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MODULATION OF GENE EXPRESSION BY TUMOR-DERIVED MUTANT p53. ROLE OF TRANSACTIVATION IN GAIN-OF-FUNCTION.

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MODULATION OF GENE EXPRESSION BY TUMOR-DERIVED MUTANT p53. ROLE OF TRANSACTIVATION IN GAIN-OF-FUNCTION.

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Dedication.

This work is dedicated to my family and friends. Dad, Mom, Brother and Sister, Pat, Earl, Ryan, B.K., Emily, Skip, Morgan, Cyndy, Jenny, Frank, Sean, Rusty, Targon, Dustin, Sabrina, Melanie, Robert, John and family, Noelle, Sarah, Jennifer and Allison, thank you for your support. I specially want to dedicate this work to my better half, Debbie. Words cannot simply describe how much I appreciate all you have done for me. I love you.

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List of abbreviations.

AMSH	Associated Molecule with the SH3 domain of STAM
ANGPT1	Angiopoietin 1
APC	Anaphase Promoting Complex
ASNase	Asparaginase
ASNS	Asparagine Synthetase
BFGF	Basic Fibroblast Growth Factor
BMV	Brome Mosaic Virus
BrdU	Bromodeoxyuridine
CAT	Chloramphenicol Acetyltransferase
CDK	Cyclin Dependent Kinase
CREB	cAMP responsive element binding protein 1
DDB2	DNA Damage Binding Protein 2
DMSO	Dimethylsulfoxide
EBAG9	Estrogen receptor binding site associated, antigen 9
EGFR	Epidermal Growth Factor Receptor
EMSA	Electrophoretic Mobility Shift Assay
ERCC5	excision repair cross-complementing rodent repair deficiency 5 (XPG)
FDR	False Discovery Rate
GADD45	Growth arrest and DNA-damage-inducible, alpha
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HBSS	Hank's Balanced Salt Solution
HDAC	Histone Deacetylase
HPV	Human Papilloma Virus
IARC	International Agency for Research on Cancer
IGF-I	Insulin-like Growth Factor 1
IL-6	Interleukin 6
ITGA6	Integrin alpha 6
LFS	Li-Fraumeni syndrome
MAPK	Mitogen Activated Protein Kinase
MAR	Matrix Attachment Regions
MBP1	Mutant p53 Binding Protein 1
MCL1	Myeloid leukemia cell differentiation protein 1
MDM2	Mouse Double Minute 2
MDR1	Multiple Drug Resistance Gene 1
NCBI	National Center for Biotechnology Information
NCI	National Cancer Institute
NER	Nucleotide Excision Repair
NF-κB1	Nuclear Factor of kappa light polypeptide gene enhancer in B-cells 1
NF-κB2	Nuclear Factor of kappa light polypeptide gene enhancer in B-cells 2
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction

PKC	Protein Kinase C
POLD2	DNA polymerase, delta 2 regulatory subunit
PUMA	p53 Up-regulated Modulator of Apoptosis
QPCR	Quantitative Polymerase Chain Reaction
RAD51	Human RecA homolog
rhoGAP	ras homologue family member G
RNA polII E	RNA polymerase II, polypeptide E
SDS-PAGE	Sodium odecyl sulfate-Polyacrylamide Gel Electrophoresis
WAF-1	Wild-type p53 Activated Fragment 1
WTp53	Wild-type p53
XPB	Xeroderma Pigmentosum B (ERCC3)
XPD	Xeroderma Pigmentosum D (ERCC2)

Abstract.

MODULATION OF GENE EXPRESSION BY TUMOR-DERIVED MUTANT p53. ROLE OF TRANSACTIVATION IN GAIN-OF-FUNCTION.

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Virginia Commonwealth University, 2005.

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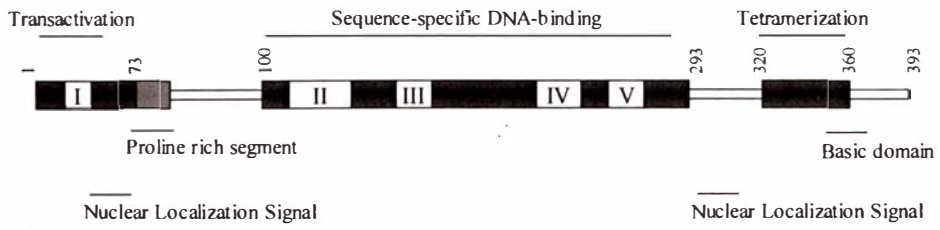
It was hypothesized that the C-terminal sequences for mutant p53 would be required for oligomerization, and oligomerization may be critical for gain-of-function. An N-terminal deletion mutant of p53 that deletes amino acids 1-293 was used as a tool to perform hetero-oligomerization studies. This mutant retains the entire oligomerization domain but dispenses off the transactivation domain and a large portion of the sequence-specific DNA-binding domain. Co-transfection experiments show that p53 del. 1-293 forms hetero-oligomeric complexes with p53-D281G. Also, co-expression of p53 del. 1-293 with p53-D281G inhibited p53-D281G-mediated transactivation of the EGFR and MDR1 promoters suggesting that hetero-oligomerization inactivates transcriptional functions of mutant p53. The interaction of p53 del. 1-293 and p53-D281G reduced transactivation potential of p53-D281G in stably transfected 10(3) murine cells. Therefore, the data presented supports the idea that proper oligomeric forms of mutant p53 are required for its transactivation function. Expression of mutant p53-D281G also resulted in increased growth rate (H1299 cells), decreased chemosensitivity (H1299 and 21PT cells) and increased plating efficiency (Saos-2 cells). Expression of a transactivation deficient mutant p53 did not induce gain-of-function properties (increased growth rate and decreased chemosensitivity). Unlike the other gain-of-function properties tested, soft agar plating efficiency in Saos-2 cells was not significantly affected by the expression of a transactivation deficient mutant p53, suggesting that transactivation may not be the only factor affecting this gain-of-function property. In order to identify the genes responsible for the observed phenotypes, global gene expression analyses were carried out using p53-null H1299 cell stably transfected to express mutant p53 (-R175H, -R273H and -D281G). A thorough and stringent analysis revealed 150 genes up-regulated by the expression of mutant p53. Up-regulation of a number of these genes was confirmed by QPCR and transient transcriptional promoter analyses; expression of the transactivation deficient mutant p53-D281G (L22Q/W23S) did not result in up-regulation of the tested genes further supporting the idea that transactivation of genes is directly related to gain-of-function phenotypes. Using the ASNS gene as a model, this transactivation by mutant p53 was concentration dependent and that the increased transcription did indeed result in increased protein levels.

H. Chapter 1. Introduction

Structure and function of p53. The tumor suppressor p53 gene is located on chromosome 17p and encodes an oligomeric, nuclear phosphoprotein that acts as a tumor suppressor. It is a sequence-specific DNA-binding protein and transcriptional activator as well as a transcriptional repressor [1-9]. The consensus DNA-binding site contains two copies of the 10 bp motif 5'-PuPuPuCWWGPyPyPy-3', separated by 0 to 13 bp [10]. (Pu denotes purine base, Py denotes pyrimidine base and W denotes A or T). All p53 genes from evolutionarily distant species contain five highly conserved regions designated domains I-V [11, 12] (Figure 1).

The sequence-specific DNA-binding domain of p53 is found in the central region covering the conserved domains II-V (residues 100-293) [13-19]. Additionally, p53 contains a transactivation domain located within residues 1-73 [20-27], and an oligomerization domain located within residues 320-360 [16, 19, 23, 28-30]. Sequences found within the central DNA binding domain are highly evolutionary conserved [19].

Figure 1. Structure of WTp53.



The tumor suppressor p53 has a very short half-life. Levels of p53 in a cell rise after stress like DNA damage, heat shock, hypoxia or hyperoxia among other stress signals. Elevated levels of wild-type p53 (WTp53) in response to cellular stress situations lead to apoptosis or cell cycle arrest [2, 31-41]. Initial studies on the cell cycle with ionizing radiation (IR)-exposed cells found that cells expressing a mutated p53 protein failed to

arrest the cell cycle at the G_1/S checkpoint [42]. Subsequently, it was found that p21/WAF-1, an integral component of this checkpoint, was under the transcriptional control of p53 [40]. Exposure of cells to IR led to an increase in WTp53 protein levels, which in turn resulted in elevated levels of p21/WAF-1 arresting the cells at the G_1/S checkpoint. Evidence exists that p53 is involved in a G_2/M checkpoint and that loss of this checkpoint contributes to an increase in polyploid cells [43].

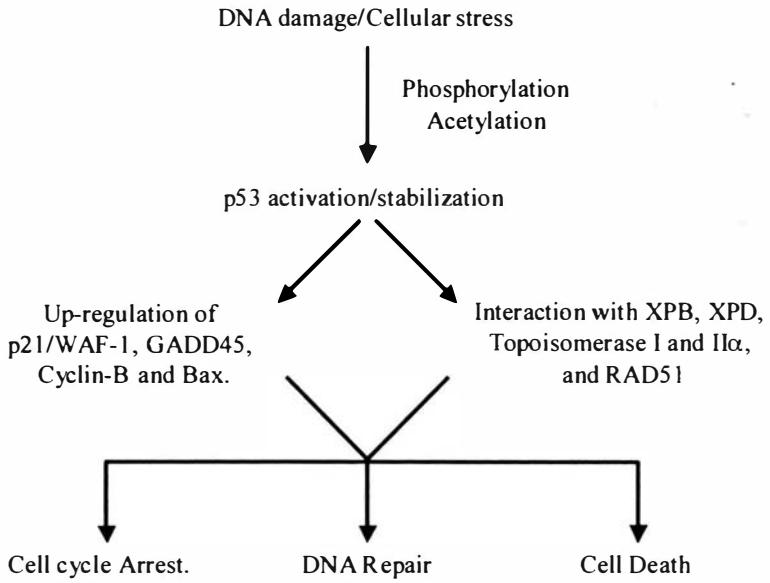
The role of p53 in apoptosis involves the transcriptional up-regulation of *Bax*, a pro-apoptotic protein [44]. It has also been shown that p53 can down-regulate expression of the anti-apoptotic protein bcl-2 by interacting with the TATA binding protein to repress transcription of the gene [44-46]. More recently, a role for p53 has been described at the mitochondrial level, where p53 is sensitive to the levels of bcl-2 and *Bax* [47, 48]. More evidence is accumulating describing a role for p53 in DNA excision repair, both as a transcription factor, up-regulating the synthesis of the repair enzyme O^6-alkylguanine transferase and the p48 subunit of the DNA damage binding protein (DDB2) and as a component of the nucleotide excision repair (NER) complex by interacting with XPB and XPD components of the NER-associated TFIIH transcription/repair complex [49-51]. The p53 protein can also interact with RAD51, topoisomerase-I and topoisomerase-II, each of which plays a role in recombination, DNA repair and DNA replication [52-55]. All in all, it is evident that p53 is involved in many crucial processes within the cell (Figure 2).

Degradation and regulation of p53. As mentioned, elevated levels of WTp53 in response to cellular stress situations leads to apoptosis or cell cycle arrest [2, 31-41]. Accumulation of p53 occurs through an increased translation rate and decreased degradation rate [56, 57]. Under normal circumstances, the levels of p53 are regulated through its interaction with the mouse double minute 2 protein (MDM2) [58, 59].

MDM2 binds to p53 and maintains relatively low levels of p53 in the cell by increasing its susceptibility to proteolysis by 26S proteosome and acting as a ubiquitin ligase [58, 59]. Under stress the MDM2–p53 interaction is blocked and the levels of p53 in the cell rise. It has also been reported that p53-MDM2 complex must be shuttled from

the nucleus to cytoplasm for p53 to be degraded. [60-63]. Also, during stress human p14 ^{ARF} is induced and it stabilizes p53 as it inhibits degradation of p53 by MDM2 [64, 65]. Additionally, aside from targeting p53 for degradation, MDM2 can also inhibit p53's transcriptional activity by physically interacting with the N-terminus of p53 [64, 65].

Figure 2. Functions of WTp53.



The p53 protein also undergoes numerous post-translational modifications during its activation. Specific phosphorylation sites have been identified within its amino and carboxy termini [9, 34, 36, 66-76]. The amino terminal domain undergoes phosphorylation by various kinases that include, casein kinase, checkpoint kinase 1 and checkpoint kinase 2, DNA-dependent protein kinase ataxia telangiectasia mutant, jun kinase and mitogen activated kinase (MAPK) [67, 70, 74]. The carboxy terminus can be phosphorylated by various cyclin dependent kinases (CDKs) and protein kinase C (PKC). Additionally, various acetylation sites have been also identified [67, 70, 74]. Phosphorylation of the N-terminus results in a conformational change that enables

transcriptional activation of genes, has some effects on DNA binding and results in a decreased interaction with MDM2 [77]. Changes in the phosphorylation state of the carboxy terminus have also been shown to affect the conformation of the protein and to modulate DNA binding activity [78].

Cancer with mutant p53 is more aggressive than cancer without p53. Over 80% of p53 mutations found in human tumors are missense mutations and in most human cancers only the mutant protein is expressed [6, 20-22, 79-84]. The percentage of missense mutations found in the p53 gene is significantly larger than that found for other tumor suppressor genes [85, 86]. The high frequency of mutation combined with the apparent selective over-expression of the mutant protein suggests that mutations in the p53 gene not only destroy the tumor suppressor function of the WT protein, but also that the mutant protein may gain oncogenic functions. This hypothesis predicts that tumors expressing mutant p53 may be more aggressive than p53-null tumors and suggests that patients with tumors expressing a mutant p53 have a poorer prognosis than patients with tumors lacking p53 altogether. This is true for a number of human cancers [87-106]. For example, cervical cancers with human papilloma virus (HPV) (which are functionally p53-null) have a better prognosis than those with mutant p53 (and without HPV) [11, 107-114].

Types of p53 mutations. While most tumor suppressor genes are inactivated by mutations leading to an absence of protein synthesis, more than 80% of alterations in the p53 gene are missense mutations that lead to the synthesis of a stable full-length protein [18]. In fact more than 50% of human cancers contain a p53 mutation [6, 20-22, 79-84].

Broadly, three types of p53 mutations can be identified [1, 5]: 1) loss-of-function, where the tumor suppressor activities of p53 are abolished; 2) dominant-negative, where hetero-oligomeric complex formation between wild-type and mutant p53 drives the wild-type p53 into a mutant conformation and results in the inactivation of wild-type p53 present in the cells; and 3) gain-of-function, where mutant p53 attains a dominant oncogenic role that does not depend on complex formation with wild-type p53.

These three categories are based on the following observations: 1) many cancers have lost wild-type p53 functions [20, 21] and p53-/- mice are highly prone to tumor formation, confirming that loss-of-function can result in tumorigenesis; 2) Patients who inherit the Li-Fraumeni cancer syndrome (LFS) and p53+/- mice develop tumors early [115]; 3) Many tumors with mutations in the p53 gene retain and over-express the mutant allele protein implying that mutant proteins perform a vital oncogenic role, and hence are selectively over-expressed [6, 20-22]. The selective over-expression of mutant p53 is believed to be required for both dominant-negative and gain-of-function activities [11, 18, 116, 117].

Walker *et al* [118] used the IARC database to define 73 "hotspots" for mutations in the p53 gene. Interestingly, most of these "hotspots" were found at CpG dinucleotides within exons 5-8 of p53. Six of these CpG "hotspots" were identified within the DNA binding domain of p53 at residues 175, 213, 245, 248, 273, and 282 [19]. These residues are either directly involved in protein-DNA contact (Class I mutants, i.e. R248W and R273H) or are critically important for maintaining protein structure (Class II mutants, i.e. R175H) [119].

Most naturally arising p53 mutations exhibit an increased stability and accumulate to high levels yet are unable to function normally and fail to induce the proper response after cellular stress [119]. Cho *et al* [17] showed through X-ray crystallography that in general mutations of p53 alter the ability of the protein to bind to the promoter regions of genes under its regulatory control. This presents a convincing explanation for loss-of-function and dominant-negative mutations, which are unable to induce genes such as p21/WAF-1 and *Bax* that would normally result in cell cycle arrest or apoptosis. However, it does not explain how mutant p53 can act as a gain-of-function mutation.

Tumor-derived p53 mutants and gain-of-function. As mentioned earlier, WTp53 is a sequence-specific transactivator of genes containing p53-binding sites on their promoter or transcriptional regulatory sequences [11]. Elevated levels of WTp53 in response to cellular stress situations lead to apoptosis or cell cycle arrest at the G_1 or G_2 boundaries by inducing expression of genes that are involved in various aspects of

cellular growth regulation [2, 31-41]. Tumor-derived p53 mutants are defective in these functions (loss-of-function).

The high frequency of missense mutation (as opposed to deletions or truncations) distinguishes p53 from other tumor suppressors and suggests that there is a selective pressure for mutant p53. Indeed, expression of mutant p53 in cells devoid of endogenous WTp53 has been shown to induce various growth promoting and proliferative functions [11, 120-130]. These activities are not shared with wild-type p53.

One of the most important discoveries with regard to mutant p53 gain-of-function was the ability of the mutant protein to enhance the tumorigenicity of cells lacking expression of p53 [11, 129-132]. Through the years, various activities have been attributed to mutant p53, which include (1) increased tumorigenicity [133, 134], (2) increased metastasis and tissue invasiveness [135], (3) increased growth in soft agar [136], (4) decreased sensitivity against chemotherapeutic drugs [137, 138], (5) increased resistance to γ-irradiation [139], (6) accelerated chemical carcinogenesis [140], (7) increased homologous recombination induced by ionizing radiation [140], (8) disruption of spindle check point [124], (9) activated topoisomerase I activity [141], (10) increased growth rate [142] and (11) induction of gene amplification [143]. Many of the gain-of-function data come from p53-null systems (eliminating the possibility of dominant-negative effects) where the expressed mutant p53 level was comparable to that observed in cancer cells [129]. This suggests the strong possibility that mutant p53 gain-of-function has a true physiological role, which may lead to aggressive cancer development and poorer prognosis.

Most of the gain-of-function properties have been demonstrated using p53-null cell culture systems. This is not an ideal system, since cells may accumulate further mutations while in culture, making the system an inaccurate representation of human tumors. To this end, mouse models have been generated in order to further understand the role of mutant p53 in oncogenesis. It has been observed that p53-null mice develop early onset lymphomas making long term studies of gain-of-function in these mice a bit complicated [144]. Using a knock-in mouse line, Liu *et al.* [145, 146] showed increased

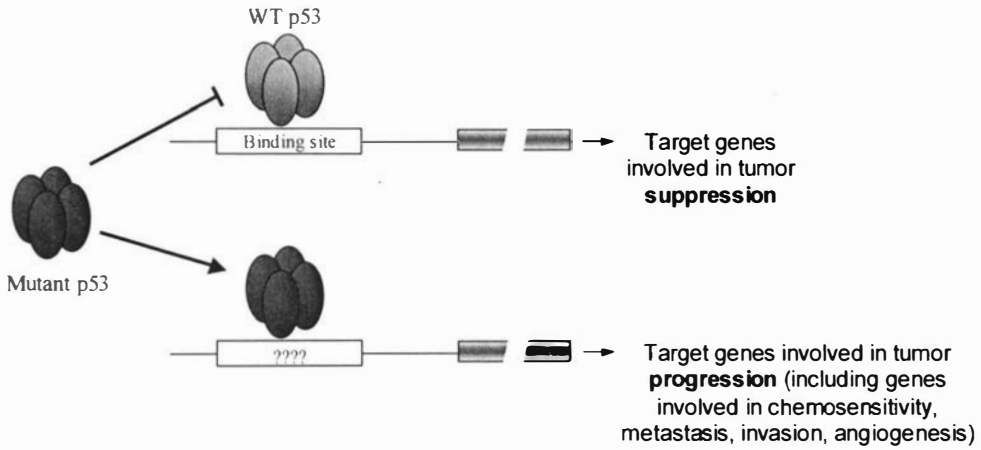
metastatic potential in mice inheriting p53-R172H (a murine p53 mutant homologous to the human p53-R175H). Using a murine p53-R172H mutant transgenic model system Wang *et al.* [124, 140] showed some evidence of gain-of-function associated with genomic instability and Murphy *et al.* [142] reached similar conclusions using the same p53 mutant in a mammary epithelial murine cell model. Hixon *et al.* [147] proposed that cells carrying mutant p53 over-express Cks1, a protein that mediates activating phosphorylation of the anaphase promoting complex (APC) by cdc2 leading to chromosomal instability as cells are unable to sustain APC inactivation. More recently, clear and strong evidence of mutant p53 gain-of-function has been demonstrated in two mouse models of the Li-Fraumeni Syndrome where higher metastatic spread and/or different tumor spectrum have been observed in the presence of mutant p53 [148, 149].

Mechanisms of mutant p53-mediated gain-of-function. Mutant p53 may achieve gain-of-function phenotypes by a direct acquisition of new functions actively involved in proliferation processes [119]. Two possible models have been proposed to explain the molecular mechanism underlying mutant p53 gain-of-function.

The first, involves transcriptional modulation by mutant p53 of specific target genes. For example, mutant p53 may transactivate growth promoting genes, disrupt DNA repair or apoptotic activities or even inhibit growth suppressive genes [150-152]. It has been shown that mutant p53 can transactivate promoters of growth-related genes *in vivo,* even in the absence of WTp53, such as human proliferating cell nuclear antigen (PCNA), epidermal growth factor receptor (EGFR), interleukin 6 (IL-6), multiple drug resistance 1 (MDR1), basic fibroblast growth factor (BFGF) and *c-myc* among others [reviewed in [85, 119], [11, 120, 134, 153-155]]. The sequence requirements for mutant p53-mediated transactivation of these genes is independent of a p53 DNA binding consensus and it is believe that mutant p53-mediated regulation of these promoters is through its interaction with other factors. Structure-function analyses indicate that the domain requirements for transactivation by WT and mutant p53 are different and that mutant p53 does not require a WTp53 DNA binding site [133, 134, 155, 156]. It is recognized that mutant p53 requires both its amino and carboxy termini for gain-of-function [133, 134]. A

transactivation deficient form of mutant p53-D281G that incorporates two additional mutations within the transactivation domain [p53-D281G (L22Q/W23S)] that disrupt TAF interactions and c-terminal modifications can no longer transactivate the MDR1 and *c-myc* promoters [133, 154]. More importantly, this transactivation deficient mutant failed to confer tumorigenic potential in p53-null fibroblasts [133]. Taken as a whole, modulation of gene expression by mutant p53 in oncogenesis remains largely uninvestigated (Figure 3).

Figure 3. Differences between mutant and WTp53.



The second model explaining mutant p53-gain-of-function involves protein-protein interactions between mutant p53 and other cellular protein(s) such as the p53 family members, p73 and p63, DNA repair machinery proteins, and/or proteins of the apoptotic pathway in the cytoplasm and/or mitochondria [157, 158]. The proteins p73 and p63 have structural and functional similarity with p53, and have been shown to transactivate promoters containing p53-binding sites [159-162]. Like WTp53, p73 can also induce apoptosis or growth suppression of cells expressing elevated levels of the protein [159-162]. Over-expression of p73 in different cancer cell lines has been detected, providing evidence that p73 may substitute for WTp53 as a tumor suppressor in cells lacking expression of p53 altogether [163, 164]. Tumor-derived mutant p53 may inactivate the

physiological functions of p73 (or p63) through hetero-oligomerization. Evidence of this phenomena exists in the literature [159, 160]. It is possible then that at least part of the oncogenic effect of mutant p53 arises from an interaction of the mutant protein with endogenous p73 (or p63). This interaction, however, is affected by a common p53 polymorphism at residue 72 (R vs. P) where a R72 form is more likely to interact with p73/p63 than the P72 form of p53 [165, 166]. Using isogenic cell lines expressing various p53 mutants in their R72 or P72 form, Bergamaschi *et al.* [167] showed that those mutants expressing the R72 form were more resistant to chemotherapy. Interestingly, the mutant background used in this study is P72.

The gain-of-function properties of mutant p53 may also be dependent on protein-protein interactions other than p53/p76-p63 interactions. A number of proteins have been identified that specifically interact with either WT or mutant p53 [119]. Mutant p53 binding protein 1 or MBP1, an oncogenic protein, has been found to interact with p53-R175H and other p53 mutants but not with WTp53 [168]. There is also evidence to indicate that mutant p53 may promote homologous recombination [53, 141]. WT and mutant p53 also interact differently with MDM2 [169]. Interestingly, the central DNA-binding domain of mutant p53 and not of WTp53, binds to MDM2. It is not clear, however, whether this interaction has any impact on gain-of-function.

Although major emphasis has been given on the transcriptional function of WTp53, functions independent of transcription have also been described [170-174]. For example, Mihara *et al.* [158] demonstrated a direct apoptogenic role of WTp53 at the mitochondria, independent of its transcriptional activity. Chipuk *et al.* [170] also showed a transcription-independent apoptotic role of WTp53 that may initiate in the cytoplasm. The impact of mutant p53 on these processes, if any, is unknown. Ohiro *et al.* [175] showed that mutant p53 inhibits stress-inducible kinase pathways, and demonstrated its anti-apoptotic activity. This activity was also independent of the transactivation function of mutant p53.

The two proposed models are not mutually exclusive and the most likely scenario is that mutant p53 disrupts cellular functions by both transcription-dependent and -

independent mechanisms. Future proteomic analysis in conjunction with functional assays will lead to a clarification of the role of protein-protein interactions in gain-of-function.

I. Chapter 2. Hypothesis

a. Major hypothesis.

The **major hypothesis** of this work was: Mutant p53 up-regulates specific genes leading to gain-of-function properties and oncogenicity of cells expressing mutant p53. This up-regulation of genes is transactivation dependent contributes to gain-of-function properties of mutant p53.

This hypothesis was based on the following observations. Experiments with WTp53 in which the tetramerization domain had been replaced by a heterologous dimerization or tetramerization domain have shown that the heterologous oligomerization domain preserves wild-type p53-mediated transactivation and growth suppression (including G_1/S inhibition) functions [176-180]. However, no information was available regarding the consequences of replacing the oligomerization domain of tumor-derived p53 mutants. Using murine non-tumorigenic p53-null 10(3) cells, it had been shown that expression of tumor-derived p53 mutants such as p53-D281G, -R175H and -R273H increase the tumorigenicity of the cells in nude mice remarkably [133, 134]. Further experiments demonstrated that the transactivation function of p53 mutants was required for its oncogenic function [133, 134]. Blandino *et al.* [128], reported that mutant p53 expression (in cells devoid of WTp53) can lead to decreased sensitivity against chemotherapeutic drugs such as doxorubicin, etoposide, cisplatin. Other groups have reported similar results [126, 137, 138, 181-186]. Several other gain-of-function phenotypes have been reported [133-143], yet, the exact role of transactivation, if any, had not examined in detail. Finally, evidence in the literature suggested that transactivation played a role in mutant p53 tumorigenicity. As such, it was important to identify target genes modulated by mutant p53 in the absence of WTp53. It was hypothesized that this type of analysis would reveal a set of target genes whose transcriptional modulation by mutant p53 would explain the gain-of-function phenotypes observed.

b. Specific Aims.

The following are the specific aims: (1) To determine whether the oligomerization domain and C-terminally located sequences are necessary for transactivation and gain-of-function properties of mutant p53; (2) To determine whether mutant p53 requires its transactivation function to induce gain-of-function properties; and (3) To identify genes modulated by tumor-derived p53 mutants.

J. Chapter 3.

Hetero-oligomerization does not Compromise Mutant p53 Gain-of-Function.

The work presented in this chapter has been published in the Oncogene research journal (Oncogene. 2002 Jan 10;21(2):176-89.). CAT assays and ^{35}S immunoprecipitations were performed by D Deb, QPCRs by KE Roth and colony forming and tumorigenicity assays by AS Chakraborti.

a. Introduction.

