IDENTIFICATION AND REGULATION OF P53 TARGET GENES IN PRIMARY HUMAN EPIDERMAL KERATINOCYTES

By

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Dedicated to my parents,
and to the memory of
Grandpa Jackson and Jennie

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53BP1	p53 binding protein 1
53BP2	p53 binding protein 2
ADR	Adriamycin
ATM	Ataxia telagiectasia mutated kinase
ATR	ATM- and Rad3-related kinase
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
BH	Bcl-2 homology
BP	Basepair
CBP	CREB binding protein
CDC2	Cell division cycle 2
CDK	Cyclin-dependent kinase
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
CIP1	Cdk-interacting protein 1 (p21)
ChIP	Chromatin immunoprecipitation
CK1	Casein kinase 1
CK2	Casein kinase 2
CREB	c-AMP-response element-binding
DBD	DNA binding domain

DDB2	Damage-specific DNA binding protein 2
DNAPK	DNA-dependent protein kinase
DMEM	Dulbecco's modified Eagle medium
FADD	Fas-associating factor with death domain
FISH	Fluorescence In Situ Hybridization
G1 PHASE	Gap 1 phase
G2 PHASE	Gap 2 phase
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde dehydrogenase
GFP	Green fluorescent protein
HAUSP	Herpes-virus-associated ubiquitin-specific protease
HSC70	Heat shock cognate protein 70
HEKs	Human epidermal keratinocytes
HPV16	Human papilloma virus 16
IR	Ionizing radiation
JNK	c-Jun N-terminal kinase
LFS	Li-Fraumeni syndrome
M PHASE	Mitosis phase
MDM2	Murine double minute 2
MEF	Mouse embryo fibroblast
MOAP1	Modulator of apoptosis 1

NEDD8	Neural precursor cell expressed, developmentally down-regulated 8
NER	Nucleotide excision repair
NES	Nuclear export signal
NLS	Nuclear localization signal
p53AIP1	p53-regulated apoptosis-inducing protein 1
p53R2	p53-inducible ribonucleotide reductase small subunit 2 homologue
PARP	Poly-ADP-ribose polymerase
PAS	Protein A sepharose
PBS	Phosphate buffered saline
PCAF	p300/CBP-associated factor
PCNA	Proliferating cell nuclear antigen
PIG3	p53-inducible gene 3
PKC	Protein kinase C
POL II	RNA polymerase II
PP	proximal promoter
PUMA	p53 upregulated modulator of apoptosis
RACE	Rapid amplification of cDNA ends
RASSF1A	Ras association domain family protein 1A
RB	Retinoblastoma protein
RRAD	Ras-related associated with diabetes
RIPA	Radioimmunoprecipitation assay lysis buffer

S PHASE	Synthesis phase
SAGE	Serial analysis of gene expression
SUMO-1	Small ubiquitin-like modifier 1
SV40	Simian virus 40
TAD	Transcription activation domain
TAF	TBP-associated factor
TBP	TATA-box binding protein
TFIID	Transcription initiation factor IID
TNFR	Tumor necrosis factor receptor superfamily
TRAIL	TNF-related apoptosis inducing ligand
TTBS	Tween-20 Tris buffered saline
UTR	Untranslated region
UV	Ultraviolet radiation
UV-DBB	UV-damaged DNA binding protein
WAF1	Wild-type p53 activated fragment 1 (p21)
XP	Xeroderma pigmentosum
XPE	Xeroderma pigmentosum group E gene
ZFP90	Zinc finger protein 90

CHAPTER I

INTRODUCTION

Almost thirty years ago, p53 was identified as a simian virus 40 (SV40) large T antigen interacting protein. The 53 kDa protein was immunoprecipitated from SV40-transformed mouse cell lysates using anti-large T serum isolated from rabbits, hamsters, mice, and monkeys (Lane and Crawford, 1979). In addition to SV40-transformed mouse cells, p53 could also be immunoprecipitated from uninfected mouse embryonal carcinoma cells in the absence of large T (Linzer and Levine, 1979). Using anti-large T serum, p53 could be immunopurified from any transformed cell examined. The presence of p53 distinguished sarcomas from normal cells (DeLeo et al., 1979). Furthermore, p53 could be immunoprecipitated out of many different SV40-transformed cell lysates by anti-large T sera isolated from several distinct species (Kress et al., 1979; Chang et al., 1979). Of note, in these early experiments, p53 was determined to be a phosphoprotein (Linzer and Levine, 1979; Kress et al., 1979; Chang et al., 1979). Analogous to the interaction of p53 with SV40 large T antigen, the interaction of p53 with another viral protein important for transformation, adenovirus E1B, was reported (Sarnow et al., 1982). The interaction of p53 with multiple viral proteins was of interest as understanding transformation by DNA tumor viruses was sought.

