta-dehydrogenase 1	+			Zhao G&D 14:981-993
	d14657	KIAA0101 gene	-			Zhao G&D 14:981-993
	X75252	Prostatic binding protein	-			Zhao G&D 14:981-993
	U33271	Pig5 normal keratinocyte mRNA	+			Polyak Nature 389:300-305
	AF010315	Pig11 unknown	+			Polyak Nature 389:300-305
	X93036	MAT8	+			Yu PNAS 96:14517-22
	NM_002081	Glypican (heparan sulfate proteoglyc)	+			Yu PNAS 96:14517-22
	NM_012101	AT group D-associated protein	+			Yu PNAS 96:14517-22
	U53786	Envoplakin	+			Yu PNAS 96:14517-22
	AA804789	PET-1	+			Yu PNAS 96:14517-22
	AA995453	PET-2	+			Yu PNAS 96:14517-22
	AF153606	PET-3	+			Yu PNAS 96:14517-22
	AF070648	PET-6	+			Yu PNAS 96:14517-22
	AI680503	PET-7	+			Yu PNAS 96:14517-22
	AI924346	PET-8	+			Yu PNAS 96:14517-22
	AB015019	BAP2-alpha	+			Yu PNAS 96:14517-22
	AI870236	PET-11	+			Yu PNAS 96:14517-22
	AI623827	PET-13	+			Yu PNAS 96:14517-22
	L17048	ETS-1	+			Madden Oncogene 15:1079-85
		EST 105829	+			Madden Oncogene 15:1079-85
		EST 110550	-			Madden Oncogene 15:1079-85
	Z50749	Sds22-like protein (regulator of PP1)	-			Okamura Oncology Res 11:281-5
	AB027289	Ceb1 (HECT+RCC1 repeats)	-	?		Mitsui BiochemBiophysResCom 266:115-22
	NM_001117	PACAP receptor	+			Hoffman Ann.NYAcadSci 865:49-58
	NM_009517	PAG608/Wig-1 p53-induced Zn-finger	+			Israeli EMBOJ 16:4384-92
	NM_005978	S100A2 calcium-binding protein	+	yes	rp,gs	Tan FEBS Lett 445:265-68
	Z20656	Cardiac alpha-myosin heavy chain	+			Zhao G&D 14:981-993
	J00231	Ig gamma 3 heavy chain disease OMM	+			Zhao G&D 14:981-993
	U06088	GALNS	+			Zhao G&D 14:981-993

H20434	H.sapiens TSC2 mRNA for tuberin.	+	Zhao G&D 14:981-993
X05615	Thyroglobulin.	+	Zhao G&D 14:981-993
T41265	ya33h07.s3	+	Zhao G&D 14:981-993
R60906	Probable nuclear antigen	+	Zhao G&D 14:981-993
U14631	11 beta-hydroxysteroid dehydrogenase type II	+	Zhao G&D 14:981-993
H28050	FK506-BINDING PROTEIN	+	Zhao G&D 14:981-993
M64098	HBP	+	Zhao G&D 14:981-993
X80200	MLN62	+	Zhao G&D 14:981-993
R49565	HSJ1	+	Zhao G&D 14:981-993
H49515	SRP	+	Zhao G&D 14:981-993
H24002	AGRIN	+	Zhao G&D 14:981-993
D14657	ORF	+	Zhao G&D 14:981-993
D21262	KIAA0035	-	Zhao G&D 14:981-993
J04977	Ku autoimmune antigen	-	Zhao G&D 14:981-993
X77956	H.sapiens Id1.	-	Zhao G&D 14:981-993
Y00705	PstI pancreatic secretory inhibitor	-	Zhao G&D 14:981-993
D13641	ORF	-	Zhao G&D 14:981-993
H29320	GTP-binding protein	-	Zhao G&D 14:981-993
R54665	Leukocyte elastase inhibitor	-	Zhao G&D 14:981-993
Z50194	PQ-rich protein	-	Zhao G&D 14:981-993
X12671	hnRNP core protein A1.	-	Zhao G&D 14:981-993
T59354	EBNA-2 nuclear protein (Epstein-barr virus)	-	Zhao G&D 14:981-993
H11374	14-3-3-like protein (Xenopus laevis)	-	Zhao G&D 14:981-993
R41873	Transcription factor	-	Zhao G&D 14:981-993
M13450	Esterase D	-	Zhao G&D 14:981-993
T70062	Nuclear factor NF45	-	Zhao G&D 14:981-993
H73758	Psoriasis-assoc FABP	-	Zhao G&D 14:981-993
X54941	Ckshs1	-	Zhao G&D 14:981-993
R91912	Placental calcium binding protein	-	Zhao G&D 14:981-993
R44863	Phenylalkylamine binding protein.	-	Zhao G&D 14:981-993
NM_002639	Maspin tumor suppressor	?	Zou JBC 275:6051-4
S43127	Ref-1		Herring Br J Cancer 78:1128-33

AF039597	KARP-1		Myung PNAS 95:7664-9
NM_005266	Connexin-40 (gap junction protein)		Genomics 46:120-6
NM_002690	DNA Pol beta	?	Yamaguchi Eur J of Bioch 221:227-37

Table Legends

Table 1

A summary of genes reported in the literature to respond to p53. Accession numbers are typically those of the human gene, although in some cases, the sequence identity in the original reference is only described in terms of homology to a gene in some other species, for example, in the case of BLAST analyses of unknown gene clones.

Upregulation of the gene is indicated by "+", while downregulation is indicated with "-". In cases where the report was equivocal, or two different groups reported different results, the symbols "+/-" or "?" are used. "mut p53" indicates that the investigators claim that the gene is specifically induced by mutated p53. "- (P21-dep)" indicates that the researchers found the gene to be altered in expression in response to $p21^{CIP}$ levels. "(+++) p73" indicates that the gene was found to be more responsive to the p73 homolog of p53.

Direct targets are those for which some sort of promoter analysis was done. The abbreviations chip, rp, gs, and fp stand for chromatin immunoprecipitation, reporter assay, gel-shift, and footprint analysis, respectively.

A representative literature reference for each of the genes is given in the final column, but it is not intended to document all reports of p53 regulation of the gene.

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Chapter 2: An Intact HDM2 RING Finger is Required for p53 Nuclear Exclusion

Table of Contents

Abstract

The p53 tumor suppressor protein is negatively regulated by HDM2. Recent reports indicate that the leucine-rich nuclear export sequence (NES) of HDM2 enables it to shuttle to the cytoplasm, and that this activity is required for p53 degradation. However, it is unclear whether HDM2 is involved in nuclear export of p53, in part because p53 has been shown to contain a functional nuclear export sequence within its tetramerization domain. We show that coexpression of HDM2 with GFP-tagged p53 causes redistribution of p53 from the nucleus to the cytoplasm of the cell. This activity is dependent on binding of p53 to HDM2 and requires an intact p53 NES but is independent of the HDM2 NES. A mutant of the HDM2 RING finger domain that is unable to ubiquitinate p53 does not cause relocalization of p53, suggesting that ubiquitin ligation or other activities of this region of HDM2 are necessary for its regulation of p53 localization.

Introduction

The p53 protein is a transcription factor that acts as a tumor suppressor by causing growth arrest or apoptosis in response to DNA damage and other forms of cellular stress (Levine, 1997). The regulation of p53 activity is largely due to post-translational events, including direct binding and occlusion of the transactivation domain, ubiquitination followed by degradation in proteasomes, and control of subcellular localization. The MDM2 protein has been implicated in all three of these processes, most recently as a mediator of export of p53 from the nucleus (Honda et al., 1997b; Lin et al., 1994b; Roth et al., 1998; Tao and Levine, 1999). At present, the data for this activity of MDM2, or the human homolog HDM2, are indirect. HDM2 proteins with mutations in their leucine-rich nuclear export sequence (NES) are unable to shuttle between nucleus and cytoplasm, as assessed in heterokaryon assays, and are incapable of decreasing p53 protein levels (Roth et al., 1998). Even when nucleus-confined HDM2 NES mutants are coexpressed with cytoplasm-confined HDM2 proteins mutated in their nuclear localization sequence, they are unable to promote p53 degradation (Tao and Levine, 1999). Similarly, the compound Leptomycin B, which inhibits nuclear export pathways requiring the CRM1 receptor for leucine-rich NES sequences, causes accumulation of p53 in the nucleus of the cell, and increased levels of p53 protein (Fornerod et al., 1997; Freedman and Levine, 1998; Fukuda et al., 1997; Ossareh-Nazari et al., 1997).

These observations have led to a model in which HDM2 shuttles rapidly between nucleus and cytoplasm, carrying p53 out of the nucleus so that it can be degraded by proteasomes in the cytoplasm. Other recent observations conflict with this interpretation, however.

The p53 protein contains its own leucine-rich NES sequence, which can act as a functional nuclear export signal when attached to a reporter protein. The putative p53 NES has been implicated in p53 nuclear shuttling in heterokaryon assays. Notably, a shuttling activity can be detected even for a p53 mutant that is unable to bind HDM2 (Stommel et al., 1999).

Here we describe experiments that dissect the regions of HDM2 and p53 required for regulation of p53 subcellular localization when the two proteins are expressed in the same cell. Localization properties of NES mutants of p53 and HDM2, and mutation of the HDM2 C-terminal RING finger indicate that HDM2 does not act as a simple nuclear export shuttle for p53, but rather activates the signal for p53 nuclear export, likely via ubiquitination of p53 (Stommel et al., 1999).

Materials and Methods

Plasmids

Human wild type p53 and p53(L22Q,W23S) were cloned upstream of EGFP in the pEGFP/N1 vector (Clontech) by PCR amplification using primers encoding PstI and BamHI endonuclease sites. HDM2 expressed from the pCMV5 plasmid was obtained from Jim Xiao (Boston University). The HDM2NES (L205A, I208A), HDM2C464A, and p53NESGFP (L348A, L350A) clones were generated using the QuikChange method (Stratagene). Constructs made with PCR were sequenced to ensure that no errors were introduced by the Pfu polymerase. HDM2 and the panel of mutant p53GFP inserts were

subcloned into pCDNA3 (Invitrogen) for *in vitro* translation experiments. pSG-E1 plasmid containing wild-type E1 was obtained from Dr. A. Schwartz (Washington University School of Medicine).

Transfection assays

NIH/3T3, U2 OS (ATCC), *ts20* and HCT116 cells were grown in DMEM supplemented with 10% calf serum and antibiotics. Cells were plated on coverslips and transfected 24 hours later using Lipofectamine Plus reagents (Gibco) according to the manufacturer's directions. 0.5 μg of p53GFP plasmids, and 2 μg of HDM2 plasmids per 6-well plate well were transfected. Total DNA was normalized in each lipofection with either pBluescript (Stratagene) or an empty yeast 2-hybrid screening vector. Cells were harvested between 20 and 24 hours after transfection. For temperature shift experiments, cells were grown at 35°C and moved to a 39°C incubator for the last 6 hours prior to harvest.

Immunofluorescence

Transfected cells were fixed in 4% paraformaldehyde in PBS, permeabilized at -20°C in methanol, and rehydrated in PBS. Following 30 minute incubation in blocking solution (1% goat serum in PBS), cells were stained with1:500 each of anti-Mdm2 (SMP14; Pharmingen) in blocking solution for 2 hrs at room temperature. Cells were washed twice with blocking solution for10 minutes each, then incubated with secondary antibody Texas-Red-X anti-mouse IgG (Molecular Probes) in blocking solution for 2 hrs at room

temperature. Finally, cells were washed in PBS and mounted on slides. Between 100 and 200 cells were counted for quantification of each assay condition. Cells with non-nucleoplasmic HDM2 were excluded from the quantitation. Endogenous p53 was detected in paraformaldehyde-fixed HCT116 cells by permeabilization with 0.1% Triton X-100 and incubation with anti-p53 Ab-7 (Calbiochem), followed by washing and incubation with Rhodamine Red-X-conjugated anti-sheep antibodies (Jackson ImmunoResearch laboratories), while HDM2 was detected using SMP14 anti-Mdm2 followed by FITC-conjugated anti-mouse antibodies (Jackson ImmunoResearch laboratories).

In vitro translation and immunoprecipitation

RNA was generated using the T7 RNA polymerase (Promega) and translated *in vitro* using reticulocyte lysates (Promega). pCDNA3 plasmids containing the indicated inserts were used as templates. Coupled transcription-translation reactions were incubated for 90 minutes at 30°C. For immunoprecipitations, lysates containing [^{35}S]-methionine-labeled proteins were mixed and incubated for 30 minutes at 30°C, then diluted with 250 µL of IP buffer (100 mM Tris pH 8.0, 100 mM NaCl, 1% Nonidet P-40), precleared with Protein G Sepharose beads (Pierce) at 4°C for 30 minutes, then incubated at 4°C with 0.2 µg of the anti-p53 antibody PAb 421 (Oncogene Research Products) for 2 hours. Protein G Sepharose beads were then added for 30 minutes, and immunoprecipitates were washed 4 times with IP buffer prior to separation by SDS-PAGE. Analysis of phosphor screen autoradiographs was conducted using ImageQuant software (Molecular Dynamics).

Immunoprecipitation and Western blotting

Cells for p53GFP protein level measurements were plated in 10 cm dishes, transfected 24 hours later with 1.5 μg of p53GFP plasmids and 3 μg of either HDM2 plasmids or empty vector plasmids, and harvested 24 hours post-transfection. Cell lysates were prepared in ice-cold NP-40 lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 5mM EDTA, 0.5% Nonidet P-40, and protease inhibitors), cleared of cell debris by centrifugation, and precleared by 30 minute incubation at 4∞C with Protein G Sepharose beads (Pierce). p53 was immunoprecipitated by incubation of lysates with the PAb421 anti-p53 antibody (Oncogene Research Products) for 2 hours at 4°C followed by addition of Protein G Sepharose beads for a final 30 minute incubation at 4°C. Beads were washed four times with lysis buffer, and precipitated proteins were separated by SDS-PAGE. p53GFP was detected on Western blots using Ab-7 anti-p53 antisera (Calbiochem) and chemiluminescence. Coimmunoprecipitation of HDM2 with p53GFP was demonstrated by immunoprecipitation from transfected U2OS cell lysates with anti-GFP antibodies (Molecular Probes) and Protein G Sepharose beads, followed by Western blotting and detection of HDM2 using SMP14 anti-Mdm2 antibodies and chemiluminescence.

Results

HDM2 increases cytosolic localization of p53

HDM2 and its murine homolog MDM2 have been characterized as mediators of p53 degradation (Haupt et al., 1997; Kubbutat et al., 1997). Recent data suggest that transit of HDM2 from nucleus to cytoplasm is required for this activity(Freedman and Levine, 1998; Roth et al., 1998; Tao and Levine, 1999). To test the hypothesis that HDM2 regulates nuclear export of p53, we conducted experiments to assess whether subcellular localization of p53 could be altered by coexpression of HDM2. NIH/3T3 cells were transfected with a plasmid encoding GFP-tagged human p53 (p53GFP). In the absence of exogenous HDM2, p53GFP was predominantly localized to the nucleus (Fig. 1, Fig.2 G). For quantitation of the assay, cells were categorized as having GFP fluorescence that was either greater in nucleus than in cytoplasm, equal in nucleus and cytoplasm, or greater in cytoplasm than nucleus. Between 100 and 200 cells were counted for each experimental condition. When HDM2 was cotransfected with p53GFP and visualized by indirect immunofluorescence, a substantial increase in the cytoplasmic p53GFP and decrease in nuclear p53GFP was observed in cells expressing both proteins (Fig. 1, Fig.2 G). HDM2 was localized to the nucleus in essentially all cells observed. We obtained similar results in the U2OS cell line (Fig.1 F-G).

To test whether the effect on p53 localization was mediated by direct interaction with HDM2, we generated a GFP fusion of the well-characterized p53(L22Q,W23S) mutant, which is unable to bind to HDM2 (Lin et al., 1994a). Cells transfected with the p53(22,23)GFP-fusion protein showed sharply defined nuclear fluorescence with negligible cytoplasmic fluorescence (Fig.2). Importantly, the exclusively nuclear localization of the p53(22,23)GFP protein was not affected by cotransfection with HDM2, indicating that export of p53 from the nucleus of the cell requires binding to HDM2 (Fig.2 C, E).

Mutation of the p53 NES prevents nuclear export by HDM2

Several models for the effects of HDM2 on p53 localization have been proposed. One study has shown that the putative NES of p53 is required for MDM2-independent transit of p53GFP from one nucleus to a second nucleus in heterokaryons (Stommel et al., 1999). Other investigations using a similar heterokaryon system have observed a shuttling activity for HDM2, and have supported a role for HDM2 as a carrier protein able to transport p53 out of the nucleus (Roth et al., 1998). We tested the localization characteristics of a GFP fusion of the p53 L348A, L350A NES mutant (p53NESGFP) (Stommel et al., 1999), in response to cotransfected HDM2. We found that the p53NES mutant was predominantly nuclear in its localization (Fig.2). Strikingly, cotransfected HDM2 was unable to alter the localization of p53NESGFP (Fig.2 D, F). This result is consistent with a requirement for the p53 NES for transit of p53 out of the nucleus, as

suggested by the earlier heterokaryon studies of this mutant, but in addition it shows that disruption of this sequence prevents HDM2-mediated relocalization of p53.

A trivial explanation for this finding could be that HDM2 is unable to bind to NES mutant p53. Earlier studies of a series of deletion mutants of the p53 C-terminus showed that truncated proteins lacking the p53 C-terminal portion containing the NES and the tetramerization domain could not bind to HDM2, leading to the suggestion that HDM2 may preferentially bind tetrameric p53 (Marston et al., 1995). The initial characterization of the p53NES mutant, whose mutations lie in the tetramerization domain, provided some evidence that the protein might be impaired in oligomerization, as assessed by *in vitro* cross-linking of tetramerization domain peptides (Stommel et al., 1999). We tested the hypothesis that failure to bind to HDM2 would explain the localization properties of the p53NES mutant by conducting co-immunoprecipitation experiments with HDM2 and the wild-type or mutant p53 fusion proteins. *In vitro* translated proteins were mixed and immunoprecipitated with the PAb 421 antibody against p53. As expected, wild-type p53 fusion protein coprecipitated HDM2, while the p53(22,23) mutant was unable to do so (Fig.2 H). The p53NES fusion protein was able to immunoprecipitate HDM2 with an efficiency comparable to wild-type p53, indicating that its inability to be relocalized by HDM2 was not the result of lack of binding between the two proteins (Fig.2 H). Quantitation of the intensity of the HDM2 band normalized to the intensity of the p53GFP band indicated that the p53NES fusion protein coprecipitated 84% as much HDM2 as did the wild-type p53GFP. We tested further the ability of GFP–p53(NES–) to bind to HDM2 by immunoprecipitating lysates of transfected U2OS cells with an anti-

GFP antibody (Fig. 2I). Wild-type p53–GFP and GFP–p53(NES–) co-precipitated with HDM2, whereas GFP–p53(L22Q,W23S) did not (Fig. 2I). These results indicate that HDM2 binding to NES-mutated p53 is largely intact, and that the inability of HDM2 to relocalize this mutant to the cytoplasm is related to impairment of some other functional interaction between the proteins, or reflects the loss of a nuclear export activity directly dependent on the p53 NES sequence.

The NES of HDM2 is not required for nuclear export of p53

Having found that alterations in the NES of p53 prevented its relocalization, we next tested whether the NES of HDM2 is required for its effects on p53. The available data on the importance of both the p53 NES and the HDM2 NES suggested a model in which both sequences might be necessary for alterations in p53 localization. In any case, a role for HDM2 as a shuttle protein is predicted to be dependent on the HDM2 NES sequence. We used the HDM2 L205A, I208A mutant (HDM2NES) which has been previously shown to be unable to shuttle in heterokaryon assays (Roth et al., 1998). Surprisingly, the HDM2NES mutant was comparable to wild-type HDM2 in its ability to increase the amount of cytoplasmic p53GFP in transfected cells (Fig.3). We examined cells by indirect immunofluorescence, and found that both wild-type and NES mutant HDM2 were expressed at comparable levels and had nucleoplasmic staining patterns that were indistinguishable from each other (Fig.3).

An intact HDM2 RING finger, and ubiquitin ligation activity are required for p53 nuclear exclusion

The unexpected ability of the HDM2NES mutant to cause p53 nuclear export led us to hypothesize that other portions of HDM2 may be required for its effects on p53 localization. The C-terminal RING finger is a candidate for this role, as it has been proposed to mediate the attachment of ubiquitin to p53, to bind specifically to RNA, and, most recently, to bind to the homologous MDMX protein (Elenbaas et al., 1996; Honda et al., 1997a; Tanimura et al., 1999). We generated a mutant of the RING finger domain, HDM2 C464A, that inactivates the ability of HDM2 to promote ubiquitination of p53 in *in vitro* assays (Fang et al., 2000; Honda et al., 1997a). This mutant was tested for its ability to delocalize p53 from the nucleus in a variety of cell lines, and was found to be completely inactive despite expression and subcellular localization comparable to wild type HDM2 (Fig.4 A, B). This experiment indicates that at least one of the activities imputed to this domain of HDM2 is required for the pathway leading to p53 nuclear exclusion.

We also measured the effect of HDM2, HDM2NES, and HDM2C464A expression on the levels of cotransfected p53GFP protein, to see whether there was a correlation between the ability of HDM2 mutants to cause relocalization of p53GFP to the cytoplasm, and to cause its degradation. Coexpression of HDM2 or HDM2NES with p53GFP in U2OS cells resulted in a substantial decrease in protein levels, while the HDM2G464A mutant did not affect p53GFP levels (Fig.4 D). Levels of the p53(22,23)GFP and p53NESGFP

mutants were not affected by coexpressed HDM2, further supporting a correlation between cytoplasmic relocalization of p53 and its degradation (Fig.4 D). The ability of HDM2 mutants to cause degradation of endogenous p53 was evaluated in the colorectal cancer cell line HCT116, which expresses wild-type p53 at levels sufficient for detection by indirect immunofluorescence (Waldman et al., 1995). Transfection of HDM2 or the HDM2NES mutant reduced the amount of endogenous p53 detected in these cells to near background levels, while the HDM2C464A mutant had no such effect (Fig.4 E-J).

One explanation for our data concerning the requirement for the p53 NES and the HDM2 RING finger for p53 relocalization is that ubiquitination of p53 by HDM2 may activate or expose the p53 NES and trigger nuclear export of the protein. Because several activities have been attributed to the HDM2 RING finger, and the structure of the RING finger would be predicted to be disrupted by mutation of one of its metal ion-coordinating residues, we sought an independent way of testing whether ubiquitin ligase activity by HDM2 was required for its effects on p53 localization. For this purpose, we made use of the *ts20* murine fibroblast cell line, which contains a temperature sensitive mutation in the E1 ubiquitin charging enzyme. At the restrictive temperature, ubiquitin conjugating pathways in these cells are inactivated due to the depletion of charged ubiquitin (Chowdary et al., 1994). We coexpressed p53GFP and HDM2 in these cells, and assessed whether HDM2 was able to alter p53 localization at the permissive and restrictive temperatures. We found that the ability of HDM2 to relocalize p53 in *ts20* cells that were shifted to 39°C for 6 hours was abrogated, whereas *ts20* cells grown at the permissive temperature throughout the experiment supported HDM2 relocalization of p53 (Fig.5 A).

Control fibroblasts showed no effect of temperature on HDM2 activity (Fig. 5 A). To ensure that the effect of increased temperature at the restrictive condition was due to inactivation of the temperature-sensitive E1 enzyme, we introduced wild-type E1 enzyme to the *ts20* cells at the restrictive temperature by transfection and observed that HDM2 relocalization of p53 was restored (Fig.5 B). These data lend further support to the idea that ubiquitination of p53 by HDM2 is required for relocalization of p53 in the cell.

Conclusions

We have examined the roles of several candidate regulatory regions of HDM2 and p53 for their ability to control the subcellular localization of p53. First, we show that expression of HDM2 shifts the usual nuclear localization of p53 to a more cytoplasmic pattern (Fig. 1). This effect requires direct binding between the two proteins, and relies on an intact p53 NES. The p53 NES is located in the tetramerization domain of the protein, and previous studies using large deletion series of the p53 C-terminus have implicated tetramerization of p53 in HDM2 binding (Marston et al., 1995). However, we find that the double point mutant p53NES fusion protein is comparable to wild type p53 in HDM2 binding, ruling out this possible explanation for the inability of HDM2 to relocalize NES mutant p53 (Fig. 2). At this point, we cannot exclude the possibility that other effects of altering the tetramerization domain may contribute to the behavior of this mutant. Previous work with heterokaryons has suggested that p53 is able to leave the nucleus without any interaction with MDM2 or HDM2 (Stommel et al., 1999); however, our data clearly show that the amount of HDM2 protein in the cell has a decisive

influence over whether p53 will be nuclear or cytoplasmic, and provide evidence for an important role of the p53 NES in the response to HDM2. It may be that a low level of p53 export from the nucleus occurs via an HDM2-independent mechanism, which is more readily detected in heterokaryon assays.

An unexpected observation in these experiments is that the NES of HDM2 is not required to promote relocalization of p53 (Fig. 3), while an intact RING finger domain is essential for this activity (Fig. 4). This finding rules out several possible models for HDM2 export activity. The simple model of an actively shuttling HDM2 protein binding to p53 and taking it out of the nucleus by forming a link between it and leucine-rich NES-binding nuclear export adapter proteins is not consistent with these data. A related model, in which both the p53 and HDM2 nuclear export sequences are required for export of a complex of the proteins, is also unlikely. Previous workers have reported that the HDM2NES mutant is ineffective at decreasing p53 levels, which, taken together with the results from HDM2 heterokaryon experiments, was suggestive evidence for the model of HDM2 acting as a NES-containing bridge between p53 and the nuclear export machinery, provided that p53 degradation occurs in the cytoplasm (Tao and Levine, 1999). However, we have found that the HDM2NES mutant is able to relocalize p53 to the cytoplasm as efficiently as wild-type HDM2, and to cause a decrease in p53 protein levels, while the HDM2C464A mutant is inactive for both of these properties (Figs.3, 4). Very recent results indicate that HDM2 with a G448S mutation in the RING finger is unable to shuttle in the heterokaryon nuclear shuttling assay, perhaps indicating that this assay is sensitive to a variety of alterations in HDM2, including mutations of the RING

finger domain (W. Tao and A. Levine, personal communication). It is also possible that previous reports of the inability of HDM2NES to decrease p53 protein levels may reflect cell or tissue variability in components of the ubiquitination, proteosomal degradation, or nuclear export pathways. Our findings are consistent with the idea that p53 must reach the cytoplasm to be degraded; however, the possibility remains that other activities of the HDM2 RING finger may be involved in its effects on p53. One possibility in this direction is that the RNA-binding activity of the RING finger may act in conjunction with HDM2 binding to the ribosomal L5 protein (Marechal et al., 1994), enabling association with ribosomal subunits during their export from the nucleus, as a mechanism for altering HDM2's, and perhaps p53's, subcellular localization. A recent study of the small ribosomal subunit nuclear export pathway in yeast has indicated that absence of the Crm1 nuclear export receptor impedes ribosomal subunit export from the nucleus (Moy and Silver, 1999). It is not yet known whether this is a direct effect, but this observation offers a possible explanation for the ability of Leptomycin B to impair HDM2-dependent proteasomal degradation of p53, even if the simple model of p53 or HDM2-p53 complexes acting as substrates for CRM1 is not accurate. In addition, this yeast study underlines the need for caution when interpreting the cellular effects of inhibition of a nuclear export receptor that is likely to have many substrates. However, our present data are consistent with the hypothesis that the p53 NES may be a direct substrate for CRM1-mediated nuclear export (Stommel et al., 1999).

