

DIVERSITY WITHIN THE MASTER REGULATORY p53 TRANSCRIPTIONAL
NETWORK: IMPACT OF SEQUENCE, BINDING MOTIFS AND MUTATIONS

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum in Genetics and Molecular Biology.

Chapel Hill
2008

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Abstract

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Diversity within the master regulatory p53 transcriptional network:

impact of sequence, binding motifs and mutations

(Under the direction of Michael A. Resnick, PhD)

In response to cellular stress and DNA damage, the *master regulatory gene* p53 directly controls the differential expression of target genes within its extensive transcriptional network through promoter response elements (REs) to elicit many biological responses, including cell cycle arrest and apoptosis. Tetrameric p53 binds the consensus sequence RRRCWWGYYY (n= 0-13) RRRCWWGYYY, where R=purine, W=A/T and Y=pyrimidine. Phenotypic diversity within the p53 transcriptional network occurs as a result of a variety of factors including cell type, post-translational modifications, stress-inducing stimuli, and mutations in p53 that alter transactivation function through changes in the strength of gene activation or spectra of genes regulated. We have developed an isogenic diploid yeast-based reporter system to evaluate the contribution of target binding sequence, organization and level of p53 on transactivation at target REs by wild-type and mutant p53. The chromosomal position for all of the human derived REs was identical and the number of p53 molecules/cell could be varied over a hundred-fold using an integrated rheostatable *GAL1::p53* promoter that is sensitive to level of galactose in the medium. We confirm transactivation by WT p53 differs between REs where small differences in sequence can

contribute significantly to levels of transactivation. Through deconstruction of the canonical RE and evaluating transactivation from various sequences and binding motifs, we have challenged the view of what constitutes a functional p53 target. We show small increases in distance between decamer half-sites greatly reduce p53 transactivation. Furthermore, we demonstrate that substantial sequence-dependent transactivation can occur from $\frac{1}{2}$ - and $\frac{3}{4}$ -site REs. Importantly, the presence of these noncanonical REs greatly expands the p53 master regulatory network. In addition, we have determined the functional fingerprints of missense mutations to demonstrate that altered function p53 mutations can occur in breast cancers and the transcriptional effects are often subtle. Finally, we have identified super-trans sequences which enhance the efficiency of p53 transactivation by greatly lowering the number of molecules required. These sequences provide a useful tool for addressing wild-type and mutant p53 function. Overall, our findings demonstrate that RE sequence, organization, level of p53 and mutations can strongly impact the ability of the master regulator p53 to transactivate downstream targets, thus diversifying its transcriptional network.

ACKNOWLEDGEMENTS

As the p53 universe is forever expanding, so are the number of individuals who I am truly thankful to for their support throughout graduate school and in the development of my scientific career. First, I would like to thank the members of my dissertation committee William Kaufmann, Charles Perou, Norman Sharpless, and Brian Strahl. I appreciate their time and scientific guidance.

I would like to thank the current and past members of the Resnick lab for their support and making the lab an enjoyable and stimulating atmosphere in which to work. A special thanks to the p53 crew, Daniel Menendez and Alberto Inga. Both are enthusiastic scientists with whom it was a pleasure to work.

I would like to acknowledge the support and guidance of my mentor Michael Resnick who has my fullest respect as a scientist, but more importantly as a person. I will remember with fondness and gratitude the hours of quality blue chair sessions that were interrupted with chocolate breaks and Cajun popcorn. I am sincerely grateful for the opportunity I was provided within his lab.

A special thanks to my family, whose support and encouragement throughout this process was genuinely appreciated.

Finally I would like to acknowledge the support of the Department of Defense for their funding through a Breast Cancer Research Program Predoctoral Traineeship Award (BC051212).

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LIST OF ABBREVIATIONS

APAF-1	apoptosis protease activating factor 1
BAX	BCL2-associated X protein
BRCA	breast cancer susceptibility gene
ChIP	chromatin immunoprecipitation
Con	consensus
CORE	COunterselectable REporter cassette
CYC1	iso-1-cytochrome C
DBD	DNA binding domain
ER	estrogen receptor
FASAY	functional analysis of separated alleles in yeast
FH	familial history
GADD45	Growth Arrest and DNA Damage-inducible
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HER2+	human epidermal growth factor-2 positive
IARC	International Agency for Research on Cancer
LFL	Li-Fraumeni-like syndrome
LFS	Li-Fraumeni syndrome
MDM2	mouse double minute
MMP2	Matrix metalloproteinase-2
nt	nucleotides
PA26	p53-activated gene 26

P53AIP1	p53-regulated Apoptosis-Inducing Protein 1
P53R2	p53-inducible ribonucleotide reductase small subunit 2
PCNA	proliferating cell nuclear antigen
PIDD	p53 induced protein with death domain
PUMA	p53-upregulated modulator of apoptosis
PWM	position-weight matrices
RE	response element
RGC	ribosomal gene cluster
RNAP II	RNA polymerase II
SAGA	Spt/Ada/Gcn5/acetyltransferase
SEM	standard error of measurement
SNP	single nucleotide polymorphism
TBP	TATA-binding protein
TP53	tumor suppressor p53
TIGAR	<u>T</u> P53 <u>i</u> nduced <u>g</u> lycolysis and <u>a</u> poptosis <u>r</u> egulator
UAS	upstream activating sequence
VEGF	vascular endothelial growth factor
WT	wild-type

CHAPTER 1
INTRODUCTION

p53 from oncogene to tumor suppressor

While the discovery of TP53 in 1979 provoked an interest in a protein involved in the role of cancer development, the natural function of p53 in tumorigenesis was thought to be that of an oncogene since it was first identified complexed with the SV40 large T protein in transformed cells [1-3]. The understanding that p53 was a tumor suppressor began to arise years later when wild type (WT) p53 was found to suppress transformation of cells, whereas mutant p53 was associated with cellular transformation [4, 5]. Furthermore, genetic alterations in p53, which mostly occurred as point mutations were found frequently in human cancers [6-9]. In agreement with Knudson's two-hit hypothesis for tumor suppressors [10], the p53 knockout mouse developed normally, but had an increased incidence of tumors where WT, heterozygous and homozygous mice developed neoplasias at ~18, 9 and 6 months, respectively [11].

Several major findings contributed to the understanding that p53 executed its role as a tumor suppressor through functioning as a transcription factor. The p53 protein was found to be induced in response to DNA damage or cellular stress to stimulate either cell cycle arrest or apoptosis [12-15]. Stimulation of these biological responses was dependent upon the ability of p53 to bind DNA, where loss in DNA binding resulted in the loss of its ability to suppress growth [16-22]. In addition, an intact transactivation domain and transactivation activity were necessary for p53 to function as a tumor suppressor [20, 21, 23, 24].

Currently, it is accepted that p53 plays a critical role as a *master regulatory gene* by directly controlling the transcriptional regulation of greater than 100 target genes in a sequence-specific manner in response to intra- and extra-cellular stresses (Figure 1.1) [25, 26]. Cell cycle regulation, apoptosis, angiogenesis, replication and repair are processes

interconnected within the p53 transcriptional network. Aberrations within the p53 transcription network are a hallmark of cancers, where mutations in p53 itself are found in greater than 50% of all cancers [27]. To date over 24,800 somatic p53 mutations and ~400 germline p53 mutations have been reported within the International Agency for Research on Cancer's (IARC) TP53 mutations database [27]. In terms of transactivation, p53 binds to a consensus binding sequence [RRRCWWGYYY (n) RRRCWWGYYY] (where R=purine, W= A or T, Y= pyrimidine and n is 0-13 bases) [28-30] to recruit the basal transcriptional machinery and additional cofactors in order to regulate the expression of the associated target gene [31]. However, p53 does not recognize and regulate each of its downstream targets in an equivalent fashion (Figure 1.2)[32]. Differential induction is dependent upon the target gene of interest, stress stimuli and cell type and is influenced by many factors including the chromatin environment, post-translational modifications, availability of transcriptional cofactors, and concentration or duration of activated p53 protein [26, 32, 33]. At the DNA level, regulation of target genes is dependent on the sequence of response elements (RE), number of REs in a promoter region, distance of the RE from the transcriptional start site and intrinsic binding with a RE [26].

The Resnick lab recently established, using an isogenic, *in vivo* yeast-based model system, that these latter influences can lead to greater than a 1000-fold variation in p53-dependent transactivation from individual REs derived from human target genes [34]. Variation in gene regulation by p53 has led to the postulation of the *master gene of diversity* concept [26] which can be applied to many sequence-specific transcription factors. Using a piano analogy, the master regulatory gene, p53 is the hand that plays a chord on the keys of a piano, the downstream target genes. The chord is the accumulation of biological responses

regulated and is dependent on both the genes regulated and strength of regulation. Functional mutations in the hand can alter the “selection of keys” by altering binding, spectrum of REs recognized, and/or the intensity of regulation [26].

Regulation and activation of p53 protein

The p53 protein is a 393 amino acid modular protein comprised of two N-terminal transactivation domains (residues 1-73), a proline rich domain (PXXP, residues 63-97), a core DNA binding domain (DBD) (residues 94 - 292), a tetramerization domain (residues 324-355) and a C-terminal regulatory domain (residues 360 – 393) [35, 36]. Within the cell, p53 is constitutively expressed, but the protein is kept at low concentrations (estimated at ~1000 molecules/cell [37]) through several negative regulators, the most classic of which is the auto-inhibitory feedback loop formed with one of its own target genes, MDM2 (mouse double minute or the human homolog, HDM2). The MDM2 protein inhibits p53-dependent transactivation by regulating p53 protein through two mechanisms. First, it can bind to the amino terminal transactivation domain of p53 (residues 18-23) to inhibit interactions between p53 and the basal transcriptional machinery or transcriptional cofactors [38-40]. Second, it can function as a ring finger E3-ubiquitin ligase to ubiquitinate p53 on its C-terminal lysines which targets p53 for proteasome degradation [41-44].

Recently, MDMX (HDMX/MDM4 in humans), a ring finger protein related to MDM2 but lacking the E3 ligase activity, has been identified as an additional negative regulator of p53. Similar to MDM2, MDMX represses p53-dependent transcription through binding the N-terminus transactivation domain of p53. Under “non-stressed” conditions, MDMX forms heterodimers with MDM2 which prevents MDM2 from targeting itself for

auto-ubiquitination thus increasing MDM2 dependent degradation of p53 [45-48]. Several additional E3 ligases (i.e., COP1 and Pirh2) and de-ubiquitinating proteins (i.e., HAUSP) have also been implicated as having a role in regulating p53 protein levels [49, 50].

Following DNA damage or cellular stress, a cascade of signaling pathways induce post-translational modifications in p53 which have been postulated to contribute to the stabilization of the protein. For example, phosphorylation of p53 at Ser15 by DNA-PK was proposed to directly interfere with interactions between p53 and MDM2 [51, 52]. Phosphorylation of Ser15, Ser20 and Thr18 has also been proposed to decrease p53-MDM2 interactions indirectly by enhancing p53 interactions with co-activators such as the histone acetyl transferases, p300 and CBP [53]. Additional post-translational modifications in the C-terminal of the protein, such as Ser392 phosphorylation were found to contribute to shifting the tetramer-monomer equilibrium towards the tetramer formation which is necessary for p53 transcriptional activity [37, 54].

However, several studies have shown that mutations which render a residue unable to be modified, such as Ser20Ala do not necessarily prevent accumulation of the p53 protein [55]. Current research has implied the rapid degradation of MDM2 following damage or stress may play as imperative a role in p53 activation as modifications in p53 itself. Following damage, phosphorylation of MDM2 at Ser395 by ATM was shown to abolish the direct interactions with p53 [56-58]. Furthermore, phosphorylation of MDM2 has been postulated to impede the MDMX-MDM2 interactions which results in the E3 ligase specificity of MDM2 being drawn away from p53 and toward itself and MDMX [46].

These findings have sparked ongoing debates as to whether post-translational modifications in p53 contribute to the activation of the protein or whether they dictate

promoter selection to specify or fine tune p53-dependent regulation and consequently a particular biological response [58]. Currently, there are approximately 40 upstream effectors identified which function to post-translationally modify p53 at over 30 residues (primarily in the N-terminal transactivation domains and C-terminal) [59]. The concept that specific post-translational modifications in p53 impact its ability to differentially regulate target genes has been coined the “barcode hypothesis” [33]. For instance, one specific barcode which occurs after p53 stabilization at high doses of DNA damage is the phosphorylation of Ser46. This post-translational modification has been shown to specifically enhance p53 binding to and activation of the apoptotic associated target, p53AIP1; mutagenesis of the residue mitigated transactivation from p53AIP1, but not other REs including p21, MDM2, p53R2 and Noxa [33, 60].

In addition to the post-translational modifications, co-factors, small molecules, and/or other interacting binding proteins have been shown to influence the promoter selectivity of p53 to elicit specific biological responses. ASPP1 and ASPP2 (apoptosis stimulating protein of p53) as well as p53 family members p63 and p73 have been shown to enhance p53 binding toward some apoptotic REs, such as Bax [61, 62]. Whereas, HZF (hematopoietic zinc-finger) binds the p53 DBD to augment p53 binding toward REs associated with cell cycle arrest, such as p21 and 14-3-3 σ [63]. Other proteins have been identified which channel or recruit p53 to selected downstream targets. For example, BRCA1 has been shown to direct p53 to genes involved in cell cycle arrest and DNA repair, but not apoptosis [64].

p53 interaction with its consensus RE

The consensus sequence which p53 binds consists of two decamer half-sites [RRRCWWGYYY (0-13)_n RRRCWWGYYY], where R = purine, Y = pyrimidine, W=A/T, and which can be spaced by up to 13 nucleotides apart [28-30]. Unlike for other transcription factor binding sites, such as NFκB, high evolutionary conservation between species is not observed for the p53 response element sequence [65]. The p53 consensus is highly degenerate where variation in sequence is tolerated at most positions with the exception of the conserved C and G at positions 4 and 7, respectively [26]. Interestingly, single nucleotide polymorphisms, or SNPs within the promoter RE sequences have been found to include or exclude sequences from the p53 transcriptional network [66].

Each decamer is comprised of two inverted quarter sites arranged in a head-to-head fashion. p53 cooperatively binds to the consensus site as a dimer of dimers, or tetramer protein where the two core DBDs of a dimer bind to consecutive quarter-sites (Figure 1.3)[67-73]. Upon binding to the RE, p53 induces a conformational change in the DNA by bending the RE. The degree of the angle is dependent upon the particular sequence of the RE, where REs with a stronger affinity toward p53 are associated with larger bending angles [71, 74]. Interestingly, various proteins such as HMG have been found to augment p53 binding to REs by inducing bends in the DNA [75].

While tetramerization is not a requirement for sequence-specific binding by the DBD [76], it is required for efficient transactivation from the consensus sequence [77-80]. Arrangement of the quarter sites in a head-to-head orientation is essential for binding which results in transactivation. p53 can bind to a RE sequence comprised of quarter sites arranged in a tail-to-tail fashion. Yet, while the tail-to-tail RE functions similar to head-to-head REs

in competitive binding assays, WT p53 was not able to transactivate from these sites [81]. These findings suggest in addition to sequence, the orientation of binding elements can also influence p53 dependent transactivation.

Previous *in vitro* studies proposed modification of the C-terminal domain (i.e., phosphorylation of Ser392 [35]) was required to alter a “latent” p53 to an “active” p53 in order to disrupt its negative effect on transactivation and stimulate p53 sequence-specific binding [16, 22, 82]. However, recent structural studies revealed the conformation of p53 was similar in both the latent and active states [73, 83]. While the C-terminal of p53 was previously believed to inhibit p53 binding, its non-sequence specific, structure-based DNA interactions, which are highly dependent on the DNA topology, are now postulated to facilitate p53 sliding on DNA as proposed by a linear diffusion based search for REs [76, 84-87].

Additionally, *in vitro* chromatin assembly and *in vivo* ChIP studies show p53 can bind to promoters prior to its induction or modification [59, 73, 88, 89]. Such active binding has favored the concept of a “selective context model” over a “selective binding model” [33]. In the selective context model, expression of a downstream target is reliant not on p53 binding per se, but on gene-specific requirements for transactivation. Thus, individual targets must overcome specific hurdles in the transcription process or “filters” which may require more or less transcriptional coregulators for activity [33]. Such filters may be reliant on whether or not the promoter region is accessible to the general transcriptional machinery. In the case where the promoter is not accessible, p53 binding can increase the promoter accessibility through recruitment of chromatin remodeling factors, histone acetyltransferases and/or methyltransferases [35]. If the promoter is accessible, p53 has been shown to facilitate pre-

initiation complexes by interacting with mediator complexes or recruiting basal transcription factors. In addition, p53 had been demonstrated to play a role in reinitiating paused polymerases [35]. Thus, downstream target genes such as p21 which have a pre-initiation complex, as well as a paused RNAP II at the promoter may require the recruitment of a smaller amount of transcription cofactors to overcome any “filter” than a RE which is lacking RNAP II. Importantly, the availability of the cofactors required to overcome such filters may be cell-type or stimuli-specific.

p53 structure

Although structures for the individual domains of the protein have been solved independently the exact structure of the p53 tetramer and how two dimer proteins interact in terms of protein-protein and protein-DNA interactions remains controversial. The highly disordered segments of the N- and C- termini which flank the structured core DNA binding and tetramerization domains, as well as the intrinsic instability of the protein has impeded the ability to form crystals of a p53 tetramer binding to a full-length p53 consensus sequence [83].

The basic structure of the core DNA binding domain (DBD) was derived from a crystal structure solved by Cho et al., which had three p53 core domain monomers bound to a consensus half-site [90]. The p53 DBD was shown to consist of a β -sandwich which provides a scaffold for two large β -loops (L2 and L3) and a loop-sheet-helix motif which compose the DNA binding surface [90, 91]. The loop-sheet-helix motif binds to the major groove of targeted DNA through several residues, whereas the L3 loop interacts with the minor groove of DNA through Arg248. The L3 loop is structurally supported by the L2 loop;

both the L2 and L3 loops are coordinated by a zinc ion (contacted by residues Cys176, His179, Cys238 and Cys242) which is required for the stabilization of the protein, as well as, the sequence-specific DNA binding [92].

Recently, Kitayner et al., solved the crystal structure of the WT p53 DBD bound to three dodecamer sequences (consensus decamer half-sites separated by a 2 nt spacer)(Figure 1.3) [73]. While the overall structure was similar to that solved by Cho et al., intriguingly, the DNA-protein interface was found to vary with the specific sequence encountered. For example, residue K120 contacts the second purine of the response element sequence; however the nature of the contact is dependent upon the specific base present [73, 93].

The DNA binding domain is linked to the tetramerization domain through ~35 flexible amino acids [68]. The tetramerization domain of p53 is composed of a β -strand (amino acids 326-333) and an α -helix (amino acids 335-354) that form a “V-shape” structure through a hinge region, or tight turn at residue 334. In the simplest terms, two monomers form dimeric proteins through interactions between the α -helices and β -strands, where the two β -strands from opposite monomers form an anti-parallel β -sheet. The dimers then associate through hydrophobic interactions between the anti-parallel α -helices to form tetramers [67, 68, 70, 94-97].

Regardless of the exact nature of the protein binding, tetrameric p53 has displayed a great capacity to bind many RE variants to differentially regulate the activity of downstream targets. However, all binding is not equivalent nor will all binding result in transactivation. The overall goal of this study is to further contribute to the understanding of how p53 functions as a sequence-specific transcription factor by further defining the “rules of p53 transactivation”. Through manipulation of the DNA sequence p53 encounters, it is possible

to address what constitutes a functional response element and how organization of the binding motif influences transactivation. Understanding the interaction of p53 with various RE sequences under conditions which render all other factors (such as chromatin and transcriptional cofactors) essentially neutral may elucidate how different p53-DNA interactions alter the potential of p53-dependent transactivation and possibly dictate the remaining factors required for efficient transactivation. Furthermore, analysis of the impact of p53 mutations upon transactivation will help to elucidate the role of p53 as a master regulator and how aberrations within its network contribute to carcinogenesis [32].

Figure 1.1

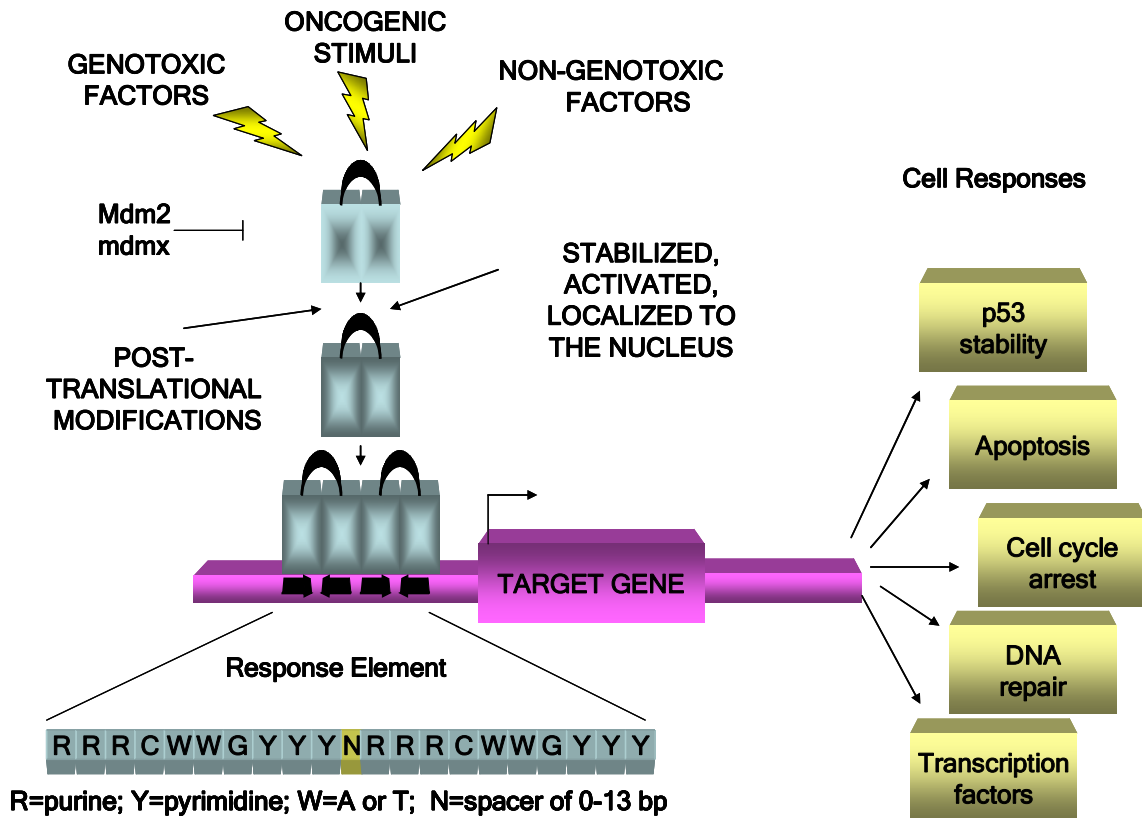


Figure 1.1 The tumor suppressor p53 is a *master regulatory gene*. As a sequence-specific transcription factor, p53 directly controls the regulation of many target genes within its transcriptional network to elicit a variety of biological responses. In response to intra- and extra-cellular stresses (including: DNA damaging agents, oncogenic stimuli, hypoxia, cell adhesion, altered ribosome pools, redox stress), the p53 protein is stabilized, activated and post-translationally modified where it binds as a homotetramer, or dimer of dimers, at a consensus site composed of two palindromic decamers [28-30]. This sequence is degenerate where alteration from the consensus may affect the ability of the p53 DBD to interact with REs thus altering expression of regulated genes.

Figure 1.2

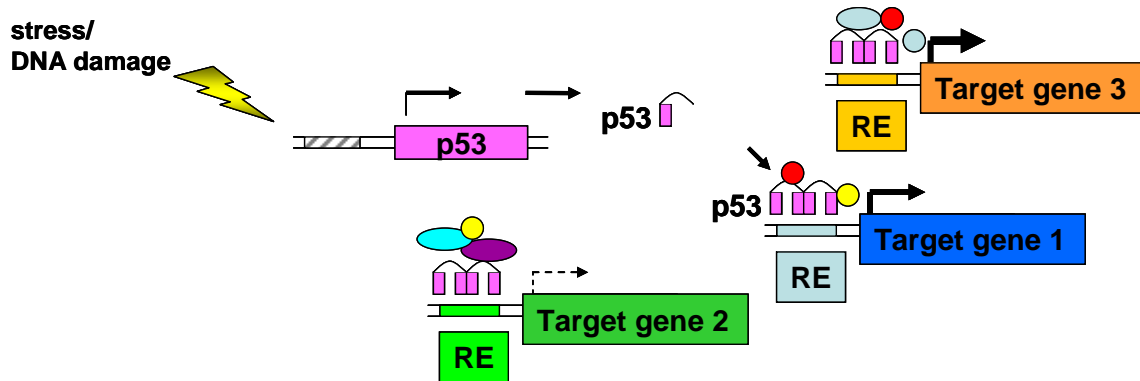


Figure 1.2 Phenotypic diversity within the p53 transcriptional network. As a sequence specific transcription factor, p53 does not interact with each response element in an equivalent fashion [32]. Rather, differential regulation of target genes within the p53 transcriptional network occurs as a result of a variety of factors including cell type, post-translational modifications, co-factor availability, target sequence and stress inducing stimuli. As a result, the activity of some downstream target genes can be strongly activated, whereas others may be weakly activated or attenuated. Such control of gene expression within the network allows for a large spectrum of p53-dependent biological responses.

Figure 1.3

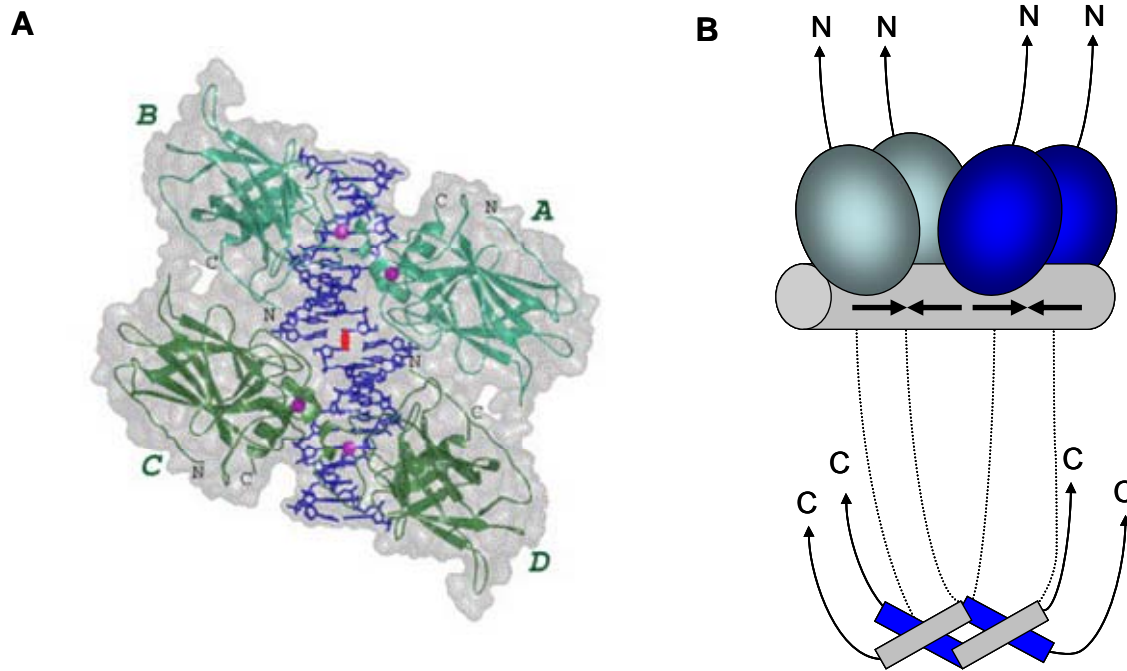


Figure 1.3 p53 binds cooperatively to response element sequences as a dimer of dimers.

(A) Kitayner et al. solved the crystal structures of p53 core DNA binding domains (residues 94-293) bound to three dodecamer sequences (comprised of consensus decamers spaced by 2 nucleotides) [73]. The exact nature of the p53-DNA interactions was dependent upon the RE sequence. Dimers (formed by monomers A-B or C-D) bound to consecutive $\frac{1}{4}$ -sites. This figure has been reproduced from Kitayner et al. [73]. (B) Tidow et al., recently solved a 3D model of the quaternary structure of human p53 bound to a RE using core DNA binding domains containing four stabilizing mutations (M133L/V203A/N239Y/N268D) and a combination of small-angle x-ray scattering (SAXS), nuclear magnetic resonance spectroscopy (NMR) and electron microscopy (EM) [83]. The results were in agreement with the Kitayner et al. crystal structures. Depicted is a cartoon model of the p53 tetramer bound to a RE modified from Shakked 2007 [98]. p53 binds in a cooperative fashion as a dimer of dimers closing upon a RE (grey tube; arrows indicate $\frac{1}{4}$ -site), where the core DNA binding domains (grey and blue ovals) and tetramerization domains (grey and blue rectangles) are on opposite sides of the DNA and linked through an unstructured regions of the protein (dashed line) [98]. The N-terminal domain in the full-length protein protruded, or extended from the core binding domain in a flexible fashion. The C-terminal domains were located in the central region of the protein surrounding the tetramerization domain [83]. This model would be consistent with the ability of p53 ability to interact with other proteins or to be post-translationally modified.

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CHAPTER 2

**ISOGENOMIC *IN VIVO* DIPLOID YEAST SYSTEM TO ANALYZE
TRANSACTIVATION CAPACITY OF THE MASTER REGULATOR p53**

Abstract

As a sequence-specific *master regulatory gene*, p53 controls the differential expression of target genes within its extensive transcriptional network. In response to cellular stress and DNA damage, p53 directly regulates genes involved in apoptosis, cell cycle, angiogenesis and DNA repair from promoter response elements (REs). However, the mechanisms of regulation from the various promoter REs targeted by p53 remain unclear, particularly the relationship between p53 binding to target RE sequence and transactivation. To specifically address the role of RE sequence variation in transactivation by WT and mutant p53 proteins at various levels of p53 expression, we developed an *in vivo* yeast-based reporter system. The chromosomal position for all of the human derived REs was identical and the number of p53 molecules/cell could be varied over a hundred-fold using an integrated *GAL1::p53* construct that is sensitive to levels of galactose in the medium. Evaluation of transactivation capacity was based on colony or enzyme based color reporters. Both reporters exploit a “rheostatable” promoter system for p53 expression and utilize the “*delitto perfetto*” *in vivo* mutagenesis approach for rapid inclusion of target REs upstream of a reporter and the development of mutant p53s. This system expands a plasmid-based haploid yeast system previously developed in the Resnick lab to systematically evaluate the contribution of RE sequence and p53 expression level towards p53 differential transactivation.

Consistent with our previous findings, transactivation by WT p53 differs between REs where small differences in the 20 to 30 bp target RE sequence can contribute significantly to levels of transactivation. Regardless of “strength of binding” or biological role, the initial induction of transactivation occurs at comparable levels of p53 expression

for all REs except from a novel sequence Con-A, described in chapter 5, which displays high levels of transactivation at low levels of p53 expression. These results challenge current notions that the ability of p53 to transactivate from various REs is simply due to differences in RE binding affinities. Our results suggest that differences in binding affinity for the various REs and on-rates may not be the principal driver of p53 transactivation specificity. Therefore, the role of sequence in p53 transactivation must be addressed using *in vivo* systems, not simply with *in vitro* binding measurements.

Introduction

Yeast as a model system to study p53

With its ease of genetic manipulation and cost effective measures as a research tool, the budding yeast *Saccharomyces cerevisiae* has become a prominent model system to study various human diseases [1]. Fundamental aspects of cancer including, but not limited to DNA replication [2], cell cycle checkpoints [3], nuclear trafficking [4] and mechanisms of drug resistance [5] have been addressed in this smaller eukaryotic *in vivo* test tube, sometimes referred to as an “honorary mammal” due to its conservation of genes, signaling and cellular pathways [6, 7].

Prior to the identification of the p53 consensus sequence, Schärer and Iggo capitalized on the finding that a p53::*GAL4* fusion protein could function as a DNA binding transactivator in mammalian cells [8] to develop a transcription assay for human p53 in yeast [9]. The assay utilized a gap repair technique which allowed a linear p53 cDNA fragment (WT or mutant p53) to fill a gapped plasmid containing flanking regions of the p53 gene by homologous recombination. This resulted in the generation of a p53 expression plasmid where p53 was under the control of the inducible *GAL1* promoter (repressed under glucose; activated in 2% galactose). Transactivation was assessed by β -galactosidase activity on X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) plates where β -gal activity was dependent on the ability of the heterologous p53, which is not found in yeast, to drive the expression of a lacZ reporter. The lacZ reporter was under the control of the minimal iso-1-cytochrome C (*CYCI*) promoter which had its upstream activating sequence (UAS) replaced by a 33 base pair sequence that p53 was previously shown to bind [10]. Binding of p53 to the DNA sequence that resulted in lacZ expression

stimulated production of the β -galactosidase enzyme which in turn hydrolyzed X-gal into galactose and 4-chloro-3-brom-indigo.

Two important findings arose from this study. First, human p53 (and murine p53) could utilize the conserved transcriptional machinery in yeast, where transactivation of a reporter was found to be dependent on the orientation and number of copies of the binding site used. Second, yeast could be used as a tool to assess the function of p53 and discriminate between alleles that could support transactivation from those that were deficient, or temperature-sensitive for transactivation. In addition, this study also demonstrated immuno-reactivity methodologies, which screened for p53 mutations based on the ability of the protein to bind conformation sensitive p53 antibodies, could not predict allele functionality. Schärer and Iggo showed some p53 mutants that were indistinguishable from WT p53 in terms of antibody binding were transcriptionally inactive. In contrast, mutants, such as E285K which preferentially recognized epitopes associated with altered protein conformation (i.e., PAb240) were partially active for transactivation [9].

Analysis of tumor associated p53 mutations in yeast

With the discovery that p53 could utilize the conserved transcriptional machinery in yeast, efforts were expanded in this model system to study additional aspects of p53's role as a transcription factor including the following: classification of cancer-associated alleles, second-site suppressors, temperature sensitivity, dominant-negative and gain-of-function activity, chemotherapeutic or small molecule reactivity, and structure-function analysis [11-14]. These studies provide isogenic conditions that are not often obtainable in

mammalian systems, to determine p53 functional status from specific binding elements through various reporters which were either integrated into the genome or plasmid-based.

The FASAY assay or functional analysis of separated alleles in yeast provides a means to screen tumor samples for p53 mutations that alter the transcriptional activity in comparison to WT p53 [15]. The first FASAY assay was originally developed as a colony growth assay [15]. RT-PCR products converted from potential carrier mRNA were used to fill a gapped plasmid resulting in placement of the p53 cDNA under the constitutively expressed *ADHI* promoter; the plasmid could be selected for with the *LEU2* marker. [Note: p53 expression levels were moderate from the *ADHI* levels in comparison to *GALI* and did not induce growth suppression as observed with expression from *GALI* [16, 17]]. Use of a centromeric yeast plasmid ensured the colonies contained plasmids derived from one replication event such that a single allele of the p53 gene was analyzed for functionality. Transforming the p53 expression vector along with a reporter plasmid containing the *HIS3* gene expressed under a p53-responsive promoter (the ribosomal gene cluster, RGC RE upstream of the *CYCI* minimal promoter) and selectable marker (*TRP1*) allowed for the functionality of p53 alleles to be determined through histidine prototrophy. Thus, only p53 alleles which were capable of transactivating from the target binding element provided proficient growth on His- plates, where inactive mutant alleles would not proliferate.

Flaman et al., further modified the yeast functional assay to screen for the presence of somatic or germline p53 mutations in tumor samples, blood samples, and cell lines [18]. Similar to Ishioka et al. [15], the approach utilized unpurified RT-PCR products from tumor or cell line p53 mRNA samples in a gap repair assay to construct a p53 expression plasmid. However, the functional status of p53 was assessed with a phenotypic assay

based on colony pigmentation. Placement of 3 copies of the RGC p53 RE upstream of the *CYCI* promoter resulted in transcription of *ADE2* driven by the interaction of p53 with the REs. Transcription of the *ADE2* gene [encoding phosphoribosylamino-imidazole-carboxylase [19]] resulted in large, white colonies. Whereas, mutations in p53 that abrogated the protein's ability to transactivate from the RGC REs rendered the *ADE2* gene inactive. This resulted in small, red colonies on plates with low, growth-limiting levels of adenine [200 ug/mL] due to the disruption in adenine biosynthesis (Figure 2.3) [19]. Interestingly, the color assay could also identify partial function or temperature-sensitive mutations which generated pink colonies, for example the mutants V272L and H214R.

Results from these yeast screens on a p53 mutant's functional status were consistent with those obtained from similar assays in mammalian cells [20]. Yet, the yeast system provided the opportunity to screen a larger number of p53 alleles for function within a simpler model. Several limitations in the system occur in that mutations in the p53 promoter are not detected nor are mutations which cause alternative splicing or those that occur in the 3' or 5' untranslated regions [12, 13]. Furthermore, these screens relied on the transcriptional activity from a single RE assuming functionality of p53 was an all-or-nothing event where the ability of p53 to bind DNA in a sequence-specific fashion equated to transactivation, a key issue addressed in the present study.

Differential transactivation from REs by WT and mutant p53

Several yeast studies expanded the FASAY assays to analyze transactivation from multiple REs factoring in the possibility that a mutant p53's transactivation potential may be influenced by the RE encountered. These studies postulated that a fraction of p53

mutations may retain some function as a transcription factor towards a subset of REs and/or under particular conditions.

Di Como and Prives analyzed WT p53 and 20 p53 mutants, including hotspot and non-hotspot residues, for transactivation activity towards 9 REs (p21, mdm2, GADD45, Cyclin G, Bax, IGF-BP3 Box A and Box B, RGC and a consensus sequence SCS [which we refer to as Con-A]) based on the HIS prototrophy growth assay [16]. Interestingly, transactivation activity for WT p53 (expressed from the ADH1 promoter) was dependent on both temperature and the RE, where p53 could transactivate from p21, SCS/Con-A, MDM2, GADD45 and cyclin G to induce normal growth rates, from RGC and Bax to induce reduced growth rates, but could not transactivate from the IGF-BP3. Assessment of mutant transactivation revealed that some mutant p53s could transactivate target genes in a manner that was dependent on the RE and temperature. Hotspot missense mutations lacked transactivation capability, whereas among non-hotspot mutations there was loss-of-function or retained function. Importantly, this study showed several cases where transactivation was not equivalent to *in vitro* binding. For example, two mutations (V143A and M160I/A161T) could transactivate from p21 *in vivo*, but in EMSA assays could not bind the RE in the presence of the RE alone or in the presence of several p53 antibodies which were thought to modify p53 to enhance its ability to bind target sequences. In addition, C277Y was found to bind, but not transactivate from the SCS or RGC REs. These observations were unexpected since the prevailing view was that strength of transactivation would reflect the strength of p53 affinity *in vitro* to a specific DNA sequence.

The hypothesis that partial function mutations would be able to transactivate from REs with a high affinity for p53 but not a low affinity was drawn from the observation that

the R175P mutation could activate p21 to induce G1 cell cycle arrest, but not Bax associated apoptosis *in vivo* mammalian cells [21, 22]. Flaman et al., explored this finding by analyzing the ability of 51 p53 missense mutations to transactivate from p21, bax or 4 consecutive bax REs and stimulate the *ADE2* reporter in yeast [23]. Thirty-nine of the mutations were loss-of-function mutations that were unable to transactivate from any of the REs examined (i.e., V272L, R249S and C277Y). However, similar to the phenotype determined for R175P, 8/51 mutations were able to transactivate from the p21 RE, but not Bax to produce white pigmented colonies with the *ADE2* reporter (i.e., K120R, R181H and R283H). In addition, 4 mutations were able to transactivate from p21 and multiple copies of the Bax RE, but not a single copy of the Bax RE (i.e., R175C, R175L and R181L).

To determine if the loss of or change in transactivation activity was explained by loss of binding to the REs, Flaman et al. performed bandshift analysis on WT p53 and several of the contact (C277Y and R283H) and conformation (R181L and V272L) mutant proteins [23]. Interestingly, R181L and R283H bound to the p21 probe to levels comparable to WT p53, whereas V272L and C277Y displayed reduced levels of binding. Examination of binding to the Bax probe revealed that all the mutations had reduced levels of binding in comparison to WT p53 with the exception of C277Y which had lost its ability to bind the Bax sequence *in vitro*. While the authors reasoned the loss of transactivation by the mutations from the Bax RE, but not p21 RE was caused by a lower affinity for the DNA sequence, the finding that binding was not completely lost, but merely abated by the mutant proteins suggests that binding alone may not be sufficient for transactivation by p53.

Campomenosi et al. continued to analyze mutations for selective or discriminant transactivation capacities from the p21, Bax and PIG3 REs with the *ADE2* assay [24]. A screen of 77 mutations revealed 12 p53 missense mutations that could transactivate from the p21 RE, but not Bax or PIG3. Four additional mutations were found to be able to transactivate from the p21 RE comparable to WT p53, but transactivated from either the Bax or PIG3 REs to reduced levels. Unexpectedly, this screen found mutations outside of the DNA binding domain resulted in discrimination towards target REs (S99Y, S99F) or cause loss-of-transactivation function (R337G and Q354E) suggesting residues in the transactivation domain or tetramerization domain also influenced the p53 tertiary configuration necessary for adequate interactions with DNA and/or other proteins. Finally, four BRCA associated p53 missense mutations (T150I, G199R, R202S and S215C) were found to be indistinguishable from WT p53 in their transactivation capacity.

Rheostatable promoter for controlled, inducible expression

While yeast systems could discriminate between alleles that were active or inactive for function, the overexpression of p53 did not allow for a discrepancy between silent or subtle, altered-function mutations as in the case of the BRCA-associated p53 mutations. In such studies, p53 was often overexpressed from either constitutively active (i.e. *ADHI*), or inducible promoters (i.e., *GALI*) which may not always recapitulate physiologically relevant conditions (Figure 2.1)[25]. The Resnick lab has contributed to the study of sequence-specific transcription factors from specific target sequences by developing an isogenic plasmid-based haploid yeast system which utilizes a *rheostatable* rather than highly expressing promoter [26]. The rheostatable promoter allows for the functional

assessment of a sequence-specific transcription factor at various protein concentrations from a single copy of a target RE upstream of a reporter (Figure 2.2) [26-28] providing the opportunity to address the responsiveness of REs to wild type and mutant p53 proteins. This can be viewed as an *in vivo* opportunity to better address biochemical properties of the two factors.

In the case of the p53 tumor suppressor, the p53 coding sequence is placed under the control of the *GALI* promoter, which provides for inducible control of p53 expression through variation in the amount of galactose in the medium. Transcription of the p53 cDNA is dependent on activation of the *GALI* promoter by *GAL4* [29, 30]. Gal4 binds to an upstream activation sequence (UAS) of *GALI*. In the absence of galactose, Gal80 interacts with Gal4 inhibiting its ability to function as an activator although it may bind the UAS. Addition of galactose leads to Gal3 inhibition of the Gal80/Gal4 interaction, allowing Gal4 to stimulate the activation of its target genes through association with the SAGA(Spt/Ada/Gcn5/acetyltransferase) complex and subsequent recruitment of the TBP (TATA-binding protein) and RNA polymerase II. Glucose prevents *GALI* expression through a catabolite repression mechanism [25]

Importantly, it should be noted that complete repression of the *GALI* promoter through *GAL4* is not entirely achievable. Evidence of “leakiness” from the *GALI* promoter becomes apparent when attempted inducible promoter fusions between *GALI* and a particular gene of interest is toxic to the yeast cells even under “repressed”, or non-inducing conditions. For example, attempts to fuse *GALI* with the bacterial DNA endonuclease genes, *PvuII* or *EcoRV* result in cell death even when glucose is supplemented within the media. The toxicity in these examples is postulated to be due to

the inefficient repair of the blunt end, double strand breaks each enzyme creates. Lewis et al., demonstrated the basal levels of expression can be attenuated without compromising the ability to strongly induce the *GALI* promoter and gene of interest when particular mutations are introduced within the UAS that Gal4 binds [31].

The rheostatable, plasmid-based haploid yeast system was utilized to systematically evaluate the contribution of RE sequence and p53 expression level towards p53 differential transactivation. In addition, it was employed to address the consequences of mutations and SNPs on the ability of p53 to function as a transcription factor [26, 27]. Twenty-six REs (22 biological targets and 4 consensus sequences) were ranked for transactivation capacity based on the *ADE2* phenotypic color reporter assay (Figure 2.3). The assay was subsequently modified to place *ADE2* under the control of the minimal *CYCI* promoter and RE of choice at its natural loci on chromosome XV.

Transcriptional capacities of the REs were determined based on the minimal relative amount of p53 required for a phenotypic change in pigmentation which ranged from red to pink to white depending on the extent of transactivation. This assessment revealed, under isogenic conditions that both the sequence and level of p53 protein contributed to transactivation, where greater than a 1,000 fold difference at various protein concentrations was observed between REs. The REs which were classified by transactivation capacity as strong binders (i.e., p21-5' and P53R2), modest inducers (i.e., NOXA and PA26) or poor transactivators (i.e., CFOS and PIG3) did not group according to biological functions. Furthermore, a correlation between functional rank and statistical prediction of binding energy of the REs was not observed. Importantly, the central core sequence in each decamer was determined to greatly affect the ability of WT p53 to

transactivate from a RE. These observations suggested that an intrinsic property of the core sequence “CATG” was required for strong levels of transactivation. (Binding studies agreed with this finding suggesting the flexibility of the CATG sequence increases the affinity of p53 towards an element [32].) Altering the CATG in the core to CTAG dramatically reduced levels of transactivation by 20-fold. Finally, the rheostatable promoter was used to *unmask* subtle transactivational differences between WT p53 and several p53 missense mutations associated with breast cancer which had previously been determined to be equivalent to WT in transactivation capacity under conditions of high expression (including the T150I, G199R, R202S and S215C mutations discussed above)[24]. Subsequent studies on p53 transactivation capacity utilizing the rheostatable promoter revealed that variation in the p53 transcriptional network result from a matrix of factors including p53 expression level, target binding sequence and mutations. These findings culminated in the view that p53 is a “*master regulatory gene of genetic diversity*” [27].

In order to further expand the understanding of p53’s role as a transcription factor and to analyze how sequence and protein alterations contribute to the variability observed within the p53 transcriptional network, we have modified the rheostatable system to develop an integrated diploid yeast system. This system allows for a more sophisticated and sensitive evaluation of the contribution of RE sequence, binding motifs and p53 expression on functionality and regulation within the p53 transcriptional network. Furthermore, the system allows for a rapid assessment of the consequences of mutations towards many REs in the p53 transcriptional network at various levels of expression.

Results

Isogenomic system to address p53 transactivation from REs in diploid yeast

While many factors may determine the ability of p53 to differentially transactivate individual genes in human cells including stress stimuli, post-translational modifications, and transcriptional co-factors, the yeast system addresses the potential for wild type and mutant p53 to bind and transactivate from various response elements derived from human genes when placed in a constant chromatin environment. We have expanded the rheostatable plasmid-based haploid yeast system to one based in diploid yeast to further assess the transactivation capacities of p53 (WT or mutant) (Figure 2.4). This system allows a single copy of a p53 variant to be rapidly assessed for transactivation capabilities from many REs simply by taking advantage of the yeast mating types.

Two panels of modified *S. cerevisiae* strains were generated. The first was a set of p53 host strains in which p53 (WT or mutant) is directed by a “rheostatable” *GALI* promoter that allows for controlled, over 200-fold, inducible expression of p53 in yeast depending on the carbon source in the media (Figure 2.6). Importantly, similar to p53 protein accumulation in mammalian cells following stress [33], expression from the *GALI* promoter in yeast displays a graded transcriptional response such that there is a range of activity from the promoter as opposed to a binary, or on/off response [34-36]. Biggar and Crabtree [34] demonstrated through fluorescence-activated cell sorting (FACS) experiments that expression of green fluorescent protein from a *GALI*-GFP reporter within a population of cells generated a single fluorescent peak where the intensity of the peak was dependent upon the concentration of galactose supplemented in the media. Thus, increases in galactose will result in a homogeneous response where the vast majority of

cells in the population respond (and within our system expressing an induced amount of p53) rather than merely increasing the percentage of cells within the population expressing the maximal level of protein.