Replacement of the oligomerization domain of WTp53 with a heterologous dimerization or tetramerization domain showed that it could preserve WTp53-mediated transactivation and growth suppression functions [176, 177, 179, 180, 187]. Similar experiments showed that the gain-of-function phenotype of tumor-derived p53 mutants also required the C-terminally located oligomerization/nucleic acid-binding domain [134]. However, no information was available regarding the consequences of replacing the oligomerization domain of tumor-derived p53 mutants and whether the C-terminal regions are only required for oligomerization or have other function(s) necessary for mutant p53-mediated transactivation and/or gain-of-function.

b. Experimental Results.

Replacement of the tetramerization domain inhibits mutant p53-mediated transactivation. Earlier, this laboratory had shown that several tumor-derived p53 mutants could transactivate promoters of a number of growth-related genes including the EGFR gene promoter [153, 155]. However, the deletion mutant p53-D281G del. 393-327, which eliminates the oligomerization domain of the protein, failed to transactivate the promoter [155], suggesting that proper oligomerization of a tumor-derived p53 mutant is required for the transactivation function of the mutant protein.

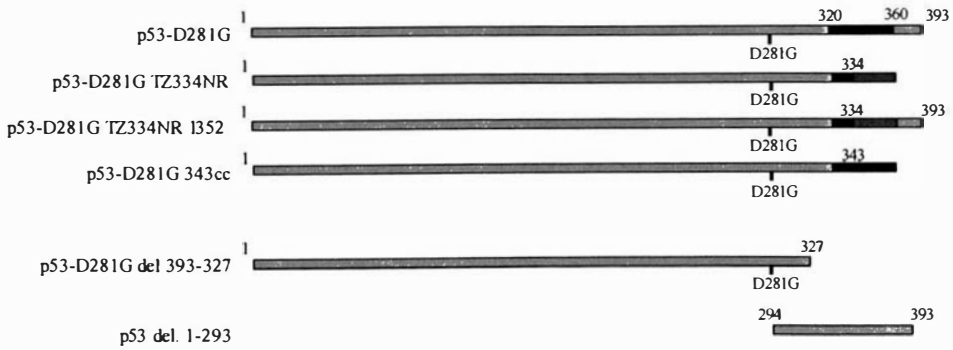
Therefore it was necessary to determine if the C-terminally located oligomerization/non-sequence-specific nucleic acid binding domain is required only for oligomerization. It was shown earlier that replacement of the tetramerization domain of WTp53 by a dimerization domain consisting of the coiled-coil structure from the yeast

GCN4 protein (or a derivative of the coiled-coil structure that results in a tetramerization domain) could effectively sustain wild-type p53-transactivation and growth suppression functions [176, 180, 187]. Performing similar experiments, p53-D281G derivatives with such a replacement of the tetramerization domain would be predicted to retain the transactivation function.

The effect of expression of p53-D281G, p53-D281G 343cc (the coiled-coil structure from GCN4 is placed after the amino acid 343) [178] and p53-D281G TZ334NR (a modified GCN4 coiled-coil structure is placed after amino acid 334 such that it functions as a tetramerization domain) [180] on the activity of the EGFR promoter was studied (Figure 4). Transfections were carried out in p53-null Saos-2 cells.

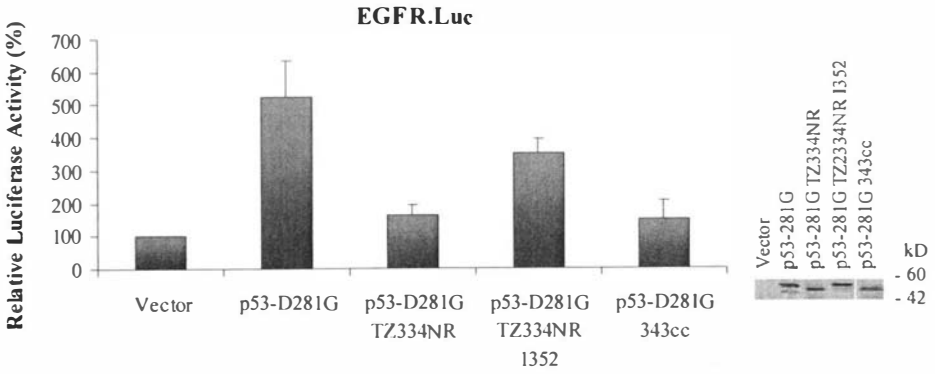
The results shown in Figure 5 from luciferase assays demonstrate that p53-D281G-mediated transactivation of the EGFR promoter is not preserved by replacing its tetramerization domain by a heterologous dimerization/tetramerization motif. This suggests that this region performs some vital function(s) required for mutant p53-mediated transactivation that cannot simply be substituted by a heterologous dimerization/tetramerization domain. Amino acid residues 320-360 of p53 code for the tetramerization domain [16, 23, 28, 188]. Besides partial substitution of this domain (starting from residue 334), the C-terminal amino acids past the oligomerization in p53-D281G TZ334NR have been deleted (residues 352-393). This segment may also play an important role in mutant p53-mediated transactivation.

Figure 4. Schematic representation of p53-D281G oligomerization derivatives.



The mutant p53-D281G TZ334NRI352, having the sequences 352-393 inserted following the TZ moiety was also tested. This mutant transactivated the best among all the domain replacement mutants. The western blot analysis shows equivalent amounts of expression by p53-D281G and p53-D281G TZ334NR, thus an expression deficiency cannot explain the inability of p53-D281G TZ334NR to transactivate. The experimental results suggest that the C-terminally located amino acids 352-393 perform a vital function for mutant p53-mediated transactivation since transactivation by this mutant was 4 fold over that of p53-D281G TZ334NR. Earlier, a similar conclusion was reached by Frazier *et al.* [154], demonstrating a requirement for amino acids 370-380 for transactivation of the c-*myc* promoter by p53-D281G. Similar experiments were carried out using WTp53 (data not shown). Replacement of the oligomerization domain of WTp53 did not affect the transactivation ability of the protein in agreement with earlier studies (data not shown) [176, 177, 179, 180, 187].

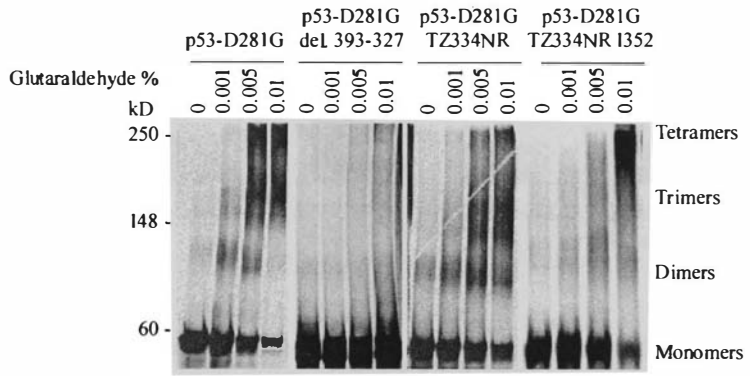
Figure 5. Replacement of the tetramerization domain of mutant p53 has a drastic effect on mutant p53-mediated transactivation.



Replacement of the tetramerization domain of mutant p53 preserves tetramerization of the protein. One possible reason for the failure of p53-D281G TZ334NR and p53-D281G TZ334NRI352 to transactivate the EGFR promoter efficiently

could be a defect in oligomerization. Using a glutaraldehyde cross-linking assay, the ability of the mutant p53 to form tetramers was tested as described [23]. The various mutant p53 forms were synthesized using ^{35}S-labeled methionine and an *in vitro* transcription-translation system. The synthetic proteins were incubated with glutaraldehyde at varying final concentrations (Figure 6). After cross-linking, the protein oligomers were analyzed using a gradient (4-20%) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE).

Figure 6. Replacement of the tetramerization domain of mutant p53 preserves tetramerization of the protein.



The data shown in Figure 6 indicates that p53-D281G, p53-D281G TZ334NRI352 and p53-D281G TZ334NR oligomerized, while p53-D281G del 393-327 could not, although there is some variation in tetramerization among p53-D281G, p53-D281G TZ334NRI352 and p53-D281G TZ334NR. Since p53-D281G TZ334NRI352 and p53-D281G TZ334NR are capable of oligomerization into tetramers, the results shown in Figure 4 demonstrate that the C-terminally located oligomerization/nucleic acid binding domain is required for p53-D281G-mediated transactivation.

Transactivation of the EGFR and MDR1 promoters by the tumor-derived mutant p53-D281G is inhibited by co-expression of p53 del. 1-293. Hetero-oligomerization of WTp53 with miniproteins containing only the C-terminal oligomerization domain results in inactivation of WTp53 function [189]. Therefore, it

was also necessary to investigate if such an interaction would inactivate mutant p53 mediated transactivation. Saos-2 cells were co-transfected with EGFR.Luc [155] or MDR1.CAT (chloramphenicol acetyl transferase) [133, 190], an expression plasmid for p53-D281G (or vector alone) and an expression plasmid for p53 del. 1-293 (or vector alone).

Figure 7. Transactivation of the EGFR promoter by tumor-derived mutant p53-D281G is inhibited by co-expression of p53 del. 1-293.

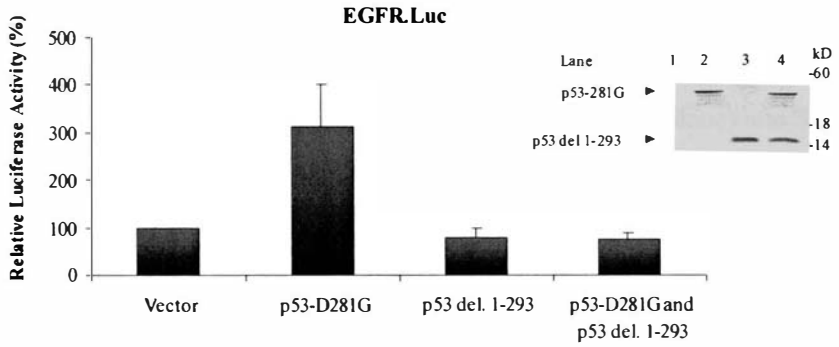
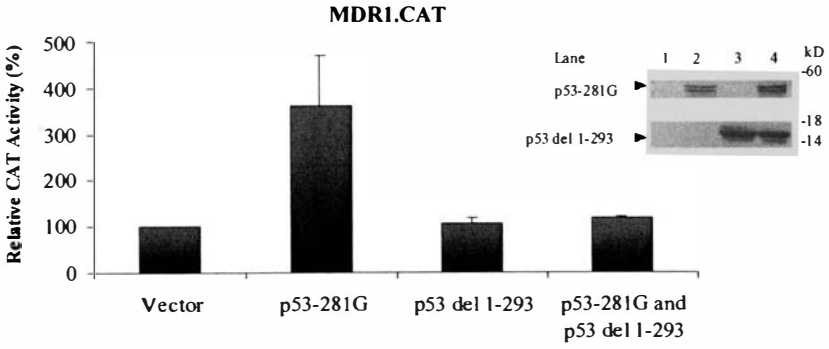


Figure 8. Transactivation of the MDR1 promoter by tumor-derived mutant p53-D281G is inhibited by co-expression of p53 del. 1-293.



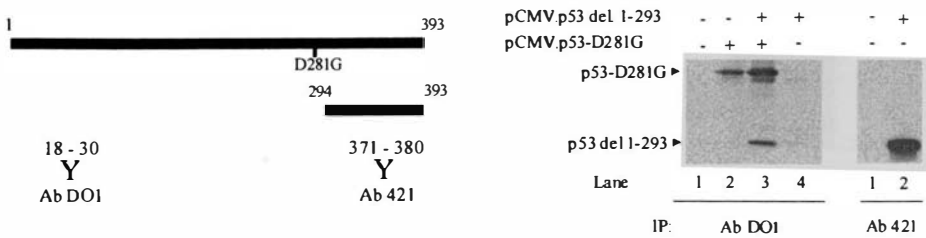
Transfections, CAT and luciferase assays were carried out as described in Materials and methods. Figures 7 and 8 show the results of the assays; it is apparent that although

the tumor-derived p53-mutant transactivated the EGFR and MDR1 promoters, this transactivation was significantly reduced by the co-expression of p53 del. 1-293. Western blot analyses examining expression of the two proteins from a representative experiment demonstrates that the proteins were adequately expressed; thus, lack of transactivation by p53-D281G in the presence of p53 del. 1-293 cannot be explained by protein expression deficiencies. It is possible then that hetero-oligomers between p53-D281G and p53 del. 1-293 are inactive in transactivation.

p53 del. 1-293 complexes with p53-D281G *in vivo*. Since co-expression of p53 del. 1-293 reduced mutant p53-D281G transactivation it was necessary to check whether these two proteins interacted *in vivo*. Saos-2 cells were co-transfected with expression plasmids for p53-D281G (or vector alone) and p53 del. 1-293 (or vector alone) into Saos-2 cells. Twenty-four hours after transfection, cells were labeled *in vivo* by ^{35}S-methionine. Cell extracts were prepared and used in immunoprecipitations (Figure 9).

Immunoprecipitations were performed using an antibody specific for the N-terminus of mutant p53 (Ab DO1, Figure 9). Western blot analysis was conducted using an antibody against the C-terminus of p53. Although the antibody DO1 could not immunoprecipitate p53 del. 1-293 alone (Figure 9, lane 4), it could precipitate the deletion mutant in the presence of p53-D281G (Figure 9, lane 3). This shows that p53 del. 1-293 binds with p53-D281G *in vivo* forming a hetero-oligomeric complex. Thus, results of these experiments (Figures 6-8) suggest that proper oligomeric forms of p53-D281G are necessary for its transactivation function.

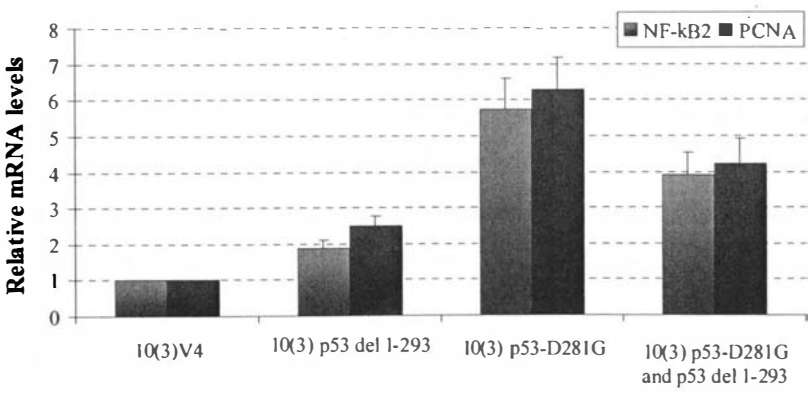
Figure 9. The protein p53 del. 1-293 co-immunoprecipitates with p53-D281G.



Co-expression of p53 del. 1-293 inhibits the endogenous transactivation by p53-D281G. Hetero-oligomerization of wild-type p53 with tumor-derived p53 mutants (or polypeptides covering the oligomerization domain of p53) inhibits wild-type p53 biological functions leading to immortalization and transformation of rat embryo fibroblasts [189]. Since co-expression of p53 del. 1-293 and p53 (wild-type or mutant) inhibited p53 transactivation [191], it was important to test whether hetero-oligomerization between p53-D281G and p53 del. 1-293 would lead to inhibition of transactivation of endogenous genes by p53-D281G.

To identify genes endogenously transactivated by p53-D281G, p53-null murine 10(3) cells stably transfected with p53-D281G were compared with cells stably transfected with vector (pCMVBamNeo, Hinds *et al.*, 1990) alone by microarray hybridization analysis (Incyte Genomics). The analysis identified a number of genes expressed at a higher level in p53-D281G expressing cells with a fold difference in expression of two or more. Two such genes, PCNA and NF-κB2, were chosen to see the effect of co-expression of p53 del. 1-293. Quantitative real time PCR (QPCR) analysis of cDNA made from total RNA shows that the levels of both the genes are considerably higher in 10(3) cells expressing the mutant p53-D281G compared to that in cells stably transfected with vector alone (Figure 10).

Figure 10. Co-expression of p53 del. 1-293 inhibits transactivation by p53-D281G.



Furthermore, co-expression of p53 del. 1-293 decreased the level of the RNAs to some extent suggesting that hetero-oligomerization indeed inhibits transactivation by mutant p53. Interestingly, 10(3) cells expressing p53 del. 1-293 expressed more of the two RNAs compared to vector-transfected cells. A possible explanation for these results may be that mutant p53 transactivates these genes through a synergistic interaction with another transactivation factor after localization of the complex on or near regulatory regions of the genes. Since p53 del. 1-293 does not posses a transactivation domain, the observed up-regulation may be due to that second transactivating protein. This possibility also suggests that the interacting factor binds/interacts with mutant p53 at its C-terminus.

p53 del. 1-293 did not inhibit colony formation by p53-D281G. Expression of p53-D281G also gives growth advantage to cells expressing the mutant protein [11]. It was therefore necessary to determine whether p53 del. 1-293 expression could influence the growth advantage conferred by mutant p53. Using a colony formation assay to assay for cell proliferation, 10(3) cells stably expressing the expression vector (pCMVBam NEO) alone or expression plasmid for p53-D281G were transfected with the following expression plasmids: (1) pCMVBam and pHyg, (2) pCMVBam p53 (wild-type) and pHyg, (3) pCMVBam FLAG-p73α and pHyg, (4) pCMVBam p53 del. 1-293 and pHyg. The data presented in Table 1 indicates p53-D281G expressing cells have a discernable growth advantage over vector-transfected cells. The deletion mutant p53 del. 1-293 had little effect, if any (compared to vector alone), on the growth properties of p53-D281G-expressing cells. Co-transfection of the cells with WTp53 or p73α decreased the number of colonies formed substantially, as expected, since both WTp53 and p73α have been shown to be growth inhibitors when expressed in cells [2, 31-41, 192]. Thus, once again we find that del. 1-293 could not significantly change the growth enhancing function of mutant p53 suggesting that hetero-oligomerization may not be enough to inhibit gain-of-function.

In an earlier report, it was shown that a deletion of the C-terminal sequences of p53-D281G overlapping the oligomerization domain disrupts gain-of-function properties.

Here experimental data suggest that hetero-oligomerization disables transactivation by p53-D281G without eliminating gain-of-function properties (tumorigenicity). Taken together this information suggests that the C-terminal sequences have a separate role in gain-of-function in addition to that in transactivation.

Table 1. Colony formation in 10(3) cells.

Cell line [10(3) expressing]	Expression plasmids used for transfection	Colonies formed (1 mm or more)
Vector alone, clone 4	pHyg and pCMVBam	0,0,2; avg. 0.67
p53-D281G, clone 4	pHyg and pCMVBam	**137, 117, 132; avg. 129**
Vector alone, clone 4	pHyg and pCMVBam WTp53	4,0,0; avg. 1.33
p53-D281G, clone 4	pHyg and pCMVBam WTp53	4,21,5; avg. 10
Vector alone, clone 4	pHyg and pCMVBam p73α	3,8,38; avg. 16.33
p53-D281G, clone 4	pHyg and pCMVBam p73α	0,8,18; avg. 8.67
Vector alone, clone 4	pHyg and pCMVBam p53 del. 1-293	1,4,0; avg. 1.67
p53-D281G, clone 4	pHyg and pCMVBam p53 del. 1-293	**113,125,107; avg. 115**

Co-expression of p53 del. 1-293 does not inhibit tumorigenicity of 10(3) cells expressing the mutant p53-D281G protein. It was important to test whether hetero-oligomerization between p53 del. 1-293 and p53-D281G would lead to inhibition of tumorigenicity of mutant p53 expressing cells. Although 10(3) cells expressing p53-D281G form tumors, cells stably expressing p53-D281G del. 393-327 (defective in oligomerization) do not [134]. This suggested a role for oligomerization in gain-of-function.

Stable cell lines were generated from 10(3) cells (1) expressing p53-D281G, (2) expressing p53 del. 1-293, (3) expressing p53-D281G and p53 del. 1-293 and (4) stably transfected with vector alone [10(3) V4]. Cells were checked for expression by western blot analysis (data not shown). Two different clones from each stable transfection were injected subcutaneously into two or three different nude mice (two sites on each mouse) as described in Materials and methods. The mice were then observed for several weeks for appearance of tumors.

As shown in Table 2, 10(3) cells stably transfected with pCMVBam vector alone or stably expressing p53 del. 1-293 did not show any tumor formation even after five months. In cells expressing p53-D281G and p53 del. 1-293 tumors appeared within 1-2 weeks of injection; a similar situation was observed with 10(3) cells expressing p53-D281G alone. Since co-expression of p53 del. 1-293 and p53-D281G did not reduce tumorigenicity, hetero-oligomerization may not interfere with mutant p53-mediated tumorigenesis.

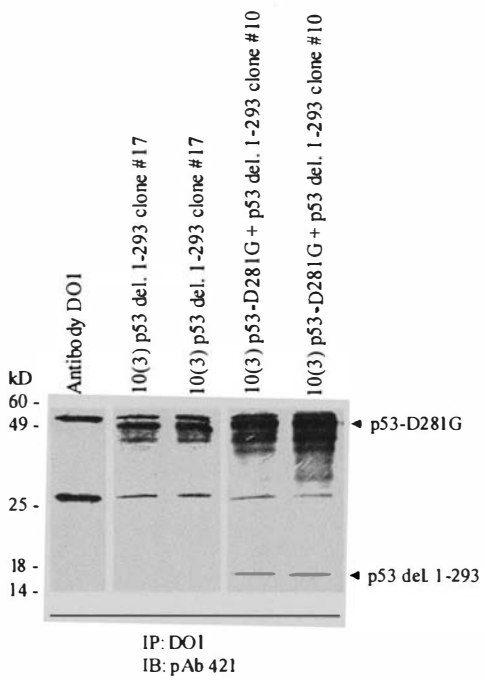
Table 2. Tumorigenicity of 10(3) cells expressing p53 derivatives.

Cells stably transfected with	Number of mice/ Number of sites injected	Number of tumors / Number of injected sites
Vector alone, clone 2	2/4	0/4
Vector alone, clone 21	2/4	0/4
p53 del. 1-293, clone 17	2/4	0/4
p53 del. 1-293, clone 19	3/6	0/6
p53-D281G, clone 13	3/6	**6/6**
p53-D281G, clone 4	2/4	**4/4**
p53-D281G, clone 4 and p53 del. 1-293 clone 22	3/6	**6/6**
p53-D281G, clone 4 and p53 del. 1-293, clone 10	3/6	**6/6**

Hetero-oligomers are produced between p53-D281G and p53 del. 1-293 in 10(3) cells. Since co-expression of p53-D281G and p53 del. 1-293 in 10(3) cells did not interfere with tumorigenicity, *in vivo* complex formation between these two proteins needed to be confirmed. Extracts from 10(3) cells expressing the p53 del. 1-293 deletion mutant alone and cells expressing p53-D281G and p53 del. 1-293 were prepared and immunoprecipitation reactions carried out as described in Materials and methods. Immunoprecipitations were performed with the antibody DO1. The immunoprecipitates were separated by SDS-PAGE, and proteins transferred to a nitrocellulose membrane. The blot was then developed with PAb421 antibody. The data shown in Figure 11 show that DO1 immunoprecipitates both p53-D281G and p53 del. 1-293 indicating that hetero-

oligomer formation between the two proteins occurs. As expected, DO1 did not immunoprecipitate p53 del. 1-293.

Figure 11. Hetero-oligomers are produced between p53-D281G and p53 del. 1-293 in 10(3) cells stably expressing the two proteins.



c. Chapter Summary.

It was hypothesized that the C-terminal sequences for mutant p53 would be required for oligomerization, and oligomerization may be critical for gain-of-function. An N-terminal deletion mutant of p53 that deletes amino acids 1-293 was used as a tool to perform hetero-oligomerization studies. This mutant retains the entire oligomerization domain but dispenses of the transactivation domain and a large portion of the sequence-specific DNA-binding domain. Co-transfection experiments followed by immunoprecipitation showed that p53 del. 1-293 forms hetero-oligomeric complexes

with p53-D281G. Also, co-expression of p53 del. 1-293 inhibited p53-D281G-mediated transactivation of the EGFR and MDR1 promoters suggesting that hetero-oligomerization inactivates transcriptional functions of mutant p53. The interaction of p53 del. 1-293 and p53-D281G reduced transactivation potential of p53-D281G in stably transfected 10(3) murine cells. Although 10(3) cells stably transfected with p53-D281G expressed 5-6-fold more PCNA and NF-κB2 mRNA compared to the cells transfected with vector alone, co-expression of p53 del. 1-293 reduced this up-regulation considerably. Therefore, the data presented supports the idea that proper oligomeric forms of mutant p53 are required for its transactivation function. The transactivation data generated supports this idea, yet tumorigenicity and colony formation assays show that co-expression of the deletion mutant in the presence of p53-D281G does not inhibit gain-of-function by mutant p53. Thus, hetero-oligomerization between p53-D281G and p53 del. 1-293 may not completely inhibit p53-D281G growth enhancing properties, although it does inhibit its transactivation ability.

K. Chapter 4.

Role of Transactivation in Mutant p53 Gain-of-Function Properties.

The work presented in this chapter has been published in the journals Oncogene and Cancer Research (Oncogene. 2002 Jan 10;21(2):176-89. and Cancer Res. 2004 Oct 15;64(20):7447-54).

a. Introduction.

Using murine non-tumorigenic p53-null 10(3) cells it had been shown that expression of tumor-derived p53 mutants such as p53-D281G, -R175H and -R273H increase the tumorigenicity of the cells in nude mice remarkably [133, 134]. Further experiments demonstrated that the transactivation function of p53 mutants was required for its oncogenic function [133, 134].

Blandino *et al.* [126, 128, 137, 138, 181-186], reported that mutant p53 expression (in cells devoid of WTp53) can lead to decreased sensitivity to chemotherapeutic drugs such as doxorubicin, etoposide, cisplatin and other groups have also reported similar results [126, 128, 137, 138, 181-186]. Several other mutant p53 gain-of-function phenotypes have been described in the literature [133-143], yet the exact role of transactivation, if any, was not examined. Here, the role of transactivation was examined in three gain-of-function phenotypes (growth rate, chemosensitivity and soft agar plating efficiency).

b. Experimental results.

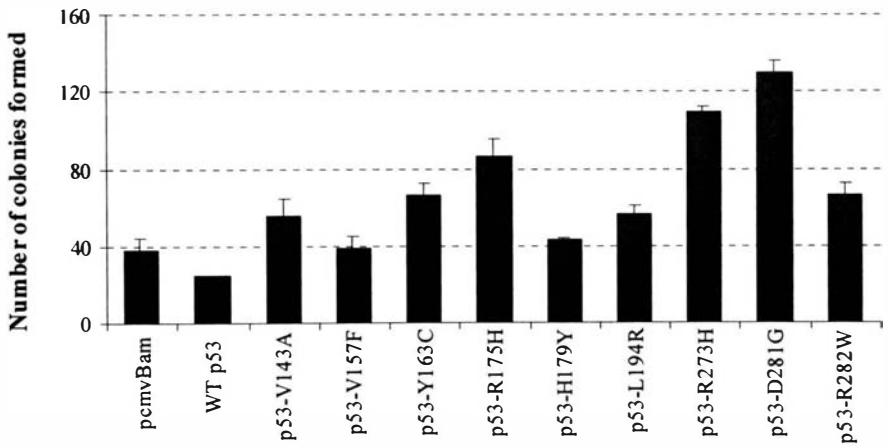
Expression of tumor-derived mutant p53 in p53-null cells confers a growth advantage. In order to explore the role of mutant p53 mediated transactivation in the various gain-of-function phenotypes it was first necessary to determine if the p53 mutants available in the laboratory could induce any gain-of-function. A series of p53 mutants found in human cancers were selected for the initial studies. For example, mutations at amino acid 175 account for 5.09% of all p53 mutations found in human cancers and mutations at 273 for 6.49%[1]. Using the colony assay described [156] we co-transfected

[1] *Data has been adapted from p53 Database (http://p53.curie.fr/). Percentages are calculated with respect to the total p53 mutations reported for all human cancers.*

10(3) cells with pCMV-Bam Neo and pCMV-Bam p53 (WT or mutant) or pCMV-Bam expression vector. Forty-eight hours after transfection the cells were subcultured and grown in selective media with periodic changes in media every three or four days. After three to four weeks of selection the cells were stained and colonies were counted. If p53 confers growth advantage and aids in transformation in 10(3) cells they should multiply faster than the vector transfected cells leading to an increased number of neomycin resistant colonies.