If the degradation of ubiquitinated proteins occurs primarily in cytoplasmic proteasomes, then it is appealing to hypothesize that ubiquitination of a nuclear protein intended for

degradation may be closely coupled to the nuclear export of that target protein. Current data for p53 are consistent with models invoking cytoplasmic degradation of p53 (Freedman and Levine, 1998). However, much of this evidence is indirect, and a population of proteasomes is detectable in the nucleus in some cell types (Brooks et al., 2000). Additional levels of regulation involving, for example, proteasome subunit composition or polyubiquitination of substrates may differ between nucleus and cytoplasm to determine the ability of an individual substrate to be degraded. The RING finger motif has recently been recognized as a common element in a variety of proteins that are members of ubiquitin ligase complexes such as those of the SCF family (Kamura et al., 1999; Skowyra et al., 1999). We have shown that an intact HDM2 RING finger is required for altering p53 localization. A simple mechanism that may be proposed for this role of this domain, based on previous studies (Honda et al., 1997a), and our additional data, is that ubiquitination may activate the p53 NES or make it more accessible to nuclear export factors in the cell. Such an effect of ubiquitination could work by altering the oligomerization state or conformation of the p53 C-terminus. In this way, the same ubiquitin tag that targets degradation by the proteasome would signal transport of the substrate to the cytoplasm where the degradation is thought to occur. By extension, it is possible that other covalent modifications of the p53 protein could similarly target p53 to the cytoplasm by activating the p53 nuclear export signal. Recent studies of cells derived from human neuroblastomas indicate that upregulation of pathways causing covalent modification of p53 may be a common means of inactivating p53 by forcing it into the cytoplasm (Zaika et al., 1999). Our results also invite comparison with the ubiquitin-dependent endocytosis and down-regulation of a variety of plasma membrane proteins in

yeast and mammalian cells, in which ubiquitin tag itself serves as a signal for altering protein localization while targeting the protein for lysosomal or proteasomal degradation (Hicke, 1997; Terrell et al., 1998). In addition, two recent papers have shown that the ubiquitin-like protein SUMO-1 can be covalently linked to p53 in cells, causing increased p53 activity (Gostissa et al., 1999; Rodriguez et al., 1999). These interesting findings point towards a model in which both positive and negative regulation of p53 can rely on covalent modification with ubiquitin or related small proteins.

In the period of time since the data presented here were published in the journal *Nature Cell Biology* (Boyd et al., 2000), several other relevant findings have appeared in the literature. Lohrum et al. demonstrated that mutation of the p53 C-terminal Lysine residues prevents Mdm2 from relocalizing p53 into the cytoplasm (Lohrum et al., 2001). The Lohrum et al. paper also showed that increased levels of Crm1 protein increase Mdm2-dependent export of p53 (Lohrum et al., 2001). Another group demonstrated that there is an independent NES signal in the N-terminus of p53 that can be inactivated by DNA-damage induced phosphorylation, thus providing an additive method of increasing p53 nuclear residence in times of genotoxic stress (Zhang and Xiong, 2001). Shirangi et al. studied the relationship between enforced p53 localization in the nucleus and its degradation by proteasomes (Shirangi et al., 2002). They found that such nuclear-restricted p53 was not degraded at the same rate after the induction of increased p53 levels as it would be in nuclear export-competent cells, but some degradation did occur, indicating that either the nuclear export block was incomplete, or else that some proteasomal degradation can occur in the nucleus. (Shirangi et al., 2002). Perhaps most

interestingly, a mechanism similar to the one posed here, coupling ubiquitination and nuclear export of a protein, has also been detected in the regulation of HIF-1α by the von Hippel-Lindau tumor suppressor protein, which is part of a Cullin-related ubiqutin ligase complex (Fabbro and Henderson, 2003). It appears that HIF-1α export from the nucleus of the cell is similarly dependent on its ubiquitination state (Fabbro and Henderson, 2003).

The findings of this study also suggest a possible reinterpretation of the mechanism of $p19^{ARF}/p14^{ARF}$ inhibition of HDM2 or Mdm2, and activation of p53. Previous workers have derived differing conclusions on this topic, with two groups finding that ARF can sequester HDM2/Mdm2 to nucleoli, possibly preventing it from acting as a nuclear shuttle protein for p53, while others attribute human $p14^{ARF}$ inhibition of HDM2 to the formation of nucleoplasmic aggregates containing $p14^{ARF}$, HDM2 and p53 (Lohrum et al., 2000; Weber et al., 1999; Zhang and Xiong, 1999). Biochemical studies of $p19^{ARF}$ have shown that it can inhibit Mdm2 ubiquitin ligase activity for p53 *in vitro* (Honda and Yasuda, 1999). This result, taken together with our data, suggests that Arf may prevent HDM2-mediated export and degradation of p53 in part by inhibiting ubiquitination of p53.

Elucidation of the mechanisms of p53 regulation, including HDM2-mediated inhibition, is critical to our understanding of the process of tumor suppression by p53. Defects in p53 nuclear/cytoplasmic localization may represent alternative mechanisms for

inactivating the protein during tumorigenesis, and, thereby, may serve as targets for p53 reactivation in cancer therapy.

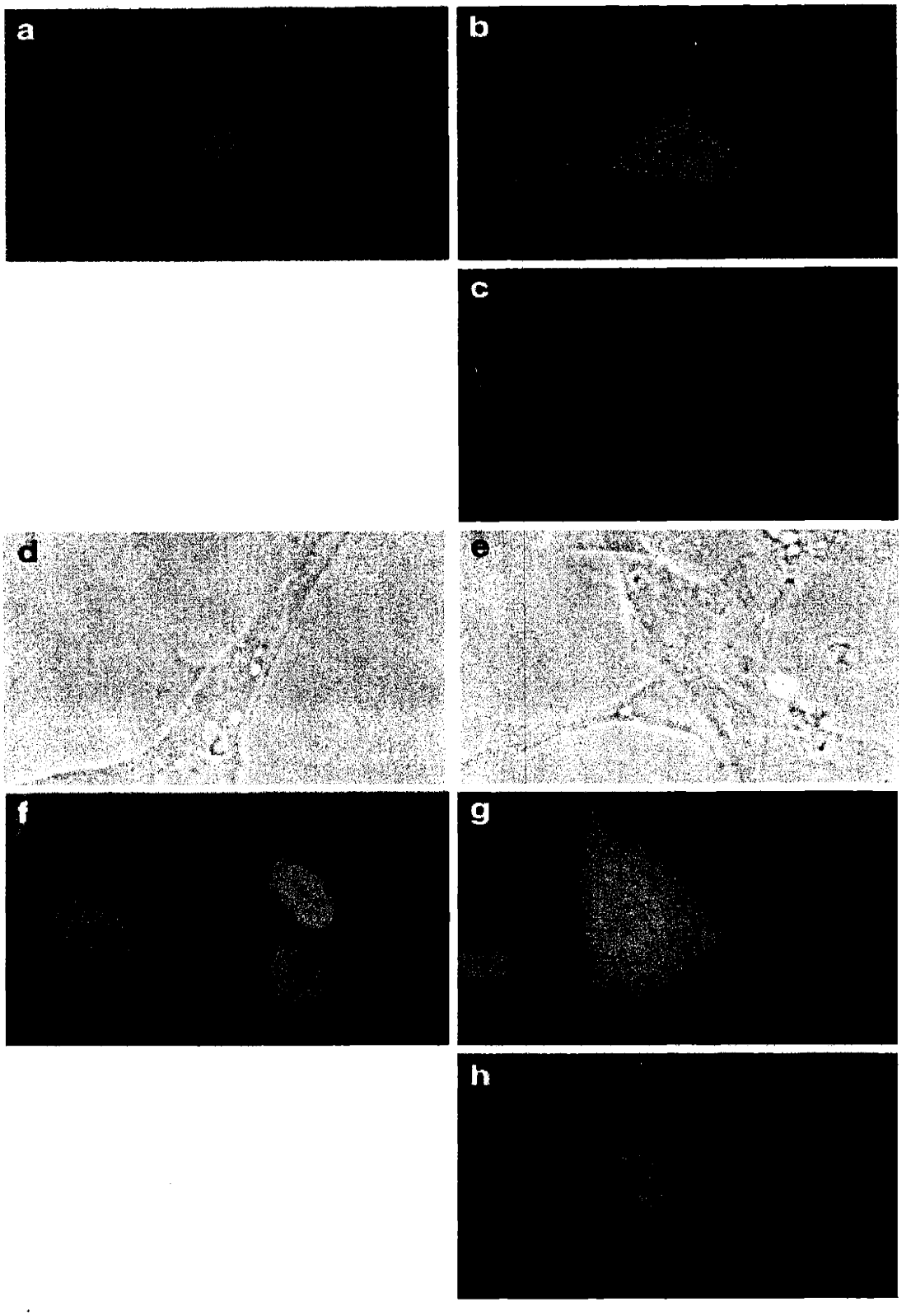
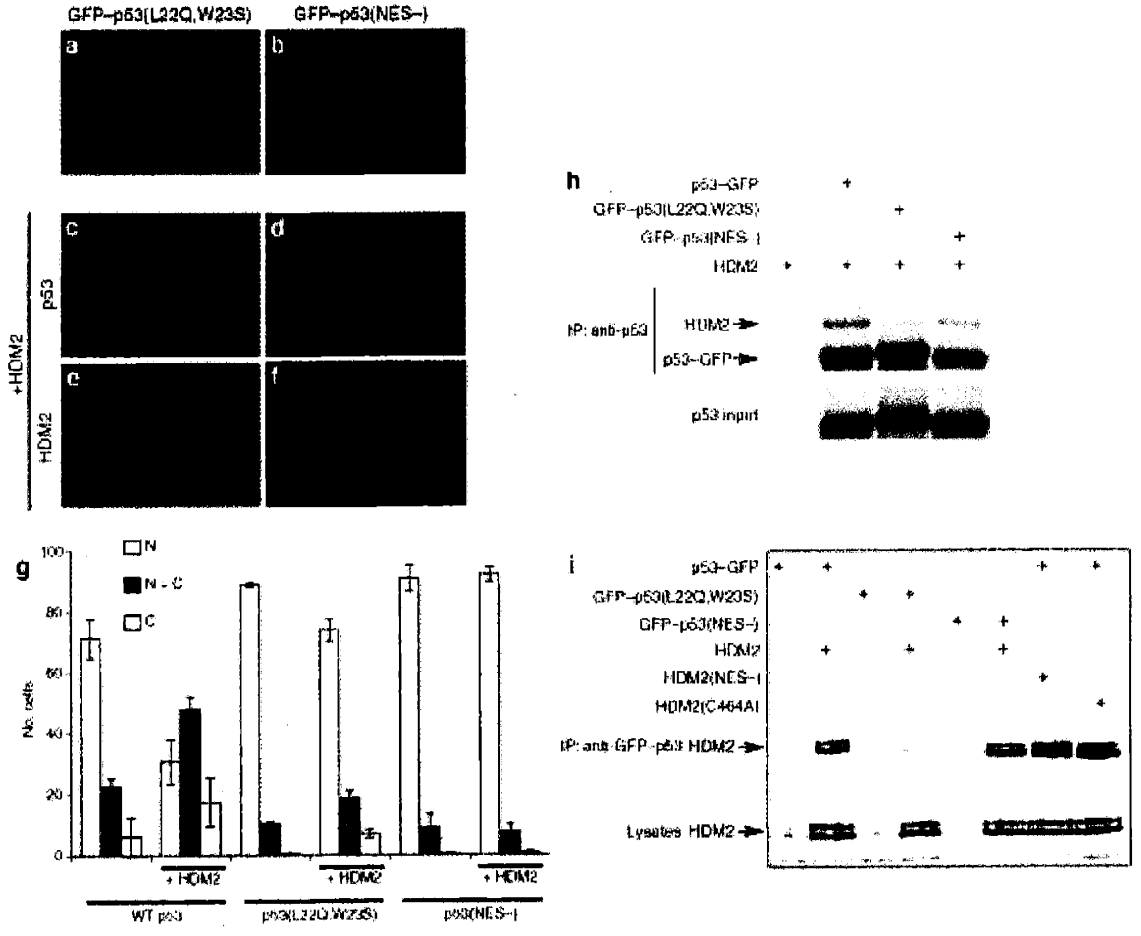


Figure 1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Lysates HDM2

Figure 2

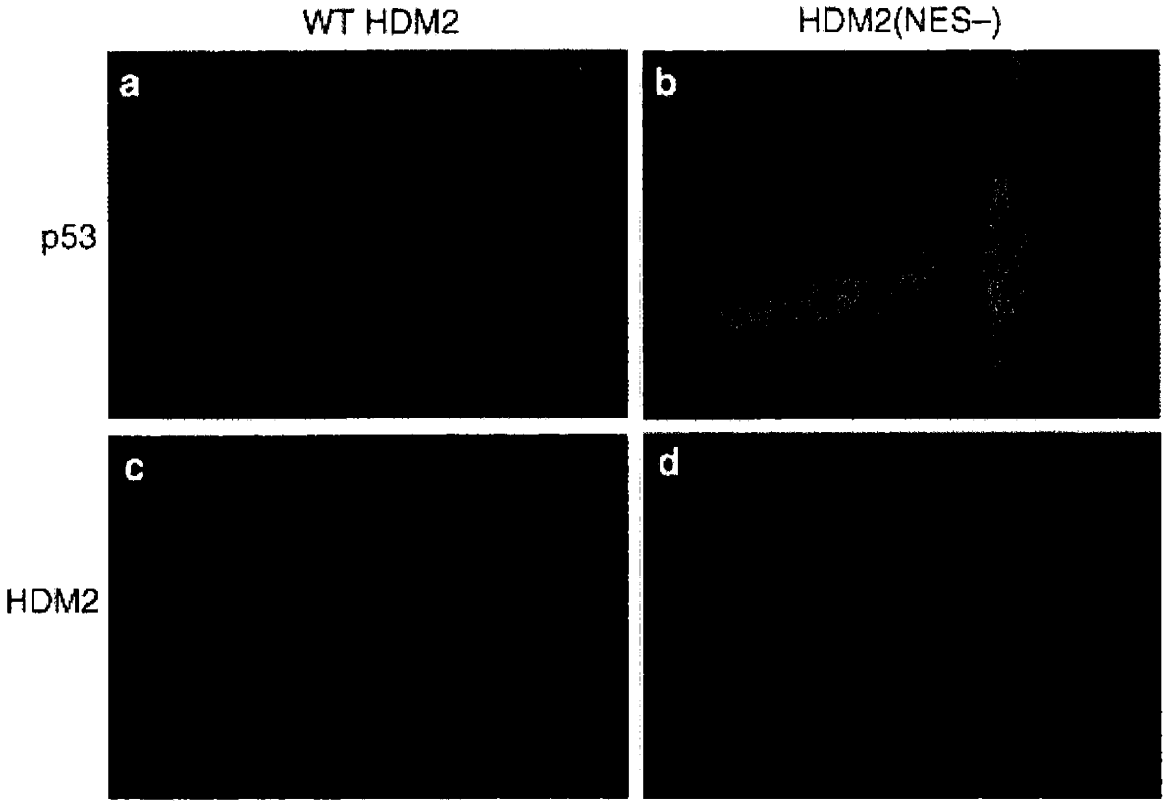

Figure 3

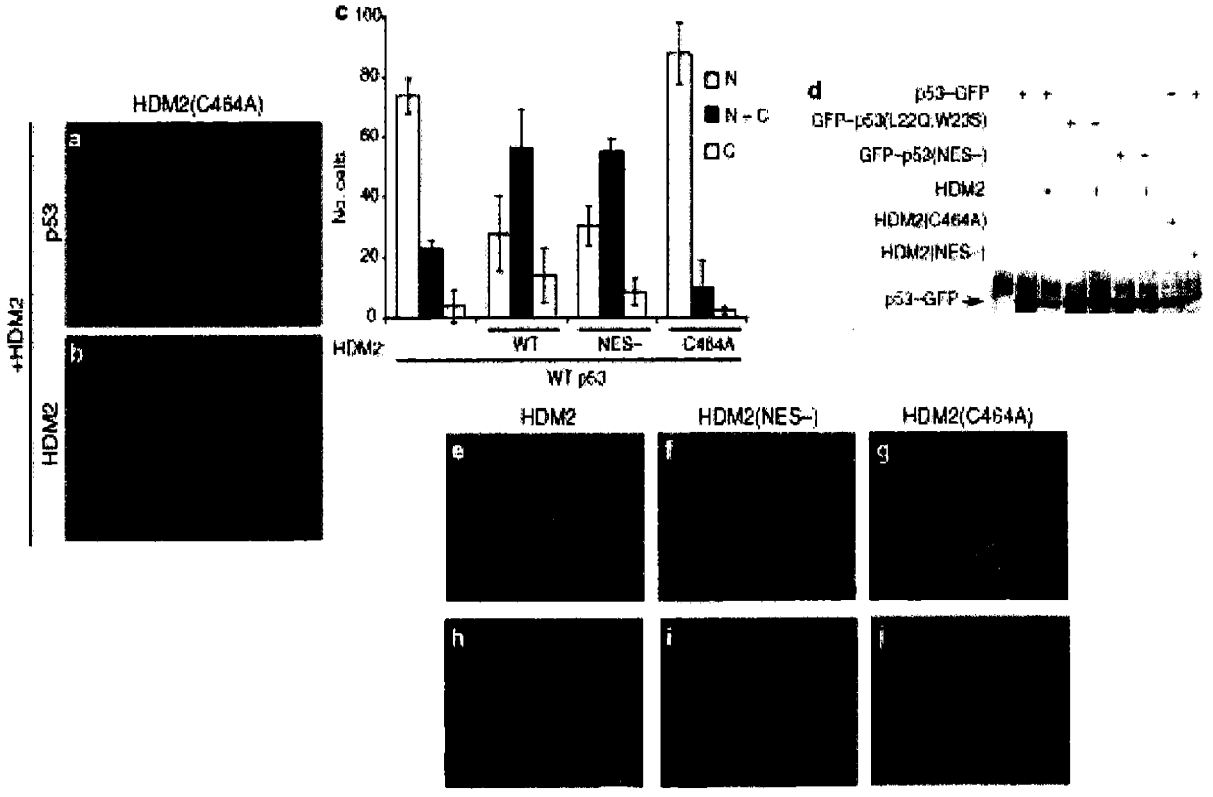
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Figure 4

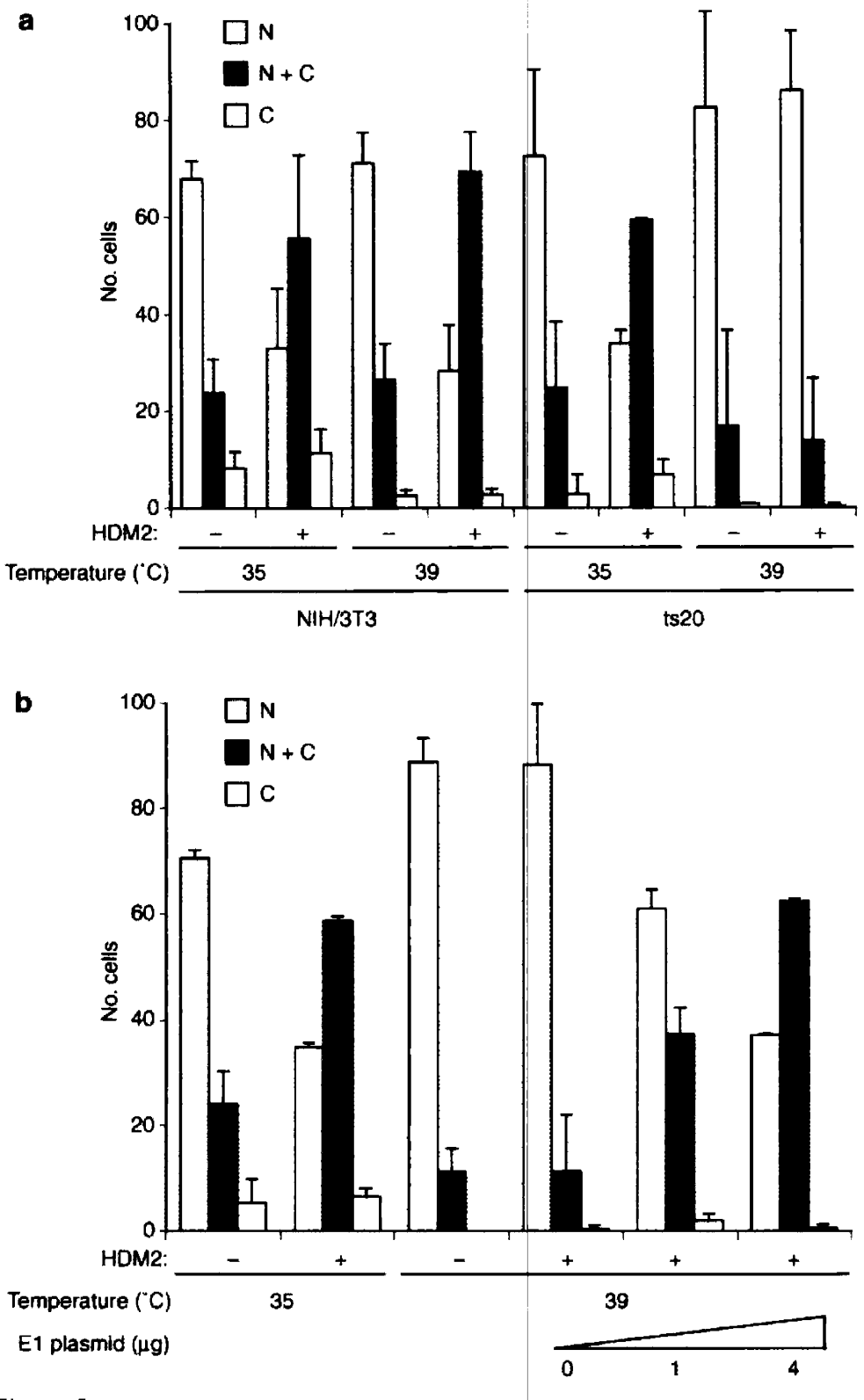
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Figure 5

Figure Legends

Figure 1 Cotransfection of p53GFP and HDM2 in NIH/3T3 fibroblasts and U2OS osteosarcoma cells. Cells were transfected with a plasmid expressing p53GFP alone (*A,D*) or plasmids expressing p53GFP and HDM2 (*B,C,E*). 24 hours later, cells were fixed, stained for HDM2 expression by indirect immunofluorescence using Texas Red-X, and visualized. Cells were examined for patterns of p53GFP fluorescence (*A,B*), Texas Red-X fluorescence (*C*), and morphology by phase contrast (*D,E*). U2OS cells (*F-H*) expressing p53GFP alone (*F*) or both p53GFP (*G*) and HDM2 (*H*) were examined in a similar fashion.

Figure 2 Cotransfection of p53(22,23)GFP and p53NESGFP with HDM2. NIH 3T3 cells were transfected with the indicated p53GFP mutant protein alone (*A,B*), or mutant p53GFP with HDM2 (*C-F*). Transfected cells were stained for HDM2 with Texas Red-X indirect immunofluorescence and visualized. Fluorescence from p53GFP (*A-D*) and Texas Red-X (*E,F*) was observed, and cells were scored as having fluorescence that was stronger in the nucleus (N), equal in nucleus and cytoplasm (N+C), or stronger in the cytoplasm (C). 100-200 cells for each condition in two independent experiments were quantitated, and means and standard deviations were calculated (*G*). (*H*) Binding of HDM2 to mutant p53GFP proteins. ^{35}S-labelled proteins were generated by *in vitro* translation, mixed, and immunoprecipitated with the PAb 421 anti-p53 antibody. 25% of the input p53GFP proteins in the assay are shown for comparison. (I) Co-immunoprecipitation of HDM2 species with wild-type or mutant p53–GFP proteins. Upper panel, lysates from U2OS cells transfected with the indicated constructs were

treated with anti-GFP antibody and protein G beads. Immunoprecipitates were analysed by western blotting using anti-HDM2 antibody. Lower panel, equal HDM2 transfection efficiencies were confirmed by western blotting of lysate samples using anti-HDM2 antibody.

Figure 3 Coexpression of HDM2NES mutant with p53GFP. NIH 3T3 cells were transfected with plasmids expressing wild type p53GFP and either HDM2 (*A, C*) or HDM2NES mutant (*B,D*). Cells were processed as in Figure 1 and fluorescence from p53GFP (*A,B*) and Texas Red-X-stained HDM2 (*C*) or HDM2NES (*D*) was visualized.

Figure 4 Coexpression of HDM2C464A with wild type p53GFP. NIH 3T3 cells transfected with plasmids expressing wild type p53GFP and HDM2C464A (*A,B*) were processed as in Figure 1. Fluorescence from p53GFP (*A*) and Texas Red-X-stained HDMC464A (*B*) was observed. (*C*) 100-200 cells for each condition of two independent experiments using the HDM2 mutants shown in Figures 3 and 4 were quantitated, and means and standard deviations were calculated. (*D*) Changes in levels of p53GFP, p53(22,23)GFP, and p53NESGFP protein in response to coexpression with HDM2, HDM2NES, or HDM2C464A. p53GFP was immunoprecipitated from lysates of U2OS cells 24 hours post-transfection and detected by Western blotting. (*E-J*) Detection of endogenous p53 and transfected wild-type or mutant HDM2 in HCT116 cells. Cells were transfected with wild-type HDM2 (*E,H*), HDM2NES (*F,I*), or HDM2C464A (*G,J*). p53 was visualized with anti-p53 Ab-7 and Rhodamine Red-X-conjugated secondary antibodies (*E,F,G*), while HDM2 was detected with SMP14 anti-Mdm2 followed by

FITC-conjugated secondary antibodies (*H,I,J*); note that images have been false colored in analysis so that p53 is indicated in green and HDM2 in red.

Figure 5 Ability of HDM2 to relocalize p53GFP in *ts20* cells with a temperature sensitive E1 ubiquitin charging enzyme. (*A*) Mutant *ts20* cells or control NIH/3T3 cells were cultured at 35°C and transfected with plasmids encoding p53GFP alone, or p53GFP and HDM2. One set of duplicate transfection cultures was maintained at 35°C for 24 hours post-transfection, while a second set was shifted to a 39°C incubator 6 hours prior to harvesting cells. Cells were processed as in Figure 1, and scored for their pattern of p53GFP fluorescence. 100-200 cells were quantitated for each condition in two independent experiments, and means and standard deviations were calculated. (*B*) Restoration of E1 activity in *ts20* cells at the restrictive temperature by cotransfection of wild-type E1 with p53GFP and HDM2. Experiments were otherwise performed as in (*A*).