The second set of strains of opposite mating type contained promoter REs upstream of the minimal *CYC1* promoter and either the *ADE2* color reporter or the firefly luciferase reporter [26]. To facilitate the construction of a large number of p53 mutants and REs at chromosomally located target loci we employed the *delitto perfetto* system for *in vivo* mutagenesis. *Delitto perfetto* utilizes oligonucleotides and targeted homologous recombination to rapidly generate *S. cerevisiae* yeast strains with specific genetic alterations [37-39].

Mating of the reporter and p53 host strains results in isogenic, diploid yeast that enable the rapid assessment of the transactivation potential for WT or mutant p53 proteins towards many individual REs in the p53 transcriptional network [27]. Importantly, all the conditions in the cells are constant, i.e., isogenic, where the only variables between strains are the RE sequence, WT or mutant p53 and level of expression. The rheostatable *GALI* promoter allows for controlled, over 100-fold, inducible expression of p53 in the diploid yeast (Figure 2.6 A and B). A rough estimate of the number of p53 molecules in each cell ranges from ~250 -500 at basal levels of expression to over 30,000 at 0.024% galactose (Figure 2.6 C).

The *ADE2* reporter provided a qualitative assay to determine transactivation potential through the appearance of pigmentation in the stationary cells of colonies over several days (Figure 2.3). It was used to differentiate WT p53 transactivation potential

from REs, as well as to assess mutant p53 transactivation potential in comparison to WT p53 and other mutants at variable levels of expression (Figure 2.7).

The luciferase reporter provided a quantitative estimate of transactivation capacity of WT p53 from the various REs in cultures of logarithmically growing cells (Figure 2.3). An example of quantitative assessment of transactivation capacity of p53 over a range of expression is shown for p53-induced transactivation from the p21-5' RE *in vivo* (Figure 2.8). Functional assessment with the luciferase reporter assay provided an indication of the *kinetics* of transactivation with a basal level of transactivation at 0.00% galactose (2% raffinose), initial induction of transactivation (between 0.004 – 0.008% galactose) and maximal level of transactivation (between 0.016% - 0.024% galactose). The p53 protein appeared stable suggesting variation in measured maximal levels of transactivation between RE was due to intrinsic properties of the RE and not a decay in protein (Figure 2.9).

Transcriptional assessment of toxic mutants to validate the diploid system

Previous findings have shown that high expression of p53 in *Saccharomyces cerevisiae* and *Schizosacchomyces pombe* can result in growth suppression [16, 17, 40]. Within haploid budding yeast, the induced expression of WT p53 from the *GAL1* promoter at 2% galactose resulted in decreased colony size in comparison to cells lacking p53 expression [17]. p53 mutants (V143A or R273H) also resulted in a growth inhibitory phenotype when expressed at high levels, but to a reduced degree (dependent upon the specific mutation) in comparison to WT p53. Highly induced levels of WT p53 increased doubling time from 4.4 to 11.6 hours, whereas no variation was observed under non-induced (2% raffinose) or repressed (2% glucose) conditions. While p53 and its family members did not evolve until

the existence of multicellular organisms it was predicted that p53 could interact with the evolutionarily conserved protein complexes involved in cell cycle progression to produce this phenotype [17]. Analysis of p53 phosphorylation status by labeling the cells with ^{32}P followed by immunoprecipitation revealed p53 was phosphorylated in yeast [17]. However, mutation analysis of the phosphorylated sites showed that differential phosphorylation (by endogenous or exogenous cell cycle dependent kinases) did not play a direct role in the growth suppressive phenotype [17].

These findings led to a screen for mutations that would exacerbate the growth suppressive phenotype at moderate to low levels of p53 expression [41]. Interestingly, several mutations, including V122A, C277R and G279R, exhibited novel phenotypes. They were toxic at modest levels of expression. However, at lower levels of expression there was enhanced activity, termed super-transactivation, towards some REs, but a reduction or loss of transactivation activity towards, resulting in an overall altered spectrum of p53 transactivation. None of the mutants were loss-of-function.

In order to validate the integrated diploid system, three toxic mutations (V122A, C277R and C279R) and one super-transactivating mutation (S121F) (described below) were assessed for transactivation capacity towards 6 REs (p21-5', P53R2, GADD45, 14-3-3 σ , CYCLIN G, AIP1) at various p53 expression levels with the *ADE2* color assay. Overall, as shown in Table 2.3, there was general agreement between the haploid and diploid yeast systems in the functional assessments. Similar to previous results in the haploid system, within the diploid system all the mutations displayed super-transactivation from the p21-5' RE which was defined as an enhanced activity from the RE in comparison to WT p53 for at least one level of expression analyzed. [It should be noted that the diploid

system required a slightly greater amount of p53 induction to generate a response. For example, WT p53 *yAFM-p21-5'* colonies were nearly white on glucose plates in the haploid system [26], however white pigmentation was not completely acquired in the diploid cells until ~0.004 – 0.008% galactose.] While the S121F mutation consistently exhibited an enhanced activity from all the REs in comparison to WT p53, the toxic mutations exhibited an altered spectrum of regulation towards the remaining REs. For example, the C279L had lost its ability to transactivate from the cyclin G and AIP1 response elements, but had a reduced ability to transactivate from the REs of P53R2 and 14-3-3 σ . Two transactivation discrepancies were found between the systems for the V122A transactivation from 14-3-3 σ and C277R transactivation from AIP1. The V122A was considered equivalent to WT p53 in its transactivation capacity from 14-3-3 σ in the diploid system, whereas it showed a slight reduction in relative transactivation within the haploid system. With the C277R transactivation from AIP1, the haploid system showed a loss in transactivation capacity whereas the diploid system scored the C277R as reduced transactivation capacity with colonies remaining pink even at maximum p53 expression. Finally, a growth inhibition phenotype was observed with all the toxic mutations at approximately 0.032 – 0.064% galactose which was consistent with the haploid results.

Transactivation capacity of WT p53 from biological REs

The quantitative luciferase assay was used to evaluate the strength and kinetics of transactivation for WT p53 from 23 biologically relevant, canonical REs and 3 consensus REs at increasing levels of induction (0 - 0.032% galactose) (Figure 2.10). The consistency between biological and experimental repeats in the luciferase assay allowed for

comparisons to be drawn between the REs using WT p53 transactivation from the p21-5' RE as a common reference. Relative transactivation capacities for the various REs were ranked according to the average strength of transactivation (calculated as the relative light units/ug protein) at maximal induction levels (0.020-0.032% galactose) (Table 2.2). Maximal levels of transactivation ranged from strong (98 -115%), moderate (50 – 77%), weak (20 – 42%), extremely weak (5-16%) to nonfunctional (below 5%).

Consistent with functional assessments in the haploid yeast system, p53 displayed high levels of activity from the Con-A, p21-5' and P53R2 REs. Synergistic transactivation from Bax A + B and MDM2 P2, (which were comprised of two adjacent weak REs), equated to or exceeded the levels of transactivation from the strong REs, respectively. Several REs (p48, zac1 and msh2) from which p53 was not able to alter the colony pigmentation at 2% galactose when assessed with the ADE2 color assay in diploid yeast were excluded from analysis with the luciferase assay. No significant difference in transactivation by WT p53 was observed between the well-known Arg or Pro polymorphisms at codon 72 with the color assay in diploid cells (data not shown), thus transactivation as measured with the luciferase reporter was only assessed for the Pro allele.

Discussion

Isogenic system to address p53 transactivation from many REs in diploid yeast

Yeast has been extensively used as an *in vivo* test tube to assess the potential role that target sequence plays in p53 transactivation. Such yeast-based assays provide the opportunity to analyze p53 [25, 27] transactivation capacities towards p53 response

elements (REs) derived from human genes. A distinct advantage of yeast is the opportunity to rapidly modify *in vivo* either the target REs or to create mutant p53 coding sequences utilizing a highly efficient recombination-based system, known as *delitto-perfetto* [Italian for “perfect murder”] that targets desired changes with oligonucleotides [37-39]. The newly developed diploid yeast approach extends previous work in the Resnick lab with a plasmid-based haploid yeast system in order to capitalize upon the opportunity to conveniently merge a large number of integrated (single copy) p53 mutants with assessment of transactivation capabilities at many response elements simply through mating of strains. Not only can the rheostatable, diploid yeast system differentiate between functional and nonfunctional REs, it can also estimate weak to strong functionality at different levels of p53 expression.

In addition, the rheostatable promoter provides the ability to address subtle mutant defects at low levels of p53 protein. We examined four p53 mutants associated with a toxic or super-transactivating phenotype in order to compare the integrated diploid yeast system with the haploid plasmid based system. Consistent with previous data, the four mutations showed altered-function towards the six REs examined, confirming the diploid yeast system can be used to differentiate transactivation potentials of altered function missense mutations. The results in yeast have proven useful in guiding studies with highly expressed WT and mutant p53 towards potential target REs in human cells and evaluations of direct DNA binding [42].

Small changes in sequence can lead to dramatic differences in transactivation

Transactivation capacities of WT p53 towards a subset of biological REs were assessed in the diploid yeast system, where sequence specific differences in transactivation can be resolved with the luciferase assay. Consistent with previous findings in the haploid plasmid-based system [26, 27], transactivation by WT p53 differed considerably between the 26 REs. Small changes in sequence were shown to dramatically affect functionality in terms of maximal levels of transactivation from a given binding element (Figure 2.10). Variation in level of p53 expression amplified these differences. Although there is considerable difference in the transactivation ability towards the various REs, level of p53 at which transcription is first detected is comparable for all REs (0.008% -0.012%) regardless of the biological role of the RE-associated genes.

p53 transactivation does not appear to follow a simple binding model

Differential modulation of transactivation has been postulated to arise due to the differences in p53 binding affinity towards various DNA sequences. To compare the *in vivo* transactivation (which is an endpoint of binding) with reported *in vitro* binding measurements, an empirical value of 0-5 derived from the maximal transactivation levels obtained with the luciferase assay, was assigned to each RE (Table 2.2). These values which represent relative strength of transactivation were compared with published binding affinities of a tetrameric p53 construct (residues 94-360 of the DNA binding domain and oligomerization domain) towards various REs [32] (Figure 2.12). The binding affinities were derived by fluorescence anisotropy which measures p53 binding to recognition elements based on the polarization of emitted light from a fluorescently labeled oligo

bound by p53 that is rotating in solution in comparison to a free, unbound oligo. The K_d value represents the concentration of p53 dimerized protein required for 50% of the DNA oligos to be bound. (Dimer concentrations were used in the study because under the experimental conditions, tetramerization of p53 did not occur until binding. Binding of the dimers to REs was reported to be co-operative with the Hill coefficients measured between 1.7 – 1.8 for all REs, where total co-operativity displays a Hill constant of 2.0 [32].) Importantly, our system based of transactivation assays reflects variation in binding with increasing p53 expression, whereas, the *in vitro* measurements determine binding with increased p53 expression. A simple binding model would suggest that REs that yield a larger K_d should require more p53 expression to initially induce transactivation. As protein concentrations increase, the values for weak binders would asymptotically reach those for strong binders. The transactivation patterns obtained from the luciferase assays (Figures 2.10 and 2.11), which show the amount of p53 required to initially induce transactivation was comparable for all REs, regardless of the K_d value, suggests that functional transactivation by p53 at REs is not simply explained by *in vitro* binding properties. The *in vivo* and *in vitro* properties differ further in that transactivation from weak REs do not reach the levels found for REs transactivated as levels of p53 increase.

Importantly, as demonstrated in Figure 2.11, we found that while the reported *in vitro* binding abilities of some REs were comparable, large differences in *in vivo* transactivation capacity could be observed. For example, cyclin G and GADD45 have binding constants measured at 7.7 ± 1.6 and 7.7 ± 1.2 , respectively, yet p53 transactivates moderately from cyclin G but weakly from GADD45. Additionally, the range of the dissociation constants overlap for three strong binders, p21-5', P53R2, and PCNA (4.9 ± 0.6 nM, 5.7 ± 0.8 nM

and 6.6 ± 1.4 nM, respectively). If transactivation simply reflects *in vitro* binding, the maximal transactivation obtained should be comparable for these REs with similar K_d s. P21 and P53R2 bind at similar rates *in vitro* and have similar “kinetics” of transactivation *in vivo* in response to increased p53. However, while PCNA has comparable binding *in vitro* to p21-5' and P53R2, it transactivates to a very different level *in vivo* (Figure 2.10B). This difference may be due to the lack of the flexible CATG at the junction of quarter sites in the RE sequence of PCNA. Conversely, several REs were shown to have similar levels of transactivation, but were significantly different in binding affinities. Figure 2.11 shows p53 transactivates from PCNA and p53AIP1 to similar weak levels where the binding constants for each are 6.5 and 11 nM, respectively.

From comparisons between *in vitro* binding and *in vivo* transactivation, levels of transactivation are not predictable without direct assessment of function *in vivo* under conditions where p53 levels of expression can be varied. This implies that factors other than just sequence and sequence organization can have a large influence on transactivation by p53 in a manner that is distinguishable from simple DNA binding, even under identical/isogenic conditions in the cell. A *semi-in vitro* system has recently been developed (Nourreddin et al, submitted; described in Chapter 3) that provides opportunities to address the additional factors influencing *in vitro* binding. This system has already been employed to address impact of spacers in REs (see Chapter 3). While not part of the present study, the yeast based system will provide the opportunity to address the mechanisms by which varying just the sequence can have such a strong effect on transactivation.

Conclusions

The sequence of target REs, level of expression and alterations in protein can greatly impact the p53 master regulatory network in a way that is not predicted by simple DNA binding. The integrated diploid yeast system has been developed as a tool to address a matrix of factors which contribute towards the variability and different levels of regulation within the p53 transcriptional network under isogenomic conditions. Key issues that have been addressed with this system include: (i) what constitutes a functional RE for transactivation, (ii) how do changes in the RE sequence affect transactivation, (iii) the importance of p53 level on transactivation and (iv) the relationship between *in vitro* binding and *in vivo* transactivation.

Furthermore, this system can also be used to characterize p53 functional mutations associated with disease to determine the impact on spectrum and intensity of transactivation and to address mechanisms. Using the yeast-based system, p53 mutations were classified into groups according to their retained transactivation capacities at various levels of p53 expression. In contrast to *in vitro* biochemical assays, the yeast assay has the advantage of comparing p53 variants in a constant chromatin environment--under identical, isogenomic conditions--for activity towards individual REs. Functional analysis of p53 missense mutations associated with disease may have diagnostic value and may also be used as a predictor of tumor behavior in response to chemotherapeutic agents. Previous results obtained with yeast functional assays appear predictive of whether a mutation will also have a biological impact in mammalian cells [25, 42]. Although the yeast-based functional assay is a powerful screening tool, assays in mammalian cells are needed to fully characterize the impact of p53 mutation. Additional promoter elements and transcriptional

cofactors in mammalian cells, beyond just RE sequence, are expected to influence p53 mediated transactivation. Importantly, the present results describe the potential role that RE sequence may play in the p53 master regulatory network with normal and mutant p53 under conditions where the level of p53 can be varied.

Figure 2.1

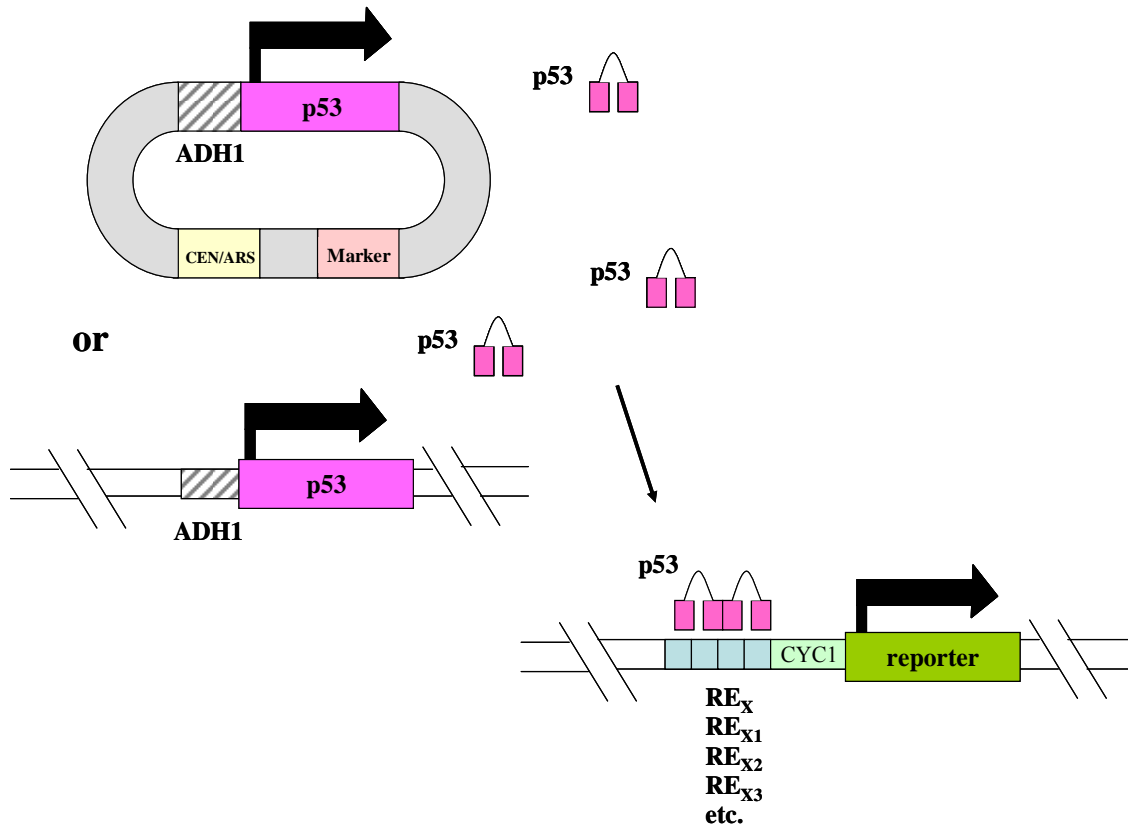


Figure 2.1 Yeast as an *in vivo* test tube to determine mammalian p53 transactivation capacity. Traditional yeast assays have determined p53 functional status under conditions where p53 cDNA is constitutively expressed at moderate to high levels from a selectable, low copy number expression vector or integrated promoter, such as ADH1. The constitutively expressed p53 can interact with individual RE sequences (derive from p53 regulated human genes) that have replaced the upstream activating sequences of a minimal promoter (ie, cytochrome C) that regulates the expression of a reporter (ie., HIS3, URA3, ADE2, beta-galactosidase, green fluorescent protein (GFP), firefly luciferase gene). Transactivation of the reporter gene is dependent on p53 and is a direct readout of p53 interaction with the specific RE sequence. Multiple yeast strains containing different RE sequences can be used to assess how specific deviations from the degenerate consensus sequence affect p53 transactivation, as well as provide functional status in terms of the transactivation capacity of mutant p53. (Reproduced from Menendez et al., 2007 *Oncogene* 26:2191-2201) [25].

Figure 2.2

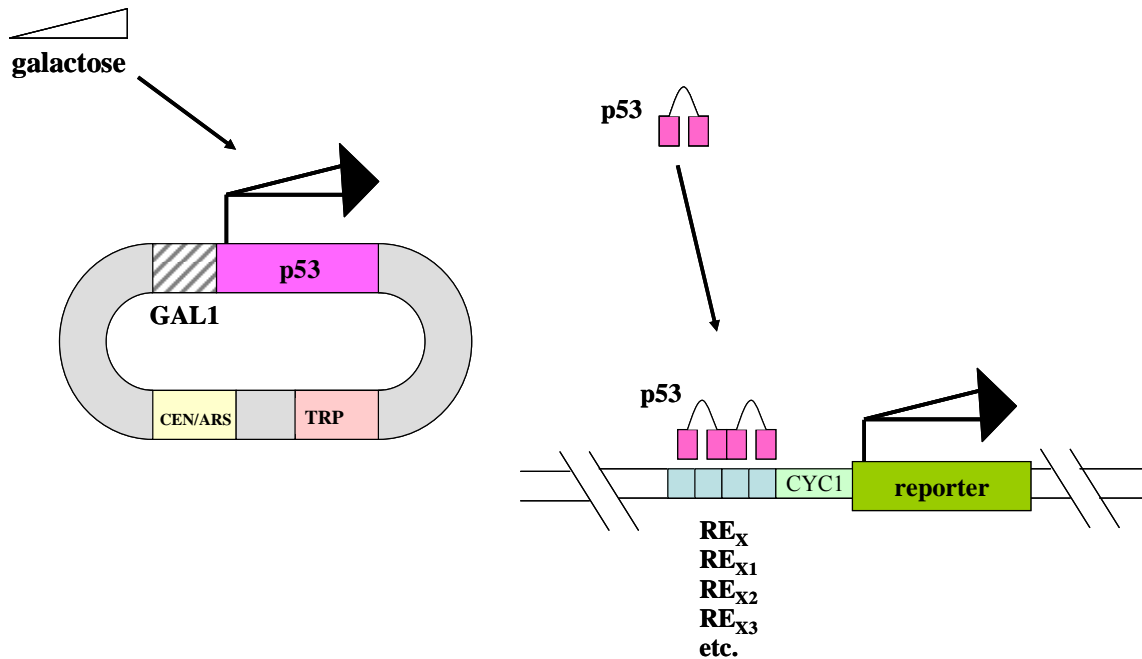


Figure 2.2 Rheostatable promoter to address p53 transactivation potentials.

The rheostatable promoter allows for the highly controlled regulation of p53 protein expression under the GAL1, 10 promoter. Under conditions where glucose is contained in the media, the GAL1, 10 promoter is repressed. Raffinose provides a basal level of expression, where the GAL1, 10 promoter is de-repressed but not induced. Increases in levels of galactose in the media as the carbon source correlates with increasing p53 expression that can vary over a 100-fold range. The controlled expression of p53 with the rheostatable promoter allows for analysis of transactivation by p53 from individual REs at various expression levels. This system may allow for a further understanding of how p53 functions as a transcription factor from specific RE sequences, especially at low levels of expression. In addition, the sensitivity of the system which provides for the expression of p53 at various low levels of expression can be used to unmask subtle phenotypic differences in transactivation capacities of mutant p53 proteins that remain undetected in traditional functional assays that utilize transient over-expression of p53. (Reproduced from Menendez et al., 2007 *Oncogene* **26**:2191-2201) [25].

Figure 2.3 A

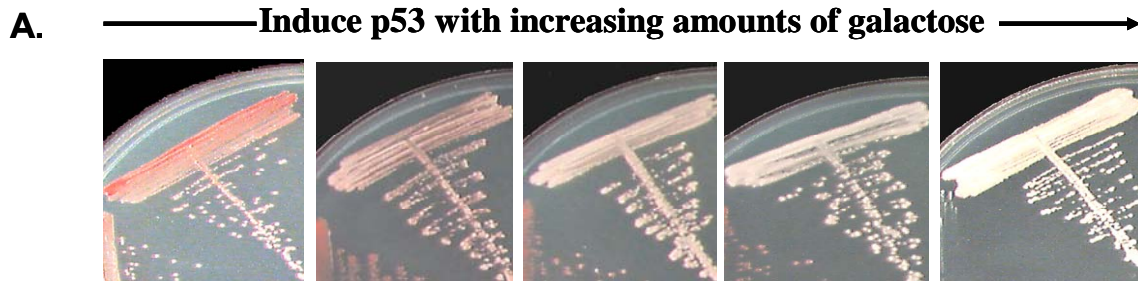


Figure 2.3A Qualitative plate color assay. The ADE2 phenotypic color assay determines the ability of p53 variants to transactivate from specific REs in stationary cells through the accumulation of pigment. The ability of p53 to interact with REs upstream of the ADE2 gene is assessed on plates containing a minimal amount of adenine. Colony pigmentation is dependent on the ability of p53 to transactivate from a specific RE sequence. If p53 (WT or mutant) is capable of strongly transactivating from a RE, colonies are large in size and white, whereas if p53 is not able to transactivate from a RE, colonies are smaller and red. Traditional functional assays with high levels of p53 expression are limited to establishing whether a p53 protein (WT or mutant) has the ability to transactivate from a RE (either red or white colonies) and cannot assess the intensity of this interaction. Inducible expression of p53 under the rheostatable GAL 1, 10 promoter enables p53 transactivation capacity to be assessed at variable levels of protein expression. Therefore, the range of pigmentation (pink to white) correlates with the extent of p53 induction from a particular RE. (Reprinted from Menendez et al., 2007 *Oncogene* **26**:2191-2201) [25].

Figure 2.3 B

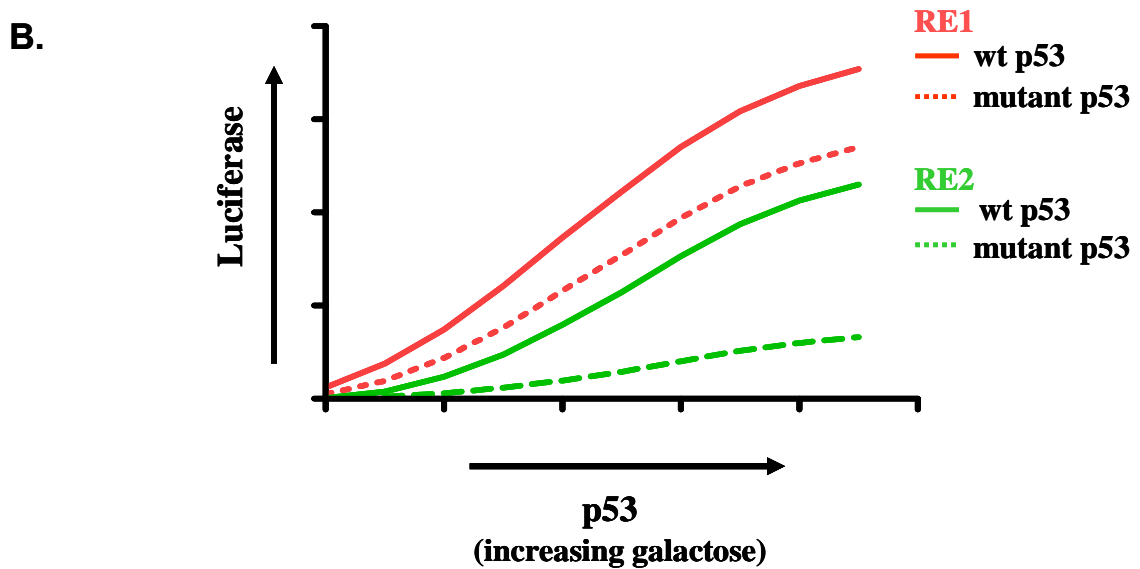


Figure 2.3B Quantitative luciferase assay. The quantitative luciferase assay is used to address *in vivo* the contribution of sequence towards the ability of wt or mutant p53 to transactivate from specific REs. The quantitative luciferase assay can be used to follow the variation in transactivation for wild type (WT) and mutant (mut) p53 over a range of p53 expression levels, from basal to high levels, in logarithmically growing cells. Transactivation by wt p53 can be shown to differ considerably between REs based on the small changes in RE sequence with the quantitative assay (i.e., RE1 verse RE2). Furthermore, the affect of specific mutations on the ability of p53 to transactivate from distinct REs can be directly compared to wild type p53 and other mutations with this sensitive assay. (Reproduced from Menendez et al., 2007 *Oncogene* 26:2191-2201) [25].

Figure 2.4

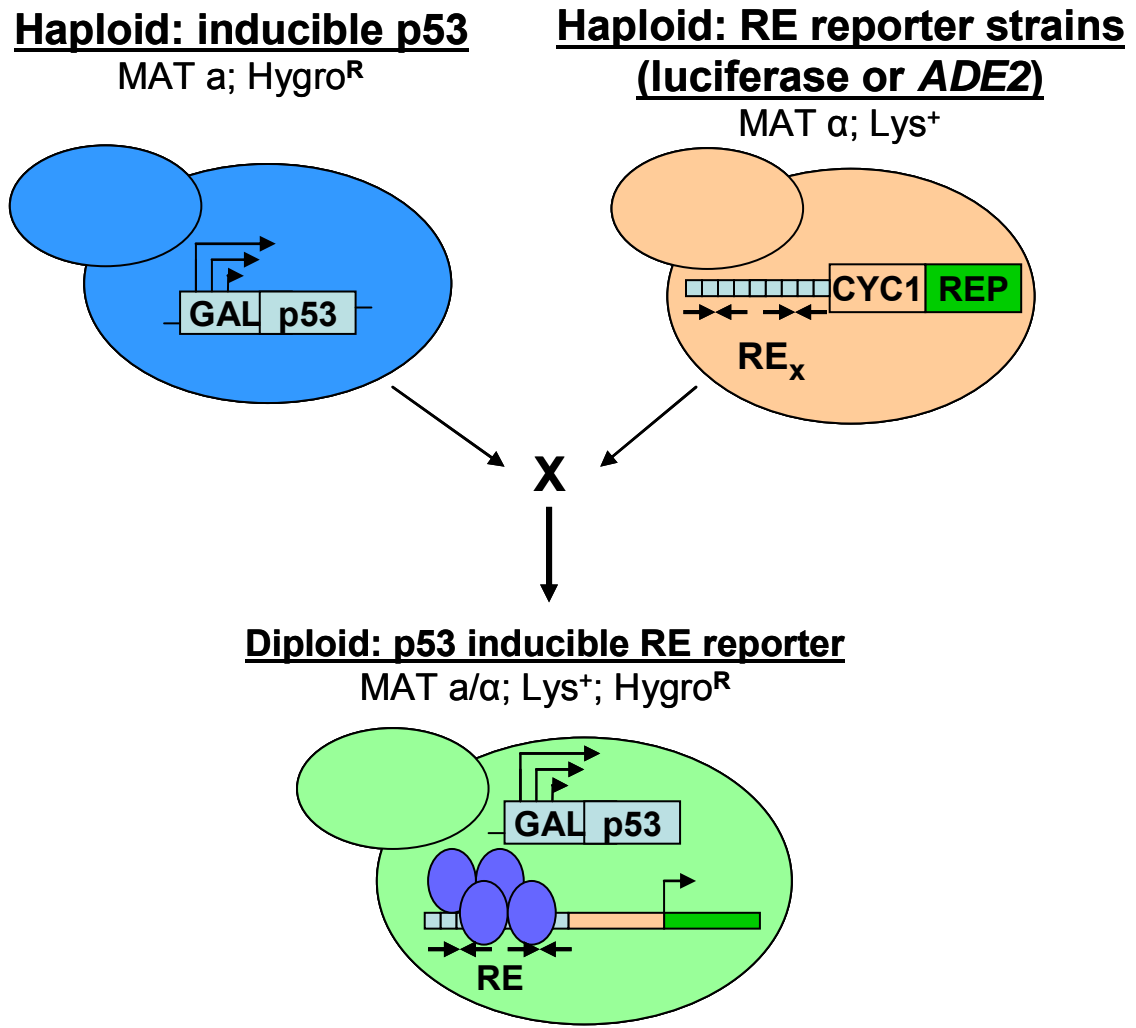


Figure 2.4 Isogenic diploid yeast system to investigate transcriptional capacity of p53 towards many REs at various p53 levels. Transactivation capacities of p53 (WT or mutant) from cognate REs were determined using diploid strains derived from haploids where p53 and reporters with upstream REs were integrated into different chromosomal loci. Two panels of haploid strains were generated. The first was a set of *p53-host* strains in which p53 (WT or mutant) was controlled by a “rheostatable” *GAL1* promoter (blue cells). The rheostatable promoter (previously described in, [41]) allows for controlled, inducible expression of p53. The second set contained promoter REs upstream of the minimal *CYC1* promoter and either the *ADE2* color reporter (*REP*) or the firefly luciferase reporter (tan-colored cells) [43]. Mating of the *REP* and *p53-host* strains results in isogenic diploid yeast that provide for rapid assessment of the transactivation potential for WT or mutant p53 proteins towards many individual REs in the p53 transcriptional network [27]. Strains are referred to as “isogenic” because they only differ by RE sequence or single base mutations in p53.

Figure 2.5

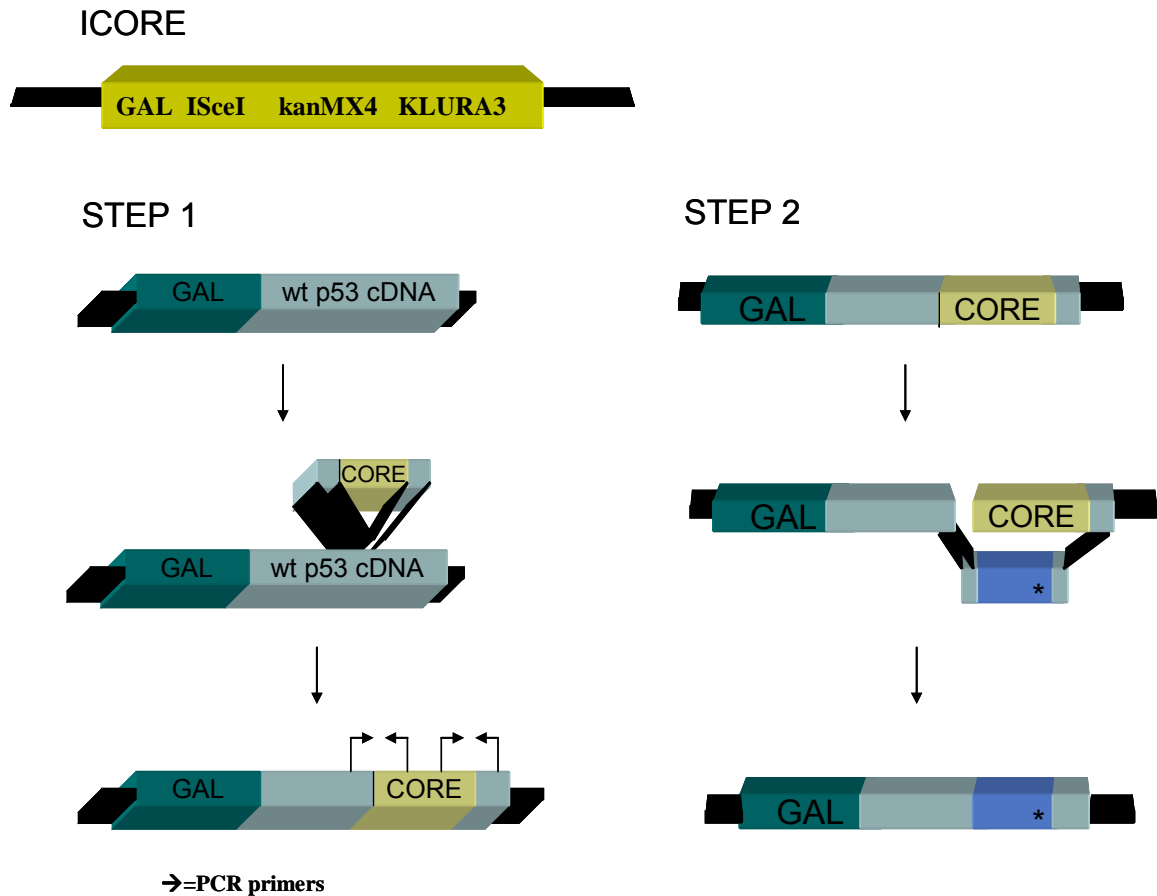


Figure 2.5 *Delitto perfetto* site-directed *in vivo* mutagenesis system. To insert the different putative p53 response elements (REs) upstream of the *ADE2* and luciferase reporters and generate specific p53 missense mutations of interest, the *delitto perfetto* site-specific *in vivo* oligonucleotide mutagenesis system was used [39]. *Delitto perfetto* is a two step process for rapid genome modification that is dependent on homologous recombination and Rad52. The first step is the integration of a COunterselectable REporter (CORE) cassette within the genome through homologous recombination. Two master reporter strains, *yAFM-ICORE* and *yLFM-ICORE*, and were constructed following the protocol previously described [26]. In addition, as depicted above, the ICORE was placed at 9 locations in the p53 cDNA, *yAT-iGAL::p53* (Table 2.1). ICORE is a modification of the CORE (*counter selectable reporter*) cassette [39] and contains, in addition to the counter-selectable *KLURA3* and the reporter *KanMX4*, the I-SceI endonuclease gene under control of the *GAL1* promoter and one copy of its unique 18-nt recognition site. Integration of the CORE can be selected for with *URA3+* and G418 (geneticin) resistance and verified with PCR. Step 2 entails replacement of CORE through a standard lithium acetate (LiAc) transformation with Integrative Recombinant Oligonucleotides (IROs). To create DNA modifications the ICORE was replaced with oligonucleotides containing the desired p53 RE (see Table 2.2) or p53 mutation of interest according to the *Delitto Perfetto* protocol [39]. Induction of I-SceI on galactose leads to the generation of a single DNA double-strand break at the ICORE site, resulting in a dramatically increased homologous DNA

targeting by oligonucleotides along with coincident loss of the ICORE. Replacement of the CORE cassette is confirmed with selection on 5-FOA plates against URA3+ (URA3 converts 5-FOA to fluorodeoxyuridine, which is toxic to yeast cells) and G418 sensitivity. Direct sequencing verifies introduction of the correct RE or mutation of interest.

Table 2.1

Table 2.1 ICORE insertion sites in p53 cDNA and window of modification.

CORE	Integrated site^a	Modification window^b
C	133 -134	118 - 149
D	163 -164	148 - 179
E	193 -194	178 - 209
F	223 – 224	208 - 239
G	253 – 254	238 - 269
A	283 – 284	268 - 299
B	313 – 314	298 - 329
C1	335 – 336	320 - 351
C2	373 – 374	358 – 389

Delitto perfetto used to generate a panel of 9 isogenic p53 host *S. cerevisiae* strains--where each contains the p53 cDNA controlled by the inducible (“rheostatable”) GAL1 promoter, integrated in a chromosomal locus, plus a CORE (COunterselectable marker and REporter marker) cassette inserted at various positions in the p53 cDNA. The cassette allows a 150 bp window for site-directed modification around the CORE with the site-directed mutagenesis system (Table 2.1). The p53 mutants under tight regulatory control are created with oligonucleotides that contain the desired mutation and replace the specific CORE cassette through homologous recombination to generate a full length mutant p53 cDNA.

^a CORE-I-SceI cassettes are strategically inserted along the p53 cDNA to allow for modification throughout the large DNA region. Indicated are the amino acids flanking the inserted CORE.

^b CORE cassettes allow for a window of modification of approximately 100 nucleotides, or as shown 30 amino acids in either direction.

Table 2.2

Table 2.2 Response element sequence, strength and rank of transactivation capacity.

Response Element	Gene Name	Transactivation			Sequence		
		Strength	% p21-5'	scale	Half-site	spacer	Half-site
MDM2 RE1+RE2 ^O	MDM2	strong	115	5	GGtCAAGTTg	N	GGACA cGTCC
					GAGCTAaqTC	c	tGACATGTCT
P53R2 ^D	RRM2B	strong	102	5	tGACATGCC		AGGCATGTCT
p21-5' ^C	CDKN1A	strong	100	5	cAACATGTTg		GGACATGTTC
CON A ^O		strong	100	5	GGGCATGTCC		GGGCATGTCC
BAX A+B ^A	BAX	strong	98	5	t cACAAGTTa	g	AGACAAGCCT
					AGACAAGCCT		GGGc gTGg gC
PA26 ^C	SENS1	moderate	77	4	GGACAAGTCT		cAACAAAGTTC
CYCLIN G ^C	CCNG1	moderate	72	3.5	AGGCTTGCCC		GGGCA gGTCT
MMP2 ^A	collagenase (IV)	moderate	70	3.5	AGACAAGCCT		GAAC TTGTCT
PUMA BS2 ^A	BBC3	moderate	68	3.5	ctGCAAGTCC		tGACTTGTCC
CON B ^O		moderate	58	3	GGGCTAGTCC		GGGCTAGTCC
CON C ^O		moderate	50	3	GGGCAAGTCT		GGGCAAGTCT
GADD45 ^D	GADD45A	weak	42	2.5	GAACATGTCT		AAGCATGTc g
MDM2 RE1 ^O	MDM2	weak	34	2	GGtCAAGTTg		GGACA cGTCC
PCNA ^D	PCNA	weak	34	2	GAACAAGTCC		GGGCATaTgT
NOXA ^A	PMAIP1	weak	28	1.5	AGGCTTGCCC		cGGCAAGTTg
P53AIP1 ^A	P53AIP1	weak	26	1.5	tctCTTGCCC		GGGCTTGTc g
14-3-3σ SITE 2 ^C	stratifin	weak	20	1	tAGCATTAGCCC		AGACATGTCC
PIDD ^A	PIDD	very weak	16.5	1	AGGcTGCCCT	gcgtgctg	GGACATGTCT
MDM2 RE2 ^O	MDM2	very weak	16	1	GAGCTAaqTC	c	tGACATGTCT
APAF1 ^A	APAF1	extremely weak	6.5	1	AGACATGTCT	ggagaccctagga	cGACAAGCCC
14-3-3σ SITE 1 ^C	stratifin	extremely weak	6	0.5	AGGCATGTgC		cAcCATGCC
P21-3' ^C	CDKN1A	extremely weak	5	0.5	GAAgAAGaCT		GGGCATGTCT
LIPHG ^O	SCGB1D2	extremely weak	5	0.5	GGtCTTGTTT		AGACTTGCTC
BAX B ^A	BAX	nonfunctional	3	0.25	AGACAAGCCT		GGGc gTGg gC
BAX A ^A	BAX	nonfunctional	0.75	0.25	t cACAAGTTa	g	AGACAAGCCT
RAD51 ^D	RAD51	nonfunctional	0.5	0	AAACTcGc gC	agg	ATCAAGCTCT

Listed are the REs examined for transactivation potential by WT p53 with the quantitative luciferase assay along with the associated target gene and biological function. (A= apoptosis, C = cell cycle response, D = DNA damage recognition and repair, O = other including p53 stability and regulation, angiogenesis, and consensus elements) Transactivation capacity is presented as strength of transactivation, percent of maximal transactivation and scale. Maximal induction was calculated as the average relative light units/ ug protein measured at 0.020% - 0.032% galactose. Percent of transactivation was relative to WT p53 transactivation from the p21-5' RE. Maximal transactivation was converted to an arbitrary scale value of 0 – 5 to represent strength of transactivation, where 0 is nonfunctional, 2 is weak transactivation and 5 is strong transactivation. Sequences of the RE are broken into decamer half-sites. Nucleotides that deviate from the consensus RE are shown in red lower case letters and blue indicates an insertion into the central sequence of a half-site.

Figure 2.6

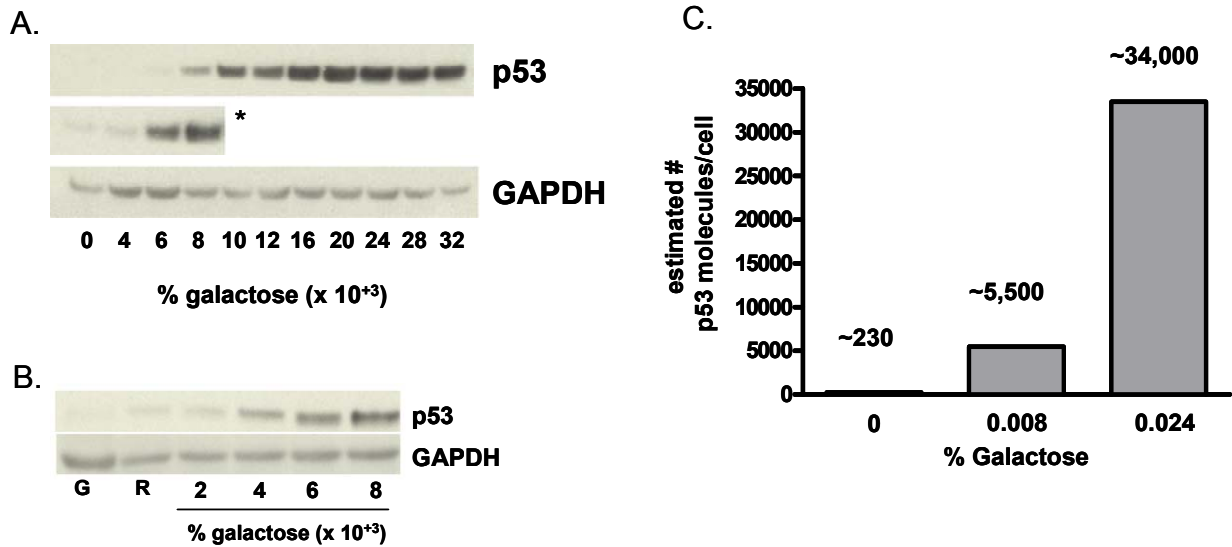


Figure 2.6 Inducible expression of p53 under the rheostatable *GAL1* promoter. (A) The *GAL1* promoter allows for controlled expression of p53 depending on the level of galactose in the media. Raffinose was added to provide a basal level of expression, derepressed from the *GAL1* promoter. Presented is a Western blot analysis of p53 expression 24 hours post-inoculation with raffinose or raffinose plus increasing amounts of galactose (.004-.032%). Increases in galactose from 0 – 0.032% correlated with an increase in p53 over a 100-fold range. The p53 protein was detected with DO-1 and pAb1801 antibodies. The asterisk (*) depicts a longer exposure to reveal protein at basal and lower galactose levels. GAPDH, identified by immunodetection provided a standard loading control. (B) Low level expression of p53 under the rheostatable *GAL1* promoter. With glucose supplemented in the media, the *GAL1* promoter is repressed. Presented is a Western blot analysis of p53 expression 24 hours post-inoculation with 2% glucose (G), 2% raffinose (R) or 2% raffinose plus increasing amounts of galactose (.002-.008%). The p53 protein was detected with DO-1 and pAb1801 antibodies. Immunodetection with GAPDH was used as a standard loading control. To determine the relative protein concentrations at repressed “G” glucose added, basal and low galactose levels, pixel values were measured from autoradiographs of increasing exposure lengths (from 1- 15 minutes). The ratio of measured pixel values were calculated between samples and compared between exposure lengths to derive the relative differences in p53 expression between media containing glucose (2%), raffinose (2%) or raffinose (2%) plus increasing concentrations of galactose (data not shown). A 3-fold induction of p53 expression was observed between samples containing glucose vs. raffinose, as well as between raffinose and raffinose + 0.004% galactose. There was a further 2-fold induction between the 0.004% galactose and 0.006% galactose samples and a 3-fold difference between the 0.006% and 0.008% galactose samples. This totaled a relative increase in p53 expression of

54-fold between repressed conditions and induction at 0.008% galactose and an 18-fold between basal level expression and induction at 0.008% galactose. (C) Estimation of the number of p53 molecules per cell at increased expression. The diploid yeast strain containing GAL1::WT p53 crossed with the p21-5' RE-luciferase reporter was grown overnight in complete medium, diluted, washed and inoculated into selective medium containing either raffinose or raffinose plus 0.008% or 0.024% galactose for 24 hours. A small sample of the overnight culture was aliquoted to estimate the concentration of cells/mL with a hemocytometer. Protein lysates were obtained and analyzed by Western analysis in comparison to known amounts of purified p53 protein (20, 30 and 40 ng) (BD Biosciences Pharmingen, San Diego, CA). Depicted is an estimate of the number of p53 molecules per cell calculated from the estimated total protein/cell and ng p53/cell.