Another observation made in early studies on p53 was that high levels of p53 were expressed in cancer cells, whereas levels of p53 in non-transformed cells were low (DeLeo et al., 1979; Rotter et al., 1980). Furthermore, p53 cooperated with activated *ras* in the transformation of primary embryonic cell cultures into tumorigenic cells (Eliyahu et al., 1984; Parada et al., 1984). Consequently, p53 was initially thought to be an oncogene. However, upon determination of the sequence of wild-type p53, comparison of the p53 protein exhibiting oncogenic properties revealed an alanine to valine mutation at amino acid 135 (A135V). Unlike the A135V mutant, the wild-type p53 protein was not able to cooperate with *ras* to transform cells (Hinds et al., 1989). Further studies demonstrated that wild-type p53 was actually a suppressor of transformation (Baker et al., 1989; Finlay et al., 1989). Soon thereafter, p53 was shown to interact with human papilloma virus 16 (HPV-16) E6 protein (Werness et al., 1993) and that the functional consequence of this interaction is ubiquitin-mediated degradation of p53 by the proteasome (Scheffner et al., 1990). That multiple DNA tumor viruses target p53 in order to allow for transformation and progression of cancer is indicative of how critical intact p53 signaling pathways are in preventing uncontrolled cell growth. This chapter will review the current understanding of the roles of p53 structure and function and target gene regulation.

p53 Alterations in Cancer

Mutations in p53 are the most common genetic alteration in human cancer. Approximately 50% of all human tumors contain p53 mutations (Vogelstein, 1990). Deletions in chromosome 17p are common in many types of tumors, including colorectal tumors. Studies on colorectal tumors determined that the "target" of the 17p deletion is the p53 gene (Baker et al., 1989). When 17p is not deleted, mutations in p53 are frequently detected (Baker et al., 1989; Nigro et al., 1989). The most common p53 mutations found in human tumors are clustered in four "hot spots" that overlap with the most conserved regions of the p53 gene (Nigro et al., 1989). The most highly conserved regions are located in the central region of the p53 protein, which contains the DNA binding domain (Pavletich et al., 1993). The crystal structure of the p53 core domain in complex with DNA provides a structural explanation regarding the nature of many mutations in p53 found in human tumors (Cho et al., 1994). The six most frequent p53 codons mutated in cancer are arginine (R) 248, R273, R175, glycine (G) 245, R249, and R282 (Hollstein et al., 1991). The crystal structure illustrates that R248 and R273 are both in direct contact with DNA (Cho et al., 1994). R175, G245, R249, and R282 are all residues critical for maintaining structural integrity of the p53 protein. Each of these four amino acids is essential for proper conformation, folding, hydrogen bonding, salt bridge interactions, and packing of the core domain of p53 (Cho et al., 1994).

The role of p53 in tumor suppression was also confirmed from the phenotype of the p53 knock-out mouse and humans with germ-line mutations in the gene. Mice lacking p53 develop and are born normally for the most part. However, spontaneous tumors, most commonly lymphomas and sarcomas, form in the p53 knock-out mouse before they are 6 months old (Donehower et al., 1992). Germ-line mutations in the p53 gene are found in people affected with Li-Fraumeni syndrome (LFS) (Malkin et al., 1990; Srivastava et al., 1990). LFS results from an inherited point mutation in a conserved region of p53 that results in cancer susceptibility in affected people. A wide range of childhood and adult cancers are observed in people with LFS (Malkin et al., 1990; Srivastava et al., 1990). The high frequency of mutations and deletions of the p53 gene in human tumors illustrates the integral role of p53 in tumor suppression.

p53 Protein Structure and Interacting Proteins

The p53 protein is comprised of 393 amino acids that account for a molecular mass of 53,000 daltons. p53 structurally consists of three functional domains: a transactivation domain (TAD), a DNA binding domain (DBD), and an oligomerization domain. Each domain is involved in processes important to p53 regulation and function.