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Chapter 3: Survey for New p53 Target Genes in the Mouse, and Analysis of DNA Repair Gene Expression

Table of Contents

Abstract

The transcriptional responses of *Trp53-/-* and *Trp53+/+* primary murine fibroblasts in response to a panel of DNA-damaging treatments were measured with cDNA microarrays. The results of these experiments reveal that the p53-dependent gene expression in untransformed murine cells does not greatly vary with the drug used for treatment, although drugs that act by related mechanisms gave rise to the most similar transcriptional responses. The data presented here also suggest that the inhibition of ribosomal RNA synthesis by cisplatin may be affected by p53 status. A number of novel putative p53 target genes were discovered, most notably, the DNA repair enzyme gene *Ercc5*, which encodes the Xeroderma pigmentosum group G gene product Xpg, a key player in nucleotide excision repair and base excision DNA repair pathways. Follow-up experiments using real-time PCR measurements revealed a second new p53-regulated DNA repair gene, *Polk*, which encodes the translesion DNA polymerase kappa enzyme, a DNA-damage inducible gene whose regulatory response is conserved from bacteria to mammals. Both Xpg and DNA polymerase kappa appear to play a role in either the repair or the error-free bypass of oxidative lesions in DNA. Thus, it appears that p53 regulates a highly conserved inducible set of downstream DNA repair enzymes that may be particularly important for the response to DNA damage by oxidizing species.

Introduction

The p53 tumor suppressor protein appears to be one of the crucial signaling nodes in the mammalian cell's response to DNA damage and other stressful events (Vogelstein et al., 2000). Several different types of cellular responses are known to be coordinated by the upregulation of target gene transcripts in response to p53 binding at their promoters or within intronic sequences. The best known branches of the p53-dependent cellular response are the arrest of the cell cycle at the G1/S boundary by induction of the $p21^{CIP/WAF}$ cyclin-dependent kinase inhibitor, and the induction of programmed cell death in severely damaged cells, most likely via the upregulation of an assembly of small Bcl-2 related proteins such as Bax, Noxa, and Puma, and other pro-apoptotic factors such as the Ced-4 homolog Apaf-1, and the Fas transmembrane receptor. However, it is clear that the p53-dependent transcriptional response is heterogeneous among different cell types in the tissues of mammalian organs, and definitive *in vivo* evidence to support the unique importance of any single p53 target gene for tumor suppression is lacking (Fei et al., 2002). For example, p21-dependent cell-cycle arrest is apparently not absolutely required to prevent most spontaneous tumors from developing, as *p21*-null mice live nearly as long as wild-type mice, and only develop tumors late in life (Martin-Caballero et al., 2001). Further, in a number of chemically-induced carcinogenesis models, p21-deficiency does not dramatically affect the tumor development (Philipp et al., 1999; Weinberg et al., 1997). Similarly, the development of tumors in mice mutant for the pro-apoptotic *Bax* gene would be expected if Bax-dependent apoptosis were a critical event required for p53 tumor suppression activity

(Knudson et al., 2001). The currently known p53 target genes most likely play partially redundant roles to account for p53-dependent suppression of tumor development. Rigorous testing of this hypothesis would require inactivation of the p53-dependent component of the expression of different combinatorial sets of its target genes.

With the advent of highly parallel assay methods in biology, a global approach to measuring the cellular consequences of expression of a particular protein has become possible. Experiments whose goal is the detection of transcriptional targets of p53 have probably detected only a portion of the total number of true target genes; even with the use of new "genomic" technologies like DNA microarrays and proteomic profiling, the transcriptional or proteomic programs of most primary cell types in the body are still well hidden due to the difficulty of obtaining pure samples of such cells in sufficient quantities to perform the necessary experiments. Indeed, many of the p53-related experiments in the current literature making use of DNA microarrays rely on transformed cells of uncertain genotype, and inducible p53-expression constructs whose levels of expression are not guaranteed to be within normal physiological bounds (Wang et al., 2001; Zhao et al., 2000). Therefore, it may be that important p53 transcriptional targets are still to be discovered. Also, the question of whether the same p53 targets are equally important in all the different tissues and cell types of the body is only beginning to be addressed. It would not be surprising, for example, to learn that different p53 target genes played important roles in male meiosis, which involves a coordinated, intricate, and still poorly understood management of a physiological kind of DNA strand breakage, in contrast to the accidental breakage events that p53 is more typically thought to respond to in other cells of the body.

Although the pursuit of biological targets for improved human cancer therapies looms large in the minds of medical researchers studying p53, there are strong arguments to be made for understanding the detailed differences in different mammals' p53 pathways: on the one hand, because non-human mammals are the current best experimental setting for rigorously testing new therapeutic candidates prior to exposing human patients to possible risks, and secondly, because the history of biological investigations has shown that the peculiarities of different organisms can highlight biological properties that are broadly shared and may be of key importance to human disease. A more extreme example of the latter case is the ciliate protist *Tetrahymena* , which, with the 200-300 subchromosomal fragments of its macronucleus, provided an initial insight into the existence of telomeres, specialized DNA sequences at the ends of eukaryotic chromosomes, whose erosion or damage in human cancer cells plays a role in genomic stability and cancer progression (Maser and DePinho, 2002; Prescott, 1994).

For these reasons, a better understanding of the genes regulated by p53 in the mouse is a desirable goal. *Trp53*-mutant and wild-type mice provide optimal reagents for studying the genomic effects of p53 expression in primary cells of otherwise unaltered genetic background. As the cellular activities of p53 are quite complicated, it is essential to control for the effects of known p53 target genes that have potent effects on the state of the cell, for example, the G1/S checkpoint arrest mediated by $p21^{CIP}$, if additional primary effects of p53 are to be uncovered. The experiments presented here reveal that several different genotoxic or chemotherapeutic drug treatments induce an overall similar p53-dependent transcriptional response in murine embryonic fibroblasts, with, however, an interesting cisplatin-specific effect on ribosomal RNA levels.

Among the p53-upregulated genes detected in these microarray experiments are many of the previously identified transcriptional targets of p53, as well as a number of genes that are candidate novel primary targets of p53 transcriptional regulation. Because one of these genes, *Ercc5*, encodes a protein that is a prominent member of the two main pathways of DNA repair in eukaryotic cells, a detailed follow-up study was conducted using real-time PCR to assess the p53-responsiveness of all DNA-repair genes available in the current annotation of the mouse genome. A second p53-responsive gene, *Polk*, encoding the translesion DNA polymerase DNA polymerase kappa, was discovered in this survey, and several previously reported p53 target genes were found to actually be responsive to p21-dependent effects, most likely via changes in the cell cycle status of the cells.

Materials and Methods

Cell Culture

Murine embryonic fibroblasts (MEFs) from wild-type, p53-null, and p21-null mice on the 129S4/SvJae background were isolated from day 13.5 embryos, and were cultured in DME media supplemented with 10% inactivated fetal calf serum, 5mM glutamine, and penicillin and streptomycin (Sigma), in humidified incubators with a 5% CO_2 atmosphere (Brugarolas et al., 1995; Lowe et al., 1993). Cells were used between passages 2 and 7. Four million MEFs were plated per 15 cm tissue culture plate, and treatment of cells commenced within 36 hours of plating.

Drug and irradiation treatment of cells

Gamma-irradiation of cells was carried out in a Gammacell irradiator at a rate of 72 rad/second. UV irradiation was administered with a Stratalinker. Cisplatin, doxorubicin, and vinblastine (Sigma) were dissolved in PBS prior to addition to cells. Untreated cell controls were mock-treated with PBS.

Harvest of RNA

For cultured MEF samples, media was aspirated from cells, and 10 mL of Trizol reagent (Gibco-BRL) was added per 15 cm plate. Cells were scraped into Trizol, and pipetted several times to lyse. The manufacturer's protocol of chloroform extraction, isopropanol precipitation, and 70% ethanol washing was then followed, and RNA was redissolved in RNAse-free water after precipitation. Mouse tissue samples were harvested rapidly, added to Trizol, and homogenized immediately using a Polytron dissociator for 30 seconds on setting 19. After letting homogenate sit at room temperature 5 minutes, the manufacturer's protocol was followed for aqueous extraction and isopropanol precipitation of RNA.

For real-time PCR analysis, RNA was further purified using RNeasy micro spin columns (Qiagen), and on-column RNase-free DNAse digestion was performed to ensure that any contaminating genomic DNA was removed. For microarray hybridizations, RNeasy Midi columns were used to clean up RNA after Trizol purification.

Microarray hybridizations and analysis

Microarrays were fabricated with PCR amplified inserts of the 15,247 clones of the National Institute of Aging's Mouse 15K arrayed library of cDNAs from pre- and periimplantation embryos, E12.5 female gonad/mesonephros, and newborn ovary, using universal primers within the pSPORT1 vector. PCR products were analyzed by agarose gel electrophoresis, purified from residual nucleotides using 96-well plate Montage filter plates (Millipore), rearrayed in 384-well plates, dried down under vacuum, resuspended in 3X saline sodium citrate solution containing 1.5M Betaine (Sigma), and spotted onto poly-L-lysine-coated microscope slides with a robotic split-pin array spotter (Diehl et al., 2001; Schena et al., 1995).

Aminoallyl-labeled cDNA was prepared from 100 μg total RNA according to the ARES labeling kit protocol (Molecular Probes). Coupling of Alexa-555 or Alexa-647 dyes to the aminoallyl cDNA was conducted for 1 hour at room temperature in weak bicarbonate buffer as per the manufacturer's recommendation. Excess dye was removed by Qiaquick columns (Qiagen), and the eluted Alexa-555 and Alexa-647-labeled cDNA populations were combined, mixed with non-specific blocking nucleic acids (2 μg each of yeast RNA and oligo-dT_{18}) briefly heated to 70°C, diluted in 50% formamide hybridization buffer, added to arrays pre-hybridized with the same buffer, and incubated at 42°C for up to 12 hours prior to washing and scanning. Scan data were analyzed with the ArraywoRx software package supplied with the scanner, with exclusion of obvious array defects and spots with poor morphology. Local background subtraction, and global intensity normalization were used in the calculation of spot intensities and ratios.

Unsupervised clustering analysis of fluorescence intensity ratios was carried out with the Cluster program written by Michael Eisen, Lawrence Berkley National Labs. Array spot intensity ratio data were log-transformed, arrays were median-centered, and hierarchical clustering was conducted with the average linkage method. Data were formatted for display with the Treeview program (M. Eisen).

Real-time PCR

5 micrograms of purified total RNA was converted to cDNA in a reaction volume of 100 microliters, using Superscript II enzyme and the commercial protocol. The template RNA was then hydrolyzed with RNase A. cDNA templates for real-time PCR were diluted in sterile water prior to addition to thermocycling plate. Primers and SYBR-green-containing reaction mixture were added, and reactions were carried out for 50 cycles, with automatic gradient denaturation testing at the end of the run to ensure that single products were obtained. Samples were assayed in triplicate in independent reactions.

Primers used for amplifying regions of p53 target genes and DNA repair genes are listed in Table 5. They were designed to generate intron-spanning amplicons of 90-150 bp size, with a Tm of 60°C using the software provided with the ABI instrument.

Results

cDNA microarray analysis of DNA-damaged Trp53 wild-type and mutant MEFs

In initial experiments, unsynchronized low-passage MEF cells were treated with a panel of DNA damaging agents for 6 or 12 hours, and then harvested for RNA isolation. The conditions of treatment and the experimental design for microarray hybridizations are presented in Table 1. A simple pairing of wild-type and *Trp53*-null MEFs for microarray analysis of each treatment condition was used in order to achieve the maximum sensitivity for detecting p53-induced or inhibited genes (Smyth et al., 2003). This experimental strategy has the limitation that the overall patterns of p53-independent gene alterations are not obtainable from the data set, as would be possible if samples were hybridized to a common reference sample; however, the experimental design is optimal for detecting p53-dependent changes, and will screen out other changes that could obscure these effects in cells (Kai and Wang, 2003). The drugs chosen for these experiments interact with cellular components in somewhat different ways: doxorubicin is chiefly a DNA intercalating compound, but is thought to interfere with Topoisomerase II activity in replication; cisplatin generates DNA strand cross-links, etoposide inhibits Topoisomerase II directly, and vinblastine is known to disrupt the mitotic spindle by interfering with tubulin polymerization, although it is possible that any of these drugs may also affect cells in currently unknown manners (Bose, 2002; Hortobagyi, 1997; Nitiss, 2002; Zhou and Rahmani, 1992). The doses of drugs used in these experiments do not cause significant levels of apoptosis in MEF cells by 12 hours (data not shown).

Analysis of the RNA transcript levels in the panel of cells was carried out by comparative cDNA hybridization on microarrays, followed by log-transforming, median-centering ratio data within

each array. Filtering of the data set to exclude genes that were not measured in at least 80% of samples, and genes that did not show at least a 1.5-fold up- or downregulation in one sample, was followed by unsupervised hierarchical clustering of the filtered data using the average linkage clustering algorithm (Eisen et al., 1998). The results of the clustering of the MEF samples, based on their p53-dependent gene expression differences are shown in Figure 1.

Several observations can be made from the hierarchical tree clustering of the MEF samples in Figure 1. The samples treated with doxorubicin cluster together, independent of the time-point of treatment, and next closest to them in the tree are the etoposide-treated samples. Although these two drugs interact with different components of the cell, their mechanisms of action are thought to converge on the disruption of normal topoisomerase II function. It may be that this common mechanistic element is the underlying reason for the close clustering of these treated samples. The ability to identify common mechanistic pathways of drug action by examining global mRNA expression profiles has in fact been one of the most exciting prospects to arise from this new technology (Ulrich and Friend, 2002). Of course, one may need to assess the cellular effects of a drug over a range of concentrations in order to be certain that apparent differences in drug action are not merely differences in delivery of active drug to the biological site of action, or differences of affinity of interaction with a common cellular target.

Two of the three cisplatin-treated samples cluster next to the doxorubicin and etoposide-treated samples, and then next further away are the untreated sample, followed by the remaining cisplatin sample and the vinblastine sample.

Close examination of the clustered data, however, indicates that most of the distinguishing features of the expression profiles for MEFs treated with different drugs, as well as the differences between the drug-treated DNA-damaged cells and the untreated cells, are mainly differences of degree rather than entirely new gene expression patterns. This is most obvious with the rows for the *cyclin G* gene, which form the bright, almost uniform band across the center of the upregulated gene cluster. Almost all of the genes that are strongly upregulated in the drug-treated samples are at least modestly upregulated in the untreated cells (Figures 1 and 2). This observation is in keeping with measurements of the previously known p53 target genes made with more sensitive real-time PCR, or Northern blotting methods. (For example, see (Attardi et al., 2000)). The fact that unstressed cells show some p53-dependent gene expression may be a reflection of the tissue culture environment, where cells are exposed to higher oxygen levels, artificial growth stimuli, and a lower cellular density, compared to the tissue environment where they would ordinarily live (Karanjawala et al., 2002).

Cisplatin treatment of MEFs may induce p53-dependent alterations in ribosomal RNA

Of the upregulated genes shown in Figures 1 and 2, one patch appears to be the main distinguishing feature of 2 of the 3 examples of cells treated with cisplatin. This cluster of genes is presented in Figure 3. This gene grouping is mostly composed of different ribosomal RNA sequences. Oligo-dT priming used for generating cDNA in a reverse transcriptase reaction can also generate substantial amounts of rDNA by priming at adenine-rich regions of the different rRNA transcripts (Gonzalez and Sylvester, 1997). Non-specific hybridization to the microarrays is supposed to be prevented by the use of blocking oligo dT and yeast RNA; however, if there

were differences in the levels of an endogenous rRNA levels between samples, it is conceivable that one could still see a differential fluorescent signal. Some recent studies into the cellular activities of cisplatin add interest to this expression result. Several different groups of researchers have found that cisplatin treatment preferentially inhibits the synthesis of rRNA even more rapidly than it affects DNA synthesis, and that this drug brings about a fairly rapid mislocalization of key rRNA transcription complex components, including the upstream binding factor UBF, TBP, RNA polymerase I, and TAFI (Jordan and Carmo-Fonseca, 1998). Furthermore, when primary neurons are treated with cisplatin, their nucleoli undergo dose- and time-dependent shrinkage that only occurs in the presence of the clinically-effective cisplatin isomer (McKeage et al., 2001).

Further studies will be required to resolve the point, particularly given the fact that the third cisplatin-treated sample, which is admittedly an outlier that clustered furthest away from all the other samples, did not show this effect, but it will be very intriguing if, as the microarray data presented here seem to hint, *Trp53-/-* cells are particularly vulnerable to the action of cisplatin on ribosomal RNA synthesis or nucleolar function. Such a mechanism might then suggest possible strategies for clinical treatment of p53-mutant cancers. For example, one could attempt the combination of cisplatin with other agents that attack the cell's rRNA machinery. In any case, this result is precisely the kind of unexpected finding that can result from the empirical measurement of many cellular components at once and demonstrates the potential for these new assay methods to give rise to interesting hypotheses that can then be further tested.

Known targets of p53 are upregulated in DNA-damaged MEFs

Reassuringly, the array data show many well characterized p53-inducible genes to be expressed at higher levels in the wild-type cells compared to the mutants. As the data in Table 2 show, most of the well-known p53 target genes are represented on the array, either as positive control cDNA spots added to the NIA Mouse 15K clone set, or else within the clone set itself. Multiple spots representing the same gene are presented to give an indication of the spot-to-spot variability in expression ratio values.

Naturally, the cDNA for p53 shows a positive signal in these experiments, as the mutant locus represents a substantial deletion of the gene (Jacks et al., 1994). The *cyclin G* gene is the most easily detectable overexpressed known p53 target gene on these arrays, but the several *Mdm2* spots also are readily observed. The *p21* gene's p53-dependent expression is seemingly under-represented by the single spot on these arrays, given the many-fold increases in its expression typically detected by Northern blotting or real-time PCR under conditions similar to those here. This observation merely underlines the fact that cDNA arrays, made up of DNA clones whose performance in binding their complementary strands has never been tested, are not capable of measuring absolute ratios of cDNA population members and will likely exhibit a spot-to-spot variation in the dynamic range of the measured fluorescence ratios.

In these experiments, *Noxa* and *Cathepsin D* were the only pro-apoptotic genes found to be induced. This is not terribly surprising, as the pro-apoptotic p53 target genes are typically less

inducible than, for example, *p21* and *Mdm2*, and under the conditions of treatment in these experiments, little apoptosis occurs.

The other known p53 target genes found in these experiments include some members of the oxidation-reduction control group, matrix metalloproteinases, the putative cell cycle control gene Btg2, and a number of previously-reported genes whose functions are currently unknown. Another strongly overexpressed gene that has received little attention thus far is the *Tel* oncogene, which is frequently found as a fusion partner in leukemias.

Potentially novel p53 target genes upregulated in response to DNA damage

Perhaps the most interesting new possible p53 target gene to appear in these experiments is the DNA repair gene, *Ercc5*, encoding the Xpg protein which is mutated in a rare sub-group of xeroderma pigmentosum (XP) patients that can either show the typical XP syndrome of sun-sensitivity and cancer development, or else much more severe, rapidly degenerative neurological symptoms, depending on how severely ablated the *ERCC5* gene is in the individual. In recent years, careful characterization of this protein has demonstrated that in addition to being the required 3' endonuclease involved in nucleotide excision repair (NER), Xpg is part of the cellular machinery of base excision repair (BER) specialized to deal with oxidative lesions such as 8-oxodG, or thymine glycol at the DNA. Notably, the BER functions of XPG do not require an active endonuclease site in the protein, indicating that the BER activities of XPG are likely to be carried out by binding to and cooperating with other proteins. XPG has received new attention in recent years because of its demonstrated role in the transcription-coupled repair of oxidative

lesions in the transcribed DNA strand, a process involving the products of the *CSA* and *CSB* genes whose function is impaired in patients with the debilitating DNA repair disorder Cockayne syndrome (Cooper et al., 1997). Chapter 4 describes further experiments to characterize p53 regulation of the *Ercc5* gene.

Other putative novel p53 target genes to note in this experiment are the redox-regulatory genes GsttL, and Gss, both involved in the glutathione pathway which regulates the level of intracellular oxidative species. These observations are in keeping with the early Vogelstein lab study that revealed a role for p53 in control of the redox state of the cell (Polyak et al., 1997). TEL, a transcription factor implicated in several chromosomal translocation breakpoints, is another gene clearly upregulated under the experimental conditions (Odero et al., 2002).

Another gene of possible interest in the *Fam*, or fat facets homolog; this gene behaves as an ubiquitin C-terminal lyase *in vitro*, and could potentially have a role to play in regulating the ubiquitination state of p53, Mdm2, or other cellular proteins in the p53 pathway. One such gene, *HAUSP*, has already been identified and appears to have some importance in regulating p53 levels (Li et al., 2002).

Also noticeable in the upregulated dataset are several genes containing the LIM protein interaction domain. Other proteins possessing these domains, for example the Fhl protein, appear to have functions in cytoskeletal regulation and possible coactivation of transcription (Morlon and Sassone-Corsi, 2003).

There are also hints in the upregulated gene expression profile of a known developmental program that some murine fibroblast lines can undertake if stressed in tissue culture, namely, adipogenesis (Tang et al., 2003). The upregulation of ADRP, an adipose differentiation-related protein, as well as the milk fat globule-EGF factor 8 protein (Mfge8), may indicate that MEFs stressed in culture under the conditions of these experiments activate part of the same cellular program that becomes active when cells have been starved in culture.

A larger than expected number of the p53-upregulated genes appear to be members of the secretory pathways in the cell; the meaning of this is unclear. One biological phenomenon that might provide an ingress for examination of this aspect of the DNA damaged cells' transcriptional phenotype is the bystander effect reported in populations of cells where some cells are irradiated, and other nearby cells are damaged via unknown mechanisms (Nagasawa et al., 2003). Soluble factors from the irradiated cells have been suspected in these circumstances. Perhaps an upregulated secretory pathway can contribute to this effect.

In addition to these genes and the others listed by name in Table 3, a number of so far unidentified EST clones showed p53-dependent upregulation in these experiments. Further characterization of these clones in the array, now that both human and mouse genome sequence projects are approaching finished status, may shed new light on further candidate p53 transcriptional targets.

p53-downregulated genes are chiefly cell-cycle regulated genes

A survey of the genes listed in Table 4 clearly shows the predominance of genes for cell cycle components, particularly genes from S-phase and G2/M, as well as the Chk1 checkpoint kinase homolog. Given the tendency for wild-type MEFs to arrest in G1/S in a p53-dependent manner when subjected to genotoxic stress, these findings are predictable.

A few down-regulated transcripts do raise interesting questions. For example, given the data in Chapter 2 of this thesis, it is intriguing that the mouse *Xpo1* homolog of the yeast *crm1* nuclear export adaptor should be down-regulated by DNA-damaging stimuli in a p53-dependent manner. However, there are reports of cell-cycle regulation of the *Xpo1* transcript, with peak expression G2/M, so again, the cell cycle explanation may account for the observations (Kudo et al., 1997).

Real-time PCR analysis of reported p53 target genes and DNA repair genes: p53 vs. p21 dependence of expression changes

The microarray experiments described above showed p53-dependent upregulation of *Ercc5* in DNA-damaged MEFs. The literature on the topic of p53 and DNA repair is full of seemingly contradictory reports and a lack of consensus about p53's importance in regulating DNA repair efficiency, and the mechanism of its action. This situation suggested the need for a detailed follow-up study examining DNA repair protein transcript regulation in *Trp53* null and wild-type cells. A clean genetic system where the gene expression profile contributions of the p53-inducible cell cycle regulator $p21^{CIP}$ could be separated from other potentially direct transactivation regulation of genes by p53 was selected. A second important consideration in

these experiments was that DNA microarrays are less sensitive than other methods, such as real-time PCR (RTPCR), and would be likely to miss transcripts expressed at very low levels, as is the case for many DNA repair genes. A third reason for undertaking these experiments was to shed light on the degree of evolutionary conservation of the inducible regulation of DNA repair proteins. Relatively recent findings in the p53 literature, for example, the regulation of 53R2 small inducible ribonucleoside reductase by p53, are at least suggestive of the hypothesis that p53 in mammals may play the role of a global coordinator of a subset of DNA repair or DNA damage response cellular programs, similar to the SOS response in bacteria or the inducible expression of some DNA repair genes in the budding yeast (Xue et al., 2003).

The current state of annotation of the murine genome makes it straightforward to design intron-spanning PCR amplicons to test the expressionon of a subset of genes like the DNA repair genes, using the highly sensitive and reproducible real-time PCR (RTPCR) method. Primers were designed for those genes for which intron and exon information was available, along with a number of positive control known p53 target genes, and a set of unconfirmed p53 target genes. In order to efficiently survey the relatively large number of genes involved in this experiment, and making use of the insights gained from the microarray experiments which indicated that most p53-responsive genes are not specific to a particular drug or modality of damage, but rather are differentially expressed to some degree even in untreated cells in culture, simplified sample conditions were adopted. Wild-type, *Trp53-/-* and $p21^{CIP}$*-/-* MEFs were cultured and treated with 10 Gy of gamma irradiation, and RNA was harvested from them at 6h post-treatment. Reverse transcription and RTPCR analysis of the product gave the results in Figures 5 to 8.

The question of the p21-dependence of changes in the expression of some putative p53 target genes was approached in the experiments presented in Figures 5 through 7. As can be seen, a number of these are either highly variable in expression, or were affected as much by p21-deficiency as they were by p53-deficiency, indicating that they are likely to be responding to a secondary or tertiary consequence of p53 expression, rather than being true direct targets of p53.

The nearly global survey of DNA repair transcripts in the mouse genome also gave some illuminating results. The great majority of these genes did not appear to be responsive to p53 in gamma-irradiated fibroblasts, or else were expressed at very low levels, causing the error in the data to be large relative to the magnitude of the changes observed.

In contrast to reports in the literature, based largely on studies in human cells, the *Xpc* gene did not appear to be p53-inducible under these conditions. Species-specific differences in repair gene expression have been described before, for example, the mouse-specific methylation of the promoter of the *Ddb2* gene that encodes the p48 component of XPE apparently prevents it from responding to p53 as it does in human cells (Hanawalt, 2001).

In contrast, a few genes did appear to be responsive to p53, and not merely because of p21-mediated effects. One, O^6-methyl guanine-DNA-methyltransferase, (Figure 6) has been known for several years, and is a candidate gene for medically-relevant phenotypes such as drug resistance in tumors (Middleton and Margison, 2003).

Most excitingly, a novel putative p53 target gene, DNA polymerase kappa (*Polk*, indicated as "kappa" in Figure 7C) was revealed in these experiments. Figure 8 shows the p53-responsive induction of this gene observed after treating MEFs with a panel of genotoxic treatments. The DNA polymerase kappa gene (*Polk*) encodes one of the "translesion polymerase" enzymes that are able to extend the polymerization of DNA across regions distorted by the presence of adducts on the opposite strand. These polymerases are the basis for irradiation-induced mutagenesis in bacteria; for example, the homolog of *Polk*, *DinB*, tends to generate small deletions at the site of the bypassed lesion (Wang, 2001). Particularly for single-celled organisms, it is thought that there may be an important survival trade-off between freeing an arrested replication complex, and experiencing a briefly increased mutagenesis rate, which may also be helpful for the survival of a population of organisms living under stressful conditions. *DinB* and *Polk* appear to be particularly suited to placing the correct base (A) opposite a thymine that has been chemically damaged by an oxidizing agent to give thymine glycol. Thus, the finding that this gene is a putative p53-regulated gene suggests further investigation of the role of p53 in the response to oxidative stress.

Conclusions

Microarray experiments

The microarray data presented here are of sufficient quality, and are measurements of a sufficiently clean genetic experimental system, to shed new light on several aspects of the p53-dependent responses of primary murine cells to genotoxic stimuli.