Co-expression of WTp53 with the pCMV-Bam Neo vector, as expected, reduced the number of colonies formed when compared to vector transfected cells (Figure 12). In contrast, cells transfected with mutant p53 showed an increased number of colonies formed, ranging from no change at all (i.e. p53-H179Y) to two- (i.e. p53-R175H) or three- (i.e. p53-D281G) fold increase, indicating that expression of most of the p53 mutants confers a growth advantage on the cells. Mutant p53-D281G formed the highest number of colonies compared to any of the other p53 mutants examined.

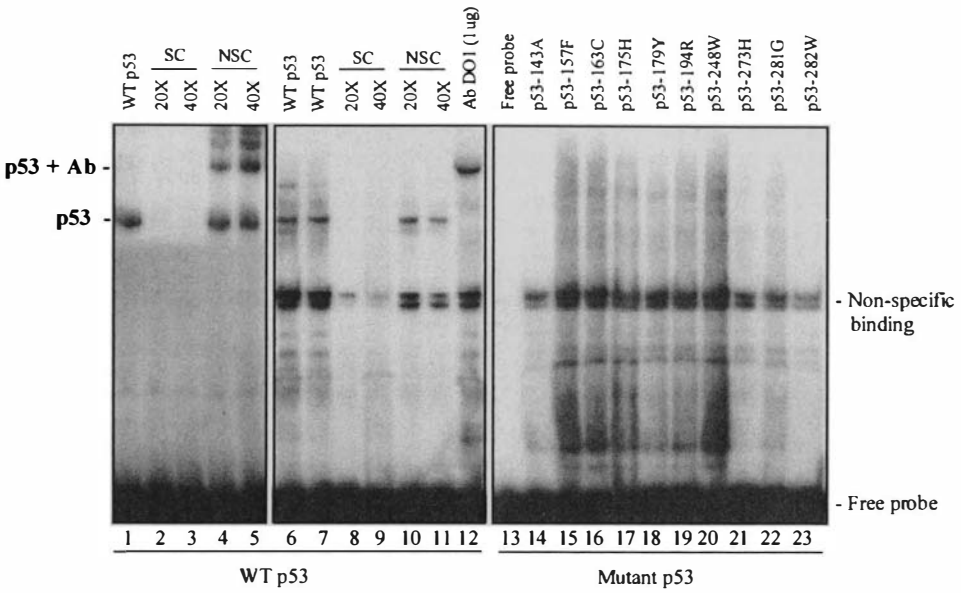
Figure 12. Expression of tumor-derived mutant p53 increases the formation of neomycin resistant colonies.



Tumor-derived p53 mutants do not bind the p53 consensus site. WTp53 function is largely dependent on the ability of the protein to bind DNA and act as a transcriptional

activator. It can bind to a consensus sequence that contains two copies of a 10 base pair motif 5'-PuPuPuCWWGPyPyPy-3' separated by 0-13bp [193]. Consequently, mutations in the DNA-binding domain of the WT protein would result in the inability of the mutant protein to bind DNA.

Figure 13. DNA binding of WT and tumor-derived p53 mutants.

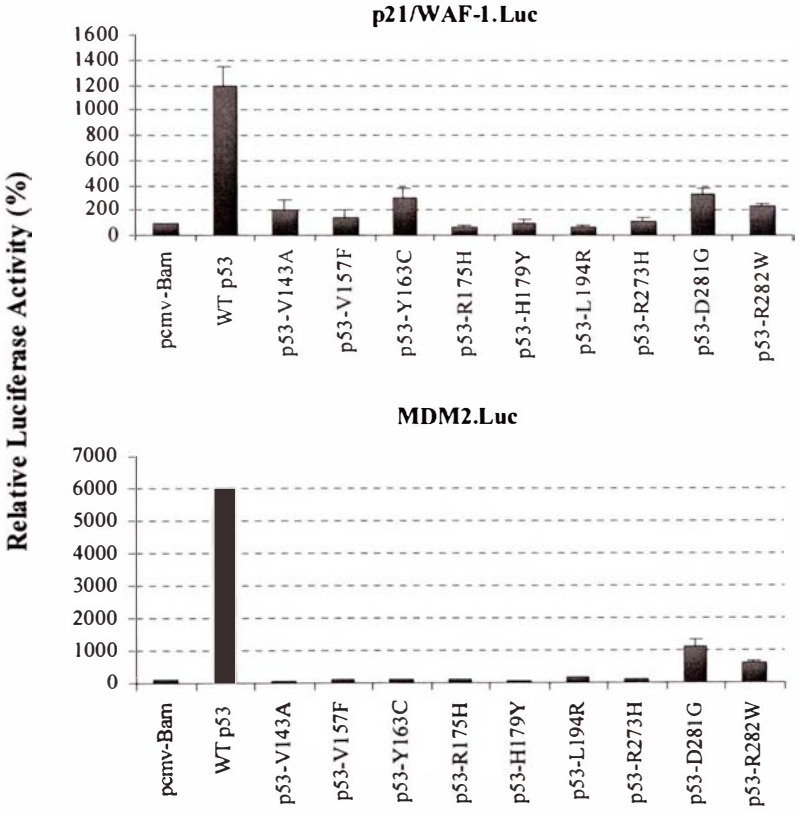


Electrophoretic mobility shift assays (EMSAs) were performed to study the ability of our p53 mutants to bind a p53 DNA-binding site. Assays were conducted using a probe containing the distal p53-binding site found in the p21 promoter to determine the ability of the p53 mutant to bind the WTp53 DNA consensus site [194] (Figure 13).

Lanes 1-5 were incubated with purified protein (100ng) [195] while lanes 6-23 were incubated with 10 μg of total protein from nuclear extracts generated after transfection of SAOS-2 cells with expression plasmids for the specified p53 protein. As expected WTp53 can bind the probe (lanes 1, 6-7) containing the p53-binding site (p21WT). The same probe added in excess as a cold competitor (lanes 2-3, 8-9) diminishes the amount of protein binding observed, and a non-specific competitor (p21MT) added in excess has

no effect in the binding (lanes 4-5, 10-11). Addition of a p53 specific antibody (DO1) produces a supershift (lane 12). Under the same conditions, any of the twelve mutant p53 species tested were unable to bind the binding site containing probe (lanes 14-23). Similar results were obtained using a probe containing the consensus p53 sequence found in the Ribosomal Gene Cluster promoter (data not shown).

Figure 14. Transcriptional activation of p53-inducible promoters by mutant p53.



Since mutations in the p53-DNA binding domain can effectively obliterate DNA-binding ability of the mutant p53 under the conditions of this assay, the question remains as to how some tumor-derived p53 mutants are capable of transactivating genes such as

MDR1, PCNA and *c-myc*, all of which are inhibited in the presence of WTp53. These facts present the possibility that the transactivation observed is mediated through the actions of another transcription (or DNA binding) factor such as Sp1 or through direct binding of the mutant protein to a site of as yet unknown sequence.

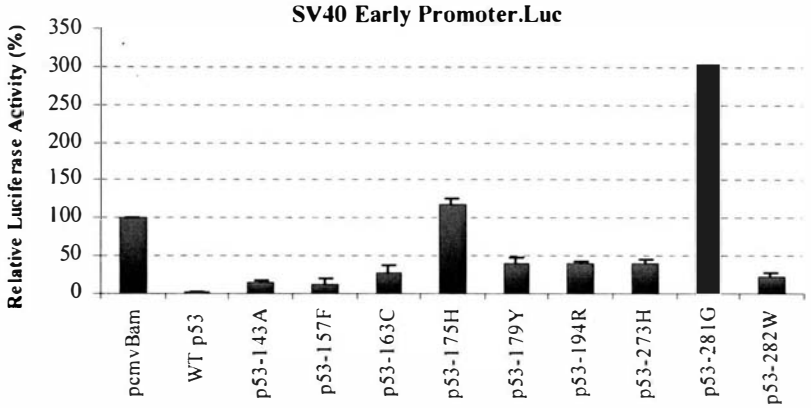
Mutant p53 does not transactivate or repress WTp53-regulated promoters. The tumor suppressor function of the p53 protein is correlated with its ability to transcriptionally activate or repress gene expression [1, 2, 34]. Promoter assays were performed to examine the ability of the various tumor-derived p53 mutants to transactivate targets of WTp53 (i.e. p21 and MDM2). WTp53 induced the p21 promoter by approximately 12-fold compared to the vector. The same amount of tumor-derived p53 mutants was unable to elicit a response of such magnitude (Figure 14). Five of our mutants, p53-V143A, p53-V157F, p53-Y163C, p53-D281G, and p53-R282W transactivated this promoter by at least 2-fold, suggesting that some mutants may have some residual WTp53 activity or can transactivate this promoter using a different molecular mechanism.

The effects of the p53 mutants on the promoter of the human mouse double minute 2 (MDM2) gene, another target of WTp53 was also examined [34, 35, 76]. As reported earlier, WTp53 transactivated this promoter approximately 50-fold compared to the vector while most of our tumor-derived mutant p53 proteins were unable to elicit any response (Figure 14) [196]. Mutant p53-D281G and p53-R282W transactivated this promoter a minimum of five to six fold, though this is 10-fold less than the transactivation observed with WTp53.

Mutants of p53 used in this study were also analyzed for their ability to repress promoters normally inhibited by WTp53 [34, 35, 76, 134, 153, 197]. For this purpose the SV40 early promoter contained within the pGL_2 vector (Promega) was used as a representative example. Again, as reported earlier [197], WTp53 transcriptionally inhibited this promoter approximately 10-fold. The inhibitory effects of mutant p53 varied appreciably, p53-R175H lost 100% of its inhibitory effects; while p53-R273H and p53-H179Y lost approximately 50% (Figure 15). Interestingly, p53-D281G

transactivated this promoter. The reasons why this occurs are still unknown. For subsequent studies mutant p53-R175H, -R273H and -D281G were selected for use as models. The first two mutants were chosen as representative examples of structural (R175H) and contact (R273H) p53 mutants. The third, D281G, was selected in order to correlate results with experimental data already available in the literature.

Figure 15. Transcriptional activation of p53-repressed promoters by mutant p53.

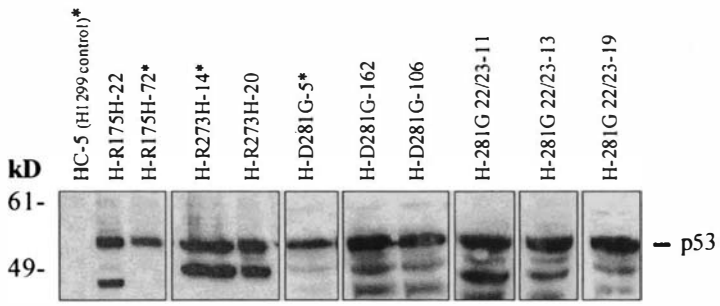


H1299 cells expressing p53-R175H, -R273H and -D281G exhibit an increased growth rate. H1299 (lung carcinoma) cells were used to generate stable transfectants expressing one of three p53 mutants (-R175H, -R273H and -D281G). Additionally cells lines expressing a transactivation deficient form, p53-R175H (L22Q/W23S) or p53-D281G (L22Q/W23S), were also generated. This mutant of p53 contains two additional mutations located within the transactivation domain of the protein [133]. Expression of p53 was determined by western blot analysis (Figure 16). Clones expressing similar levels of p53 were selected for further experiments; asterisks in Figure 16 indicate clones used in microarray hybridization analyses. 21PT (breast carcinoma) and Saos-2 (osteosarcoma) cells expressing mutant p53 were also generated (data not shown).

Earlier data indicated that expression of mutant p53 in cells normally devoid of p53 may give a growth advantage [11, 119, 134]. This notion was tested using a growth rate

assay. Cells were plated at equal densities (1 x 10^5 cells per plate); one plate of each cell line was seeded for each day of the assay. The data in Figure 17 show that mutant p53-expressing H1299 cells proliferate at a higher rate compared to the vector control cell line (HC-5). This increased growth rate was also observed with different clones of H1299 expressing the same p53 mutant and H1299 cells expressing different mutant p53 forms (R175H or R273H). At least two clones of each cell line were tested with similar results, decreasing the possibility that this characteristic is not due to a clonal variation (data not shown).

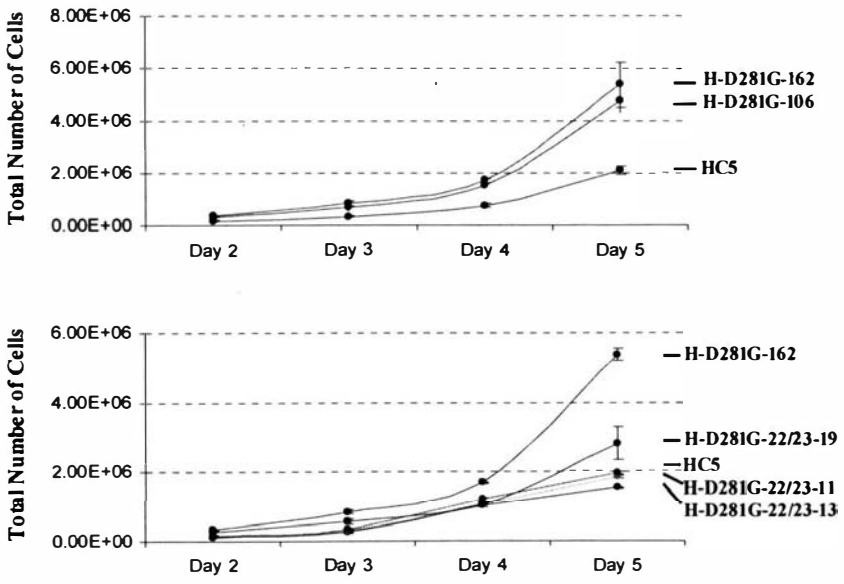
Figure 16. Expression of mutant p53 in H1299 cells.



Expression of the transactivation deficient mutant p53-D281G (L22Q/W23S) did not increase the growth rate of cells as well as those expressing p53-D281G. This mutant p53 is defective in gain-of-function as measured by the lack of increased tumorigenicity when constitutively expressed in 10(3) cells, which become tumorigenic if p53-D281G is stably expressed in these cells [133]. It has also been shown that this particular p53 mutant is defective in transactivation of the MDR1 and *c-myc* promoters, which are up-regulated by p53-D281G [133, 134]. Since p53-D281G (L22Q/W23S) is defective in transactivation, the lack of increased growth rate in cells expressing this mutant implies that transactivation is necessary for this gain-of-function property of mutant p53.

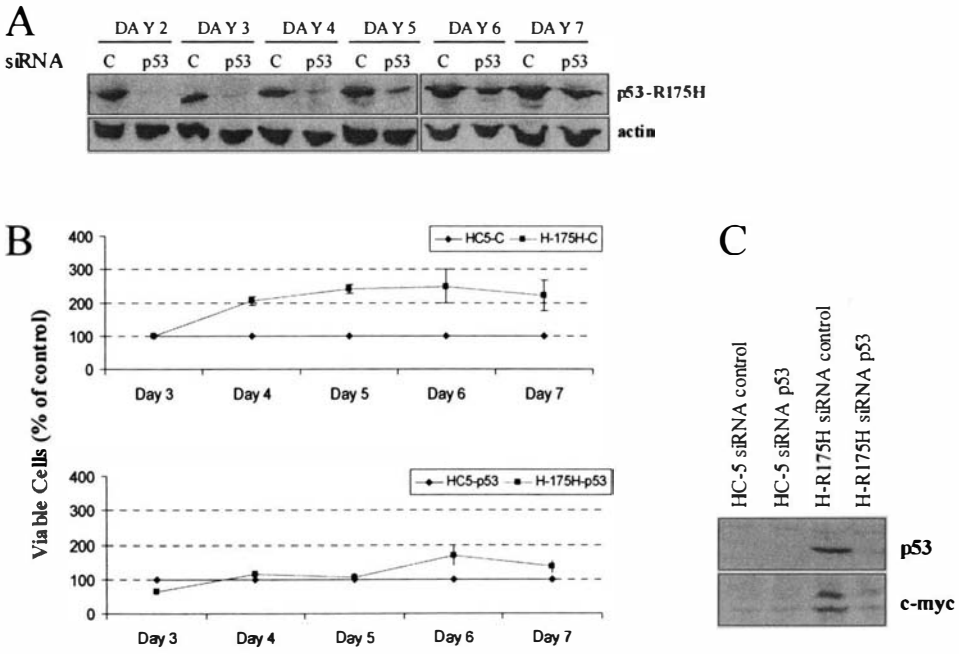
RNAi against p53 decreases mutant p53 enhanced growth. To determine whether expression of mutant p53 remains directly related to the increased growth rate in H1299 cells, we used p53-specific siRNA. H1299 cells expressing p53-R175H were transfected with a double-stranded ribonucleotide sequence specific for p53 (or a non-specific sequence as a control) 24 and 48hrs prior to plating [198]. Cell viability and level of p53 were measured every day for up to five days after plating. Data presented in Figure 18 show that as the mutant p53 protein level decreases after siRNA transfection (18A, days 2-5), the number of viable cells remains similar to that of the control cells (18B, bottom panel, days 3-5). This continues up to day 5, after which the transient effect of the siRNA diminishes. As the mutant p53 protein level begins to increase (18A, days 6-7), cell viability also rises (18B, bottom panel, days 6-7), indicating that continuous mutant p53 expression is necessary for the observed phenotype.

Figure 17. Mutant p53 expression results in enhanced growth rates in H1299 cells.



Thus, there is a direct relationship between mutant p53 level and growth enhancement in H1299 cells. Control siRNA itself does not affect mutant p53 mediated growth enhancement compared to vector control cells (18B, upper panel). In a parallel experiment, levels of *c-myc*, a target of mutant p53, were examined after transfection with siRNA directed against p53 (Figure 18C). Western blot analysis shows that both p53 and *c-myc* levels decrease after transfection. Since the growth rate of mutant p53 expressing cells decreases after siRNA treatment, this suggests a possible involvement of *c-myc* in mutant p53 mediated increased growth rate phenotype in H1299 cells.

Figure 18. The increased growth rate phenotype is dependent on mutant p53 expression.

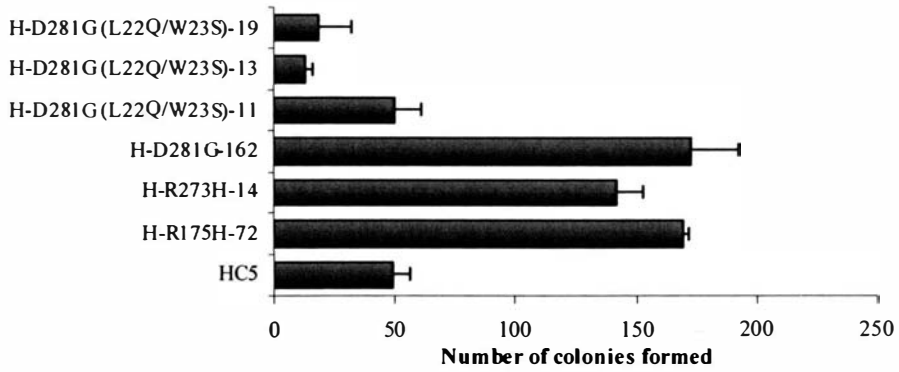


H1299 cells expressing mutant p53 show decreased sensitivity to etoposide. Earlier work by others has also shown that expression of mutant p53 in cells results in a decreased sensitivity to chemotherapeutic drugs [128]. H1299 cells stably expressing

p53-R175H, -R273H, -D281G (or transfected with vector alone) were tested for decreased sensitivity to etoposide using colony formation assay. Data shown in Figure 19 indicate that cells expressing p53 mutants -R175H, -R273H and -D281G are less sensitive to etoposide than the cells stably transfected with vector alone. This is in accord with results published by others using H1299 and other cell lines [128]. Expression of the transactivation deficient mutant p53-D281G (L22Q/W23S) in H1299 cells did not affect chemosensitivity of the cells to etoposide.

These results were recapitulated with H1299 pools and with 21PT cell lines expressing the same three p53 mutants (Figure 20 and data not shown). Furthermore, a bromodeoxyuridine (BrdU) incorporation assay using the H1299 stable transfectants shows increased DNA replication in cells expressing mutant p53 (but not the L22Q/W23S transactivation deficient protein) after drug exposure (data not shown).

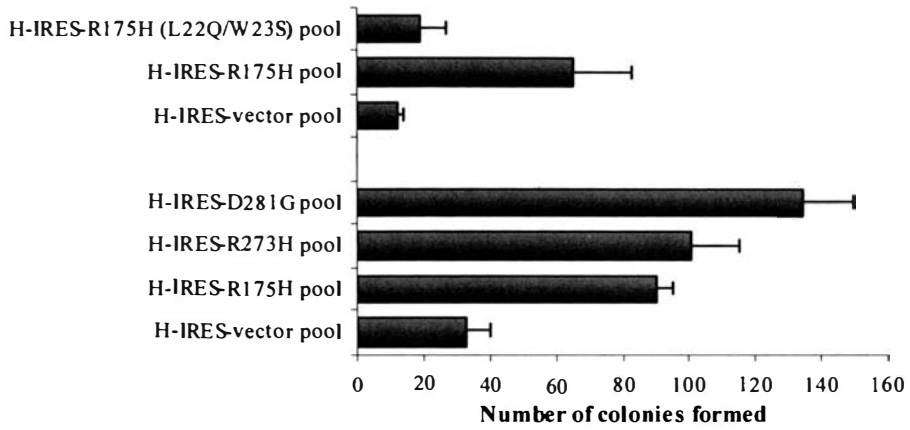
Figure 19. Expression of mutant p53 in H1299 results in decreased sensitivity to etoposide.



As shown, cell lines expressing the transactivation deficient mutant p53 have a disadvantage in etoposide-induced cell death suggesting a correlation between mutant p53-mediated transactivation and the decreased chemosensitivity observed in cancer cells expressing p53 mutants. The mechanism behind this observation is unknown. This decreased ability of the cells to survive is perhaps due to the inability of the

transactivation deficient mutant to up-regulate genes whose expression provides cells protection against etoposide induce cell death.

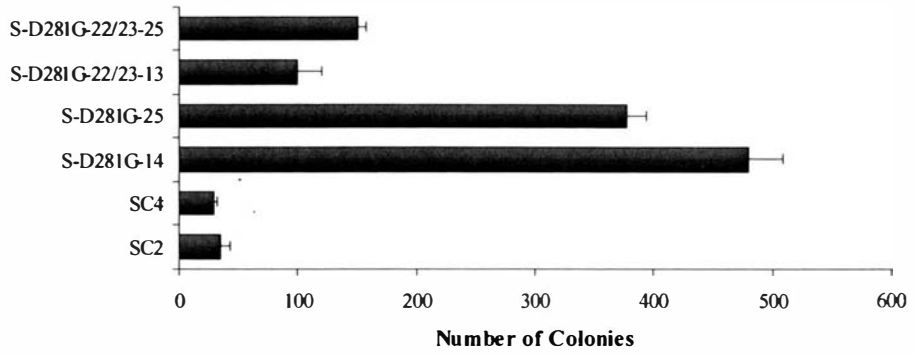
Figure 20. Pools of mutant p53-expressing H1299 cells recapitulate results obtained earlier.



Mutant p53 enhances the plating efficiency of Saos-2 cells in soft agar. The ability of Saos-2 cells to form colonies in soft agar was assayed as described earlier by Dittmer *et al.* [11]. Once again, a transactivation deficient mutant p53 expressing cell line was included in the assay. Saos-2 cells stably expressing mutant p53-D281G, p53-D281G (L22Q/W23S) or vector alone, were plated at a density of 3000 cells/well in 6-well plates over a 0.5% agar plug. Cells were grown in 0.25% agar medium for 4-5 weeks with periodic additions of media. Approximately 1% of vector-transfected cells initially plated formed colonies, whereas mutant p53-D281G displayed an increased ability to grow in soft agar (5-10% above vector control cells). Cells expressing mutant p53-D281G (L22Q/W23S) had a comparative growth of 2-3% (Figure 21). Additionally, colonies of cells expressing mutant p53-D281G were larger (approximately 3-4 fold) than those formed by either vector or p53-D281G (L22Q/W23S) expressing cells (data not shown). Although, mutant p53 enhances the plating efficiency of Saos-2 cells, expression of the transactivation deficient mutant did not completely reduce plating

efficiency. This suggests that for this gain-of-function phenotype, transactivation may not be the only factor involved.

Figure 21. Mutant p53-expressing Saos-2 cells exhibit increased plating in soft agar.



c. Chapter Summary.

Expression of mutant p53 resulted in increased growth rate (H1299 cells), decreased chemosensitivity (H1299 and 21PT cells) and increased plating efficiency (Saos-2 cells). Expression of a transactivation deficient mutant p53 did not induce gain-of-function properties (increased growth rate and decreased chemosensitivity). This supports the idea that genes transactivated by mutant p53 may be responsible for these two gain-of-function phenotypes.

Unlike the other gain-of-function properties tested, soft agar plating efficiency in Saos-2 cells was not significantly affected by the expression of the transactivation deficient mutant, suggesting that transactivation may not be the only factor affecting this gain-of-function property. Perhaps this property is the result of both altered protein-protein interactions and transactivation.

L. Chapter 5.

Modulation of Gene Expression by Tumor-Derived Mutant p53.

The work presented in this chapter has been published in the journals Oncogene and Cancer Research (Oncogene. 2002 Jan 10;21(2):176-89. and Cancer Res. 2004 Oct 15;64(20):7447-54).

a. Introduction.

Since the data described above and earlier suggested that mutant p53-mediated transactivation is necessary for its growth promoting functions, gene expression profiles analyses were conducted using the H1299 cell clones expressing mutant p53-R175H, -R273H, or -D281G in order to identify a possible pathway used by the mutant p53 proteins in inducing oncogenesis [119, 133, 134].

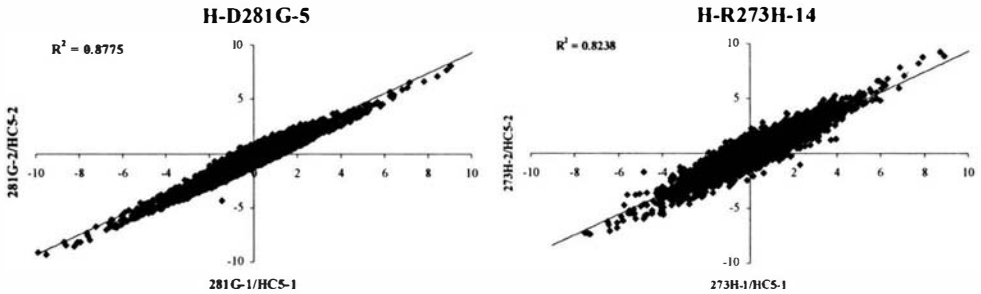
b. Experimental results.

Mutant p53-R175H, -R273H and -D281G expression in H1299 cells affects a common set of genes, including a number of growth-related genes. The gene expression profile of vector transfected H1299 cells was used as controls. Affymetrix U95Av2 arrays (HG-U95Av2) were used for the analyses. Hybridization signals were normalized and filtered and changes in gene expression were detected in terms of S-score for a given gene between two compared microarrays [199, 200]. The magnitude of the S-score represents the significance of change, thus an absolute value S-score greater than 2 represents a p-value < 0.045, uncorrected for multiple comparisons. Scatter plot analyses (Figure 22) of the S-scores show a very high degree of consistency of changes in gene expression between biological duplicates. The scatter-grams also indicate a relatively large number of changes in gene expression in each of the mutant p53-expressing cell lines versus the control cell lines.