p53 N-terminus

The first 73 N-terminal amino acids of p53 contain the acidic TAD (Fields and Jang, 1990). The TAD is also the site where many interacting proteins bind to p53. Important to the ability of p53 to activate transcription, components of the transcription initiation factor IID (TFIID) associate with the N-terminus of p53. Specifically, TATA box binding protein (TBP) (Seto et al., 1992) and TBP-associated factors (TAFs) $TAF_{II}32$ and $TAF_{II}70$ interact with the N-terminus of p53 (Lu and Levine, 1995; Thut et al., 1995). In addition, cAMP response element-binding protein (CREB)-binding protein (CBP)/p300 complex is known to interact with p53 at its N-terminus. CBP/p300 serves as a transcriptional coactivator and potentiates p53-mediated transcription (Gu et all, 1997; Lill et al., 1997; Avantaggiati et al., 1997). CBP/p300 has histone acetyltransferase (HAT) activity and is able to acetylate histones in regions of transcriptionally active chromatin (Bannister and Kouzarides, 1996; Ogryzko et al., 1996). CBP/p300 also acetylates the p53 C-terminus, which enhances its sequence specific binding ability (Gu and Roeder, 1997). The adenovirus E1b protein binds to p53 at its N-terminus and inhibits transcriptional activation (Kao et al., 1990). One of the most important p53 interacting proteins binds to p53 at the N-terminus: MDM2 (Oliner et al., 1993). MDM2 negatively regulates p53 by associating with the TAD and inhibiting p53 transactivation (Oliner et al., 1993) and also targets p53 for ubiquitination and rapid degradation by the 26S proteasome (Haupt et al., 1997; Kubbutat et al., 1998). A region of the p53 protein near the N-terminus

and adjacent to the TAD is referred to as the proline-rich domain, which consist of amino acids 61-94 and contains five repeats of the sequence PXXP, where P = proline, and X = any amino acid (Walker and Levine, 1996). The proline-rich region of p53 was found to be important for the ability of p53 to induce apoptosis (Sakamoro et al., 1997). A common polymorphism of p53, either P or R at amino acid 72, is located in the proline-rich region (Matlashewski et al., 1987). There are differences in the functioning of the p53 protein depending on which polymorphism is encoded. The p53 protein with R72 is able to induce apoptosis better than p53 with P72 (Dumont et al., 2003), whereas the presence of P72 renders p53 able to induce increased levels of G1 arrest than p53 with R72 (Pim and Banks, 2004). The processes involving the p53 N-terminus demonstrate the importance of its role in p53-mediated transcription.

p53 Central Core

Another important functional domain of p53 is the DBD, located in the central region of the p53 protein (amino acids 102-292). As mentioned earlier, the DBD contains four conserved regions that are mutational “hot-spots” in human cancers (Pavletich et al., 1993). In 1991, p53 was found to have the ability to bind to DNA in a sequence-specific manner (Kern et al., 1991). Soon thereafter, the same group defined the sequence of the p53 consensus binding site to be 2 repeats of RRRC(A/T)(T/A)GYYY (R = A or G, Y = C or T) separated by 0-13 basepairs of random sequence (El-Deiry et al., 1992). The sequence of

the p53 binding site is highly degenerate in the p53 target genes identified to date. p53 was then shown to directly activate transcription through this consensus binding site (Farmer et al., 1992). Later studies proved that p53 requires this sequence-specific transcriptional activity to function as a tumor suppressor (Pietenpol et al., 1994). As with the N-terminus, proteins that interact with the DBD of p53 can affect the function of p53. The SV40 large T antigen binds to the DBD of p53 to inhibit its function (Tan et al., 1986). The HPV E6 protein also binds to the core domain of p53 to promote its degradation (Li and Coffino, 1996). Two additional cofactors, p53 binding protein 1 (53BP1) and p53 binding protein 2 (53BP2), also interact with the p53 DBD (Iwabuchi et al., 1994; Gorina and Pavletich, 1996). 53BP1 and 2 have the ability to act as transcriptional cofactors and enhance p53 transactivation (Iwabuchi et al, 1998). Also, heat shock cognate protein 70 (hsc70) binds to the DBD of p53 mutant proteins containing mutations in residues such as R175 and V143 that alter the structure of the protein (Fourie et al., 1997; Hinds et al., 1990). Such properties of the p53 DBD illustrate its essential role in p53-mediated signaling.