Figure 2.7

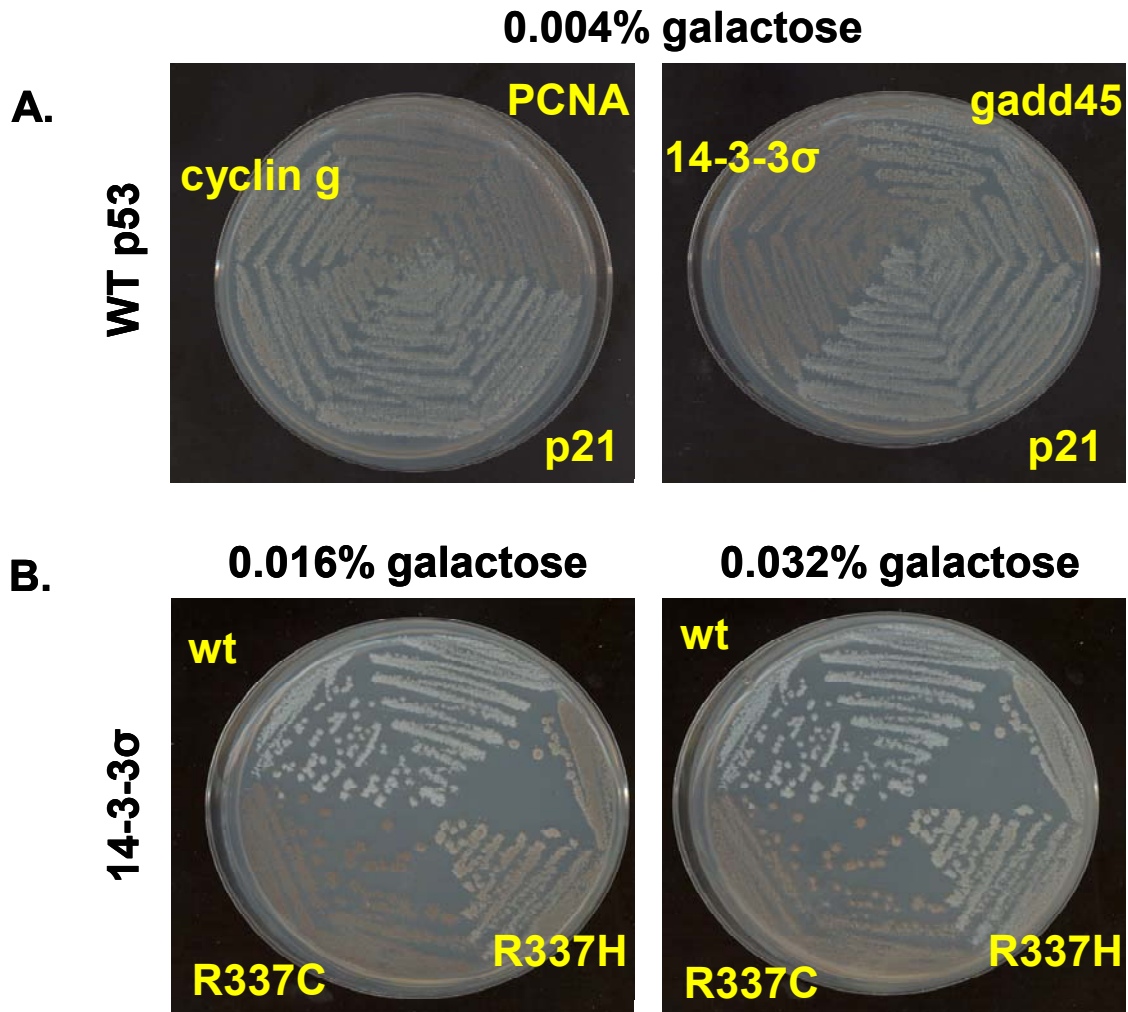


Figure 2.7 Differential transactivation capacities of WT and mutant p53 towards REs at variable expression observed with the phenotypic color assay. The ADE2 reporter provides a qualitative assay to determine transactivation potential through the accumulation of colony pigmentation in stationary cells over several days. WT or mutant p53 cDNA is expressed from the GAL1 promoter at variable levels dependent on the amount of galactose supplemented in the media. The color assay can be used to differentiate WT p53 transactivation potential from distinct REs at comparable expression (A), or to distinguish mutant p53 transactivation potential in comparison to WT p53 and other mutants at variable levels of expression (B).

Figure 2.8

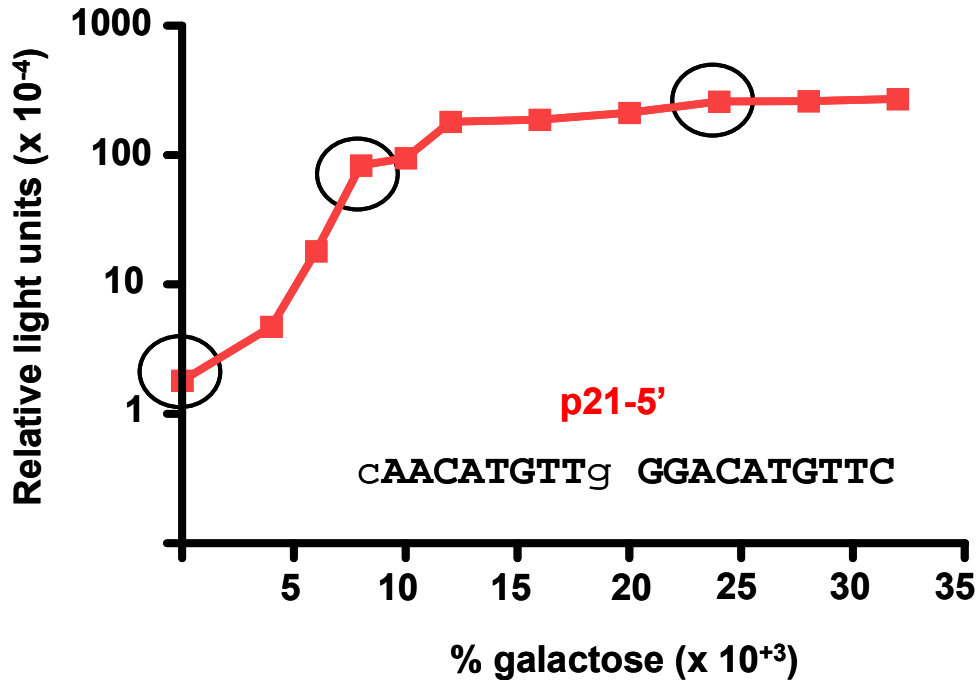


Figure 2.8 Quantitative assessment of p53-induced transactivation from the p21-5' RE *in vivo*. The diploid yeast strain containing GAL1::WT p53 crossed with the p21-5' RE-luciferase REP was grown overnight in complete medium, diluted, washed and inoculated into selective medium containing either raffinose or raffinose plus increasing galactose (0 – 0.032%) for 24 hours. Protein lysates were obtained and a luciferase assay was used to determine the transactivation capacity of p53 from the p21-5' RE'. The strength of transactivation was calculated as relative light units/ug protein. Circled are the basal, linear-increase, and plateau phases of the transactivation response as a function of galactose concentration and are referred to as basal, moderate and high levels of p53 expression.

Figure 2.9

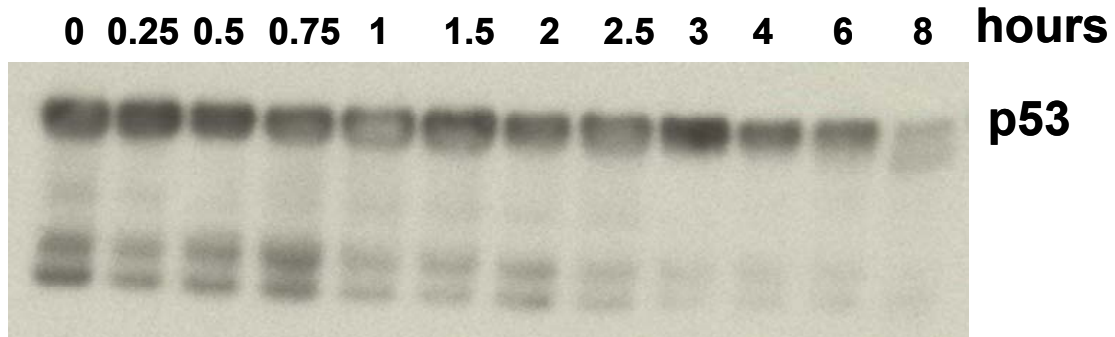


Figure 2.9 p53 protein stability following a glucose pulse. To determine the stability of p53 within the diploid system, the half-life of p53 was followed after addition of glucose, which represses the GAL1 promoter, to the media. WT p53 crossed to the p21-5' RE::luc was grown overnight in YPDA complete media, diluted and grown in synthetic media supplemented with 2% raffinose plus 0.024% galactose overnight with vigorous shaking at 30°C. Eighteen hours after inoculation into induction media, 2% glucose was added to the cultures. Samples were harvested at the time points indicated and levels of p53 protein were assessed by Western analysis after adjusting to common overall protein levels. Induction of p53 was stable within the inducing media, where p53 levels remained elevated until approximately 4 hours after the addition of glucose when a decrease in protein levels was observed. The two lower bands appear to be product of protein degradation.

Table 2.3

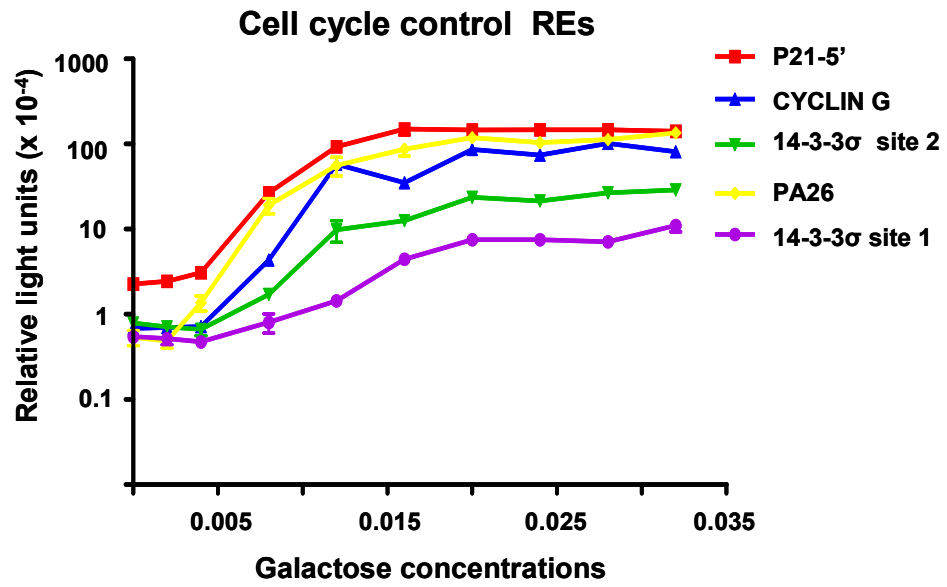
Table 2.3 Toxic and super transactivating mutations display altered function in the haploid and diploid rheostatable systems.

MUTANT	V122A		C277R		C279R		S121F	
	2n	1n	2n	1n	2n	1n	2n	1n
P21-5'	↑	↑	↑	↑	↑	↑	↑	↑
P53R2	=	=	↓	↓	↓	↓	↑	↑
GADD45	↑	↑	↓	↓	↑	↑	↑	↑
14-3-3σ	=	↓	=	=	↓	↓	↑	↑
CYCLIN G	↓	↓	L	L	L	L	↑	↑
AIP1	↓	↓	↓	L	L	L	↑	↑
	TOXIC						SUPERTRANS	

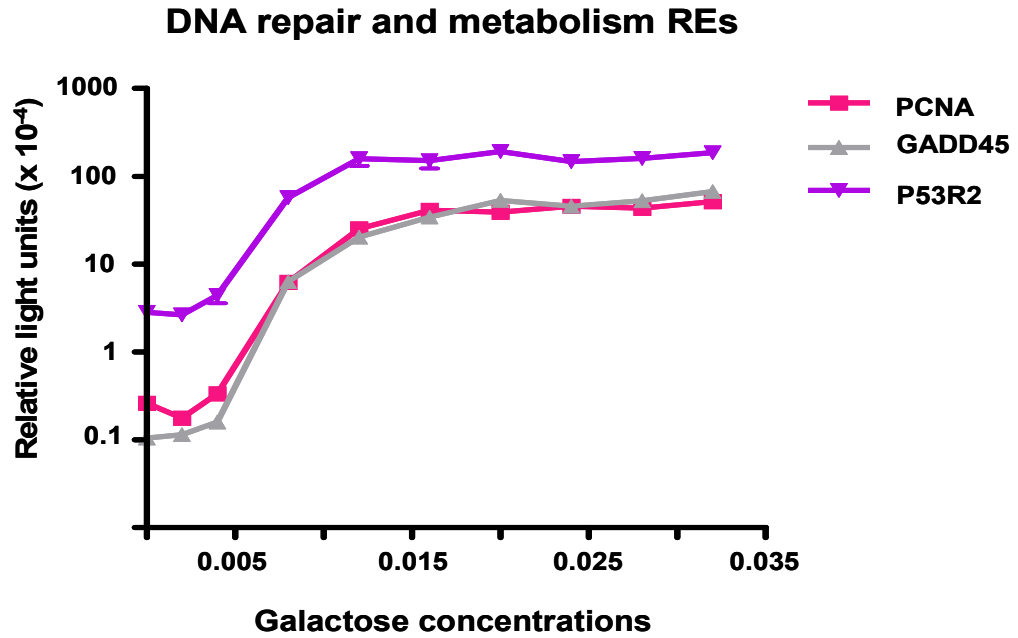
Relative transactivation capacities were assessed with the ADE2 color assay in the diploid (2n) integrated rheostatable system and compared to data previously obtained in the haploid (1n) plasmid-based system [27, 41]. Transactivation capacities were determined to be equal (=) to WT p53, lost (L) or increased (↑) or decreased (↓) in comparison to WT p53 at one or more expression level analyzed.

Figure 2.10

A.



B.



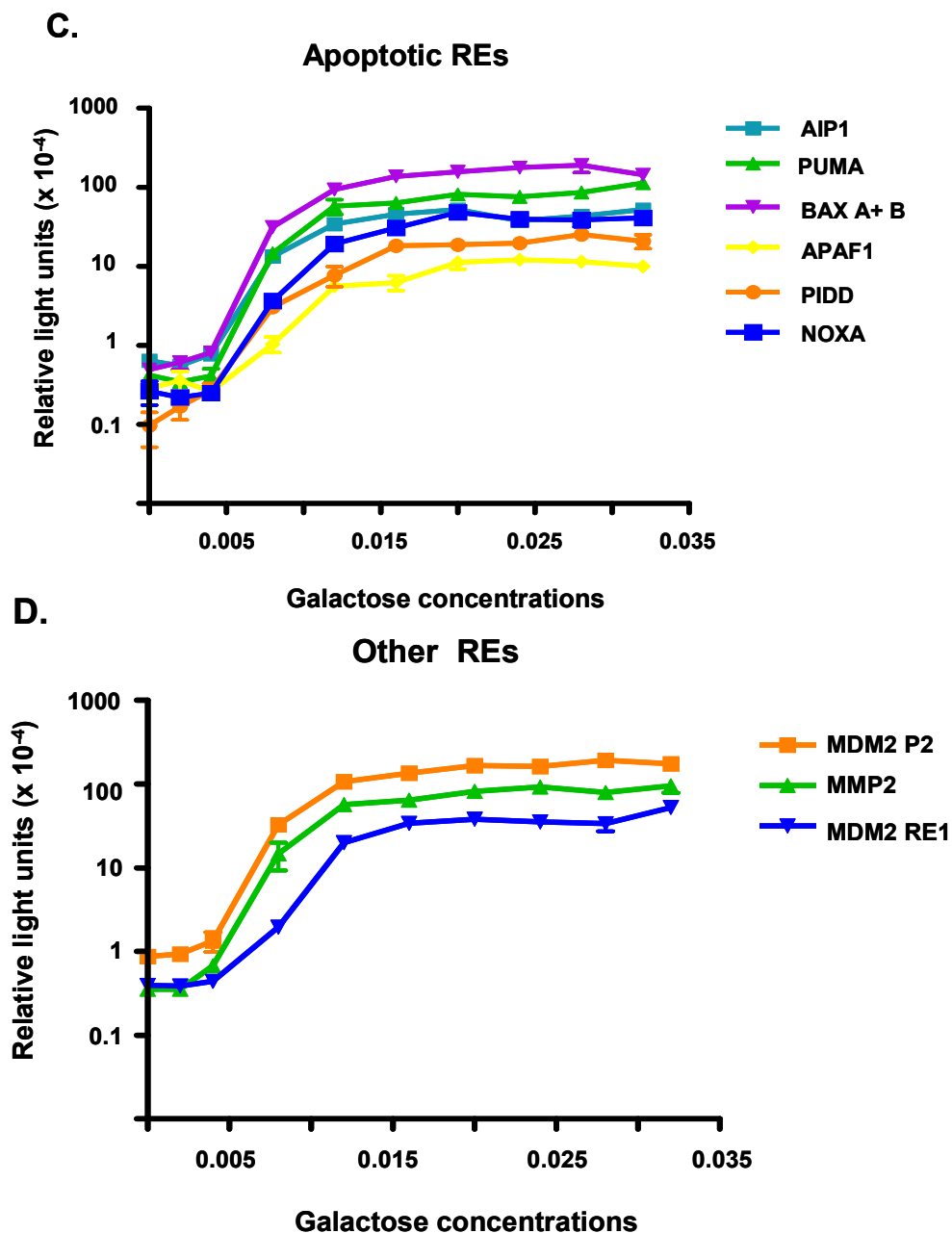


Figure 2.10 Sequence specific differences in WT p53 transactivation capacity from REs does not correlate to the biological function of the target gene. The quantitative luciferase assay was used to resolve sequence specific differences in transactivation by WT p53. Isogenic diploid yeast strains were grown in increasing concentrations of galactose (0 – 0.032%) to induce p53 protein. The ability of WT p53 to transactivate from RE sequences was measured by a luciferase assay 24 hours after inoculation into the galactose supplemented media. Induction from each RE was compared relative to the ability of p53 to transactivate from the p21-5' RE (as quantified in Table 2.2). Presented is the mean and standard error of measurement (SEM) of 8 independent repeats graphed with REs of similar biological function. WT p53 transactivates from p53 target REs to various degrees, where small differences in sequence can contribute significantly to levels of transactivation. Regardless of transactivation capacity or biological role, the initial induction of transactivation occurs at comparable levels of p53 expression for all REs.

Figure 2.11

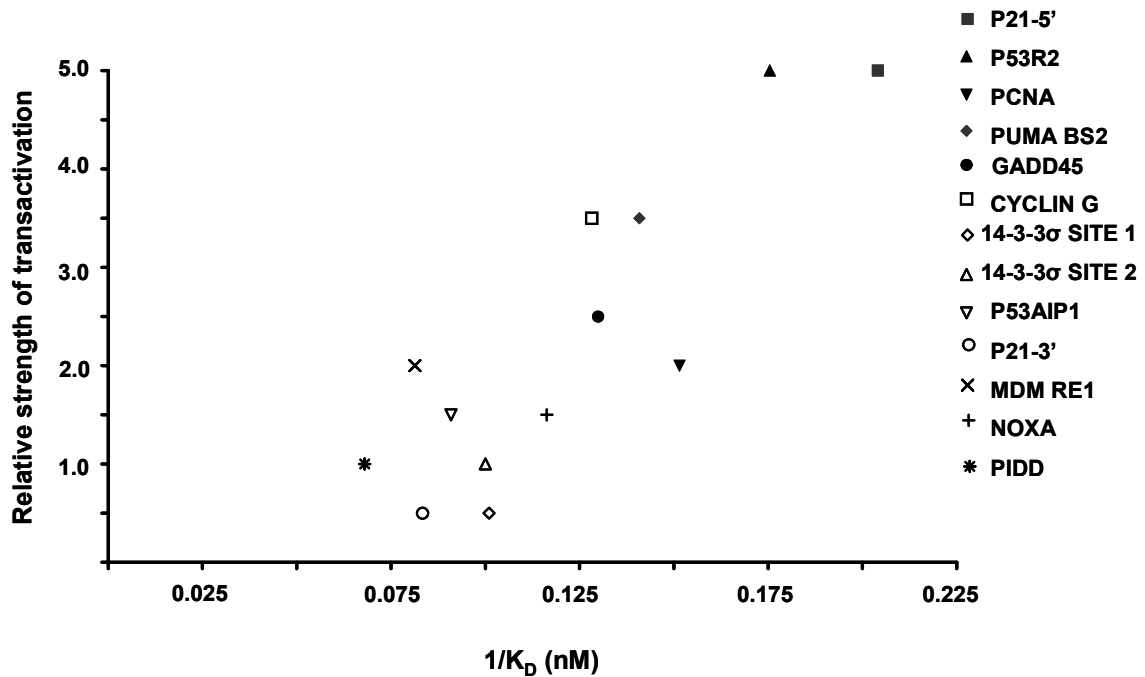


Figure 2.11 *In vivo* transactivation vs. *in vitro* binding. We have asked whether the ability of p53 to transactivate from target RE sequences *in vivo* simply reflects its ability to bind to DNA based on *in vitro* assays or whether there are additional intrinsic factors that contribute to *in vivo* transactivation. The “kinetics” of transactivation from several REs (scored as 0-5) was compared to the dissociation constants (1/K_D) recently determined using fluorescence anisotropy [32]. While there seems to be a general trend in strong transactivators having smaller K_D values, the *in vivo* transactivation data for the different REs under isogenic conditions argue that differential transactivation by WT p53 is not simply due to the variation in p53 binding affinities. In spite of comparable p53 binding *in vitro*, transactivation *in vivo* can vary dramatically under isogenic conditions (i.e., cyclin G vs. Gadd45). Furthermore, functional assays show WT p53 transactivates to similar levels from REs despite differences in binding affinities (i.e., MDM RE1 vs PCNA).

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CHAPTER 3
NONCANONICAL DNA MOTIFS AS TRANSACTIVATION TARGETS BY
WILD TYPE AND MUTANT p53

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Abstract

Sequence-specific binding by the human p53 master regulator is critical to its tumor suppressor activity in response to environmental stresses. p53 binds as a tetramer to two decameric half-sites separated by 0–13 nucleotides (nt), originally defined by the consensus RRRCWWGYYY (n= 0-13) RRRCWWGYYY. To better understand the role of sequence, organization and level of p53 on transactivation at target response elements (REs) by wild type (WT) and mutant p53, we deconstructed the functional p53 *canonical* consensus sequence using budding yeast and human cell systems. Contrary to early reports on binding *in vitro*, small increases in distance between decamer half-sites greatly reduces p53 transactivation, as demonstrated for the natural TIGER RE. This was confirmed with human cell extracts using a newly developed, semi-*in vitro* microsphere binding assay. These results contrast with the synergistic increase in transactivation from a pair of weak, full-site REs in the MDM2 promoter that are separated by an evolutionary conserved 17 bp spacer. Surprisingly, there can be substantial transactivation at noncanonical ½- (a single decamer) and ¾-sites, some of which were originally classified as biologically-relevant canonical consensus sequences including PIDD and Apaf-1. p53 family members p63 and p73 yielded similar results. Efficient transactivation from noncanonical elements requires tetrameric p53, and the presence of the carboxy terminal, non-specific DNA binding domain enhanced transactivation from noncanonical sequences. Our findings demonstrate that RE sequence, organization and level of p53 can strongly impact p53-mediated transactivation, thereby changing the view of what constitutes a functional p53 target. Importantly, inclusion of ½- and ¾-site REs greatly expands the p53 master regulatory network.

Introduction

The tumor suppressor p53 (OMIM no. 191170) is a sequence-specific *master regulatory gene* that controls an extensive transcriptional network providing for genome integrity in response to cellular and environmental stresses or damage [1-3]. p53 differentially regulates the expression of target genes, as well as microRNAs associated with cell cycle control, apoptosis, DNA repair, angiogenesis, senescence and carbon metabolism [4, 5]. A variety of factors such as stress and cell-type dependent, post-translation modifications and transcriptional co-factors can influence p53-induced transcriptional changes [6-8]. Paramount to p53 transcriptional function is the direct interaction between p53 and its targeted DNA sequence. The nature of this interaction could *per se* determine transactivation capacity, as well as influence p53-mediated biological processes [9]. Such activities are often altered during human cancer development as highlighted by the frequent appearance of p53 missense mutations in its sequence-specific DNA binding domain [10, 11] which can abrogate or alter p53 transactivational activity that result in changes in biological responses, such as the balance between apoptosis and survival in response to DNA damage. [3, 10, 12-15].

Through *in vitro* studies, a consensus p53 DNA binding sequence has been derived comprising a motif of two decamers (half-sites) RRRCWWGYYY (n) RRRCWWGYYY, (where R=purine, W= A or T, Y= pyrimidine and n is 0-13 bases) where each decamer is composed of two adjacent p53 monomer binding sites (quarter-sites) in inverted orientation [16-18]. p53 binds cooperatively to the consensus RE as a dimer of dimers, where a tetramer is the accepted functional unit required for full transcriptional activity [19-26]. Most functional response elements (REs) identified in association with p53 target genes depart

from this consensus, where base changes are tolerated at each position with the exception of the C and G at positions 4 and 7 in each half-site [27, 28].

Recent crystal structures show that the interactions between p53 and DNA are influenced by the base pairs present in the RE sequence [29] while binding assays in solution established a wide range of dissociation constants among natural p53 REs [23]. Functional studies in model systems demonstrate that the RE sequence and amount of p53 expression can dramatically influence the level to which p53 can transactivate from a specific RE [3, 30]. However, results of those various approaches do not fully overlap and it is still not well-understood how the loose consensus, in terms of base variation and spacer length between half-sites affects p53 binding to DNA, or how differential binding relates to transactivation specificity [31-33].

More recently, several studies have refined the accepted, or canonical p53 consensus binding sequence based on presumed unbiased chromosome- or genome-wide *in vivo* binding assays using chromatin immunoprecipitation (ChIP) [34-37]. However, the findings may be skewed towards stronger p53 interacting sequences or influenced by the agent used to induce p53 protein, as well as the human cell lines examined. While ChIP provided a powerful tool for identifying sequences that p53 could bind and the results pointed to a refined p53 consensus with more restrictive sequence features [34], these assays could not address the strength of p53 binding. Furthermore, the experimental approach failed to retrieve known p53 binding sites, indicating that the technique could miss interactions between p53 and weaker response elements which might include noncanonical sites that do not match the accepted consensus motif.

Bioinformatics studies to identify p53 REs are guided by identified sequences. Based on the consensus sequence, computational algorithms have exploited the base pair composition of known p53 REs to determine binding probabilities of p53 towards sequences and generate position-weight matrices (PWM) or “logos” [38, 39]. While such algorithms may guide the identification of p53 binding sites in the genome, they cannot assess the strength of p53 binding to these sequences. The ability of p53 to bind (and presumably transactivate from) a sequence is often related to compliance with the canonical consensus sequence where each position in the motif is assumed to be equivalent and mutually exclusive in terms of affecting p53 binding [40]. However, PWM values appear not to be a good predictor of p53 transactivation [30].

While the canonical consensus sequence has guided studies of the p53 network, an additional layer of complexity was identified recently suggesting that the canonical motif is not limited to two decamers in human cells. p53 dependent-transactivation was detected in association with a decamer half-site created by a single nucleotide polymorphism (SNP) in the promoter of the Flt-1 gene (the vascular endothelial growth factor (VEGF) receptor-1 gene) [41]. These results showing that p53 can function from a noncanonical consensus sequence imply that the number of potential p53 target sites within the genome may be much greater than anticipated. Current methods that identify and/or define putative p53 REs would overlook such noncanonical binding elements.

Stimulated by the observation of a transcriptionally active p53 half-site, as well as by the finding that even a single nucleotide change can greatly impact transactivation potential of a RE [42, 43], we have systematically deconstructed the canonical p53 RE sequence to address the requirements for a functional p53 binding site. Utilizing *in vivo* systems we

developed in the budding yeast *Saccharomyces cerevisiae* (see chapter 2), the transactivation potential of p53 was first examined from p53 canonical consensus REs containing base changes and/or variations in organization of binding motifs (*i.e.*, multiple REs and various spacer lengths) and then assessed transactivation from 1/2- and 3/4-site REs, which we refer to as noncanonical REs. For these noncanonical REs the surrounding sequence does not resemble a p53 binding sequence and the motif itself would not be recognized within standard descriptions of a p53 canonical consensus sequence. Within the yeast system, we have addressed not only the ability of p53 to function from specific target sequences (*i.e.*, on/off), but also the extent of transactivation from these sites at variable levels of expression. These studies have been extended to transactivation capacity and p53 promoter occupancy in a human cell system, and the *in vivo* functionality evaluations were compared with results obtained in a recently developed semi-*in vitro* binding assay using human cell nuclear extracts. Several structural mutants were examined to assess the p53 structural requirements for transactivation from noncanonical REs. Overall, the findings expand our understanding, as well as the anticipated size of the human p53 master regulatory network.

Results

Increases in distance between weak, full-site REs can lead to synergistic transactivation

A common feature of many p53 target genes, including p21, PUMA, BAX, and DDB2 (p48), is the presence of multiple p53 binding sites [44-50]. The distance between binding sites is variable and can even be overlapping. The *Mdm2* gene, which targets p53 for degradation by the ubiquitination pathway through a negative feedback loop, is an example of a p53 target gene that contains two promoters. The upstream promoter is constitutively

active and does not rely on p53 for transactivation, whereas the second is in the first intron and is p53-dependent [51, 52]. The second promoter contains two full-site p53 REs separated by 17 nucleotides (nt). Using our diploid yeast-based p53 rheostatable system, we investigated the interaction between these two REs and the impact of this spacer by changing the distance between the murine MDM2 REs.

The induction from the individual MDM2 REs at high p53 expression was much weaker than observed with the strong p21-5' RE (Figure 3.1A): ~33% (RE1) and ~18% (RE2) relative to p21-5' RE. However, transactivation from the natural MDM2, containing the 17 base spacer, was much higher than the sum of the individual REs, reaching the p21-5' RE levels. The synergy was apparent at both moderate and high p53 levels. To determine the impact that a spacer may play for full-site REs, the distance was reduced to either 10 or 5 nt. As shown in Figure 3.1B, decreasing the separation to 10 nt had little effect. However, a decrease to 5 nt resulted in a substantial reduction in transactivation suggesting that synergistic interactions are lost as full REs become closely spaced, although transactivation from the two sites remain additive.

Increase in spacer between half-sites decreases p53 transactivation

While weak full-site REs can interact synergistically when separated by 17 bases, previous studies have indicated that spacers between decamer half-sites of the canonical consensus RE can alter the ability of p53 to transactivate from a RE [18, 53, 54]. We systematically investigated the role that spacers may play on p53 transactivation using the yeast-based rheostatable promoter and a system based on expression following transfection into human cells. Addition of a one-base spacer between the p21-5' half-sites resulted in a

dramatic 60% decrease in p53 transactivation (Figure 3.2A) in yeast. Addition of a second nucleotide further decreased p53 transactivation to approximately 25% of transactivation at high p53 expression. Importantly, at lower p53 levels transactivation was essentially abolished, demonstrating that the impact of spacer is markedly affected by p53 expression level. Further increases in spacer resulted in decreased transactivation and at 5 nt there was almost no detectable luciferase activity at high p53 expression levels. Similar findings were observed in a plasmid-based haploid yeast system when p53 transactivation was measured from p21-5' REs containing a spacer of 0, 2, 5, or 10 nt (Supplemental Figure 3.1A) [3, 28, 30]. Thus, the length of spacer sequence between decamer half-sites combined with level of p53, greatly influences the ability of p53 to transactivate from the p21-5' sequence. Interestingly, transactivation by the p53 family members p63 β and p73 β was also compromised when p21-5' RE contained spacers (Supplemental Figure 3.1B); a 2 nt spacer essentially abolished transactivation.

To determine the effect of spacers upon weak target REs in the p53 transcriptional network, transactivation was assessed from the RE of the human apoptosis gene Noxa [55] with and without a 5 nt spacer between the decamer half-sites. In comparison to the p21-5' RE, transactivation from the Noxa RE was ~30% of the levels of p21-5' at high p53 expression (Figure 3.2A). This was comparable to p53 transactivation from the p21-5' RE containing a spacer of 1 or 2 bases. A spacer of 5 bases between the Noxa half-sites abolished p53 responsiveness (also found with the haploid yeast system; Supplemental Figure 3.1A).

Finally, we wanted to establish the impact that naturally occurring spacers in REs might have on transactivation. TIGAR (TP53 induced glycolysis and apoptosis regulator) contains a

p53 target RE with a two-base spacer between decamers. This gene reduces levels of glycolysis, decreases free reactive oxygen species and attenuates the apoptotic response [56-58]. Interestingly, the natural TIGAR RE is one of the few examples in the genome where the binding element matches the canonical p53 consensus sequence precisely (i.e., no mismatches). However, p53 could only induce transactivation from this sequence to ~ 20% of the levels with the p21-5' RE, which does not precisely match the consensus p53 sequence (Figure 3.2A). When the spacer was removed, p53 could transactivate from TIGAR to levels comparable to the p21-5' RE. Similar results were obtained with the haploid yeast system (Supplemental Figure 3.1A).

The impact of spacers on the ability of p53 to function from a RE was also assessed in human cells under conditions of high p53 expression. Luciferase reporter vectors containing the p21-5' RE with spacers of increasing length were generated in the vector pGL3 promoter (pGL3-P). The ability to transactivate from transfected p21-5' REs was assessed in p53 null SaOS2 cells (derived from a human osteosarcoma line) that were transfected with a cytomegalovirus (CMV) based p53 expression plasmid [59]. Consistent with the results observed in yeast, p53-dependent transactivation decreased with increasing spacer length between decamer half-sites. As shown in Figure 3.2B, expression of WT p53 resulted in an ~35-fold induction of transcription from the natural p21-5' RE as compared to transfection with a p53 deficient plasmid, whereas transactivation from the p21-5' RE containing a one- or two-base spacer resulted in a 45% and 67% reduction in relative luciferase activity, respectively. Similar to the situation in yeast, additional nucleotides resulted in >90% net reduction in transactivation. It is interesting that within the three

systems--haploid and diploid yeast and human cells--transactivation from a RE with a spacer of 10 bases was slightly increased in comparison to a RE with a 5 base spacer.

Spacers between half-sites impact p53 binding *in vivo* and *in vitro*

To determine if the difference in p53-dependent transactivation from REs containing spacers is simply due to a reduction in p53 promoter binding, we investigated *in vivo* occupancy using chromatin immunoprecipitation (ChIP) assays and *in vitro* binding using a newly developed microsphere binding assay (Noureddine et al., submitted). ChIP assays were performed on the luciferase reporter plasmids containing the p21-5' RE with spacers of varying lengths which had been transfected into SaOS2 cells along with the p53-expressing plasmid. As shown in Figure 3.2C, a one nt spacer decreased occupancy at the p21-5' RE by two-fold. Further increases in spacer length reduced p53 occupancy. No occupancy was observed in mock-transfected cells. Thus, the pattern of occupancy by p53 mirrored that for transactivation. Consistent with the transactivation results, p53 occupancy at the p21-5' RE with a spacer of 10 nucleotides was slightly increased in comparison with a spacer of 5 nucleotides (0.6% vs 0.4%).

To further characterize the impact of spacer on p53 interactions with a RE, we utilized a fluorescent microsphere binding assay to evaluate sequence-specific p53-DNA binding interactions (Noureddine et al., submitted). Briefly, this assay addresses p53 binding to individual beads with specific RE test sequences, where each bead "type" (*i.e.*, beads with specific REs) is identified with a unique double stranded oligonucleotide 24-nt "tag" sequence. Several bead types are then multiplexed in a binding assay and analyzed using Luminex technology to determine the amount of p53 bound to each bead type. We generated

a series of oligos with the p21-5' RE sequence of interest that contained various spacer lengths flanked by non-specific DNA. Each of these RE sequences was conjugated to beads. The bead types were combined and incubated for 60 minutes with nuclear cell extracts obtained from human lymphoblastoid cell lines that were either not induced or induced for p53 expression with doxorubicin (Dox). The level of p53 expression was approximately 15-fold greater in extracts from Dox-treated versus nontreated cells (data not shown). The p53 interaction with each bead type (*i.e.*, each p21-5' RE variant) was determined after incubation with p53 antibodies and secondary antibodies conjugated with phycoerythrin.

As displayed in Figure 3.2D, the mean relative binding of p53 to the p21-5' RE with a spacer of 1 nucleotide (0.83 ± 0.09) was comparable to that for the p21-5' RE (1.0 ± 0.11) with no spacer. However, p53 binding to the p21-5' REs was affected when the spacer between half-sites was ≥ 2 nucleotides, with a dramatic decrease at > 4 nucleotides. The sequence of the spacer used to increase the distance between the decamer half-sites had no apparent effect on binding activity (data not shown). Furthermore, p53 did not bind to the negative control (NC) sequence which had the p21-5' RE replaced with a scrambled sequence. Interestingly, p53 displayed the same residual binding to the individual half-sites of the p21-5' RE [p21-5' left (L) and right (R)] as it did towards REs containing a spacer of 4 nucleotides or more (0.06 and 0.08, respectively).

p53 can transactivate from half-site REs in yeast and human cells

Since a low level of binding or transactivation was observed with widely separated decamers, we investigated p53 binding and transactivation from single decamer sequences in the yeast diploid and human cell systems. Transactivation was barely detectable from the left

or right decamers of the p21-5' RE (Figure 3.3A). However, two additional complete consensus half-sites, designated Con G and Con D, were able to support transactivation at a level corresponding to 2.4% and ~10%, respectively, of that from the full p21-5' RE. Importantly, transactivation was only observed at high levels of p53 expression. By way of comparison, transactivation from the con D half-site was comparable to levels obtained from the low-responding 14-3-3 σ RE.

To assess the ability of p53 to transactivate from decamer half-sites in a mammalian system, luciferase reporter vectors containing the p21-5' RE half-sites were transiently transfected with or without a vector containing p53 under the CMV promoter into p53 null SaOS2 cells [59]. As shown in Figure 3.3B (also see Figure 3.2B), WT p53 induced transactivation from the full p21-5' RE was 35-fold greater than with an empty pGL3-P vector. However, there was clear induction from the left and right half-sites, 4- and 6-fold, respectively. These results correlated well with the relative p53 occupancy assessed by ChIP: 4% for the full RE, 0.4% for the left decamer and 0.9% for the right decamer and agree with the previous findings in Menendez et al. [60] that p53 can function from a half-site RE. Based on these findings, the p53 transcriptional network may incorporate more downstream targets than previously predicted.

p53 transactivation from decamer half-sites is sequence-dependent

The sequence requirements for p53-dependent transactivation were examined further, utilizing a plasmid-based haploid yeast system with rheostatable p53 expression (previously used to assess transactivation capacity of canonical full-site REs; see Materials and Methods and [30]). Similar to results from mammalian cells, p53 was able to weakly transactivate

from p21-5' half-site REs at a level that was ~1% of the levels of transactivation from the p21-5' full-site RE (Figure 3.3C). This result differed somewhat from that of the diploid yeast system where transactivation from the p21-5' half-site REs was <0.5% of the transactivation from the full-length p21-5' RE (Figure 3.3A). Transactivation from Con D was greater than p53 transactivation from either of the p21-5' half-sites and Con D was greater than Con G in both systems.

We also examined the impact of changes in the WW of the core CWWG (W = A or T). The 3 bases on either side consisted of GGG and TCC which had been shown to enhance binding at full-site REs [33] (Figure 3.3C). Decamers required the central CATG motif at the junction of the monomer binding sites for modest levels of transactivation, ~7% of the levels from the p21-5' RE (Figure 3.3C). Altering the motif from CATG to CAAG or CTTG decreased transactivation another 2- to 3-fold whereas changing this motif to CTAG nearly eliminated transactivation.

Transactivation was also assessed with various combinations of sequences surrounding the CATG core domain. As shown in Figure 3.3C, transactivation with the GGG/CCC flanking sequence was comparable to the GGG/TCC sequence tested with the CWWG motifs. However, transactivation was reduced when other alterations were made. For example, a change in flanking sequence to GGG/CTC or GGA/CTC resulted in almost no transactivation.

p53 functionality from noncanonical, 3/4-site REs in yeast

We also addressed the ability of p53 to transactivate from 3/4-site REs. A consensus binding site was created for each of the two possible configurations of a 3/4-site RE. The first,

designated Con J, consists of a $\frac{1}{4}$ -site directly adjacent to a $\frac{1}{2}$ -site, whereas the second, designated Con K, contains a 5 base spacer between the $\frac{1}{4}$ -site and the $\frac{1}{2}$ -site. Transactivation in the diploid yeast system from the Con J and Con K $\frac{3}{4}$ -site REs was 25% and 18%, respectively, of the full p21-5' RE level (Figure 3.4A), at high p53 expression (0.024% galactose). This was substantially more than observed for half-site REs. Similar findings were observed in the haploid yeast system (Supplemental Figure 3.2A). Further evaluation of the sequence requirements for p53 transactivation from $\frac{3}{4}$ -sites with the haploid yeast system showed p53 could transactivate from a $\frac{3}{4}$ -site RE containing a CTTG core (Supplemental Figure 3.2B). Transactivation was also effected by surrounding flanking sequences.

Canonical REs containing spacers may actually be noncanonical $\frac{3}{4}$ -site REs

In our examination of full-site RE sequences containing spacers, several REs contradicted our finding that large spacers between decamer half-sites abolish p53 transactivation. For example, the PIDD RE (p53 induced protein with death domain) [61] which promotes apoptosis has an 8 nt spacer in its RE. Contrary to our expectation, high expression of WT p53 resulted in ~20% of the level of transactivation from the p21 5' RE. As expected, removal of the 8 nt spacer between the decamer half-sites increased the levels of p53 transactivation (Figure 3.4B).

Examination of the PIDD RE sequence suggested that rather than functioning from a canonical full-site RE, p53 might transactivate from a noncanonical $\frac{3}{4}$ -site RE. The canonical PIDD element was first separated into two noncanonical $\frac{3}{4}$ -site REs (designated PIDD $\frac{3}{4}$ A and PIDD $\frac{3}{4}$ B), both of which utilized the “spacer” sequence as part of the binding element.

The noncanonical REs were comprised of a $\frac{1}{4}$ -site directly adjacent to a $\frac{1}{2}$ -site, but differed in the central CWWG motif in the half-site and number of mismatches from the consensus binding sequence. Transactivation assays revealed p53 could function from the PIDD $\frac{3}{4}$ A sequence to levels equivalent to the canonical PIDD RE containing the 8 nt spacer at high expression (Figure 3.4B). In contrast, WT p53 could not transactivate from the PIDD $\frac{3}{4}$ B RE. These findings showed that the PIDD RE was neither a true canonical consensus RE nor an exception to the “spacer” rule, but rather a noncanonical $\frac{3}{4}$ -site RE which can support p53 transactivation.

The results of the PIDD RE sequence analysis suggested that other known p53 REs previously identified as canonical full-site REs are actually $\frac{3}{4}$ -site REs. Based on an empirically derived set of RE rules previously established in our lab to predict p53 transactivation capacity [14, 30, 62], several established p53 target REs were re-examined to determine if their responsiveness to p53 was actually due to the presence of a noncanonical $\frac{3}{4}$ -site RE instead of a full-site RE. As shown in Figure 3.4C, the REs of p21-3', PCNA, 14-3-3 σ (site 2) and Apaf-1 REs were actually comprised of a genuine $\frac{3}{4}$ -site binding element (containing a $\frac{1}{2}$ -site directly adjacent to a $\frac{1}{4}$ -site) followed by an additional sequence that vastly deviated from a consensus $\frac{1}{4}$ -site. Luciferase assays in the diploid yeast strains revealed that p53 transactivation from these sites was clearly reduced in comparison to transactivation of p21-5' RE (Figure 3.4C), but comparable to the levels of transactivation from the consensus $\frac{3}{4}$ -site REs, Con J and Con K (see Figure 3.4A). These results are consistent with p53 transactivation capacities towards the p21-3', PCNA, and Apaf-1 REs assessed with the haploid yeast system in a recent study focused on examining conservation of RE sequence and conservation of RE functionality [62].

Structural requirements for p53 transactivation from 1/2- and 3/4- site REs

We examined several p53 mutations that could address the structural requirements for transactivation from noncanonical REs (1/2- and 3/4-site REs) in comparison to canonical REs (weak and strong full-sites). The following REs were examined: 1/2-site REs (con G, con B, p21-5' left, and p21-5' right), 3/4-site REs (con J and con K), as well as weak (TIGAR and Gadd45) and strong (p21-5' and TIGAR-spacer) full-site REs. Mutations in the oligomerization domain were analyzed for transactivation capacity to determine the extent to which the tetramerization of p53 facilitated functionality from noncanonical REs. Also examined were N-terminal transactivation domain and C-terminal regulatory domain mutants. The results are summarized in Table 3.1 and presented relative to transactivation from the p21-5' RE. The variation in transactivation by the p53 mutants towards the REs was not due to differences in levels of expressed p53 protein (Supplemental Figure 3.3).

The Arg337 residue is located on the surface of the tetramerization domain and plays a role in tetramer stabilization through a salt bridge with Asp352 on the opposite monomer [63-67]. The mutations R337C and R337H, associated with Li-Fraumeni-like syndrome (LFL) and adrenal cortical carcinoma (ACC), respectively, appear to compromise the ability to form tetramers. The impact of the R337C mutation (previously described as a partial function mutation) on p53 functionality has been postulated to arise by a change in thermodynamic stability, shifting the equilibrium from the tetramer to dimer and/or monomeric states [68, 69]. The R337H mutation is considered to have a subtle functional effect causing a pH dependent effect on folding [63, 68, 70-73] (Storici and Resnick, unpublished data).

In agreement with previous findings by Lomax et al.[68], R337C was found to be an altered function mutation in the diploid yeast and SaOS2 cell systems, significantly diminishing transactivation from both canonical and noncanonical REs. Transactivation from the strong, full-site REs was reduced to less than 25% of the levels of WT p53 (“+” in Table 3.1), whereas transactivation from the weaker full-sites and the $\frac{3}{4}$ -site REs was barely detectable (“+/-” in Table 1) with no transactivation from the $\frac{1}{2}$ -site REs.

Similar to previous results [68], the p53 R337H mutant could transactivate from the p21-5' RE to levels comparable to WT p53 in the haploid yeast system. However, although R337H could function from the p21-5' RE in the diploid yeast and SaOS2 cell transactivation assays, the level was reduced in comparison to WT p53. Similar results were found for transactivation from the weaker full-site REs and $\frac{3}{4}$ -site REs. The differences between WT and mutant p53 were not as clear for the $\frac{1}{2}$ -site REs.

L344 is one of five residues that comprise the hydrophobic core of the α -helices which form the interface for p53 dimer-dimer interactions [64, 66, 67, 74, 75]. The mutation L344A prevents tetramer formation although stable dimers are formed [66, 74, 76, 77]. The missense mutation L344P disrupts the helix [68, 78-80] and is associated with Li-Fraumeni syndrome (LFS). This alteration prevents dimer formation resulting in monomeric p53 protein [68, 69, 80-83]. Within the haploid and diploid yeast systems, the L344A protein had reduced transactivation activity towards the strong canonical full-site REs and noncanonical $\frac{3}{4}$ -site REs (Table 3.1) and no activity towards half-sites. However, the L344A protein was capable of transactivating to low levels from the p21-5' half-site REs within mammalian cells. Similar results were seen in SaOS2 cells with an N345S dimer mutation, where

transactivation was measurable, but significantly reduced in comparison to WT p53 transactivation (data not shown).

Consistent with previous findings showing L344P is a loss-of-binding mutation, the monomeric p53 L344P was not able to transactivate from the full-site, $\frac{3}{4}$ -site, or $\frac{1}{2}$ -site REs tested within the 2n yeast or SaOS2 cells (Table 3.1) [68, 69, 80-83]. Similarly, the germline mutation L330H, which is predicted to form only monomers through destabilizing the β -strand of the tetramerization domain [74, 81, 84], was also inactive for transactivation from the p21-5' full-site RE and half-site REs in the mammalian system (data not shown).

Finally, deletion of the tetramerization domain (Δ 325-357) or a terminal truncation (331 stop: similar to the p53 β isoform) resulted in complete loss of transactivation from any of the binding sequences examined, including full-site REs, in the yeast or SaOS2 cell systems (data not shown). These results were in agreement with previous findings that the tetramerization region is necessary for p53 binding to and transactivation from a RE [77, 85].

Previously, we reported that the terminal deletion Δ 368 resulted in reduced transactivation capacity towards full-site REs when assessed with a yeast *ADE2* reporter plate assay (on plates) in haploid yeast [30]. Those studies have been extended to address its ability to transactivate from full-, $\frac{3}{4}$ - and $\frac{1}{2}$ -site REs. We found that removal of the C-terminal tail resulted in at most a modest transactivation from the noncanonical and canonical REs (Table 3.1). The inhibition varied with the strength of the RE sequence, as well as with level of p53 expression (data not shown) for all REs. Thus, in comparing WT to mutant, the C-terminal domain of p53 appears in several cases to actually enhance rather than repress transactivation from canonical, as well as noncanonical REs.

Finally, we also examined a deletion in the N terminal domain, $\Delta 1-39$, to assess if transactivation from $3/4$ - and $1/2$ -site REs was differentially affected. The mutation resulted in <25% residual transactivation from the full-site REs (strong: p21-5' and TIGAR – spacer; moderate: GADD45) at low expression in the haploid yeast system (data not shown). At the high expression levels required to examine noncanonical REs (0.128% galactose), transactivation was also reduced from $3/4$ -site REs (con J and con K) and a $1/2$ -site RE (con G) suggesting that the canonical and noncanonical REs are similarly affected by defects in the transactivation domain.