In order to identify genes reproducibly affected by the expression of any mutant p53 in these cell lines, the S-score data was analyzed by a permutation method computer program, Significance Analysis of Microarray (SAM) [201]. A SAM analysis of the S-scores has the advantage that small and subtle consistent changes reproduced across the

array samples can be detected. A one-class analysis and a stringent False Discovery Rate (FDR) of 0.3% were used so as to focus on the most reproducible changes evoked by the p53 mutant across all hybridizations. One-hundred and eight (88 up-regulated, 20 down-regulated) genes were identified as having an altered expression in the mutant p53-expressing cell lines. We found no WTp53 regulated genes such as p21, MDM2, PUMA, DDB2, ERCC5, GADD45 or *Bax* [37, 38] included in the list. On the other hand, *c-myc* and PCNA, which have been reported as up-regulated by mutant p53, were present in our list of up-regulated genes. MDR1, BFGF, IL-6 and Bcl-2 were up-regulated minimally and therefore are not included in our final list of genes [119, 153, 156, 190, 202, 203].

Figure 22. Scatter-gram analysis of S-scores generated.



This type of analysis excludes those genes differentially affected by the p53 mutant proteins, therefore, a cluster analysis of this list only reveals two major clusters containing genes that are up-regulated or down-regulated in common by all three p53 mutants (Figure 23). In parallel, microarray data obtained from H1299 cells infected with adenovirus expressing WTp53 (or β-galactosidase control) were analyzed in a similar manner (data not shown) and compared to the transcriptional profiles from the mutant p53 expressing cell lines in order to determine the transcriptional response of the genes in the presence of WTp53. Table 3 shows a representative example of the genes identified as up-regulated in H1299 grouped in functional categories such as metastasis (i.e. human myeloid cell differentiation protein or MCL1), cell cycle (i.e. ASNS and

E2F-5) [204, 205], amino acid homeostasis (i.e. ASNS) [204], transcriptional regulation (i.e. Nuclear Factor-IL6) [206, 207], and cell survival (i.e. NF-κB2) [208, 209]. Among the identified targets were the ASNS [204, 210-212] and the transcription factor NF-κB2 [208, 209]. Asparagine is a non-essential amino acid that has been reported as regulating expression of genes involved in cell growth [211, 212]. Depletion of asparagine can result in apoptosis and treatment of cancer with Asparaginase (ASNase), an enzyme used in combination chemotherapy, can reportedly induce an apoptotic response presumably through depletion of asparagine and asparagine synthetase expression alone has been found to be sufficient to induce ASNase resistance in cells [213, 214]. The transcription factor NF-κB2, on the other hand, has been implicated in cell survival and chemoresistance, presenting a role for NF-κB2 [208, 209].

Figure 23. Expression of mutant p53 in H1299 changes the cellular transcriptional profile.

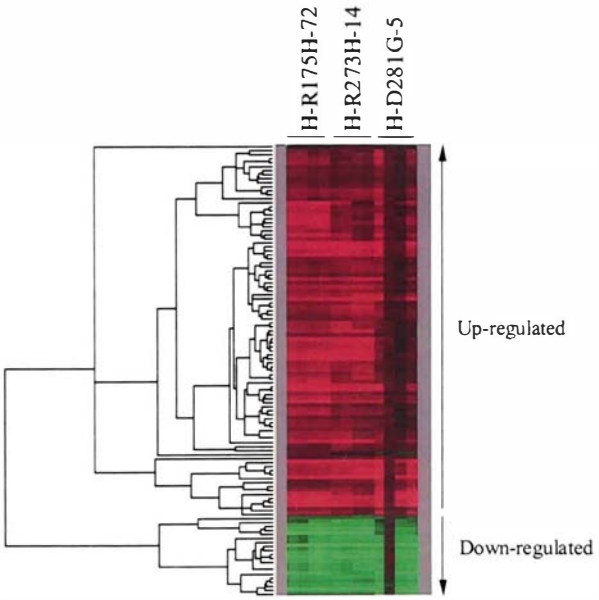


Table 3. Representative example of genes up-regulated by mutant.

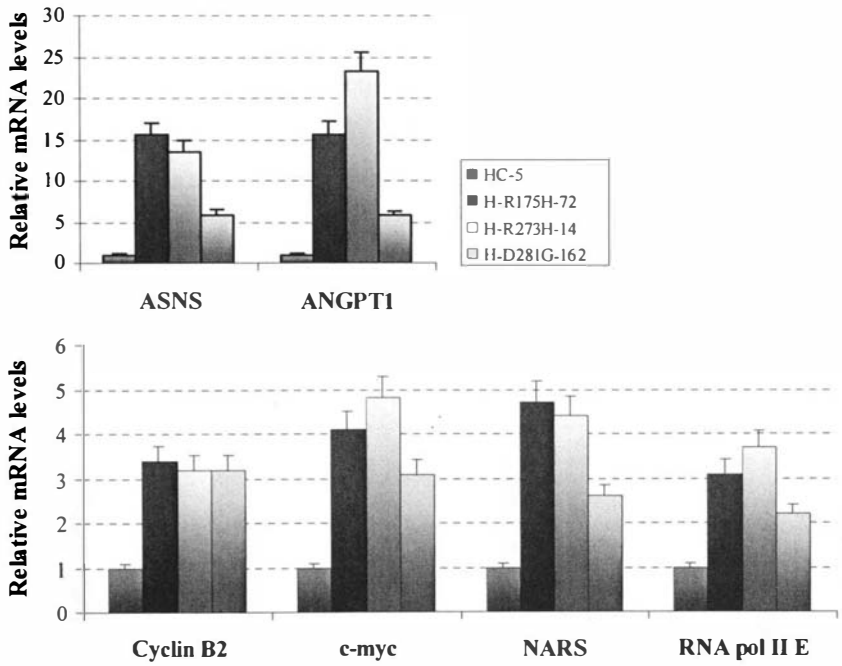
Function	Gene Name	Gene bank No.	S-score value	Effect by WTp53
Amino acid and Protein Synthesis	glycyl-, threonyl-, asparaginyl -tRNA synthetases	U09510; M63180; D84273	5.248; 3.691; 2.880	Repressed
	Asparagine synthetase* (ASNS)	**M27396**	5.071	Repressed
General Metabolism/ Cellular component	type II inosine monophosphate dehydrogenase (IMPDH2)	L33842	3.036	Repressed
Cell Cycle	Cyclin H	U11791	2.035	Repressed
	Cyclin B2	**AL080146**	3.327	Repressed
	cdc25A	**M81933**	2.478	Repressed
	Associated molecule with the SH3 domain of STAM (AMSH)	**AF052135**	2.199	Repressed
Oncogenesis-Transformation-Invasion-Metastasis	*c-myc*	V00568	2.749	Repressed
	Human myeloid cell differentiation protein (MCL1)	L08246	3.607	Repressed
	Apoptosis Inhibitor 5 (API5)	**U83857**	2.057	Repressed
	ras homologue family member G (rhoGAP)	**X61587**	1.045	Repressed
	EBAG9	**AB007619**	1.798	Repressed
	Angiopoietin 1 (ANGPT1)	**NM_001146**	2.719	NA#
	Integrin alpha 6 (ITGA6)	**X53586**	3.917	Repressed
DNA Replication	**DNA polymerase delta-2 (POLD2)**	**U21090**	2.783	Repressed
Survival-Apoptosis	NF-kB, p52	X61498	2.664	Repressed
	NF-kB, p52	**X61498**	2.664	Repressed
Transcription	**Transcription factor E2F-5**	**D82348**	3.260	Repressed
	RNA polymerase II, polypeptide E (RNA polII E)	**D38251**	3.171	Repressed

* Transcriptional up-regulation of genes in bold lettering has been confirmed by either QPCR or *in vivo* transcriptional assays. # NA = Not Affected.

The authenticity of changes in gene expression observed in the microarray analyses was verified by quantitative polymerase chain reaction (QPCR) for a representative

group of gene targets (Figure 24). Gene specific primers were designed to detect and determine relative mRNA levels in cells expressing mutant p53 compared to control cells. QPCR was performed using the Light Cycler (Roche) with the DNA-binding dye SYBR Green (Molecular Probes) that binds to the minor grooves of the DNA double helix. Total RNA was isolated from stably transfected H1299 cell lines expressing the mutant p53 protein (-D281G-5, -R273H-14 and –R175H-72). The isolated RNA was DNAse treated and reverse transcribed using commercial kits from Invitrogen to generate the cDNA used in the experiment as described earlier [156]. Primers were designed using Oligo 5 (Molecular Biology Insights).

Figure 24. QPCR analysis of cells expressing mutant p53.

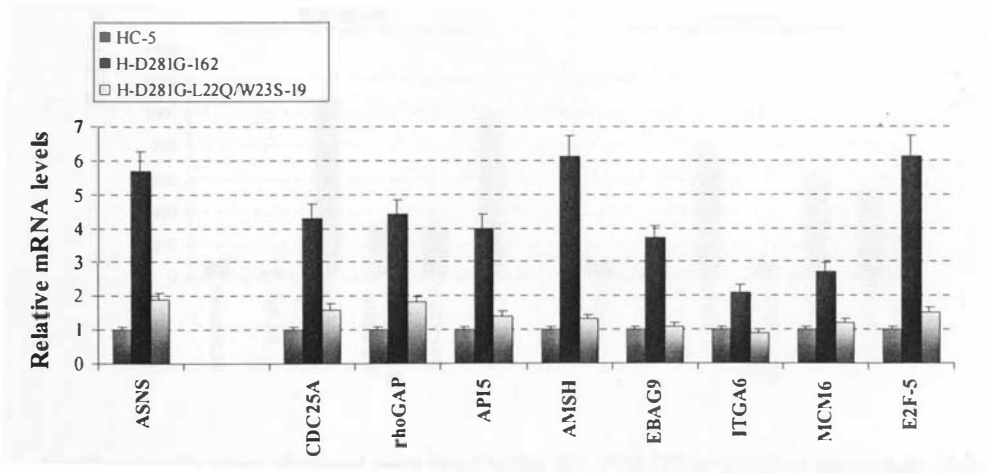


H1299 cells expressing a transactivation deficient mutant p53-D281G (L22Q/W23S) are deficient in up-regulating mutant p53 targets. Cells expressing

the triple mutant p53-D281G (L22Q/W23S) were also examined by QPCR. This transactivation deficient mutant did not retain the ability to up-regulate expression of mutant p53 target genes identified by the microarray expression analysis. As shown in Figure 25, in all the cases examined, the presence of the two additional mutations within the transactivation domain resulted in a significant loss of up-regulation compared to the transactivating counterpart. The combined data presented indicates that transactivation by tumor-derived p53 mutants is directly related to the mutant p53 protein's ability to induce gain-of-function properties in cells in which they are expressed.

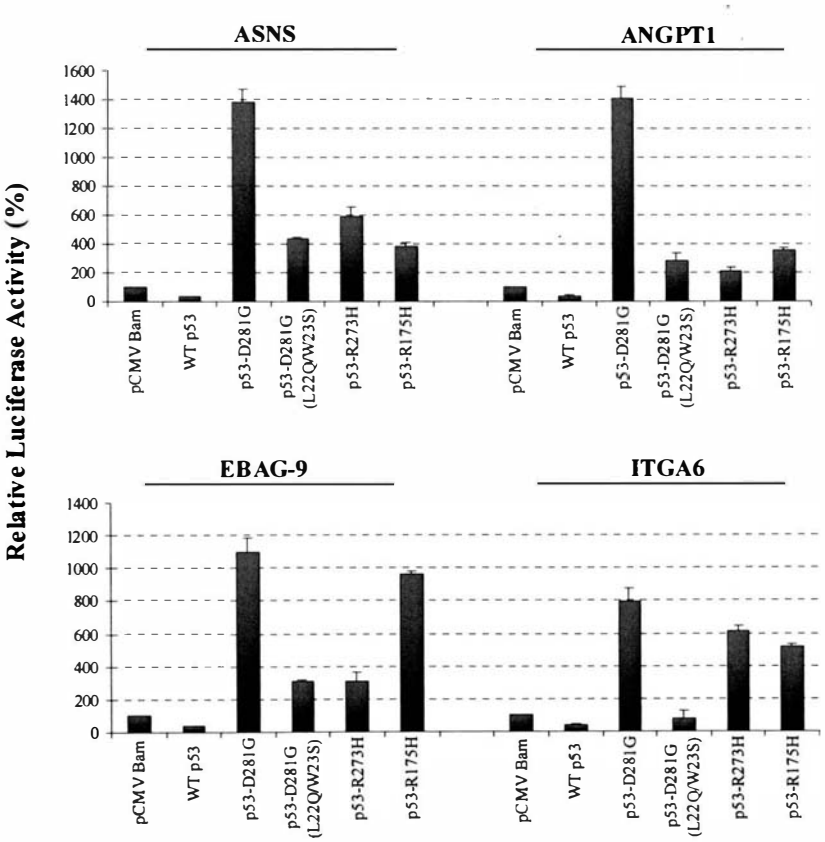
Promoters and cloned upstream sequences of a number of genes up-regulated by p53 mutants are transactivated in transient transcriptional assays. To determine whether the promoters of some of the genes whose expression was found to be up-regulated are transactivated by mutant p53 in transient transcriptional assays, seven promoters or presumptive promoters of the up-regulated genes were assayed. The presumptive promoters for angiopoietin 1 (ANGPT1), integrin α6 (ITAG6), cyclin B2, polymerase delta subunit 2 (POLD2) and E2F-5 were cloned from genomic DNA as described in Materials and Methods using information obtained in the NCBI database.

Figure 25. Transactivation deficient mutant p53 fails to up-regulated genes *in vivo*.



Functions of the promoters of estrogen responsive A9 (EBAG9) [215] and asparagine synthetase (ASNS) [212], and the presumptive promoters for ANGPT1, ITGA6, Cyclin B2, POLD2 and E2F-5 were studied using a luciferase reporter plasmid and p53 null human osteosarcoma Saos-2 cells [156] in the presence and absence of WT or mutant p53 expression plasmids. The results obtained show that mutant p53 can transactivate the promoters tested (Figure 26).

Figure 26. Promoter analysis of genes identified by microarray analysis.



Similar results were obtained with the Cyclin B2, POLD2 and E2F-5 promoters (data not shown). Although there is some variation among the p53 mutants, they all have the

capacity to transactivate the promoters, suggesting a uniformity of function. WTp53 repressed all the promoters tested. The transcriptional data from these promoters further verifies the earlier results of the microarray hybridization analysis.

Other p53 mutants were also tested for transactivation of the ASNS promoter (Figure 27). As can be seen in the figure, the different mutants up-regulated the ASNS promoter to various degrees, further suggesting that p53 mutants may induce oncogenic properties through a common molecular mechanism.

Figure 27. Mutant p53 mediated transactivation of the ASNS gene.

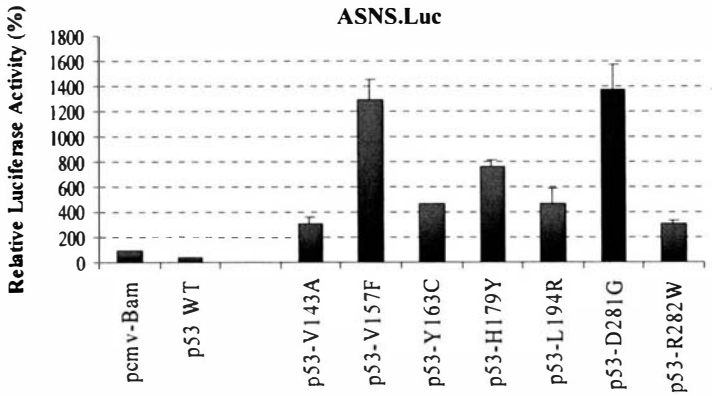
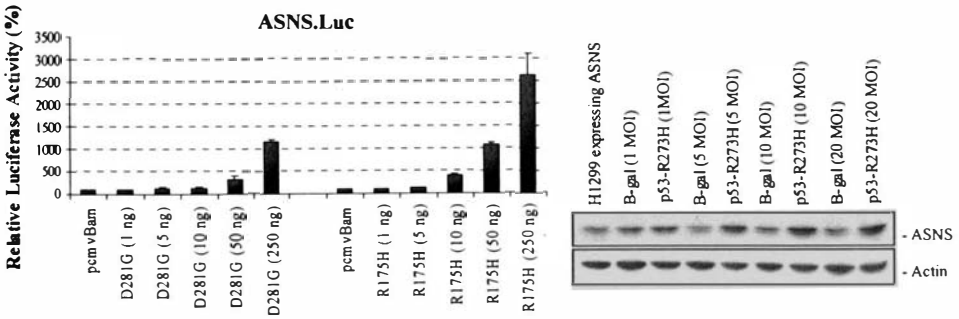


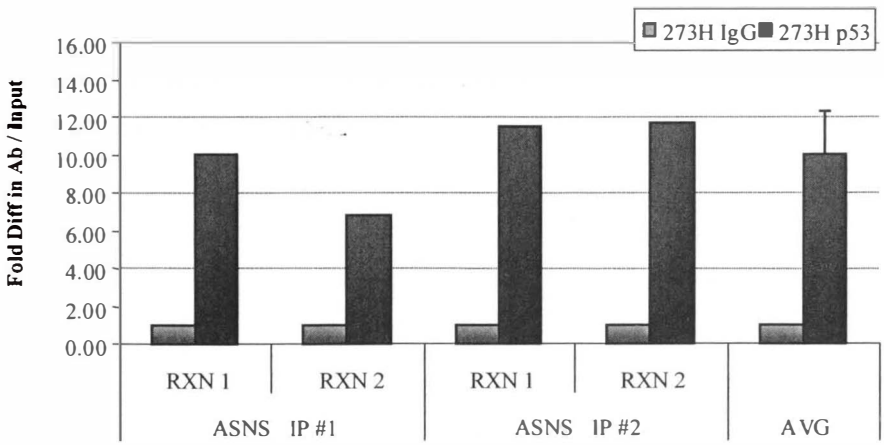
Figure 28. Mutant p53-R273H increases protein level expression of ASNS.



Transactivation of the ASNS promoter by mutant p53 was concentration dependent for p53-R273H and -D281G. Furthermore, adenoviral infection of H1299 cells with an adenovirus expressing mutant p53-R273H at increasing multiplicities of infection (MOI) resulted in increased asparagine synthetase protein levels (Figure 28).

Chromatin immunoprecipitation reveals the presence of mutant p53 on the ASNS and hTERT promoters. We carried out Chromatin Immunoprecipitation (ChIP) [216] assays to determine the presence of mutant p53 in the promoter region of the ASNS genes using antibodies specific for p53 or an IgG control. Cell extracts prepared from the mutant p53-R273H expressing H1299 cell line were used for immunoprecipitation as described in Experimental Methods. The presence of an ASNS promoter fragment in the immunoprecipitated DNA was detected using specific PCR primers. QPCR analysis of the immunoprecipitate reveals that mutant p53 localizes on the ASNS promoter (Figure 29). The figure shows results from two independent immunoprecipitations. Each PCR reaction was performed in duplicate.

Figure 29. Mutant p53 localizes on the ASNS promoter.



c. Chapter Summary.

Gain-of-function phenotypes are most likely the result of transcriptional changes induced by the expression of mutant p53 in cells. It had been shown earlier that transactivation deficient forms of mutant p53 did not increase the tumorigenic potential of 10(3) when injected into nude mice [133, 156]. Moreover, inhibition of mutant p53 through RNA interference also decreased gain-of-function phenotypes [148]. Therefore, in order to identify the genes responsible for the observed phenotypes, global gene expression analyses were carried out using p53-null H1299 cells stably transfected to express mutant p53 (-R175H, -R273H and -D281G).

A thorough and stringent analysis revealed 150 genes up-regulated by the expression of mutant p53. These genes were inhibited or unaffected in the presence of WTp53. Up-regulation of a number of these genes was confirmed by QPCR and transient transcriptional promoter analyses; expression of the transactivation deficient mutant p53-D281G (L22Q/W23S) did not result in up-regulation of the tested genes, further supporting the idea that transactivation of genes is directly related to gain-of-function phenotypes. Using the ASNS gene promoter as a model, it was shown that transactivation by mutant p53-R273H and -D281G was concentration dependent and that the increased transcription did indeed result in increased ASNS protein levels. Finally, using chromatin immunoprecipitations, localization of the mutant p53-R273H protein was detected on the ASNS promoter.

M. Discussion.

Since the C-terminal region of wild-type p53 harbors the oligomerization domain, and oligomerization is necessary for the biological functions of wild-type p53 [6, 23, 180, 191, 217], it was hypothesized that the C-terminal sequences for mutant p53 would be required for oligomerization, and the oligomerization may be critical for gain-of-function [134]. This hypothesis was tested using several different approaches.

First, the tetramerization domain of p53-D281G was replaced with either a coiled-coil dimerization domain from the yeast GCN4 protein (p53-D281G 343cc) or a modified coiled-coil domain that can act as a tetramerization motif (p53-D281G TZN4). The transcriptional data generated with these heterologous constructs showed that mutant p53 required the C-terminally located oligomerization and non-sequence-specific nucleic acid-binding domain for its transactivation properties (Figure 5). This requirement was sequence-specific and not met by merely allowing the protein to oligomerize (Figure 6). Similar experiments were carried out using WTp53 (data not shown). However, in the context of WTp53, replacement of the tetramerization domain of WTp53 by the dimerization domain (WTp53 343cc) could effectively sustain transactivation and growth suppression functions of WTp53 [176, 178-180, 218], data not shown].

Interestingly, the replacement of WTp53's tetramerization domain by a derivative of the coiled-coil structure resulting in a tetramerization domain could not induce transcription from the human p21 promoter as well as p53 343cc (data not shown). The reason for this apparent discrepancy is unknown, but could be related to the DNA concentrations used in the different transfections [23]. Also, the wild-type p53 derivatives inhibited the CMV immediate-early promoter significantly; the extent of inhibition is comparable to that mediated by wild-type p53 (data not shown). Thus, the functions of wild-type p53 were preserved using the various oligomerization domain derivatives as was shown earlier [176, 178-180, 218].

As a second approach, a N-terminal deletion mutant of p53 was used. This deletion mutant retained the oligomerization domain but dispenses off the transactivation domain and a large portion of the sequence-specific DNA-binding domain, inactivating the

transactivation and DNA-binding activities of wild-type p53 [23]. Co-transfection followed by immunoprecipitation analysis showed that p53 del. 1-293 forms hetero-oligomeric complexes with p53-D281G (Figure 9). This interaction also inhibited mutant p53-D281G-mediated transactivation of the EGFR and MDR1 promoters, suggesting that hetero-oligomerization inactivates transcriptional functions of mutant p53 (Figures 7 and 8). Interestingly, this decreased transactivation was only partial in stably transfected murine 10(3) cells and although 10(3) cells stably transfected with p53-D281G express 5 to 6-fold more PCNA and NF-κB2 mRNA compared to the cells transfected with vector alone, co-expression of p53 del. 1-293 reduced this transactivation only partially (Figure 10). The data, therefore, support the idea that proper oligomeric forms of mutant p53 are required for its transactivation function.

Since replacement of the oligomerization domain of p53-D281G inhibited the transactivation ability of the mutant protein while having little of no effect in the context of WTp53, the data strongly suggest that the C-terminal region of WTp53 mainly functions in oligomerization whereas in the context of mutant p53 it seems to perform additional functions. Thus, there is a clear distinction in the requirement of the C-terminal domain for wild-type and mutant p53-mediated transcriptional functions. A number of possible explanations for this requirement can be offered: 1) WTp53 has a nucleic acid binding activity associated with its C-terminal region [9, 179]. It is possible that under appropriate conditions mutant p53 utilizes this activity to interact with promoter elements and other factors to drive transcription from a promoter. 2) A number of post-translational modifications, such as phosphorylation and acetylation have been reported to occur in specific sites located near the C-terminus of WTp53 [34, 35, 219]; perhaps mutant p53 requires these modifications for its transactivation function. 3) Finally, the C-terminal region could be a site for protein-protein interactions [i.e., c-*abl*, [220]] needed for mutant p53-mediated transactivation. The over-expression of p53 del. 1-293 would compete for such modifications or functions with the intact protein resulting in inhibition or decrease in transactivation. Analyses involving more specific mutations near the C-terminus along with functional assays are required before a comprehensive

idea and solid conclusions about the function of the C-terminus in mutant p53-mediated transactivation can be made.

Since co-expression of p53 del. 1-293 with p53-D281G inhibited transactivation, it was expected that this interaction would also inhibit gain-of-function properties. Results shown in Tables 1 and 2, however, indicate that this is not the case. Co-expression of p53 del. 1-293 did not inhibit colony formation and tumorigenicity induced by mutant p53-D281G. Co-immunoprecipitation experiments showed that indeed these proteins interact with each other *in vivo* (Figure 11). Thus, hetero-oligomerization between p53-D281G and p53 del. 1-293 may not inhibit the growth enhancing properties of the full-length p53-D281G, although it does inhibit its transactivation property. This observation seems to go against the notion that transactivation by tumor-derived p53 mutants may be responsible for the gain-of-function.

It is necessary then to point out that before reaching such a conclusion, it must be first shown that all the mutant p53 protein molecules are complexed with p53 del. 1-293, and that every complex in every stably transfected cell contains at least one molecule of p53 del. 1-293 capable of inhibiting transactivation by the tetrameric complex. Although p53 del. 1-293 and p53-D281G can form hetero-oligomeric complexes in 10(3) cells, it is possible that any free mutant p53 tetramer could induce the gain-of-function properties observed. It is also possible that because of extended number of passages the cells have gone through, the 10(3) cells expressing p53-D281G became spontaneously transformed independent of the involvement of mutant p53-D281G; in such a case inhibiting mutant p53 transactivation activity would not decrease its oncogenic potential. Murine 10(3) cells indeed have a tendency to spontaneously transform when cultured for prolonged periods of time (Deb S., unpublished data). However, the conclusions that can be drawn from this portion of the research are that mutant p53 requires both its C-terminal sequences and proper oligomerization for transactivation and gain-of-function properties.

Expression of gain-of-function p53 mutants has also been shown to increase the growth rate of cells, decrease sensitivity to chemotherapeutic drugs, interfere with p53-independent apoptosis, and induce centrosome abnormalities in cells among other

properties [85, 124, 128, 142, 221]. Gualberto *et al.* [123] reported that some p53 mutants confer a dominant gain-of-function phenotype that disrupts the spindle checkpoint control. They have also shown that this property did not require the transactivation function of mutant p53. Thus, it is possible that some gain-of-function properties are achievable even in the absence of the transactivation function of mutant p53 and may be solely due to protein-protein interactions.

This makes it necessary to identify which gain-of-function properties are dependent on transactivation and which involve other mechanisms such as protein-protein interactions. A study of various p53 mutants available in this laboratory using a colony formation assay, revealed that the p53 mutants vary in their ability to induce a growth advantage suggesting that these mutants have a tumorigenic potential (Figure 12). Transcriptional reporter assays and EMSA analyses also showed that these mutants did not bind the WTp53 DNA binding consensus site and were unable to properly transactivate the human p21 and MDM2 promoters (Figures 13 and 14), showing that the mutants lack WTp53 activity.

Using p53-R175H, -R273H and -D281G as models, the data generated show that expression of all three of these p53 mutants can induce increased growth rates in H1299 and 10(3) cells, decreased chemosensitivity in H1299 and 21PT cells, and increased soft agar plating efficiency in Saos-2 cells (Figures 17, 19-21 and data not shown). In all three of the phenotypes studied, expression of a transactivation deficient mutant p53 decreased or nullified the observed phenotypes. Interestingly, in Saos-2 cells, expression of this transactivation deficient form of mutant p53 did not completely nullify the ability of the cells to plate in soft agar, suggesting that transactivation is not the only factor involved in mediating this phenotype. This further supports previously reported data that certain mutant p53 gain-of-function phenotypes can be achieved independent of transactivation [170-175].