p53 C-terminus

The C-terminus of p53, amino acids 300-393, is also important for several aspects of p53 function and regulation. Amino acids 300-318 constitute a flexible linker region that connects the central core and the C-terminus of p53 (Cho et al., 1994). The oligomerization domain consists of amino acids 323-355. When

binding to DNA, p53 oligomerizes to form tetramers by means of the oligomerization domain (Wang et al., 1994). Tetramerization is necessary for p53 to proficiently transactivate targets and suppress growth of cancer cell lines (Pietenpol et al., 1994). The C-terminus of p53 also contains three nuclear localization signals (NLS) in amino acids 316-325, 369-375, and 379-384. The NLS in amino acids 316-325 contains the greatest nuclear localization ability. Correspondingly, this NLS is also conserved in several species and the sequence best matches the consensus sequence of a typical NLS (Dang and Lee, 1989; Shaulsky et al, 1990). Stommel et al. (1999) identified a nuclear export signal (NES) in the C-terminus of p53, located specifically within the oligomerization domain. They proposed that when p53 tetramerizes and binds DNA, the NES is concealed, preventing p53 export from the nucleus (Stommel et al., 1999). Finally, amino acids 363-393 of the p53 C-terminus constitute a basic domain has the ability to negatively regulate p53 sequence-specific DNA binding. Deletion of the C-terminal domain or binding of the p53 monoclonal antibody PAb421 to the p53 C-terminus counteracts the negative regulation and activates sequence-specific binding. The PAb421 antibody relieves the allosteric inhibition of DNA binding by inducing a conformational change in the protein (Hupp and Lane, 1994). Post-translational modifications in this basic domain in the C-terminus domain also activate sequence-specific DNA binding (Hupp et al., 1992; Hupp et al., 1993; Hupp and Lane, 1994). In summary, the C-terminus of p53 is also involved in several important aspects of p53 function.

p53 Post-translational Modifications

During conditions of basal cellular functioning, p53 protein levels are very low due to its short half-life (Maltzman and Czyzyk, 1984) and rapid turnover mediated through its interaction with MDM2, as mentioned above. However, when cells encounter genotoxic stress, the p53 protein is activated as a result of many different types of post-translational modifications (Appella and Anderson, 2001).

Phosphorylation

Phosphorylation is one of the most important modifications of p53 and increases its sequence-specific binding ability (Hupp and Lane, 1994). In the N-terminus of p53, serines (S) 6, 9, 15, 20, 33, 37, and 46 and threonines (T) 18 and 81 are phosphorylated in response to genotoxic stress. The phosphorylation of S15 is well-studied and important for several reasons. Phosphorylation of S15 on the N-terminus of p53 contributes to the disruption of the p53/MDM2 complex resulting in the stabilization of p53 protein (Shieh et al., 1997). Of note, post-translational modification of MDM2 also contributes to disruption of the p53/MDM2 complex (Brooks and Gu, 2003). Zhang and Xiong (2001) reported the presence of another NES in p53 consisting of amino acids 11-27 in the N-terminus. Upon the phosphorylation of S15 within this region, p53 is no longer exported from the nucleus (Zhang and Xiong, 2001). Phosphorylation of p53 at S15 increases the ability of p53 to bind to p300/CBP, and the subsequent

acetylation at the C-terminus of p53 (Lambert et al., 1998). Overall, S15 phosphorylation of p53 results in enhanced transcriptional activation ability, which is exemplified by the aforementioned properties.

Phosphorylation events that occur in the p53 C-terminal basic domain also have implications in the regulation of p53 activity. p53 is phosphorylated at S315, S376, S371, S378, and S392 in its C-terminal domain. Casein kinase 2 (CK2) is able to phosphorylation S392 in the C-terminus of p53 (Keller et al., 2001). In addition, protein kinase C (PKC) phosphorylates S371, S376, and S378 in the basic domain in the C-terminus (Takenaka et al., 1995). Phosphorylation of these serines in the C-terminal basic domain stabilizes p53 tetramerization and activates sequence-specific DNA binding (Hupp et al., 1992; Hupp et al., 1993; Hupp and Lane, 1994).

Many different protein kinases have been implicated in phosphorylating specific serines and threonines in the p53 protein. Examples include ataxia telangiectasia mutated (ATM), ATM- and Rad3-related (ATR), checkpoint kinases 1 and 2 (Chk1, Chk2), c-jun N-terminal kinase (JNK), casein kinase 1 and 2 (CK1, CK2), protein kinase C (PKC), p38 stress activated kinase, and DNA-dependent protein kinase (DNAPK) (Bode and Dong, 2004). Some kinases are able to phosphorylate more than one site in p53, and some sites can be phosphorylated by more than one kinase. In general, the sites in p53 that are phosphorylated in response to a genotoxic stress are dependent on which kinase is activated by that particular stress. In addition, the particular kinase that gets

activated depends on the type and extent of the stress and the sort of cellular outcome that needs to occur to deal with that stress (i.e. cell cycle arrest vs. apoptosis) (Bode and Dong, 2004). In summary, though the mechanistic details of phosphorylation of the p53 protein are numerous and convoluted, this type of post-translational modification plays an important role in the p53-mediated response to genotoxic stress.