Discussion

Given the role for p53 in assuring genome stability, it is important to understand how this master regulator functions as a sequence-specific transcription factor. The interactions of p53 bound to DNA, as well as the mechanisms by which WT or mutant p53 can transactivate to different extents from the many variants of the consensus motif are not completely understood. In this work, we have developed a matrix of factors influencing transactivation that include WT and mutants, sequence and motif, and DNA binding. We have established that p53 can function from many noncanonical $1/2$ - and $3/4$ -site REs that are common to the genome. Importantly, functionality of a sequence is not directly predictable by relation to the consensus sequence.

Spacers can have opposite effects on transactivation

Previous studies have shown that widely-separated full-size REs associated with the same p53 target gene, such as the muscle creatine kinase (MCK), can interact to

synergistically transactivate the associated target gene [86]. The mechanism has been suggested to involve looping out of the intervening DNA so that multiple p53 tetramers can “stack” and concentrate the basal transcription machinery [86, 87]. While this mechanism of transactivation may hold for REs separated up to 3 kb, the synergy was proposed to be lost when the distance was less than 25 bases due to steric hindrance [86], an arrangement that holds for several p53 target genes including MDM2. We found that p53 could function synergistically from the two weak MDM2 REs separated by a 17 and 10 nt spacer, but further reduction to 5 nt resulted in additivity.

Interestingly, the length of the spacer between the two REs within the promoter of *Mdm2* has been conserved between mouse and human although the sequence of the spacer is 50% diverged. In the haploid yeast system, p53 was found to function similarly from the human and murine MDM2 REs in terms of synergistic transactivation [62]. Given the divergence in the sequence of the spacer, it is unlikely that an additional transcription factor binding site would be found in the 17 nucleotides that would allow a second transcription factor to interact cooperatively with p53 to induce the observed synergistic transactivation from the two REs. Instead, there may be a functional conservation that assures the level of p53-mediated transactivation. This is supported by sequence analysis across 14 species, corresponding to at least 70 million years of evolution, where 12 had maintained the 17 nucleotide spacer (with varying degrees of sequence divergence) between RE1 and RE2 (including chimp, rat, rabbit, dog, elephant, armadillo and hedgehog), while opossum and bat had a one nucleotide indel [88, 89]. From this preliminary search, it appears that the spacer sequence is under stabilizing selection such that variations in the length of the spacer which would affect the ability of p53 to synergistically function from these sites were not observed.

Interestingly, a similar phenomenon, is observed in the DNA sequences within and between (but not flanking) cis-regulatory elements of the *otx*, *delta*, *wnt8*, and *brachyury* genes where insertions or deletions of random sequence do not occur as assessed by a comparison between the orthologous cis-regulatory regions and flanking sequences of the sea urchins, *Strongylocentrotus purpuratus* and *Lytechinus variegatus* [90]. We are currently investigating whether functional conservation of spacer length as a mechanism for regulating p53 functionality holds with other closely-spaced full-sized p53 REs.

The impact of the spacer between full-sites acts opposite to that of a spacer between half-sites. In previous studies, it was proposed that REs had a “rotational specificity” where spacers between half-sites could abrogate p53-dependent transactivation if they were on opposing faces of the DNA helix, but had little impact on transactivation when the half-sites were on the same face [18, 53, 54]. Recently, in electrophoretic mobility shift binding assays (EMSA), a 2 nt spacer did not significantly alter p53 affinity (K_d) towards a consensus RE [29]. Furthermore, in a 3D model derived through cryoelectron microscopy of full length p53, where p53 dimers are proposed to form through interactions of alpha-helices on the N- and C-termini rather than alpha helices on the C-termini, the best fit RE sequence had a spacer length of 6 nucleotides [91]. These *in vitro* findings imply if p53 transactivation is primarily dependent on the ability of p53 to bind target REs, a small spacer would not affect p53-dependent transactivation.

The effect of spacer upon transactivation from a RE at varying levels of p53 was first analyzed with the WAF1/Cip1 p21-5' RE. The present results are in agreement with an earlier report showing that increases in spacer length of 4 or 14 bases between half-sites decreases p53 transactivation in yeast [18]. However, there is no evidence for rotational

specificity influencing transactivation. This is the first demonstration that a spacer of 1 or 2 nt between half-sites can dramatically affect the ability of p53 to transactivate from a RE. Importantly, the impact of spacer on the ability of p53 to transactivate from a RE is greatly influenced by the intrinsic potential transactivation strength of the RE, as shown for NOXA, as well as level of p53 expression. We also established with the haploid yeast system that a spacer had a similar negative effect on the ability of p53 family members, p63 β and p73 β , to transactivate from a RE.

The biological importance of spacer as a means for affecting response to p53 was clearly demonstrated for the TIGAR RE. We propose that unlike the implications from the established consensus RE, a spacer between half-sites in natural elements serve to modulate the levels to which p53 can regulate the associated target gene and may play an important mechanistic role in the evolution of RE responsiveness to p53. In the case of TIGAR, the opportunity to address evolutionary implications is limited due to the apparent recent emergence of the putative p53 binding site in the primate lineage, however, the orthologous TIGAR RE in *Pan troglodytes* (chimpanzee), and *Macaca mulatta* (rhesus monkey) also contain a 2 nt spacer [88, 92].

Binding vs. transactivation

Several modes of binding have been postulated for p53 to a RE containing a spacer that include shifting of the tetramer on the DNA to compensate for the reduced protein-protein interactions, bending and/or kinking of the DNA within the spacer and/or RE and rotational motions of p53 to accommodate supercoiling of the spacer sequence [24, 27]. In any of these, binding would modify either the conformation of the p53 tetramer and/or the

DNA sequence itself in a fashion that is not predictable from current structural studies. While functional studies demonstrate that the RE sequence can dramatically influence the level to which p53 can transactivate from a specific RE [3], the relationship between *in vitro* binding and efficiency of *in vivo* transactivation had not been established prior to this study.

We utilized a novel fluorescent microsphere, semi-*in vitro* binding assay to determine the effect of spacer upon p53 binding. Recently, we demonstrated a good correspondence between binding and *in vivo* transactivation for several human p53 target REs (Noureddine et al., submitted). While differences were observed between the *in vivo* and *in vitro* assays for p53 binding to the p21-5' RE containing a single nucleotide spacer, increasing the length of a spacer between the decamer half-sites beyond one nucleotide had a large impact on the ability of p53 to bind in the semi-*in vitro* assays, contrary to reports with pure components [22, 23]. Interestingly, p53 binding to a RE with a ≥ 3 nt spacer was nearly equivalent to binding to the individual p21-5' half-site REs, suggesting that p53 may recognize each decamer sequence as a distinct binding motif once a spacer increases beyond a certain length. These findings strongly argue that the 0-13 nucleotide spacer in the established p53 consensus sequence, which is based on interactions between purified protein and REs, should be reduced to no more than a few bases. It appears that factors present in nuclear extracts or cells limit the ability of p53 to recognize REs with spacers between half sites. It will be interesting to identify these putative spacer-discriminating factor(s).

p53 functionality from 1/2-site and 3/4-site REs

The observations that p53 can function, as defined by binding and transactivation, from consensus half-site REs in yeast and from the p21-5' half-site REs in mammalian cells

indicate the p53 transcriptional network is comprised of many more downstream targets than previously predicted. Transactivation from half-sites is strongly dependent upon the targeted sequence and level of p53 expression. Similar to full-site REs [30], the central binding motif at the monomer junction (*i.e.*, CATG) had the greatest impact upon the ability of p53 to function from half-sites. The difference between the yeast and mammalian systems in functionality from the half-sites indicates that additional co-factors in human cells may assist in p53 interactions with weaker binding sequences. Tetrameric p53 can bind full-site and half-site binding elements *in vitro*, but the p53 bound to a half-site had a much higher disassociation rate *in vitro* as indicated through “trap” assays that had measured dissociation of p53 from a labeled RE sequence [22].

Given the higher probability of a decamer sequence occurring in the genome compared to a 20 base sequence, it will be interesting to determine the number of functional p53 half-site REs in the human genome which is composed of ~3 billion base pairs. In a preliminary genomics screen, we have identified over 1,400 “perfect” p53 half-sites containing a CATG core motif in the genome within 2 kb of a transcriptional start site. However, the question remains as to which of these sites will have a relevant biological function in p53-dependent stress responses or whether the sequences are merely “noise” within the genome.

Half-sites could serve many roles in the genome. For example, a collection of half-sites could affect chromatin accessibility to transcriptional machinery (*i.e.*, loosening the chromatin or recruiting and sequestering chromatin modifiers). Such sites might function in the proposed opening of the genome to make it more available for repair [93]. On the other hand, half-sites might also serve to titrate p53. Additionally, p53 half-sites may play a role in

bringing together different transcriptional networks as described for a SNP in the FLT1 promoter [41, 60]. Subsequent studies of the Flt-1 half-site have shown that p53 bound to the T-SNP can cooperate with estrogen receptor (ER), also bound to a half-site of its own consensus sequence, to synergistically transactivate from the Flt1-T promoter [60]. Such co-regulation may be advantageous for precise fine-tuning of p53-regulated responses and may make activity from the noncanonical $\frac{1}{2}$ -site REs more dependent on availability of cooperating transcription factors or additional cofactors and on levels of nuclear p53.

Within yeast, p53 was able to transactivate from noncanonical consensus $\frac{3}{4}$ -site REs containing a $\frac{1}{4}$ -site adjacent to a $\frac{1}{2}$ -site or a $\frac{1}{4}$ -site separated from a $\frac{1}{2}$ -site by a 5 nt spacer. The lack of a significant difference between the transactivation from both $\frac{3}{4}$ -site REs imply that p53 encounters the $\frac{3}{4}$ -site RE as a tetramer protein. Transactivation from $\frac{3}{4}$ -site REs was moderate relative to full-sites. Furthermore, the hierarchy of transactivation, in terms of the level to which p53 could bind and transactivate *in vivo*, was comparable to earlier *in vitro* studies that measured p53 binding efficiencies towards $\frac{1}{2}$ -sites, $\frac{3}{4}$ -sites and full-site sequences with competitive gel retardation assays [53]. The impact of a $\frac{3}{4}$ -site was revealed in our study of the RE for the PIDD gene where p53 transactivation was substantial even though the RE contained a spacer greater than 3 nt. Transactivation assays revealed the responsiveness of the originally described PIDD RE was actually due to a noncanonical $\frac{3}{4}$ -site RE. Transactivation from the PIDD $\frac{3}{4}$ A RE, but not the PIDD $\frac{3}{4}$ B reinforces the requirement for a strong core element for functionality and that a functional RE cannot contain greater than 3 mismatches within a half-site. There are likely to be many functional $\frac{3}{4}$ -site REs in the genome, among which are sequences that were originally considered as canonical, full-site REs containing >2 nt spacers. Furthermore, as recently pointed out in our

functional conservation analysis of p53 REs from several species, there are clear examples of weaker, apparently noncanonical $\frac{3}{4}$ -site REs, such as p21-3' and APAF1 being conserved in evolution [62].

While this report is the first to systematically evaluate p53 function from noncanonical $\frac{1}{2}$ - and $\frac{3}{4}$ -site REs, there are other reports of a transcription factor binding to noncanonical binding sites within the genome. Johnson *et al.* [94], recently mapped the *in vivo* interactome for the transcription factor neuron-restrictor silencing factor (NRSF) using a large scale CHIP analysis and found that NRSF bound to noncanonical half-site binding motifs. A variety of approaches such as ChIPSeq or FAIRE, formaldehyde-assisted isolation of regulatory elements, may be able to reveal interactions at noncanonical sites under different stress conditions and in various cell types [95, 96] to better understand the dimensions of the p53 master regulatory universe.

Transactivation from $\frac{1}{2}$ -site and $\frac{3}{4}$ -site REs require tetrameric p53

Having established WT p53 interacts with sequences that do not fully conform to the canonical p53 binding sequence, a panel of p53 mutants was analyzed to determine what structural features of p53 play a role in transactivation from $\frac{1}{2}$ - and $\frac{3}{4}$ -site REs versus full-site REs. Together, the mutations suggest that common functional aspects of p53 are required for the ability of p53 to function from full, $\frac{3}{4}$ -, and $\frac{1}{2}$ -site REs. In general, the observed transactivation of oligomerization mutations towards full-site REs was in agreement with previous reports of mutants able to form tetramers or dimers retaining transactivation towards strong REs, while monomeric proteins were inactive [83].

Having established WT p53 interacts with noncanonical sequences, a panel of p53 mutations was analyzed to determine what structural features of p53 play a role in transactivation from 1/2-site and 3/4-site REs versus full-site REs. The observed transactivation of oligomerization mutations towards full-site REs has confirmed previous reports of mutants able to form tetramers or dimers retaining transactivation towards strong REs, while monomeric proteins were inactive [83]. Together, the mutations suggest that common functional aspects of p53 are required for the ability of p53 to function from full, 3/4, and 1/2-site REs.

The DNA binding domain of p53 has been shown *in vitro* to bind DNA in the absence of the oligomerization domain and that dimeric p53 can bind to half-sites in a cooperative fashion independent of tetramerization [22, 97]. While able to transactivate at a low level from strong full-site REs and 3/4-site REs, the L344A dimerization mutation was unable to transactivate from the weak full-site REs or consensus half-site REs in yeast. In agreement with these findings, Waterman *et al.* previously showed that the L344A protein could bind *in vitro* to oligonucleotides containing an optimal consensus binding site and/or half-site, but not to an oligonucleotide containing a suboptimal full-site [77].

The impact of the dimer mutation L344A, as well as N345S, was less severe in mammalian cells. Similarly, a designed dimer mutant (Met340Gln/Leu344Arg) was capable of transactivating from a p21 full-size RE within SaOS2 cells to half the level of WT p53 suggesting a direct correlation between the oligomeric state and transactivation activity [69]. While L344A dimeric proteins may bind to the sites in a cooperative fashion, tetramerization which is a necessity for efficient transactivation may be required to stabilize the binding and

reduce the off-rate of p53 from the DNA in yeast. It is possible that other factors in SaOS2 cells may contribute to this stabilization accounting for the difference between the systems.

Since altered tetramer proteins and dimers exhibited a reduced ability to transactivate from noncanonical REs, transactivation is not simply an additive process determined by the number of available pentamer sequences. It is possible that binding and transactivation *in vivo*, but not binding alone, may require a conformational change of the p53 protein that is only obtainable with a tetrameric protein. Several transcription factors have displayed such a characteristic including the heat shock protein which can bind chromatin weakly as monomer, but is not sufficiently active to cause a biological response until after it is induced and forms a trimer [98, 99]. Furthermore, in the case of a half-site, where there is not a sequence to which the second p53 dimer can bind, tetramerization may assure the second dimer is in the vicinity of the p53 binding element in order to interact with other factors required for transactivation.

The finding that the p53 $\Delta 368$ mutation reduced transactivation from both canonical and noncanonical REs agrees with studies showing that p53 does not require modification of the C-terminal to engage its target binding sites [31, 32]. Furthermore, these results indicate that the C-terminal is not absolutely essential for function from canonical or noncanonical sites, but may play a role in regulating the level of transactivation.

The deletion of the first N-terminal transactivation domain (TAD) which mimics a naturally occurring alternatively spliced form of p53 that is differentially expressed in breast tumors, had a more severe impact upon transactivation than previously reported with the qualitative *ADE2* color reporter (data not shown) [30, 100]. The results may reflect the greater quantitative assessment of transactivation with the luciferase assay. Nevertheless,

both assays revealed that the decreased opportunity to interact with the transcriptional machinery through the loss of the transactivation domain strongly affects p53 transactivation from both canonical and noncanonical REs.

Conclusions and Implications

Through deconstructing the canonical consensus sequence and assessing functionality, as determined by binding and transactivation within three *in vivo* model systems and a semi-*in vitro* binding assay, it has been possible to refine the requirements for functional p53 binding elements. The organization and arrangement of binding motifs, as well as the level of p53 expression, have a large impact on the ability of p53 to transactivate from a RE sequence. It is interesting that p53 may not function from reported canonical consensus REs yet functions from noncanonical REs that are common to the human genome. Half-sites and spacers between full-sites may provide additional levels of regulation in p53 transcriptional network. Truly functional REs may be restricted to decamers separated by <3 nucleotides. When the spacer increases beyond 3 nucleotides, we suggest p53 recognizes the half-sites as separate binding entities.

The ability of p53 to function from noncanonical decamer half-sites introduces a new realm of target sequences and genes into the p53 transcriptional network and expands the universe of p53 regulated genes. Noncanonical sequences might provide p53 responsiveness at high levels of expression or in combination with other transcription factors, as for the case of the *Flt-1* gene [60]. As discussed in the “piano model” for transactivation from a broad range of REs [14], variation in the level of p53, as well as various mutants can markedly affect the spectrum of p53 responses. Determining the relationship between expression levels

and responsiveness at canonical and noncanonical target sequences is important in understanding how p53 implements cellular fate in response to stress, such as cell cycle arrest or apoptosis. It is also important for addressing the consequences of p53 alterations, particularly those mutations that retain transactivation capabilities, as well tailoring individual therapies.

Figure 3.1A

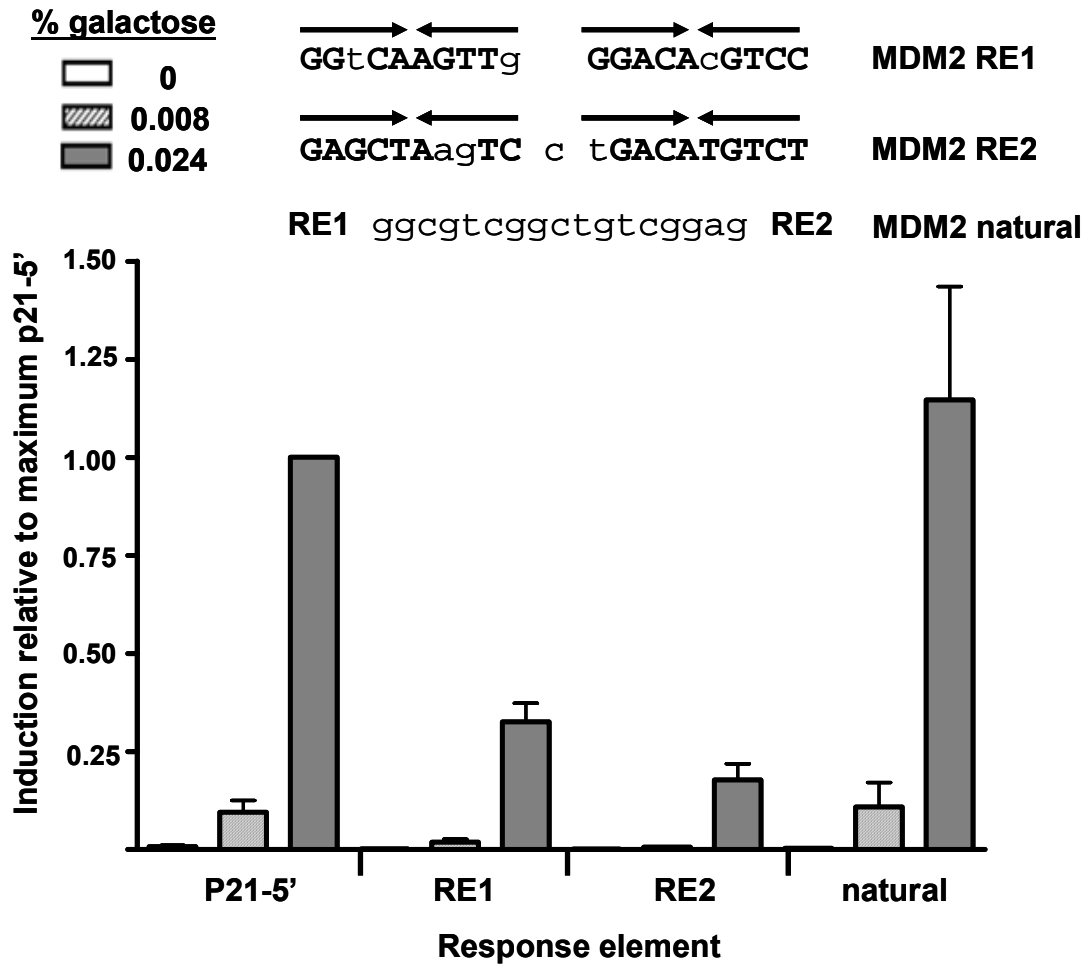
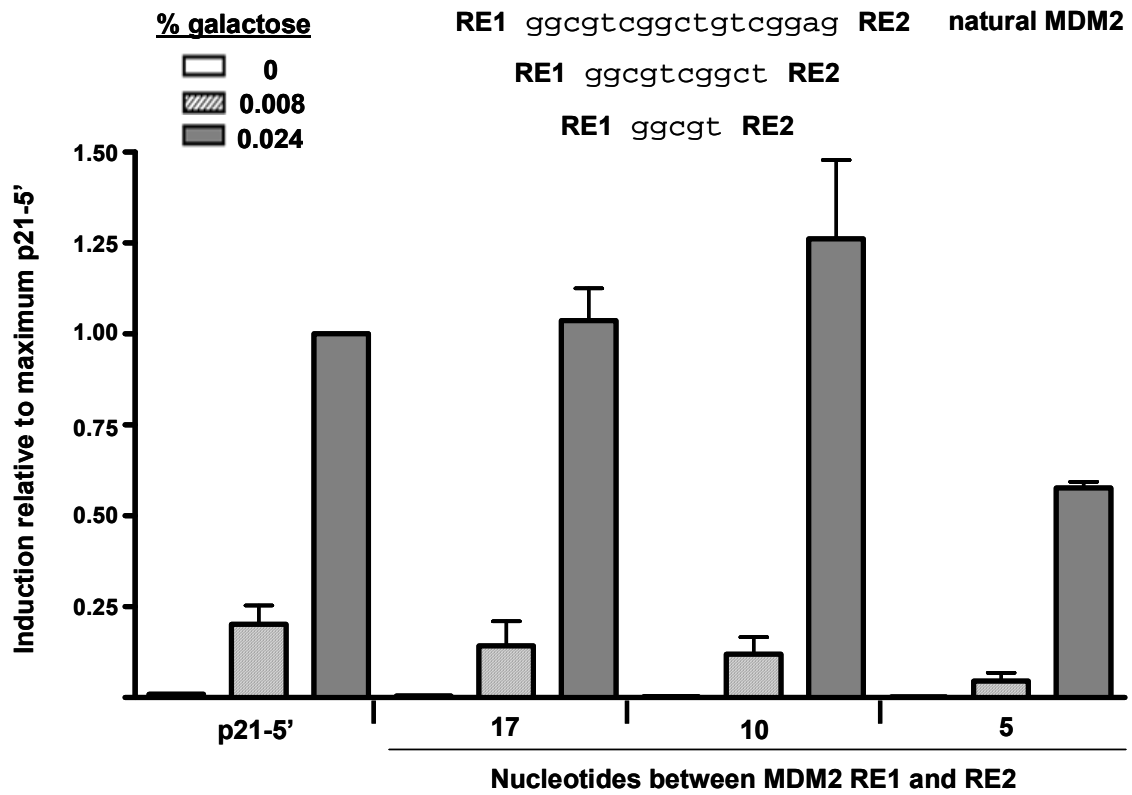


Figure 3.1 Weak REs can function synergistically when separated by a spacer. (A) To ascertain if p53 functions from the two full-site REs of MDM2 independently or if the REs interact, p53 transactivation from the isolated REs, as well as the natural MDM2 RE containing a 17 nucleotide spacer were evaluated. Isogenic diploid yeast strains containing the p21-5' and MDM2 REs, as indicated, were grown in increasing concentrations of galactose to induce p53 protein to basal, moderate and high levels of expression. The ability of wt p53 to transactivate from RE sequences was measured by a luciferase assay 24 hours after inoculation into the galactose supplemented media. Induction from each RE was compared relative to the ability of p53 to transactivate from the p21-5' RE at 0.024% galactose and is depicted as the mean and standard error of measurement (SEM) of 6 independent experiments. The average light units/ug protein from p21-5' at 0.024% galactose was 2.1 million. Solid arrows over the sequences indicate a $\frac{1}{4}$ -binding site.

Figure 3.1B



(B) Impact of reducing the spacer to 10 and 5 nucleotides. The average light units/ug protein for p21-5' at 0.024% galactose was 2,800,000.

Figure 3.2A

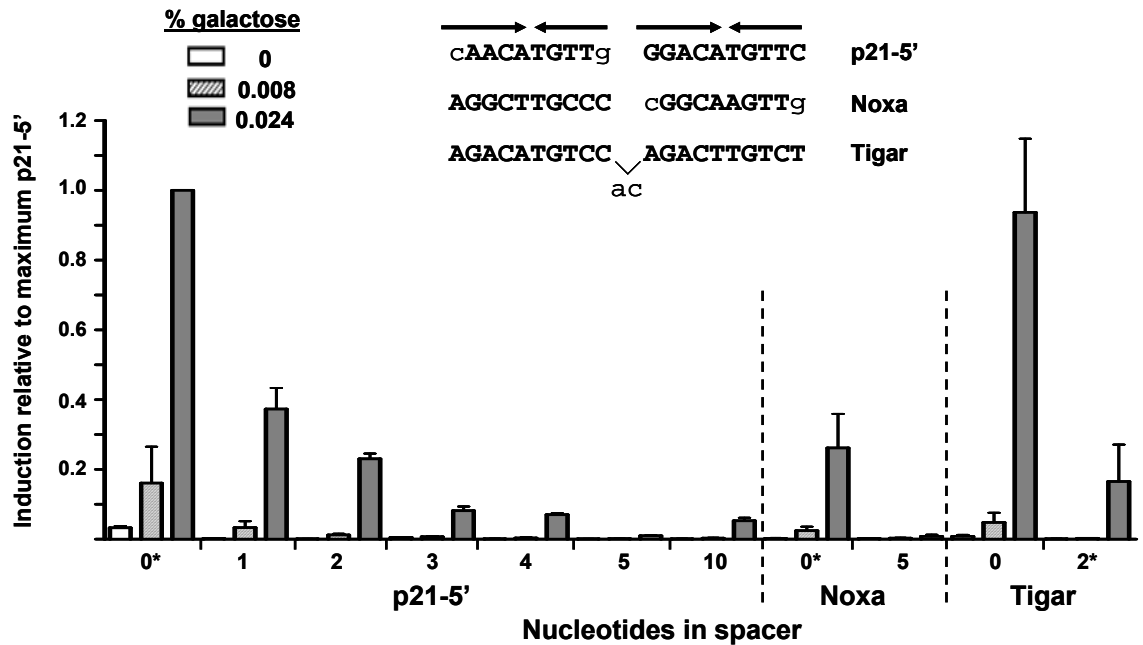
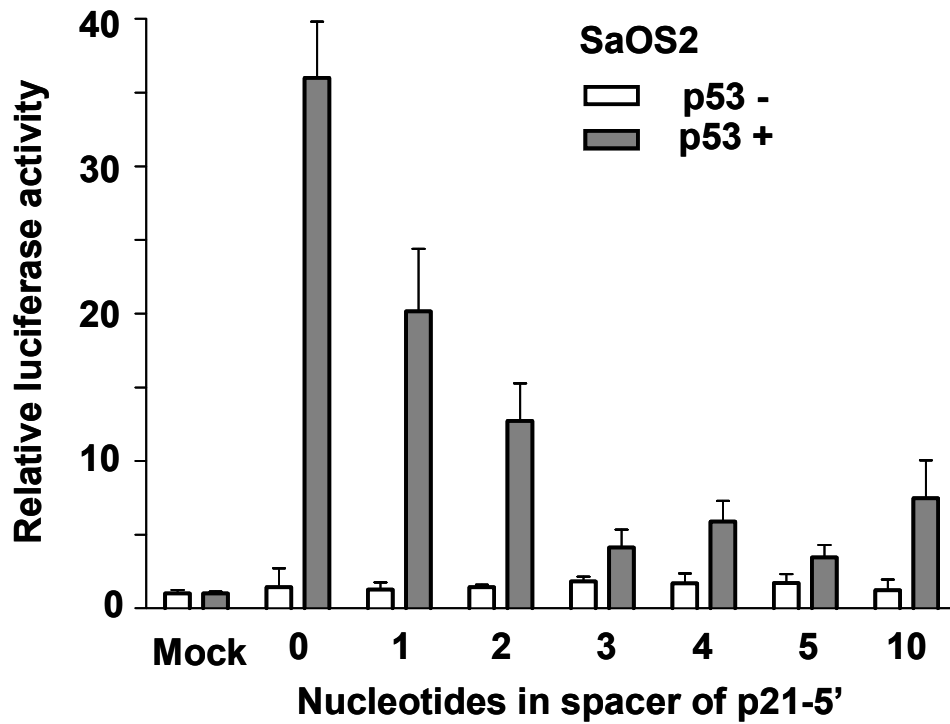


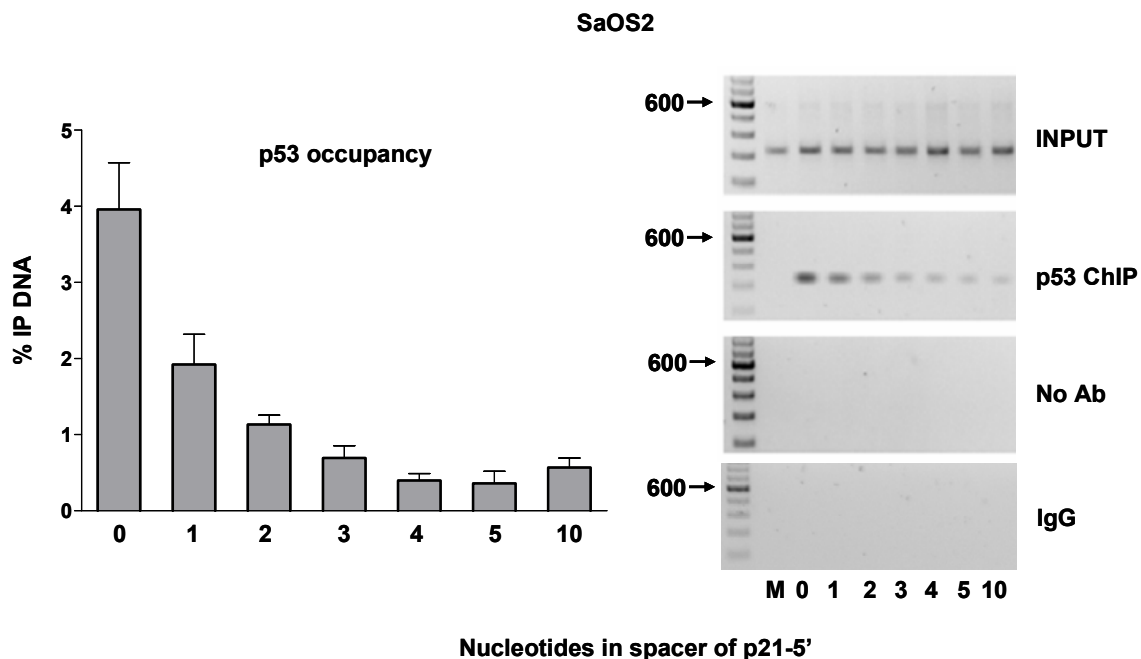
Figure 3.2 Spacer decreases p53 transactivation, promoter occupancy and binding in yeast and mammalian cells and *in vitro* (A) Transactivation in yeast. The ability of p53 to transactivate from REs containing spacers of variable nucleotide length sequences was measured 24 hours after p53 induction with a quantitative luciferase assay. Induction from each RE at various p53 expression levels were compared to the induction from the p21-5' RE at 0.024% galactose. The average light units/ug protein for WT p53 towards p21-5' at 0.024% galactose for a minimum of 6 biological repeats was 1.26 million. *indicates the number of nucleotides in the spacer of the natural RE. Solid arrows identify a 1/4-binding site.

Figure 3.2B



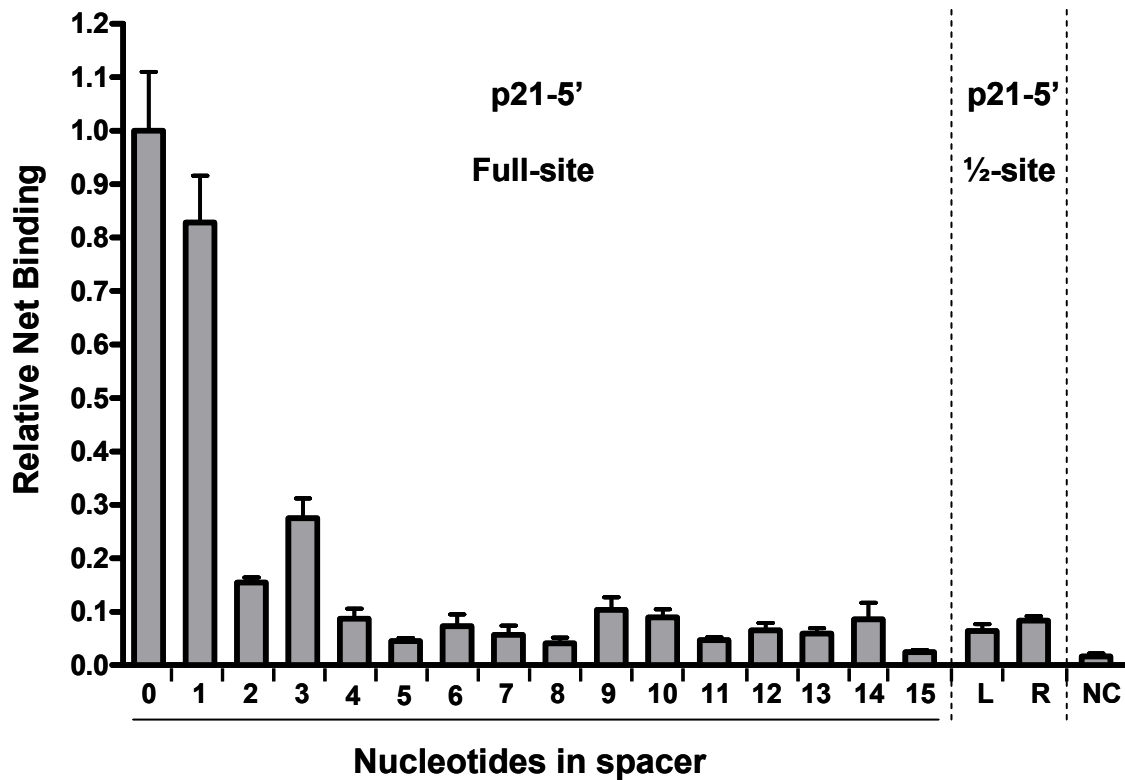
(B) Human SaOS2 cells were transfected with p21-5'::luciferase reporter constructs containing spacers of increasing length between decamer half-sites in the presence (solid bars) or absence (open bars) of the high expressing pCMV-p53wt vector. At 48 hours post transfection, induction of the luciferase reporter was assessed. Relative luciferase activity was compared to the pGL3-P plasmid lacking the p53 RE (mock) and is represented by the average and standard deviations of three independent experiments, each containing three replicates.

Figure 3.2C



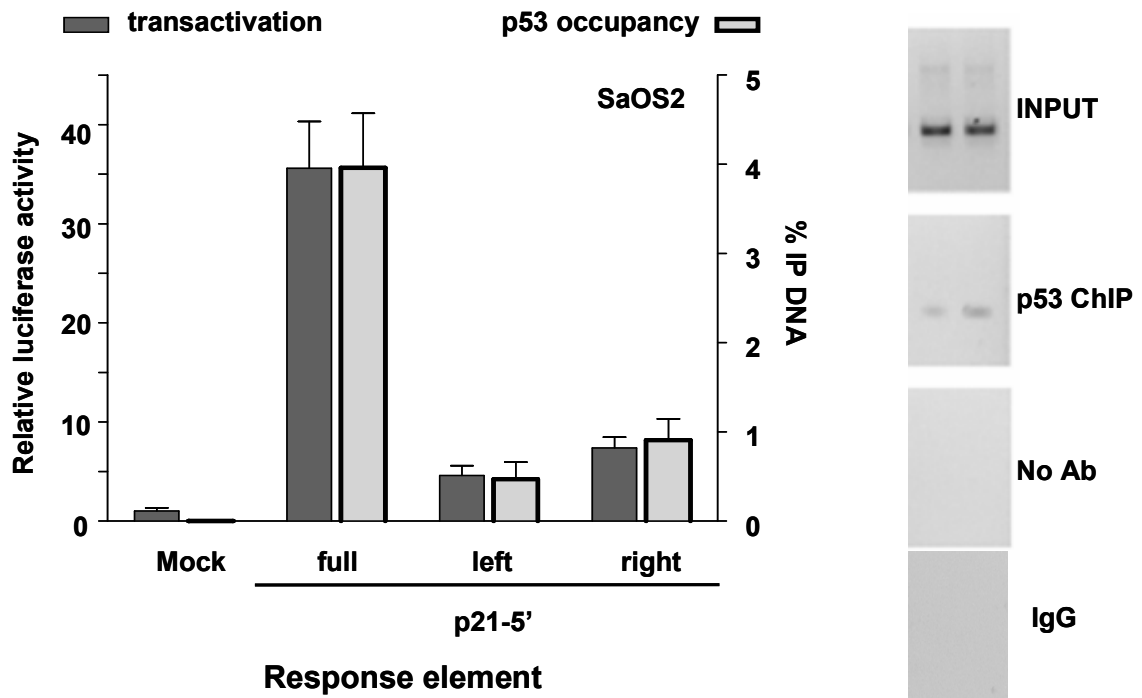
(C) Occupancy of p53 at p21-5' REs in human cells. The p21-5' promoter constructs containing the increasing spacers between half-sites were co-transfected with p53 into SaOS2 cells (as described in (B)). Twenty-four hours later, ChIP analysis was performed. Presented are the average and standard deviation from 4 independent experiments (left). PCR products of the Input DNA (input) and ChIP DNA (p53, IgG, or no antibody, Ab) are shown (right). The "M" corresponds to a pGL3-P plasmid control lacking the p53 RE. No bands were observed above the 600 bp markers.

Figure 3.2D



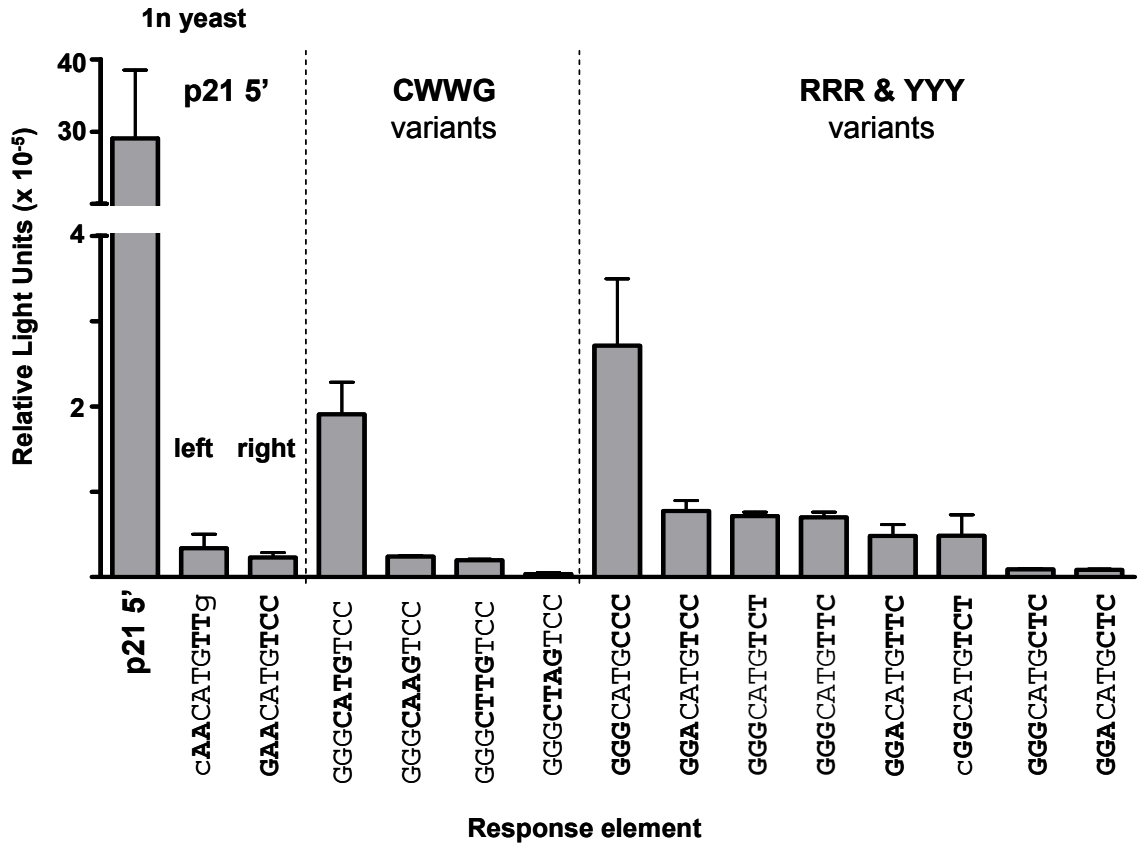
(D) *In vitro* fluorescent microsphere binding assay to evaluate sequence-specific p53-DNA binding interactions (see Materials and Methods). Fluorescent microspheres bearing double stranded DNA fragments were multiplexed and incubated in the presence of nuclear extracts from non-treated (NT) or Doxo-treated (0.6ug/mL [1mM] Doxorubicin for 18 hours at 37°C) lymphoblastoid cells. The DNA fragments contained the p21-5' RE, p21-5' RE with spacers of increasing length (0-15), p21-5' half-site RE (left or right), or a scramble sequence.

Figure 3.3B



(B) Transactivation in human SaOS2 cells. The cells were co-transfected with WT p53 along with either the full, left or right half-sites of the p21-5' RE containing reporter construct. Transactivation was assessed with the luciferase assay 48 hours later. Relative luciferase activity was compared to a mock transfection containing the promoter-less pGL3 plasmid. Presented are the averages and standard deviations of 3 independent experiments that were each done in triplicate. PCR products of the input DNA (input) and ChIP DNA (p53, IgG or no antibody, Ab) are shown (right). Input and ChIP PCR products for the mock and p21-5' full-site are shown in Figure 3C. No bands were observed above the 600 bp markers.

Figure 3.3C



(C) Sequence dependence of p53 transactivation from decamer half-sites. The extent to which p53 transactivation from half-sites is sequence dependent at high expression levels (2% galactose) was determined with a plasmid-based haploid yeast system [30]. Relative light units/ug protein from 1/2-sites were compared to transactivation from the yLFM strain containing the p21-5'; full-site RE. The average light units/ug protein from p21-5' at 2% galactose was 2.9 million.

Figure 3.4A

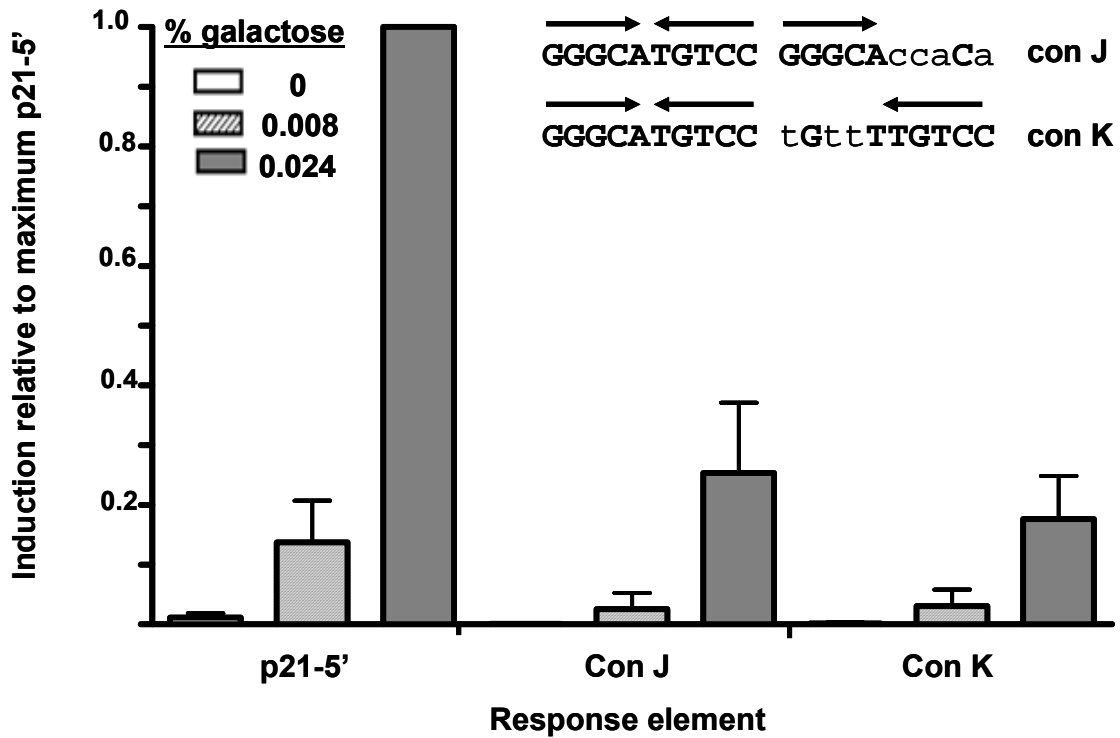
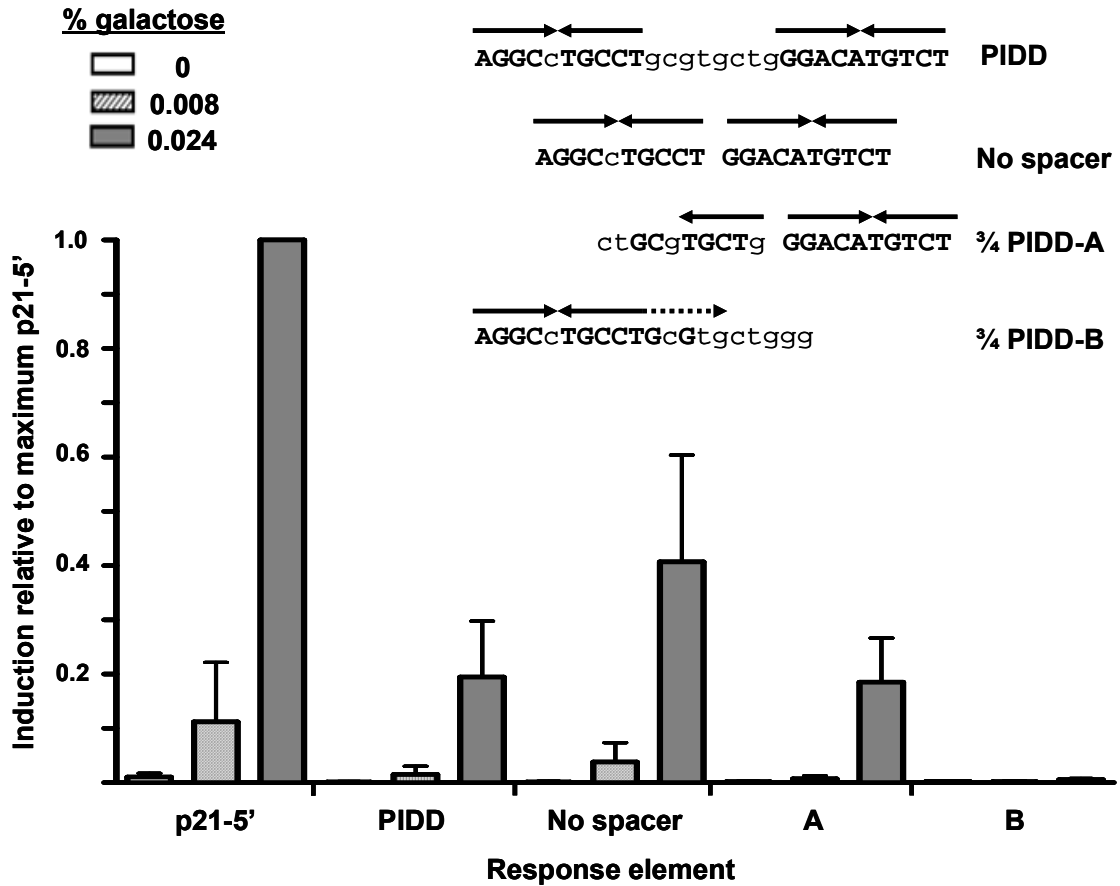


Figure 3.4 A $\frac{3}{4}$ -site can function as a noncanonical RE in p53 transactivation.