Expression of mutant p53 remained directly related to the observed growth rate in H1299 cells (Figure 18). Inhibition of mutant p53 expression through RNA interference decreased the growth rate of cells to a rate comparable to that of control cells. Thus, the

data generated not only suggest that expression of mutant p53 was responsible for the phenotype, but that continuous expression of the mutant protein is necessary to maintain the gain-of-function phenotype observed in these cells. It was also observed that decreasing the mutant p53 protein levels also decreased protein levels of *c-myc*, suggesting a possible role for this protein in mutant p53-mediated enhanced growth in H1299 cells. Further experiments using this approach could be used to determine the exact role of mutant p53 expression in other gain-of-function phenotypes.

In order to understand the mechanism behind the phenotypes observed in cells expressing mutant p53, gene expression analyses were performed to study the transcriptional changes that occur in cells when mutant p53 is expressed in them. Using microarray hybridization analyses, a group of genes was identified as regulated by the p53 mutants in the absence of WT. QPCR data (Figure 24) suggests that our microarray hybridization analysis is reliable. Some of the identified genes were found to be involved in cell growth, survival, adhesion and angiogenesis (Table 3). The finding that all three p53 mutants transactivate a common set of genes (Figure 23) in the absence of WTp53 is unique and provides highly significant information toward understanding mutant p53-induced gain-of-function suggesting the existence of a common pathway utilized by p53 mutants to aid in oncogenesis. Of the genes identified in our microarray, MCL1, EBAG9, ANGPT1, ITGA6, NF-κB2 and E2F-5 have been reported as up-regulated in various cancer types and established cell lines [205, 222-226]. Since information is not available in the literature, it remains to be seen whether these genes are also up-regulated in the natural context of the p53 mutation, i.e. in human cancers carrying a p53 mutation. It is important to point out that some of the genes identified in these analyses function as transcription factors (i.e. E2F-5 and NF-κB2), which suggests the possibility that some of the genes modulated by p53 may be modulated through these transcription factors. Further analyses will be necessary in order to sort out the array of genes modulated by mutant p53 into primary and secondary targets. Nevertheless, it is very likely that the genes modulated by mutant p53 identified here may play a role in the various gain-of-function phenotypes that have been attributed to mutant p53 in the literature. For

example, the transcription factor NF-κB2 has been implicated in cell survival and chemoresistance, presenting a role for NF-kB2 [208, 209]. It is then possible that this factor is somehow involved in chemoresistance brought about by the expression of mutant p53 in cells (Appendix A).

Although the exact molecular mechanism behind the gain-of-function properties of p53 mutants is yet to be completely clarified, transactivation of growth promoting genes remains a strong candidate. Another possibility that has been proposed for the observed gain-of-function properties is the neutralization of p73 and p63, two p53 family members [227-229]. Both p73 and p63 share significant sequence similarity with p53, and are able to transactivate promoters containing a WTp53 consensus site in their promoter region, such as p21/WAF1 and MDM2. As with WTp53, over-expression of p73 or p63 can result in apoptosis and cell cycle arrest [230]. Although there is sequence similarity between the oligomerization domains of the three proteins, WTp53 does not hetero-oligomerize with p73 and p63 [231]. More recent reports, on the other hand, suggest that p53 mutants can interact with p73 and p63 and that these interactions occur via the DNA-binding domain (amino acids 100-293) of the mutant p53 protein [232-234]. This functionally inactivates p73 (and p63) and there is evidence to suggest that this interaction is responsible for the decreased sensitivity to chemotherapeutic drugs that is observed in cells over-expressing mutant p53 [232, 233, 235]. However, data presented in the literature also suggest that transactivation by mutant p53 is directly related to its gain-of-function properties. The transactivation deficient mutant p53-D281G (L22Q/W23S) contains the same amino acid mutation within the DNA-binding domain as its transactivating counterpart (p53-D281G). This mutant then should be able to interact with p73 and p63 and its expression in cells should result in decreased sensitivity to chemotherapeutic drugs. This is not the case in the H1299 cell culture model presented above. Therefore, it is possible that this phenotype may require a combination of transcriptional activation of certain genes as well as protein-protein interaction with p73, p63 and perhaps other proteins. Preliminary studies performed in the laboratory suggest that although p73 is present in H1299 cells, its interaction with mutant p53 is

minimal [[236] and data not shown]. Additionally, this transactivation deficient mutant failed to promote tumorigenicity suggesting further complexities to mutant p53 gain-of-function [133].

In general, single amino acid changes have an effect in both the transactivation and transrepression activities of p53 (Figures 14 and 15). This is likely due to the presence of mutations within the DNA-binding domain [234]. Thus, a decreased ability to bind DNA would result in a decrease in the transactivation and repression activities of p53. This does not explain however, the ability of certain p53 mutants to transactivate promoters of genes (i.e. PCNA, IL-6, BFGF, IGF-I, and others) [reviewed in [119, 153, 190, 202]]. It is known that mutant p53-mediated transactivation does not require WTp53 DNA-binding sites, showing that the mechanism of transactivation by mutant p53 is distinct from that of the WTp53 protein [119, 134, 155].

Three possible molecular explanations exist for mutant p53-mediated transactivation:

1) It may be that p53 mutants differ in their ability to recognize and bind responsive elements, although there is no strong evidence so far for any mutant p53 consensus sequence in the literature. In this case, mutant p53 proteins may possess a DNA binding ability altogether distinct from the WT protein. Analyses of mutant p53 DNA binding has revealed that the mutant p53 protein can physically associate with certain responsive promoters indicating that mutant p53 proteins are targeted to DNA in a specific manner [152, 237]. Intriguingly, sequence analysis of mutant p53 responsive promoters has yet to show any sequence homology nor describe a mutant p53 specific response element. The question still remains, if mutant p53 can associate with DNA, then how is this occurring? It appears that the interaction of mutant p53 with DNA is determined by the structure of DNA and not by sequence specific motifs [238, 239]. This possibility arises from observations that mutant p53, but not WTp53, binds with high affinity to Matrix Attachment Regions (MAR) elements. MAR elements are polymorphic, have no known consensus sequence and are distributed throughout the genome. *In vitro* binding of mutant p53 to MAR elements is largely dependent on the length of the DNA and not the sequence [240]. This is further supported by findings that mutant p53 proteins bind with

high affinity to DNA sequences with a propensity to undergo structural transitions [241, 242]. The finding that transactivation of some promoters by mutant p53 requires both the DNA binding and C-terminal domains seems to support this idea [134, 154, 156, 243]. DNA structure-dependent recognition would explain the lack of sequence similarity between the various mutant p53 responsive promoters. Searching for structural similarities in the DNA rather than sequence motifs may be a more meaningful approach to understanding mutant p53-mediated transactivation.

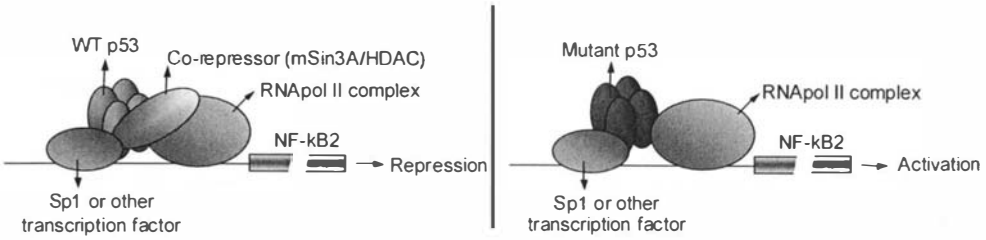
2) A second possibility is that mutant p53-mediated induction of mRNA, as judged by microarray or QPCR analysis, is the result of stabilization of RNA by a post-transcriptional mechanism, although no direct evidence exists for this mechanism either. The results of the promoter analyses suggest that RNA stabilization is not a mechanism of mutant p53 mediated gene up-regulation, at least for the target genes identified here.

3) A third explanation for mutant p53-mediated transactivation is the possibility that mutant p53 targets promoters regulated by other transcription factors. Mutant p53 has been shown to interact with other sequence specific transcription and nuclear factors such as Sp1, ATF/CREB or NF-κB [244-246]. Sp1 can interact with both WTp53 and mutant p53 [247, 248]. While the interaction between WTp53 and Sp1 may be dynamic, mutant p53 may become tethered to Sp1 resulting in the up-regulation of genes. As such, differences in levels of transcription can be accounted for by the identity of the transcription factors involved, or by the relative affinity of the mutant p53 for the transcription factor. This model of transactivation is presented in Figure 30.

Since different promoters behave differently towards p53 (WT or mutant), it is possible that the arrangement of other transcription factors present on a promoter influence the effects of p53 on the overall transcription of that promoter. While WTp53 inhibits Sp1-mediated transcription by interfering with DNA binding of Sp1, mutant p53 seems to enhance the activation potential of Sp1 [249] suggesting that stereo-specific complexes containing either WT or mutant p53 are functionally different. On the other hand, the regulation of WT and mutant p53 of MDR1 involves different promoter regions [250]. Taken together, proximity, arrangement and/or physical contact of the mutant p53

with transcriptional factor(s) or complex(es) may determine the outcome of the interaction.

Figure 30. Proposed model of transactivation.

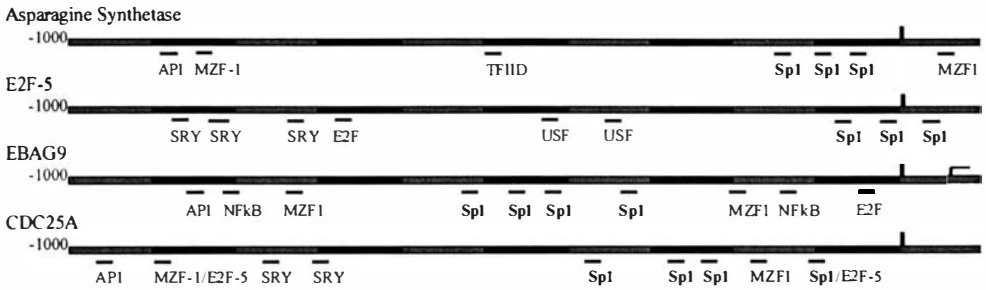


In fact, a computer-based sequence analysis of genes listed in Table 3 reveals that several of the genes identified here contain Sp1 sites reportedly involved in the transcriptional regulation of the gene (Figure 30). Mutant p53 transactivation may indeed function through these sites. Using an algorithm designed by Hoh *et al.* [251] to find presumptive p53-binding sites on the upstream sequences (approximately 2000bp up-stream from the 5'end of the mRNA) of genes listed in Table 1 it was observed that only in a few cases there were reasonable sites that may bind WTp53 (data not shown). These sites may play a role in WTp53-transcriptional repression of these promoters, a prediction that remains to be tested in the future. In other cases WTp53 must be repressing by utilizing protein-protein interactions [252, 253]. Whether mutant p53 utilizes any of the WTp53 binding sites or not is an open question, although perhaps unlikely.

The observations reported here may help formulate the role of mutant p53 in oncogenesis and attempts to explain why tumors expressing mutant p53 have a poorer prognosis. In a normal cell both the alleles of p53 are WT and p53 has full potency as a "protector" against stress situations such as DNA damage principally acting as a transcriptional activator. Because of a mutation in one of the alleles affecting the coding sequence of p53, a faulty mutant protein with one amino acid substitution is produced. Mutant p53 becomes stabilized weakening the transcriptional function of the WT protein. This may eventually lead to the loss of the remaining WTp53 allele, a question that has

yet to be answered in the literature. This loss of heterozygosity has been reported in many human cancers expressing mutant p53 [6, 20-22, 79-84]. The mutant protein then begins working as a potential oncogenic agent transcriptionally activating growth promoting, angiogenic and invasive genes leading to aggressive oncogenic phenotypes observed in these cells.

Figure 31. Presumptive promoters of ASNS, E2F-5, EBAG9 and CDC25A.



If one considers transcriptional regulation by WTp53 to be complex, then transcriptional regulation by mutant p53 appears to be even more complex. This complexity seems to arise from the fact that several rather distinct mechanisms appear to play a role in mutant p53-mediated regulation of transcription, resulting in the altered gene expression. This would explain the cell type specific gene expression changes associated with mutant p53 expression and the apparent difficulties in deciphering the mechanisms underlying mutant p53 oncogenicity and gain-of-function properties (Appendix B). This makes it important to identify transcriptional targets common to multiple p53 mutants. Definition of this pathway and the molecular mechanisms dictating it will provide strong candidates for cancer therapy development.

N. Figure and Table Legends.

Figure 1. Structure of p53. Graphical representation of the various identified domains with the p53 protein.

Figure 2. Functions of p53. Cellular damage results in the stabilization of p53 as a result of phosphorylation and acetylation. The p53 protein can then act as a transcription factor inducing up-regulation and down-regulation of various genes or as a structural component of protein complexes. Cells are halted at various checkpoints during the cell cycle or targeted for apoptosis.

Figure 3. Differences between mutant and WTp53. Transcriptional activation by WTp53 leads to modulation of genes involved in tumor suppression. This is largely dependent on the ability of WTp53 to bind to its consensus sequence. Mutant p53 expression, on the other hand, can lead to modulation of genes involved in tumor progression even in the absence of WTp53 (gain-of-function) [254].

Figure 4. Schematic representation of p53-D281G oligomerization derivatives. Schematic representation of p53-D281G derivatives used in the domain exchange experiments. The tetramerization domain of p53 amino acid 320-360 is shown by a black box. p53-D281G TZ334NR has sequences from amino acids 334 to 393 replaced by a modified coiled-coil structural domain (green box), p53-D281G TZ334NRI352 has the sequences 352-393 inserted after amino acid 352 following the TZ moiety, p53-D281G 343cc has sequences from amino acids 344 to 393 replaced by the coiled-coil structural domain (red box) coding for a dimerization motif from the yeast GCN4 protein.

Figure 5. Replacement of the tetramerization domain of mutant p53 has a drastic effect on mutant p53-mediated transactivation. Saos-2 cells were co-transfected with EGFR.Luc (200ng) and the expression plasmid for tumor-derived mutant p53-D281G, one of its derivatives or empty vector (800ng) in a 24-well plate using Lipofectamine 2000. Cell lysates were prepared 48hrs after transfection, and luciferase activity measured. Western blot analysis from a representative transfection showed equal protein expression (right inset).

Figure 6. Replacement of the tetramerization domain of mutant p53 preserves tetramerization of the protein. *In vitro* translated p53-D281G and its derivatives (p53-D281G TZ334NR, p53-D281G TZ334NRI352 and p53-D281G del. 393-327) were incubated with the indicated concentrations of glutaraldehyde [23]. Samples were separated on a gradient (4-20%) SDS-PAGE after cross-linking. The gel was dried and autoradiographed. Positions of presumptive monomers, dimers, trimers and tetramers of p53 derivatives are indicated.

Figure 7. Transactivation of the EGFR promoter by tumor-derived mutant p53-D281G is inhibited by co-expression of p53 del. 1-293. Saos-2 cells were plated at equal densities in 24-well plates and transfected 24hrs later. Each well was transfected with 170ng of EGFR.Luc, 170ng of pCMVBam p53-D281G (or vector alone) and 510ng of pCMVBam p53 del. 1-293 (or vector alone). Forty-eight hours after transfection, cell extracts were prepared and luciferase activity measured. Western blot analysis was carried out with equal amounts of protein from a representative transfection to evaluate protein expression (right inset).

Figure 8. Transactivation of the MDR1 promoter by tumor-derived mutant p53-D281G is inhibited by co-expression of p53 del. 1-293. Saos-2 cells were plated at equal densities in 10cm plates and transfected the following day using the calcium phosphate precipitation method. Cells were transfected with 2.5μg of MDR1.CAT, 2.5μg of pCMVBam p53-D281G (or vector alone) and 7.5μg of pCMVBam p53 del. 1-293 (or vector alone). After transfection, cells were treated as described in Experimental Methods and CAT assays performed. Western blot analysis was carried out with equal amounts of protein from a representative transfection to evaluate protein expression (right inset).

Figure 9. p53 del. 1-293 co-immunoprecipitates with p53-D281G. Saos-2 cells were transfected in 10cm plates with 3.2μg of the expression plasmid for p53-D281G (or expression vector alone) and 9.6μg of an expression plasmid for p53 del. 1-293 (or expression vector alone). Twenty-four hours after transfection, cellular proteins were labeled *in vivo* by ^{35}S-methionine. Extracts were then prepared and immunoprecipitated

with monoclonal antibodies as depicted in the figure. The immunoprecipitates were resolved in a 15% SDS-polyacrylamide gel followed by autoradiography. The left panel shows the binding sites for monoclonal antibodies PAb421 and DO1 on p53-D281G.

Figure 10. Co-expression of p53 del. 1-293 inhibits transactivation by p53-D281G. Total RNA was prepared from 10(3) cells stably transfected with vector alone, p53-D281G, p53 del. 1-293, or p53-D281G and p53 del. 1-293. Quantitative real time PCR was performed to determine the relative amounts of mRNA for NF-κB2 and PCNA genes. Graphs show the relative levels of PCNA and NF-κB2 mRNAs (normalized to GAPDH levels within the cells) in different stable transfectants of 10(3) cells.

Table 1. Colony formation in 10(3) cells stably transfected with vector or p53-D281G. 10(3) cells stably transfected with the vector pCMVBam Neo [255] or stably expressing p53-D281G [134] were transfected with 1μg of pHyg and 5μg of the indicated p53 or p73 expression plasmid [23, 188, 197]. Transfections were carried out by the calcium phosphate precipitation method [134]. After 48hrs, cells were sub-cultured 1:4 and allowed to form colonies in the presence of hygromycin (200 μg/ml). The media was changed every 2-3 days. After 3 weeks, cells were fixed with methanol and stained by methelyne blue.

Table 2. Tumorigenicity of 10(3) cells expressing p53 derivatives. The tumorigenic potential of 10(3) cells expressing p53 derivatives was tested using nude mice. Cells (5×10^6 per site) were subcutaneously injected into flanks of athymic nude mice (National Cancer Institute) and tumor development was monitored as described [11];[134].

Figure 11. Hetero-oligomers are produced between p53-D281G and p53 del. 1-293 in 10(3) cells stably expressing the two proteins. Cell extracts from 10(3) cells expressing p53 del. 1-293 alone or expressing p53-D281G and p53 del. 1-293 were prepared and immunoprecipitation reactions performed with the antibody DO1 (Oncogene, Ab-6). The immunoprecipitates were then separated by SDS-PAGE followed by western blot analysis. The blot was developed with PAb421. Antibody DO1 was run as a control to depict the positions of light and heavy chains of IgG. Positions of

molecular weight markers are shown. The figure shows results from duplicate immnoprecipitations.

Figure 12. Expression of tumor-derived mutant p53 increases the formation of neomycin resistant colonies. 10(3) cells were plated at equal densities (10^6 cells per plate) 24 hrs prior to transfection. Cells were transfected with pCMV-Bam Neo and WTp53 or mutant p53 (or vector alone) using the calcium phosphate precipitation method. The cells were subcultured 1:4 48 hrs after transfection and Neomycin (G418) was added to start the selection process. After 3 weeks, cells were fixed with methanol and stained; colonies containing more than thirty cells were counted. The final counts were as follows: (1) pCMV-Bam: 41, 31, 42 Avg. 38 (2) pCMV-Bam WTp53: 24, 25, 24 Avg. 24.3, (3) pCMV-Bam p53-V143A: 55, 48, 65 Avg. 56, (4) pCMV-Bam p53-L157F: 39, 34, 45 Avg. 39.3, (5) pCMV-Bam p53-163C: 64, 63, 73 Avg. 66.7, (6) pCMV-Bam p53-R175H: 88, 77, 95 Avg. 86.7, (7) pCMV-Bam p53-179Y: 42, 44, 51 Avg. 43, (8) pCMV-Bam p53-194R 60, 58, 51 Avg. 56.3, (9) pCMV-Bam p53-R273H: 112, 110, 106 Avg. 109.3, (10) pCMV-Bam p53-D281G: 124, 129, 137 Avg. 130, and (11) pCMV-Bam p53-R282W: 72, 60, 68 Avg. 66.7. The figure shows the number of average colonies formed.

Figure 13. DNA binding of WT and tumor-derived p53 mutants. Saos-2 cells were transfected with 10μg of the expression plasmid containing WT or mutant p53, whole cell extracts were prepared as described in Experimental Methods and DNA binding reactions carried out as described in the text. The probe (p21WT) used contained the p53 binding sequence found in the p21 promoter (distal site). Lanes 1-5 were incubated with purified p53 protein (100 ng), lanes 6-23 were incubated with an extract obtained from whole cell lysates containing approximately 10μg of total protein. WTp53 can bind the probe sequence containing the p53-binding sites (Lanes 2-12). A cold competitor (p21WT, lanes 2, 3, 8, 9) and a non-specific competitor (p21MT, lanes 4, 5, 10, 11) were also used. A p53-specific antibody (Ab-6, Oncogene) was used to generate a supershift of the p53-bound probe (lane 12). Tumor-derived p53 mutants were

analyzed for DNA binding to the same probe set (lanes 13-23). No binding was detected for these tumor-derived p53 mutants

Figure 14. Transcriptional activation of p53-inducible promoters by mutant p53. Human osteosarcoma Saos-2 cells were transfected using Lipofectamine 2000 (Invitrogen) with p21.Luc or MDM2.Luc (200ng), and the expression vector containing the WT or mutant p53 cDNA (25ng with p21.luc, 100ng with MDM2.Luc). Cells were grown for forty-eight hours, cell lysates prepared, and luciferase activity measured. The vector reading was set to 100 and the relative luciferase activity in the remaining extracts calculated with respect to the vector. Western blot analysis was performed to confirm equal expression of the protein after transfection in a representative experiment using the specific antibody PAb1801 (data not shown).

Figure 15. Transcriptional activation of p53-repressed promoters by mutant p53. Human osteosarcoma Saos-2 cells were transfected using Lipofectamine 2000 (Invitrogen) with SV40.Luc (200ng), and the expression vector containing the WT or mutant p53 cDNA (800ng). Cells were grown for forty-eight hours, cell lysates prepared, and luciferase activity was measured. The vector reading was set to 100 and the relative luciferase activity in the remaining extracts calculated with respect to the vector. Western blot analysis was performed to confirm equal expression of the protein after transfection in a representative experiment using the specific antibody PAb1801 (data not shown).

Figure 16. Expression of mutant p53 in H1299 cells. Human p53-null H1299 cells were transfected using Lipofectamine 2000 (Invitrogen) with expression plasmids for mutant p53 (or vector alone) as indicated. Forty-eight hours after transfection, cells were sub-cultured 1:3 and neomycin added at a concentration of 400 μg/ml to begin the selection process. After 3-4 weeks of selection, individual colonies were cultured independently and checked for p53 expression using the antibody Pab1801. Clones with similar expression levels were selected for microarray hybridization analyses. Asterisks indicate cell lines used for microarray hybridizations analyses.

Figure 17. Mutant p53 expression results in enhance growth rates in H1299 cells. Cells were seeded at a density of 1x 10^5 cells per 60mm plate. Five plates of each cell line were plated initially (Day 0). One plate of each was trypsinized, and cells suspended in 1ml of serum containing media. Cells were counted and the total number of cells calculated and plotted for each day. Standard deviations result from three independent experiments run simultaneously. Mutant-p53 expressing cell lines were compared to the control cell line HC-5. Only days 2-5 are shown.

Figure 18. The increased growth rate phenotype is dependent on mutant p53 expression. H1299 cells expressing p53-R175H or vector alone (HC-5) were transfected using Lipofectamine 2000 with siRNA directed against p53 ('p53') or a non-specific control sequence ('C'). Cells were transfected a second time with the same siRNAs 24hrs later. **A.** Mutant p53 protein levels were detected 2 to 7 days after the first transfection using the antibody PAb1801 in a parallel experiment; β-actin was used as a loading control. **B.** Transfected cells were plated at equal density 48hrs after the first transfection (Day 2) in 96-well plates. Cell viability was measured using an MTS assay kit (Promega) for the following five days. Viability of cell lines transfected with control siRNA **(top)** or siRNA directed against p53 **(bottom)**. Cell viability is expressed as a percentage of HC-5 control cells on the indicated day. **C.** In a separate experiment, H1299 cells transfected with siRNA against p53 were harvested 48hrs after transfection and equal amounts of protein separated by SDS-PAGE. *C-myc* and p53 were detected using the specific antibodies (Oncogene, Ab-6 and Ab-1).

Figure 19. Expression of mutant p53 in H1299 results in decreased sensitivity to etoposide. Cells were plated at 5 x 10^4 cells/plate and treated with 6μM etoposide (final concentration) for 48hrs. After treatment, cells were washed with HBSS and the surviving cells allowed to recuperate for three weeks with periodic changes of media. The colonies formed were fixed with methanol, stained with methylene blue and counted. The data shown is representative of three independent experiments run simultaneously; colony numbers were adjusted to account for plating differences based on control plates. Control plates were plated at one-tenth the density and treated with an equal amount of

vehicle control (DMSO) for 48hrs with less than 10% plating difference (data not shown). Clones H1299 cells expressing mutant p53-R175H, -R273H, -D281G, p53-D281G (L22Q/W23S) or vector alone were used.

Figure 20. Pools of mutant p53-expressing H1299 cells recapitulate results obtained with individual clones. Cells were plated at 5 x 10^4 cells/plate and treated with 6μM etoposide (final concentration) for 48hrs. After treatment, cells were washed with HBSS and the surviving cells allowed to recuperate for three weeks with periodic changes of media. The colonies formed were fixed with methanol, stained with methylene blue and counted. The data shown is representative of three independent experiments run simultaneously; colony numbers were adjusted to account for plating differences based on control plates. Control plates were plated at one-tenth the cell density and treated with an equal amount of vehicle control (DMSO) for 48hrs with less than 10% plating difference (data not shown). Pools of H1299 cells expressing p53-R175H, p53-R175H (L22Q/W23S), -R273H, -D281G, or vector alone were used.

Figure 21. Mutant p53-expressing Saos-2 cells exhibit increased plating in soft agar. Saos-2 cells expressing mutant p53-D281G, the transactivation deficient p53-D281G (L22Q/W23S) or vector alone were plated at a density of 3000 cells/well in 6-well plates over a .5% agar plug. Cells were grown in .25% agar medium for 4-5 weeks with periodic additions of media. Approximately 1% of vector-transfected cells formed colonies, whereas mutant p53-D281G displayed an increased ability (5-10%) to grow in soft agar. Cells expressing mutant p53-D281G (L22Q/W23S) had a comparative growth of 2-3%.

Figure 22. Scatter-gram analysis of S-scores generated. Scatter plot analyses of the S-scores obtained after analysis of the microarray hybridization data. The plot represents two unpaired comparisons (mutant p53 expressing vs. vector control cells) graphed against each other. The plots show a very high degree of consistency of changes in gene expression between biological duplicates. The scattergrams also indicate a relatively large number of changes in gene expression in each of the mutant p53-

expressing cell lines versus the control cell line. Clustering of the points around zero would have indicated few or no changes occurring.

Figure 23. Expression of mutant p53 in H1299 changes the cellular transcriptional profile. Cluster analysis of the output gene list obtained from our SAM analysis of the H1299. Microarray hybridization analysis of cell clones expressing mutant p53 (-R175H, -R273H and -D281G) derived from H1299 cells using Affymetrix GeneChip™ System U95Av2 chip reveals the up-regulation of a set of 150 genes up-regulated in the presence of all three p53 mutants, but identified as inhibited or unaffected by WTp53 (not shown). Changes in gene expression were detected in terms of statistical significance (S-score) of change in expression for a given gene between two microarrays. WTp53 hybridizations were generated from WTp53 infected cells compared to β-galactosidase infected samples (data not shown). The Cluster and TreeView programs (http://rana.lbl.gov/) were used to provide graphical displays of the gene expression patterns [256, 257]. Gene names and accession numbers have been removed for ease of viewing. FDR = 0.3%

Table 3. Microarray hybridization analysis of RNA from H1299 cells expressing mutant p53-R175H, -R273H and -D281G in comparison to vector transfected cells. Only up-regulated genes are listed on the table. Initial functional grouping of the genes was performed with the EASE program [258].