The overall conclusion is that in these cells, p53 regulates a common set of target genes that are not particularly specific to the identity or mechanism of action of the drug used. Indeed, even in untreated cells, a detectable small upregulation of most of the genes that are strongly induced in response to genotoxic damage is observed. Therefore, if there are p53 target genes in the genome that are specifically responsive to mitotic spindle-damaging agents, for example, they are likely to be few in number, or may require experimental conditions beyond those tested here, to be revealed. Naturally, the cellular response to any pharmacological agent is expected to follow a time-course and characteristics that will be partially specified by the details of the interaction of the drug with the cell: how quickly it can access the cellular target structures, whether it is active initially or needs to be metabolized by the cell, its stability, and its affinity and specificity for the target structures. Thus, it is difficult to find a single set of conditions for fairly testing diverse drugs in controlled experiments, without doing extensive dose- and time-course tests for each substance evaluated. The experiments here are a first approach to evaluating the genomic p53-dependent responses of primary fibroblasts. The conditions used here should favor the detection of novel primary p53-regulated target genes, since relatively early time-points were measured. This approach has the disadvantage that such time-points might not be optimal for detecting the overall effects of the spindle poison drug vinblastine, which might be expected to require a time-course on the scale of the full cell cycle to cause its effects in all cells treated.

The most noticeable drug-specific effect in these experiments was the effect of cisplatin to cause *Trp53* wild-type cells to contain higher detectable levels of some rRNA transcripts than *Trp53* null cells. Although these data should be treated with caution before they are confirmed in

further experiments, this finding, which initially evoked skepticism in the investigator, turned out to have a plausible link to newly reported biological activities of cisplatin specifically targeting the rRNA synthesis pathways (Jordan and Carmo-Fonseca, 1998; McKeage et al., 2001). If it turns out to be the case that p53-mutant cells are vulnerable to cisplatin treatment for this mechanistic reason, it will be an interesting example of an analogy between cancer chemotherapy and the many antibacterial drugs that target differences in prokaryotic and eukaryotic ribosomes. Another avenue that would merit further exploration if these findings are validated would be any connections between the functions of the known p53 upstream regulator protein, $p14^{ARF}$, which is associated with the nucleolus of the cell, and has recently been shown to be able to alter ribosomal RNA synthesis pathways (Sugimoto et al., 2003).

p53-downregulated genes

Consistent with the known effects of p53 in stopping the cell cycle at the G1/S and G2/M boundaries under appropriate conditions, the great majority of the genes found to be expressed at lower levels in the *Trp53-/-* cells in these experiments were cell-cycle associated genes involved in control of the cycle, replication, and other essential processes for coordinated cell division (Table 4). This is no surprise, for many of these genes have long been documented to be transcriptionally regulated in eukaryotes (Spellman et al., 1998). As convincing data for a direct role of p53 in repressing gene expression are scant in the huge literature about this gene, most of the expression changes observed here are likely to be secondary, tertiary, and further removed effects from the primary regulation of genes in the genome by p53.

p53-induced genes

The previously known p53-induced genes listed in Table 2 show that the array experiments were sensitive enough to detect a variety of confirmed p53-regulated genes, and that most duplicate spots for a given target gene measured similar levels of induction. It is also apparent in surveying this list that the p53-induced genes whose function is still unknown outnumber the genes of known function. This observation, coupled with the history of the study of even the best understood p53 target genes like $p21^{CIP}$, suggests that there are likely to be many surprises ahead in the unraveling of the details of actual p53 functions in the cells of mice and humans. For example, the simple paradigm of p21 as a cyclin-dependent kinase inhibitor has had to be revised, following studies that showed that in fact it also functions as an essential stabilizer of cyclin-D associated CDK complexes (Sherr and Roberts, 1999).

Surveying the list of confirmed upregulated p53 target genes, the previously reported role of p53 in pro-apoptotic, DNA repair, redox-control, other stress response, and some extracellular functional pathways is confirmed. When these data are combined with the putative novel upregulated genes in Table 3, some additional experimental hypotheses can be proposed.

The upregulation of microsomal epoxide hydrolase, as well as a glutathione S-transferase homolog and glutathione synthetase, suggests that part of the p53-dependent response may be explicitly geared toward responding to xenobiotic compounds, which would comprise most of the genotoxic substances encountered by animals evolving in a pre-technological age. These

known drug-metabolizing enzymes are thought to play important roles in the effectiveness of a variety of different drug treatments in outbred human populations, and it would be consistent with p53's known role in responding to the consequences of genotoxic damage, if it were also able to assist the cell in metabolizing and breaking down compounds that can damage the DNA (Orphanides, 2003).

Another notable difference in the gene expression profiles of cells differing in their p53 status was the p53-dependent increased expression of genes of the protein secretory pathways in the cell. Other examples of differentially expressed genes fall into many different functional areas of cell biology, including some protein kinases, and a tyrosine phosphatase, as well as a number of sequences whose functions are totally unknown at present.

Perhaps the most notable gene that was found to be induced in a p53-dependent manner in these experiments was *Ercc5*, which encodes the xeroderma pigmentosum disease gene product Xpg, a key member of the nucleotide excision repair complex, and a newly-recognized mediator of base excision repair of oxidative damage to the DNA. Further experiments and discussion dealing with Xpg are presented in Chapter 4.

The finding that the DNA polymerase kappa gene *Polk* is a new p53-responsive target in the cell is also striking, for several reasons. Although eukaryotic cells and prokaryotic cells have many differences in their DNA metabolism and genomic regulation strategies, it is becoming apparent that there is recognizable conservation of at least some of the components of the DNA repair

network in the cell that are transcriptionally induced in response to genotoxic damage. Surprisingly, p53 appears to have become responsible in eukaryotic evolution for the induction of a number of DNA repair genes whose homologs in bacteria are not all controlled by a single regulator. For example, the p53-regulated *Mgmt* demethylation direct repair enzyme gene is the homolog of the *E. coli* gene *ada+* gene. The Ada protein in *E. coli* is trancriptionally induced in response to methylating agents, and the gene product actually has two functions, one as a transcription factor that regulates its own gene upon activation by being methylated, and the second function is as a direct repair enzyme for alkylated DNA. The induction of Ada is responsible for the adaptive response that bacteria exhibit if they are pre-treated with an alkylating agent (Friedberg et al., 1995). p53 now appears to be responsible for a comparable induction of the homologous enzyme in mammalian cells.

Similarly, the well-studied SOS response to DNA damage in bacteria involves the inactivation of the LexA repressor protein by proteolytic cleavage mediated by activated RecA protease. One of the more than 20 genes released from repression by this mechanism is *DinB*. As a side-note, analysis of the genome of the most highly-DNA-damage-resistant organism so far observed, the bacterium *Deinococcus radiodurans* reveals that the most prominent expanded gene family is the *DinB*-related polymerase group, consistent with an important role for this family in surviving DNA damaging treatment (Friedberg et al., 1995).

The broader biological conservation of the induction of Pol kappa in response to genotoxic stress is highlighted by recent results in yeast, where it was found that the yeast *DinB* homolog is also a DNA-damage induced gene, whose activation depends on intact checkpoint pathways including

the Cds1 kinase that is homologous to the CHK2 kinase implicated in p53 activation (Kai and Wang, 2003). Taken together with the observations that the *Ercc5* gene homolog, *Rad2*, and the p53R2 ribonucleotide reductase gene homolog *RNR2* are also DNA-damage-inducible genes in the yeast, these findings sketch out a remarkable conservation of DNA-damage inducible genes whose control has become dependent on p53 in mammals.

The functional importance of Pol kappa in mammalian cells is highlighted by some initial results from mouse knockout studies. *Polk*-null ES cells are extremely sensitive to the mutagen benzo-*a*-pyrene compared to wild-type cells; however, they are only somewhat sensitive to UV and X-irradiation (Ogi et al., 2002). Better understanding of the specificity of the enzyme is beginning to arise from *in vitro* studies as well. It appears that Pol kappa is particularly well-suited to placing an adenine base opposite the oxidative DNA thymine glycol type of lesion, so that it could be responsible for error-free replication of this sort of common DNA adduct (Fischhaber et al., 2002). On undamaged DNA, however, the enzyme shows poor fidelity, which may account for the observation that its homolog in bacteria has been known for years to be responsible for a pathway of error-prone bypass replication (Friedberg et al., 2002). This link to the repair of oxidatively damaged DNA is particularly interesting in light of the discovery of p53-dependent regulation of *Ercc5* in the same data set. A theme of the p53-coordinated response to oxidative DNA damage is suggested by these data. Such a response would make eminent sense if, as has been suggested, damage to the DNA by endogenous production of oxidizing chemical species, for example, in mitochondrial metabolism, is one of the most common harmful events in the lifetime of the cell (Tsutakawa and Cooper, 2000). Impaired p53 responses to oxidative DNA damage could help to account for some currently mysterious aspects of the *Trp53-/-* mouse

phenotype, for example, the decreased fertility of null male mice. In humans, male-factor infertility has been clearly linked to decreased resistance to oxidative stress in the developing germ cells (Agarwal et al., 2003; Sikka, 2001). This is in contrast to the overproliferative phenotype followed by atrophy which occurs in the male germ cells of mice mutant for Apaf-1 or Bax (Honarpour et al., 2000; Russell et al., 2002).

Other DNA damage repair transcripts

The remainder of the RTPCR survey of p53-dependence of DNA repair gene transcripts showed that most of these genes are apparently not subjected to p53 control, at least within the error of the measurements in this experiment.

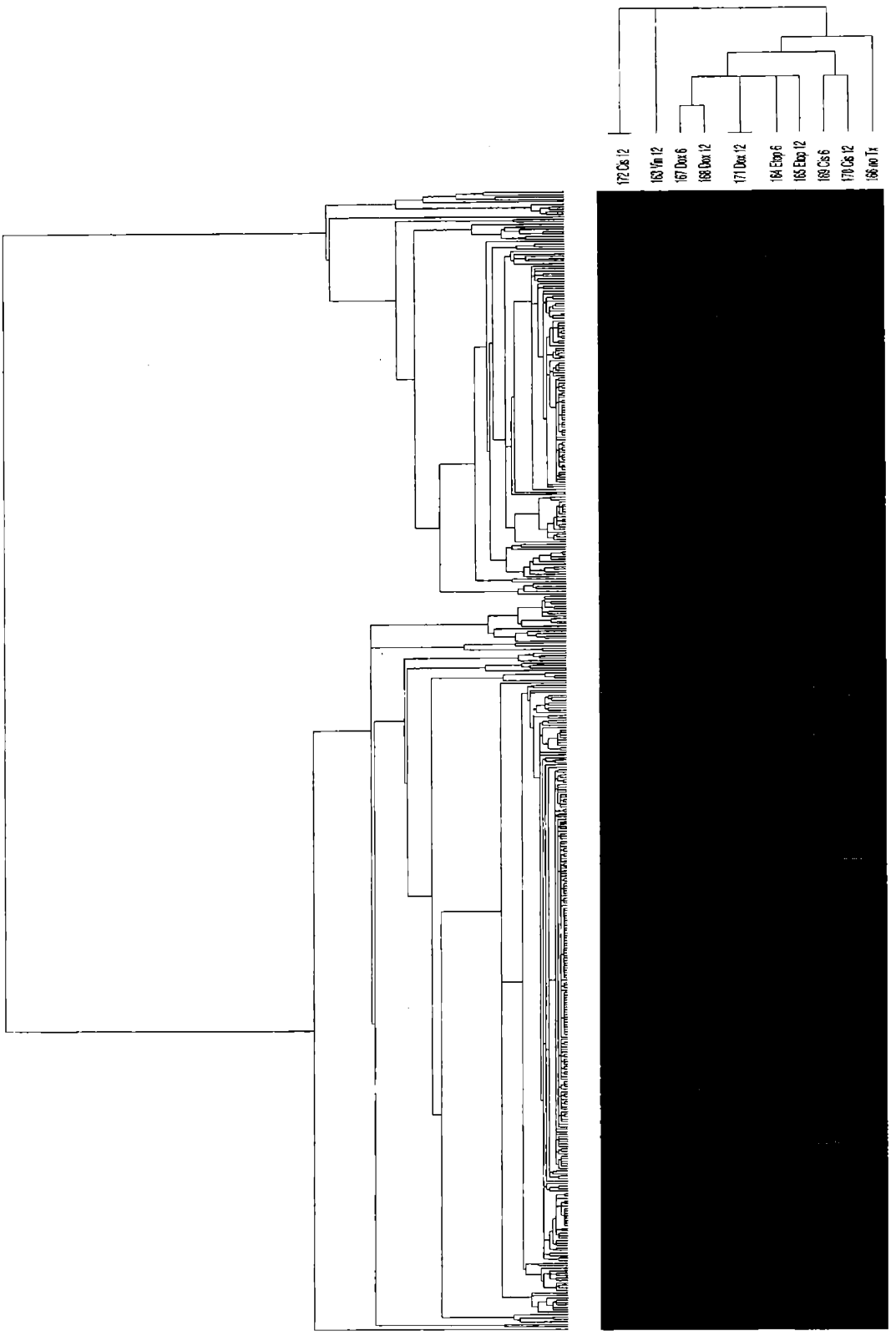
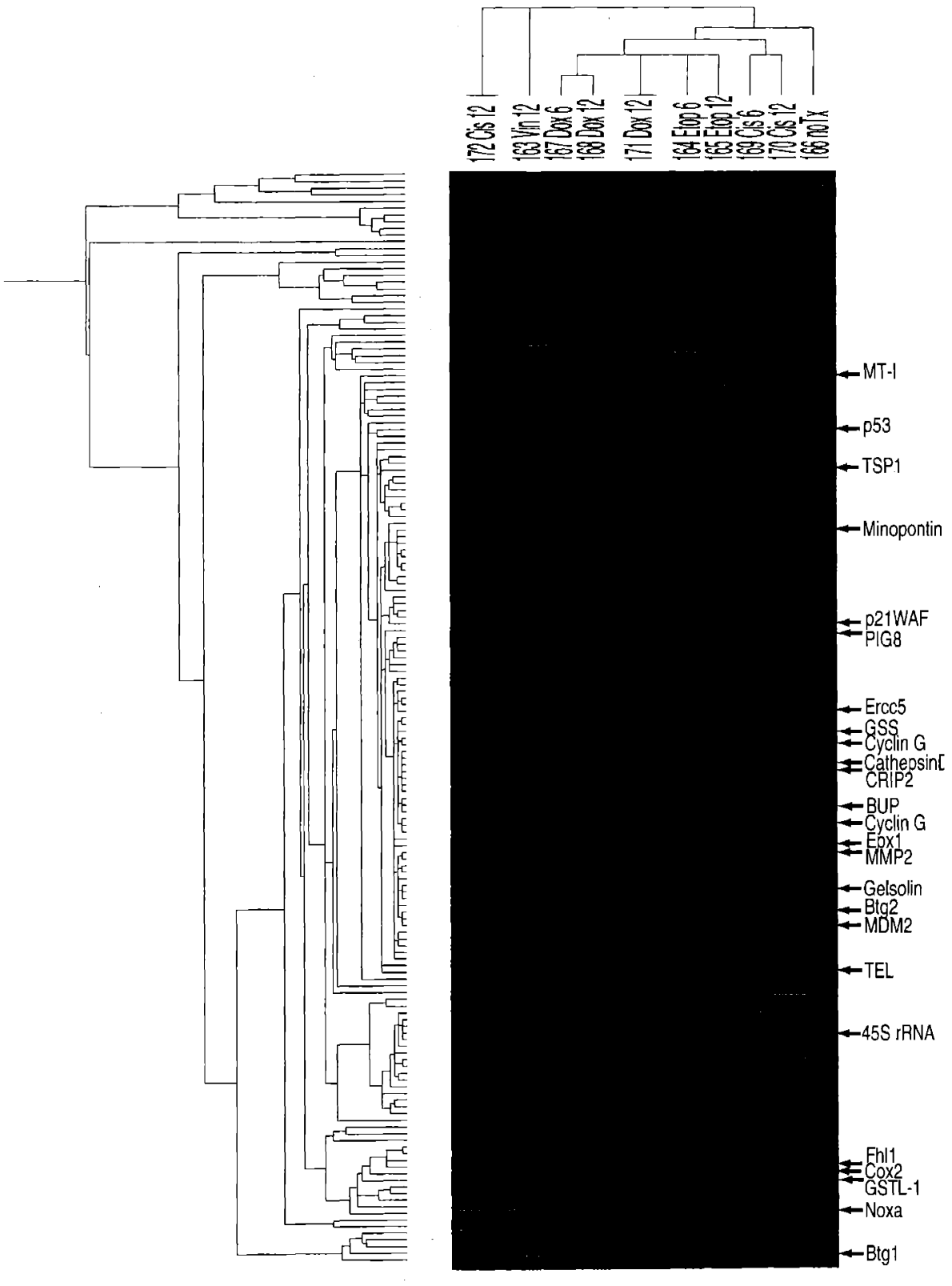

172 Cis 12
163 Vin 12
167 Dox 6
168 Dox 12
171 Dox 12
164 Etop 6
165 Etop 12
169 Cis 6
170 Cis 12
166 no Tx

Figure 1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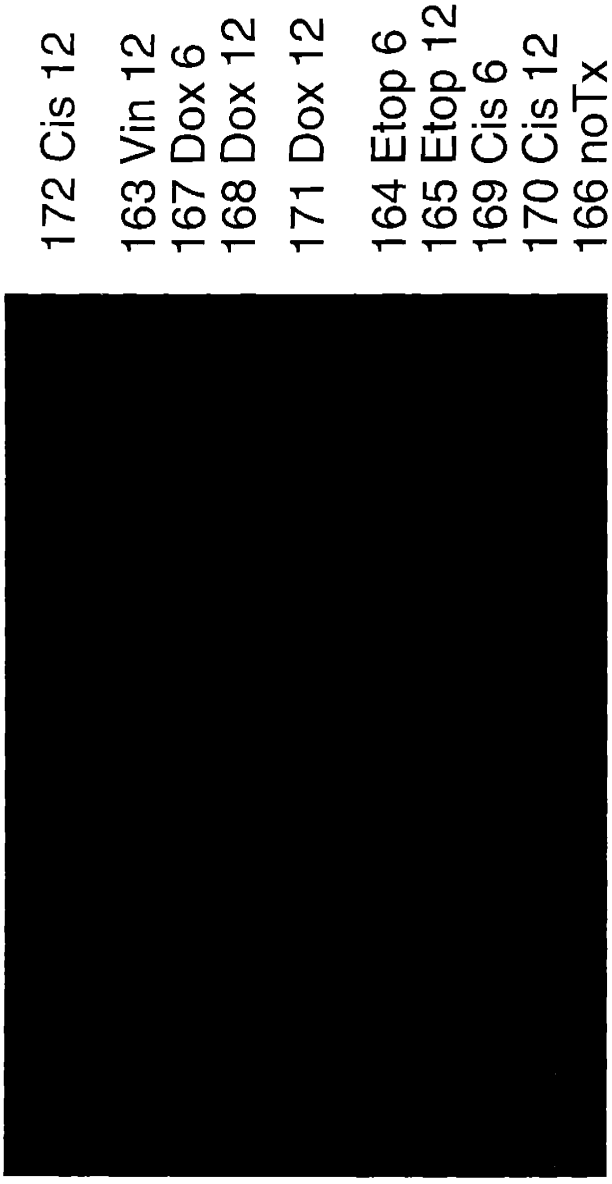
163 Vin 12
167 Dox 6
168 Dox 12
171 Dox 12
164 Etop 6
165 Etop 12
169 Cis 6
170 Cis 12
166 no Tx
MT-I
p53
TSP1
Minopontin
p21WAF
PIG8
Ercc5
GSS
Cyclin G
CathepsinL
CRIP2
BUP
Cyclin G
Epx1
MMP2
Gelsolin
Btg2
MDM2
TEL
45S rRNA
Fhl1
Cox2
GSTL-1
Noxa
Btg1

Figure 2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163 Vin 12
167 Dox 6
168 Dox 12
171 Dox 12
164 Etop 6
165 Etop 12
169 Cis 6
170 Cis 12
166 noTx
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Mm.200324
45S pre rRNA
AP-3
8968
45S pre rRNA
45S pre rRNA
ARK2
45S pre rRNA
Kcnn4
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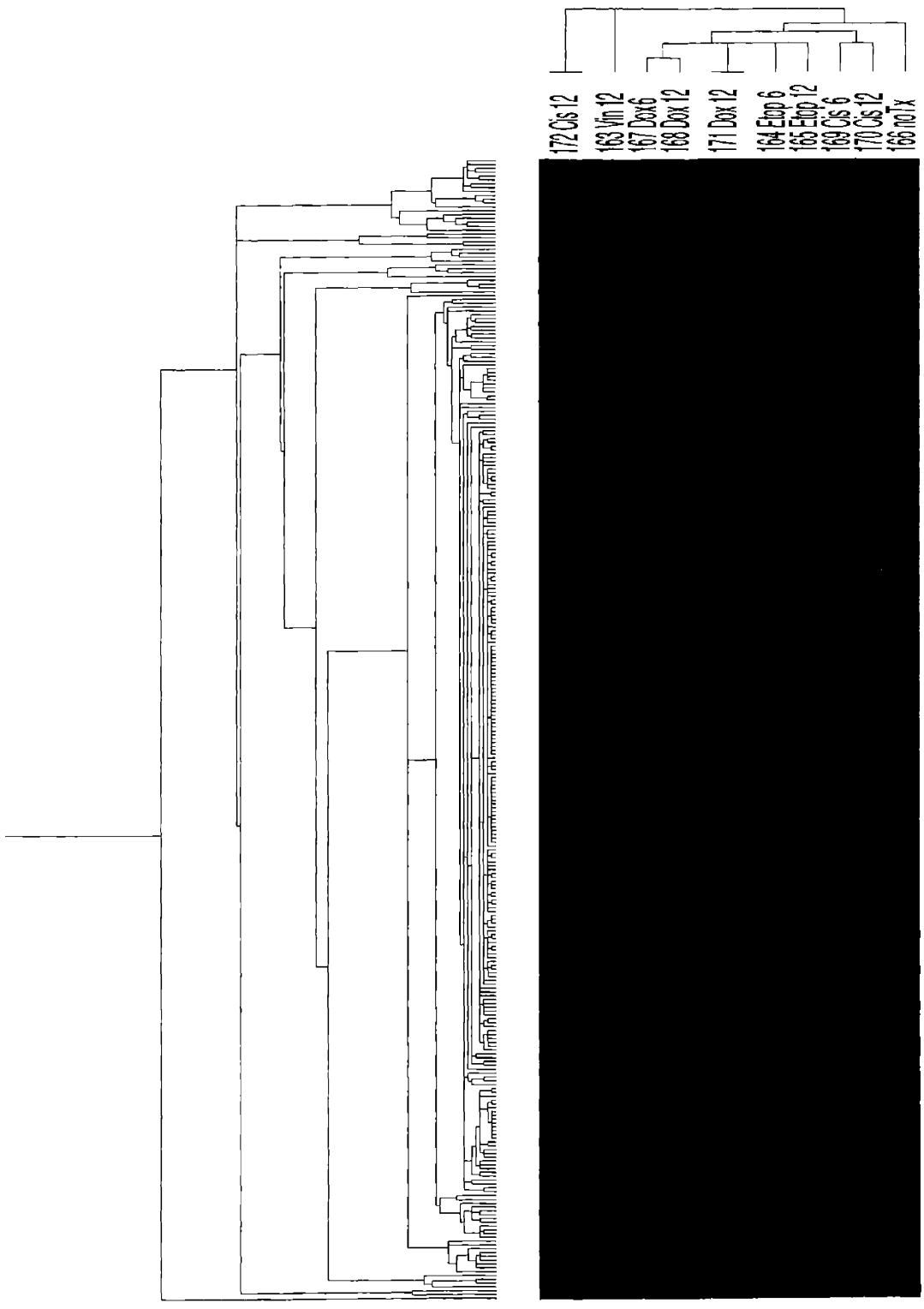
8304
45S pre rRNA
FLJ10783
Ramp2
28S rRNA

Figure 3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163 Vin 12
167 Dox 6
168 Dox 12
171 Dox 12
164 Etop 6
165 Etop 12
169 Cis 6
170 Cis 12
166 no Tx

Figure 4

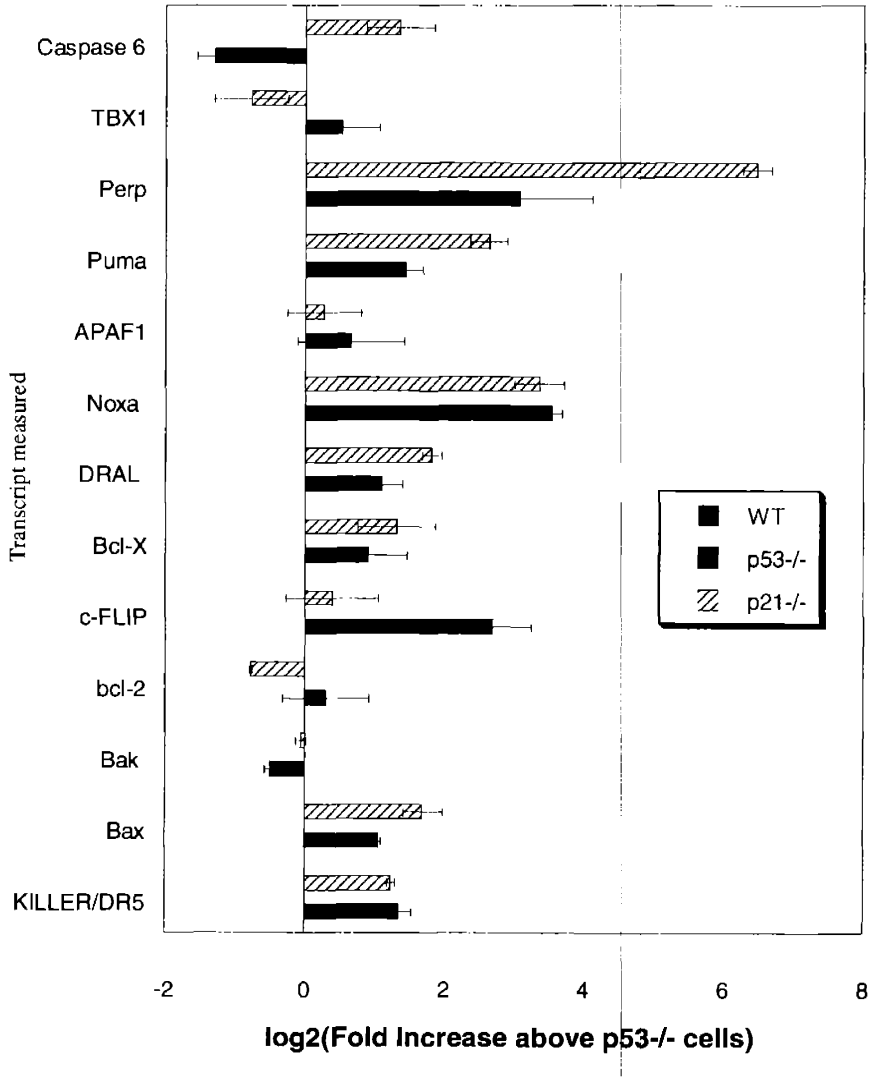
Caspase 6
TBX1
Perp
Puma
APAF1
Noxa
DRAL
Bcl-X
c-FLIP
bcl-2
Bak
Bax
KILLER/DR5
Transcript measured
WT
p53-/-
p21-/-
-2
0
2
4
6
8
log2(Fold Increase above p53-/- cells)