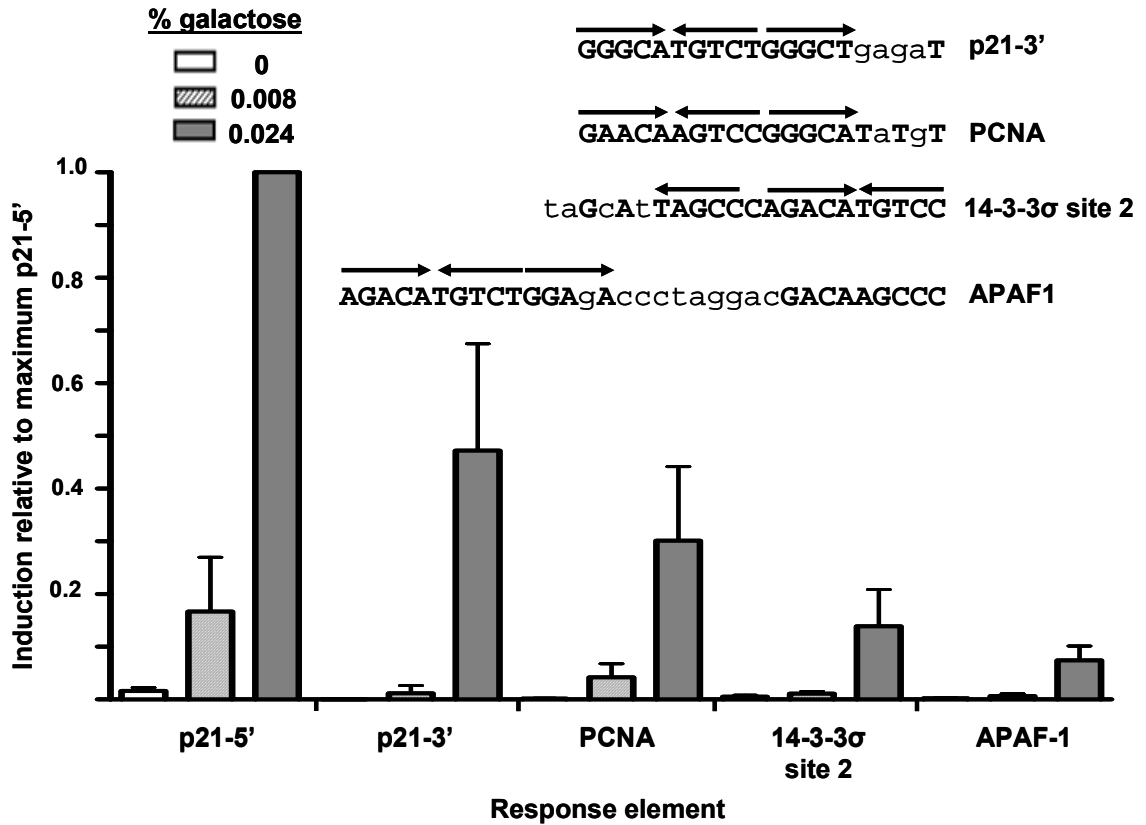
(A) Transactivation was assessed from $\frac{3}{4}$ REs Con J and Con K. The ability of WT p53 to transactivate was measured with the diploid yeast luciferase assay 24 hours post inoculation into the galactose-supplemented media. Induction from each RE was compared relative to the ability of p53 to transactivate from the p21-5' RE at 0.024% galactose and is depicted as the mean and SEM of 6 independent experiments. The average light units/ug protein from p21-5' at 0.024% galactose was 2.1 million. Solid arrows indicate a $\frac{1}{4}$ -binding site.

Figure 3.4B



(B) p53 functions from a $\frac{3}{4}$ -site RE in the PIDD RE. Removal of the natural 8 bp spacer increased transactivation as expected; however, p53 was also able to transactivate from the natural PIDD RE containing the spacer. To determine the true functional p53 binding element, the canonical PIDD RE was broken into two noncanonical sites, $\frac{3}{4}$ PIDD-A and $\frac{3}{4}$ PIDD-B, which incorporated the spacer sequence into the RE and assessed for transactivation capacity with the luciferase assay. Solid arrows indicate $\frac{1}{4}$ -binding site; dashed arrows indicate putative $\frac{1}{4}$ -binding sites.

Figure 3.4C



(C) Analysis of p53 transactivation from various p53 targets in the genome, that may be functioning $\frac{3}{4}$ -sites. Shown are the identified p53 target sequences for p21-3', PCNA, 14-3-3 σ site 2 and APAF1 with the noncanonical $\frac{3}{4}$ -site RE contained in these sites (identified by solid arrows).

Table 3.1

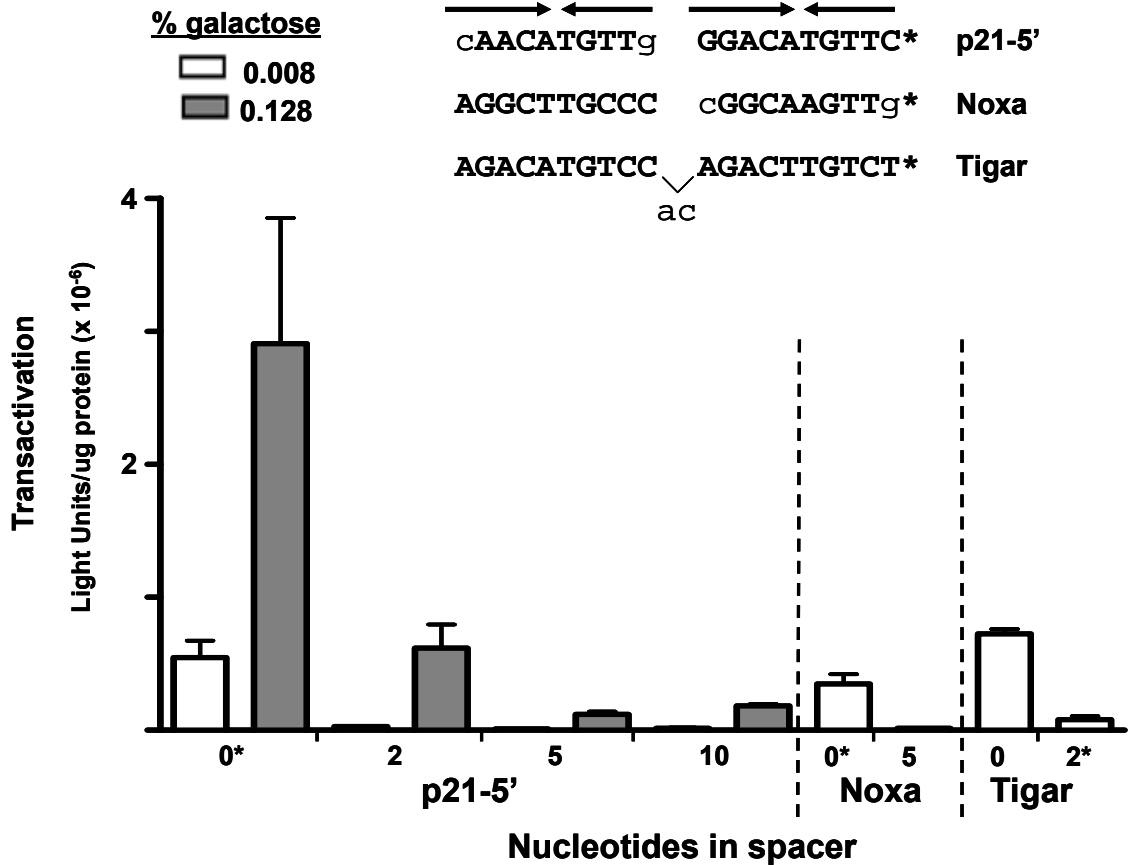
Table 3.1 Transactivation capacity of WT and mutant p53 towards canonical and noncanonical REs.

SYSTEM	p53 ALLELE:						RE
	<u>tetramer</u>	<u>altered tetramer</u>		<u>dimer</u>	<u>monomer</u>	<u>terminal Δ</u>	
	WT	R337C	R337H	L344A	L344P	Δ368	
1n	+++		+++	+/- ^a	+/- ^a	+++	FULL RE: p21-5' Tigar no spacer Tigar natural Gadd45
2n	+++	+	+	+/- ^b	-	++	
mammalian	+++	+	++	++ ^d	-	++	
1n	+++					++	
2n	+++	+	+	+/-	-	+++	
mammalian	+					+/-	
1n	+					+/-	3/4 RE: Con J Con K
2n	+	+/-	+/-	-	-	+	
1n	++		+	+	-	+/-	
2n	+	+/-	+/-	+/-	-	+	
1n	++		+	+	-	+/-	
2n	+	+/-	+/-	+/-	-	+	
1n	+/-			-	-	+/-	1/2 RE: Con G Con B p21-5' left p21-5' right
2n	+/-	-	+/-	-	-	+/-	
1n	+/-			-	-	-	
2n	+/-	-	+/-	-	-	+/-	
mammalian	+ [y +/-]	-	+/-	+/-	-	-	
mammalian	+ [y +/-]	-	+/-	+/-	-	-	

WT p53 and several structural mutations were examined in three *in vivo* systems (haploid [1n] and diploid [2n] yeast and human SaOS2 cells) for transactivation from REs representing full, 3/4-sites and 1/2-sites. The ability of the p53, WT or mutant, to transactivate from a RE was measured with a luciferase assay and compared to the ability of WT p53 to transactivate from the p21-5' RE at 0.008% and 0.024% galactose in 1n and 2n yeast, respectively, or compared to the pGL3 plasmid lacking the promoter REs in human cells. Relative transactivation by a p53 variant in comparison to WT p53 towards the p21-5' RE is represented by +++ (75-100%), ++ (25-75%), + (7.5-25%), +/- detectable but weak response, or - (no detectable response). The R337C and R337H mutations are compromised for their ability to form tetramers (the equilibrium between dimers and tetramers is altered in comparison to WT p53) [80]. The L344A and L344P mutations result in p53 protein being present as a dimer and monomer, respectively. The

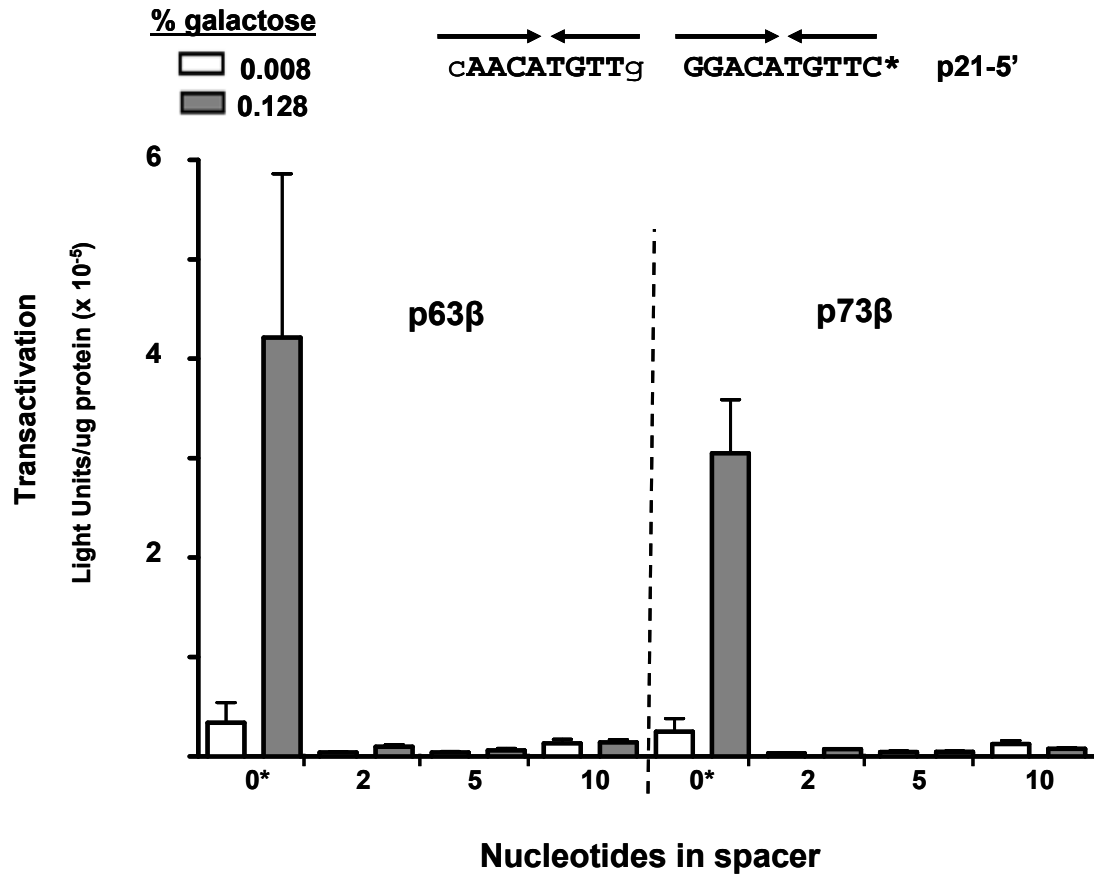
$\Delta 368$ mutation deletes the C-terminal basic domain required for structure-specific and non-sequence specific binding but has no effect on dimer or tetramer formation. “a” corresponds to significantly higher response at high galactose concentrations (0.128 and 2%). “b” indicates that the mutant was capable of transactivation from RE in an *ADE2* plate assay at high galactose concentrations. “d” indicates that the dimer mutation N345S was also analyzed in the mammalian system and yielded the same results as L344A.

Supplemental Figure 3.1A



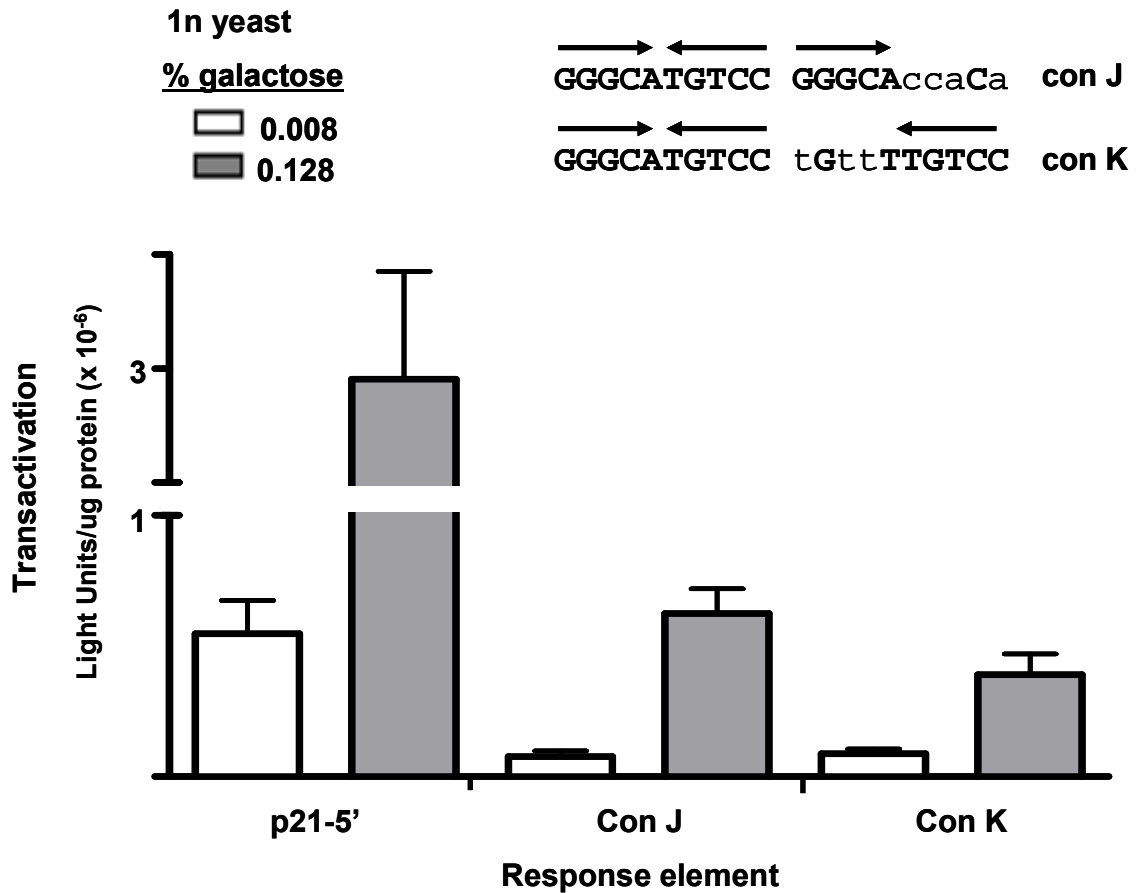
Supplemental Figure 3.1 Increase in spacer decreases transactivation by p53 family members. (A) The plasmid-based haploid yeast system [30] was utilized to determine the extent to which p53 transactivation was impacted by a spacer between decamer half-sites. Transactivation was measured as relative light units/ug protein at 0.008% and 0.128% galactose and compared to WT p53 transactivation from the p21-5' RE. Depicted are the average light units/ug protein and standard deviations from 3 biological repeats. The average light units/ug protein for WT p53 towards p21-5' at 0.128% galactose was 2.9 million.

Supplemental Figure 3.1B



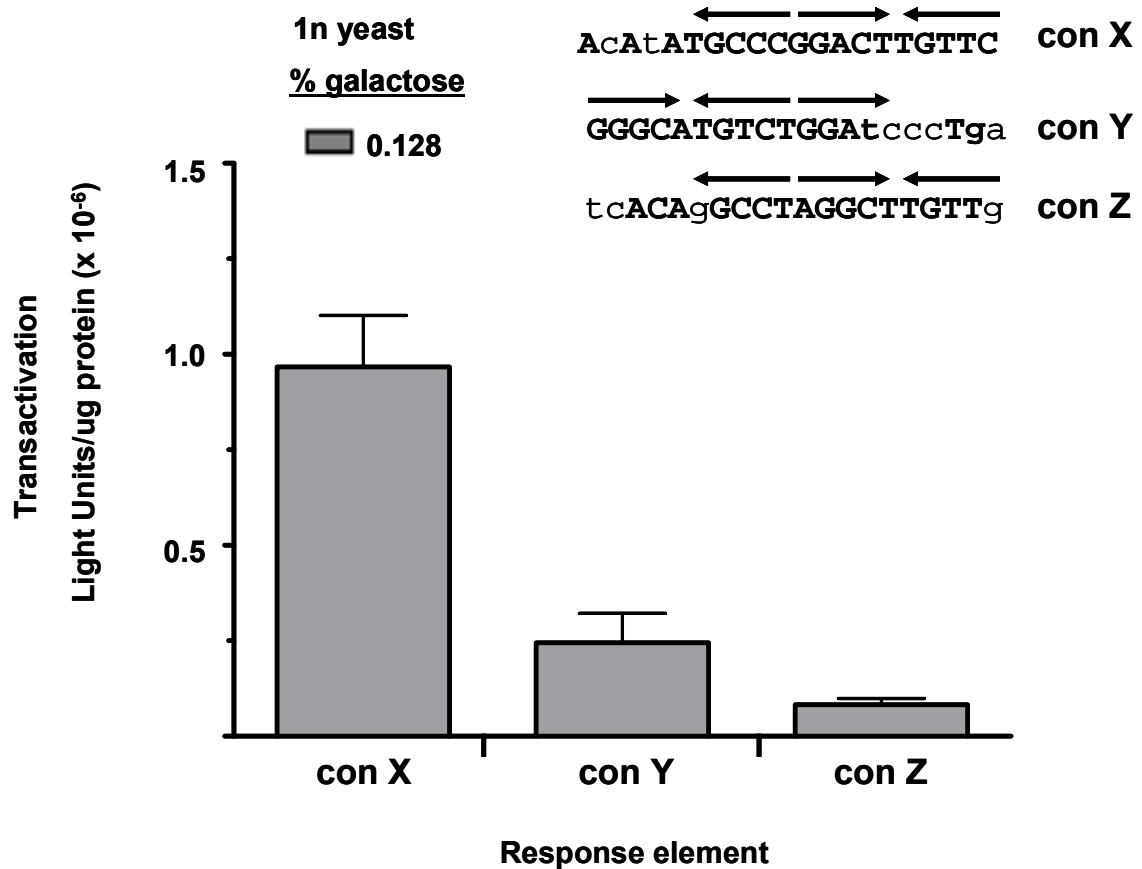
(B) The transactivation capacity of p63β and p73β from the p21-5' RE containing spacers of 0, 2, 5 and 10 nt was measured to determine the affect of spacers on transactivation by p53 family members. Transactivation was measured at moderate and high levels of p53 expression.

Supplemental Figure 3.2A



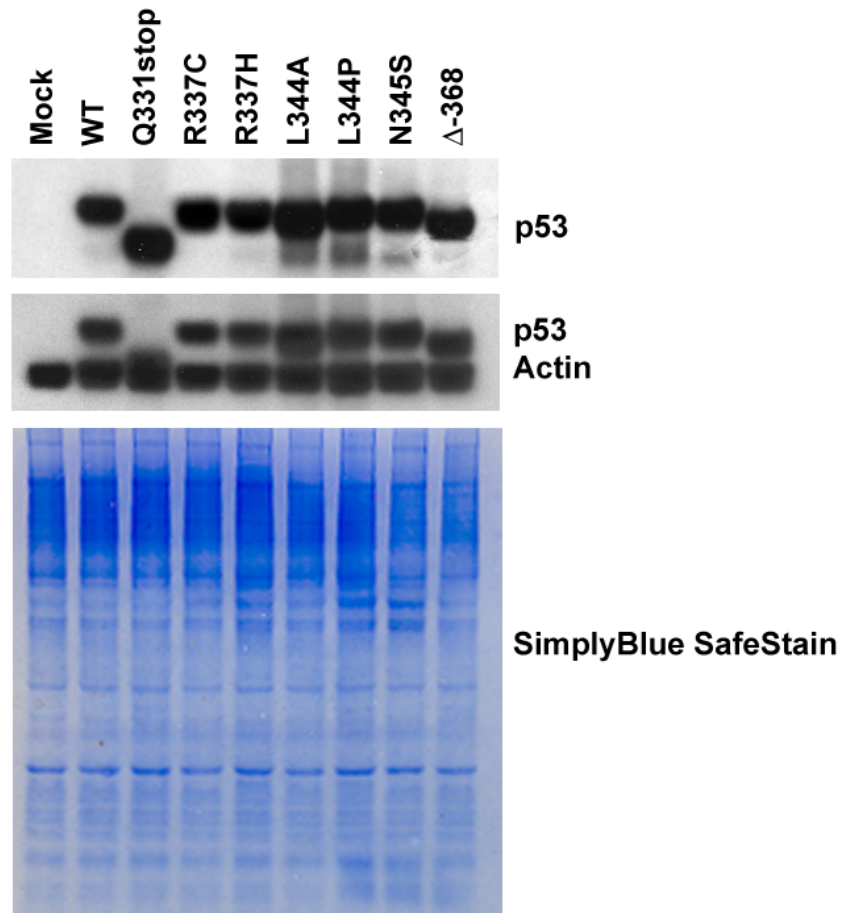
Supplemental Figure 3.2 Transactivation from noncanonical $\frac{3}{4}$ -site REs in haploid yeast. (A) The ability of p53 to transactivate from the consensus $\frac{3}{4}$ -site REs, Con J and Con K were determined in the haploid yeast system, as described in Inga *et al.* [30]. The ability of WT p53 to transactivate from the $\frac{3}{4}$ -site REs was measured 24 hours post inoculation into the galactose-supplemented media by a luciferase assay. The ability of WT p53 to transactivate from each $\frac{3}{4}$ -site RE was compared to the ability of WT p53 to transactivate from the p21-5' RE at 0.128% galactose. Transactivation is depicted as the light units/ug protein and presented as the mean and standard deviation of 3 independent experiments. The average light units/ug protein from p21-5' at 0.128% galactose was 2.9 million. Solid arrows indicate a $\frac{1}{4}$ -binding site.

Supplemental Figure 3.2B



(B) Sequence dependence of p53 transactivation from $\frac{3}{4}$ -site REs. The plasmid-based haploid yeast system [30] was utilized to determine the sequence requirements for transactivation from noncanonical $\frac{3}{4}$ -site REs at high expression levels (0.128% galactose). The central core “CWWG” motif was altered from CATG to CTTG and flanking regions were changed as described. Transactivation from $\frac{3}{4}$ -site REs was measured with a luciferase assay and calculated as light units/ug protein.

Supplemental Figure 3.3



Supplemental Figure 3.3 Mutant p53 expression. To exclude the possibility that variation in transactivation capacity towards the canonical and noncanonical REs by mutant p53 was due to variable protein levels, the relative expression of p53 mutants was compared to WT p53. Western analysis was performed 24 hours following transient transfection of mock, WT p53 or mutant p53 pCMV plasmid vectors into SaOS2 cells. Thirty-five ug of total protein was run on 4-12% BisTris NuPAGE as described in the Materials and Methods. Staining of the gel with SimplyBlue SafeStain (Invitrogen) for total loading protein was performed to discriminate possible overlap of protein between the loading control actin and the truncated p53 protein Q331stop which run at comparable distances.

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CHAPTER 4

ALTERED FUNCTION p53 MISSENSE MUTATIONS ASSOCIATED WITH BREAST CANCER CAN HAVE SUBTLE EFFECTS ON TRANSACTIVATION

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Abstract

Mutations in the *master regulator* p53 that alter transactivation function could result in changes in the strength of gene activation or spectra of genes regulated, thereby leading to dramatic phenotypic changes and diversification of cell response. Mutations in p53 are associated with ~25% of sporadic breast cancers, a rate lower than the frequency in other cancers, such as lung and colorectal carcinomas. However, p53 mutations occur at much higher frequencies in BRCA1/2 germline-associated and in familial breast cancers (especially in the autosomal dominant Li-Fraumeni syndrome, LFS). We have determined functional fingerprints of breast cancer related p53 mutants particularly those associated with LFS families and BRCA1/2 related cancers. The ability of p53, wild type and mutants, to transactivate from 11 human target REs has been assessed at variable expression levels with an *in vivo*, isogenic yeast model system that allows for the rapid analysis of p53 function using a qualitative and a quantitative reporter. Among 50 missense mutants, 29 were classified as loss-of-function p53 mutations. The remaining 21 (42%) retained transactivation towards at least one RE. Interestingly, at high levels of p53, 12 of the 21 mutants that retain function appear similar to WT in their transactivation capacity. However, when the levels of these p53 mutants were reduced, subtle transactivation defects were revealed. Our results demonstrate that altered function p53 missense mutations can occur in breast cancers although the transcriptional effects associated with some of these mutations are often subtle. Understanding the consequences of altered function mutations on the p53 transcriptional network will help elucidate how specific mutations contribute to the development, penetrance, and phenotype of breast cancers, as well as the response to chemotherapeutic agents.

Introduction

The tumor suppressor p53 is a *master regulatory gene* that regulates the differential expression of target genes in a sequence-specific manner in response to cellular and environmental insults [1-5]. Cell cycle regulation, apoptosis, angiogenesis, replication and repair are processes interconnected within the p53 transcriptional network. p53 exerts itself as a transcription factor by binding as a homotetramer, or dimer of dimers, to a consensus response element (RE) sequence comprised of two decamer half-sites. These decamer half-sites [RRRCWWGYYY]₂ (where R= purine; W= A/T; Y=pyrimidine) vary between downstream target genes and can be separated by up to 2 nucleotides for efficient transactivation (Jordan et al, submitted). Activation of a downstream target is dependent on a matrix of factors including cell type, stimuli, post-translational modifications and transcriptional co-factors [6].

The importance of p53 as a tumor suppressor and sequence-specific transcription factor in human cells is highlighted by the occurrence of p53 mutations in the majority of cancers [7]. Interestingly, p53 is unique in comparison to other transcription factors in that over 75% of mutations that occur in this tumor suppressor are single amino acid changes which result in missense mutations [8]. These missense mutations predominantly occur in the DNA binding domain of the protein (>80%) [8]. At the molecular level, p53 mutations found in cancers, including breast cancer, are usually associated with loss of the ability to maintain proper cell cycle checkpoints, suppress transformation caused by oncogenes, induce apoptosis and maintain the integrity of the genome [1, 9].

Specific mutations in p53 are known to denature the native protein or abrogate its ability to bind DNA thus completely abolishing its function. Such mutations are thought to

provide a selective advantage within cancerous cells by forming a hetero-tetramer with WT p53 thus providing the opportunity for either dominant-negative or gain-of-function effects [10-12]. However, recent studies have demonstrated that other p53 missense mutations retain their ability to function as a sequence-specific transcription factor towards some downstream targets such that the ability to regulate cellular responses is mitigated but not completely lost [4, 13, 14], sometimes resulting in a change-of-spectrum of genes regulated rather than a simple modulation. These findings have led to a “master gene of diversity” concept which draws an analogy between sequence-specific transcription factors, in this case p53, and a hand playing the keys of a piano, the downstream target genes [4, 5]. The chord is the accumulation of biological responses regulated and is dependent on both the genes regulated and strength of regulation. Functional mutations in the hand (i.e., p53) can alter transactivation functions by altering binding, strength of activation, and/or spectrum of genes regulated which may lead to changes in the transcriptional network.

To date, functional p53 mutations have been identified as super-transactivating, change-in-spectrum and those that proportionately modulate the levels of transactivation downward. For example, mutant p53 with altered function may be capable of regulating genes containing a strong RE, such as p21, but unable to regulate those with a weak RE, such as Bax. This is consistent with the observation of mutant p53s that still induce cell cycle arrest, yet lose the ability to activate apoptosis [15-19]. In addition, p53 mutations may alter the active binding sites of potential transcriptional cofactors, thus diminishing the potential maximal level of transcriptional response. Modifications in the transcriptional network may result in cellular responses that impact genome stability, repair, replication, and programmed cell death. Varying patterns of cellular responses, including apoptosis and survival have

been elicited in human cells as a consequence of distinct altered function p53 mutations [20]. Furthermore, the aberrant biological consequences of the specific altered function mutations seemed to be influenced by the specific cell type and activating stimuli.

Mutations in p53 are associated with approximately 25% of sporadic cases of breast cancer, a rate lower than the frequency in other sporadic cancers, such as lung and colorectal carcinomas. However, p53 mutations occur at much higher frequencies in BRCA1/2 germline-associated [21, 22] and in familial breast cancers (especially in the autosomal dominant Li-Fraumeni syndrome, LFS) [23-26] where family members are predisposed to early onset cancers. BRCA1/2 and p53 are involved in maintaining genome stability by controlling aspects of homologous recombination and repair, centrosome regulation, cell cycle checkpoints and transcription [27] where loss of either increases the occurrence of carcinogenesis [28, 29]. Interestingly, a significant increase in the frequency of sporadic p53 mutations occurs with an inherited mutation in BRCA1, possibly due to a decreased efficiency to repair damage [22, 30, 31]. BRCA1-associated cancers have an altered spectrum of p53 mutations which may reflect changes in mutagenesis and/or selection for the acquired mutations [22]. While BRCA1 mutations are largely absent in somatic breast tumors, silencing of the gene through hypermethylation has been reported in sporadic cases [32]. Such epigenetic changes have been reported to associate with estrogen receptor negative (ER-) tumors and occur concomitantly with p53 mutations [32]. In addition, a recent hierarchical clustering analysis of immuno-histochemistry profiling has established that degrees of genomic instability correlate with specific subtypes of breast cancers, where the basal subtype had the highest number of genomic aberrations in both sporadic and familiar BRCA-associated cases [33-35].

Li-Fraumeni and Li-Fraumeni-like (LFL) germline disorders--which often harbor a p53 mutation--display an array of early onset, tissue specific tumors of which breast tumors are most frequently observed [8, 36]. The specific p53 mutation carried in the germline, particularly structural-based mutations, may influence the tumor type and penetrance of the disease [36-39]. Recent studies have determined the severity of a particular inherited p53 missense mutation on its ability to function as a sequence specific transcription factor correlates with clinical manifestations [8, 40]. Partial deficiency alleles are associated with less severe family history, lower number of tumors, later onset of disease in comparison to severe deficiency (loss-of-function) alleles and higher risk of breast tumors. Similar findings were observed with somatic p53 mutations associated with breast cancers; however, it was postulated that the majority of these missense mutations were devoid of transcriptional function and contributed to a clinical phenotype through a dominant-negative effect [41].

At the clinical level, p53 mutations in breast cancer have been associated with poor prognosis, earlier on-set, increased aggressiveness of tumors, aneuploidy, and adverse responses to chemotherapeutic treatments [42, 43]. Studies which classify breast cancers based on gene expression profiling have shown p53 mutations are more frequent in the hormone receptor-negative subtypes such as the HER2+/ER- [human epidermal growth factor-2 positive/estrogen receptor negative] and the basal-like subtypes [ER-, PR- (progesterone receptor), HER2-, cytokeratin 5/6+, and/or HER1+] [44-47]. Based on a recent population based study, these subtypes were prevalent among African American and/or premenopausal women and correlated with a more aggressive disease and shortened survival, irrespective of lymph node status [46]. Regardless of subtype, p53 status (WT or mutant) also displays a signature expression profile in breast tumors which is a prognostic

indicator of patient survival, where WT p53 associates with a more favorable outcome [44, 48, 49].

Based on previous findings that several p53 missense mutations associated with breast cancers display subtle transactivation defects when examined in mammalian cell-based or yeast functional assays [50, 51], many other reported mutations are likely to retain function. However, functional consequences of mutations may not be predicted simply by assessments of conservation, topology, or structural models. Using functionality assays based in the budding yeast *Saccharomyces cerevisiae*, it is possible to assess the transcriptional capacities of p53 mutants towards various promoters. We have employed a newly developed diploid yeast model system to analyze the functional consequences of p53 missense mutations found in breast cancers on gene activation endpoints in the p53 transcriptional network at various levels of p53 expression (Jordan et al., submitted). Transactivation capacities of WT and mutant p53 have been determined using a qualitative and a quantitative reporter to establish a functional fingerprint for each mutant towards a subset of human REs that are representative of p53-dependent cellular responses. Using a “rheostatable” promoter, we have ascertained functional discrepancies between WT and mutant p53 transactivation at variable expression levels. We have determined that p53 missense mutations found in sporadic and familial breast cancers can retain function and the alterations in transactivation are often subtle where differences can be exaggerated by changes in p53 levels.

Results

Functional fingerprinting of p53 missense mutations associated with breast cancers

Budding yeast, which lack endogenous p53, have been used previously as an *in vivo* test tube to analyze directly interactions between p53 and REs elements in a cellular environment [4, 5]. Recently, we developed a diploid yeast model system to address the contribution of RE sequence, organization and level of p53 upon p53-mediated transactivation (Jordan et al. submitted). We have utilized this system, described in Figure 2.4, to assess the consequences of p53 missense mutations found in breast cancers upon transactivation and ultimately clinical manifestations.

We sought to define how specific p53 missense mutations interfere with p53 function by assessing the ability of mutant p53 to transactivate from a panel of REs associated with p53-dependent downstream target genes. The 50 mutations associated with breast cancer examined were chosen based upon the following criteria: 1) appear in Li-Fraumeni syndrome (LFS), Li-Fraumeni-like syndrome (LFL), or familial history (FH) cancers; 2) present in the L2 loop, L3 loop or zinc binding region of the protein; 3) associated with familial BRCA1/2 cancers; 4) multiple amino acid changes at the same codon; and/or 5) identified in patients undergoing neoadjuvant treatment for locally advanced breast tumors (Table 4.1). Within criteria group 5, p53 mutational analysis was performed on untreated breast cancer tissues obtained from women participating in two clinical-translational trials, a single institution study from the University of North Carolina – Lineberger Comprehensive Cancer Center (UNC-LCCC 9819), and a multiinstitutional cooperative group trial sponsored by the National Cancer Institute (CALGB 150007). Both trials involved acquisition of breast cancer tissue before treatment with neoadjuvant anthracycline-based chemotherapy in order to

correlate biomarkers with response to primary chemotherapy. (These studies were approved by the institutional review boards of the University of North Carolina at Chapel Hill and participating institutions through the Cancer and Leukemia Group B (CALGB). All study subjects gave written informed consent to participate in the clinical trial and the correlative science studies.)

Twenty-one missense mutations identified in patients participating in these trials (referred to as neoadjuvant treatment) are assessed within the present study for functional status and later will be correlated to therapeutic responses to determine if transactivational activity can be used as a prognostic tool for implementing treatment. Forty-nine of the fifty p53 missense mutations are listed in the International Agency for Research on Cancer (IARC) p53 mutation database as being detected in somatic breast tumors or identified as a germline mutation in LFS, LFL, or FH families (Table 4.1) [8]. The single mutation not reported in the IARC database, M246A, was identified in a patient participating in the multi-institutional prospective trial (see group 5); p53 status in the neoadjuvant treatment patients (group 5) was confirmed by single-stranded conformational polymorphism analysis (SSCP) and validated with direct sequencing [52].

The *ADE2* plate color assay was used to determine *functional fingerprints* for WT and the p53 missense mutations based on their ability to transactivate from 11 different REs at variable levels of protein expression (Figure 4.1 and Supplemental Figure S4.1). The REs analyzed are associated with known p53 target genes involved in cell cycle, DNA repair, apoptosis, angiogenesis, and p53 regulation (Supplementary Table S4.1). Transcription of *ADE2* is dependent on the ability of p53 (WT or mutant) to interact with and transactivate using the specific RE sequence upstream of the reporter and the minimal *CYCI* promoter.

The *ADE2* color assay scores p53 transactivation capacity from a RE based on colony pigmentation which ranges from red (none) to pink (weak to moderate) to white (strong) depending on the extent of *ADE2* transcription. The level of p53 expression was controlled by replica plating strains onto plates containing rich media, raffinose (2%) plus various amounts of galactose (0 – 0.128%) in the presence of low levels of adenine (5mg/L). Mutations were classified as loss-of-function if they were unable to transactivate from a single RE at any protein concentration. Mutations were categorized as functional if they were equivalent to WT p53 in transactivation capacity or altered function if the allele retained the ability to function from a RE(s), but deviated from WT p53 in transactivation capacity from several REs at any level of p53 expression.

Among the 50 missense p53 mutations, 29 were classified as loss-of-function due to their inability to transactivate from a single RE (Table 4.1). The remaining 21 (42%) mutations were able to function from at least one biological RE. Among the 21 functional mutations, 9 were clearly altered in transactivation capacity at high levels of expression (0.128% galactose) (Supplemental Figure S4.1) and displayed a change-in-spectrum as exemplified by reduced or absence of transactivation from the 14-3-3 σ and PCNA REs by P151A and R283P, respectively, in Figure 4.1. Of the 9 mutations, 3 (Y220C, M237I and P278A) retained the ability to transactivate from only the strongest REs, p21-5' and P53R2 (and, in the case of P278A, MDM2 which contains 2 full-site REs) (Supplemental Figure S4.1).

At high levels of p53, the remaining 12 of the 21 mutants (L130V, A138V, C141W, P151H, R174K, R174W, P190L, H214R, R267Q, V272L, E285K, and R337H) looked similar to WT in their transactivation capacity (L130V in Figure 4.1 and Supplemental

Figure S4.1). Transactivation from three biological replicates was either indistinguishable from WT p53 or nearly identical with the exception of 1 or 2 REs at 0.128% galactose. However, when the levels of p53 were reduced, subtle transactivation defects were revealed that further differentiated 6 of the 12 mutants (underlined) from WT p53 at 3 or more REs. Similar to previous studies [4], the variation from WT p53 at lower levels of expression consisted of both reduced and enhanced transactivation capacities from specific REs. The remaining 6 mutants that appeared similar to WT p53 at high levels of expression remained similar to WT p53 in transactivation capacity at low levels of expression with the exception of subtle variation at 1 or 2 REs. For example, A138V was only observed to be slightly reduced in transactivation compared to WT p53 towards PCNA at 0.008% galactose (Supplemental Figure S4.1). The 9 missense p53 mutants that were identified as change-in-spectrum at higher levels of expression also displayed aberrant transactivation patterns at lower p53 expression albeit to a greater extent. Consistent with previous results [4, 52], the change-in-spectrum mutation T125R suppressed growth at low expression levels in the diploid yeast (0.032-0.064% galactose).

Luciferase assays confirm transcriptional anomalies

The functional status of the 21 p53 missense mutations that retained function was examined with a luciferase assay that provides the opportunity to quantitate transactivation from REs in late log phase growing cells. This is illustrated by transactivation from the p21-5' RE over a range of WT p53 levels (Figure 4.2; see also Figure 2.7).

Assessment of the altered function mutants with the luciferase assay showed variable degrees of functionality from the p21-5' RE, where the maximal level of transactivation was

dependent on the specific mutation. Similar to the results with the plate assay, several mutations (*i.e.*, R174W and R267Q) did not differ from WT p53 in their ability to transactivate from the p21-5' RE at high expression levels. However, decreasing the p53 expression revealed a reduction in transactivation for several mutants in comparison to WT p53 (*i.e.*, R267Q) (Figure 4.2). Other mutations, such as L194P, displayed a decreased ability to transactivate from the p21-5' RE in comparison to WT p53 at low and high levels of expression. Several mutations that were similar to WT p53 in terms of maximal levels of transactivation from the strong p21-5' RE showed an altered transactivation potential when assessed for transactivation from the weaker GADD45 RE (Figure 4.3). For example, H214R showed an increased ability to transactivate from the RE in comparison to WT p53. Others such as A138V and R174W were indistinguishable from WT p53.

The transactivation with increasing levels of p53 (*i.e.*, increase galactose) was similar between the DNA binding domain mutations and WT p53 where the initial induction (between 0.004 – 0.008% galactose) and maximal levels of transactivation (plateauing between 0.016% - 0.024% galactose) occurred over the same range of p53 induction. This was not observed for two mutations at the same codon in the tetramerization domain, R337C and R337H. In the luciferase assay, R337H clearly altered transactivation from the p21-5', 14-3-3 σ and GADD45 REs, requiring higher levels of p53 expression than WT to initiate transactivation, although maximal levels of transactivation appeared similar to WT p53 (Figure 4.4). The R337C mutation resulted in overall dampening of p53 transactivation. The requirement for increased p53 levels necessary for initial transactivation by the tetrameric mutants appeared dependent on the strength of the RE.

Change-in-spectrum mutations which retained transactivation function from some REs, but were devoid of function from others, were also verified with the luciferase assays. As shown in Figure 4.5, Y220C was able to transactivate from the strong p21-5' RE, but to reduced levels; the maximal level of transactivation was comparable to that for WT p53 transactivating from the weaker 14-3-3 σ RE. The Y220C mutant was deficient in transactivation from the 14-3-3 σ RE.

Protein levels were analyzed at 0.024% galactose (within the range of expression where transactivation was shown to plateau in the luciferase assays) by Western analysis for the 21 mutations that retained function (Supplemental Figure S4.2). Thirteen of the twenty-one mutants displayed similar levels of protein in comparison to WT p53. However, seven mutants (C141W, L194P, Y220C, M237I, P278A, 285K and R337C) displayed a significant reduction in expression compared to WT p53. Although detectable amounts of p53 were present, the finding was puzzling since several of these mutants were shown to efficiently function from multiple REs in the *ADE2* color assay. Detection of the p53 protein with additional antibodies which recognized different epitopes was consistent with most of these mutants having reduced levels of protein in comparison to WT p53 (Supplemental Figure S4.3). Detection of R337C protein increased with the use of the PAb420 and Ab-1 antibodies suggesting there was an issue with the recognition of the epitope; additionally, an increased ratio of degraded protein was apparent relative to R337H. Transactivation from the p21-5' (assessed with the exact samples used in the Western blots) was significantly reduced for the L194P, Y220C, M237I and P278A mutants in comparison to WT p53 which reflected the decreased expression of protein (Supplemental Figure S4.3). However, C141W and

E285K induced activity from the p21-5' RE to ~26 and 38% of the levels of WT p53, respectively which remained disproportionate to the levels of protein expressed.

Discussion

Single amino acid changes can differentially impact transactivation at various targets and affect biological outcomes [20]. The specific changes may result in selective advantage of specific p53 mutations in certain tissue types or stages of neoplastic transformation as well as alter the responsiveness to or the efficacy of chemotherapeutic agents.

We have used a diploid yeast system to analyze the potential transactivation capacity for a set of p53 missense mutations associated with breast cancers. The 50 missense p53 mutations examined represent approximately 18% of all somatic p53 mutations reported in the IARC p53 mutation database and 20% of all p53 missense mutations documented in breast tumors [8]. Among the 50 mutations analyzed, one mutation occurs at an acetylation site in a non-structured portion of the protein, 2 mutations are in the tetramerization domain and 47 of the mutations are distributed across the sequence-specific DNA binding domain (DBD). Among the mutations, 12 have been associated with LFS, LFL, or FH syndromes, 5 have been found within BRCA-associated tumors and 21 have been identified in locally advanced tumors of patients undergoing neoadjuvant treatment.

Breast cancer associated mutations in the DBD can modulate p53 transactivation

The DBD of p53 consists of a β -sandwich which provides a scaffold for two large β -loops, L2 and L3 that are stabilized by a zinc ion and a loop-sheet-helix motif [53, 54]. Mutations in the DBD have been postulated to affect the binding affinity of p53 towards REs

by abolishing DNA contacts, decreasing the thermodynamic stability of the protein, causing local distortions in the DNA binding surface, or enhancing the loss of the Zn ion [55, 56]. Mutations in the L2/L3 loops are predicted to be highly destabilizing to the tertiary structure of p53 and/or cause chemical shifts which alter the DNA binding surface [8, 57, 58]. Alterations in this region have been correlated with a progressive breast cancer that is often nonresponsive to chemotherapeutic treatments including doxorubicin, tamoxifen, and/or combined therapies of 5-fluorouracil and mitomycin. [27, 59, 60]. Of the 47 DBD mutations analyzed for transactivation potential, 10 are in residues within the L2 loop, 16 are in the L3 loop and 6 are zinc-binding residues. All 6 mutations (C176F, H179R, C238F, C242F, C242S, C242Y) which interfere with the histidine or cysteine side chains involved in coordination of the Zn ion rendered the protein nonfunctional in terms of transactivation, reiterating the vital role of this molecule in site-specific DNA binding and stabilization of the p53 protein [55, 61]. Similarly, all the missense mutations analyzed in the L3 loop which binds the minor groove of DNA and partakes in the dimerization interface between core domains [56] were loss-of-function mutations with the exception of M237I. M237I showed weak transactivation from the p21-5' RE which was only slightly above the background levels of transactivation observed with a loss-of-function mutation and partial transactivation from the p21-5' and P53R2 REs in the plate color assay (data not shown). Among the 10 mutations in the L2 loop, four mutations (R174K, R174W, P190L and L194P) retained function.

Functional fingerprinting of the mutations shows single nucleotide changes occurring in breast cancer can markedly affect the ability of p53 to transactivate from REs. For example, different missense mutations at the same residue affect p53 function differently as

for the case of L194R which results in loss-of-function whereas L194P results in altered function. Importantly, each altered-function p53 missense mutation had a unique functional fingerprint (Supplemental Figure S4.1). Overall, the majority of the change-in-spectrum mutations do not seem to be the result of a general reduction in transactivation (i.e, a proportional reduction in transactivation capacity from all REs). Rather, the p53 mutants appear to impact the REs differentially where the variation in transactivation capacity is distinct to a specific RE.

Differences in transactivation could not be attributed simply to protein stability since most of the p53 mutants were expressed at levels comparable to that for WT protein. Of the six DBD mutations that had a reduced level of protein expression, the altered function Y220C and M237I mutants were severely compromised for transactivation capacity. In agreement with these findings, previous reports which analyzed folding of WT and mutant core p53 proteins (residues 94 – 312) showed Y220C and M237I were thermodynamically unstable and were estimated to significantly decrease the percent of protein folded, as well as reduce the binding affinity towards the GADD45, p21, cyclin G and Bax REs relative to WT p53 [62]. However, C141W and E285K mutants which also expressed low levels of protein were only modestly compromised for transactivation capacity in comparison to WT p53. Based on the transactivation and protein expression results, there is no obvious explanation for the observation that these mutants can transactivate from the majority of the REs analyzed with such a compromised protein level and would suggest the mutants were more active than WT p53 based on unit of protein. However, Schärer and Iggo published similar results concerning E285K where the levels of protein in the yeast were decreased in comparison to WT p53, yet when assessed for transactivation from a 33 base pair sequence at

29°C, E285K activity was ~75% of WT p53; this finding was attributed to protein instability [63]. It is possible, the reduced levels of protein may be due to an increased level of degradation within the cell due to conformation changes associated with temperature sensitive alleles; the Y220C, M237I, and E285K have been reported as temperature sensitive [62-65].