Figure 24. QPCR analysis of cells expressing mutant p53. mRNA was extracted from exponentially growing plates of the indicated cell lines and cDNA prepared. The cDNA was then analyzed by QPCR using gene-specific primers for various genes identified through microarray hybridization analysis. The degree of expression was quantitated using a relative standard curve and normalized to an internal control [Brome Mosaic Virus (BMV) RNA] corresponding to the cDNA batch [156]. The normalized mRNA level in the HC-5 control cell line was arbitrarily set to 1 and the relative fold difference calculated for the remaining samples.

Figure 25. Transactivation deficient mutant p53 fails to up-regulate genes *in vivo*. mRNA was extracted from exponentially growing plates of the indicated cell lines

and cDNA prepared. The cDNA was then analyzed by QPCR using gene-specific primers for various genes identified through microarray hybridization analysis as up-regulated by mutant p53. The degree of expression was quantitated using a relative standard curve and normalized to an internal control [Brome Mosaic Virus (BMV) RNA] corresponding to the cDNA batch [156]. The normalized mRNA level in the HC-5 control cell line was arbitrarily set to 1 and the relative fold difference calculated for the remaining samples.

Figure 26. Promoter analysis of a group of representative genes identified by microarray analysis. The presumptive promoters for ANGPTl and ITGA6 were cloned from genomic DNA using information obtained in the NCBI database (see Materials and Methods). The promoters of ASNS and EBAG9 were obtained from outside sources [212, 215]. Promoters were studied using reporter luciferase constructs and p53 null human osteosarcoma Saos-2 cells as described in Experimental Methods. The vector reading was arbitrarily set to 100.

Figure 27. Mutant p53 mediated transactivation of the ASNS gene. Human osteosarcoma Saos-2 cells were transfected with 200ng of ASNS.Luc and 250ng of the expression vector for WT or mutant p53 as indicated. Cell lysates were prepared 48hrs after transfection and luciferase activity was measured. The vector reading was set to 100 and the relative luciferase activity in the remaining extracts calculated with respect to the vector. Western blot analysis was performed to confirm equal expression of the protein after transfection in a representative experiment using the specific antibody PAb1801 (data not shown).

Figure 28. Mutant p53-R273H increases protein level expression of ASNS. Right panel. Saos-2 cells were transfected with 200ng of ASNS.Luc and increasing amounts of mutant p53 expression plasmid (p53-R273H or –D281G) as indicated. Cell lysates were prepared and luciferase activity was measured 48hrs after transfection. The vector reading was set to 100. Left panel. H1299 cells were plated at equal densities and infected with increasing multiplicities of infection 24 hrs later using an adenovirus expressing mutant p53-R273H (or β-gal as a control). Cell lysates were prepared 48hrs

later and western blotting performed to detect protein levels of ASNS (upper panel). Samples were normalized based on total protein readings. Actin was used as a loading control (lower panel). H1299 cells transiently transfected with an expression plasmid containing the ASNS cDNA were used as a positive control.

Figure 29. Mutant p53 localizes on the ASNS promoter. Exponentially growing cells of H-273H-14 cell line were crosslinked, and harvested as described in Experimental Methods. p53 was immunoprecipitated using the specified antibody (DO1). Normal IgG was used in the control immunoprecipitation. The presence of the ASNS promoters in the immunoprecipitate was detected using QPCR specific primers. The graph shows the fold difference in Antibody/Input ratio of the QPCR signals obtained for the specified antibody in two independent immunoprecipitation experiments. The average fold difference for the signals and standard deviations were also calculated (right).

Figure 30. Proposed model of transactivation. Left panel: Schematic representation of WTp53 repressed NF-κB2 promoter. WTp53 localizes on the promoter region by binding to other transcription factors (i.e. Sp1, NF-κB, etc). Repression of transcription occurs through the action of a co-repressor protein such as mSin3 or Histone deacetylase (HDAC) [259]. **Right panel:** We propose that mutant p53 retains the ability to bind some transcription factors. Therefore, it is possible for mutant p53 to localize on the promoter by binding to Sp1 or another transcription factor but is unable to repress transcription since it has lost the ability to bind the co-repressor. The intact transactivation domain of the mutant protein can then interact with the RNA polymerase complex, independently or through a co-activator thus increasing transcription of the gene.

Figure 31. Presumptive promoters of ASNS, E2F-5, EBAG9 and CDC25A. The transcription factor binding sites were identified using a world wide web base search program (TFSEARCH- Transcription Binding search) found at http://www.cbrc.jp/research/db/TFSEARCH.html). Only those binding sites with high

probability (>90%) are shown. The presumptive transcription initiation site is denoted by a black upright bar and the ATG codon by a horizontal bar.

O. Experimental Methods.

General Methods.

Plasmids and DNA. The p53 and p73 expression plasmids contained the human p53 and p73 cDNAs under the regulation of the CMV immediate-early promoter in the pCMVBam expression vector [188, 255]. The N-terminal deletion derivative p53 del. 1-293 was generated in the context of the human wild-type p53 cDNA as described earlier [23]. p73α and p73β cDNAs were cloned in such a way that they have a FLAG epitope at the N-terminus when converted into the respective proteins [197].

The p53 C-terminal derivatives have been described earlier [178, 180]. The p53 343cc had sequences from amino acids 344-393 replaced with the coiled-coil structural domain from the yeast GCN4 protein, while p53 TZ334NR had sequences from amino acids 334-393 replaced by a modified coiled-coil structure defining a tetramerization domain. The p53-D281G forms of these derivatives contained the amino acid substitution at the position 281 from Arg to Gly; these clones were constructed by standard sub-cloning techniques by replacing a restriction fragment of the wild-type p53 cDNA overlapping the sequence coding for amino acid 281 with the corresponding fragment from p53-D281G cDNA. All coding sequences were cloned in the pCMVBam vector in the correct orientation downstream of the CMV immediate-early promoter.

The chloramphenicol acetyltransferase (CAT) plasmids utilized the *Escherichia coli* CAT gene under the transcriptional control of the MDR1 [134] promoter. The luciferase plasmids used the firefly luciferase (luc) gene under the control of the human EGFR and p21 promoters [40, 197]. The p53 mutants (p53-V157F, p53-Y163C, p53-H179Y, p53-L194R, p53-R282W) in pCMV-Bam used in this study were generated using the Quick Change Site directed Mutagenesis (Stratagene) following the manufacturer's protocol. Sequencing was performed after mutagenesis to confirm the presence of the desired mutation. pCMV-Bam WTp53, p53-V143A, p53-R175H, p53-R273H, and p53-D281G were described earlier [156]. MDM2.Luc, p21.Luc, and *c-myc*.Luc [40, 197], ASNS.Luc [211, 212] and hTERT.Luc [260] have been described earlier.

Generation of stable cell lines expressing mutant p53. The p53-null human lung carcinoma cell lines H1299 [261], human 21PT [262], human Saos-2 [156] and the murine fibroblast 10(3) cell line were transfected using Lipofectamine 2000 or the calcium phosphate method [188] with pCMVBam Neo or pCMVBam Neo-mutant p53 expression plasmid as indicated [255]. Control and mutant p53-expressing stable cell lines were generated: 10(3) Vector 4 [10(3)-V4], 10(3) p53-D281G-clone 4 [10(3)-D281G-4], 21PT-control vector 5 [21PT-V5], 21PT-p53-R175H-clone 22 [21PT-R175H-22], 21PT-p53-R273H-clone 15 [21PT-R273H-15], 21PT-p53-D281G-clone 46 [21PT-D281G-46], H1299-control 5 [HC-5], H1299-p53-D281G-clone 5 [H-D281G-5], H1299-p53-R273H-clone 14 [H-R273H-14], H1299-p53-R175H-72 [H-R175H-72], p53-D281G (L22Q/W23S)-clones 11, 13 and 19 [H-D281G (L22Q/W23S)-11, 13 and 19], Saos-2 control vector 2 and 4 [SC2 and SC4], Saos-2-p53-D281G clones 14 and 25 [S-D281G-14 and 25] and Saos-2-p53-D281G (L22Q/W23S) clones 13 and 25 [S-D281G (L22Q/W23S)-14 and 25]. Additionally, stable pools of H1299 expressing mutant p53-R175H, -R273H, -D281G and –D281G (L22Q/W23S) were generated using the pIRES puro$_3$ expression vector from Clontech. Forty-eight hours after transfection the cells were subcultured 1:4 and grown in selective medium containing neomycin (G418) at a concentration of 200 μg/ml [10(3)], 400 μg/ml [H1299 and 21PT] or 1 mg/ml [Saos-2] to start the selection process. The medium was changed periodically and after three weeks of selection individual colonies were selected and analyzed for expression of p53 by Western blotting as described [191] using the monoclonal antibody PAb1801. Several clones from each transfection were expanded into cell lines. The H1299 pools were generated using 2 μg/ml of puromycin.

Murine 10(3) cells [11] or 10(3) cells stably expressing p53-D281G [134] were transfected with pCMVBam p53 del. 1-293 and pCMVBam NEO [255] or pCMVBam p53 del. 1-293 and pHyg (Clontech), respectively, using the calcium phosphate precipitation technique [188]. Forty-eight hours after transfection, cells were washed, trypsinized, subcultured at 1:4, and plated into selective medium supplemented with 200 μg/ml G-418 (active concentration) for previously untransfected 10(3) cells and 200

μg/ml G-418 and 200 μg/ml hygromycin for p53-D281G-expressing cells. Cells were maintained in the selective medium with twice weekly changes of medium. Cells from individual antibiotic-resistant colonies were isolated and expanded into cell lines.

Cell culture. Human H1299 cells were maintained in RPMI 1640 plus 10% Fetal Bovine Serum (FBS). 21PT cells were maintained in α-MEM plus 10% FBS and indicated growth factors as described [262]. Saos-2 cells were grown in DMEM plus 10% FBS and 5% Calf Serum (CS). 10(3) cells were grown in DMEM plus 10% FBS. 10(3) stable clones were grown in complete medium supplemented with the corresponding selection agent.

Western blot analysis. Western blot analysis was carried out after SDS-PAGE using monoclonal antibodies against p53 (Oncogene, Ab-6, pAb1801 or pAb421 as indicated) or FLAG (Sigma) and a commercial kit (Promega) for labeling with an alkaline phosphatase-conjugated secondary antibody following the manufacturer's protocol. Alternatively, proteins were detected using the ECL system (Amersham) following the protocol prescribed by the vendor. Actin was detected with a mouse monoclonal β-actin specific antibody (Sigma). *C-myc* was detected using a specific antibody (Oncogene, Ab-1) and ASNS was detected using an antibody obtained from Dr. Michael Kilberg's laboratory.

Chapter 3 Methods.

Luciferase assay. Luciferase assays were carried out using a commercial kit from Promega as suggested by the vendor, and luminescence was measured in a Luminometer made by Turner Designs. Cells were harvested 36-40hrs after transfection, and extracts were prepared as suggested. As described for the CAT assay, equal amounts of protein from each extract were used for the luciferase assay. Relative luciferase activity was plotted on the Y-axis and the value of the vector control was set to 100%.

Glutaraldehyde cross-linking. Analyses for the presence of oligomeric forms of p53-D281G and its derivatives were carried out as described [23]. Human p53-D281G and its derivatives were generated by a coupled *in vitro* transcription-translation system

(Promega) and were incubated with glutaraldehyde at varying final concentrations at 37°C for 15 minutes. After cross-linking, proteins were boiled in 2X Laemmli loading dye [23], subjected to gradient (4-20%) SDS-PAGE, and visualized by autoradiography.

Chloramphenicol acetyltransferase assay. Cells were harvested 36-40hrs post-transfection and lysed by three successive cycles of freezing and thawing. Extracts were normalized for protein concentration and assayed for CAT enzyme activity as described earlier [155]. Multiple independent experiments (three or more) were done to determine the standard deviations. CAT activity was detected by thin-layer chromatographic separation of [^{14}C]-chloramphenicol from its acetylated derivatives followed by autoradiography. Quantitation was done using a Phosphorimager (Molecular Dynamics).

***In vivo* labeling and immunoprecipitation.** Saos-2 cells were transfected with p53 expression plasmids (or expression vector alone) as indicated. Twenty-four hours post-transfection labeling of cellular proteins was carried out with ^{35}S-methionine for 2hrs after which cells were harvested and cell extracts prepared [188]. Immunoprecipitations were carried out for 14hrs at 4°C using the indicated monoclonal antibodies [188]. PAb421 [263, 264] was used to bind p53 proteins with an intact C-terminus (intact p53-D281G as well as p53 del. 1-293) and DO1 [263, 264] was used to detect p53 proteins with an intact N-terminus (p53-D281G but not p53 del. 1-293).

RNA isolation and microarray hybridization analysis. Total RNA was isolated from stably transfected 10(3) cells using Trizol (Gibco) reagent following manufacturer's protocol. Quality of the RNAs was checked by 1.2% agarose TBE gel electrophoresis [236]. Microarray analysis from RNA from 10(3) cells stably transfected with (1) vector alone and (2) p53-D281G was done by Incyte Genomics, Inc. (Palo Alto, CA) starting from whole RNA. Briefly, polyadenylated RNA was isolated with the OligoTex (Qiagen) RNA kit. Polyadenylated RNA was reverse transcribed to generate Cy3- and Cy5-labeled cDNA probes, respectively. cDNA probes were competitively hybridized to a mouse Unigene 1 cDNA microarray (Incyte Genomics, Inc.) containing immobilized cDNA fragments (average cDNA length, 500-5000 base pairs). Cy3 and Cy5 fluorescence were imaged individually, and the normalized ratios of Cy3/Cy5

fluorescence at a given spot on the microarray were used to calculate differential gene expression.

Quantitative real-time PCR. Relative quantitation of gene expression was performed using quantitative real-time PCR (QPCR). The relative amount of each sample was calculated from a standard curve after employing a Fit Points algorithm. This expression value was normalized to a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [265]. This method is described in detail by Johnson *et al* [266] and Tanguay *et al.* [267]. Following is a brief description of the entire process.

(a) cDNA synthesis. Total RNA was isolated from stably transfected 10(3) cells using Trizol reagent (Gibco BRL) following the manufacturer's instructions. Ten micrograms of total RNA was treated with RQ1 RNAse-free DNase (Promega), followed by phenol-chloroform extraction and ethanol precipitation. cDNA was synthesized with random hexamers using the Thermoscript RT-PCR System (Gibco BRL) according to the manufacturer's protocol.

(b) Primers. Primers were designed using Oligo 5.0 Software (National Biosciences, Inc) and synthesized by Integrated DNA Technologies. Primer optimization was performed to determine the optimal concentration of $MgCl_2$ and annealing temperature for each primer set. Relative standard curves were constructed for each target gene. Serial dilutions were made, and given arbitrary values corresponding to the dilutions.

(c) Quantitative real-time PCR. Quantitative real-time PCR was performed using a LightCycler (Roche). Each reaction (20µl) contained 2µl of the respective diluted cDNA, primers (0.5µM), and 4mM $MgCl_2$, and FastStart Reaction Mix SYBR Green I, (Roche) which includes FastStart Taq DNA polymerase, reaction buffer, dNTPs, and SYBR Green I dye. A negative control was included in each QPCR run.

Except for the composition of the standard curves, each reaction was performed in triplicate. Samples were investigated with murine GAPDH: 5'- CCA GCC TCG TCC CGT AGA CA -3' and 5'- GCC TCA CCC CAT TTG ATG TTA GTG -3', murine PCNA: 5'- GAT AAA GAA GAG GAG GCG GTA A -3' and 5'- ACA CGC TGG CAT

CTC AGG AGC A -3', and murine NF-κB2: 5'- TGG CCC CTA TCT GGT GAT TGT -3' and 5'- ACA CTC CCA ACT CTG AAC ACT GCT -3'. Agarose gel electrophoresis and melting curve analysis were used to demonstrate the presence of a specific product.

Colony formation assay. 10(3) cells stably transfected with the vector pCMVBam NEO [255] or stably expressing p53-D281G [134] each in 10cm plates were transfected with 1μg of pHyg and 5μg (1) pCMVBam [188], (2) pCMVBam WTp53 [188], (3) pCMVBam FLAG-p73α [197], (4) pCMVBam FLAG-p73β Deb [197], or (5) pCMVBam p53 del. 1-293 [23]. Transfections were done by calcium phosphate precipitation technique as described earlier [134]. After 48hr of transfection cells were subcultured 1:4; hygromycin was added next day at 200 μg/ml to start the selection process. Neomycin (G418 at 200 μg/ml) was also added to those cells selected earlier for neomycin resistance as described before [134]. Every 2-3 days media were changed. After 3 weeks, cells were fixed with methanol and stained by methylene blue.

Tumorigenicity assay. The tumorigenic potential of 10(3) cells expressing p53 derivatives was tested using nude mice. Cells (5 x 10^6 per site) were subcutaneously injected into flanks of athymic nude mice (National Cancer Institute) and tumor development was monitored as described [11]; [134].

Immunoprecipitation. *In vivo* complex formation between p53-D281G and p53 del. 1-293 in 10(3) cells stably transfected to express p53-D281G and p53 del. 1-293 was done by immunoprecipitation of p53-D281G with an antibody (DO1) that recognizes a motif on the N-terminal part of the protein that is absent in p53 del. 1-293. Cells from a 10 cm dish were harvested in 4 ml of 10S buffer (containing 250mM HEPES, pH 7.2, 1.5% NP40, 0.5% Triton X-100, 0.025% SDS, 50mM sodium phosphate buffer, pH 7.0, 5mM NaF, 0.5mM PMSF and 2.5 mM DTT) as described. One ml of the extract was then incubated with 2μg of p53 antibody DO1 and 16μl of protein A agarose (Invitrogen) and tilted overnight at 4°C. The following morning, the immunoprecipitate was washed three times with 10S buffer and once with a buffer containing 10mM Tris pH 7.5, 50mM NaCl, 0.5% sodium deoxycholate and 0.5% Triton X-100. The pellet was then suspended in 20 μl of 2X Laemmli loading buffer, boiled and analyzed by 15% SDS

polyacrylamide gel electrophoresis, followed by Western blot analysis using PAb421 as the probe.

Chapter 4 Methods.

Colony formation assay. 10(3) cells were plated at a density of 6 x 10^5 cells per plate in 10cm dishes 24 hrs prior to transfection and transfected using the calcium phosphate method as described earlier [188, 191]. Cells were transfected with 1 μg of pCMVBam NEO and 5 μg (1) pCMVBam [188, 255], (2) pCMVBam WTp53, (3) pCMVBam p53-V143A, (4) pCMVBam p53-V157F, (5) pCMVBam p53-Y163C, (6) pCMVBam p53-R175H, (7) pCMVBam p53-H179Y, (8) pCMVBam p53-L194R, (9) pCMVBam p53-R273H, (10) pCMVBam p53-D281G, or (11) pCMVBam p53-R282W. After 48 hr of transfection cells were subcultured 1:3; neomycin (G418) was added next day at 200 μg/ml to start the selection process. Every 3-4 days the media was changed. After three weeks, the colonies formed were fixed with methanol and stained with methylene blue [191]. Only colonies containing more than thirty cells were counted.

Purification of p53. WTp53 was purified as described [195]. Recombinant baculovirus expressing WT was used to infect SF9 cells. The virus was prepared as described [195]. The protein was purified by affinity chromatography using a column of Affigel-10 coupled with the purified monoclonal antibody PAb421 [268]. Purity of the preparation was checked by SDS-PAGE and subsequent analysis by Coomassie [195].

Electrophoretic Mobility Shift Assay. Saos-2 cells were transfected using the calcium phosphate precipitation method [188] with an expression plasmid containing the cDNA for the WT or mutant p53 protein as indicated in the figure. After 48hrs, nuclear extracts were prepared following the method described in Yang *et al* [269] using a solution containing 20mM HEPES, pH 7.8; 25% glycerol; 520mM NaCl; 1.5mM $MgCl_2$; 0.1mM EDTA; 0.5mM DTT, 0.5mM PMSF; 2% Nonidet P-40; and protease inhibitor cocktail 1 (Calbiochem). The probes used were radiolabeled with [α^{32}P]-dCTP using Klenow fragment of DNA polymerase I of *E. coli* (New England Biolabs). Approximately 10μg of total protein [100ng of purified p53 [195, 270] in the control gel]

were incubated at 18°C for 30 minutes in binding buffer [4% glycerol, 1mM $MgCl_2$, 0.5mM EDTA, 0.5mM DTT, 50mM NaCl, 10mM Tris-HCL, pH 7.5, and 1μg of poly dI:dC (Pharmacia)] with 5,000 cpm of radiolabeled probe. For the competition and supershifting studies, proteins were pre-incubated for 15 minutes in binding buffer with either an excess of unlabeled competitor oligodeoxynucleotide containing a p53-binding site (p21WT) or a probe containing the same site mutated in two places to prevent binding of p53 (p21MT). The labeled probes were added after the pre-incubation and the binding reactions were carried out as described above. The antibody DO1, specific for detecting p53, was used for supershifting. The extracts were then loaded into a 4% polyacrylamide gel and separated by electrophoresis at 200 volts at 12°C. The gel was dried and the presence of radioactively labeled DNA was detected by autoradiography. Probes used: 1) p21WT: 5'- GAT TCG TCA GGA ACA TGT CCC AAC ATG TTG AGC TCT GGA -3' and 5'- AGC TTC CAG AGC TCA ACA TGT TGG GAC ATG TTC CTG ACG -3'; p21MT: 5'-GAT TCG TCA GGA AAA TTT CCC AAA ATT TTG AGC TCT GGA -3' and 5'- AGC TTC CAG AGC TCA AAA TTT TGG GAA ATT TTC CTG ACG -3'.

Transfections and luciferase assay. For transient transcriptional promoter assays human osteosarcoma (Saos-2) cells were cultured and transfected using Lipofectamine 2000 (Invitrogen) in 24-well plates following the manufacturer's protocol. Each well was transfected with 200ng of the indicated promoter-luciferase construct and 25ng (p21.Luc), 100ng (MDM2.Luc), 250ng (ASNS.Luc and hTERT.Luc), or 800ng (SV40.Luc) of the p53 expression plasmid. Puc18 DNA was used as filler DNA up to 1μg. Cells were harvested 36-48 hours after transfection, and extracts were prepared using Reporter Lysis Buffer (Promega). Equal amounts of protein from each extract were used for the luciferase assay. Value of the vector control was set to 100 and relative luciferase activity form the remaining samples calculated. All assays were repeated three times or more.

Growth rate assay. Cells were seeded at a density of $1x10^5$ cells per 60-mm plate. Five plates of each cell line were plated initially. Whenever needed one plate of each

was trypsinized, and cells were suspended in 1 ml of serum containing medium; cells were counted each day using a hemocytometer and the total number of cells calculated and plotted. Media in the remaining plates were changed every day. Standard deviations were calculated from three independent experiments run simultaneously.

siRNA transfection and cell viability assay. H1299 cells ($3x10^5$) expressing p53-R175H (H-R175H-72) or vector alone (HC-5) were transfected by Lipofectamine 2000 with siRNA directed against p53 or a non-specific sequence (33nM final concentration) 24hrs after plating. Cells were transfected a second time 24hrs later. Cells were then plated at equal density (3000 cells per well) 48hrs after the first transfection (Day 2) in 96-well plates. Cell viability was measured using an MTS assay kit (CellTiter® 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit, Promega) for the following five days following the manufacturer's protocol. Mutant p53 protein levels were detected using antibody PAb1801 from a parallel experiment; β-actin (Sigma, AC-15) was used as loading control. Cell viability was expressed as a percentage of HC5 control cells transfected with the same siRNA. siRNA sequences used were: 1) control siRNA ('C'): 5'- CAU GUC AUG UGU CAC AUC UCT T -3' and 5'- GAG AUG UGA CAC AUG ACA UGT T -3' 2) p53 siRNA ('p53'): 5'- GCA UGA ACC GGA GGC CCA UTT -3' and 5'- AUG GGC CUC CGG UUC AUG CTT -3' [198].

Drug sensitivity assays. H1299 (or 21PT) cells stably expressing p53-R175H, -R175H (L22Q/W23S), -R273H, -D281G, -D281G (L22Q/W23S) or transfected with vector alone (as indicated in the figure), were plated at equal densities and treated with a final concentration of 3-6μM etoposide (Sigma) for 48hrs as specified in the text. Chemosensitivity of the cells was then measured by two different methods: (1) colony formation or (2) bromodeoxyuridine (BrdU) incorporation. Colony formation assay: Cells were plated at ($5x10^4$ cells/10 cm dish) and exposed to a final concentration of 6μM etoposide for 48hrs. After treatment, plates were washed and the media replaced. The surviving cells were allowed to form colonies for 2-3 weeks with periodic changes of media. Colonies were fixed with methanol, stained with methylene blue and counted as described earlier [156]. Control plates were plated at $5x10^3$ cells/10 cm dish and treated

with an equal amount of DMSO. Control plates were assessed for plating efficiency and DMSO effects on cell growth. BrdU incorporation assay: Cells were plated at a density of $1x10^6$ in a 10 cm dish in the presence of etoposide (6μM final concentration) or DMSO as control for 48-72h. Cells were incubated in the presence of 10μM BrdU for 40 minutes. After washing with Dulbecco's Phosphate Buffer Solution (DPBS), cells were trypsinized, counted, fixed by vigorous vortexing and addition of cold absolute ethanol (ETOH) drop-wise. Cells were then stored at 4°C for at least 18hrs. Samples were washed with DPBS plus 0.5% BSA to remove EtOH. Fixed cells were treated with 400 μl 2N HCl for 20 minutes, followed by incubation in 0.1M sodium borate pH 8.5 for 2 minutes. Samples were washed and resuspended in DPBS plus 0.5% BSA and 0.5% Tween 20 followed by incubation with anti-BrdU antibody coupled with fluorescein isothiocyanate (FITC) for 1h in the dark with tilting. After washing to remove excess antibody, cells were resuspended in propidium iodide staining solution for at least 1hr prior to fluorescence-activated cell sorting (FACS) analysis [271, 272]. Samples were gated for sub-G1 DNA containing cells.

Soft agar plating efficiency assay. Saos-2 cells expressing mutant p53-D281G, the transactivation deficient p53-D281G (L22Q/W23S) or vector alone were plated at a density of 3000 cells/well over a .5% agar-medium plug. Assays were carried out in 6-well plates. Cells were plated in .25% agar-medium for 4-5 weeks with periodic additions of growth media. After four weeks, the colonies formed were counted under a light microscope. Colonies with an apparent cell number >30 were counted. The agar-medium used in the assay contained the appropriate selection agents.

Chapter 5 Methods.

DNA microarray hybridization. Expression profiles of mutant p53-expressing cells were compared with H1299 cells stably transfected with vector alone. Cells (approximately $3x\ 10^6$ cells at the time of preparation) were plated 36-40hrs prior to RNA preparation. To compare to the mutant p53 expression profiles, H1299 cells were also infected with recombinant adenovirus expressing WTp53 and compared to cells infected

with adenovirus expressing β-galactosidase as a control. One culture (approximately $3x10^6$ cells) per microarray sample was used. All microarray hybridization analyses were performed with biological duplicates of each mutant p53 expressing cell line using Affymetrix U95Av2 chips by either the GeneChip Core lab at UCSD or Virginia Commonwealth University's institutional Nucleic Acid Research Facility. The U95Av2 arrays represent 12,625 human gene sequences and expressed sequence tags (ESTs) previously characterized, in which each gene is represented by a probe set consisting of 16-20 probes. The general procedures for microarray hybridization and analysis have been described elsewhere [273].