Figure 5

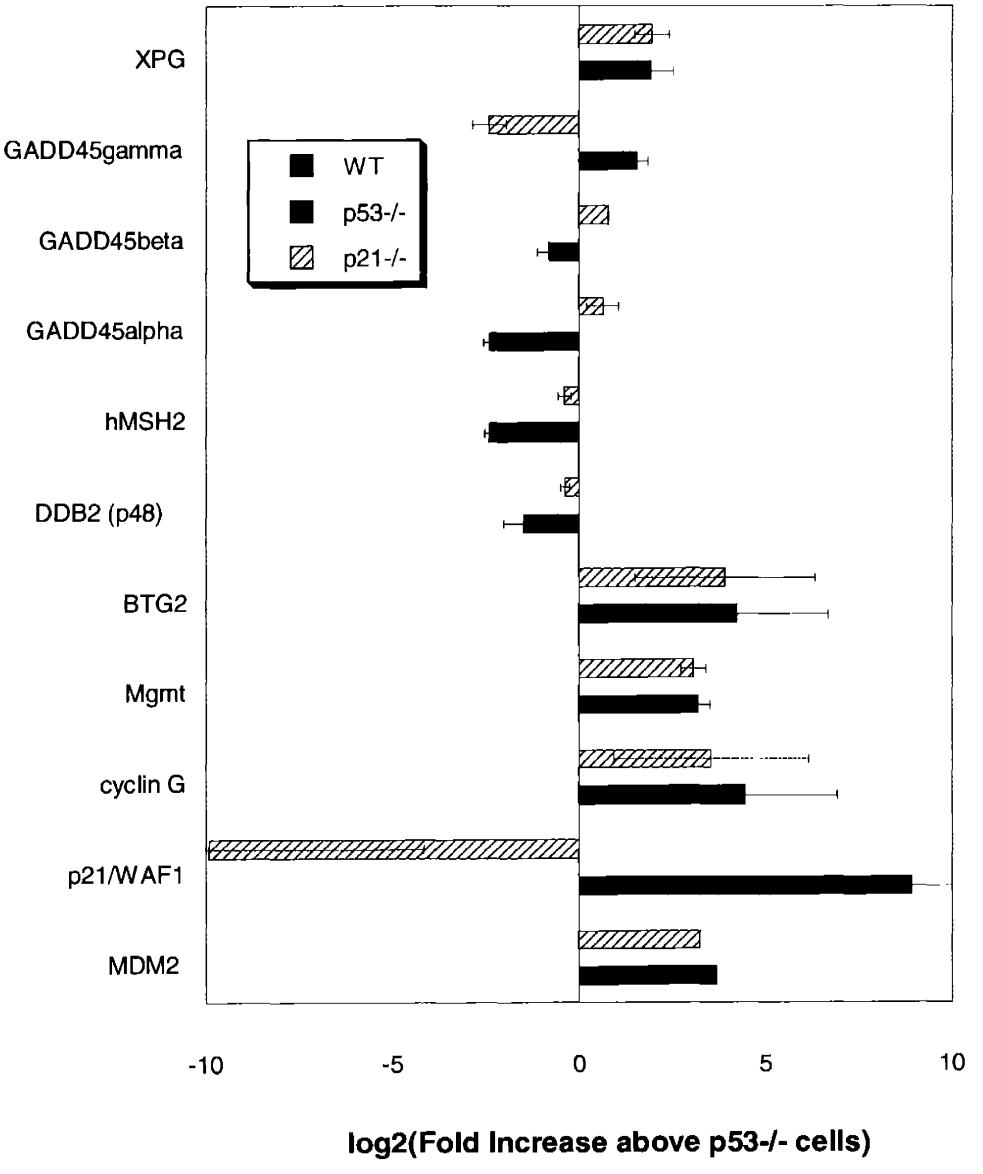
XPG
GADD45gamma
GADD45beta
GADD45alpha
hMSH2
DDB2 (p48)
BTG2
Mgmt
cyclin G
p21/WAF1
MDM2
WT
p53-/-
p21-/-
-10
-5
0
5
10
log2(Fold Increase above p53-/- cells)

Figure 6

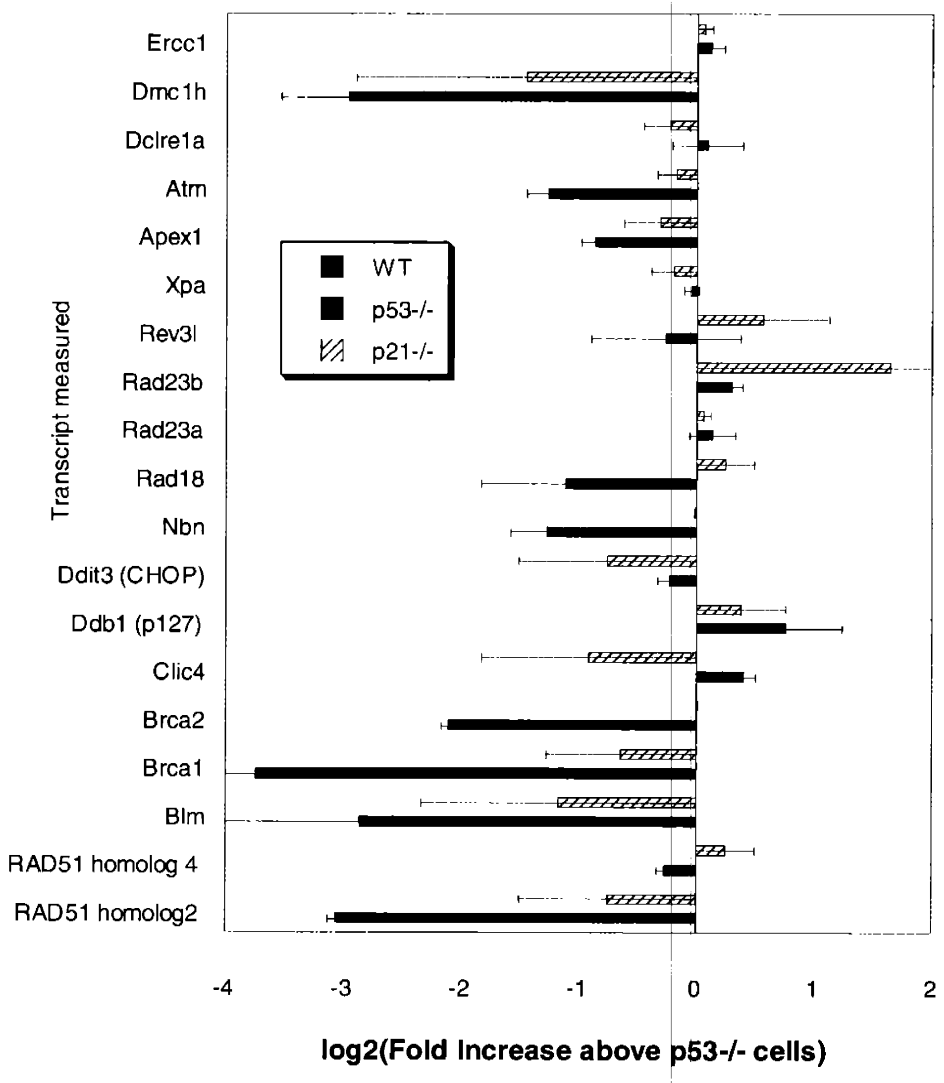
Ercc1
Dmc1h
Dclre1a
Atm
Apex1
Xpa
Rev3l
Rad23b
Rad23a
Rad18
Nbn
Ddit3 (CHOP)
Ddb1 (p127)
Clic4
Brca2
Brca1
Blm
RAD51 homolog 4
RAD51 homolog2
WT
p53-/-
p21-/-
Transcript measured
-4
-3
-2
-1
0
1
2
log2(Fold Increase above p53-/- cells)

Figure 7A

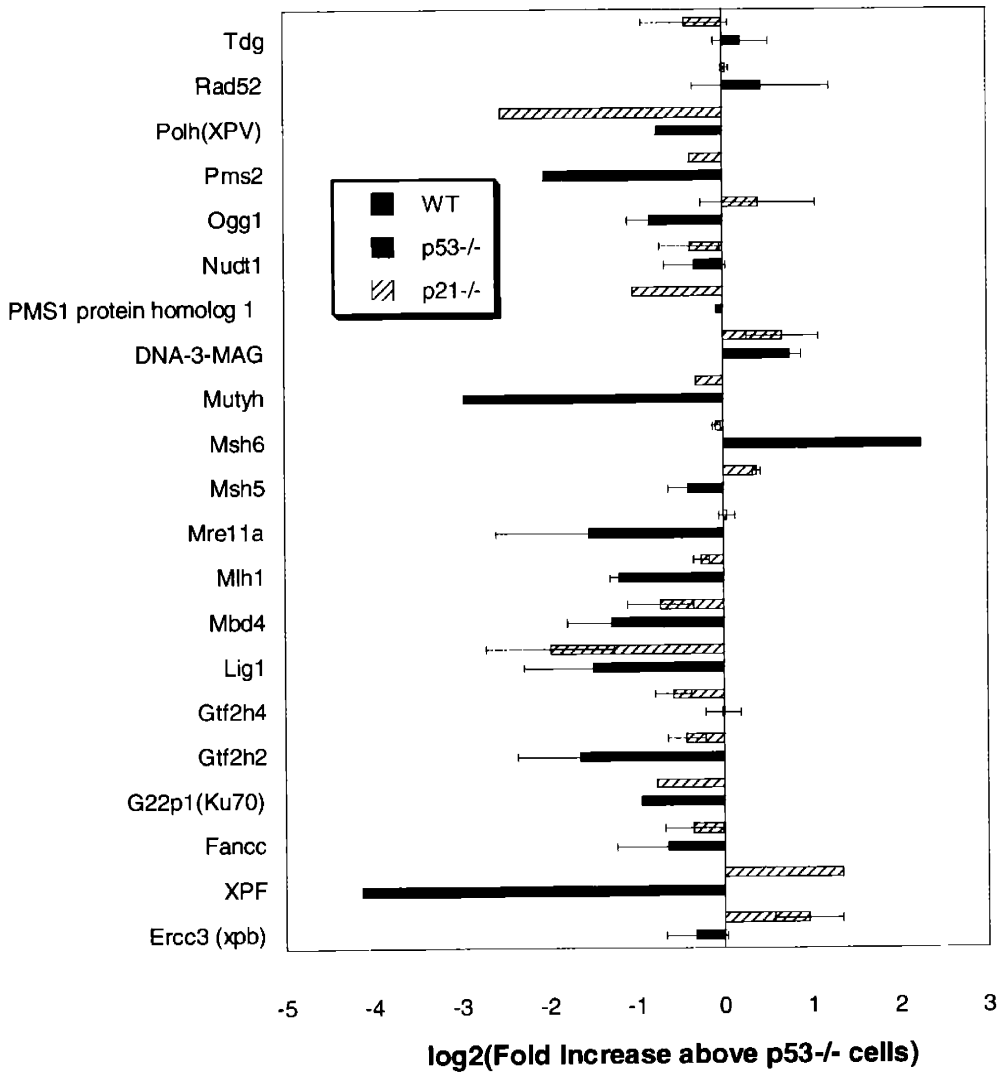
Tdg
Rad52
Polh(XPV)
Pms2
Ogg1
Nudt1
PMS1 protein homolog 1
DNA-3-MAG
Mutyh
Msh6
Msh5
Mre11a
Mlh1
Mbd4
Lig1
Gtf2h4
Gtf2h2
G22p1(Ku70)
Fancc
XPF
Ercc3 (xpb)
WT
p53-/-
p21-/-
-5
-4
-3
-2
-1
0
1
2
3
log2(Fold Increase above p53-/- cells)

Figure 7B

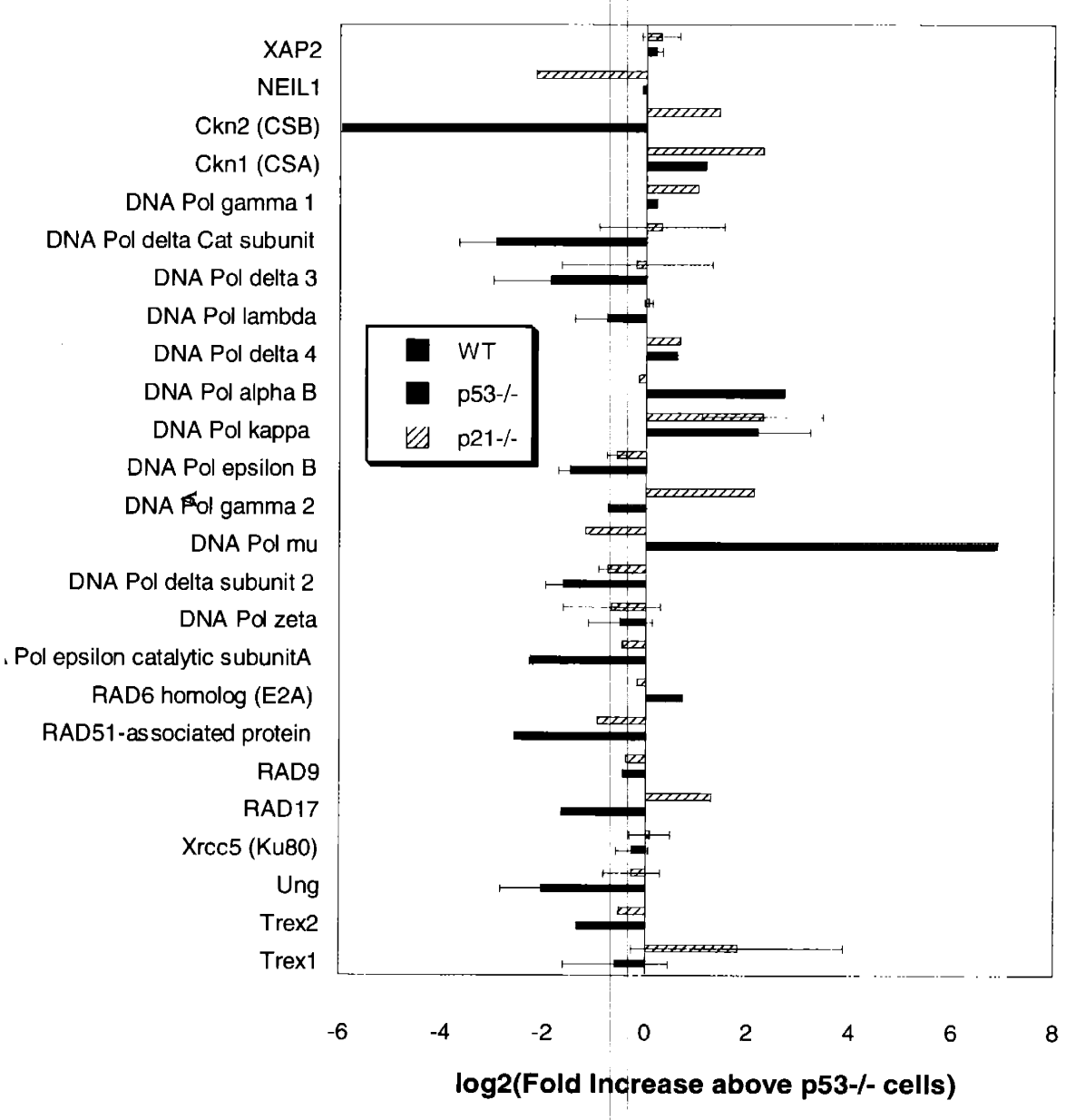
XAP2
NEIL1
Ckn2 (CSB)
Ckn1 (CSA)
DNA Pol gamma 1
DNA Pol delta Cat subunit
DNA Pol delta 3
DNA Pol lambda
DNA Pol delta 4
DNA Pol alpha B
DNA Pol kappa
DNA Pol epsilon B
DNA Pol gamma 2
DNA Pol mu
DNA Pol delta subunit 2
DNA Pol zeta
Pol epsilon catalytic subunitA
RAD6 homolog (E2A)
RAD51-associated protein
RAD9
RAD17
Xrcc5 (Ku80)
Ung
Trex2
Trex1
WT
p53-/-
p21-/-
-6
-4
-2
0
2
4
6
8
log2(Fold Increase above p53-/- cells)

Figure 7C

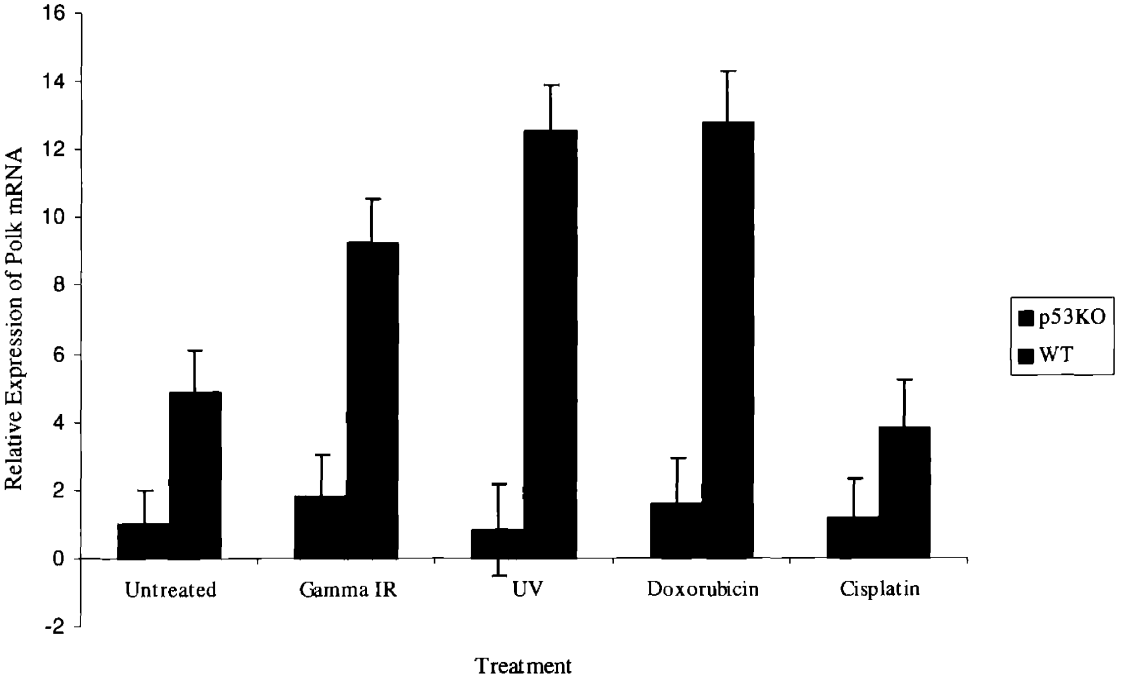
16
14
12
10
8
6
4
2
0
-2
Relative Expression of Polk mRNA
Untreated
Gamma IR
UV
Doxorubicin
Cisplatin
Treatment
p53KO
WT

Figure 8

Figure Legends

Figure 1

The results of unsupervised hierarchical clustering of the transcriptional profiles of MEFs treated with various chemotherapeutic drugs is shown. Drugs used for treatment were cisplatin (Cis) at 40 μM, doxorubicin (Dox) at 200 ng/mL, etoposide (Etop) at 20 μM, and vinblastine (Vin) at 10 μM, and RNA samples were harvested at the indicated times in hours after the start of treatment. Untreated (noTx) MEFs were treated with PBS. Standard methods for purification of RNA, reverse transcription reactions with aminoallyl-dUTP nucleotides, cDNA cleanup, coupling of ARES fluorescent dyes to the aminoallyl-cDNA, excess dye removal, and hybridization to the microarrays were used, and are described in the Methods section. In the clustering diagram, red indicates genes where *Trp53*+/+ cells expressed higher levels of the transcript than did *Trp53*-/- cells, and green indicates genes where the *Trp53*+/+ cells expressed less of the mRNA than did *Trp53*-/- cells. The intensity of the color indicates the magnitude of the expression difference. Gene identities are not shown in this diagram because of space constraints. The length of branches of the clustering tree diagram indicates the relative distance between genes or array samples in their respective data spaces.

Figure 2

The p53-upregulated genes from Figure 1 are shown in expanded form. The identity of these genes, as well as some additional p53-induced genes from additional arrays whose overall quality precluded their inclusion in the clustering analysis, is presented in Tables 2 and 3. The identities of some of the better-known p53 target genes are indicated on the figure. In most cases where a gene was represented by more than one spot on the arrays used for the experiments, the fluorescence values for the various spots clustered adjacent to, or nearby each other. The most marked example of this was cyclin G, which was represented by 4 spots on the array; 3 of these clustered next to each other, while the 4^{th} clustered a few short branches away.

Figure 3

A cluster of relatively cisplatin-specific upregulated genes from Figure 2 is shown. The various rRNA spots were verified to be dispersed widely on the array, confirming that this expression difference can not be accounted for by a regional artifact of hybridization on the array. All RNA purification steps, amino-allyl-cDNA synthesis, purification, non-specific hybridization blocking, washing, and scanning of arrays were carried out identically for all samples.

Figure 4

This figure shows the expression pattern of genes from Figure 1 whose expression levels inversely correlate with p53 status in the MEFs. The vast majority of these genes are identifiable as cell-cycle regulated genes, whose expression changes are likely to be the result of induction of

cell cycle inhibitory factors such as $p21^{WAF}$, whose impact on the cell cycle is well understood. The identities of the genes in this figure are listed in Table 4.

Figure 5

Transcript levels of known genes described in the literature as being p53 regulated and implicated in apoptosis were measured by real-time PCR in wild-type, *Trp53-/-*, and *p21-/-* cells. RNA samples were harvested 6 hours after treating cells with 10 Gy gamma irradiation. Purification of RNA following Trizol harvest, and preparation of cDNA template were carried out as described in the Methods section. Real-time PCR analysis of samples was carried out using TaqMan probes for the 18S rRNA sequence to normalize for input RNA amounts. In separate reactions, levels of the various cDNA sequences listed were assayed, using SYBR green fluorescent dye intercalation for detection of amplified sequences, according to the manufacturer's protocol. A denaturing step was conducted at the end of the reaction sequence to ensure that products with the appropriate melting temperature were generated in the reaction.

The expression levels measured in these experiments are relative measurements that can only be used to compare expression levels of a particular mRNA among the different cell lines used. Both the wild-type cells and the *p21-/-* cells are compared to the *p53-/-* cells in these experiments. Thus, any genes that are regulated in a p53-dependent manner, but are not affected by p21-dependent cell cycle changes should show a similar induction or repression in the wild-type cells and the *p21-/-* cells. Although the data for numerous genes are plotted in Figures 5-8, it should be remembered that one cannot infer the absolute levels of each of these genes from the data provided. Because of the relatively wide range of expression level differences seen in these experiments, the data are plotted on a log scale for easier visualization. The expression level of each mRNA in the *p53-/-* cells is set to a value of 1; therefore, on the log scale, the *p53-/-* bars have a length of 0.

Figure 6

Gamma-irradiated MEF samples were prepared as indicated for Figure 5, and transcript levels of reported p53 target genes involved in p53 regulation, cell cycle control, and DNA repair were measured by real-time PCR as described for Figure 5. In these experiments, no evidence was found for p53-dependent expression of *Ddb2*, *Msh2*, or the *GADD* genes in cultured primary mouse fibroblasts.

Figures7A-7C

DNA repair gene levels were measured in samples treated identically to those in Figure 5. A number of these genes had low levels of expression, which increases the relative error of measurement, as seen by the error bars in the figures. The most convincing positive result for p53-dependent, but p21-independent mRNA expression was that for DNA polymerase kappa (found in Figure 7C).

Figure 8

Induction of DNA polymerase kappa gene (*Polk*) in response to 10Gy gamma irradiation, 20 J/m^2 UV irradiation in a Stratalinker, cisplatin at 40 μM, and doxorubicin at 200 ng/mL was measured by real time PCR of samples harvested 6 hours after treatment. The harvest and real-time PCR methodologies were performed exactly as outlined in the legend for Figure 5. Note that the scale of this figure is linear and not a logarithmic scale as for Figures 5-7.