Tetramerization mutations that alter p53 transactivation

Although the majority of p53 missense mutations occur in the DNA binding domain, several missense mutations have been found in the tetramerization domain that are associated with germline syndromes and are found in sporadic breast tumors. R337C is a partial function mutation associated with LFS [66]. R337H has been associated with pediatric cases of adrenocortical carcinoma (ACC); however, it may be a low penetrant LFS or LFL allele as well [25, 26, 67, 68] (also see chapter 3).

The functional fingerprints varied between the two mutations in that R337C had a greater impact on p53 transactivation from the various REs which may reflect a greater instability of the protein as depicted in the protein analysis (Supplemental Figure S4.1). Contrary to previous reports, the p53 missense mutation R337H was not a silent mutation but displayed altered function when examined in the *ADE2* phenotypic assay (Supplemental Figure S4.1). At high expression levels, R337H looked identical to WT p53 in the color assay. At low levels of p53 expression, R337H has a reduced ability to transactivate from REs in comparison to WT p53, presumably due to its reduced ability to form tetramers. Interestingly, the luciferase assay revealed a pattern of transactivation that was unique to R337H where higher levels of protein expression were required to detect transactivation.

This altered pattern of transactivation may be a manifestation of the novel features R337H which include a pH-dependent instability and a higher propensity to form amyloid-like fibrils [69, 70]. R337C clearly demonstrated an altered transactivation capacity which was distinct in comparison to WT p53 displaying an overall dampening effect on transactivation.

Change-in-spectrum mutations can alter the selection of target REs

Another yeast-based functional assay which uses an *ADHI*-p53 expression plasmid and high copy RE reporter plasmid system has been developed by Kato *et al.* [13]. Functional analysis of all but one (M246A) of the 50 mutations examined in the present study is available on the IARC p53 mutation database [8]. There was less than 65% agreement in terms of overall transactivation functionality. Only 8/49 or 16% were identified as functional (6 partially functional; 2 functional) as compared to 21 mutations in the present study system (16 altered function; 5 functional) (Supplemental Table S4.2). The large discrepancy between the two systems may be in part due to the method of classifying a mutation as retaining function. In that study, functional classification was determined based on the median of 8 RE specific activities, expressed as % WT p53 activity for transactivation [8, 13]. In order for a mutation to be considered partially functional, the median for the 8 REs had to be between 20 - 75% of WT activity, whereas the mutant was considered functional if the median was between 75 -140%. In the present study, a mutant was considered at least partially functional if there was a level of expression for which there was transactivation from at least one of 11 REs, which may be why more mutants are described as retaining function in the present study. For example, we concluded the R337C was an altered function missense mutation whereas, Kato *et al.*, classified it as a nonfunctional

mutation although it did display transactivation equating to at least 20% of WT activity for 3 of the 8 REs analyzed (WAF1, MDM2 and P53R2). Because of the wide range of p53 expression, the present study also provides greater opportunity to identify change-of-spectrum mutants, as well as subtle changes in transactivation.

Our results suggest several downstream targets are eliminated from the p53 transcriptional network under some biological conditions for several of the p53 mutants resulting in a change-in-spectrum of RE targets. For example, R337C was not capable of transactivating from AIP1, 14-3-3 σ or PCNA at moderate levels of expression (Supplemental Figure S4.1). However, the observation that transactivation is retained under other conditions should not be overlooked because it suggests *a priori* that the mutant may be an ideal candidate for drug rescue [57]. For example, the Y220C mutation which lies in the periphery of the β -sandwich and plays a role in stabilizing its hydrophobic core [58] was found in the present study to be capable of transactivating from only the p21-5' and P53R2 target REs at higher levels of expression. Recent crystal structures of the p53 core domain containing the Y220C mutation revealed that although the mutation was thermodynamically destabilizing, the overall conformation of the core remains intact [58]. The Y220C mutation results in a cavity or cleft in the protein away from the RE binding site which can be specifically targeted by a small molecule to rescue transcriptional function [56, 58].

Subtle variation in transactivation capacity of altered function missense mutations

A particularly interesting observation in this study was that many of the altered function mutations when examined at higher levels of p53 expression looked similar to WT p53 in terms of transactivation capacity. Importantly, these mutations would not have been

distinguished from WT p53 in typical yeast functional assays where p53 is expressed at high levels from a constitutive promoter. By reducing the level of transcription with the rheostatable promoter, it was possible to unmask subtle transcriptional discrepancies. These mutants might have unique properties in terms of biological consequences. Possibly, the mutants function similarly to WT p53 for gene expression from target genes under high stress and chemotherapeutic conditions, but differently under conditions of low p53 expression. Other transcription factors could further modify the response. It is also possible that these mutations are acquired early in tumor development and in combination with mutations in other genes contribute in an additive fashion to the complex cancer disease. Also, mutations in p53 may be an underlying contributor to the genomic instability observed in BRCA1-associated breast cancer cases and the functional status of individual p53 missense mutations may impact the degree of genetic imbalance.

The functional relevance of p53 at low expression levels is beginning to be elucidated and highlights the need to understand mutants under conditions of low expression. Espinosa *et al.* [71, 72] observed that p53 occupies some REs, (*i.e.*, p21) prior to overall p53 stabilization. There is transcriptional initiation from these REs, but the transcriptional machinery stalls prior to elongation. Such regulation may be required for a rapid response to cellular stress and may be lost in mutations that have an aberrant low level response. The loss of transactivation function at low levels may promote an alternative mode of promoter selectivity and/or provide the opportunity for a competing transcription factor to bind promiscuously to p53 target elements. Although mutations in p53 are often associated with nuclear accumulation of the protein, recent observations from the Lozano group have shown this is not necessarily the case *in vivo* [73]. These observations suggest a scenario can exist

within the cell where aberrant low level p53 response can affect a biological outcome. Based on our transactivation analysis, interactions between the subtle p53 missense mutations and the MDM2 RE remain intact, suggesting this feedback regulatory loop can be initiated by the p53 mutants.

Conclusion and Implications

Given the heterogeneity of breast cancers, understanding the consequences of altered function mutations on the p53 transcriptional network will help elucidate how specific mutations predispose and/or contribute to the development, penetrance, and phenotype of breast cancers. Our results demonstrate that altered function p53 missense mutations can occur in breast cancers. Among 50 p53 mutations identified in breast cancers, 21 had altered function—not simply complete loss—towards at least one RE. Although the transcriptional effects associated with these mutations are often subtle, by reducing the level of transcription with the rheostatable promoter, it is possible to address the retained functions of mutant p53 and novel features in transcriptional networks. Importantly, structure-based analysis of mutations in the DBD was not predictive of the *in vivo* transactivation capacity, where greater than 45% of the predictions based on computational geometry (which assessed residual score profiles derived from Delaunay tessellations) disagreed with the results of the transactivation assays (Supplemental Table S4.2) [8, 74]. This finding emphasizes the need to address function of p53 mutations *in vivo*. It is important to emphasize that the yeast-based results indicate the potential for transactivation and that many factors can come into play in p53 mediated transactivation in human cells.

Results obtained with the yeast functional assay appear predictive of whether a mutation will also have a biological impact in mammalian cells [20]. While the yeast-based assay can predict the *potential* for specific p53 mutations to display altered function, assays in mammalian cells can ascertain the full impact of the functional mutations in the presence of p53 transcriptional cofactors and in the endogenous chromatin context of the RE. However, we propose that assessments of functional fingerprints for p53 missense mutations associated with breast cancer in yeast provide diagnostic value and with further study may also be used as a predictor of tumor behavior in response to chemotherapeutic agents. This would be particularly relevant to the tailoring of individual therapies, especially when the treatment agents impact p53-dependent biological responses.

Table 4.1

Table 4.1 p53 missense mutations associated with breast cancers: functional status, frequency, and features.

p53 MUTATION	FUNCTIONAL ^a STATUS	IARC DATABASE ^b			FEATURES ^c
		SOMATIC		GERMLINE FAMILIES	
		TOTAL	BREAST		
T125R	ALTERED	2	1	0	
L130V	ALTERED	21	3	0	Neo.
C135F	LOSS	49	3	0	
C135Y	LOSS	70	11	0	Neo.
A138V	FUNCTIONAL	48	7	0	
C141W	ALTERED	14	1	0	Neo.
P151A	ALTERED	17	3	0	BRCA
P151H	FUNCTIONAL	35	4	0	Neo.
G154S	ALTERED	11	0	0	Neo.
V173L	LOSS	85	10	0	L2; Neo.
R174K	ALTERED	9	1	0	L2
R174W	FUNCTIONAL	14	2	0	L2
C176F	LOSS	181	7	0	L2-Zn
H179R	LOSS	139	16	0	L2-Zn; Neo.
R181P	LOSS	22	2	1	L2; LFS, LFL, FH
S183L	LOSS	3	1	0	L2
P190L	ALTERED	48	4	0	L2; BRCA
L194P	ALTERED	14	1	0	L2; BRCA
L194R	LOSS	55	9	0	L2
H214R	ALTERED	72	5	0	BRCA
Y220C	ALTERED	315	41	4	LFS, LFL, FH; Neo.
M237I	ALTERED	172	26	1	L3; LFS, LFL, FH
C238F	LOSS	38	5	0	L3-Zn; BRCA
N239D	LOSS	43	5	0	L3; Neo.
N239T	LOSS	9	2	0	L3
S241C	LOSS	33	4	0	L3
C242F	LOSS	86	7	0	L3-Zn
C242S	LOSS	32	5	0	L3-Zn
C242Y	LOSS	51	5	3	L3-Zn; LFS, LFL, FH; Neo.
M243T	LOSS	8	2	0	L3
G244V	LOSS	23	2	1	L3; LFS, LFL, FH
G245S	LOSS	396	35	18	L3; LFS, LFL, FH; Neo.
M246A	LOSS	0	0	0	L3; Neo.
M246T	LOSS	12	3	0	L3
R248L	LOSS	112	6	0	L3, Neo.
R249G	LOSS	43	7	0	L3
R249S	LOSS	374	14	0	L3, Neo.
D259V	LOSS	17	2	0	Neo.
G266R	LOSS	65	5	0	Neo.
R267Q	ALTERED	12	3	3	LFS, LFL, FH
V272L	FUNCTIONAL	47	1	1	LFS, LFL, FH
R273C	LOSS	611	28	9	LFS, LFL, FH; Neo.
R273H	LOSS	733	76	16	LFS, LFL, FH; Neo.
P278A	ALTERED	24	5	0	Neo.

D281E	LOSS	48	3	0	
R283P	ALTERED	34	3	0	Neo.
E285K	FUNCTIONAL	156	20	0	Neo.
K305M	LOSS	2	2	1	
R337C	ALTERED	17	4	3	LFS, LFL, FH
R337H	ALTERED	2	0	69	LFS, LFL, FH; ACC

^aBased on transactivation capacity from 11 human p53 target REs assessed with the plate color assay and the luciferase assay.

^bNumber of reported allele specific mutations in the R12 release of the IARC p53 mutation database. Total number of somatic mutations reported 24,810; total number of missense mutations occurring in breast tumors 1984; total number of germline mutations reported is 399; however, families may have multiple tumors in various tissues with the same mutation.

^cCriteria used to select the specific missense mutation for transcriptional analysis. BRCA, associated with BRCA1/2 cancers; LFS, Li-Fraumeni syndrome; LFL, Li-Fraumeni-like syndrome; FH, familial history; L2, L2 loop; L3, L3 loop; Zn, zinc binding; neo., identified in patients undergoing neoadjuvant treatment for locally advanced breast tumors.

Figure 4.1

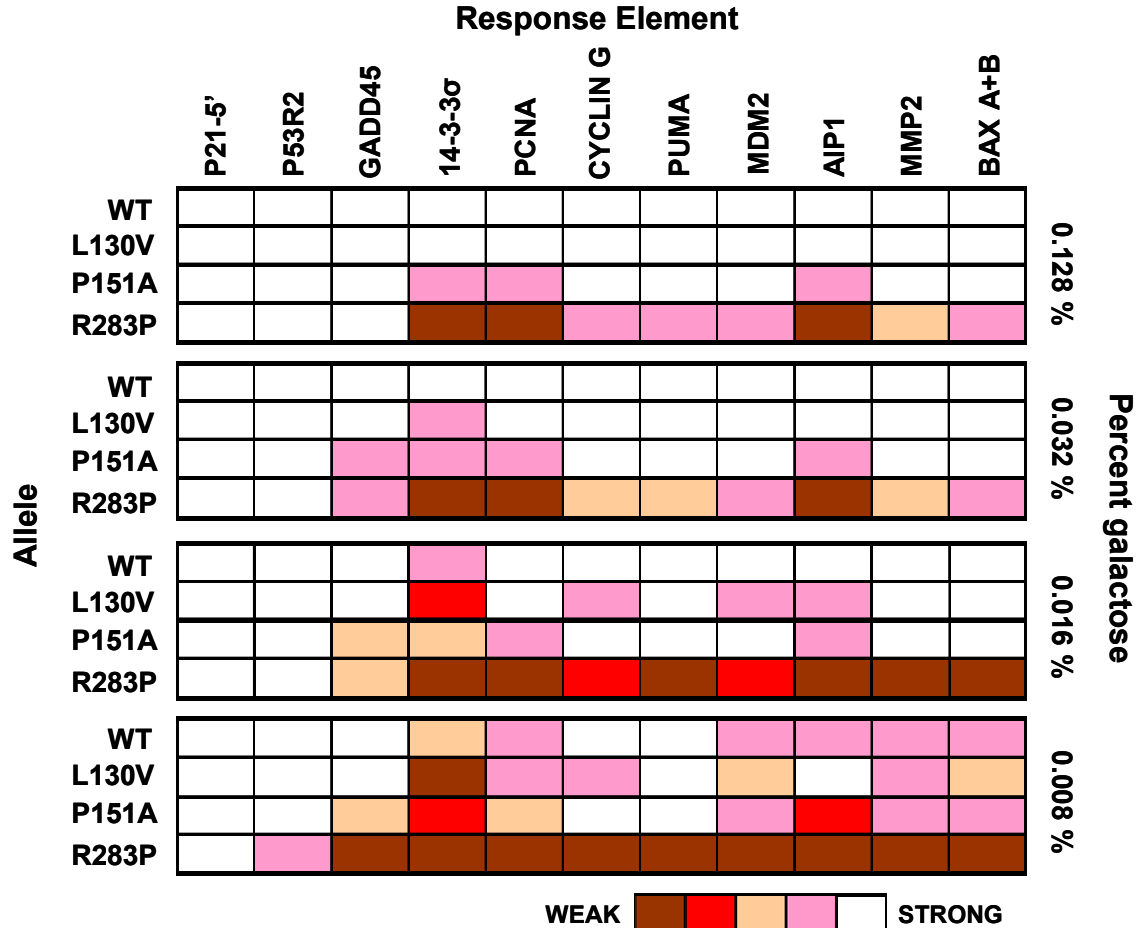


Figure 4.1 Functional fingerprints of mutant p53 reveal subtle transactivational differences. The *ADE2* plate color assay was used to assess the transactivation capacity of WT and mutant p53 towards 11 human target REs at various protein concentrations. Single colony isolates of yeast strains containing the mutation and RE of interest were streaked onto YPDA plates containing glucose and high levels of adenine and grown at 30°C. The plates were then replica plated onto a series of plates containing synthetic media with low levels of adenine [5 mg/L], 2% raffinose and various amounts galactose (0, 0.001, 0.002, 0.004, 0.008, 0.016, 0.032, 0.064 and 0.128%). Transactivation capacity was scored from weak to strong according to the amount level of color (red being little transactivation and white being strong transactivation) for three biological replicates. Shown are examples of functional fingerprints for WT p53 and several change-in-spectrum mutations at 4 levels of protein expression. At high expression levels (0.128%) several mutations were found to display an altered spectrum of REs regulated in comparison to WT p53, for example, P151A and R283P. However, several mutants, such as, L130V, appear indistinguishable from WT p53 in transactivation capacity. Reducing the levels of expression with the rheostatable promoter, exaggerated the subtle transcriptional effects of these mutations and distinguished them from WT p53 and other mutations in transactivation capacity.

Figure 4.2

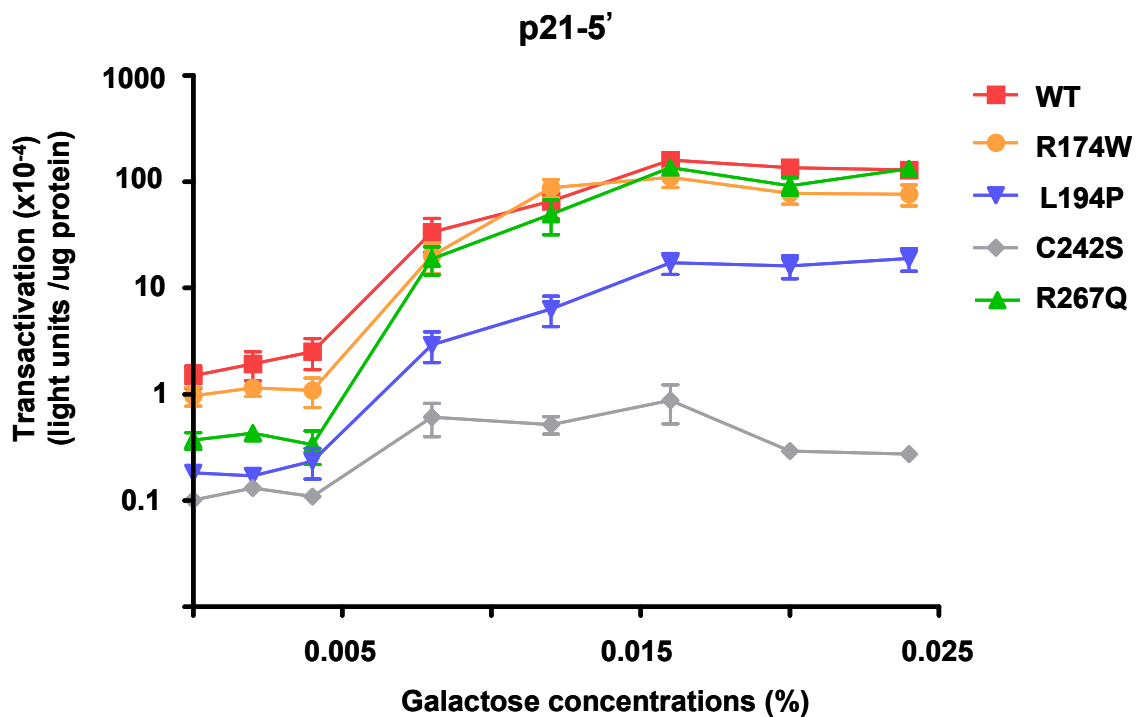


Figure 4.2 Assessment of WT and mutant p53 transactivation towards the p21-5' RE using a luciferase assay in growing cells. Diploid yeast strains containing *GALI::p53* (WT or mutant) crossed with the p21-5' RE-luciferase reporter strain were grown overnight in complete medium, diluted, washed and inoculated into selective medium containing either raffinose (2%) or raffinose (2%) plus increasing concentrations of galactose (0 – 0.024%) overnight at which point the cells are late log. Protein lysates were obtained and a quantitative luciferase assay was used to determine the transactivation capacity for the p53 variants from the p21-5' RE'. The strength of transactivation was calculated as relative light units/ug protein. Depicted are the mean and standard error of measurement (SEM) for 7 independent experiments. Transactivation kinetics show basal, linear-increase, and plateau phases of the transactivation response which is dependent on the p53 variant. Many p53 missense mutations associated with breast cancers, i.e. R174W and R267Q, do not affect the maximal level of transactivation towards the strong p21 5' RE in comparison to WT p53. However, transactivation can be altered at low levels of p53 expression. Several mutants, such as L194P, were shown to modulate the levels of transactivation at all concentrations of expression. C242S is a loss-of-function mutation.

Figure 4.3

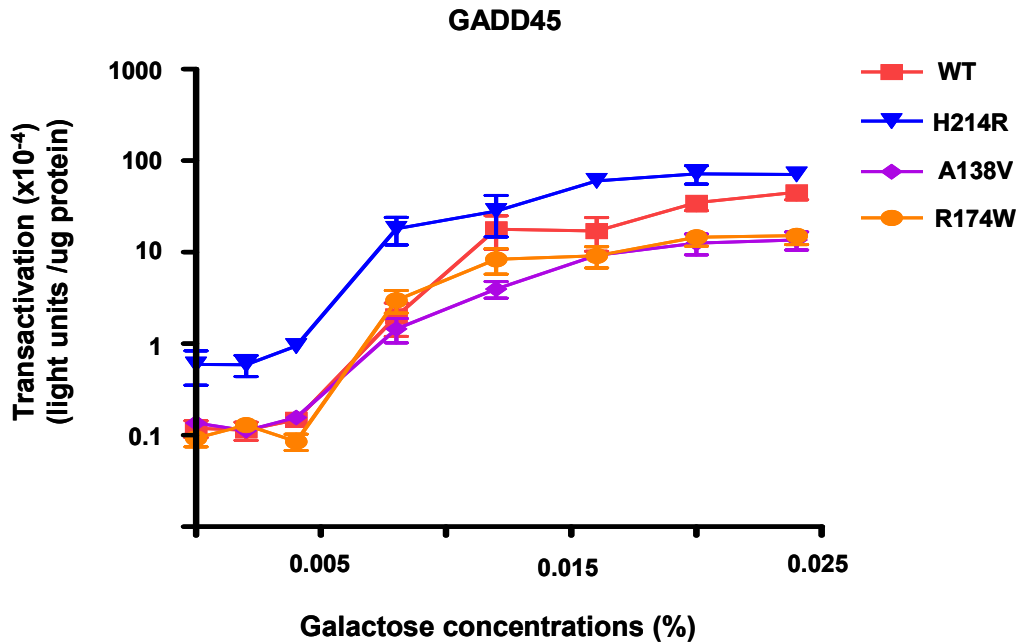


Figure 4.3 Transactivation from the GADD45 RE distinguishes altered function mutants from WT p53. The ability of p53 (WT and mutant) to transactivate from the GADD45 RE was measured 24 hours after inoculation into increasing concentrations of inducing media with a quantitative luciferase assay (see Figure 4.2). The strength of transactivation was calculated as relative light units/ug protein. Presented are the mean and standard error of measurement (SEM) for 6 independent experiments. Interestingly, transactivation from the GADD45 RE can differentiate mutant p53 alleles that looked similar to WT p53 in the *ADE2* plate assay. H214R has an increased ability to transactivate from the RE in comparison to WT p53 at low and high levels of expression.

Figure 4.4

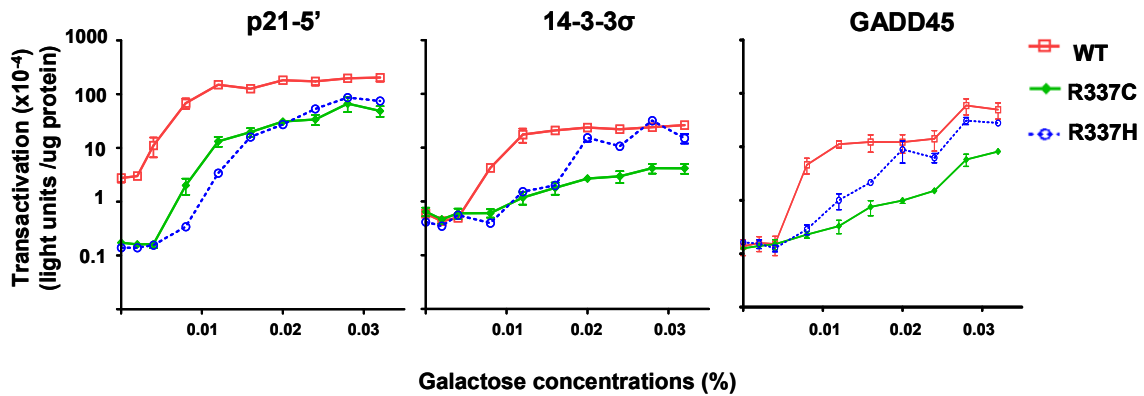


Figure 4.4 Altered function mutations at the same codon in the tetramerization domain display different transactivation capacities. Transactivation capacities were obtained for the R337C and R337H mutations towards the p21-5', 14-3-3σ, and GADD45 REs at increasing levels of expression with the quantitative luciferase assay. The levels of transactivation were severely diminished with R337C. R337H required an increased amount of p53 expression to stimulate transactivation. The magnitude of effect upon transactivation capacity for the individual mutations appeared dependent on the RE.

Figure 4.5

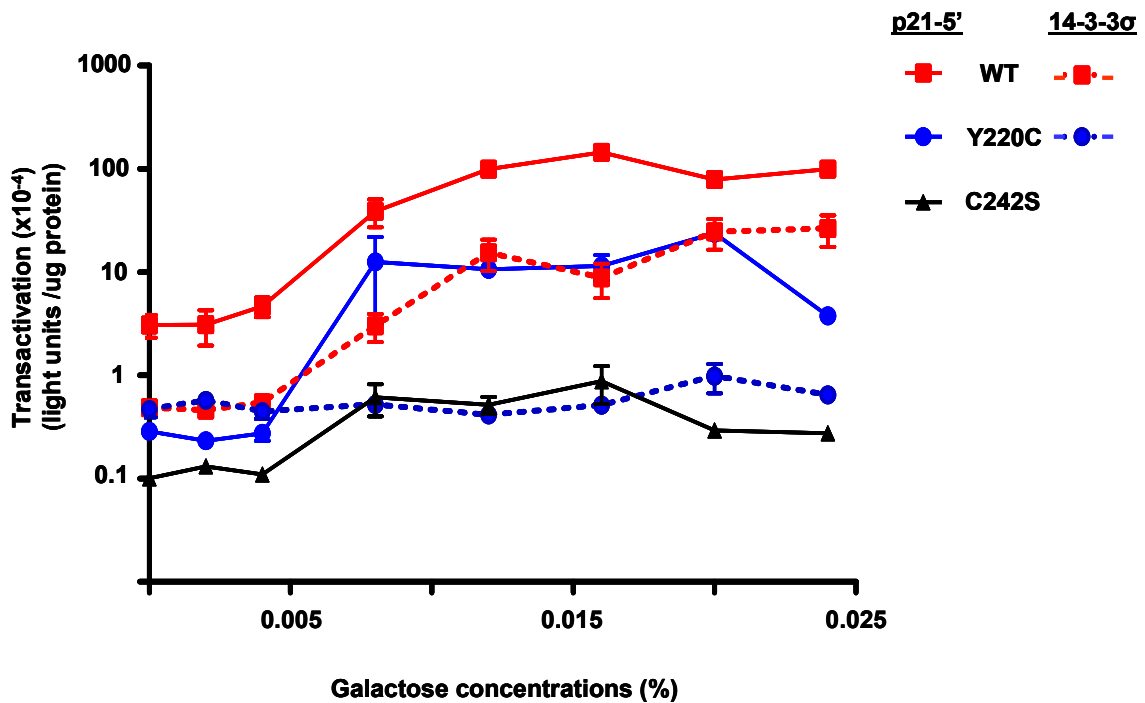


Figure 4.5 Change-in-spectrum p53 missense mutations can eliminate REs from the p53 transcriptional network. To verify that change in spectrum mutations can alter the transcriptional network through eliminating several REs under certain conditions *in vivo*, transactivation was assessed for WT and Y220C p53 from the p21-5' and GADD45 REs. Diploid yeast strains were grown overnight in complete medium, diluted, washed and inoculated into induction media (see Figure 4.2). Protein lysates were obtained from overnight cultures and a quantitative luciferase assay was used to determine the transactivation capacity for the p53 variants from the p21-5' RE'. The strength of transactivation was calculated as relative light units/ug protein. Depicted are the mean and standard error of measurement (SEM) of 6 independent experiments. Y220C is capable of transactivating from the p21-5' RE albeit to levels comparable to WT p53 transactivation from the weaker 14-3-3σ RE. However, Y220C was not capable of transactivating from the 14-3-3σ suggesting this target gene may be eliminated from the p53 transcriptional network in the presence of this particular mutation.

Supplemental Figure S4.1

0.128% galactose

WEAK  STRONG

RE	P21	P53R2	GADD45	14-3-3 σ	PCNA	CYCLIN G	PUMA	MDM2	AIP1	MMP2	BAX A + B
Allele											
WT											
T125R											
L130V											
A138V											
C141W											
P151A											
P151H											
G154S											
R174K											
R174W											
P190L											
L194P											
H214R											
Y220C											
M237I											
R267Q											
V272L											
P278A											
R283P											
E285K											
R337C											
R337H											

0.032% galactose

RE	P21	P53R2	GADD45	14-3-3 σ	PCNA	CYCLIN G	PUMA	MDM2	AIP1	MMP2	BAX A + B
Allele											
WT											
T125R											
L130V											
A138V											
C141W											
P151A											
P151H											
G154S											
R174K											
R174W											
P190L											
L194P											
H214R											
Y220C											
M237I											
R267Q											
V272L											
P278A											
R283P											
E285K											
R337C											
R337H											

Supplemental Figure S4.1

0.016% galactose

WEAK  STRONG

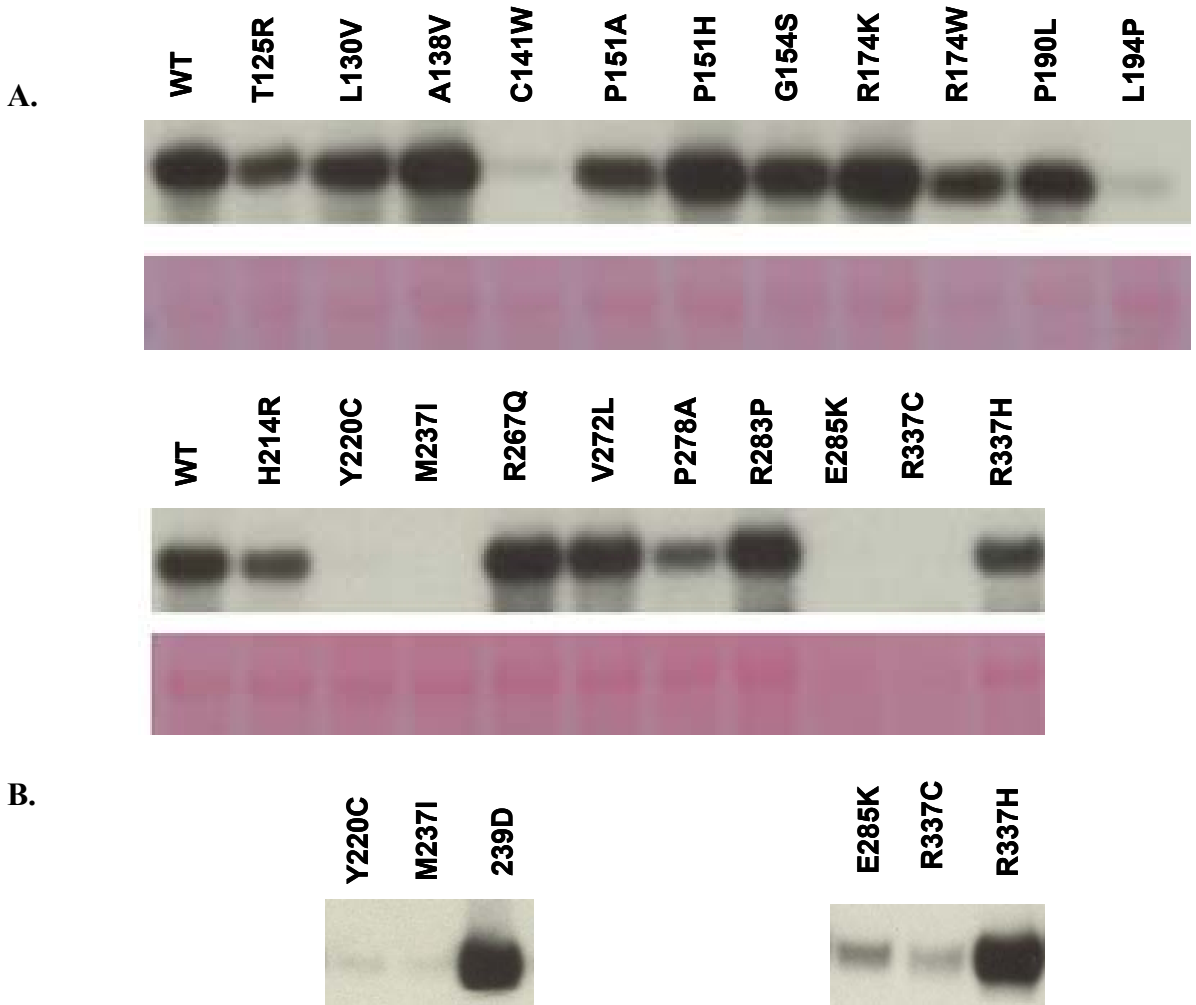
RE Allele	P21	P53R2	GADD45	14-3-3 σ	PCNA	CYCLIN G	PUMA	MDM2	AIP1	MMP2	BAX A + B
WT											
T125R											
L130V											
A138V											
C141W											
P151A											
P151H											
G154S											
R174K											
R174W											
P190L											
L194P											
H214R											
Y220C											
M237I											
R267Q											
V272L											
P278A											
R283P											
E285K											
R337C											
R337H											

0.008% galactose

RE Allele	P21	P53R2	GADD45	14-3-3 σ	PCNA	CYCLIN G	PUMA	MDM2	AIP1	MMP2	BAX A + B
WT											
T125R											
L130V											
A138V											
C141W											
P151A											
P151H											
G154S											
R174K											
R174W											
P190L											
L194P											
H214R											
Y220C											
M237I											
R267Q											
V272L											
P278A											
R283P											
E285K											
R337C											
R337H											

Supplemental Figure S4.1 Functional fingerprint of 21 altered-function p53 missense mutations. Transactivation capacities of WT and mutant p53 were assessed from 11 REs with the *ADE2* plate color assay at variable levels of p53 induction. Single colony isolates grown on YPDA plates containing glucose and high levels of adenine and grown at 30°C were replicated onto a series of plates containing synthetic medium with low levels of adenine [5 mg/L], 2% raffinose and incremental increases in galactose. Transactivation capacity was assessed through colony pigmentation and scored according to the extent of transactivation from weak to strong (see Figure 4.1).

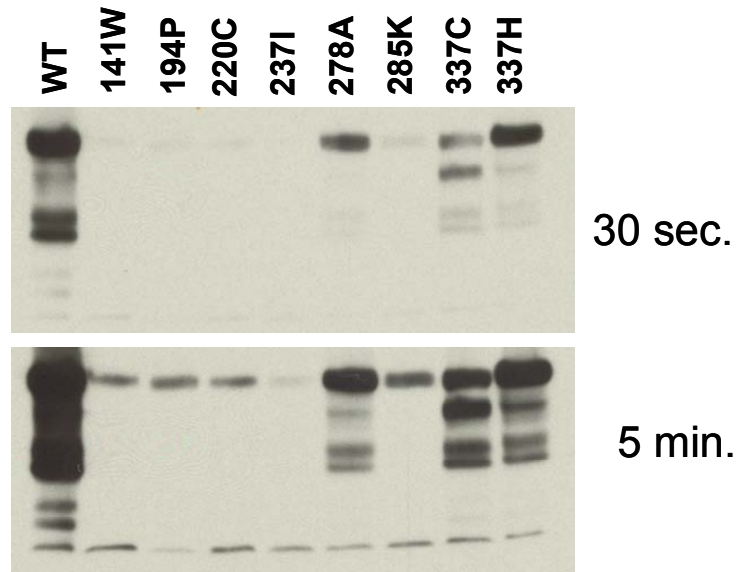
Supplemental Figure S4.2



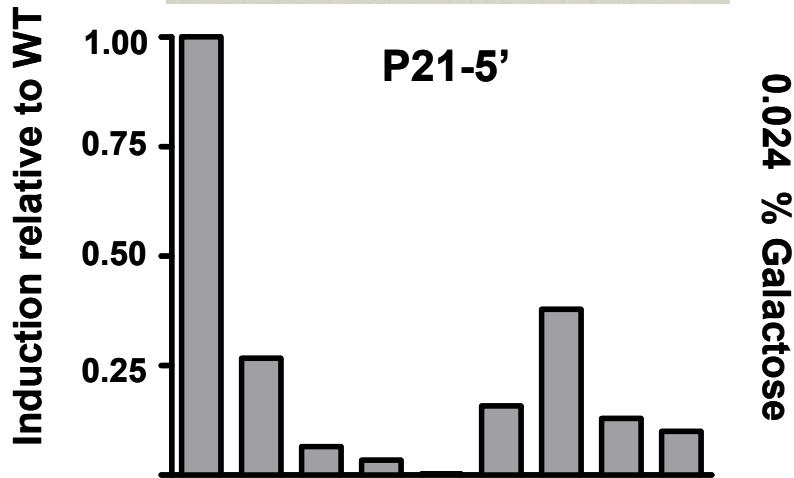
Supplemental Figure S4.2 WT and mutant p53 expression. A. To assess if the variation in transactivation capacity towards the biological REs by mutant p53 was due to variable protein levels, the relative expression of p53 mutants was compared to WT p53. Diploid yeast strains containing *GALI::p53* (WT or mutant) crossed with the p21-5' RE-luciferase reporter were grown overnight in complete medium, diluted, washed and inoculated into selective medium containing raffinose (2%) plus 0.024% galactose. Overnight cultures, corresponding to late log phase, were harvested and 50 ug of total protein was run on 4-12% BisTris NuPAGE as described in the Materials and Methods. The p53 protein was detected with a mix of DO7 (BD BioSciences Pharmingen) and pAb1801 (Santa Cruz) antibodies. Bands were detected using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) and the enhanced chemiluminescence (ECL) detection system (Amersham, Cleveland, OH, USA). Membranes were stained with Ponceau S to determine efficiency of protein loading. B. Longer exposures lengths show there are detectable levels of p53 protein with the Y220C, M237I, E385K and R337C mutants. N239D is a loss-of-function mutation.

Supplemental Figure S4.3

A.



B.



Supplemental Figure S4.3 Transactivation from the p21-5' RE by p53 missense mutations with reduced protein expression. A. Western analysis was performed on the p53 mutants expressing reduced levels of p53 in comparison to WT and R337H using the combined D0-7 (BD BioSciences Pharmingen), pAb240 (Santa Cruz) and p53 (Ab-1) (Santa Cruz) antibodies to detect p53. The pAb240 and P53 (Ab-1) antibodies recognize different epitopes from those previously used. Similar to previous results, the protein expression was detectable, but reduced in comparison to WT p53. B. Transactivation from the p21-5' RE was assessed for WT and p53 mutants with the luciferase assay.

Supplemental Table S4.1

Supplemental Table S4.1 Response element sequence and biological function of associated target gene.

Response Element	Function	Sequence		
		Half-site	Spacer	Half-site
p21-5'	Cell cycle	cAACATGTTg		GGACATGTTC
P53R2	DNA repair	tGACATGCCC		AGGCATGTCT
PCNA	DNA repair	GAACAAGTCC		GGGCATaTgT
PUMA BS2	Apoptosis	ctGCAAGTCC		tGACTTGTCC
GADD45	DNA repair	GAACATGTCT		AAGCATGCTg
CYCLIN G	Cell cycle	AGGCTTGCCC		GGGCagGTCT
14-3-3σ	Cell cycle	tAGCATTAGCCC		AGACATGTCC
AIP1	Apoptosis	tctCTTGCCC		GGGCTTGTcG
BAX A+B	Apoptosis	tcACAAGTTa	G	AGACAAGCCT
		AGACAAGCCT		GGGCgTGggC
MDM2 RE1+RE2	p53 regulation	GGtCAAGTTg		GGACAcGTCC
		GAGCTAagTC	C	tGACATGTCT
MMP2	angiogenesis	AGACAAGCCT		GAACTTGTCT

Supplemental Table S4.2

Supplemental Table S4.2 Functional status determined by *in vivo* transactivation assays vs. structure based predictions.

MUTATION	FUNCTIONAL STATUS^a Jordan et al.	FUNCTIONAL STATUS^b Kato et al.	STRUCTURE BASED PREDICTION^c	RESIDUE FUNCTION^d
T125R	ALTERED	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
L130V	ALTERED	NONFUNCTIONAL	NONFUNCTIONAL	PARTIALLY EXPOSED
C135F	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
C135Y	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
A138V	FUNCTIONAL	PARTIALLY	FUNCTIONAL	PARTIALLY EXPOSED
C141W	ALTERED	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
P151A	ALTERED	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
P151H	FUNCTIONAL	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
G154S	ALTERED	PARTIALLY	NONFUNCTIONAL	PARTIALLY EXPOSED
V173L	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
R174K	ALTERED	FUNCTIONAL	FUNCTIONAL	PARTIALLY EXPOSED
R174W	FUNCTIONAL	NONFUNCTIONAL	NONFUNCTIONAL	PARTIALLY EXPOSED
C176F	LOSS	PARTIALLY	NONFUNCTIONAL	ZN BINDING
H179R	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	ZN BINDING
R181P	LOSS	NONFUNCTIONAL	FUNCTIONAL	EXPOSED
S183L	LOSS	FUNCTIONAL	FUNCTIONAL	PARTIALLY EXPOSED
P190L	ALTERED	PARTIALLY	NONFUNCTIONAL	PARTIALLY EXPOSED
L194P	ALTERED	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
L194R	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
H214R	ALTERED	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
Y220C	ALTERED	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
M237I	ALTERED	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
C238F	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	ZN BINDING
N239D	LOSS	NONFUNCTIONAL	FUNCTIONAL	DNA BINDING
N239T	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	DNA BINDING
S241C	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	DNA BINDING
C242F	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	ZN BINDING
C242S	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	ZN BINDING
C242Y	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	ZN BINDING
M243T	LOSS	NONFUNCTIONAL	FUNCTIONAL	DNA BINDING
G244V	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	EXPOSED
G245S	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
M246A	LOSS	NO RESULTS	NONFUNCTIONAL	BURIED
M246T	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	BURIED

R248L	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	DNA BINDING
R249G	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
R249S	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
D259V	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
G266R	LOSS	NONFUNCTIONAL	FUNCTIONAL	BURIED
R267Q	ALTERED	PARTIALLY	NONFUNCTIONAL	BURIED
V272L	FUNCTIONAL	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
R273C	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	DNA BINDING
R273H	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	DNA BINDING
P278A	ALTERED	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
D281E	LOSS	NONFUNCTIONAL	NONFUNCTIONAL	BURIED
R283P	ALTERED	NONFUNCTIONAL	NONFUNCTIONAL	DNA BINDING
E285K	FUNCTIONAL	NONFUNCTIONAL	NONFUNCTIONAL	PARTIALLY EXPOSED
K305M	LOSS	NONFUNCTIONAL	N/A	ACETYLATION SITE
R337C	ALTERED	NONFUNCTIONAL	N/A	TETRAMERIZATION
R337H	ALTERED	PARTIALLY	N/A	TETRAMERIZATION

All mutations reside in the DNA binding domain of the protein except for K305M which is an acetylation site and R337C and R337H which are in the tetramerization domain.

^aBased on transactivation capacity from 11 biological REs assessed with the plate color assay and luciferase assay.

^bTransactivation assessed by Kato et al. [8, 13], based on median transactivation, represented as a percentage of WT p53, from 8 REs. A mutation was considered partially functional if the median for the 8 REs was greater than 20%.

^cData for the structure based predictions was acquired from the IARC p53 mutant database and derived by Mathe et al. [8, 74], using a four-body potential score from Delaunay tessellations.

^dResidue function information was acquired from the IARC p53 mutation database [8, 74].