Data management and analysis. Data analysis was done using Affymetrix Microarray Analysis Suite version 4 (MAS 4). All arrays were normalized by a correction to a set value of median total hybridization intensity. Changes in gene expression were detected in terms of statistical significance of change in expression for a given gene between two compared microarrays (S-score) [199, 200]. S-score analysis takes into account signals detected by 16-20 multiple probe pairs for individual genes, as well as intensity-dependent and -independent noise. The general procedures are described elsewhere [273]. Briefly, the normalized files generated by the MAS 4 software were analyzed by the S-score program [199]. Two hybridizations from each cell line were performed and changes in gene expression were detected in terms of S-score [199, 200]. S-scores are derived to have a mean value of zero (representing no change) with a standard deviation of 1. Genes with an average intensity difference value of less than 50 in at least one sample were filtered out, resulting in 1724 genes. The S-scores generated from our mutant p53-expressing cell lines were then analyzed for significance across replicate experiments by using a permutation method performed with the Significance Analysis of Microarray (SAM) program from Stanford University [201]. The settings for this analysis were: one-class response, unlogged data, and 300 permutations, K-nearest neighbor imputer of 10 and a random number seed of 123456789. Once the program reported the list of ranked genes, the "delta value" was adjusted to a stringent False Discovery Rate (FDR) of 0.3 %. Cluster analysis was done

using the Cluster and TreeView programs (http://rana.lbl.gov/) to provide a graphical display of the expression patterns [256, 257]. Genes reported by SAM were analyzed by hierarchical clustering with average linkage grouping. For our analysis the arrays themselves were not clustered. Functional grouping of the identified genes was done by manual editing of GeneOntology categories obtained through the DAVID annotation tool (http://david.niaid.nih.gov/david/ease.htm) [258].

Quantitative PCR (QPCR). QPCR was conducted using the LightCycler (Roche). Details of this method are described elsewhere [156]. Briefly, cDNA was synthesized using the Thermoscript RT-PCR system (Invitrogen). Primers were designed using Oligo 5 (Molecular Biology Insights) and synthesized by Sigma Genosys. Reactions were performed in triplicate utilizing SYBR Green dye. . The degree of expression was quantitated using a relative standard curve and normalized to an internal control (BMV) corresponding to the cDNA batch [156]. Primer used were: 1) *Asparagine synthetase* (ASNS): 5' AGA GAT TCT CTG GCG ACC AAA AGA –3' and 5'- CTG GGT AAT GGC GTT CAA AGA CTT -3'; 2) *Angiopoietin 1* (ANGPT1): 5'- GAT GTC AAT GGG GGA GGT T -3' and 5'- CTC TGA CTG GTA ATG GCA AAA ATA -3'; 3) *Cyclin B2*: 5'- CTG CCA CGC TTT TTC TGA TG -3' and 5'- GAC TTG TAC CAG CCA ATC CA -3' 4) *c-myc*: 5'- GCC GCC GCC AAG CTC GTC TCA GAG -3' and 5'- GCT GCT GGT GGT GGG CGG TGT CTC -3'; 5) *Asparaginyl-tRNA synthetase* (NARS): 5'- GCC GGA TGA GTT GTG TC -3' and 5'- ACC CCA ATT AGT TCC CAG AA -3'; 6) *RNA polymerase II, polypeptide E* (RNA polII E): 5'- CCC ACC GAC CAG ATC TTT G -3' and 5'- GAC GAC GTG CTC AGG GAC TC -3'; 7) *Cell division cycle 25A* (CDC25A): 5'- AAG GCC CAT GAG ACT CTT -3' and 5'- AAA CTT GCC ATT CAA AAC AG -3'; 8) *ras homologue gene family member G* (rhoGAP): 5'- AGT ACA TCC CCA CCG TGT TC -3' and 5'- GGA CTG GCA ATG GAG AAA C -3'; 9) *Apoptosis inhibitor 5* (API5): 5'- CCG ACC TAG AAC AGA CCT T –3' and 5'- GCC AAC AAT TTC AAT ACC TC -3'; 10) *Associated molecule with the SH3 domain of STAM* (AMSH): 5'- TAG ATG TGT TCC CAA CCT TA -3' and 5'- GTT GGC ACT GGC TAA CTG -3'; 11) *estrogen receptor binding protein site associated antigen 9* (EBAG9): 5'- TGC CTT

TTA TTC ATC AGT CTT C -3' and 5'- CGG CTG CTC TCT TTT CTC T -3'; 12) *integrin α6* (ITGA6): 5'- GTC CAG AGC CAA GGT CCA G -3' and 5'- CTC AAT CGC CCA TCA CAA AA -3'; 13) *minichromosome maintenance protein 6* (MCM6): 5'- ATC CCT CTT GCC AAG GAT TT -3' and 5'- GAA AAG TTC CGC TCA CAA GC -3'; 14) *transcription factor E2F-5* (E2F-5): 5'- CCC CCA CCT GAT GAC CTC AC -3' and 5'- CTG CCG GGG TAG GAG AAA GC -3'; and 15) *C-Syn proto-oncogene* (C-SYN): 5'- TGA ACA GCT CGG AAG GA -3' and 5'- CCC AAT CAC GGA TAG AAA GT -3'.

Cloning of presumptive promoters. Cloning of the presumptive gene promoters was done using genomic data sequence available in the NCBI database. Briefly, sequence specific primers were designed to amplify (by PCR) a genomic DNA fragment of up to 1Kb upstream sequences containing the transcriptional start site for each of the promoters. Genomic DNA from MCF-7 cells was extracted using DNAzol reagent (Invitrogen) and used in the PCR reaction as template. Primers were designed using the Oligo 5 (Molecular Biology Insight) program. The PCR fragment was then purified and cloned into the pGL_3 Luciferase reporter plasmid (Promega) using the indicated restriction enzymes. Sequences of the fragment were confirmed by DNA sequencing (Amplicon Express); clones bearing no base pair mutations were selected for further use. The following primers sets were use: 1) Angiopoietin 1 (ANGPT1): 5'- CGG GGT ACC CAG GAG GTT TTT ATG TGG AA -3' and 5'- CCC AAG CTT AAT GGC AGC GAG GAA -3' (KpnI and HindIII); 2) Integrin α6 (ITGA6): 5'- GGA AGA TCT AGC CTT CAT GCC ACC TAC AC -3' and 5'- CCC AAG CTT GCC ACC TTC GCC TCC TC -3' (BglII and HindIII); 3) Cyclin B2: 5'-CGG GGT ACC TGG GCT GAT TAT TAG ACG AA -3' and 5'- GGA AGA TCT ACG GGG AAG GCA AGA -3' (KpnI and BglII); 4) DNA polymerase delta subunit 2 (POLD2): 5'- CGG GGT ACC AGG CCC AGG GAA GTA GCA GA -3' and 5'- GGA AGA TCT GCC CAC CGA CCC AGG AG -3' (KpnI and BglII); 5) Transcription factor E2F-5 (E2F-5): 5'- CGG GGT ACC ATG AAA ACC AAC CCT AAA ACT CCA -3' and 5'- CCC AAG CTT GAT CCA GAA CGC CGT CCT T -3' (KpnI and HindIII). Luciferase constructs for the Asparagine synthetase

and EBAG9 were kind gifts of Michael Kilberg and Satoshi Inoue respectively [212, 215].

Transfection and luciferase Assays. Luciferase assays were done as described [156]. Saos-2 (p53-null human osteosarcoma) cells were plated at equal density 24 hrs prior to transfection. Culture media was changed on the day of transfection. Cells were transfected with 100ng of the appropriate promoter-luciferase plasmids and 250ng of an expression plasmid (pCMVBam) for WTp53, p53-R175H, -R273H, -D281G -D281G (L22Q/W23S) or vector alone [156]. Cells were harvested 48hrs after transfection using Reporter Lysis Buffer (Promega). Luciferase activity was detected using a Luminometer from Turner Designs. Cell extracts were normalized based on total protein concentration; the vector reading was arbitrarily set to 100. Alternatively, cells were transfected with increasing concentrations of the expression plasmid for p52-R273H or -D281G and 100ng of the ASNS promoter-luciferase construct. Assays were then conducted as described above.

Adenovirus preparation and infection. A) The p53 (WT or mutant) gene was first cloned in a plasmid vector downstream of the human cytomegalovirus promoter, pZEROtg CMV. The viruses were generated by recombination in bacteria as described [274]. For control, we generated a recombinant adenovirus expressing β-galactosidase where the β-galactosidase gene is cloned downstream of the CMV promoter. In a 10 cm dish 3x 10^6 H1299 cells [261] were infected with the recombinant adenoviruses expressing WTp53, p53-D281G or β-galactosidase at an MOI of 5. The infection was conducted in the absence of serum. Total RNA was harvested at 24 or 48 hours after adenoviral infection from H1299 cells using Trizol (Invitrogen) reagent following manufacturer's protocol. Quality of the RNAs was checked by 1.2% agarose Tris-borate-EDTA gel electrophoresis. **B)** H1299 cells were infected with increasing MOIs (1, 5, 10 and 20) with a recombinant virus expressing p53-R273H (or β-galactosidase). Cell lysates were prepared 48 hrs after infection. Lysates were normalized based on total protein concentration and resolved by SDS-PAGE. ASNS was detected using an antibody specific for the protein (a kind gift from Dr. Michael Kilberg's laboratory).

Chromatin immunoprecipitation. Chromatin immunoprecipitations were done as described [216, 275, 276]. Briefly, exponentially growing H1299 cells expressing mutant p53-R273H ($1x10^7$) were crosslinked with 1% formaldehyde for 15 minutes and the reaction stopped by addition of glycine to a final concentration of 0.125M for 15 more minutes. Cells were collected in and washed once with PBS, once with Wash buffer 1 (0.25% Triton X-100, 10mM EDTA, pH 8, 0.5 mM EGTA, pH7.5, HEPES, pH 7.5), once with Wash buffer 2 (0.2M NaCl, 1mM EDTA, pH 8, 0.5mM EGTA, pH 7.5, 10mM HEPES, pH 7.5) and lysed in 500µl of Lysis buffer (150mM NaCl, 25mM Tris-HCl, pH 7.5, 5mM EDTA, pH8, 1% Triton X-100, 0.1% SDS, 0.5% Sodium Deoxycholate). Cells were subjected to sonication and the chromatin sonicated to 500-100bp length fragments. DNA from approximately $2.5x10^6$ cells was used in each immunoprecipitation. The immunoprecipitation volume was increased to 1ml by addition of IP buffer (250mM NaCl, 25mM Tris-HCl, pH 7.5, 5mM EDTA, pH 8, 1% Triton X-100, 0.1% SDS, 0.5% Sodium Deoxycholate), precleared with 30 µl of protein A agarose (Calbiochem) and the indicated proteins immunoprecipitated overnight at 4°C with gentle tilting using specific antibody for p53 or a control antibody (p53, Oncogene, Ab-6; Normal rabbit IgG, Santa Cruz) following the manufacturer's recommendations. The antibody was captured the following day with 15 µl of 50% slurry protein A agarose with gentle tilting at room temperature. The immunoprecipitate was pelleted and washed three times with Immunoprecipitation buffer, once with a High Salt wash (500mM NaCl, 50mM Tris-HCl, pH 8, 0.1% SDS, 1% NP-40), once with a LiCl wash (800mM LiCl, 50mM Tris-HCl, pH 8, 0.5% Sodium Deoxycholate, 1% NP-40) and three times with 1X TE. The supernatant from the IgG immunoprecipitation was saved and labeled as input. The antibody-DNA complexes were eluted with two 200µl washes of Elution buffer (2% SDS, 10mM DTT, 100mM $NaHCO_3$). The two elution fractions were combined and the crosslinking reversed by incubation at 65°C for six hours. Ten nanograms of pGEM(3z)f- were added to each sample to act as an internal control and the DNA was then ethanol precipitated overnight. Samples were then dissolved in 180µl of 1X TE and RNAse (10µg) and protease K (20µg) treated. Proteins were removed by phenol-chloroform extraction and

the isolated DNA ethanol precipitated overnight. The immunoprecipitated samples were dissolved in 12μl and the input sample in 200μl of 1X TE. QPCR was carried out as described above. The input DNA was diluted approximately 1:100 prior to PCR. Relative amounts of PCR product for the genes in this study were normalized to the relative amounts of the pGEM(3z)f- DNA in that sample and the Antibody/Input ratio calculated using the normalized amounts. The primers used were: 1) ASNS: 5'- AAT TTA TTT CGG TGC TG -3' and 5'- GAA GCC AAA ATA CAT CAG -3' 2) pGEM(3z)f-: 5'- GTA ATA CGA CTC ACT ATA GGG -3' and 5'- ATT TAG GTG ACA CTA TAG AAT AC -3'

P. Appendices

Appendix A.

The work presented in this appendix has not been published at the present time. It explores the role of the NF-κB2 gene in decreased chemosensitivity induced by mutant p53. This work was originally started by Katherine E. R. Stagliano and completed by myself. Although not directly related to the work in this dissertation, it includes and supports some of the data presented above. Only the results, figures, figure legends and methods sections from the manuscript are reproduced here in an edited form.

Appendix B.

During the process of writing this dissertation, new microarray data was generated using cell lines expressing the transactivation deficient mutant p53-D281G (L22Q/W23S) derived from H1299 cells. Presented here is a more comprehensive analysis of the microarray data presented above in conjunction with the newly generated data. Included is also data obtained from analyses of 21PT cells expressing mutant p53-R175H, R273H or -D281G.

Appendix A.

Tumor-derived p53 mutants induce NF-κB2 gene expression.

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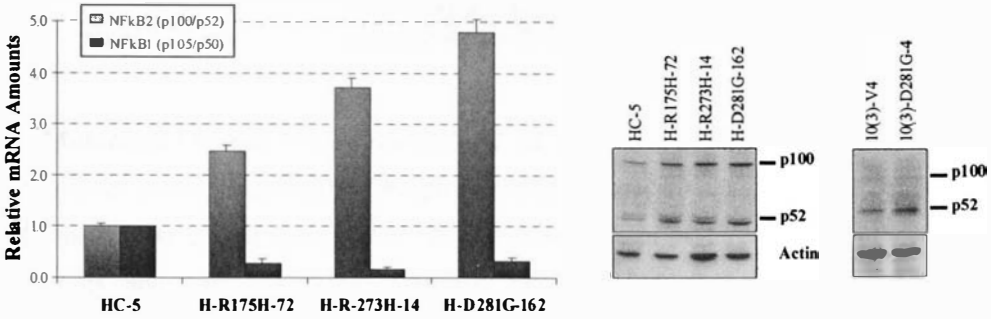
Experimental Results.

H1299 and 21PT cells expressing mutant p53 over-express a common set genes, including NF-κB2, which are inhibited or unaffected by WTp53. Since data described earlier suggested that mutant p53-mediated transactivation is necessary for its growth promoting functions, microarray hybridization analyses of three H1299-derived and three 21PT-derived cell clones expressing p53 mutants –R175H, -R273H, and -D281G were performed. The gene expression profiles of vector transfected H1299 and 21PT cells were used as controls. Affymetrix U95Av2 Arrays (HG-U95Av2) representing ~12,000 sequences were used. Changes in gene expression were detected as described earlier [199, 243]. A common set of approximately 150 genes was found to be significantly upregulated by all three p53 mutants in both the cell lines. Approximately 100 of those genes were found to be inhibited or unaffected by WTp53 but up-regulated in the presence of mutant p53. (Table 1A shows a partial list). This common set of genes includes genes involved in cell growth, survival and adhesion (i.e. E2F-5, NF-κB2, integrin α6, etc.), often implicated in cancer.

This up-regulation was verified by QPCR for a representative group of gene targets and expression of the triple mutant p53-D281G (L22Q/W23S) in H1299 cells did not result in up-regulation of the same genes [133] (data not shown). Interestingly, the group of up-regulated genes includes a member of the NF-κB family, NF-κB2 (p100/p52),

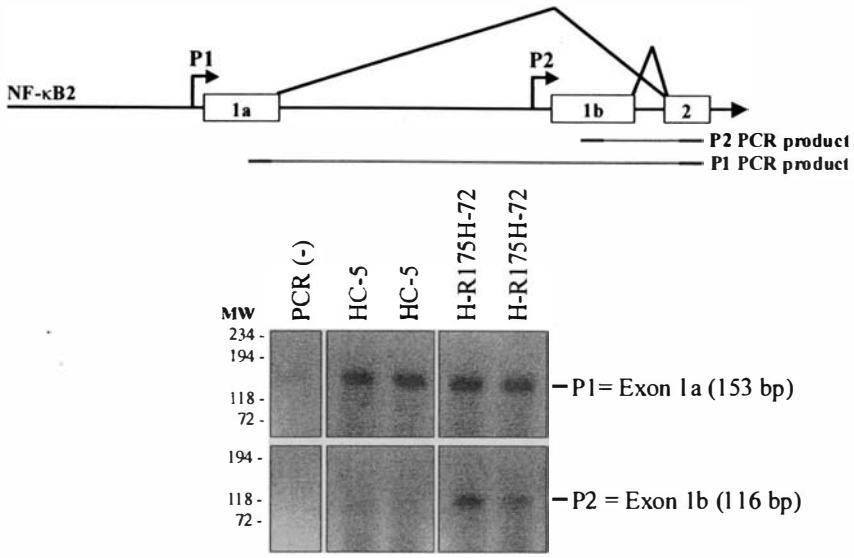
which has earlier been shown by our laboratory to be up-regulated in murine 10(3) cells constitutively expressing tumor-derived p53-D281G [156]. Figure 1A (left panel) shows QPCR analysis demonstrating up-regulation of expression NF-κB2 (p100/p52) in different mutant p53 expressing cell clones. This up-regulation was also confirmed by Western blot analysis using a specific antibody against NF-κB2 (p52) in the H1299 mutant p53-expressing cell lines (Figure 1A; right panel) [156]. Expression levels of NF-κB1 (p105/p50) were also determined by QPCR and found to be unaffected (Figure 1A). NF-κB2 has been implicated in survival and anti-apoptotic functions in cells [208, 209]. It is possible then, that mutant p53 utilizes the NF-κB2 pathway to reduce drug sensitivity.

Figure 1A. Expression of mutant p53 up-regulates genes in H1299 cells.



Specific NF-κB2 promoters are upregulated by mutant p53 in H1299. NF-κB2 has been shown to have two different promoters (P1 and P2) both of which can be active in a cell [277]. Using promoter specific primers, PCR using the conditions described by Lombardi *et al* [277] with cDNA prepared from H1299 cells expressing p53-R175H or vector control (HC-5) was performed. The data shown in Figure 2A demonstrate that in H1299 cells mutant p53 up-regulates NF-κB2 (p100/p52) expression using the P2 promoter, whereas the P1 promoter remains unaffected.

Figure 2A. Mutant p53 up-regulates NF-κB2 using the P2 promoter in H1299 cells.

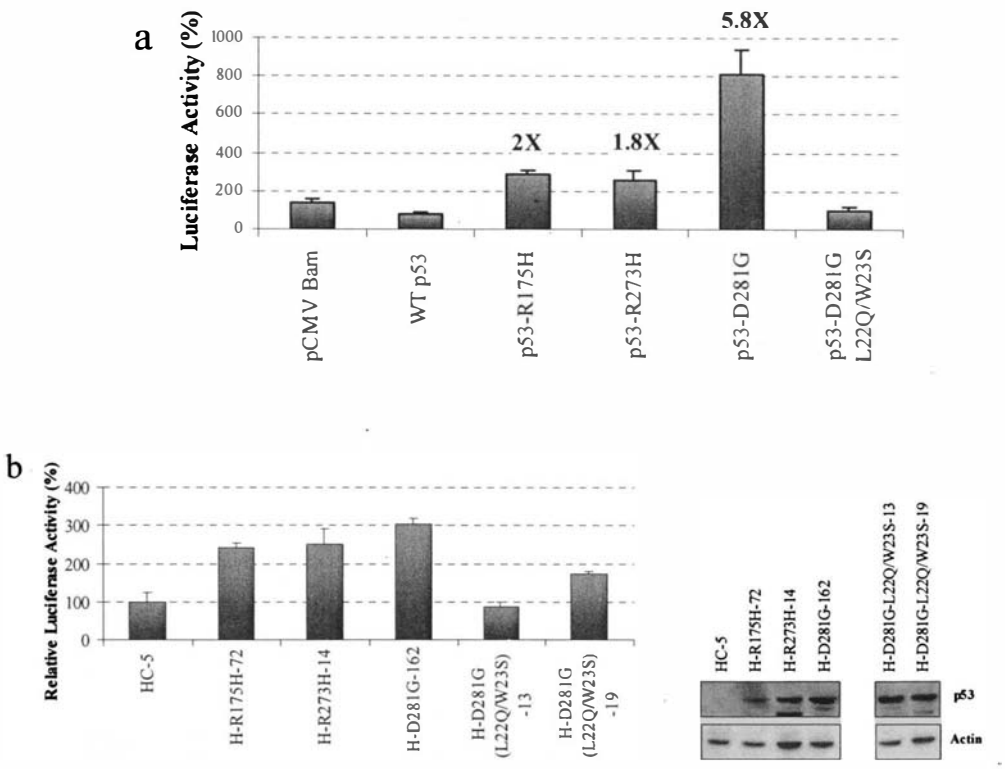


Mutant p53 transactivates the NF-κB2 P2 promoter *in vivo*. After ascertaining that mutant p53 up-regulates the P2 promoter of NF-κB2 (p100/p52), the P2 promoter sequences were cloned upstream of the luciferase gene in pGL3 vector as described in Materials and Methods using published sequence information [277]. Transient transcriptional assays were carried out in p53-null Saos-2 cells. Transcriptional results shown in Figure 3A-a demonstrate that tumor-derived p53 mutants p53-R175H, -R273H and D281G transactivate the NF-κB2 P2 promoter, while WTp53 represses, in agreement with our *in vivo* expression data. Furthermore, transfection of cells with mutant p53-D281G (L22Q/W23S) did not show up-regulation of the NF-κB2 P2 promoter (Figure 3A-a).

The NF-κB pathway is activated in cells expressing mutant p53. To determine whether expression of mutant p53 results in activation of the NF-κB pathway, NF-κB-specific transcription was assayed using a synthetic construct containing five NF-κB-binding sites upstream of a TATA box cloned upstream of a luciferase reporter gene

[278]. If the NF-κB pathway is active, more NF-κB2 should result in higher promoter activity in cells expressing the p53 mutants.

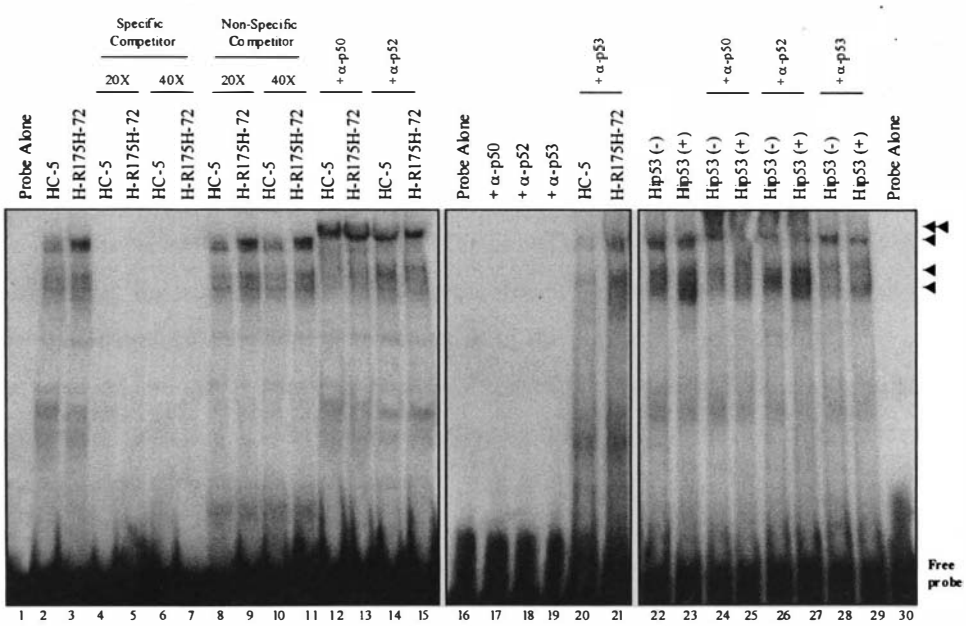
Figure 3A. Mutant p53 up-regulates NF-κB2 P2 promoter.



As expected, cells expressing mutant p53-R175H, -R273H and -D281G had higher promoter activity in comparison with the HC-5 (stably transfected with vector alone) cell line (Figure 3A-b) demonstrating that the NF-κB pathway is more active in cells expressing mutant p53 and that this activation is dependent on transactivation by tumor-derived p53 mutants. Western Blot analysis showed similar levels of expression of p53 in these cells (Figure 3A-b, right inset). Interestingly, at least one of the mutant p53 target genes, *c-myc*, has also been found to be a target of NF-κB suggesting the

possibility that mutant p53 may be transactivating *c-myc* through NF-κB [279]. However, it is also possible that both mutant p53 and NF-κB independently up-regulate *c-myc* expression. Protein expression levels of Rel A and c-Rel, two other members of the NF-κB protein family was also examined. Western blot analysis using extracts from vector control H1299 cells (HC-5) and H1299 cells expressing mutant p53 show no appreciable difference at the protein level for either of these proteins (data not shown).

Figure 4A. H1299 cells expressing mutant p53-R175H show increased NF-κB binding.

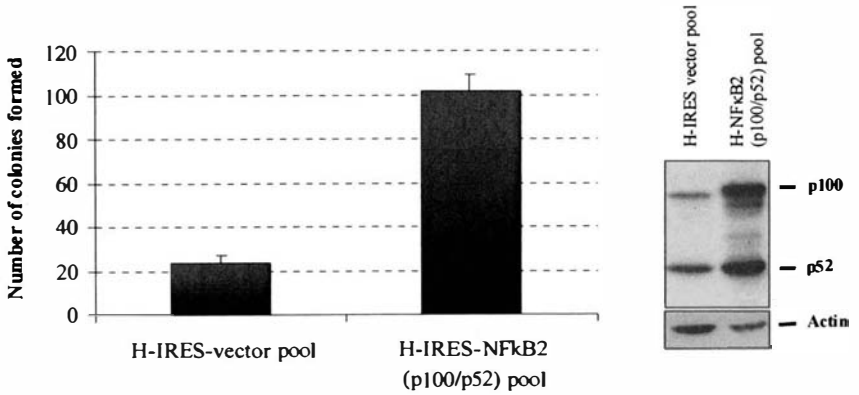


H1299 cells expressing mutant p53 show increased NF-κB DNA binding. EMSAs were performed to determine whether NF-κB binding activity changed in cells expressing mutant p53 compared to the control cells. Nuclear extracts from HC-5 control and mutant p53-R175H expressing H1299 cells were prepared and binding reactions performed using double-stranded DNA oligonucleotide probe containing the NF-κB DNA binding site as described [236, 280, 281]. The experimental data obtained suggest that there is an increase in DNA binding activity in cells expressing the mutant p53-

R175H (Figure 4A, single arrows indicate NF-κB complexes). Antibody supershift using specific antibodies against p50 or p52 suggests that the complexes formed contain p50 or p52 (double arrow). This supershift is not observed in the presence of antibody alone (lanes 17-19). Thus, data shown so far indicate that mutant p53 expression leads to an activation of the NF-κB pathway. In parallel, using a WTp53 ecdysone (Ponasterone A) inducible cell line [282], similar DNA binding reactions were carried out. Induction of WTp53 resulted in increased DNA binding. Unlike in the case of mutant p53 expressing cells, these complexes contained p50 in agreement with the results reported by Bohuslav *et al* [283] but not p52 (compare lanes 12-15 with 24-27). Sp1 and p53 were detected to determine equal loading of protein and proper WTp53 induction using specific antibodies as indicated (data not shown).