Tables

Table 1a : Samples for MEF microarray experiments in Figures 1 to 3

Sample code	Alexa555 sample (green fluorescence)	Alexa647 sample (red fluorescence)	Treatment	Time point
167	Trp53-/- MEF	Trp53+/+ MEF	doxorubicin	6 hours
168	Trp53-/- MEF	Trp53+/+ MEF	doxorubicin	12 hours
171	Trp53-/- MEF	Trp53+/+ MEF	doxorubicin	12 hours
169	Trp53-/- MEF	Trp53+/+ MEF	cisplatin	6 hours
170	Trp53-/- MEF	Trp53+/+ MEF	cisplatin	12 hours
172	Trp53-/- MEF	Trp53+/+ MEF	cisplatin	12 hours
164	Trp53-/- MEF	Trp53+/+ MEF	etoposide	6 hours
165	Trp53-/- MEF	Trp53+/+ MEF	etoposide	12 hours
163	Trp53-/- MEF	Trp53+/+ MEF	vinblastine	12 hours
166	Trp53-/- MEF	Trp53+/+ MEF	no treatment	6 hours

Table 1b: Additional samples incorporated into the full analysis in Tables 2, 3, and 4

Sample code	Alexa555 sample (green fluorescence)	Alexa647 sample (red fluorescence)	Treatment	Time point
142	Trp53-/- MEF	Trp53+/+ MEF	gamma IR	6 hours
146	Trp53-/- MEF	Trp53+/+ MEF	gamma IR	6 hours
147	Trp53-/- MEF	Trp53+/+ MEF	UV	6 hours
148	Trp53-/- MEF	Trp53+/+ MEF	doxorubicin	6 hours
149	Trp53-/- MEF	Trp53+/+ MEF	cisplatin	6 hours
158	Trp53-/- MEF	Trp53+/+ MEF	vinblastine	12 hours
159	Trp53-/- MEF	Trp53+/+ MEF	no treatment	6 hours

Table 2: Known p53 target genes induced in MEF array experiments

Function	Spot	Locus Link ID	Gene	Mean induction	Standard Deviation
	2540	Mm.222	p53	2.21	0.60
Cell Cycle	10112	pos. control	p21/WAF1	2.13	0.60
Protein Stability	1596	pos. control	MDM2	1.92	0.87
	5377	Mm.22670	MDM2	3.18	1.51
	13522	Mm.22670	MDM2	3.20	1.74
Cell Cycle	8704	pos. control	cyclin G	7.69	3.64
	5667	Mm.2103	cyclin G	8.92	3.57
	7323	Mm.2103	cyclin G	7.59	4.89
	1073	Mm.2103	cyclin G	7.66	3.49
Apoptosis	9584	pos. control	Noxa	1.64	0.91
	400	Mm.2147	Cathepsin D	2.63	0.72
	3508	Mm.2147	Cathepsin D	1.93	0.42
	3552	Mm.2147	Cathepsin D	2.27	0.57
Oxidative stress	8880	pos. control	Pig8	1.93	0.69
	10533	Mm.192991	Mouse metallothionein-I	1.74	0.53
Other stress response	2608	pos. control	alpha-B2-crystallin	1.77	0.58
	7326	Mm.178	alpha-B2-crystallin	2.08	0.78
	3020	Mm.9075	Microsomal epoxide hydrolase	2.79	1.37
Extracellular Matrix	936	pos. control	MMP-2	1.77	0.55
	1134	Mm.29564	MMP-2	1.83	0.53
"Growth					

Regulatory"	1684	pos. control	CGR11	2.01	0.72
	7183	Mm.903	Btg2	3.18	1.44
Signal Transduction	8440	pos. control	Ephrin A1	1.32	0.66
Angiogenesis	1508	pos. control	thrombospondin 1	1.87	0.75
Transcription factors/coactivators	5544	Mm.3126	four and a half LIM domains 1	2.28	1.68
RNA-binding	980	pos. control	PAI-1	1.45	0.59
Channels	921	Mm.6404	(MDR/TAP) (Abcb1)	1.41	0.51
Extracellular matrix	10256	Mm.321	Minopontin (osteopontin)	3.37	1.26
Cytoskeletal	3791	Mm.21109	gelsolin (Gsn)	2.50	0.95
Translation	318	Mm.5286	(Arbp)	1.58	0.50
Unknown	5027	Mm.14486	BUP protein (BUP)	2.75	0.74
	7082	Mm.14486	BUP protein (BUP)	2.36	0.75
	6728	Mm.133825	cysteine-rich protein 2 (CRIP2)	3.90	1.49

Table 3: Potential novel p53 target genes induced in MEF array experiments

Function	Spot	Locus Link ID	Gene	Mean -fold induction	Std Dev
DNA Repair	8244	Mm.2213	ERCC5 (XPG)	2.38	1.04
	1725	Mm.4143	SCID complementing gene 2	1.58	0.49
Oxidative stress	12100	Mm.282	glutathione S-transferase like (Gsttl-pending)	1.40	0.69
	9107	Mm.7504	glutathione synthetase (Gss)	1.71	0.69
	1843	Mm.27154	vanin 1 (Vnn1)	1.64	0.68
Other stress response	5718	Mm.29852	HSPC254 , partial cds	1.67	0.57
	6569	Mm.21549	heat shock protein 20-like protein	1.60	0.41
	4652	Mm.195950	HSP40-like protein , partial sequence	1.60	0.55
Transcription factors/coactivators	10483	Mm.193459	ets variant gene 6 (TEL oncogene) (Etv6)	3.74	1.75
RNA Stability/RNA binding	1003	Mm.86786	Pur alpha	1.70	0.49
	15166	Mm.19101	DEAD box polypeptide 5 (Ddx5)	1.26	0.50
	4686	Mm.85253	Miwi like (MILI)	1.35	0.47
Protein Stability	13897	Mm.396	fat facets homolog (Fam) ubiquitin protease	2.04	0.68
	654	Mm.28867	tripeptidyl peptidase II (Tpp2)	1.89	0.70
Metabolic	15313	Mm.19834	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8	1.34	0.46
	15314	Mm.33084	CTP:phosphocholine cytidylyltransferase (Ctpct)	1.96	0.93
	4485	Mm.3798	fumarylacetoacetate hydrolase (Fah)	1.62	0.68
	5835	Mm.18675	hypoxanthine guanine phosphoribosyl transferase (Hprt)	1.67	0.49
Channels/Transporters	4931	Mm.90003	gap junction membrane channel protein beta 3 (Gjb3)	1.47	0.58
	2924	NA or unknown	potassium channel, subfamily N, member 4 (Kcnn4)	1.61	0.82
Cell Cycle	5895	Mm.89918	cyclin D2 (CCND2)	1.70	0.75

	9717	NA or unknown	aurora-related kinase 2 (ARK2)	1.52	0.74
	13271	Mm.30120	cyclin ania-6a gene	1.95	0.41
	5337	Mm.141567	CC1 coiled-coil protein	1.48	0.60
	2162	Mm.44604	Septin2	1.69	0.89
"Growth Regulatory"	1128	Mm.16596	B-cell translocation gene-1 protein (BTG1)	1.44	0.61
Extracellular Matrix	9542	Mm.24293	mannosyl transferase (MGAT2)	1.87	0.55
	15295	Mm.43831	Bgbp (heparin binding protein)	1.50	0.49
	8962	Mm.193099	M.musculus fibronectin	1.70	0.53
	15280	Mm.7281	procollagen, type V, alpha 1 (Col5a1)	1.71	0.54
	10458	Mm.35439	secreted acidic cysteine rich glycoprotein (Sparc)	1.73	0.41
	300	Mm.3093	serine protease inhibitor 4 (Spi4)	1.83	0.54
Cytoskeleton	14945	Mm.5567	carboxyl terminal LIM domain protein 1 (Clim1)	1.48	0.69
	11479	Mm.18626	capping protein (actin filament), gelsolin-like (Capg)	1.85	0.52
	333	Mm.44004	KRIT1 (Krit1) , complete cds	1.65	0.55
	12152	Mm.21990	plakoglobin	1.49	0.62
	1849	Mm.25168	CRIP3 thymus LIM protein	2.02	0.64
Membrane proteins	5080	Mm.29647	G protein-coupled receptor 49 (GPR49)	2.00	0.88
	7043	Mm.198	transmembrane protein 5 (TMEM5)	1.64	0.49
	2342	Mm.381	Adipose differentiation-related protein (ADRP)	1.74	0.81
	2362	Mm.1451	milk fat globule-EGF factor 8 protein (Mfge8)	1.61	0.53
Secretory pathways	15358	Mm.18634	Sec31 protein , complete cds	1.43	0.47
	15322	Mm.27600	signal recognition particle 72kD (SRP72)	1.50	0.48
	6867	Mm.28463	adaptor-related protein AP-3, delta subunit (Ap3d)	1.56	0.66
	10559	Mm.181287	hippocalcin-like 1 (Hpcal1)	1.51	0.64
	1422	Mm.1574	receptor (calcitonin) activity modifying protein 2 (Ramp2)	1.45	0.58
	12515	Mm.1574	receptor (calcitonin) activity modifying protein 2 (Ramp2)	1.59	0.92
	10363	Mm.29771	vacuolar adenosine triphosphatase subunit A gene	1.81	0.47
Signal Transduction	2582	Mm.138866	GAC-1 (GAC-1) , complete cds	1.21	0.46

	464	Mm.27118	IGF-II -binding protein 2 (IMP-2)	1.25	0.45
	129	Mm.23575	retinoic acid induced 3 (RAI3)	1.56	0.60
	11309	Mm.200379	gene for LKB1 serine/threonin kinase, exon 2-10, partial cds	1.91	0.88
	280	Mm.28130	PCTAIRE-motif protein kinase 3 (Pctk3)	1.50	0.54
	1674	Mm.153891	protein tyrosine phosphatase 4a3 (Ptp4a3)	1.50	0.50
Growth Factors/ Receptors	8768	Mm.2675	platelet derived growth factor, alpha (Pdgfa)	1.79	0.62
Immune system-related	1509	Mm.1401	CXCR-4	1.58	0.42
	9486	Mm.20236	decay accelerating factor 1 (Daf1)	1.48	0.90
Unknown	15359	Mm.34366	HBV pX associated protein-8 (LOC51773)	1.25	0.55
	2243	Mm.182419	hypothetical protein (TR2/D15 gene)	1.41	0.62
	6611	Mm.24756	KIAA0580 protein, partial cds	1.65	0.53
	473	Mm.35807	KIAA1247 protein, partial cds	2.73	1.00
	10567	Mm.23921	KIAA1370 protein, partial cds	1.82	0.67
	2092	Mm.172947	fragment	2.71	1.26
	8351	Mm.200927	cDNA DKFZp761L0516	1.66	0.80
	6604	Mm.182921	PP1201 protein (PP1201),	2.04	0.76
	12707	Mm.35796	ecotropic viral integration site 5 (Evi5)	2.23	0.56
	498	Mm.10272	leucine rich repeat (in FLII) interacting protein 1 (Lrrfip1)	1.56	0.57
	4836	Mm.648	short incubation prion protein Prnpa gene	1.87	0.59
	4118	Mm.5675	NA or unknown	2.46	1.29
	5268	Mm.6149	NA or unknown	1.72	0.92
	14567	Mm.12746	NA or unknown	1.53	0.49
Unknown	2220	Mm.21171	NA or unknown	1.42	0.55
	10989	Mm.24670	NA or unknown	1.43	0.50
	1471	Mm.25370	NA or unknown	1.66	0.46
	10600	Mm.25647	NA or unknown	1.77	1.07
	4150	Mm.26610	NA or unknown	1.76	0.64
	15296	Mm.28347	NA or unknown	1.18	0.49
	91	Mm.28492	NA or unknown	1.54	0.62
	1119	Mm.28771	NA or unknown	2.51	0.95
	5864	Mm.28951	NA or unknown	1.85	0.75

	3808	Mm.29116	NA or unknown	1.57	0.83
	7636	Mm.29464	NA or unknown	2.04	0.51
	6678	Mm.29616	NA or unknown	1.97	1.28
	4777	Mm.29694	NA or unknown	1.57	0.58
	11153	Mm.30163	NA or unknown	1.34	0.59
	524	Mm.32497	NA or unknown	1.65	0.66
	1502	Mm.34175	NA or unknown	1.52	1.57
	3687	Mm.75160	NA or unknown	1.88	0.65
	7805	Mm.86550	NA or unknown	2.18	0.91
	14787	Mm.87581	NA or unknown	2.49	1.75
	15350	Mm.87673	NA or unknown	1.37	0.47
	5767	Mm.100529	NA or unknown	2.12	0.39
	15114	Mm.102939	NA or unknown	1.42	0.73
	2428	Mm.102976	NA or unknown	1.31	0.75
	15138	Mm.103099	NA or unknown	1.29	0.48
	14920	Mm.103269	NA or unknown	1.64	0.54
	5111	Mm.155205	NA or unknown	1.25	0.57
	11279	Mm.171798	NA or unknown	1.59	0.81
	2598	Mm.173203	NA or unknown	1.42	0.60
	2067	Mm.173561	NA or unknown	1.36	0.50
	7246	Mm.174130	NA or unknown	1.39	0.54
	14655	Mm.192260	NA or unknown	1.41	0.57
Unknown	1885	Mm.200324	NA or unknown	1.75	0.66
	9363	Mm.11862	Dahl salt resistant strain clone etb satellite DNA	1.68	0.79
	12019	Mm.22448	hypothetical protein FLJ10749 (FLJ10749)	1.69	0.90
	9073	Mm.28771	hypothetical protein FLJ12816 (FLJ12816)	1.81	0.83
	15342	Mm.34109	cDNA FLJ12484 fis, weakly similar to Human HEM45	1.52	0.75
	11542	Mm.22240	cDNA: FLJ21231 fis, clone COL00744	1.86	0.84
	8638	Mm.9671	cDNA: FLJ22272 fis, clone HRC03192	1.88	0.71
	9458	Mm.29247	cDNA: FLJ22378 fis, clone HRC07430	1.34	0.46
	13050	Mm.27114	cDNA: FLJ22394 fis, clone HRC07882	1.78	0.53
	15341	Mm.39995	cDNA: FLJ22679 fis, clone HSI10687	1.21	0.59
	592	Mm.41937	cDNA: FLJ23461 fis, clone HSI07757	1.40	0.44

Table 4: Genes down-regulated in response to p53 in DNA-damaged MEFs

Spot	Locus Link	Gene	Mean ratio	Std. Dev.
387	Mm.29546	actin-like 6 (Actl6),	0.80	0.21
12928	Mm.22430	activator of S phase kinase (Ask-pending),	0.53	0.19
3164	Mm.22430	activator of S phase kinase (Ask-pending),	0.59	0.23
5621	Mm.22430	activator of S phase kinase (Ask-pending),	0.61	0.19
8801	Mm.22430	activator of S phase kinase (Ask-pending),	0.57	0.20
42	Mm.29460	adenylate kinase 2 (AK2),	0.73	0.28
13890	NA or unknown	apolipoprotein B apoB , 3' end	0.54	0.20
4734	Mm.11738	aurora-related kinase 1 (ARK1)	0.61	0.30
1060	Mm.182628	beta-1,4-galactosyltransferase	0.66	0.40
10725	Mm.196331	calcium-sensitive chloride conductance protein-1 (mCLCA1)	0.73	0.24
9769	Mm.12940	calmodulin binding protein 1 (Calmbp1),	0.56	0.19
11162	Mm.3049	CDC28 protein kinase 1 (Cks1),	0.67	0.20
8091	Mm.326	CDC28 protein kinase 2 (CKS2),	0.60	0.17
2300	NA or unknown	cdc2a	0.42	0.19
8203	Mm.18923	CDC47	0.72	0.26
8572	NA or unknown	CDK2	0.61	0.17
411	Mm.102479	CDC2L5	0.68	0.24
8334	Mm.4761	cell division cycle 2 homolog A (S. pombe) (Cdc2a),	0.60	0.20
1267	Mm.20912	cell division cycle 6 homolog (S. cerevisiae) (Cdc6),	0.73	0.31
8198	Mm.181792	centromere autoantigen H (Cenph),	0.61	0.22
9613	Mm.1654	CGI-48 protein (LOC51096),	0.77	0.18
2331	Mm.16753	checkpoint kinase 1 homolog (S. pombe) (Chek1),	0.60	0.21
11379	Mm.16753	checkpoint kinase 1 homolog (S. pombe) (Chek1),	0.55	0.19
12402	Mm.27609	chromatin assembly factor 1, subunit A (p150) (Chaf1a),	0.60	0.22
14506	Mm.28148	chromobox homolog 3 (Drosophila HP1 gamma) (Cbx3),	0.57	0.19
971	Mm.28385	Clast1	0.56	0.18
3914	Mm.4189	cyclin A2 (Ccna2),	0.39	0.19
12062	Mm.4189	cyclin A2 (Ccna2),	0.38	0.21
12401	Mm.4189	cyclin A2 (Ccna2),	0.37	0.18

5451	Mm.22569	cyclin B1	0.54	0.23
1016	Mm.22592	cyclin B2 (Ccnb2),	0.69	0.20
377	Mm.1987	decorin (Dcn),	0.80	0.32
7595	Mm.28343	DEK oncogene (DNA binding) (DEK),	0.63	0.23
7993	Mm.28343	DEK oncogene (DNA binding) (DEK),	0.60	0.16
12759	Mm.196110	DNA for alpha globin gene and flanking regions	0.81	0.19
207	Mm.29381	DNA for MUSPUR,	0.69	0.47
14560	Mm.9199	DNA polymerase epsilon small subunit ,	0.68	0.18
4684	Mm.2903	DNA primase, p49 subunit (Prim1),	0.77	0.43
4766	Mm.19726	dynein, axon, heavy chain 11 (Dnahc11),	0.75	0.24
2496	Mm.173031	endogenous retroviral-like element MuERVC-C105	0.59	0.27
6468	Mm.4303	enhancer of zeste homolog 2 (Drosophila) (Ezh2),	0.58	0.22
13478	Mm.27836	ERIC1 (Eric1)	0.52	0.16
7681	Mm.22269	exportin 1 (CRM1, yeast, homolog) (XPO1),	0.62	0.27
4717	Mm.197520	F-box only protein 5 (FBXO5),	0.57	0.15
6804	Mm.2999	fibroblast growth factor inducible 16 (Fin16),	0.53	0.28
2854	Mm.20315	fidgetin-like 1 (Fignl1),	0.74	0.24
13264	Mm.12239	geminin (Geminin-pending),	0.53	0.31
12425	Mm.1399	glial cells missing homolog (Drosophila), related sequence 2 (Gcm1-rs2),	0.69	0.27
3369	Mm.33356	glutamate transporter (GLAST-1) , 3'end	0.94	0.37
14543	Mm.2011	glutathione S-transferase, mu 1 (Gstm1),	0.71	0.17
6116	Mm.22701	growth arrest specific 1 (Gas1),	0.65	0.35
7103	Mm.29786	HCAP-H , partial cds	0.61	0.21
3266	Mm.57223	helicase, lymphoid specific (Hells),	0.57	0.27
7007	Mm.28275	heterogeneous nuclear ribonucleoprotein G	0.82	0.27
4840	Mm.16421	high mobility group 1 protein (HMG-1)	0.71	0.20
3823	Mm.911	high mobility group protein 17 (Hmg17),	0.72	0.18
5804	Mm.1693	high mobility group protein 2 (Hmg2) gene	0.55	0.23
11130	Mm.6642	highly expressed in cancer, rich in leucine heptad repeats (HEC),	0.67	0.26
11262	Mm.31730	highly expressed in cancer, rich in leucine heptad repeats (HEC),	0.60	0.22
11010	Mm.16421	HMG-1	0.50	0.17
2212	NA or unknown	IGFBP-3	0.61	0.27
11278	Mm.29254	IGF-BP3	0.64	0.29

2978	Mm.578	insulin-like growth factor binding protein 5 (IGFBP5)	0.55	0.16
11552	Mm.28386	kinesin-like 5 (mitotic kinesin-like protein 1) (KNSL5),	0.49	0.21
13190	Mm.52266	L2DTL protein (L2DTL),	0.70	0.23
13546	Mm.171388	L2DTL protein (L2DTL),	0.66	0.26
111	Mm.4538	lamin B receptor (LBR),	0.75	0.33
7710	Mm.19980	LEK1 ,	0.60	0.31
11106	Mm.99	M2 subunit ribonucleotide reductase	0.75	0.24
8660	NA or unknown	Mad2	0.48	0.17
8040	Mm.5048	mCDC46 protein	0.60	0.22
11716	Mm.171319	MCM10 homolog	0.60	0.18
892	NA or unknown	MCM3 homolog	0.52	0.25
13607	Mm.25544	meiotic recombination 11 homolog A (S. cerevisiae) (Mre11a),	0.59	0.23
393	Mm.27141	MgcRacGAP for GTPase activating protein	0.64	0.26
7763	Mm.27141	MgcRacGAP for GTPase activating protein	0.51	0.20
4971	Mm.16711	mMCM2	0.62	0.15
8162	Mm.4933	mMIS5	0.54	0.18
11119	Mm.46316	monocyte/neutrophil elastase inhibitor gene	0.82	0.31
1612	Mm.906	Mouse retinoic acid-responsive protein (MK-2)	0.56	0.26
9161	Mm.28479	Mouse stathmin gene sequence	0.62	0.26
6936	Mm.7141	Murine PCNA	0.66	0.22
10682	Mm.4594	myeloblastosis oncogene-like 2 (Mybl2),	0.67	0.22
7508	Mm.12508	nuclear pore-targeting complex	0.69	0.28
12051	Mm.12508	nuclear pore-targeting complex	0.66	0.30
11914	Mm.27584	nucleolar protein ANKT	0.61	0.28
888	Mm.20450	nucleosome binding protein 1 (Nsbp1),	0.68	0.32
14213	Mm.29709	origin recognition complex subunit 6 (Orc6),	0.64	0.26
2172	Mm.4502	P1 protein (P1.m)	0.67	0.26
2114	Mm.6856	pituitary tumor-transforming 1 (Pttg1),	0.56	0.26
11740	Mm.6856	pituitary tumor-transforming 1 (Pttg1),	0.57	0.20
10019	Mm.3063	pleiotrophin (Ptn),	0.62	0.22
13284	Mm.2770	preproinsulin-like growth factor IA	0.65	0.16
1920	Mm.30160	PRO2730	0.73	0.25

8370	Mm.2185	protein kinase ,	0.42	0.21
10329	Mm.2185	protein kinase ,	0.55	0.24
2388	NA or unknown	RAD51	0.68	0.24
1634	Mm.32022	RAD54, S. cerevisiae, homolog of, B (RAD54B),	0.78	0.32
8548	Mm.2870	replication protein A2 (Rpa2),	0.63	0.19
8529	Mm.200680	replication protein A2 (Rpa2),	0.63	0.27
3503	Mm.1603	retinoblastoma binding protein 7 (Rbbp7),	0.60	0.14
3458	Mm.200478	s17 protein	0.84	0.38
13649	Mm.3794	serine/threonine kinase (sak-a)	0.59	0.25
4310	Mm.37801	Shc SH2-domain binding protein 1 (Shcbp1),	0.60	0.23
11347	Mm.27945	smooth muscle protein phosphatase type 1-binding subunit	0.68	0.21
12595	Mm.9086	solute carrier family 16 member 1 (Slc16a1),	0.63	0.25
5164	Mm.7516	somatic histone binding protein NASP	0.54	0.17
9057	Mm.7516	somatic histone binding protein NASP	0.53	0.22
12053	Mm.43444	spindle assembly checkpoint protein (Mad2a) gene	0.50	0.23
363	Mm.193096	stearoyl-Coenzyme A desaturase 2 (Scd2),	0.79	0.27
14144	Mm.4172	stem-loop binding protein (Slbp),	0.59	0.28
14703	Mm.8552	survivin40, survivin121, and survivin140 genes	0.56	0.19
4362	Mm.36676	Swi/SNF related (Smarcal1),	0.55	0.23
4574	Mm.3680	TERF1-interacting nuclear protein 2 (Tin2)	0.66	0.20
7258	Mm.124	thymopoietin beta	0.63	0.20
13074	Mm.24337	TOPK	0.39	0.18
530	Mm.4237	topoisomerase (DNA) II alpha (Top2a),	0.48	0.20
12409	Mm.4237	topoisomerase (DNA) II alpha (Top2a),	0.36	0.21
13787	Mm.11516	TRAF4 associated factor 1 ,	0.62	0.28
8372	Mm.925	transcription factor Dp 1 (Tfdp1),	0.74	0.18
4525	Mm.26069	transferrin receptor (Trfr),	0.69	0.41
12310	Mm.26069	transferrin receptor (Trfr),	0.62	0.21
9292	Mm.14455	transforming growth factor, beta induced, 68 kDa (Tgfbi),	0.53	0.16
2380	Mm.27836	transforming, acidic coiled-coil containing protein 3 (Tacc3),	0.51	0.19
13371	Mm.27836	transforming, acidic coiled-coil containing protein 3 (Tacc3),	0.59	0.21
11833	Mm.1904	Ttk protein kinase (Ttk),	0.52	0.21
5301	Mm.1703	tubulin, beta 5 (Tubb5),	0.72	0.24

7956	Mm.89830	ubiquitin carrier protein E2-C (UBCH10),	0.74	0.18
3809	Mm.27496	ubiquitin specific protease 1 (USP1),	0.60	0.14
8274	Mm.27496	ubiquitin specific protease 1 (USP1),	0.74	0.20
11361	Mm.35830	Y1-globin gene	0.70	0.16
8439	Mm.7952	zinc finger protein (Peg3)	0.55	0.19
394	Mm.182931	similar to SAICAR synthetase and AIR carboxylase (ADE2H1),	0.73	0.24

Table 5: Genes Tested for p53-responsiveness by Real-time PCR

Function	Gene	WT log_2 (Mean Induction)	WT StDev	p21KO log_2 (Mean Induction)	p21KO StdDev
Apoptosis					
	KILLER/DR5	1.3	0.2	1.2	0.1
	Bax	1.1	0.0	1.7	0.3
	Bak	-0.5	0.1	-0.1	0.1
	bcl-2	0.3	0.6	-0.8	0.0
	c-FLIP	2.7	0.5	0.4	0.7
	Bcl-X	0.9	0.6	1.3	0.6
	DRAL	1.1	0.3	1.8	0.1
	Noxa	3.5	0.1	3.4	0.4
	APAF1	0.7	0.8	0.3	0.5
	Puma	1.4	0.2	2.6	0.3
	Perp	3.1	1.0	6.5	0.2
	TBX1	0.5	0.5	-0.8	0.5
	Caspase 6	-1.3	0.3	1.4	0.5
Regulation/Cell Cycle					
	MDM2	3.7		3.2	
	p21/WAF1(mouse)	8.9	1.3	-9.9	5.7
	cyclin G	4.4	2.5	3.5	2.6
DNA repair					
	Mgmt	3.2	0.3	3.0	0.3
	BTG2	4.2	2.5	3.9	2.4
	DDB2 (p48)	-1.5	0.5	-0.4	0.1
	hMSH2	-2.4	0.1	-0.4	0.2
	GADD45alpha	-2.4	0.2	0.6	0.4
	GADD45beta	-0.8	0.3	0.8	0.0
	GADD45gamma	1.5	0.3	-2.4	0.5
	XPG	1.9	0.6	2.0	0.5

Untested DNA Repair				
RAD51 homolog2	-3.1	0.1	-0.7	0.3
RAD51 homolog 4	-0.3	0.1	0.2	0.2
Blm	-2.9	1.7	-1.2	0.1
Brca1	-3.7	0.3	-0.6	0.0
Brca2	-2.1	0.1	0.0	0.5
Chek1	0.0	0.0	0.0	0.0
Clic4	0.4	0.1	-0.9	0.1
Ddb1 (p127)	0.8	0.5	0.4	0.4
Ddit3 (CHOP)	-0.2	0.1	-0.8	0.0
Nbn	-1.3	0.3	0.0	0.2
Rad18	-1.1	0.7	0.2	0.4
Rad23a	0.1	0.2	0.1	0.2
Rad23b	0.3	0.1	1.6	0.0
Rev3l	-0.3	0.6	0.6	0.9
Xpa	0.0	0.1	-0.2	0.2
Apex1	-0.9	0.1	-0.3	0.1
Atm	-1.3	0.2	-0.2	0.2
Dclre1a	0.1	0.3	-0.2	0.0
Dmc1h	-3.0	0.6	-1.4	1.3
Ercc1	0.1	0.1	0.1	0.2
Ercc3 (xpb)	-0.3	0.3	1.0	0.4
(XPF)"Ercc4"	-4.1		1.3	
Fancc	-0.6	0.6	-0.4	0.3
G22p1(Ku70)	-0.9		-0.8	
Gtf2h2	-1.6	0.7	-0.4	0.2
Gtf2h4	0.0	0.2	-0.6	0.2
Lig1	-1.5	0.8	-2.0	0.7
Mbd4	-1.3	0.5	-0.7	0.4
Mlh1	-1.2	0.1	-0.3	0.1
Mre11a	-1.5	1.1	0.0	0.1
Msh5	-0.4	0.2	0.4	0.0
Msh6	2.2	0.0	-0.1	0.0

Mutyh	-3.0		-0.3	
DNA-3-methyladenine glycosylase	0.8	0.1	0.7	0.4
PMS1 protein homolog 1	-0.1		-1.0	
Nudt1	-0.3	0.3	-0.4	0.3
Ogg1	-0.8	0.3	0.4	0.6
Pms2	-2.0		-0.4	
Polh(XPV)	-0.8		-2.5	
Rad52	0.4	0.8	0.0	0.0
Tdg	0.2	0.3	-0.4	0.5
Trex1	-0.6	1.0	1.8	2.1
Trex2	-1.3		-0.5	
Ung	-2.0	0.8	-0.3	0.6
Xrcc5 (Ku80)	-0.3	0.3	0.1	0.4
RAD17	-1.7		1.3	
RAD9	-0.4		-0.4	
RAD51-associated protein	-2.6		-0.9	
RAD6 homolog (E2A)	0.7		-0.2	
DNA Pol alpha catalytic subunit	1.5		1.7	
DNA Pol epsilon catalytic subunitA	-2.3	0.0	-0.5	0.0
DNA Pol zeta	-0.5	0.6	-0.7	0.9
DNA Pol delta subunit 2	-1.6	0.3	-0.7	0.2
DNA Pol mu	6.9		-1.2	
DNA Pol gamma 2	-0.7		2.1	
DNA Pol epsilon B	-1.5	0.2	-0.6	0.2
DNA Pol kappa	2.2	1.0	2.3	1.2
DNA Pol alpha B	2.7		-0.1	
DNA Pol Delta 4	0.6		0.7	
DNA Pol lambda	-0.8	0.6	0.0	0.1
DNA Pol Delta 3	-1.9	1.1	-0.2	1.5
DNA Pol beta	0.9	0.9	-2.9	8.0
DNA Pol delta catalytic subunit	-2.9	0.7	0.3	1.2
DNA Pol gamma 1	0.2		1.0	
Ckn1 (CSA)	1.2		2.3	
Ckn2 (CSB)	-6.0		1.4	

NEIL1	-0.1		-2.2	
XAP2	0.2	0.1	0.3	0.4

Table Legends

Table 1

Table 1a shows MEF cell genotypes and treatment of the samples used to generate the microarray data in Figures 1 to 4. RNA harvesting, and sample preparation for microarray hybridization are described in the Methods section, and in the Figure legends.