Supplemental Table S4.3

Supplemental Table S4.3 Oligonucleotides with p53 mutation of interest for CORE replacements

MUTATION	OLIGONUCLEOTIDE 5' → 3'
T125R	CAC AGG GCA GGT CTT GGC CAG TTG GCA AAA CAT CTT GTT GAG GGC AGG GGA GTA CCT GCA AGT CAC AGA CTT GGC TGT CCC AGA ATG
L130V	AAG TCT GTG ACT TGC ACG TAC TCC CCT GCC GTC AAC AAG ATG TTT TGC CAA CTG GCC AAG ACC TGC CCT GTG
C135F	ACT TGC ACG TAC TCC CCT GCC CTC AAC AAG ATG TTT TTC CAA CTG GCC AAG ACC TGC CCT GTG CAG CTG TGG GTT
C135Y	ACT TGC ACG TAC TCC CCT GCC CTC AAC AAG ATG TTT TAC CAA CTG GCC AAG ACC TGC CCT GTG CAG CTG TGG GTT
A138V	ACT TGC ACG TAC TCC CCT GCC CTC AAC AAG ATG TTT TGC CAA CTG GTC AAG ACC TGC CCT GTG CAG CTG TGG GTT GAT
C141W	TGC ACG TAC TCC CCT GCC CTC AAC AAG ATG TTT TGC CAA CTG GCC AAG ACC TGG CCT GTG CAG CTG TGG GTT GAT TCC ACA CCC
P151A	TGC CCT GTG CAG CTG TGG GTT GAT TCC ACA GCC CCG CCC GGC ACC CGC GTC CGC GCC ATG GCC ATC TAC AAG CAG TCA CAG CAC ATG ACG GAG GTT GTG A
P151H	C AAC AAG ATG TTT TGC CAA CTG GCC AAG ACC TGC CCT GTG CAG CTG TGG GTT GAT TCC ACA CAC CCG CCC GGC A
G154S	CAG CTG TGG GTT GAT TCC ACA CCC CCG CCC AGC ACC CGC GTC CGC GCC ATG GCC ATC TAC AAG CAG TCA CAG CAC ATG ACG GAG GTT GTG
V173L	GGC ACC CGC GTC CGC GCC ATG GCC ATC TAC AAG CAG TCA CAG CAC ATG ACG GAG GTT CTG AGG CGC TGC CCC CAC CAT GAG CGC TGC TCA GAT
R174K	GGC ACC CGC GTC CGC GCC ATG GCC ATC TAC AAG CAG TCA CAG CAC ATG ACG GAG GTT GTG AAG CGC TGC CCC CAC CAT GAG CGC TGC TCA GAT
R174W	GGC ACC CGC GTC CGC GCC ATG GCC ATC TAC AAG CAG TCA CAG CAC ATG ACG GAG GTT GTG TGG CGC TGC CCC CAC CAT GAG CGC TGC TCA GAT
C176F	GGC ACC CGC GTC CGC GCC ATG GCC ATC TAC AAG CAG TCA CAG CAC ATG ACG GAG GTT GTG AGG CGC TTC CCC CAC CAT GAG CGC TGC TCA GAT AGC GAT
H179R	TGC CCC CAC CGT GAG CGC TGC TCA GAT AGC GAT GGT CTG GCC CCT CCT CAG CAT CTT ATC CGA
R181P	GGC ACC CGC GTC CGC GCC ATG GCC ATC TAC AAG CAG TCA CAG CAC ATG ACG GAG GTT GTG AGG CGC TGC CCC

	CAC CAT GAG CCC TGC TCA GAT
S183L	GTG AGG CGC TGC CCC CAC CAT GAG CGC TTA GAT AGC GAT GGT CTG GCC CCT CCT CAG CAT CTT ATC CGA GTG GAA GGA AAT TTG CGT GTG
P190L	GAG CGC TGC TCA GAT AGC GAT GGT CTG GCC CTT CCT CAG CAT CTT ATC CGA GTG GAA GGA AAT TTG CGT GTG GAG
L194P	GAG CGC TGC TCA GAT AGC GAT GGT CTG GCC CCT CCT CAG CAT CCT ATC CGA GTG GAA GGA AAT TTG CGT GTG GAG
L194R	GAG CGC TGC TCA GAT AGC GAT GGT CTG GCC CCT CCT CAG CAT CGT ATC CGA GTG GAA GGA AAT TTG CGT GTG GAG
H214R	GAG TAT TTG GAT GAC AGA AAC ACT TTT CGA CGT AGT GTG GTG GTG CCC TAT GAG CCG CCT GAG GTT GGC TCT GAC TGT ACC ACC ATC CAC TAC
Y220C	AAC ACT TTT CGA CAT AGT GTG GTG GTG CCC TGT GAG CCG CCT GAG GTT GGC TCT GAC TGT ACC ACC ATC CAC TAC
M237I	CGA CAT AGT GTG GTG GTG CCC TAT GAG CCG CCT GAG GTT GGC TCT GAC TGT ACC ACC ATC CAC TAC AAC TAC ATA TGT AAC AGT TCC TGC ATG GGC GGC
C238F	T GTG GTG GTG CCC TAT GAG CCG CCT GAG GTT GGC TCT GAC TGT ACC ACC ATC CAC ATG TTT AAC AGT TCC TGC ATG GGC GGC ATG AAC CGG
N239D	TAC ATG TGT GAC AGT TCC TGC ATG GGC GGC ATG AAC CGG AGG CCC ATC CTC ACC ATC ATC ACA CTG
N239T	TGT ACC ACC ATC CAC TAC AAC TAC ATG TGT ACC AGT TCC TGC ATG GGC GGC ATG AAC CGG AGG CCC ATC CTC ACC ATC ATC ACA CTG GAA GAC TCC AGT G
S241C	ACC ATC CAC TAC AAC TAC ATG TGT AAC AGT TGC TGC ATG GGC GGC ATG AAC CGG AGG CCC ATC CTC ACC ATC ATC ACA CTG GAA GAC TCC AGT GGT AAT
C242F	ATC CAC TAC AAC TAC ATG TGT AAC AGT TCC TTC ATG GGC GGC ATG AAC CGG AGG CCC ATC CTC ACC ATC ATC ACA CTG GAA GAC TCC AGT GGT AAT
C242S	ATC CAC TAC AAC TAC ATG TGT AAC AGT TCC TCC ATG GGC GGC ATG AAC CGG AGG CCC ATC CTC ACC ATC ATC ACA CTG GAA GAC TCC AGT GGT AAT
C242Y	ATC CAC TAC AAC TAC ATG TGT AAC AGT TCC TAC ATG GGC GGC ATG AAC CGG AGG CCC ATC CTC ACC ATC ATC ACA CTG GAA GAC TCC AGT GGT AAT
M243T	ATC CAC TAC AAC TAC ATG TGT AAC AGT TCC TGC ACG GGC GGC ATG AAC CGG AGG CCC ATC CTC ACC ATC ATC ACA CTG GAA GAC TCC AGT GGT AAT
G244V	ATC CAC TAC AAC TAC ATG TGT AAC AGT TCC TGC ATG

	GTC GGC ATG AAC CGG AGG CCC ATC CTC ACC ATC ATC ACA CTG GAA GAC TCC AGT GGT AAT
G245S	ATC CAC TAC AAC TAC ATG TGT AAC AGT TCC TGC ATG GGC AGC ATG AAC CGG AGG CCC ATC CTC ACC ATC ATC ACA CTG GAA GAC TCC AGT GGT AAT
M246A	TAC ATG TGT AAC AGT TCC TGC ATG GGC GGC GCG AAC CGG AGG CCC ATC CTC ACC ATC ATC ACA CTG GAA GAC TCC AGT GGT AAT
M246T	TAC ATG TGT AAC AGT TCC TGC ATG GGC GGC ACG AAC CGG AGG CCC ATC CTC ACC ATC ATC ACA CTG GAA GAC TCC AGT GGT AAT
R248L	TGT AAC AGT TCC TGC ATG GGC GGC ATG AAC CTG AGG CCC ATC CTC ACC ATC ATC ACA CTG GAA GAC TCC AGT GGT AAT
R249G	AAC AGT TCC TGC ATG GGC GGC ATG AAC CGG GGG CCC ATC CTC ACC ATC ATC ACA CTG GAA GAC TCC AGT GGT AAT
R249S	AAC AGT TCC TGC ATG GGC GGC ATG AAC CGG AGT CCC ATC CTC ACC ATC ATC ACA CTG GAA GAC TCC AGT GGT AAT
D259V	ATG GGC GGC ATG AAC CGG AGG CCC ATC CTC ACC ATC ATC ACA CTG GAA GTC TCC AGT GGT AAT CTA CTG GGA CGG AAC AGC TTT GAG GTG CGT GTT TGT G
G266R	ATG GGC GGC ATG AAC CGG AGG CCC ATC CTC ACC ATC ATC ACA CTG GAA GAC TCC AGT GGT AAT CTA CTG CGA CGG AAC AGC TTT GAG GTG CGT GTT TGT G
R267Q	ATG GGC GGC ATG AAC CGG AGG CCC ATC CTC ACC ATC ATC ACA CTG GAA GAC TCC AGT GGT AAT CTA CTG GGA CAG AAC AGC TTT GAG GTG CGT GTT TGT G
V272L	GGT AAT CTA CTG GGA CGG AAC AGC TTT GAG TTG CGT GTT TGT GCC TGT CCT GGG AGA GAC CGG CGC ACA GAG GAA GAG AAT CTC CGC AAG AAA GGG GAG
R273C	AGC TTT GAG GTG TGT GTT TGT GCC TGT CCT GGG AGA GAC CGG CGC ACA GAG GAA GAG AAT CTC CGC AAG AAA GGG
R273H	AGC TTT GAG GTG CAT GTT TGT GCC TGT CCT GGG AGA GAC CGG CGC ACA GAG GAA GAG AAT CTC CGC AAG AAA GGG
P278A	AAC AGC TTT GAG GTG CGT GTT TGT GCC TGT GCT GGG AGA GAC CGG CGC ACA GAG GAA GAG AAT CTC CGC AAG AAA GGG
D281E	GAG GTG CGT GTT TGT GCC TGT CCT GGG AGA GAG CGG CGC ACA GAG GAA GAG AAT CTC CGC AAG AAA GGG
R283P	GTT TGT GCC TGT CCT GGG AGA GAC CGG CCC ACA GAG GAA GAG AAT CTC CGC AAG AAA GGG
E285K	GTT TGT GCC TGT CCT GGG AGA GAC CGG CGC ACA AAG

	GAA GAG AAT CTC CGC AAG AAA GGG
K305M	CAG TGG TTT CTT CTT TGG CTG GGG AGA GGA GCT GGT GTT GTT GGG CAG TGC TCG CAT AGT GCT CCC TGG GGG CAG CTC GTG GTG AGG
R337C	CC CTT CAG ATC CGT GGG CGT GAG TGC TTC GAG ATG TTC CGA GAG CT
R337H	CC CTT CAG ATC CGT GGG CGT GAG CAC TTC GAG ATG TTC CGA GAG CT

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CHAPTER 5

Con-A: A SUPER-TRANSACTIVATING RESPONSE ELEMENT FOR p53

TRANSACTIVATION

Abstract

Based on *in vitro* binding studies, the ability of p53 to function as a transcription factor from target response elements (REs) is expected to be a function of the amount of p53 with stronger binding elements requiring less protein. Using a recently developed yeast-based model system that addresses the *in vivo* potential for p53 to transactivate from target RE sequences, we established that low basal levels of p53 expression could result in transactivation of a linked reporter and that regardless of the RE a common level of p53 was needed before increased induction of transactivation could be first detected. Further increases in p53 led to increased transactivation in a manner that was dependent on the specific RE. A surprising response was obtained with the previously reported RE sequence GGGCATGTCC GGGCATGTCC known as Con-A. Transactivation was exceedingly high under conditions of very low levels of p53 as compared with p21-5' RE, one of the strongest REs known. While it is possible the perfect consensus sequence Con-A may induce structural features similar to a palindrome, the basal level response was not apparent when transactivation was assessed from a related perfect palindrome sequence. This novel *in vivo* interaction between p53 and the Con-A target which is much less dependent on p53 concentration than other targets requires p53 tetramerization. Because of its strong capability for transactivation, Con-A and a related sequence provide a useful tool for addressing p53 function and the functionality of p53 mutant proteins. In light of previous studies with super-transactivating mutants, these findings suggest novel interactions that can drive transactivation at greatly reduced levels of WT and mutant p53s. The absence of Con-A and the related sequence within the human genome may reflect its strong potential for binding and/or transactivation.

Introduction

Transcription factors and target response element sequences (REs) are central to any master regulatory network. The combination of a rheostatable promoter and luciferase reporter that we developed (described in Chapter 2) provides a highly sensitive system for assessment of WT and mutant p53 function, as well as interactions with target REs. Based on *in vitro* binding experiments, if all factors are constant the *in vivo* responses are expected to strongly depend on intracellular concentration of p53 as well as the specific RE examined, especially since p53 acts as a tetramer. Within the cell, other factors such as chromatin and associated transcription components also play a role in p53-dependent transactivation, but under the isogenic conditions used in the yeast-based system described in Chapter 2, their effects are proposed to be relatively constant (although further studies would be warranted to specifically address this issue).

As shown in Chapter 2 and presented in Figure 5.1, there are three phases to transactivation that can be revealed by analyzing function at increasing protein concentrations with the rheostatable promoter system in yeast. The first phase is a p53 dependent, low level response that is due to basal levels of expressed p53 when only a small number of molecules are present. Importantly, this low level response would not be observed with typical over-expression systems or with *in vitro* binding assays. The 2nd phase corresponds to substantial increases in amount of p53 and the 3rd corresponds to a plateau in transactivation due to a constant level of p53 (discussed in Results).

We have found that the initial induction of transcription, corresponding to the beginning of the 2nd phase, extrapolates to comparable levels of p53 protein for 26 REs examined, regardless of the biological role of the associated target gene or calculated *in vitro*

binding affinity. However, the *kinetics* or increase in the 2nd phase with increasing p53 and maximal level of transactivation at the 3rd phase of transactivation by p53 is strongly dependent on RE sequence. The common transition/transactivation point to the 2nd phase suggests an additional factor (possibly a conformational change in the binding sequence or tetrameric protein) is required for efficient activity from specific binding elements with increased levels of p53.

We discovered a sequence, referred to as Con-A [1], had unusual properties for p53 driven transactivation. It was extremely sensitive to basal levels of p53 such that high levels of transactivation were observed relative to all other REs examined and yet the maximum response (3rd phase) was comparable to that for transactivation from other highly responsive REs such as p21-5'. Thus, sequence itself can strongly affect p53 transactivation even under conditions where the number of p53 molecules is small and suggests novel interactions with p53. Previously, we had described super-trans mutants that can increase transactivation from a RE, also under low expression conditions. This study complements those findings in demonstrating that both sequence and protein can be modified to greatly enhance transactivation under conditions of low levels of p53 expression.

Results

RE-dependence of transactivation with varying levels of p53

The rheostatable system provides a unique opportunity to address p53 interactions with target sequences at variable levels of expression and define transactivation activity at low levels of p53 expression. Analysis of the kinetics of transactivation from 23 biological REs (see chapter 2) in terms of the three phases of transactivation revealed several distinct

patterns of transactivation for WT p53 relative to transactivation from the p21-5' RE (Figure 5.1). The three patterns of transactivation reiterated the existence of a low level phase of transactivation followed by induction and plateau of activity. First, *overlapping patterns of transactivation* at basal, inducing and maximal levels of expression were observed for several REs, as demonstrated with the p21-5' and P53R2 REs. Second, *variable basal transactivation followed by similar maximal transactivation* was observed (i.e., p21-5' vs. PUMA and PA26). The strength of induction from the RE with a weaker basal response appears to reach levels similar to the p21-5' RE quickly. However, measurements at additional low level protein concentrations between 0.004% and 0.012% will be required to verify this observation. Finally, *multipliers of transactivation* were observed where transactivation from a specific RE was proportionately reduced at all levels of p53 expression (i.e., p21-5' vs. MDM2 RE1). Importantly, variation in basal level response at low p53 expression appeared to be dependent upon the specific sequence of the RE. For example, the p21-5' and P53R2 REs which have overlapping low level response both contain strong core sequences containing "CATG" in both decamers, whereas, the REs with a decreased basal response lacked a strong core in either half-site. As shown in Figure 5.1, MDM2 RE1 which has a mismatch within the "WW" of the core motif had the weakest basal response of those depicted.

Con-A: a highly responsive RE to very low levels of p53

In addition to the above REs, which correspond to target sequences in human promoters, we also examined sequences that were fully matched to the established p53 consensus (*i.e.*, two decamers of RRRCWWGYYY). One of these, Con-A [GGGCATGTCC

GGGCATGTCC] had previously been used in structure-studies of p53 bound to REs [1]. This sequence, which does not exist in the human genome, was derived from three tandem repeats of the upstream p53 binding element of the human RGC sequence (CCAGGCAAGT)₃ with all possible nucleotide substitutions in an attempt to determine the DNA binding sequence specificity of wild-type p53 [1]. Murine WT p53 bound with the highest affinity to this sequence in competitive gel shift assays.

High levels of WT p53 can transactivate Con-A and p21-5' RE to similar levels in the *ADE2* color plate assay (data not shown) and luciferase transactivation assays in diploid yeast (Figure 5.2). However, unlike all other REs, Con-A is highly transactivated by p53 at basal levels of expression when p53 is grown in raffinose or at low levels of galactose (0.002%, 0.004%, and 0.008%), as shown in Figure 5.3A which summarizes results from over 75 experiments. Furthermore, within the *ADE2* plate color assay, WT p53 transactivation from the Con-A RE resulted in white colonies when cells were grown on raffinose plates (data not shown).

Low level transactivation from the Con-A sequence was p53-dependent (Figure 5.3B). Transactivation activity at non-induced conditions (2% raffinose) was significantly increased in comparison to activity under repressed conditions (glucose). Furthermore, if the p53 cDNA was interrupted by a CORE cassette (see Figure 2.5), transactivation activity from the sequence was not observed, suggesting the activity from the Con-A RE at low levels of p53 was due to p53 and not other factors interacting with the sequence.

The basal level of p53 in the cells grown in raffinose media corresponds to only ~250-500 p53 molecules per cell, compared to ~5,500 p53 molecules at the point (0.008% galactose) where increases in p53 lead to increased transactivation (Figure 2.6C). The

number of molecules for cells grown on glucose is likely to be <50 to 100. The strong ability of a tetramer protein to transactivate with small numbers of molecules suggests novel properties of the Con-A RE that cannot be simply explained from *in vitro* measurements of K_d where the K_d is only a factor of 1.5 different from that for the strongly binding p21-5' RE (Veprintsev and Fersht, personal communication). Other REs often require at least ten times more p53 to achieve even low levels of transactivation. Furthermore, several weak REs required approximately 100 times more p53 protein over basal level to reach transactivation levels comparable to that obtained from Con-A at basal amounts of p53 expression. We conclude that there is a special relationship between p53 and the Con-A target sequence in terms of transactivation at low levels of p53 expression. Because of its highly responsive nature, we refer to Con-A as a *super-transactivating* RE. Previously, we had described super-trans p53 mutants that were highly efficient at transactivation at low levels of p53 [2].

Con-A basal level response requires a p53 tetramer functioning from a full-site RE

While the p53 tetramer is the functional unit essential for high level transactivation, dimers have been shown to bind consensus sequences *in vitro* albeit with a high off-rate [3]. To ascertain if the basal response was the result of p53 dimer binding and functioning from the Con-A sequence at low levels of p53 expression, we analyzed transactivation by p53 mutations that are compromised for oligomerization: Δ Tet, L334A and L334P. Only monomeric p53 protein is formed with Δ Tet (deletes residues 325-357), while the L344P and L344A mutations result in monomeric and dimeric proteins, respectively (see Chapter 3). As shown in Figure 5.4A, these mutant proteins were not able to drive transactivation from the

Con-A sequence at low or even high levels of p53. Thus, low level transactivation is not due to p53 functioning as a dimer protein.

Transactivation by WT p53 was also examined from $\frac{3}{4}$ -site and $\frac{1}{2}$ -site noncanonical versions of Con-A. While WT p53 could transactivate from the $\frac{3}{4}$ -site consensus elements to approximately 15-25% of the maximal levels of transactivation obtained from the full element at high levels of transactivation, there was very little response at low levels of p53 (Figure 5.4B). Similarly, WT p53 was not able to produce a functional response at low expression from a half-site consensus where the response at high levels (0.024% galactose) was ~1.7% of the response achieved from the full-site RE at maximal induction. [Transactivation from the Con-A half-site relative to the Con-A full-site yielded a slightly greater response in comparison to that for WT p53 transactivation from either of the p21-5' half-sites relative to the p21-5' full-site (see Figure 3.3).] Therefore, the low level p53 capability for transactivation from the super-trans Con-A RE is dependent on a tetrameric p53 and a full-site RE.

Low level transactivation is sequence dependent

We next investigated the sequence specific requirements necessary for efficient basal level transactivation by assaying several additional consensus sequences for low level response. As shown in Figure 5.5A, changing the core from CATG to CTAG or CAAG decreased transactivation at high levels of p53 by ~45 - 50% (average light units per ug protein at 0.024% galactose for 8 biological repeats were ~1,250,000, ~ 698,000, ~640,000 for Con-A, Con B and Con C, respectively). In addition, there was a dramatic reduction in the responsiveness to basal levels of p53, where altering the core and/or flanking sequence

diminished the lower level transactivation to ~ 5% or less of that obtained from the Con-A sequence at low levels of p53 expression (2% raffinose, 0.002% and 0.004% galactose).

Although the core CATG is necessary for basal levels of transactivation, analysis of the flanking sequences revealed that it was not sufficient for the super-trans low level response. While there is still a complete match to consensus, a change in the flanking sequences GGG...TCC to AGG...CCT or GGA...TCC resulted in a reduction of transactivation at basal p53 levels to that found for p21-5' as shown in Figure 5.5B. However, the flanking sequence GGG...CCC also resulted in strong transactivation at low levels of p53 (Figure 5.6). Similar to Con-A, this RE [GGGCATGCCC GGGCATGCCC], referred to as crystal I [4] was not found in the genome. Subsequently, based on fluorescence anisotropy titration experiments, Veprintsev and Fersht identified the sequence GG(A/G)CATGCCC GGGCATG(T/C)CC (which includes the crystal I sequence) as having the highest affinity for p53 [5].

Spacers between Con-A half-sites greatly decrease its super-trans response

The crystal I RE that contained a 2 nt spacer between the decamers was recently employed in crystal studies that addressed the structure of p53 tetramer bound to target sequence [4]. We, therefore, determined the impact that the 2 nt spacer might have on transactivation. Contrary to transactivation from full-site p21-5', REs containing a 1 or 2 nt spacer (Fig. 3.2A), a 2 nt spacer between the decamers did not diminish maximal transactivation from the crystal I sequence (Figure 5.6). However, the 2 nt spacer abolished the low, basal level p53 response. Importantly, the loss of basal transactivation by the (crystal I + 2 nt) RE indicates that the super-trans response at Con-A to low level p53 is not

related to some palindrome-forming feature of the sequence since the 2 nt insertion would have enhanced any secondary structure of this complete palindrome. In agreement with this view, transactivation was also low towards the consensus RE referred to as “Con cam” composed of two half-sites that are mirror images of itself (Figure 5.6). Furthermore, the crystal II and crystal III sequences (Figure 5.5B) which also create complete palindromes were shown to have a weaker basal level response in comparison to the quasi-palindrome Con-A sequence.

Since the 2 nt spacer did not affect maximal transactivation from the crystal I sequence, the impact of spacer upon WT p53 transactivation from the Con-A sequence was systematically investigated for the impact of spacers. Addition of a single nucleotide spacer between the Con-A half-sites did not dramatically affect maximal levels of transactivation as seen for the p21-5' RE (see Figure 3.2); however, the 1 nt spacer caused a 50% decrease in response to low levels of p53 (Figure 5.7). Similarly, addition of a second nucleotide did not significantly reduce transactivation at higher levels of expression; however, it further decreased p53 transactivation at low p53 levels. Increasing the spacer to 5 nt affected transactivation at both low and high levels of p53: 5% and 30%, respectively, when compared with no spacer. Thus, the Con-A super-trans response is strongly dependent on spacer and specific consensus sequences.

Transactivation capacity of p53 mutants from Con-A

Since the Con-A sequence strongly responds to low levels of WT p53, we addressed the ability of various p53 mutants to transactivate from this sequence versus other target REs, in addition to the altered structure mutants described above. Mutants with very low

transactivation capabilities may be more responsive with the super-trans Con-A RE. We examined the 50 missense p53 mutants associated with breast cancer which had been analyzed for functional fingerprints in response to 11 human target REs (Chapter 4) for their ability to transactivate from the Con-A RE. Within that group none of the 29 loss-of-function mutants were capable of transactivating from Con-A in the *ADE2* color assay (Table 2.1; data not shown). However, all but one of the 21 p53 missense mutations that retained any function were capable of nearly WT transactivation at high levels of p53 induction (Figure 5.8). While WT p53 was effective at transactivation from Con-A at very low and basal levels, only 2 (P151H and R174K) among the 21 mutants were capable of near WT levels of transactivation at basal levels (0% galactose) of p53 expression. There was considerable variation in response between the mutants at intermediate levels of p53 expression (Figure 5.8). Thus, transactivation from the Con-A can reveal subtle transcriptional defects of p53 mutants.

Transactivation of Con-A was also examined in the luciferase assay for 13/21 of the functional p53 mutants (T125R, L130V, A138V, C141W, P151A, G154S, R174W, P190L, M237I, R267Q, E285K, R337C and R337H). As shown in Figure 5.9A, many p53 missense mutations including C141W, G285K, and P151A eliminated the basal level response yet retained good induction at higher levels of p53. None of the breast cancer associated mutants exhibited a super-trans response at basal levels of p53 followed by no increase with increased p53 levels. For many mutants such as L194P and R337C, there was a general modulation of transactivation in comparison to WT (data not shown and Figure 5.9B). As expected due to the compromised ability to form tetramers, neither of the tetramerization mutants at R337 displayed a high activity from Con-A at low p53 expression. However, transactivation

analysis from Con-A further distinguished the different pattern of transactivation by R337H in comparison to WT p53 and other mutants, represented by R174W, where greater levels of R337H protein were required for transactivation. Similar to results from other REs, R337C appeared to diminish overall transactivation (Figure 5.9B).

We also examined an additional 3 mutants (not among the 50 mutants in the breast cancer study), V122A, C277R, and G279R which had previously been described as “super-trans” because of their ability to transactivate from the p21-5’ RE at lower levels of expression in comparison to WT p53 when assessed with the plate assay (see Table 2.3)[2]. At high levels of expression, all three of these mutants were also toxic [6]. Similar to several of the breast cancer mutants, C277R and G279R had a reduced low level response from Con-A in comparison to WT p53, but similar transactivation at higher expression (data not shown). Interestingly, the response for V122A towards Con-A did not differ from wild type (Figure 5.9C); all three phases of transactivation were equivalent. Preliminary results suggests V122A has an elevated basal level response from p21-5’ in comparison to WT p53 (data not shown).

Discussion

Many studies have addressed the function of p53 mutants towards a variety of REs that have different capabilities in supporting transactivation. Using a system that examines p53 function over a broad range of protein expression, we have discovered that the previously described Con-A sequence was highly effective at supporting transactivation even under extremely low expression conditions, thereby greatly increasing the functionality of p53.

The super-transactivation nature of Con-A requires a full-site consensus binding element and is dependent on the ability of p53 to form a tetramer, the same structural requirement necessary for high level response. As reported for other REs, a CATG core domain is required for a strong response by the super-trans RE. Flanking sequences that match consensus are important, but not sufficient for low level response. Surprisingly, the super-trans RE response does not require that the sequence be a palindrome although a second sequence, crystal I, had this feature. In fact, the addition of a spacer consisting of 2 complementary bases negated the super-trans effect. Since the closely related palindromic consensus sequences crystal II, crystal III and Con cam were not super-trans REs, sequence is important to the strong transactivation responses.

The observation that neither of the super-trans REs are found in the genome (the closest sequence found has 17 of the 20 nucleotides without a mismatch) suggests that these are sequences from which p53 would not be able to sufficiently regulate activity. Binding predictions suggest that approximately 1.5 fold more p53 protein would bind Con-A in comparison to p21-5' under identical conditions [5] (Veprintsev and Fersht, personal communication). However, the 1.5 fold difference in binding does not explain the large discrepancy in low level transactivation between the two sequences. [In agreement with the predictive binding constants, fluorescence anisotropy *in vitro* binding assays have determined p53 binding affinity towards Con-A was comparable to that for other strong binding REs including p21-5' at high levels (Veprintsev and Fersht, personal communication)[5].] Interestingly, the "Con cam" sequence which did not have a basal response comparable to Con-A was proposed to be 7-fold more active than Con-A based on theoretically predictions of binding affinities (Veprintsev and Fersht, personal

communication). Thus, the basal transactivation results may indicate a novel *in vivo* binding mechanism at low levels of p53 protein. Possibly the super-trans response at low p53 levels is due to unique chromatin structures influenced by the Con-A or crystal I sequences.

The super-trans Con-A sequence appears diagnostic of residual transactivation function of mutant p53 proteins. If a mutant is able to transactivate from Con-A, then it can also transactivate from at least one additional RE. However, if a mutant p53 fails to transactivate from Con-A, even at high p53 expression, it is also incapable of transactivating from any of an additional 11 REs studied. Importantly, transactivation from the Con-A sequence appears to distinguish the transcriptional capacity between many p53 mutations associated with breast cancers and WT p53. The observation that no mutants were identified that affected transactivation at high p53 levels but not super-trans activity at low expression levels, indicates that there are common features of the p53 protein required for the two activities.

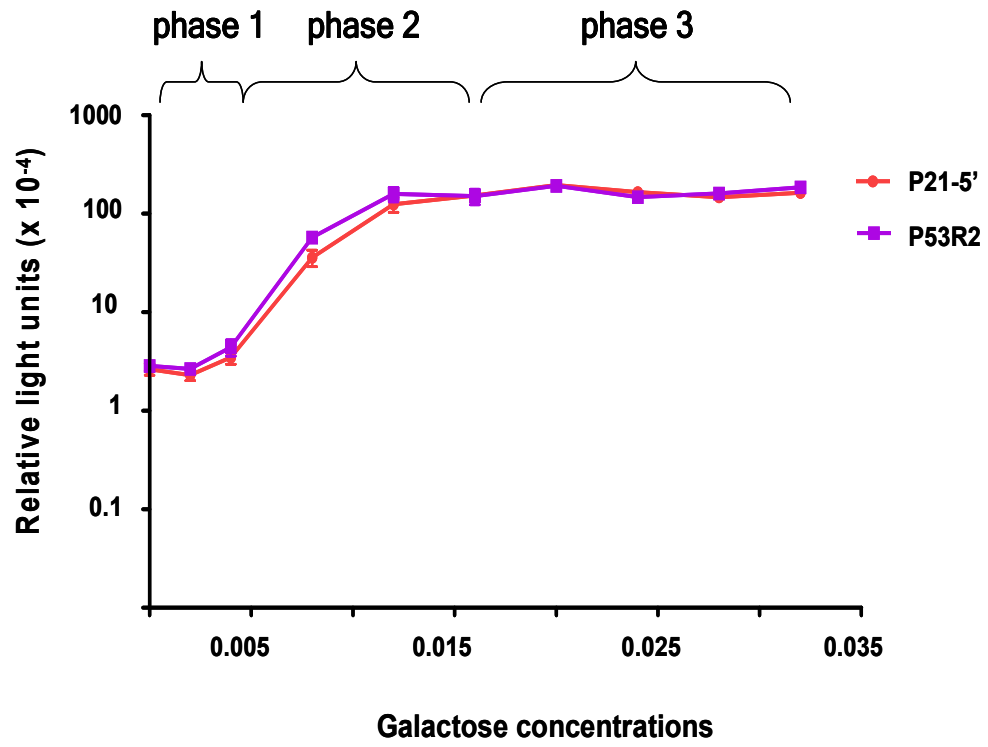
The mutation V122A which exhibits high basal level response with Con-A had previously been identified as a change-in-spectrum mutation that was also toxic to the yeast. The toxicity may indicate a gain in binding affinity towards additional binding sites within the genome in comparison to WT protein. This may be plausible since V122A is positioned within the L1 loop of the protein which contacts the major groove of DNA. (The two other toxic mutants, C277R and G279R are located adjacent to or in the H2 helix). It is interesting to speculate if the V122A mutation can increase low level response from other REs, then the ability to regulate the associated downstream targets within the network will be compromised. Although C277R and G279R appeared super-trans for the p21-5' RE, these mutations did not display high levels of basal activity from Con-A. This suggests the high

basal response is not a general phenomenon to change-of-spectrum, toxic mutations. Yet, it will be interesting to evaluate basal response for mutations such as S121F that are found to be super-trans for all the REs. Furthermore, the variation in Con-A transactivation by V122A and C277R or G279R may differentiate toxic mutants that enhance a proteins affinity towards DNA from those mutations that increase protein-protein interactions within the cell.

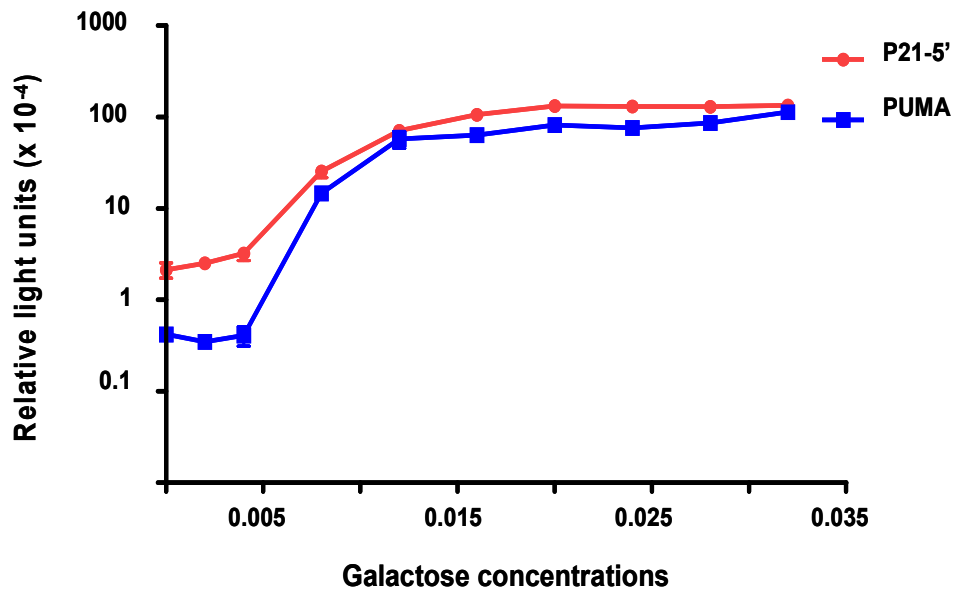
The super-trans REs along with super-trans mutants establish that small amounts of p53 can be highly effective at transactivation. Thus, alterations in the genome or p53 can dramatically change the rules of engagement [7] and make p53 much more efficient at transcription. While the underlying mechanism remains to be established, it is possible that either of these two super-trans conditions arise from novel interactions that greatly reduce the off-rate of the protein. We are currently exploring this and other possible mechanisms in yeast and human cells.

Figure 5.1

A.



B.



C.

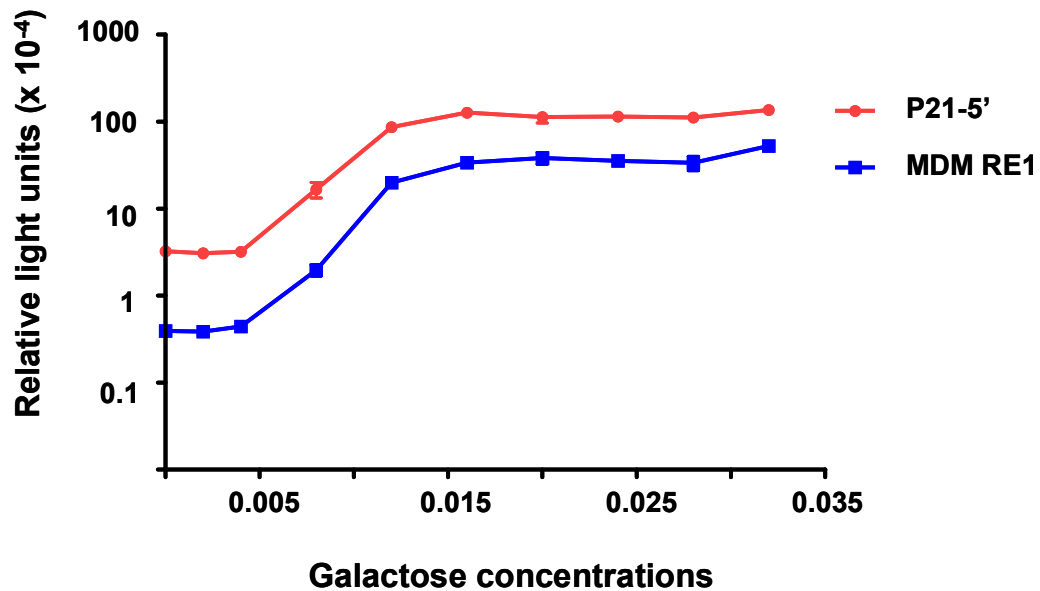


Figure 5.1 Three phases to transactivation by p53. Luciferase assays reveal distinct patterns of transactivation kinetics for WT p53 towards REs. Despite sequence specific differences in the maximal levels of transactivation obtainable from a specific RE, there are three phases of transactivation. Phase 1 was classified as a low level response where sequence-dependent basal levels of transactivation were observed. Phase 2 corresponds to the increase in transactivation associated with increasing amounts of p53. Phase 3 was a high level p53 response, where sequence dictated the maximal transactivation levels. Three patterns of transactivation were identified between REs: a) similar overall response; b) differing basal transactivation with similar maximal transactivation; c) simple modulator (multiplier) of transactivation.

Figure 5.2

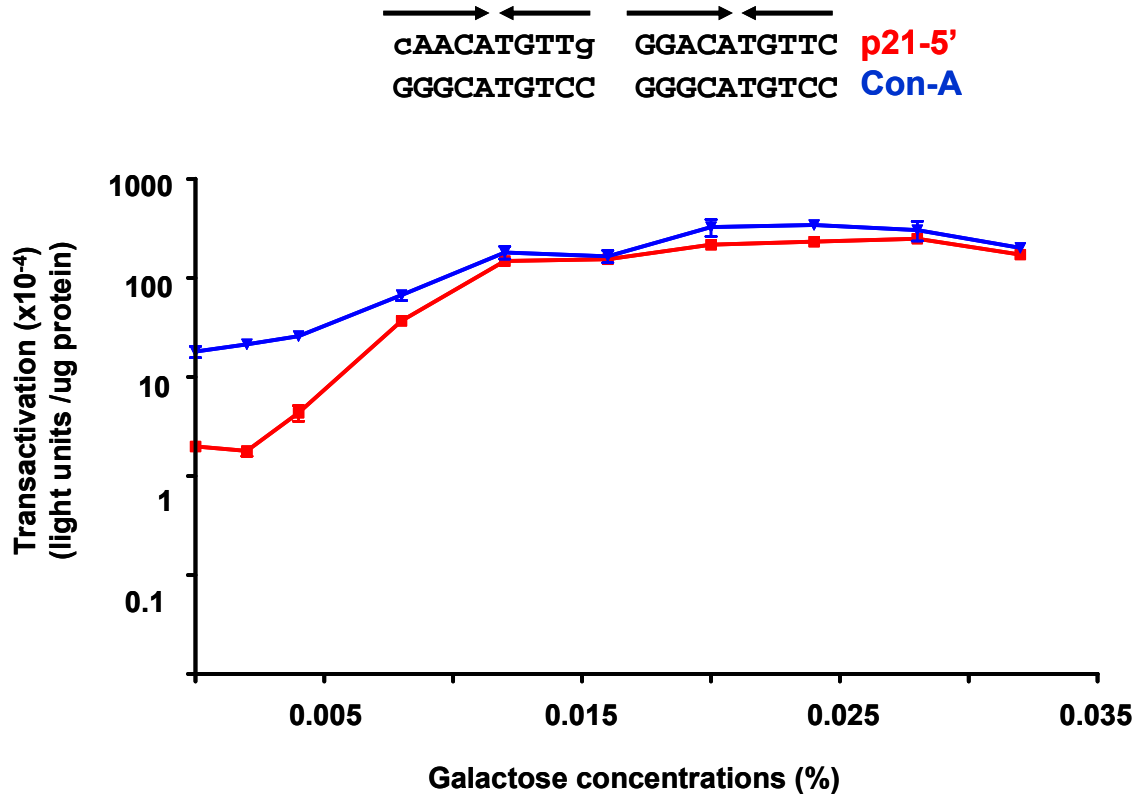


Figure 5.2 WT p53 transactivation is similar from the Con-A and p21-5' REs at high levels of p53 expression. The ability of WT p53 to transactivate from the Con-A and p21-5' REs was measured 24 hours after incubation in media containing increasing amounts of galactose. The luciferase assay shows WT p53 transactivates from both sequences to a similar level at high levels of transactivation. However, WT p53 has the ability to transactivate from Con-A at low levels of p53 expression. Presented are the mean and SEM for 4 biological repeats.

Figure 5.3A

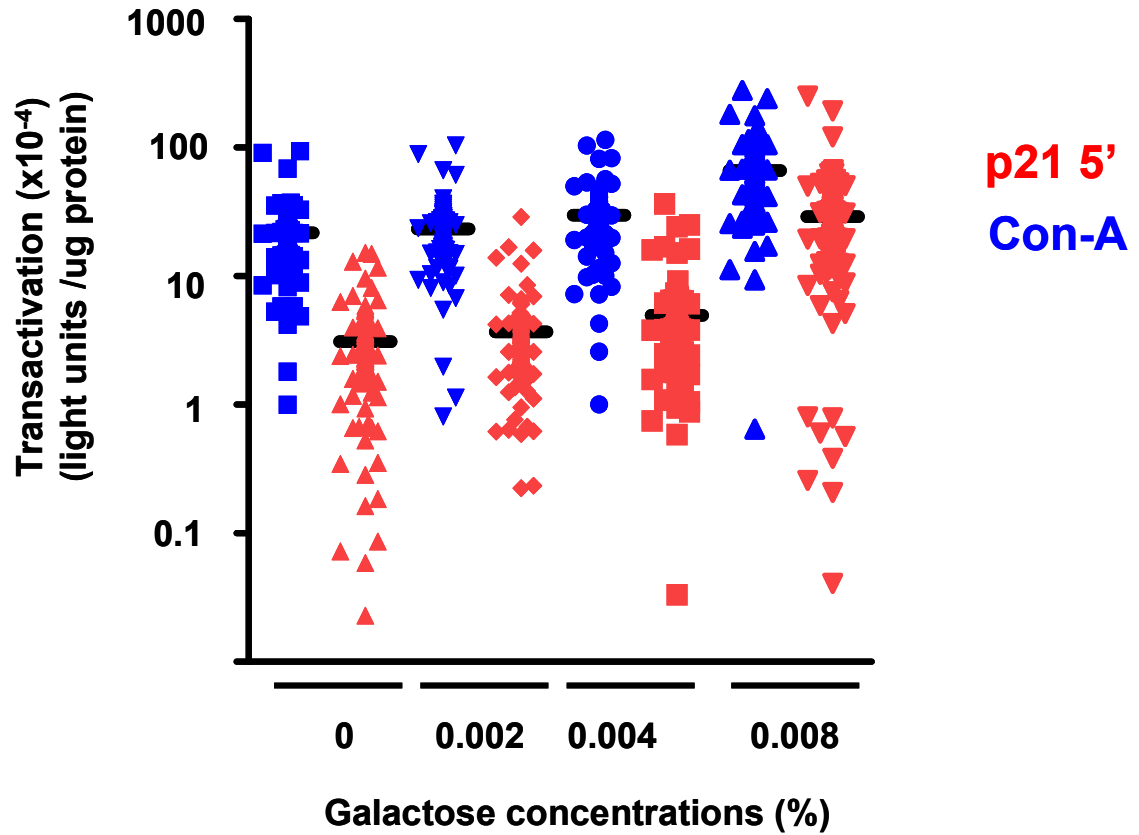


Figure 5.3A WT p53 transactivation from Con-A vs p21-5' REs at low levels of expression. To address the reproducibility of high basal level by p53, transactivation from the Con-A and p21-5' REs was compared at levels of p53 induced by 0, 0.002, 0.004, and 0.008% galactose. Presented is the strength of transactivation calculated as light units/ug protein for a minimum of 75 biological repeats at each concentration of galactose. The black line indicates the mean value of the biological repeats.

Figure 5.3B

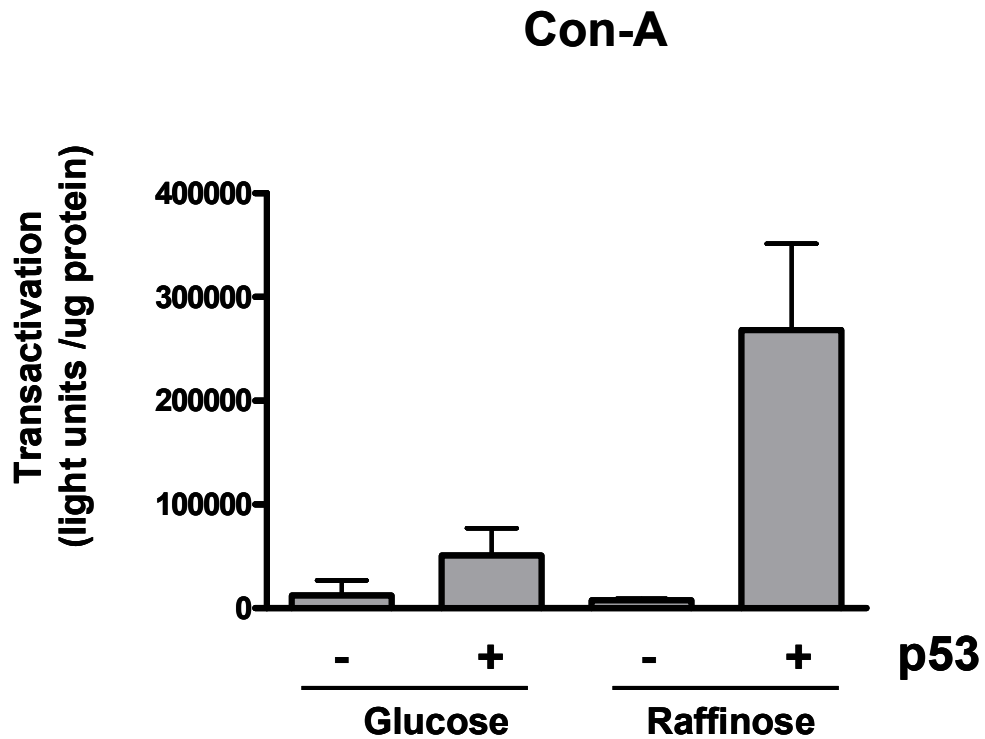


Figure 5.3B Low level transactivation is p53-dependent. To ascertain if the low level transactivation from the Con-A sequence was dependent on p53, transactivation from Con-A was assessed for WT p53 (+) and a strain where the p53 DNA contained a CORE cassette (-). Individual diploid colonies were grown overnight in YPDA complete media, washed, diluted and inoculated into synthetic media containing 2% glucose or 2% raffinose which corresponds to repressed and non-induced conditions, respectively. Overnight cultures were harvested and transactivation was assessed with a quantitative luciferase assay. Depicted is the mean and SEM for 8 biological replicates

Figure 5.4A

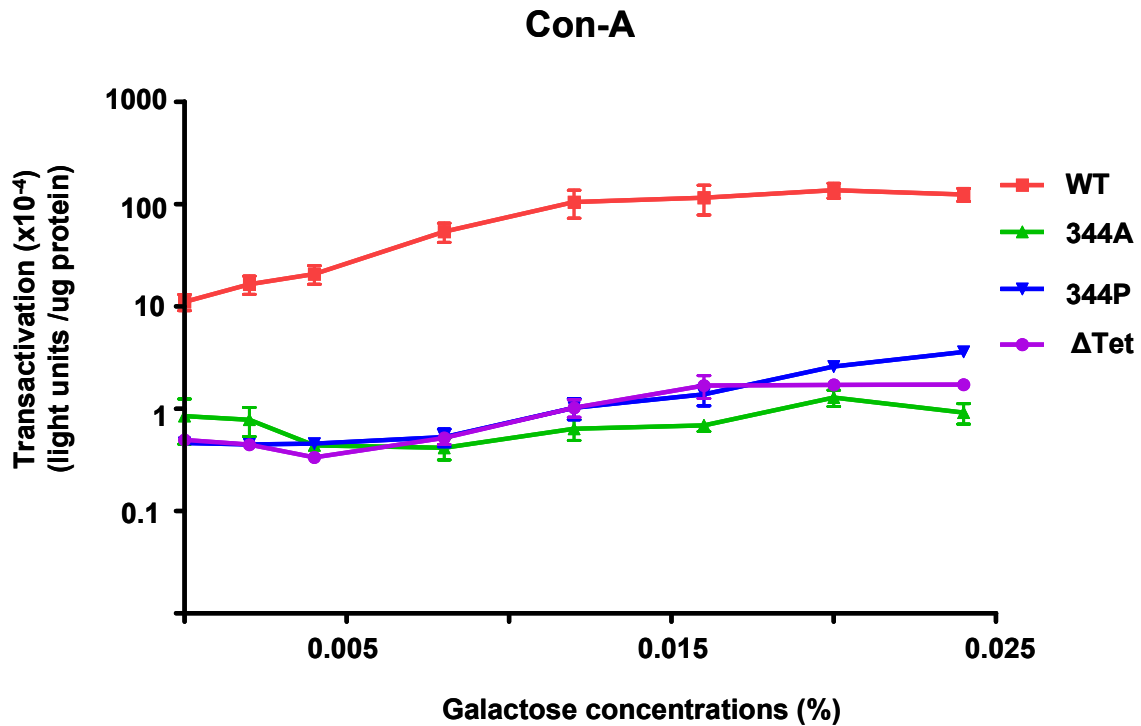


Figure 5.4A p53 tetramerization is required for the super-transactivation at low levels of p53. To determine if the low level p53 response was due to a p53 dimer binding and transactivating from the Con-A sequence, several mutations that affected the ability of the protein to oligomerize were assessed for transactivation. 344A is a dimer protein, 344P is a monomer and Δ Tet eliminates the tetramerization domain. As determined with a luciferase assay, the three mutations were not able to function from the Con-A RE at any level of p53 analyzed. Presented are the mean and SEM of 8 biological repeats.

Figure 5.4B

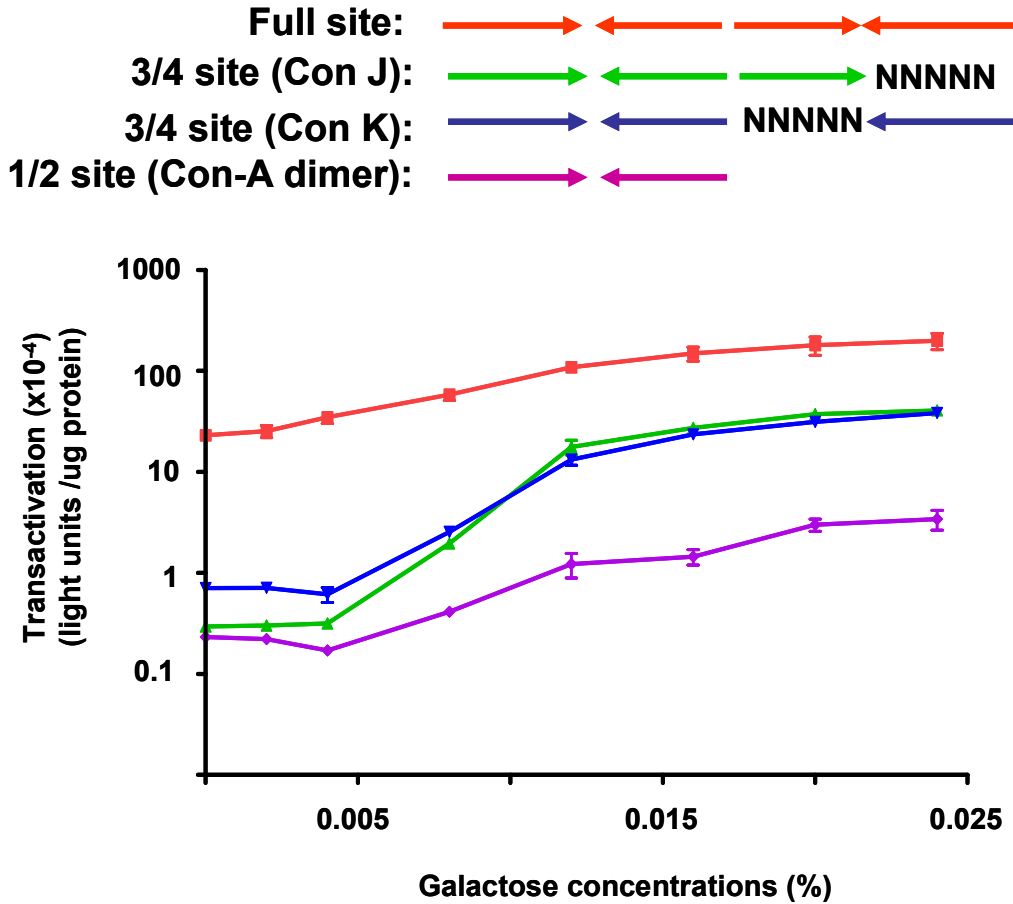


Figure 5.4B Super transactivation at basal levels of p53 requires a full-site RE. To assess if WT p53 could maintain a low level response with noncanonical REs, the ability of WT p53 to transactivate from a Con-A half-site or either derivation of the $\frac{3}{4}$ -site was compared to its ability to transactivate from the full-site RE. Arrows indicate a $\frac{1}{4}$ -site, GGGCATGTCC. Depicted is the mean and SEM of 8 biological replicates analyzed by a luciferase assay.