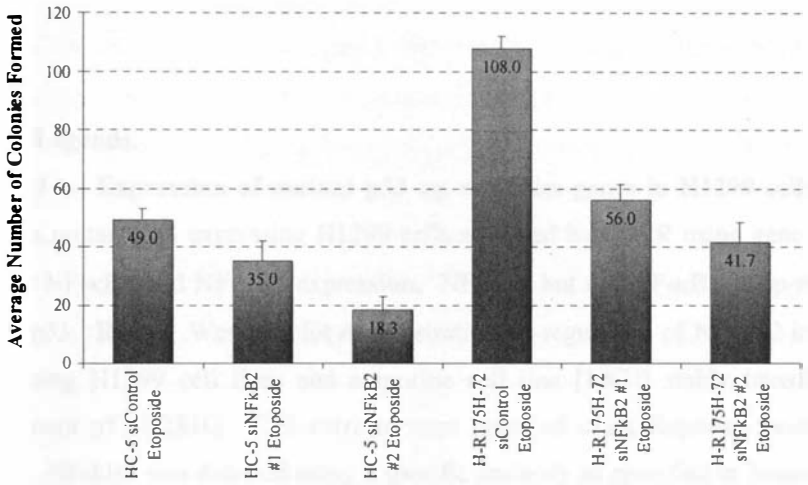
Over-expression of NF-κB2 in H1299 cells leads to loss of sensitivity to etoposide. The human NF-κB2 (p100/p52) gene [284] was cloned into pIRESpuro3 (Clontech), a bi-cistronic expression vector under the CMV immediate-early promoter where the NF-κB2 gene shares the same promoter with the puromycin resistance gene. Because of the bi-cistronic nature of this cloning vector, most of the puromycin-resistant colonies express the gene of interest. Using this NF-κB2 expression plasmid and the empty expression vector pools of H1299, cells expressing NF-κB2 (or stably transfected with vector alone) were generated (Figure 5A, right panel).

Figure 5A. Expression of NF-κB2 confers H1299 cells with a decreased sensitivity to etoposide.



Cells were exposed to 6μM etoposide (final concentration) for 48hrs as before. Over-expression of NF-κB2 in these cells led to an increase in the number of surviving cells compared to the vector control cell line (Figure 5A, left panel). The data presented demonstrate that NF-κB2 over-expression in H1299 cells imparts a lack of sensitivity to etoposide. That mutant p53 expression in H1299 cells leads to induction of NF-κB2 expression as well as lack of sensitivity to etoposide suggests that mutant p53 desensitizes the cells to etoposide via the NF-κB2 signal transduction pathway.

Figure 6A. siRNA against NF-κB2 increases the sensitivity of cells to etoposide.



NF-κB2 specific siRNA reduces the level of NF-κB2 in H1299 cells expressing p53 and inhibits mutant p53-induced lack of sensitivity towards etoposide. Observations reported in Figures 1, 3, 5 and 7 suggest that mutant p53 may increase chemoresistance of cells by inducing over-expression of NF-κB2. We tested this notion by using siRNA capable of reducing expression of NF-κB2 specifically and determined whether this would lead to increased etoposide sensitivity in H1299 cells expressing mutant p53 (-R175H). We reasoned that a decrease in NF-κB2 protein levels during the incubation period with etoposide would increase the sensitivity of the cells to the drug. Two different siRNAs against NF-κB2 were used. After transfection of siRNA specific

for NF-κB2, the level of NF-κB2 protein is significantly reduced (Figure 9B). The parallel nonspecific siRNA had little or no influence (data not shown).

A reduction in NF-κB2 level led to a reduction in the number of surviving cells (colonies formed) after etoposide regardless of the p53 status in the cells. The average number of colonies formed is depicted in the figure. In parallel, cells transfected with the same siRNAs were plated and treated with vehicle (DMSO) as indicated in Materials and Methods. Plating efficiency differed by less than 10% (data not shown). The siRNA experimental data corroborates our idea that at least one pathway through which mutant p53 induces chemoresistance in cancer cells is by up-regulating expression of genes such as NF-κB2.

Figure Legends.

Figure 1A. Expression of mutant p53 up-regulates genes in H1299 cells. Left: cDNA from mutant p53 expressing H1299 cells analyzed by QPCR using gene specific primers for NF-κB1 and NF-κB2 expression. NF-κB2 but not NF-κB1 is up-regulated by mutant p53. **Right:** Western blot demonstrating up-regulation of NF-kB2 in mutant p53-expressing H1299 cell lines and a murine cell line [10(3)] stably transfected to express mutant p53-D281G. Cell extracts were prepared using Reporter Lysis Buffer (Promega). NF-kB2 was detected using a specific antibody as specified in Materials and Methods

Figure 2A. Mutant p53 up-regulates NF-κB2 using the P2 promoter in H1299 cells. Using promoter specific primers, PCR was conducted using the conditions described by Lombardi *et al* [277] with cDNA prepared from clones of H1299 cells expressing mutant p53-R175H (or the vector control cell line HC-5). PCR primers are designed to detect mRNA products resulting from the two NF-κB2 promoters. PCR reactions were performed in duplicate. The data shown demonstrates that in H1299 cells mutant p53 up-regulates NF-κB2 using the P2 promoter, whereas the Pl promoter remains unaffected.

Figure 3A. Mutant p53 up-regulates NF-κB2 P2 promoter. a. Saos-2 cells were transfected with 200ng of pGL3 vector containing the NF-kB2 P2 promoter upstream of the luciferase reporter gene, 200ng of Renilla luciferase (TK.Renilla.luc) control plasmid and 600ng of the indicated p53 expression plasmid. Cells lysates were prepared 48hrs after transfection and luciferase activity detected using the Dual Luciferase Reporter Assay System (Promega). Reporter luciferase readings were normalized based on TK.Renilla.luc control plasmid readings. Fold induction for p53-R175H, -R273H and D281G, respectively. b. H1299 cells expressing mutant p53-R175H, -R273H, -D281G, -D281G (L22Q/W23S) or vector alone were transfected with 500ng of a reporter plasmid containing five NF-κB sites. Cells were harvested 48hrs after transfection, lysates prepared and luciferase activity detected. Cell extracts were normalized based on total protein concentration; the vector reading was arbitrarily set to 100. Inset shows western blot analysis demonstrating similar levels of p53 expression in the cell lines. Protein amounts were normalized based on total protein concentration.

Figure 4A. H1299 cells expressing mutant p53-R175H show increased NF-κB binding. Nuclear extracts of HC-5 and H-R175H were incubated as described in Materials and Methods with a ^{32}P-labeled probe containing the NF-κB DNA binding site sequence. Competition studies were done using a specific competitor (SC, lanes 4-7) and a non-specific competitor (NSC, lanes 8-11) in 20x and 40X molar excess (lanes 4-7). The single arrow indicates the DNA complexes containing NF-κB complexes. Increased NF-κB activity is observed in the presence of mutant p53 (lanes 2-3, 8-11). The double arrow indicates the supershifted complex in the presence of an antibody specific for NF-κB1 (p50), NF-κB2 (p52) or p53 (lanes 12-15, 20-21, 24-29). Equal amounts of protein were added to each lane.

Figure 5A. Expression of NF-κB2 confers H1299 cells with a decreased sensitivity to etoposide. H1299 cells stably expressing NF-κB2 were treated with 6μM etoposide final concentration for 48hrs. After treatment the surviving cells were washed and allowed to form colonies for three weeks with periodic changes of media. Colonies were then fixed in methanol, stained and counted. The data shown are representative of

three independent experiments; colony numbers were adjusted to account for plating differences based on control plates. Control plates were plated at one-tenth the density and treated with DMSO. Expression of NF-κB2 protein was checked by western blot (Right). Actin was used as a loading control.

Figure 6A. siRNA against NF-κB2 increases the sensitivity of cells to etoposide. H1299 cells expressing mutant p53-R175H (or the control cell line HC5) were plated at equal densities in 12-well plates. Cells were transfected 24 and 48hrs after plating using siRNAs against NF-κB2 (or a non-specific control) and plated as described in Materials and Methods 24hrs after the second transfection. Cells were then exposed to 6μM etoposide (final concentration) or vehicle control (DMSO) for 48hrs. Cells were allowed to grow and form colonies. Colonies were then fixed with methanol, stained with methylene blue and counted. **A.** Graphical representation of the results. The average number of colonies formed is depicted in the figure. Plating efficiency differed by less than 10% (data not shown). **B.** Western blot analysis of cells from a parallel experiment transfected with siRNAs to evaluate the effectiveness of the RNAi constructs. Actin was used as a loading control.

Experimental Methods.

Generation of stable cell lines. Stable cell lines were generated after transfection of p53-null H1299 and 21PT cells with mutant p53 expression plasmids (or expression vector alone), containing a neomycin resistance gene as described [134]. The following p53 mutant cell lines were generated: p53-R175H, p53-R273H and p53-D281G using G418 selection (400 μg/ml for H1299 cells and 500 μg/ml for 21PT cells). The p53-D281G (L22Q/W23S) clones were only generated in H1299 cells. H1299 cells were also transfected with pIRES-puro3 vector alone or pIRES-puro3 containing NF-κB2, p53-R175H, p53-R273H, p53-D281G or p53-D281G (L22Q/W23S) cDNA. Cells were selected with 2.5μg/ml of puromycin for two weeks. The resulting colonies were then pooled and used for further assays. 10(3) cell clones were generated earlier at 200 μg/ml

G418 [134]. Hip53, a wild-type p53 inducible cell line, was generated earlier [282]. None of the cells lines used in our assays Saos-2, H1299, 21PT or 10(3) express detectable p53 [11, 128, 133, 262]. 21PT expresses an N-terminally truncated and functionally inactive p53 [285].

Recombinant adenoviruses, adenoviral infection and RNA preparation. Recombinant adenoviruses expressing WTp53 and β-galactosidase were generated by the laboratory of Dr. Kristoffer Valerie, Massey Cancer Center (Richmond, Va) and by the lab of Atsushi Miyanohara, UCSD Gene Therapy Program. In a 10 cm dish 3x 10^6 cells were infected with the recombinant adenoviruses expressing either WTp53 or β-galactosidase at 10 multiplicity of infection (MOI)/cell ratio. At 20 hours, RNA was extracted for microarray analysis. Total RNA was isolated using Trizol (Invitrogen) reagent following the manufacturer's recommendations and checked by 1.2% agarose Tris-borate-EDTA gel electrophoresis.

Drug sensitivity assays. H1299 (or 21PT) cells stably expressing p53-R175H, -R175H (L22Q/W23S), -R273H, -D281G, -D281G (L22Q/W23S) or transfected with vector alone (as indicated in the figure), were plated at equal densities and treated with a final concentration of 3-6μM etoposide (Sigma) for 48hrs as specified in the text. Chemosensitivity of the cells was then measured in two different ways: (1) colony formation after 2-3 weeks, and (2) bromodeoxyuridine (BrdU) incorporation. Colony formation assay: Cells were plated at a density of $5x10^4$ cells in 10cm dishes and exposed to a final concentration of 6μM etoposide for 48hrs. After treatment, plates were washed and the media replaced. The surviving cells were allowed to form colonies for 2-4 weeks with periodic changes of media. Colonies were fixed with methanol, stained with methylene blue and counted as described earlier [156]. Control plates were plated at a density of $5x10^3$ cells in 10 cm plates and treated with an equal amount of dimethyl sulfoxide (DMSO). Control plates were assessed for plating efficiency and DMSO effects on cell growth. BrdU incorporation assay: Cells were plated at a density of $1x10^6$ in a 10cm dish in the presence of etoposide (6μM final concentration) or DMSO as control for 48-72h. Cells were incubated in the presence of 10μM BrdU for 40 minutes.

After washing with Dulbecco's Phosphate Buffer Solution (DPBS), cells were trypsinized, counted, fixed by vigorous vortexing and addition of cold absolute ethanol (ETOH) drop-wise. Cells were then stored at 4°C for at least 18hrs. Samples were washed with DPBS plus 0.5% BSA to remove EtOH. Fixed cells were treated with 400 µl 2N HCl for 20 minutes, followed by incubation in 0.1M sodium borate pH 8.5 for 2 minutes. Samples were washed and re-suspended in DPBS plus 0.5% BSA and 0.5% Tween 20 followed by incubation with anti-BrdU antibody coupled with fluorescein isothiocyanate (FITC) for 1h in the dark with tilting. After washing to remove excess antibody, cells were re-suspended in propidium iodide staining solution for at least 1hr prior to florescence-activated cell sorting (FACS) analysis [271, 272]. Samples were gated for sub-G1 DNA containing cells.

siRNA transfection. H1299 cells expressing mutant p53-R175H (or the control cell line HC-5) were plated at equal densities in 12-well plates (3×10^5). Cells were transfected 24 and 48hrs after plating using siRNA against NF-κB2 (or a non-specific control). Sequences used were: Control (siControl): 5'- CAU GUC AUG UGU CAC AUC ACT T -3'and 5'- GAG AUG UGA CAC AUG ACA UGT T -3', NF-κB2 (siNFkB2 #1): 5'- GAC AAG GAA GAG GUG CAG CTT -3' and 5'- GCU GCA CCU CUU CCU UGU CTT -3' and NF-κB2 #2 (siNFkB2 #2): 5'- GCC CUG AGU GCC UGA AUC U -3' and 5'- AGA UCC AGG CAC UCA GGG CTT -3'. Twenty-four hours after the second transfection, cells were trypsinized, counted and plated in 10 cm plates at a density of $5x10^4$ cells per plate (control plates were plated at a density of $5x10^3$ cell per plate). Cells were then treated with 6µM etoposide (final concentration) or equivalent vehicle control (DMSO). Cells were allowed to form colonies for 2 to 4 weeks. Colonies were then fixed with methanol, stained with methylene blue and counted as described earlier [156].

DNA microarray hybridization, data management and analysis. Expression profiles of mutant p53-expressing cells were compared with H1299 (or 21PT) cells stably transfected with vector alone. Cells infected with recombinant adenovirus expressing WTp53 were compared to cells infected with adenovirus expressing β-galactosidase as a

control. All microarray hybridization analyses were performed in duplicate using Affymetrix U95Av2 chips by either the GeneChip Core lab at UCSD or Virginia Commonwealth University's Institutional Nucleic Acid Research facility. U95Av2 Array represents ~12,000 human gene sequences that have been previously characterized. The general procedures for microarray hybridization and analysis are described elsewhere [273]. Data analysis was done using Affymetrix Microarray Analysis Suite 5 (MAS 5). Changes in gene expression were detected in terms of statistical significance (S-score) of change in expression for a given gene between two compared microarrays. S-score analysis takes into account signals detected by 16 multiple probe pairs for individual genes, as well as intensity-dependent and -independent noise [199]. Briefly, the files generated by the Affymetrix software were first filtered to eliminate genes with an average intensity value less than 50 in at least one of the samples, resulting in 2417 genes for further study. The filtered data was then analyzed with the S-score program. S-scores are derived to have a mean value of zero (representing no change) with a standard deviation of 1. The S-scores generated were then analyzed for significance across replicate experiments by using a permutation method performed with the significance analysis of microarray (SAM) program from Stanford University [201]. The settings for this analysis were: unlogged data, 300 permutations, K-nearest neighbor imputer of 10 and a random number seed of 123456789. Once the program reported the list of ranked genes, the "delta value" was adjusted to a stringent False Discovery Rate (FDR) of 0.3% resulting in the identification of 149 genes upregulated by all three p53 mutants in both cell lines. Clustering analysis was done using the Cluster and TreeView programs (http://rana.lbl.gov/) to provide a graphical display of the expression patterns [256]. Genes reported by SAM were analyzed by hierarchical clustering with average linkage grouping. For our analysis the arrays were not clustered. Functional grouping of the identified genes was done by manual editing of Gene Ontology categories obtained through the DAVID annotation tool (http://david.niaid.nih.gov/david/ease.htm) [258].

Quantitative PCR. QPCR was conducted using the LightCycler (Roche) as described [286]. cDNA was synthesized using the Thermoscript RT-PCR system

(Invitrogen). Primers were designed using Oligo 5 (Molecular Biology Insights) and synthesized by Sigma Genosys. Reactions were performed in triplicate utilizing SYBR Green dye, which exhibits a higher fluorescence upon binding of double-stranded DNA. The QPCR primers used were, *NF-κB2*: 5'- GGG GCA TCA AAC CTG AAG ATT TCT -3' and 5'- TCC GGA ACA CAA TGG CAT ACT GT -3'; *NF-κB1*: 5'- CAC TTA GCA ATC ATC CAC CTT -3' and 5'- AGC CCT CAG CAA ATC CT -3'.

NF-κB2 Promoter PCR. NF-κB2 promoter PCR was performed using the conditions described by Lombardi et al. [277] with cDNA prepared form H1299 cells expressing mutant p53 (or the control cell line HC-5). The promoter specific primers used were: P1: 5'- AGA GCA GCA GCT GCA CAC AG -3' and 5'- GCT CTG TCT AGT GGC TCC -3'; P2: 5'- AAC TCC GGA TCT CGC TCT CC -3' and 5'- GCT CTG TCT AGT GGC TCC -3'. Note that the reverse primer for the P1 and P2 promoters is the same; while the forward primer is different, thus generating two distinct PCR products of 153 bp (P1) and 116 bp (P2).

Cloning of presumptive promoter. The presumptive promoter 2 (P2) sequence of NF-kB2 was cloned in the pGL3 basic vector upstream of the luciferase gene using the available genomic sequences in the NCBI database and genomic PCR using a commercial kit (Invitrogen). The primers used were: 5'- CGC TAG CAA CTC GCG CCT GGT GTC CGT -3' and 5'- CCA AGC TTG CGG CAT GAC TCA CTG GGT TGT AG -3' (forming NheI and BglII sites at the ends, respectively). The restriction enzymes in parenthesis indicate the location of the insert in the multiple cloning site of the pGL3 reporter vector. The P2 promoter was cloned using Saos-2 cells, and contains a single mismatch (A to G). We found the same mismatch in multiple analyzed clones and did not investigate any further. It should be noted though, that an analysis with the program TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) indicates that the sequence does not fall on any known transcription factor-binding site (not shown).

Transient transcriptional assays. Promoters were tested by transient transcriptional assays using p53-null Saos-2 (human osteosarcoma) cells. Transfections and luciferase assays were carried out as described [156]. Saos-2 cells were plated at equal densities in

24-well plates and transfected with 200 ng of the promoter luciferase construct, 200 ng of Renilla luciferase control (TK Renilla.luc, used for normalization of transfection) and 500 ng of the corresponding p53 expression plasmid 24hrs after plating. H1299 cells stably expressing mutant p53 were plated at a density of $3x10^5$ cells/well in 6-well plates and transfected with 500 ng of a reporter plasmid containing NF-κB sites upstream of the luciferase gene (kind gift from Dr. Valentine Andela of the University of Rochester) [278]. Transfections were carried out using the Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's recommendations. Cell lysates were prepared 48hrs after transfection using Reporter Lysis Buffer (Promega). Luciferase activity was detected using a Luminometer from Turner Designs. Transcriptional assays were repeated at least three times.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared in the following manner. Cells grown to confluency were washed twice in PBS and lyzed in 700 µl of Dignam buffer [281] [10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1% Nonidet P-40, 0.5 mM PMSF, plus protease inhibitor (cocktail III from Calbiochem)] by adding the buffer directly onto the plate and incubating on ice for 20 minutes. Cells were then scraped and transferred into a pre-chilled eppendorf tube and further incubated on ice for 20 minutes. Nuclei were then pelleted at 4000 rpm, 4°C for 10 minutes, the supernatant (cytoplasmic extract) stored at -80°C. The nuclei were resuspended in 100 µl Nuclear extraction buffer [(20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, 1% Nonidet P-40, plus protease inhibitors] and incubated on ice for 1 hour. The samples were pelleted at 10,000 rpm, 4°C for 10 minutes and the supernatant (nuclear extract) stored at -80°C. Protein concentrations were determined using Biorad's Protein assay reagent and equal amounts of protein were used in each binding reaction. EMSAs were performed as described [283] using 15µg of nuclear extract protein per binding reaction. Reactions were pre-incubated at 25°C for 20 minutes prior to the addition of the oligonucleotide probe. After pre-incubation, approximately 20,000 counts of labeled probe were added to each reaction tube and incubated at 25°C for an additional 20 minutes. For the supershift

experiments, antibodies (1μg) were added after incubation with the probe and incubated for an additional 15 minutes. Samples were then loaded and complexes separated on a 6%, 0.5X Tris-Borate-EDTA gel run at 200volts for approximately 1.5-2hrs. The gel was then fixed, dried and exposed to film. Probes were annealed and ^{32}P-dCTP labeled using Klenow fragment as described [236]. The oligonucleotide sequences used were: Wild-type (WT) 5'- GAT CCG AGG GCT GGG GAT TCC CAT CTC CCA CGT TTC ACT TCA -3' and 5'- AGC TTG AAG TGA AAC GTG GGA GAT GGG AAT CCC CAG CCC TCG -3'; Mutant (MT) 5'- GAT CCG AGG GCT TTT TAT GAA AAT CTC CCA CGT TTC ACT TCA -3' and 5'- AGC TTG AAG TGA AAC GTG GGA GAT TTT CAT AAA AAG CCC TCG -3'. For competition studies an unlabeled WT probe was use as the specific competitor (SC) and the MT probe as the non-specific competitor (NSC) at 20X and 40X molar excess. Hip53 cells were induced with 10μM Ponasterone A. NF-κB1 (p50), NF-κB2 (p52) and p53 specific antibodies were used for the supershift experiment (Santa Cruz, sc-114X; Rockland, cat# 4185; Oncogene, Ab-6, respectively).

Western blotting. NF-κB2 and Sp1 levels were detected using antibodies from Santa Cruz Biotechnologies (sc-7386 and sc-59 respectively). Actin levels were detected using an antibody from Sigma (AC-15). NF-kB1 (p105/p50), Rel A and c-Rel were detected using an antibody kit from Calbiochem (cat# ASK20). p53 was detected using the p53 antibody PAb 1801. Western blots were developed by the ECL method (Amersham). In western blots for the siRNA and colony forming assay the H-IRES NF-κB2 (p100/p52) cell line, NF-κB2 protein was detected using an antibody from Upstate Biosciences (cat # 05-361).

Appendix B.

The microarray data generated was analyzed as before [236, 243]. S-cores generated by comparing hybridization signals from mutant p53 expressing cell lines and the corresponding controlled were analyzed for significance across replicates. The data were analyzed using the following SAM settings: multiclass analysis, 300 permutations, K-nearest neighbors imputer of 10, and a random seed number of 123456789. This type of analysis identifies genes that are differentially regulated between groups of data. In this case, data from H1299 cells expressing mutant p53 was labeled as group 1; data from 21PT cells was labeled as group 2; and data from cells expressing the transactivation deficient p53-D281G (L22Q/W23S) was group 3. The FDR was set to 0.025%. The Cluster and TreeView programs were used to generate a visual representation of the results.

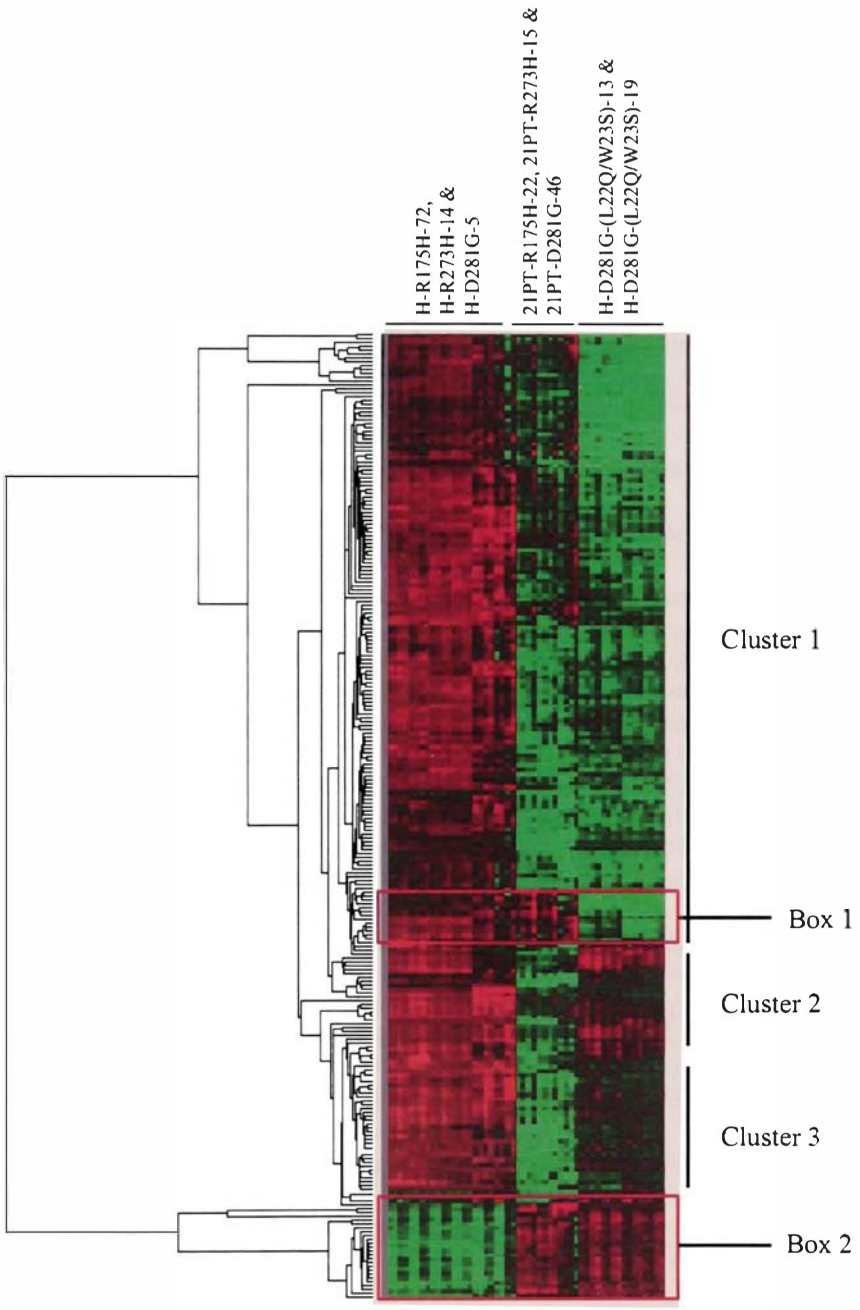
There are several, striking features to this figure regarding the modulation of gene expression by mutant p53 in H1299 and 21PT cells. First, a larger number of genes is up-regulated by mutant p53 rather than down-regulated. This suggests that up-regulation of genes by mutant p53 may play a significantly more important role than down-regulation of genes in mutant p53 oncogenicity. Second, genes up-regulated in the H1299 cells by the transactivating forms of mutant p53 are repressed (Cluster 1) or unaffected (Clusters 3) by the transactivation deficient mutant p53-D281G (L22/Q/W23S).

Thirdly, a number of genes are up-regulated by the mutant proteins regardless of their transactivation status (Cluster 3). This suggests that perhaps some transcriptional up-regulation may be due to factors other than the transactivation ability of mutant p53, such as protein-protein interactions. Also, it appears that genes repressed in H1299 cells are up-regulated by the transactivation deficient form of p53-D281G (Box 2). The reasons for this are unknown. Perhaps, in order to induce repression of genes, mutant p53 requires an interaction with another factor. The two additional mutations, which disrupt TAF interactions and c-terminal modifications, may be involved in this interaction. In

short, mutating these two residues may prevent or disrupt the interaction between mutant p53 and this second factor resulting in up-regulation of genes rather than repression.

Finally, it is clear from the figure that although mutant p53 up-regulates a number of genes in H1299 cells, this is not the case when the mutant protein is expressed in 21PT cells (Box 1). This is most likely due to the level of stringency used during the analysis. A less stringent analysis reveals a larger number of genes similarly regulated in both 21PT and H1299 cells (data not shown). Nonetheless, it is evident that expression of mutant p53 in H1299 does not lead to a similar transcriptional regulation of genes in H1299 and 21PT cells. Perhaps mutant p53 regulation of genes is partially dependent on the genetic background of the cells.

Figure 1B. Microarray hybridization analysis.



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