Table 1b lists the samples and conditions for other microarray experiments whose filtered data were incorporated into the analysis listed in Tables 2 to 4. These microarrays were not of the same overall quality as those of Table 1a, but detected many of the same upregulated genes.

Table 2

Known p53 target genes whose levels were upregulated in the microarray experiments. Mean ratios are the fold induction relative to the level of expression in *Trp53-/-* cells, taken from globally normalized microarray spot fluorescence values as described for Figure 1.

Table 3

Previously unreported genes whose transcript levels are higher in *Trp53+/+* MEFs than in *Trp53-/-* MEFs under the conditions of the microarray experiments. Mean ratios are the fold- inductions as in Table 2. The identity of the genes was obtained from the annotation provided for the 15K Mouse Clone set from the National Institute of Aging, and was compared to the p53-responsive genes in the scientific literature, as listed in Table 1 of Chapter 1 of this thesis.

Table 4

Genes whose transcript levels were lower in *Trp53+/+* MEFs compared to the *Trp53-/-* MEFs. Mean ratios are fold-inductions, as in Table 2.

Table 5

Real-time PCR measurements of the transcript levels for various p53 target genes, and previously untested DNA repair genes. As indicated in the legends for Figures 5 to 8, the induction values are expressed as log to the base 2 values of the fold induction, i.e., a value of 1 indicates 2-fold induction. All values are relative to the expression in *Trp53-/-* cells.

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Chapter 4: *Xpg* is a Direct Target of p53

Abstract

The p53-dependent regulation of *Ercc5*, the gene encoding the mouse homolog of the *ERCC5 or XPG* xeroderma pigmentosum group G disease gene, was analyzed. Microarray data indicating that this gene is induced by p53 were verified by real-time PCR analysis. *Xpg* is upregulated by p53 in response to several different DNA-damaging stimuli, and it appears to be a $p19^{ARF}$-dependent gene. Upregulation of *Xpg* transcript was also observed in several tissues of gamma-irradiated mice. Xpg protein levels increase under induced conditions, as measured by a single-cell FACs assay. The *Ercc5* gene contains a p53-responsive site in the first intron; the site is a near-match for the p53 consensus sequence, and mutating the core base-pairs of one half site ablates the p53-inducibility of the construct. Finally, preliminary data indicate that *Trp53-/-* cells have a modest deficiency in repair of an oxidatively-damaged plasmid, suggesting that p53 regulation of *Ercc5* can have consequences for cellular responses to damaged DNA.

Introduction

The p53 transcription factor is best known as a regulator of genes such as *Cdkn1a*, which encodes the cyclin-dependent kinase inhibitor p21, and for inducing cell death under some conditions by upregulating small pro-apoptotic Bcl-2 homologous genes such as *Bax*, *Noxa*, and *Puma*. An accumulating body of evidence is beginning to support the notion that p53 is involved in DNA repair as a third major role for preventing cells from becoming cancerous.

In vivo data mainly derive from mouse strains that enable facile detection of mutations. Mice carrying an integrated *LacZ* transgene in their genome show p53-dependent differences in the accumulation of mutations at older ages, although no effect was seen in young mice unless they were given mild irradiation (Giese et al., 2002). However, even in untreated mice, in similar experiments, the mutational spectrum in neurons was found to differ between *Trp53* wild-type and null mice, indicating that a more subtle perturbation of the normal repair pathways was taking place (Buettner et al., 1997). A different mouse system for mutation detection, the pink-eyed-dilution unstable strain (p^m), carries a tandem duplication region in the *P* gene. Intrachromosomal recombination of the tandem duplication restores wild-type function to the *P* gene, and permits melanocytes to develop normally and produce pigment in the skin, giving rise to a visible region of coat color change. Surprisingly, *Trp53-/-* mice had a decreased rate of intrachromosomal recombination at this locus, suggesting that some aspect of the DNA

homeostasis of the p53-null cell is abnormal, but underlining how complicated the topic is. This observation is reminiscent of the sorts of variable phenotypes that can arise from mutating different translesion polymerase genes in a variety of organisms: some phenotypes show increased mutation rates, others have lowered mutation rates, and still others show only a change in the spectrum of mutations (Friedberg et al., 2002).

From the point of view of the different canonical pathways of DNA repair, p53 has been suggested to play a role in both nucleotide excision repair (NER) and base excision repair (BER) (Seo et al., 2002). A different study, based on lymphoid cell lines expressing wild-type or mutant p53, showed that p53 was required for full induction of BER after irradiation (Offer et al., 2001).

A very interesting recent publication from the Hanawalt lab concerns the response of human fibroblasts to low levels of benzo-*a*-pyrene and related compounds, the chief carcinogens in tobacco smoke. The researchers used a very sensitive assay to measure the removal of these adducts from the DNA by an NER mechanism after exposure levels that were similar to those faced by the fibroblasts in a smoker's lung. A clear p53-dependent difference in the rate of removal of these lesions was observed, indicating in this highly physiological setting that p53 could be a major determinant of the mutagenesis rate of these cells (Lloyd and Hanawalt, 2002). These studies add to earlier data from this lab which showed that p53 was required for efficient NER removal of both major pyrimidine dimer products produces by UV irradiation of DNA (Ford and Hanawalt, 1997).

Mechanistic links between p53 and the regulation of DNA repair have been proposed, based on the p53-dependent expression of a few genes: *GADD45*, *XPC*, *DDB2*, O^6-*MGMT*, and the inducible ribonucleotide reductase gene *p53R2*. There are interesting differences in the mouse and human p53-induction of these genes. For example, *DDB2*, encoding an important component of the nucleotide-excision repair (NER) complex best known for removing bulky lesions like UV-irradiation-induced thymine dimers from the DNA, is apparently a methylated and silenced gene in mice, although it is a p53-regulated gene in humans. It has been speculated that this difference is related to the differential evolutionary selection pressures on the DNA repair pathway genes of diurnal hairless humans compared to nocturnal furry rodents (Hanawalt, 2001). *XPC*, which encodes an early component in the assembly of the NER complex, is also reported to be p53-inducible in human cells (Amundson, 2002). *GADD45*α appears to be involved in maintaining genomic stability, based on the phenotype of the null mouse (Hollander et al., 1999). The exact nature of links between this protein and repair processes are not known, however. *p53R2* behaves in a manner similar to its prokaryotic homologs' inducible role in the SOS response, and assists the cellular response to damaged DNA by supplying increased amounts of the raw material of DNA repair, deoxy nucleotides, in times of need. Finally, there are some data in the literature, based on *in vitro* assays, suggesting that p53 protein itself has some intrinsic nuclease activity that can participate in DNA repair.

This report describes evidence that the gene for Xpg, a key member of the two main DNA repair pathways in the cell, with a recently discovered role in the repair of oxidative

lesions to the DNA, is directly regulated by p53 in response to DNA damage and other stimuli.

The *XPG* gene has been found to be mutated in a rare subset of xeroderma pigmentosum patients who can have clinical features ranging from the early neurodegeneration of Cockayne syndrome to the more conventional photosensitivity and skin cancer incidence of XP patients. A possible reason for this observation was discovered in the past several years: in addition to playing a catalytic role as the 3'endonuclease member of the NER complex, it appears that XPG plays a separate role in potentiating the BER removal of oxidative lesions from damaged DNA, particularly from the transcribed strand whose integrity is essential for the faithful production of mRNA (Le Page et al., 2000). This second activity does not require that the endonuclease domain be functional, so it would appear that XPG may act as a coordinator of other BER proteins via protein-protein interactions (Nouspikel et al., 1997). It is clear from the severely runted phenotype of *Xpg-/-* mice that this gene is required for development or maintenance of the cells in a wide variety of organs, particularly the GI tract (Harada et al., 1999).

The implications for p53 regulation of XPG in mammals are considerable; it is notable that the yeast homolog of *Xpg, Rad2*, was shown to be a DNA-damage-inducible gene, indicating that there has been long-term evolutionary conservation of the inducibility of this gene (Siede and Friedberg, 1992). Also, the removal of oxidative damage from the DNA of mammalian cells has been shown to be an inducible phenomenon that can be upregulated by low "priming" doses of X-irradiation (Weinfeld et al., 2001). p53-

mediated upregulation of *Xpg* in the mouse would be an excellent mechanistic candidate for this induction.

Materials and Methods

Cell Culture

Murine embryonic fibroblasts (MEFs) were isolated from day 13.5 embryos, and were cultured in DME media supplemented with 10% fetal calf serum, 5mM Glutamine, and penicillin and streptomycin (Gibco-BRL), in humidified incubators with a 5% CO2 incubator. Cells were used between passages 2 and 7. Retroviral infections with the pMCSV-hygro vector or pMCSV-XPG, containing the full-length *Ercc5* cDNA, were conducted following Calcium phosphate transfection of Phoenix cells. Infected MEFs were selected with hygromycin for 7 days following infection and then used for experiments.

Drug and irradiation treatment of cells

Gamma-irradiation of cells was carried out in a Gammacell irradiator at a rate of 72 rad/second. UV irradiation was administered with a Stratalinker. Cisplatin (Sigma) and doxorubicin (Sigma) were dissolved in PBS prior to addition to cells.

Harvest of RNA

To harvest RNA from cultured MEF samples, media was aspirated from cells, and 5 mL of Trizol was added per 10 cm plate. Cells were scraped into Trizol, and pipetted several times to lyse them. The manufacturer's protocol of chloroform extraction and isopropanol precipitation was then followed, and RNA was redissolved in RNAse-free water after precipitation and washing. Mouse tissue samples were harvested rapidly, added to Trizol, and homogenized immediately using a Polytron dissociator for 30 seconds on setting 19. After letting the homogenate sit at room temperature for 5 minutes, the manufacturer's protocol was followed for extraction and precipitation of RNA.

For real-time PCR analysis, RNA was further purified using RNeasy micro spin columns (Qiagen), and on-column DNAse digestion was performed to ensure that any contaminating genomic DNA was removed.

Real-time PCR

5 micrograms of DNase-treated and cleaned total RNA was converted to cDNA in a reaction volume of 100 microliters, using Superscript II enzyme and the commercial protocol. RNA was hydrolyzed with RNase, then template was diluted in sterile water prior to addition to thermocycling plate. Primers and SYBR-Green-containing reaction mixture were added, and reactions were carried out for 50 cycles in an ABI 7000

instrument, with automatic gradient denaturation testing at the end of the run to ensure that single products were obtained.

Northern blotting

RNA samples were diluted to equalize concentrations, then concentrated formamide loading buffer was added, samples were heated for 1 minute at 70°C, briefly chilled on ice, then spun down and loaded on formaldehyde gels. After electrophoresis, gels were stained with ethidium bromide solution, then washed in RNase-free water, photographed to verify RNA quality, equilibrated in 20XSSC, and placed in capillary transfer apparatuses to transfer RNA to Hybond N membranes. RNA was fixed to membranes using a Stratalinker with two auto-crosslinking cycles. Prehybridization in ExpressHyb (Clontech) for at least 3 hours at 65°C was followed by ^{32}P-labeled cDNA probe incubation at 65°C in fresh Expresshyb buffer for at least 4 hours. Blots were washed at 65°C in 2XSSC 0.1% SDS solution with several changes, then wrapped in Saran Wrap and exposed to film with enhancer screens at –80°C for several hours to overnight. Probes for murine *Xpg* were PCR amplified from mouse cDNA using primers within the final 1500 bp of the *Xpg* sequence, and were radioactively labeled with random primers, ^{32}P-αdCTP, and Klenow enzyme. Probes for other genes were as described in Flores *et al.* (Flores et al., 2002).

Flow cytometric measurement of Xpg

These assays were conducted in collaboration with the laboratory of Dr. Cilla Cooper, Lawrence Berkley National Labs. MEFs differing in *Trp53* gene status were cultured, treated with DNA damaging agents, briefly trypsinized, and fixed with 70% ethanol. After washing in PBS, cells were blocked with 5% goat serum in buffer for 2 hours at room temperature, then were incubated with commercially available anti-XPG antibodies (Santa Cruz). After washing cells, fluorescently-labelled secondary antibodies were incubated with the cells, and then after final washing, data were acquired with flow cytometry. Cells that had been treated with only the secondary antibody were compared to cells treated with primary and secondary antibodies, to control for non-specific antibody binding to the cells.

Luciferase assays

Promoter fragments were cloned into the pGL3-luciferase vector (Promega) and sequenced. Mutation of the putative p53-responsive site in the "0 kb" construct insert was carried out by overlap PCR mutagenesis and cloning into the identical site in the pGL3 vector (Pogulis et al., 1996). Cells used for the assay were the *TP53*-null cell line NCI-H1299. Variation in transfection efficiency between cell samples was controlled for by cotransfection of the pRL Renilla luciferase expressing plasmid (Promega). Cells were transfected with 0.5 μg of the respective pGL3 plasmid, 50 ng of the control pRL plasmid, and 0.5 μg of either plasmid pCMV-NB (for uninduced samples) or plasmid pCMV-p53 which expresses human p53 under the control of the pCMV IE promoter. Transfection was carried out with Fugene 6 reagent, according to the manufacturer's

protocol (Roche). Assays were harvested at 15 hours post-transfection, and cells were washed once with PBS before being lysed in the passive lysis buffer from Promega and analyzed with a dual fluorescence luminometer.

Plasmid reactivation assay

The upstream 1 kb promoter sequence of the murine *Tubulin β3* gene was cloned into plasmids phRL-B, and pGL3 –basic, which are designed to give minimal background activity in transcriptional reporter assays (Promega). The pGL3-Tubβ3 vector was treated with 2% osmium tetroxide for 30 minutes at room temperature, after carrying out a dose-response study to find a treatment level that would significantly, but not completely, inactivate the activity of the plasmid in the reporter assay. Oxidatively-damaged plasmid was purified away from osmium tetroxide using a gel-filtration spin columns, followed by Qiagen DNA cleanup columns (Qiagen). Damaged plasmid concentration was then measured in an uv spectrometer, and samples were prepared for transfection by pre-mixing with the transfection control Renilla plasmid in the appropriate ratio. Passage 3 MEFs within 24 hours of plating were transfected with 0.5 μg of each plasmid per 6-well plate well of cells, using Fugene 6 with no variation from the recommended ratios of DNA, cell media, and tranfection reagent (Roche). At 10 hours post-transfection, cells were harvested as described for the promoter reporter assays, and luciferase and Renilla luciferase activity levels were determined. Duplicate experiments with triplicate transfection replicates were used to generate the data for each condition.

Results

Xpg/Ercc5 is a p53-regulated, partially p19^{ARF}-dependent gene in murine fibroblasts

Initial results from a large number of cDNA microarray experiments indicated that *Ercc5* gene was upregulated in *Trp53+/+* MEFs compared to *Trp53-/-* MEFs in response to several different genotoxic stimuli, and also, to a lesser degree, in untreated MEFs (Chapter 3, this thesis). To further validate these data by the use of an independent method, real-time PCR was carried out on samples of MEFs treated with DNA-damaging agents. The data in Figure 1 show that *Ercc5* is in fact upregulated in a p53-dependent manner in response to the DNA-damaging agents used here.

The Northern blot of the *Xpg* transcript shown in Figure 2 demonstrated that only one transcript form was detected, and further revealed that the upregulation of the *Xpg* transcript is not affected by p21^{CIP}-dependent effects on the cell cycle of irradiated cells. The inclusion of cells deficient in the p53 upstream regulator p19^{ARF} showed that the *Xpg* transcript, like most of the p53-responsive genes in the cell, shows lesser induction in the absence of p19^{ARF} than in its presence. In this sense, then, the *Xpg* transcript behaves as a typical p53-responsive gene. The overall levels of induction of the *Xpg* message are similar to those observed for pro-apoptotic target genes like *Bax* and *Noxa*, and are less extreme than the induction of the *p21^{CIP}* gene.

Primary mouse tissues show p53-dependent Xpg upregulation

To assess the induction of the *Xpg* transcript in primary mouse tissues, *Trp53* wild-type and null mice were treated with 5 Gy of gamma irradiation, and sacrificed at 6 hours post-treatment. RNA harvested from tissues showed modest levels of increased expression in *Trp53+/+* mice compared to the *Trp53-/-* samples (Figure 3). The tissues showing the greatest increase in *Xpg* transcript levels were the thymus, spleen, small intestine, and liver. Interestingly, the first three are among the most irradiation-sensitive tissues in the body, and the intestine is the site of the most dramatic degenerative phenotype in the *Xpg-/-* mouse (Harada et al., 1999). These findings suggest that the p53-responsiveness of *Xpg* in these tissues might be of some functional significance.

The Xpg gene is directly responsive to p53

To ascertain whether or not the effects of p53 on *Ercc5* expression were direct, rather than being some secondary consequence of other cellular alterations caused by p53, the *Ercc5* promoter was dissected and tested for its ability to respond to p53 directly. Prior to this analysis, putative p53-binding sites were identified in the promoter with the assistance of the laboratory of Dr. Wing Wong at the Harvard School of Public Health. A 6 kb-long genomic fragment containing the promoter, the 304 bp first exon, and the proximal region of the first intron was shown to be able to confer p53-inducibility to the luciferase plasmid, and when this region was dissected into smaller roughly 1 kb-long fragments, it was found that the proximal-most 1kb fragment conferred the p53-

responsiveness to the plasmid. A putative p53-binding site beginning at position +903 relative to the beginning of the first exon was mutated, and the resulting construct was tested for p53-responsiveness (Figure 6). It was determined that the construct with the mutated binding site was now entirely unresponsive to the presence of p53, indicating that it is the likely mediator of this response. The p53 binding site at this position perfectly matches the originally-derived consensus sequence for the core 4 nucleotides of each half site. It varies from the consensus at the two outermost base pairs of the 3' half site, and at one basepair in the left-most half-site; however, these outermost basepairs are generally less well conserved in p53-sites found in a variety of other well-studied target genes (see the TRANSFAC database at http://transfac.gbf.de). The position of this site is in good agreement with an increasingly obvious tendency for p53 binding sites to be located in the first introns of the target genes, as is the case for *Bax*, *Perp*, *Killer*, and a number of other genes (Thornborrow et al., 2002).

Modestly decreased repair of oxidatively-damaged DNA in p53-null cells

The oxidizing agent osmium tetroxide efficiently catalyzes the formation of thymine glycol modifications in DNA in vitro. In fact, DNA treated with this reagent has been used as the antigen for generating antibodies specific for this particular DNA modification (Chen et al., 1990). As an initial approach for testing *Trp53-/-* cells for phenotypes that might be the result of impaired levels of Xpg protein, plasmid activity rescue experiments were conducted, where the ability of cells transfected with a damaged plasmid to restore its function is tested. The choice of treatment concentration and time

were dictated by prior dose-response experiments that indicated that higher concentrations of OsO_4 damaged the plasmid irreparably, while shorter treatments did not give significant damage for appreciable rescue in the assay. In order to control for cell-cycle differences between the unsynchronized *Trp53-/-* and wild-type MEF populations, *p21-/-* MEFs were also included in the experiment. Furthermore, to test for the *Xpg*-dependence of the plasmid rescue, exogenous *Xpg* was introduced into the *Trp53-/-* MEFs by retroviral infection to see if this could augment any deficiencies in the repair ability measured in the assay.

The results shown in Figure 7 are consistent with the notion that *Trp53-/-* cells lack sufficient *Xpg* to repair the plasmid as well as wild-type or *p21-/-* MEFs. The effect observed is modest, but this is not unexpected, given that the cells under consideration are not null for the gene, but merely show decreased upregulation of it. These data can thus be taken as an initial indication that *Trp53-/-* cells exhibit a deficiency in this kind of DNA repair, which can be rescued by adding additional *Xpg* .

Conclusions

These experiments establish that *Ercc5*, the gene encoding the essential DNA repair protein Xpg, is directly regulated by the p53 tumor suppressor in primary cells of mice. This finding is of considerable interest, for in combination with the previously mentioned p53 regulation of DNA polymerase kappa, a possible mediator of error-free replication bypass over oxidative lesions in the DNA, it appears that a new branch of p53 effector functions may have been uncovered. Although the phenotypic data from the plasmid

rescue assay presented here do not show a total deficiency of repair activity, a complete loss of *Xpg* function was not anticipated, since it appears that only the induced response levels of *Xpg* expression are impaired in p53-null cells. It is quite possible that an impaired pathway of this sort could contribute to the effects of loss of p53 on tumor development, as *Xpg* also contributes to the removal of a wide variety of other sorts of DNA lesions; indeed, such repair deficiencies in p53-null cells have already been reported in the literature, but the genes responsible for them are not yet clear. *Xpg*, could, for example, be a candidate p53 target gene involved in the removal of benzo-*a*-pyrene, a clearly p53-dependent phenotype in human cells (Lloyd and Hanawalt, 2000; Lloyd and Hanawalt, 2002).

The p53-regulated portion of the *Ercc5* gene is found within the first intron of the gene, as is typical for many p53 targets, and contains a sequence showing reasonable resemblance to the reported p53 binding consensus site (el-Deiry et al., 1992). It should be noted that the familiar RRRCWWGYYY...RRRCWWGYYY consensus site for p53 was derived from only the first p53-binding sites that were discovered, and in the years since then, a number of variant sites that are bound well by p53 have become known, for example, the unusual repeat sequence bound in the *Pig3* promoter (Contente et al., 2002).

Some aspects of the phenotype of *Trp53-/-* could also be accounted for in part by impaired responses to oxidative stresses, for example, the decreased male fertility, and possibly also the phenotype of the partial *Trp53* deletion strain that was reported to have an early aging phenotype (Tyner et al., 2002). It would be interesting to investigate the

phenotype of p53 null mice as well for signs of early aging. To carry out this experiment, however, thymectomy of newborn mice would have to be done to prevent the very early onset thymomas that *Trp53-/-* mice develop. It is possible that other tumor development would occur too rapidly to enable a phenotype related to aging to be observed.

It is challenging to sort out whether the mutagenic effects of losing a multi-functional protein with complicated cellular activities like p53 are due to the loss of its DNA-repair enhancing activities, or are rather a side-effect of the pro-apoptotic effects of the p53's action in damaged cells, or the increased cell cycle delay caused when p53 is activated. Recent papers have given an indication that neither cell-cycle arrest, nor apoptotic mechanisms are necessarily essential for p53 and its homologs in other organisms to fend off genomic instability. The Lowe lab recently showed that lymphomas developing in a *Myc*-transgenic mouse model are not under selective pressure to mutate their p53 genes if they overexpress the *Bcl-2* gene or a dominant negative *Caspase 9* construct (Schmitt et al., 2002). Further, the lymphomas in such mice are aggressive, but do not develop aneuploidy as *Trp53-/-* tumors do, suggesting that loss of p53 leads to aneuploidy through a mechanism distinct from the suppression of cell death (Schmitt et al., 2002). Interestingly contrary results, and a questionable interpretation of the data of the Schmitt *et al.* paper come from Sogame *et al.*, studying the phenotype of Drosophila with a deletion in the *Dmp53* homolog of p53. These researchers find that *Dmp53-/-* flies are susceptible to irradiation-induced genomic instability, and since the fly p53 does not cause G1/S arrest of the cell cycle, they conclude that their results agree with those of Schmitt et al. in saying that apoptotic mechanisms (which can be executed by *Dmp53*)

account for the normal genomic stability of *Dmp53* wild-type flies (Sogame et al., 2003). Of course, these are two very different experimental systems. It is certainly possible that different organisms might require a different one of the two main p53 activities, cell cycle arrest or apoptosis, to prevent genomic instability. For example, *Myc*-driven lymphomas might need only cell cycle arrest to be able to preserve their genomic DNA, while flies may rely on an apoptosis program to protect themselves against genomic mishaps. However, it seems plausible and even likely that other p53-inducible cellular systems might be involved in DNA maintenance; clearly, DNA repair would be at the top of a list of candidates for such a role. It will of considerable interest to see how much of the network of p53-regulated genes has been conserved in the evolution of the many organisms that possess p53-homologous genes.