Figure 5.5A

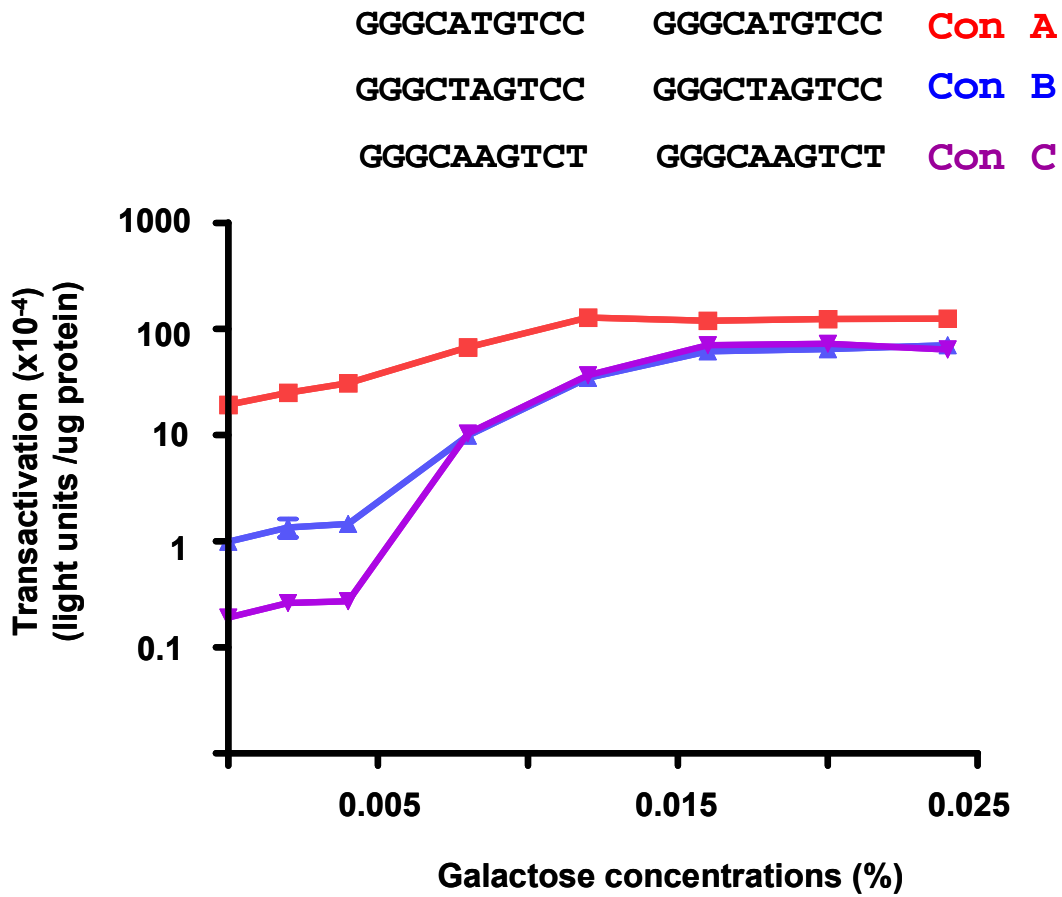


Figure 5.5A Super-transactivation at low levels of p53 requires a CATG core. To determine the sequence requirements for basal transactivation, the central core of the dimer was altered from CATG to with CTAG or CAAG. WT p53 transactivation was assessed with a luciferase assay using cells from overnight cultures growing in media with various levels of galactose. Depicted are four biological replicates mean and SEM.

Figure 5.5B

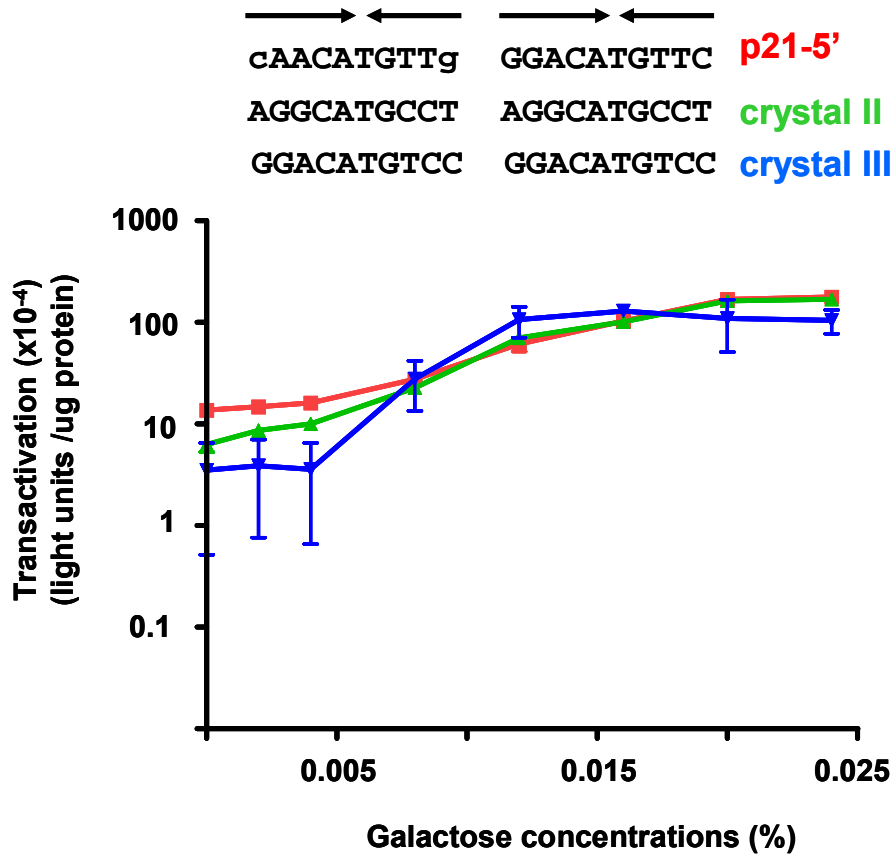


Figure 5.5B Flanking sequences affect basal transactivation by WT p53. To assess the sequence dependent nature of the super-trans response of p53 to Con-A, the flanking sequence surrounding a CATG core was altered. Presented are the mean and SEM of 4 biological repeats for WT p53 transactivation from the p21-5', crystal II and crystal III REs as determined with a luciferase assay. Crystal II and crystal III are sequences used in recent crystal structures where human p53 complexed to various binding sequences was analyzed [4]. Transactivation from crystal II and crystal III REs show comparable levels of transactivation to the p21-5' RE at low levels of expression. These results suggest CATG is essential, but not sufficient for super-transactivation at low levels of p53. Depicted is the mean and SEM of 4 biological replicates.

Figure 5.6

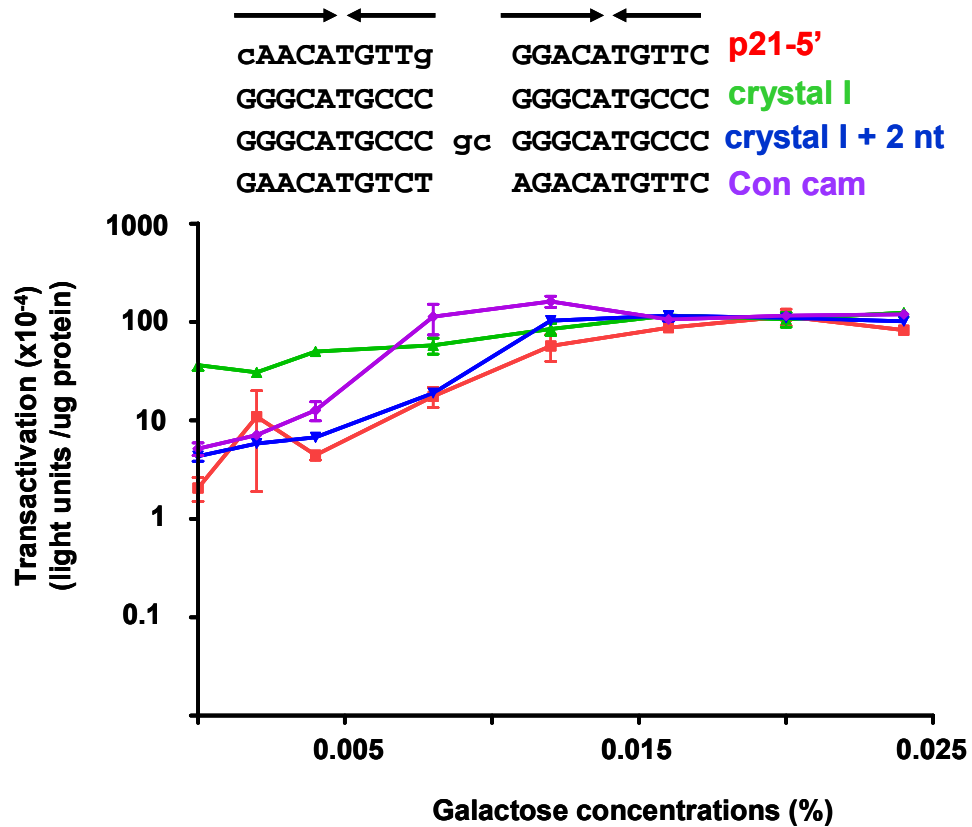


Figure 5.6 Crystal I: a second super-trans RE from which p53 can transactivate at low levels. Super-transactivation, as assessed with a luciferase assay, was also observed when the CATG flanking region consisted of GGG...CCC, but not for GAA...TCT (Con cam). A 2 nt spacer between the crystal I decamer half-sites, which would increase a secondary structure of the perfect palindrome, did not decrease the maximal levels of transactivation, but did reduce the low level response. Depicted is the mean and SEM of 4 biological replicates. [4].

Figure 5.7

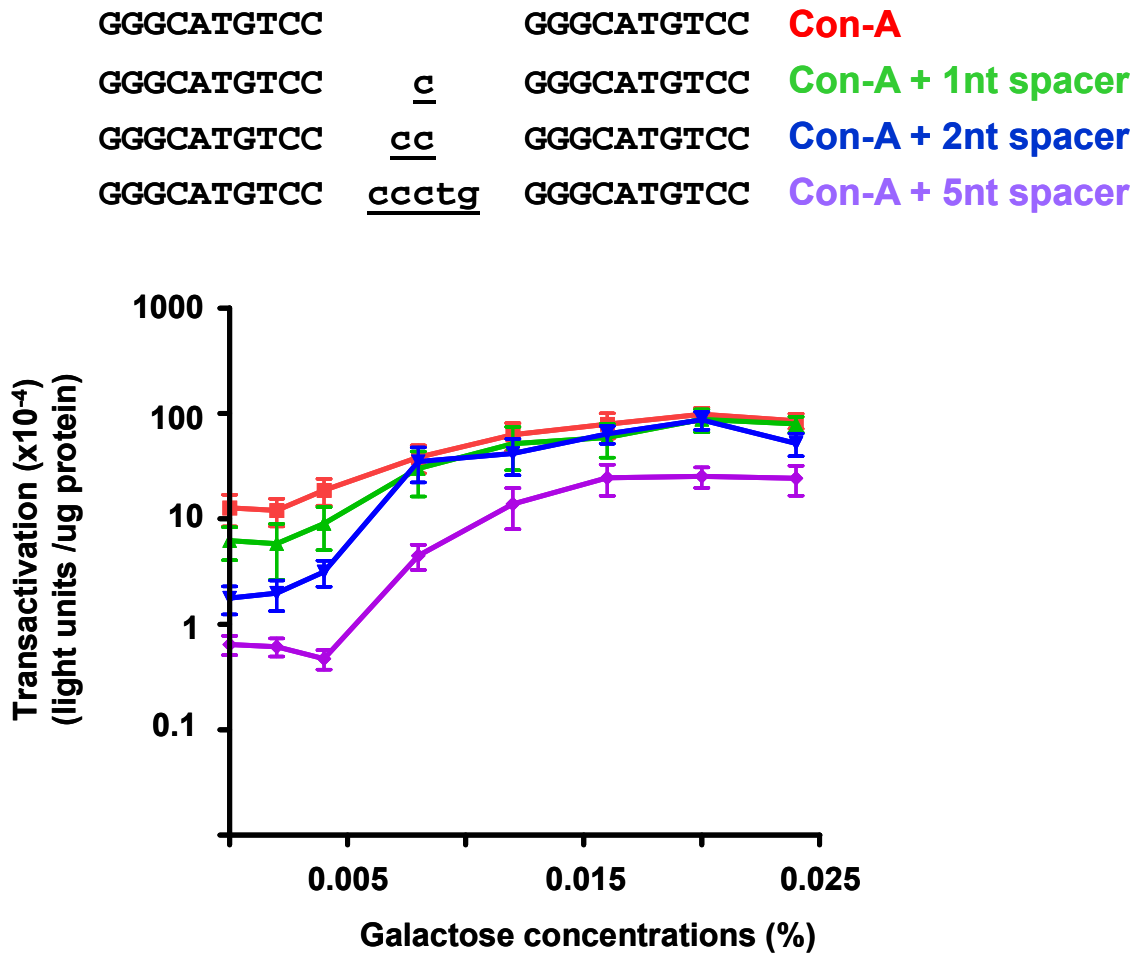


Figure 5.7 Small increases in spacer affects Con-A super-trans response at low levels of p53. Spacers of 1, 2 and 5 nucleotides were inserted between the decamer half-sites of the Con-A RE to determine the impact of spacer on p53 transactivation. The ability of p53 to transactivate from these sequences was measured with a luciferase assay 24 hours after p53 induction. Presented is the mean and SEM for 8 biological repeats. Addition of a 1 and 2 nt spacer between the decamer half-sites of Con-A did not effect maximal transactivation, but reduced basal level transactivation to increasing degrees. Insertion of a 5 nt spacer decreased transactivation with basal levels of p53 as well under conditions of maximal p53 expression.

Figure 5.8

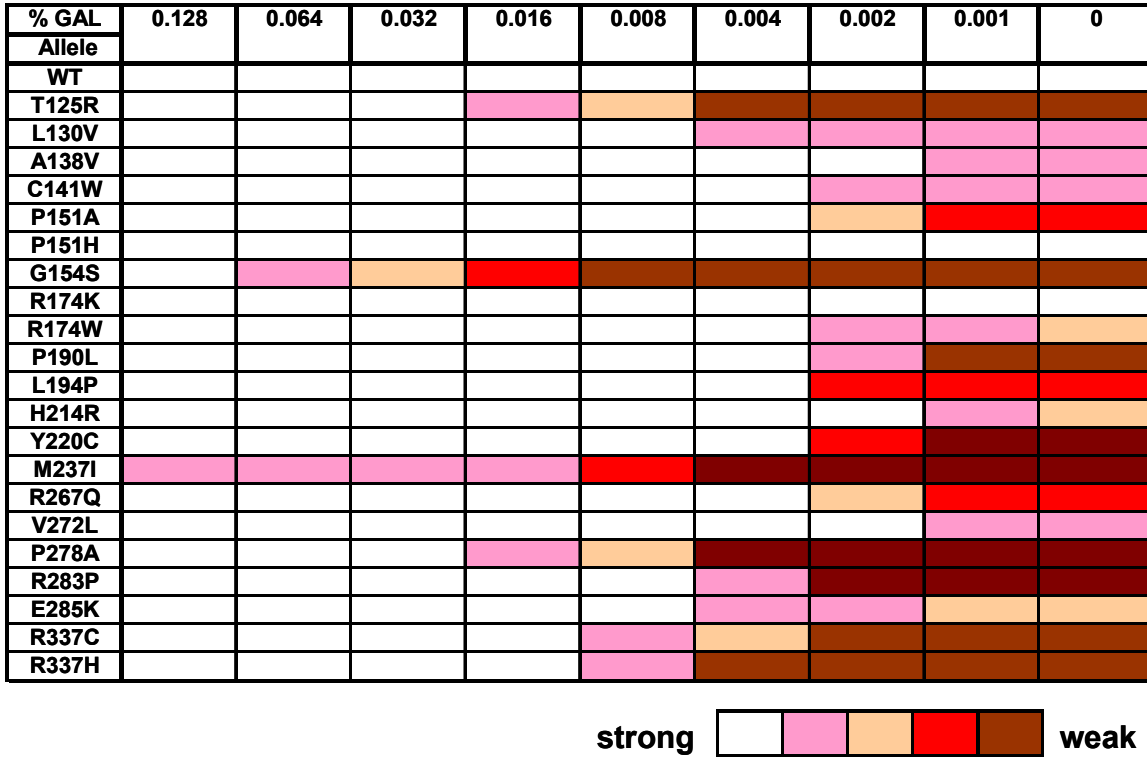


Figure 5.8 Subtle defects of p53 missense mutations revealed by transactivation from Con-A. Transactivation capacities of WT and mutant p53 were assessed from the Con-A RE using the *ADE2* plate color assay at decreasing levels of p53 induction. Single colony isolates grown at 30°C on YPDA plates containing glucose and high levels of adenine were replica plated onto a series of plates containing synthetic medium with low levels of adenine [5 mg/L], 2% raffinose and incremental increases in galactose. Transactivation capacity was assessed through colony pigmentation and scored according to the extent of transactivation from strong to weak responses.

Figure 5.9A

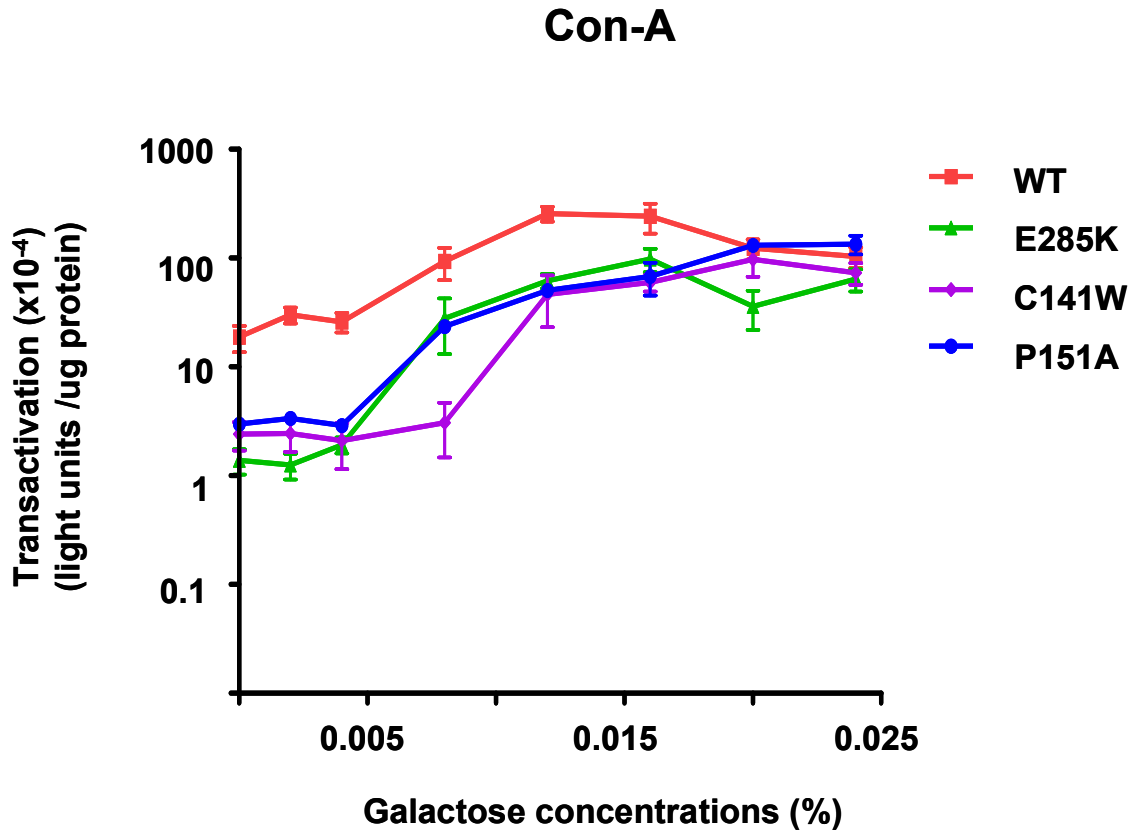


Figure 5.9A Impact of p53 missense mutations transactivation from Con-A. Transactivation capacities of several p53 missense mutations from the Con-A sequence were assessed with the luciferase assay. Several p53 mutations (including data not shown) in the DNA binding domain of the protein reduced transactivation from Con-A only at low levels of p53. Presented are the mean and SEM of 4 biological.

Figure 5.9B

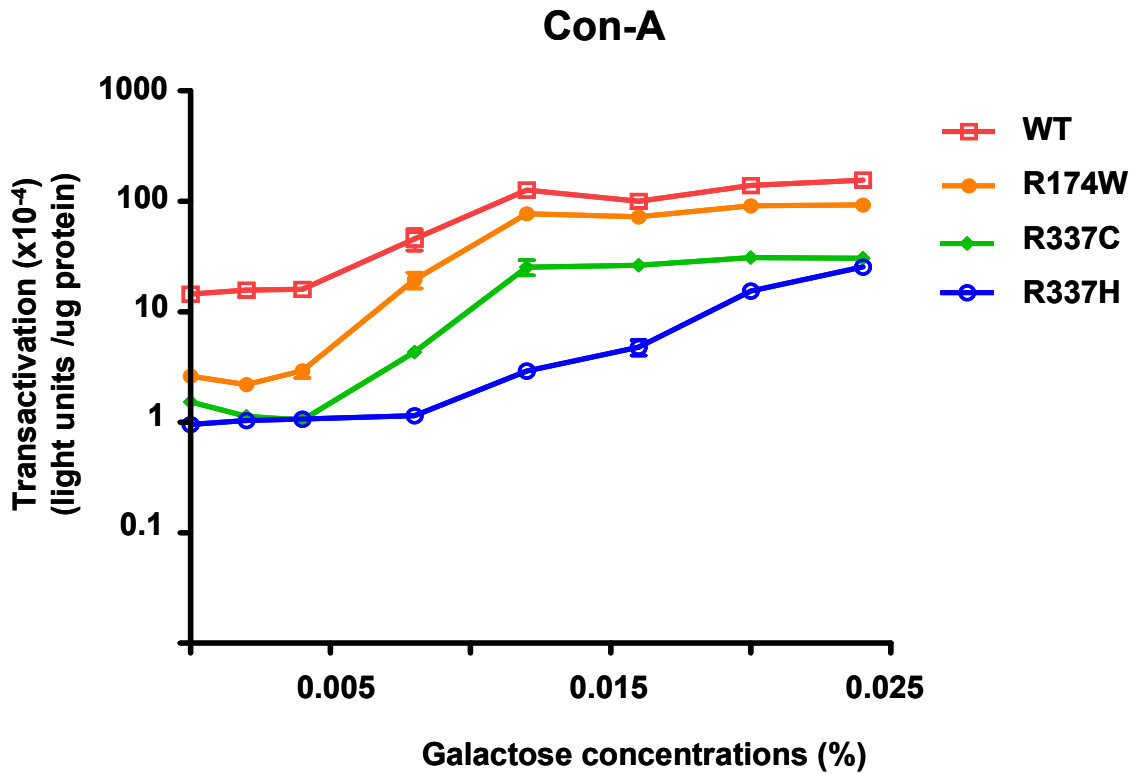


Figure 5.9B Compromised tetramerization diminishes basal response. Mutations at R337 which compromise tetramerization were assessed for the ability to transactivate from Con-A. As expected, both mutants lack a low level response. Presented are the mean and SEM of 4 biological repeats. Interestingly, transactivation from Con-A further differentiates the kinetics of transactivation for R337H which requires greater protein for transactivation (see Chapter 4). While R337C diminishes transactivation from the site, it appears to begin to induce transactivation at similar protein levels as WT p53 and R174W.

Figure 5.9C

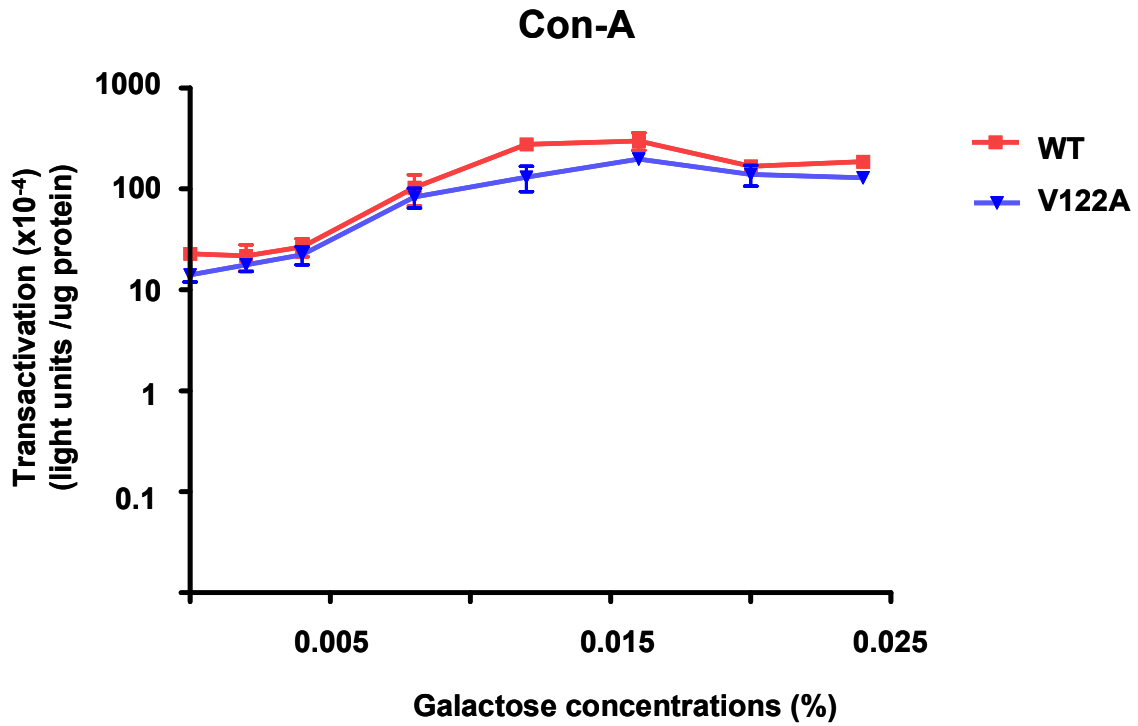


Figure 5.9C Transactivation by the toxic mutant V122A. The toxic mutant p53 protein is super-transactivating towards several REs including p21-5' at low levels of p53 [2, 6]. However, transactivation from Con-A was similar to WT p53. Shown are the mean and SEM of four biological repeats.

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CHAPTER 6
DISCUSSION

Phenotypic diversity within the p53 transcriptional network occurs as a result of a variety of factors including cell type, post-translational modifications, co-factor availability, target sequence and stress inducing stimuli. We have sought to understand how p53 expression level, target binding sequence and mutations in the p53 protein contribute to the differential regulation of gene expression within the p53 transcriptional network. Based on this concept, the integrated diploid yeast rheostatable system was developed to determine the influence of binding motifs and sequence on transactivation capacity when REs were assessed outside of their endogenous genomic context. The diploid yeast system expands a previously established plasmid-based haploid yeast system by integrating both p53 and the REs into specific loci, allowing a single copy of a p53 variant to be rapidly assessed for transactivation capabilities from many REs simply by taking advantage of the yeast mating types. In contrast to *in vitro* biochemical assays, the yeast assay has the advantage of comparing p53 variants in a constant chromatin environment--under isogenomic conditions--for activity towards individual REs. Although additional factors (i.e., post-translational modifications or protein binding partners) not available in the yeast cell may further influence a biological response in mammalian cells analysis, the isogenomic model system addresses the potential for wild type and mutant p53 to bind and transactivate from various response elements derived from human genes.

Small changes in sequence can lead to dramatic differences in transactivation

When analyzed in isogenic systems, the sequence of the RE has been shown to contribute to the ability of p53 to differentially transactivate target genes, where variation in sequence can greatly affect transactivation capacity [1]. The Resnick lab has focused on determining the “rules of p53 transactivation” to understand what constitutes a functional p53 target sequence, as well as what attributes are required for strength of transactivation [2]. Results from the integrated diploid yeast system are consistent with the rules of transactivation previously determined in a haploid-plasmid based system and mammalian cell lines for transactivation from canonical REs. Interestingly, the number of mismatches in the consensus sequence does not correlate with strength of activity. For example, WT p53 can only moderately transactivate from the MMP2 RE which does not deviate from the consensus sequence, but can strongly transactivate from the P53R2 and p21-5' RE which contain mismatches. Previous studies have determined no more than 4 alterations from the consensus sequence are associated with functionality [3]. Within the decamer half-site [RRRCWWGYYY], changes in the conserved C and G at the 4th and 7th position greatly decreases transactivation (i.e., p21-3'MDM2 RE2, and PCNA). In addition, the nucleotides in the CWWG motif influence the level of transactivation where CATG is most active followed by CAAG and CTTG then CTAG which is associated with inhibitory effects. GADD45 and 14-3-3 σ site 1 are the only two canonical REs examined that contain CATG in both half-sites which did not display strong transactivation however, the reduced transactivation is attributed to negative affects of the flanking sequence. The two inner most purines and pyrimidines flanking the core affect transactivation more than the outer nucleotides with GG/CC being the most active and

AG/CT most inactive. Finally, most functional REs occur within 5-10 kb of transcriptional start sites [1, 3, 4].

Noncanonical REs expand the p53 transcriptional network

The present study expands upon the “rules of p53 transactivation” by deconstructing and reconstructing the *canonical* p53 consensus sequence in an attempt to determine the essential requirements within a binding element from which p53 can function *in vivo*. In agreement with previous results [5, 6], we have shown a large spacer may allow for the synergistic transactivation between full REs. However, the spacer length between decamer half-sites strongly influences strength of transactivation, where small increases in distance between decamer half-sites were shown to greatly reduce p53 transactivation. These results are particularly interesting because spacers would not have been predicted to affect the binding affinity of p53 towards the sites through established *in vitro* binding assays. When binding was measured for p53 towards the p21-5' RE containing a 0, 1, 2, 5, or 10 nucleotide spacer with fluorescence anisotropy there was not a significant difference between the binding affinities (data not shown; personal communication with D. Veprintsev and A. Fersht). In fact, both the predicted and measured binding affinities for the p21-5' RE containing a 1 nt and 10 nt spacer were identical. Thus, while *in vitro* biochemical assays may predict the potential for binding under ideal conditions with purified components, it appears p53 function must be assessed within the context of a cellular environment. Interestingly, the effect of spacer upon binding could be distinguished with a newly developed, semi-*in vitro* microsphere

binding assay that utilizes human cell nuclear extracts containing induced levels of p53 and additional transcriptional co-factors.

Further *in vivo* functional assays within the yeast and mammalian systems revealed there can be substantial sequence dependent transactivation from noncanonical $\frac{3}{4}$ -site and $\frac{1}{2}$ -site REs. Similar to transactivation from full site REs, efficient transactivation from these noncanonical elements requires a tetrameric p53. Through deconstruction and transactivation assays of REs originally classified as biologically-relevant REs, including PIDD and APAF1, it becomes apparent many canonical REs are actually noncanonical $\frac{3}{4}$ -site REs. This suggests $\frac{3}{4}$ -site REs play a significant role within the p53 transcriptional network. Furthermore, a recent study from the Resnick lab showed such weaker elements have been evolutionarily conserved and selected for in terms of function [7]. Ongoing studies are focusing on the functional assessment of $\frac{1}{2}$ -site REs and the role of half-sites in p53-dependent response. Intriguingly, it has been determined that p53 can function from $\frac{1}{2}$ -site REs such as FLT1 in a synergistic manner with other sequence specific transcription factors to co-regulate the activity of target genes [8, 9].

Currently, the accepted or canonical consensus element consists of two decamer half-sites separated by up to 13 nucleotides. However, our studies should encourage a reevaluation of transcriptional targets of p53 in two ways. First, when considering functional targets, the parameters of a full-site sequence should limit the spacer length between decamers to 3 nucleotides. Second, the p53 binding site should be extended to include $\frac{1}{2}$ -site and $\frac{3}{4}$ -site REs as possible downstream targets. Importantly, while our research has expanded the universe of possible p53 downstream target genes, functional

assessment from $\frac{1}{2}$ - and $\frac{3}{4}$ -sites will be required before an associated target gene is placed within the p53 transcriptional network. It is interesting to speculate that these noncanonical sites have been selected for as a means to fine-tune the p53-dependent response at upper levels of expression and/or longer durations of the accumulated protein. Furthermore, if p53 transactivation follows a “selective context model” [10] it is reasonable to speculate the primary interaction between p53 and the DNA, which is impacted by the sequence and organization of the RE, will influence the remaining factors or “rules of engagement” required to elicit a functional and/or unique response from a given RE. As shown by recent crystal structures [11], the RE sequence dictates the p53-DNA interactions and subsequently its binding surface interfaces. The identification of the super-trans consensus sequence Con-A which displays a unique basal level response and super-trans mutants which appear more efficient than WT p53 towards some REs can be used as tools to understand the most efficient interactions between p53 and DNA to achieve function.

Functional mutations diversify the p53 transcriptional network

Consistent with its important biological role, p53 status and level of expression have been considered promising prognostic factors in multiple forms of cancer, including breast cancer. Functional assays estimate that approximately 20 – 30% of mutations in p53 will mitigate the protein’s ability to regulate its downstream targets rather than render it inactive [3, 12]. Modifications in the p53 transcriptional network are hypothesized to change genome stability, repair, replication, apoptosis and the efficacy of chemotherapeutic agents. We have employed the rheostatable diploid system to

determine the functional fingerprints of 50 p53 missense mutations found in breast cancers, particularly those associated with BRCA1/2 cancers and LFS, and define how p53 mutants impact the ability of p53 to transactivate from target gene REs. Among the selected pool of mutants, 42% retained function and had a unique functional fingerprint in comparison to WT p53 and in terms of transactivation from 12 target REs, including Con-A. Interestingly, most of the mutations displayed subtle transactivation defects that were only apparent at low levels of p53. Importantly, while assessing p53 function, the Con-A sequence has been identified as a possible diagnostic tool for identifying p53 mutations with retained function.

These studies which are aimed at correlating p53 status in breast cancer to prognosis should be expanded to consider the impact of specific mutations upon tumorigenesis. Presently, we are participating in a collaborative attempt to correlate the functional status of p53 mutants with physiological response to treatment. Furthermore, the biological consequences of alterations in p53 regulation towards a variety of cellular endpoints, such as apoptosis and survival, can be analyzed in human cell functional assays. It is predicted the biological impact will be dependent upon the specific mutation and the degree of functionality retained by the mutant [13]. This study will further assess the predictability of yeast assays towards the effects of p53 mutations on human cellular responses that may be important in the onset or advancement of tumorigenesis.

Conclusions

This research which utilizes the combination of transactivation results with various mutants and sequences along with information about semi-*in vitro* binding is

providing unique opportunities to address the p53 master regulatory network. Based on results with the various REs obtained under isogenic conditions, we demonstrate that RE sequence, organization and level of p53 can strongly impact p53-mediated transactivation, thereby changing the view of what constitutes a functional p53 target. In addition, mutations in the protein itself have been found to further diversify the network by altering the spectrum of target genes, as well as the intensity to which the downstream targets can be regulated. The results will provide valuable insight toward understanding the biological role this prominent tumor suppressor plays in the multiple pathways it can influence as a master regulatory gene.

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CHAPTER 7
MATERIALS AND METHODS

Construction of isogenic diploid yeast strains containing p53 inducible reporters

The *delitto perfetto* site-directed mutagenesis system (Figure 2.5) [1, 2] was used to generate a panel of isogenic “p53-host” strains and a panel of response element (RE) reporter strains in the budding yeast, *S. cerevisiae*. Each p53-host strain, yAT-iGAL::p53 (*MATa leu2-3,112 trp1-1 his3-11,15 can 1-100 ura3-1, trp5::pGAL1:p53:cyc1-Ter, lys2::Hygro^R*), contains the wild type p53 cDNA controlled by the inducible, “rheostatable” *GAL1* promoter [3] integrated at the *TRP5* locus on chromosome VII. p53 mutations were constructed using a derivative of the p53 host strain containing a CORE cassette (CO, counterselectable, *KLURA3*; RE, reporter, KanMX4 resistance gene) integrated within the p53 cDNA at various nucleotide positions (Table 2.1) [4]. Modifications of p53 were performed using the *delitto perfetto* approach [1, 2] where the CORE cassette was replaced with an oligonucleotide containing the mutation of interest to generate a full-length mutant p53 cDNA or the desired deletion of the C-terminus. Replacement of the CORE was confirmed by selection on 5-FOA and kanamycin sensitivity. Specific p53 alterations were confirmed by colony PCR and sequencing (Big dye, Applied Biosystems, Foster City, CA).

The second panel of isogenic strains containing p53 REs upstream of the *CYCl* minimal promoter and the firefly luciferase reporter were obtained starting from the yLFM-ICORE strain, as previously described [5]. The reporter strains are also isogenic with the p53 host strains, but *LYS2* and Hygro^S.

Mating of the reporter and p53-host strains followed by selection for diploid cells on Lys- Hygro+ plates, results in isogenomic, yeast that enable the assessment of the transactivation potential for WT or mutant p53 proteins towards individual REs in the p53 transcriptional network. Strains differ only by the mutation of interest and nucleotide variation in the RE (note that a half-site or tandem REs are more nucleotides).

Qualitative *ADE2* color assay

Single colony isolates of the p53-inducible RE-*ADE2* reporter strains were streaked onto an YPDA control plate containing glucose and high levels of adenine and grown to equivalent amounts at 30°C. The plates were then replica plated onto a series of 9 experimental plates containing selective media with low levels of adenine [5 mg/L], 2% raffinose and incremental increases in galactose (0, 0.001, 0.002, 0.004, 0.008, 0.016, 0.032, 0.064 and 0.128%). Transactivation capacities for the p53 mutants were determined after three days of growth at 30°C by the ability of the mutant to produce a change in colony pigmentation. Transactivation of the *ADE2* gene, which is a direct readout of p53 interaction with the specific RE, results in large white colonies where decreased or loss-of-transactivation of the *ADE2* results in pink and red colonies, respectively. Transactivation capacities for the p53 WT and mutants were determined after three days of growth at 30°C by the ability of the mutant to produce a change in colony pigmentation. Colony pigmentation was scored on a scale of 1 to 5, where 1 is no apparent transactivation (red colonies) and 5 is strong transactivation (white colonies).

Quantitative luciferase assay

Individual colonies of cells with the p53-inducible RE reporter were inoculated into 5ml rich media, YPDA plus adenine [200 mg/L], and grown overnight at 30°C with shaking. The overnight culture was diluted 1:50 in H₂O. For each measurement, 1 ml of the diluted culture was spun down, washed of residual glucose with H₂O and re-suspended in 2mL synthetic complete - LYS media (plus 2% raffinose or raffinose supplemented with increasing amounts of galactose (0, 0.002, 0.004, 0.008, 0.010, 0.012, 0.016, 0.020, 0.024, 0.028 or 0.032%). These cultures were grown overnight (~ 18 hr) at 30°C to ~2 – 4 x 10⁷ per ml (late log early stationary). The 2 ml cultures were spun down and the supernatant was aspirated. The remaining pellet was resuspended in 100 µl reporter lysis buffer (Promega, Madison, WI) and an equivalent amount of 425-600 micron acid-washed, glass beads was added (Sigma, St. Louis, MO). Samples were homogenized for 30 seconds in the Biospec Products, Inc. mini-bead beater (Bartlesville, OK), briefly incubated on ice and spun for 20 minutes at 16k relative centrifugal force (rcf) in an Eppendorf 5415R centrifuge (Batavia, IL) to separate the soluble protein fraction. The standard protocol recommended by the manufacturer (Promega; Madison, WI) was performed for the luciferase assay system starting with 10 µl of protein extract. Luciferase activity was measured from 96-well, white optiplates (Perkin Elmer, Waltham, MA) in a Wallac Victor² multilabel counter (Perkin Elmer, Waltham, MA). Light units were standardized per µg protein as determined by a Bio-Rad protein assay (Bio-Rad; Hercules, CA). Luciferase assays in haploid strains were performed as previously reported [6].

Human cell lines

Human SaOS2 (HTB-85, ATCC) osteosarcoma cells were grown in McCoy's A5 medium supplemented with 10% FBS and 1X penicillin /streptomycin (Gibco, Carlsbad, CA). All cultures were incubated at 37°C with 5% CO₂. Human lymphoblastoid cells were grown in RPMI 1640 media supplemented with 15% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) and incubated at 5% CO₂ at 37°C with 1% penicillin-streptomycin antibiotics (Invitrogen). The lymphoblast cell lines GM12824 and GM12825 used in the semi-*in vitro* binding assay were purchased from Coriell Cell Repositories (Camden, NJ). Results with human cells included in the noncanonical paper of Chapter 3 were largely accomplished through a collaboration with Dr. Daniel Menendez in the Resnick lab and were guided by results in yeast.

Transfections and luciferase assays in human cell lines

Plasmids pC53-SN3 [7] coding for human p53 cDNA under the control of CMV promoter and the control vector pCMV-Neo-Bam were kindly provided by Dr. Bert Vogelstein. p53 mutations within the pC53-SN3 vector were generated by site-directed mutagenesis (QuickChange site-directed mutagenesis kit, Stratagene) and were confirmed by sequencing. Luciferase reporter constructs containing the p53 REs were constructed in pGL3-Promoter backbone (Promega, Madison, WI). Partially complementary oligonucleotides containing the RE and five additional flanking nucleotides from both sides were annealed *in vitro* to yield double-stranded molecules that would be compatible for an *in vitro* ligation reaction with XhoI/BamHI double digested pGL3-promoter vector. Ligation products were transformed into XL1 blue E. coli cells, purified, amplified and sequenced.

pRL-SV40, a reporter plasmid coding for *Renilla reniformis* luciferase (Promega, Madison, WI) was used as a control of transfection efficiency in the luciferase reporter assay.

For luciferase assays SaOS2 cells were seeded in 24-well plates 24 hours before transfection. Cells were transfected using Fugene-6 (Roche, Indianapolis, IN) according to manufacturer's instructions at ~80% confluence (with 200 ng of reporter constructs). When appropriate, 25ng of the p53 was co-transfected. Total plasmid DNA per well was adjusted to an equal level by adding the empty vector pCMV-Neo-Bam. Forty-eight hours post transfection, extracts were prepared using the Dual Luciferase Assay System (Promega) following the manufacturer's protocol and luciferase activity was measured on a Victor Wallac multilabel plate reader (PerkinElmer). For each construct, relative luciferase activity is defined as the mean value of the firefly luciferase/*Renilla* luciferase ratios obtained from at least three independent experiments.

Chromatin Immunoprecipitation (ChIP) assays

SaOS2 cells were seeded in 10-cm dishes and transfected at 80% confluence with WT p53 expression vector along with the pGL3-P reporter plasmid containing the p21 5'RE or its derivatives. ChIP on plasmid assays were performed as described previously [8] using the ChIP kit (Upstate Biotechnology) following the manufacturer's instructions. A mouse monoclonal anti-p53 antibody DO7 (Pharmigen, BD) was used. PCR amplifications were performed on immunoprecipitated chromatin using a pair of primers to amplify a specific region in the pGL3-P backbone containing the p53 REs (5'-ATAGGCTGTCCCCAGTGCAA-3' and 5'-TGG AATAGCTCAGAGGCCGA-3'). The PCR cycles were as follows: an initial 10 min Taq Gold polymerase at 95°C followed by 40 cycles

of 95°C for 15 s and 60°C for 1 min. The PCR products were then run on a 1.8% agarose gel and quantified with IMAGEQUANT V5.1 (Molecular Dynamics-GE, Piscataway, NJ).

Semi-*in vitro* binding assay with human nuclear extracts

To evaluate the sequence-specific p53-DNA binding interactions, the semi-*in vitro* fluorescent microsphere binding assay as previously described [9] was used. Briefly, lymphoblast cells were grown to $\sim 8.5 \times 10^5$ cells/mL before exposing to 0.6 μ g/mL (1mM) doxorubicin (Sigma, St. Louis, MO) for 18 hours at 37°C. Nuclear protein was extracted from non-treated and treated cells using a Nuclear Extraction Kit according to manufacturer's protocol (Active Motif, Carlsbad, CA) and protein concentration was measured in triplicate using the BCA Protein Assay Kit (Pierce, Rockford, IL), followed by a plate read using a Perkin Elmer HTS 7000 BioAssay Reader. All the oligonucleotides reported in this study were synthesized by Invitrogen (Carlsbad, CA). Fluorescent microspheres bearing double stranded DNA fragments containing a sequence of interest, were multiplexed and incubated for 1 hour in the presence of 1.75 μ g of nuclear protein extracts from either treated or non-treated cells. Following incubation in cell extracts, the beads were incubated with p53 antibodies (DO-7, BD Biosciences, San Jose, CA) and secondary antibodies conjugated with phycoerythrin(R-phycoerythrin-coated goat anti-mouse) for 30 minutes. The p53 interaction with each bead was measured on a BioPlex Machine (BioRad) as raw fluorescence intensity signal generated from phycoerythrin-conjugated secondary antibody to mouse anti-p53. For signal normalization, an aliquot of the DNA-conjugated beads was treated separately with phycoerythrin-conjugated streptavidin for 20 minutes in the absence of any nuclear extracts. All binding reactions were conducted in

triplicates. For each bead type in every multiplex set of beads, relative binding signal was obtained by normalizing the absolute binding signals of extract-treated beads (mean of 3 replicates) to mean signals obtained from the same set of beads that had been independently treated with phycoerythrin-conjugated streptavidin (mean of 3 replicates). This normalization accounts for bead type-specific oligo content. Net binding for each oligo is the numerical difference between NT and DOX-treated signal obtained for this oligo. Results with the bead system included in the noncanonical paper of Chapter 3 were largely accomplished through a collaboration with the Doug Bell lab at NIEHS and were guided by results in yeast.

Western analysis

Yeast cells. Individual yAT x yLFM diploid colonies were inoculated into YPDA media and grown overnight. Overnight cultures were diluted 1:50 in H₂O and 1 ml of the culture was spun down for each sample treatment, washed with 2 ml of H₂O and inoculated into 2 ml synthetic media (lys-) with either glucose (2%), raffinose (2%) or raffinose plus increasing amounts of galactose in media (0.002 – 0.032% galactose). Cultures were grown 24 hours at 30°C with vigorous shaking. Protein extracts were prepared in 35 ul reporter lysis buffer (Promega, Madison, WI) plus 2% protease inhibitors (cocktail for use with fungal and yeast extracts; Sigma, St. Louis, MO). An equivalent amount of 425-600 micron acid-washed, glass beads (Sigma, St. Louis, MO) were added prior to the samples being homogenized for 30 seconds in the Biospec Products, Inc. mini-bead beater (Bartlesville, OK). Following homogenization, samples were briefly incubated on ice and spun for 20 minutes at 16k rcf in an Eppendorf 5415R centrifuge (Batavia, IL) to purify soluble proteins. Protein concentrations were measured with the Bio-Rad protein assay according to the

standard protocol (Bio-Rad; Hercules, CA). 50 µg of total protein extracts were run on SDS-PAGE gels and transferred as previously described [3]. p53 was detected with the mouse monoclonal antibodies DO-1 and pAb1802 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) according to the manufacturer's protocol. GAPDH was detected with the rabbit polyclonal GAPDH antibody (HRP) (Abcam, Cambridge, MA).

Human cells. Whole cell extracts from SaOS2 transfected cells were obtained using a cell culture lysis reagent (Promega) following the manufacturer's instructions. Equal amounts of whole cell extracts were separated on 4-12% BisTris NuPAGE and transferred to polyvinylidene difluoride membranes (Invitrogen). The blots were probed with primary antibodies (Santa Cruz) for p53 (pAb1801 and DO-1) and Actin (C-11). Bands were detected using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) and the enhanced chemiluminescence (ECL) detection system (Amersham, Cleveland, OH, USA).

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