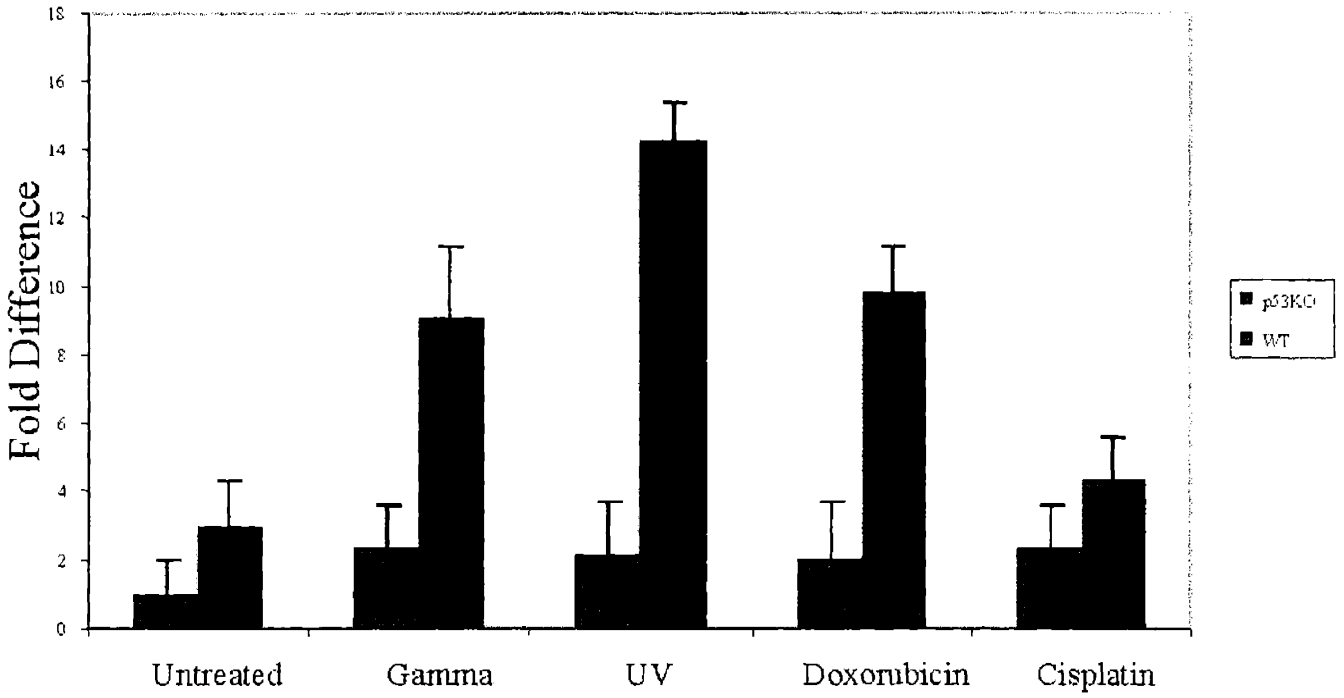
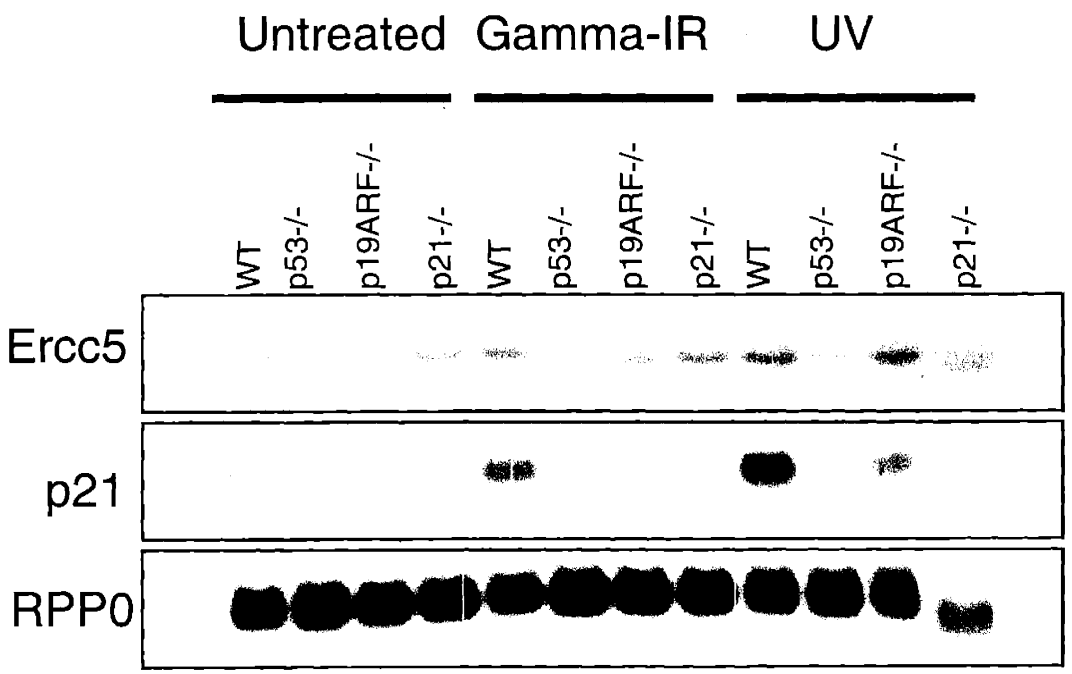

Fold Difference
18
16
14
12
10
8
6
4
2
0
Untreated
Gamma
UV
Doxorubicin
Cisplatin
p53KO
WT

Figure 1


Untreated
Gamma-IR
UV
WT
p53-/-
p19ARF-/-
p21-/-
WT
p53-/-
p19ARF-/-
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p21-/-
Ercc5
p21
RPP0

Figure 2

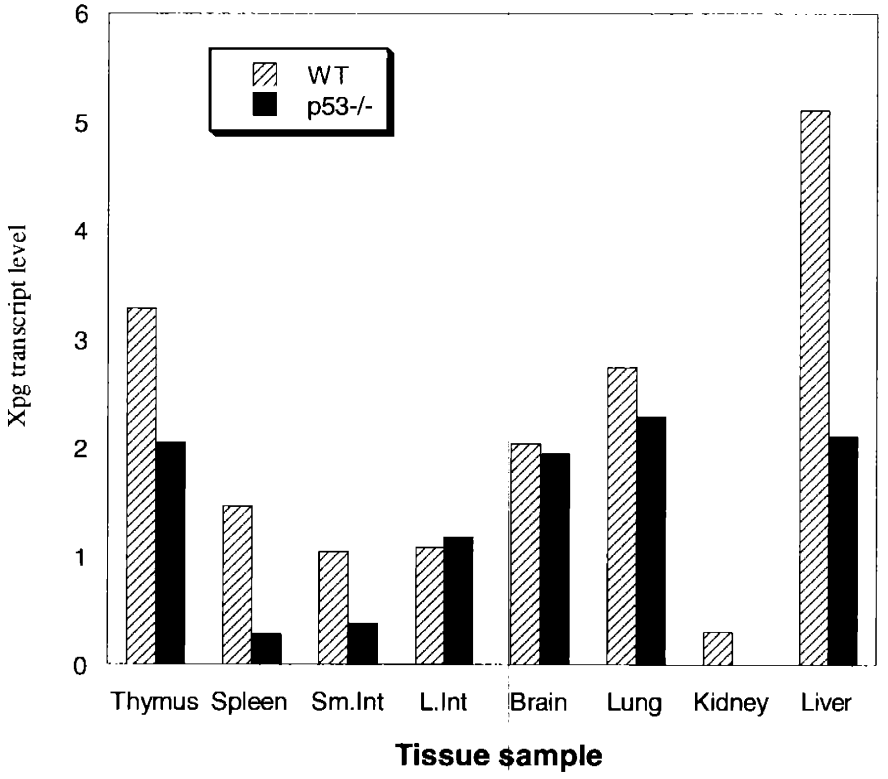

6
5
4
3
2
1
0
WT
p53-/-
Xpg transcript level
Thymus
Spleen
Sm.Int
L.Int
Brain
Lung
Kidney
Liver
Tissue sample

Figure 3

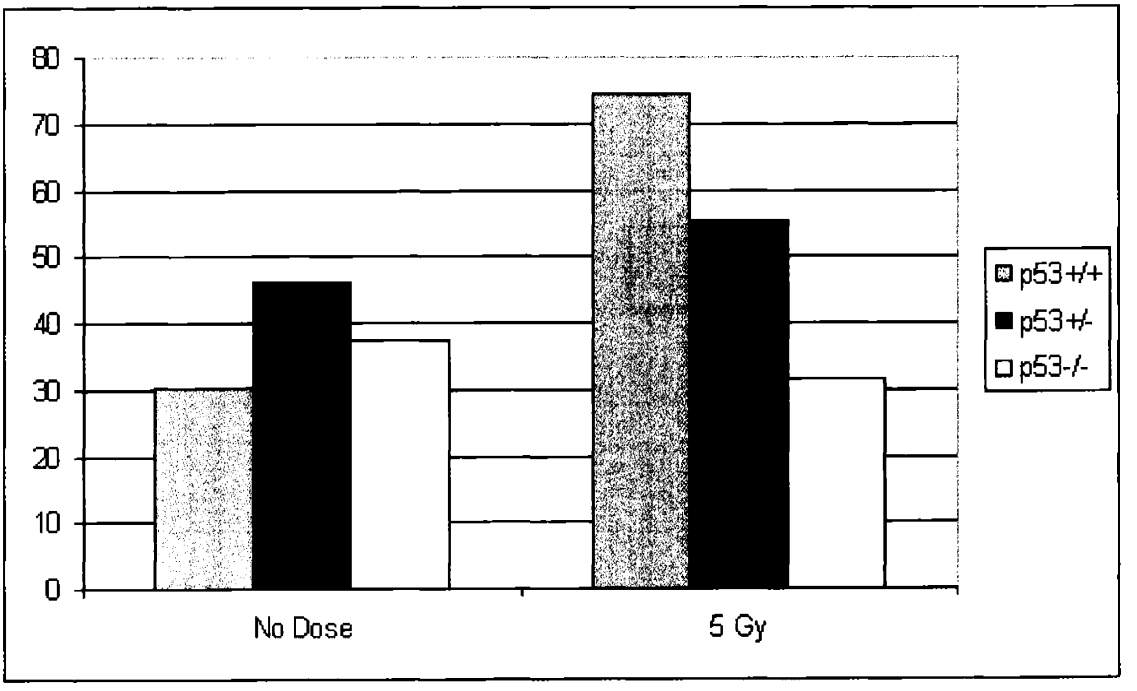


Figure 4

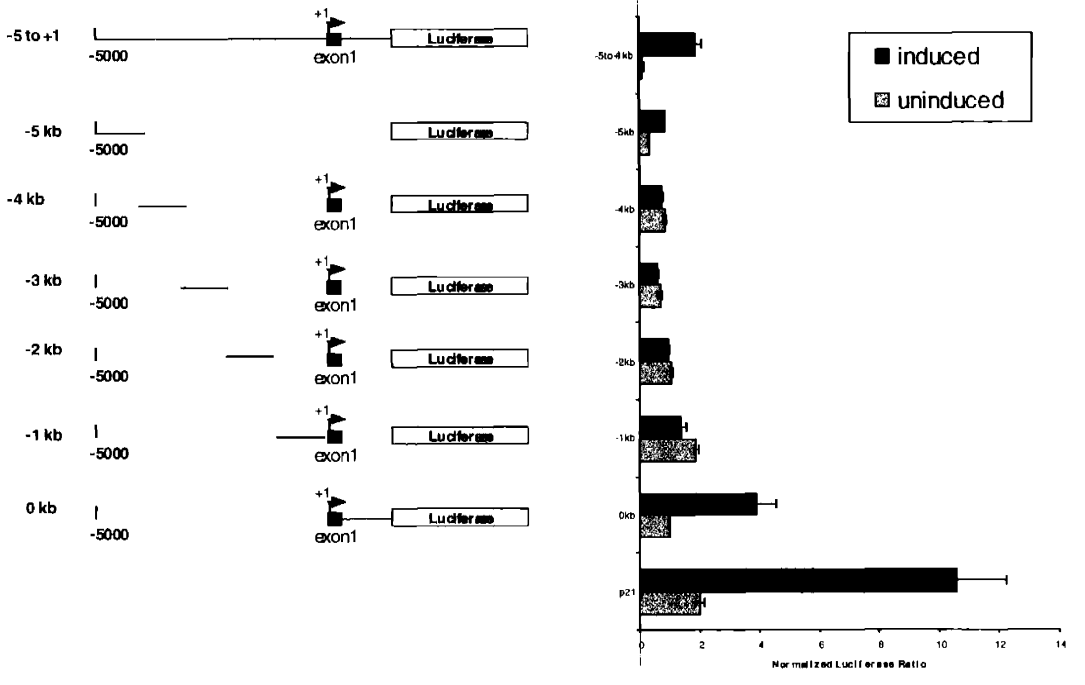
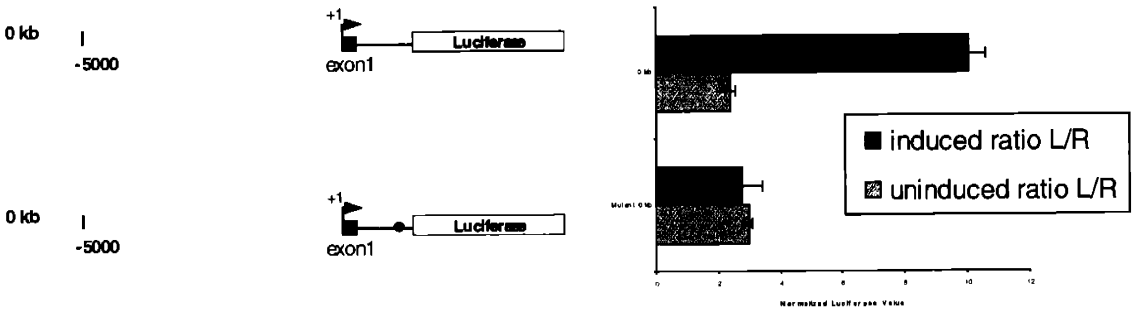

-5 to +1
-5000
+1
exon1
Luciferase
-5 kb
-4 kb
-3 kb
-2 kb
-1 kb
0 kb
induced
uninduced
-5to-4 kb
-5kb
-4kb
-3kb
-2kb
-1kb
0kb
p21
0
2
4
6
8
10
12
14
Normalized Luciferase Ratio

Figure 5



Site at bp 907: gtaCatGcct ggacgtgtgtccgtaggtgg tggCatGcca
Mutated site : gtaaaaacct ggacgtgtgtccgtaggtgg tggCatGcca

Figure 6

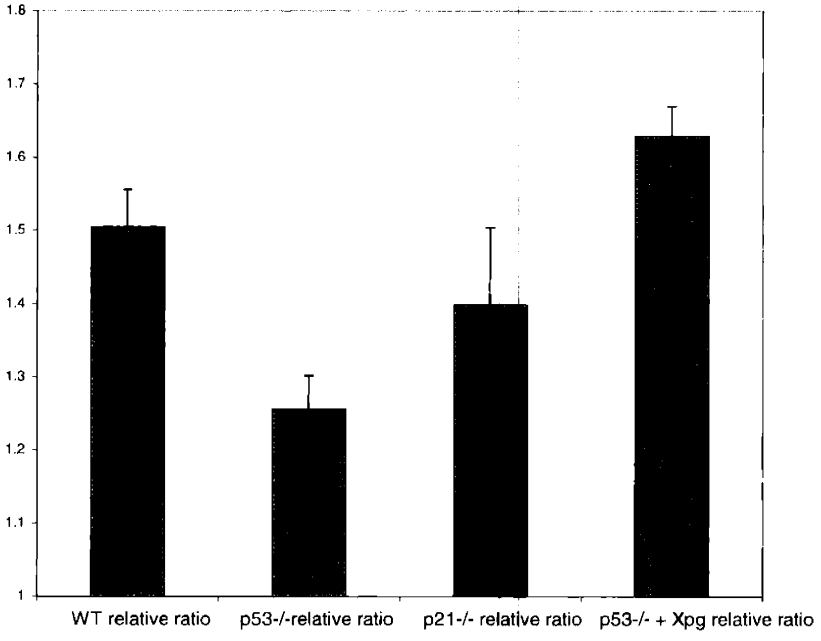

1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
WT relative ratio
p53-/-relative ratio
p21-/- relative ratio
p53-/- + Xpg relative ratio

Figure 7

Figure Legends

Figure 1 *Real-time PCR measurement of Xpg transcript*

Unsynchronized MEFs were cultured as indicated in the Methods section, then were treated with either 10 Gy gamma irradiation, 10 J/m^2 of ultraviolet radiation, 40 μM cisplatin, or 200 ng/mL doxorubicin. RNA was harvested at 6 hours post-treatment, and purified of contaminating genomic DNA. Reverse transcription from total RNA was used to generate template for real-time PCR analysis, which was carried out as described in the Methods section.

Figure 2 *Northern blotting of Xpg*

MEFs were treated with Gamma irradiation and UV irradiation as in Figure 1, and total RNA was harvested after 6 hours and probed with a 3' probe within the *Ercc5* gene cDNA sequence. *p21* and the loading control *ARPP0* were then hybridized with the stripped blot to confirm induction of a known p53-target gene in the case of *p21*, and to be sure that gel loading and transfer occurred evenly, in the case of *ARPP0*.

Figure 3 *Primary mouse tissue RNA analyzed by real-time PCR*

Mice were sacrificed 6 hours after treatment with 5Gy of gamma irradiation, and RNA from tissues was processed for real-time PCR as in Figure 1. Levels of expression are normalized to the lowest sample level measured in the experiment, so that relative levels in different tissues could be displayed.

Figure 4 *Xpg protein induction*

FACs analysis of Xpg levels in ethanol-fixed cells was carried out by incubation with anti-XPG antibody, washing, and fluorescent secondary antibody staining. Levels of Xpg in cells were assessed by comparison of fluorescence values with a second sample that was identical except for having been stained with matched non-specific primary antibody.

Figure 5 *Analysis of p53-responsiveness of the Xpg sequences*

Portions of the *Ercc5* genomic locus were PCR-amplified and cloned into a luciferase reporter vector. H1299 cells were transfected with the constructs as well as a Renilla luciferase plasmid, to control for variations in transfection efficiency, and either a control pCMV plasmid, (for the "uninduced" samples) or pCMVNB-p53, which expresses p53 (for the "induced" samples). 15 hours after transfection, cells were harvested and assayed

for luciferase and renilla luciferase activity. Luciferase activities were normalized to the cotransfected renilla luciferase value. Data were gathered in triplicate for each condition.

Figure 6 *Mutation of the putative p53-responsive site in Xpg exon 1*

Experiments were conducted as for Figure 5. The mutated plasmid alters 2 of the 4 core bases in the 5'-most half site, as shown.

Figure 7 *Rescue of oxidatively-damaged plasmid activity varies with p53 status*

The plasmid pGL3β3Tub was treated with Osmium tetroxide and then repurified. *Trp53+/+*, *Trp53-/-*, and $p19^{ARF}$-/- MEFs, and *Trp53-/-* MEFs infected with XPG-expressing retrovirus were transfected with the oxidatively-damaged plasmid and, as a transfection control, undamaged Renilla luciferase plasmid containing the same regulatory sequences. Cells were harvested 10 hours later and cell lysates were prepared. Cell lysates were assayed for luciferase activity and Renilla luciferase activity. Normalized luciferase ratios relative to the undamaged plasmid are plotted for each genotype. Increasing ratios represent the increased reactivation of the oxidatively-damaged plasmid by the host cells.

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Chapter 5: Conclusions

Table of Contents

Regulation of p53 by ubiquitination-coupled nuclear export

The findings of these experiments, and subsequent studies published in the literature, indicate that p53 localization in the nucleus is a dynamically regulated process where the rate of nuclear export may determine the overall amount of time spent in the nucleus. As the majority of p53 functions are thought to be mediated by its transactivation of target genes, this localization control is likely to be functionally significant. The previous model in the literature suggesting that HDM2 acts in a manner similar to the HIV Rev protein, as a nuclear shuttling protein with p53 as its passive cargo, is not supported by the findings here, as mutation of the HDM2 nuclear export sequence (NES) had no effect on its ability to relocalize p53 to the cytoplasm. The p53 nuclear export sequence, however, was essential for this location change. A unifying link between HDM2-mediated ubiquitination of p53, and control of its localization, was provided by the observation that HDM2 mutants unable to carry out ubiquitination, but still competent to bind p53, were completely inactive for the nuclear export of p53. This finding is consistent with the known structure of the p53 C-terminus, where a NES-like sequence is predicted to be obscured by tetramerization of the oligomerization domain, and where the lysine residues targeted by ubiquitin are located immediately next to the oligomerization domain, where they could conceivably alter the conformation or stability of this region of the protein. The data presented here do not imply that the tetramerization of p53 must be disrupted

completely to expose the NES; a change in conformation or accessibility could potentially serve as well for the proposed mechanism.

The link between p53 ubiquitination and control of its localization within the cell parallels other results gathered in the past few years indicating that ubiquitination, similar to modification with other small ubiquitin-like proteins, can serve other purposes aside from being a signal for proteasomal degradation. For example, internalization of the beta-adrenergic receptor from the plasma membrane, and the degradation of the receptor, are controlled by ubiquitination mediated by the beta-arrestin protein. A surprising study was published recently that provided some evidence that Mdm2 might actually play a role in this ubiquitination and relocalization event as well (Shenoy et al., 2001).

The story of p53 localization control continues to grow more involved as additional studies are published. For example, in addition to the likely nuclear export-mediated mechanism involving the C-terminus of p53 presented here, a detailed study of the p53 N-terminus uncovered a second nuclear export sequence that could be concealed by DNA-damage-induced phosphorylation of the protein (Zhang and Xiong, 2001). Furthermore, a cytoplasmic Parkin-related ubiquitin ligase protein termed Parc has been identified that can sequester p53 in the cytoplasm, and which is a candidate mediator of the p53 mislocalization observed in neuroblastoma cells (Nikolaev et al., 2003).

Microarray analysis of p53-dependent gene expression following different genotoxic treatments

As a global survey of p53-responsive gene expression in untransformed cells, the data presented in Chapter 3 both confirm and broaden current understanding of p53. Looking at this sort of genomic data set, gathered in an unbiased manner, emphasizes the artificial quality of the linear pathways used to simplify the presentation of functional relationships between biological molecules, for it is evident that alterations in p53 status affect a wide variety of genes associated with many of the diverse activities of the cell. Many of these cellular effects are expected to be indirect consequences of primary activities of the p53 protein, but by studying early time-points, and comparing cells whose only genetic difference is the deletion of the *Trp53-/-* gene, the chances of detecting new direct transcriptional targets are maximized.

The most striking results of the microarray experiments presented in Chapter 3, and the subsequent real-time PCR experiments, are the discovery of two novel p53-regulated genes, *Polk* and *Ercc5*. These two genes play a role in DNA repair and the maintenance of genomic stability. Together with previous findings that indicated that the inducible ribonucleotide reductase gene *p53R2* is also regulated by p53, these findings show that in mammals, p53 has become responsible for some very ancient inducible DNA damage responses, for the homologs of these genes are similarly DNA-damage-inducible in organisms from bacteria to yeast, albeit by the SOS response in bacteria, and by DNA-damage response element-binding proteins in the yeast (Siede and Friedberg, 1992; Tang

et al., 2000). In the case of *Polk* and *Ercc5*, characterization of the biological activity of these proteins suggests that they both may play an important role in the response to oxidative lesions to the DNA of the cell (Le Page et al., 2000; Weinfeld et al., 2001). In the case of DNA polymerase kappa, the enzyme has been shown to specifically insert the correct base (A) opposite a damaged thymine glycol, thus preventing the fixation of a mutation (Fischhaber et al., 2002). Furthermore, the XPG-dependent base-excision repair removal of oxidative lesions from the DNA is known to be inducible by X-irradiation; p53 is a strong candidate for the mediator of this regulatory function.

The organism most divergent from humans so far found to possess a DNA-damage-inducible, p53-homologous gene is *Entamoeba histolytica*; it will be interesting to see which genes are regulated by the putative p53-ancestral protein in this organism, as well as in the fruit fly and nematode worm. At least in the fruit fly, initial data suggest that the p53-homologous protein Dmp53 is required for genome stability in response to irradiation, and that the protein does not control the cell cycle, but is capable of inducing apoptosis (Sogame et al., 2003). It would be revealing to know whether DNA repair pathways in these divergent organisms are p53-responsive or not; if they are, it would be an indication that p53-regulation of DNA repair is part of an ancestral program of p53 genome defense. Given that *Entamoeba histolytica* is a single-celled organism, it might be unexpected if its p53 pathway caused cell death in response to DNA damage. In any case, it can be expected that future experiments will uncover the settings in which p53-mediated upregulation of DNA repair genes may be important for preventing the development of cancer in the cells and tissues of animals

In contrast to *Ercc5* and the known p53 induced DNA repair gene O^6-*Mgmt*, the vast majority of the other DNA repair genes screened for p53-responsiveness in RTPCR assays did not show any strong p53-specific upregulation, further supporting the notion that any DNA repair defects in p53-mutant cells may be fairly specific.

Among the other p53-dependent upregulated genes presented in Chapter 2 are two regulators of the glutathione pathway, the microsomal epoxide hydrolase gene, and a seemingly large number of genes involved in protein secretory pathways. The former two groups of genes could conceivably be involved in a p53-dependent response selected to inactivate mutagenic chemicals from the environment. However, additional experimentation will be required to see if these genes are directly regulated by p53, and exactly what the consequences of their inadequate upregulation would be for the life of the cell. p53 regulation of the several glutathione-related genes and epoxide hydrolase are consistent with the general findings of Polyak *et al.*, who described the initial instances of p53 target genes with apparent roles in controlling the redox state of the cell, although their interpretation was different from that presented here (Polyak et al., 1997).

As one contemplates the list of even the best known p53 target genes, it is daunting to consider the sort of experiments that will be required to definitively assess the function of each of these genes, and the importance of the interactions between each of these genes, in the settings of normal biology in the diverse tissues of the organism, and in the various stages of development of different malignancies. Technological means for asking the question of the contribution of different genes to a cellular phenotype in mammals have,

up until now, essentially been limited to the testing at most 2 or 3 gene deficiencies through the use of homologous recombination and breeding to generate compound knockout animals. These strategies have been constrained by the fact that gene mutations that interfere with any stage of development or the biology of reproduction, cannot be easily handled, except with conditional knockout strategies that are limited by the number of site-specific recombinase enzymes available for such experiments. In addition, the making of null gene alleles, although allowing the cleanest test of essential functions of a gene *in vivo*, are not the best suited changes for answering other biological questions. For example, in the case of the functions of p53-target genes, the key question to be answered from the point of view of p53 biology are: 1. What are the contributions of the p53-mediated *induction* of these genes to the prevention of tumor development in animals? 2. How do the functions of these genes interact with each other, in all pairwise and larger-order combinations? It is possible that the new experimental biological tools made available with RNA interference technology may be helpful in approaching some of these questions, if larger combinations of genes can be manipulated than is currently feasible with homologous knockout techniques in the mouse. One can even imagine using a transcription factor such as p53 as the tool for inducing the expression of siRNA constructs or some other technological means of guiding the destruction of its own target genes, as a way to try to eliminate the p53-dependent induction of subsets of genes and study their respective importance for different cellular outcomes.

Direct regulation of *Ercc5*, the XPG disease gene, by p53

More detailed study of the *Ercc5* gene regulation by p53 revealed that this DNA repair gene does appear to be a direct target of p53, with a near-consensus binding site found within the first intron of the gene. These features are typical of a number of p53-inducible genes; indeed, it seems to be the case that a substantial number of the genuine p53-responsive genes are regulated by elements in their first introns, for example, *Bax*, *Perp*, *Fas*, *Killer* and *Cyclin G* (Ruiz-Ruiz et al., 2003; Takimoto and El-Deiry, 2000; Yardley et al., 1998; Yoon et al., 2002).

Initial functional characterization of *Xpg*-dependent deficiencies in *Trp53-/-* cells using an assay that measures rescue of the activity of a DNA-damaged plasmid revealed a modest impairment in the mutant cells that was restored by infection with an XPG-expressing retrovirus. Although only the first step in exploring the relevance of p53-regulated *Xpg* expression to the normal and cancerous biology of the mouse, these results are encouraging, and suggest that other experiments should be conducted to observe the genomic consequences of exposing *Trp53*-deficient cells to increased oxidatively damaging stimuli. Direct measurement of the rate of removal of oxidative lesions from the DNA of *Trp53*-mutant and wild-type cells would also be an important follow-up to these experiments, to see if the cells lacking p53 have the same sort of deficiency of repair as they have been reported to have after benzo-*a*-pyrene treatment, and treatment with methylating agents (Lloyd and Hanawalt, 2002; Seo et al., 2002).

It has been difficult to establish the extent and mechanisms of the role of p53 in maintaining genomic stability in normal cells of the body. Interestingly, there is some

indication from analysis of the growing number of recorded Li-Fraumeni cancer-prone families that the genetic phenomenon of anticipation may be a feature of this disease (Trkova et al., 2002). Given the variable quality of clinical data, and changing regimens of screening and care of such patients, the data on this point are not yet extensive enough to definitively say that there is earlier onset of tumor development in successive generations of Li-Fraumeni families, but there are suggestions of such a trend, which would be a good indication of the importance of p53 function for maintaining genome stability (Trkova et al., 2002).

Circumstantial evidence from evolutionary considerations might lead one to think that the mutations that result in tumors commonly found in human children might be the sort of events that the function of the p53 gene would have been evolutionarily optimized to prevent (Leroi et al., 2003). As the hallmark of many childhood cancers is gross chromosomal rearrangements of various kinds, it will be important to assess whether any novel p53 target genes, including *Xpg*, possess the ability to prevent such events from occurring,

If it should happen to be the case that p53-regulation of *Xpg* is an important determinant of the ability of cells to withstand oxidative DNA damage or other genotoxic treatments, then it is conceivable that this protein could be a candidate target for drug development efforts. For example, a drug that would impair Xpg function somewhat might not lower the functional levels of the protein enough to cause harm to p53-inducing wild-type cells, but might lower the uninduced levels of the p53-mutant cells below a threshold so that they would be more vulnerable to other treatment agents. Alternatively, novel therapies

using oxidizing mechanisms might have tumor specificity. These are speculative comments, but as out knowledge of the molecular details of the cellular tumor suppression pathways continues to grow, new drug targets will continue to present themselves; and some of them may prove to be useful in the clinical setting.

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