Acetylation

Other post-translational modifications of p53 include acetylation, ubiquitination, sumoylation, neddylation, and methylation. These modifications occur at lysine residues in the C-terminal domain of p53. p300/CBP acetylates p53 at lysines (K) 370, 372, 373, 381, and 382. These five lysine residues are highly conserved (Gu and Roeder, 1997). K320 is acetylated by p300/CBP-associated factor (PCAF) (Liu et al., 1999). Acetylation of these lysines in the C-terminus of p53 occurs in response to DNA damage. Like phosphorylation, acetylation of the C-terminal basic domain results in an increase in p53 sequence-specific binding ability in both *in vitro* binding assays and at the p21 promoter in human lung carcinoma H460 cells using chromatin immunoprecipitation (ChIP) (Gu and Roeder, 1997; Luo et al., 2004). In addition, there is coordination of the phosphorylation and acetylation of p53 in response to DNA damage. Phosphorylation at S15 happens first, stabilizing p53 and allowing for phosphorylation at other serines (Lambert et al., 1998). Also, p300/CBP has

higher affinity for p53 phosphorylated at S15 (Lambert et al., 1998). Following association with p53, p300/CBP acetylates lysines in the C-terminal domain, resulting in upregulation of sequence-specific binding of p53 to DNA (Sakaguchi et al., 1998). Interplay between phosphorylation and acetylation modifications results in a rapid response to DNA damage.

Ubiquitination

Another post-translational modification of p53 important to its regulation and function is ubiquitination. In unstressed cells, p53 protein levels are kept low through modulation by the ubiquitin-proteasome system. MDM2, an important negative regulator of p53, is the E3-ubiquitin ligase in the pathway to the degradation of p53 (Honda et al., 1997). In addition to targeting p53 for degradation, MDM2 also inhibits the transcriptional activity of p53 by binding to its N-terminal TAD (Oliner et al., 1993), as mentioned earlier. Mutational analysis on the C-terminal domain of p53 revealed six lysine residues ubiquitinated by MDM2: K370, K372, K373, K381, K382, and K386. Mutating all six of these lysine residues to arginines renders p53 resistant to MDM2-mediated degradation (Rodriguez et al., 2000). Ubiquitination of p53 was shown to be a reversible process from the identification of herpes virus-associated ubiquitin-specific protease (HAUSP). HAUSP binds to p53, deubiquitinates it, resulting in p53 protein stabilization (Li et al., 2002). Further studies in which expression of HAUSP is knocked-down using RNAi revealed that HAUSP is required for the

stability of MDM2 during basal cell activity. Therefore, when expression of HAUSP was almost completely eliminated, p53 was stabilized and activated (Cummins et al., 2004; Li et al., 2004). The function of a HAUSP regulatory pathway maintaining the balance between MDM2 and p53 levels is another level of protection against tumorigenesis.

Sumoylation

Sumoylation and neddylation are covalent post-translational modifications that resemble ubiquitination. Sumoylation involves the addition of a small ubiquitin-related modifier (SUMO-1) through a pathway similar to that of the ubiquitin-conjugation process. Sumoylated-p53 accumulates in response to ultraviolet radiation (UV). The SUMO-1 modification is specifically conjugated to p53 at K386 (Rodriguez et al., 1999; Gostissa et al., 1999). Unlike ubiquitination, sumoylation of p53 does not lead to p53 protein degradation. Rather, the presence of this modification in the basic C-terminal domain of p53 results in an increase in p53 sequence-specific binding (Rodriguez et al., 1999; Gostissa et al., 1999), as is the case with phosphorylation and acetylation.

Neddylation

NEDD8 is another ubiquitin-like covalent modification and is the most homologous to ubiquitin out of all ubiquitin-like proteins. Neddylation (the addition of NEDD8) also occurs through a pathway similar to ubiquitin-

conjugation. MDM2 can function as an “E3 NEDD8 ligase” and neddylate p53 on K370, K372, and K373 of the basic C-terminal domain (Xirodimas et al., 2004). Of note, these three lysines are also sites that can be ubiquitinated as mentioned above. MDM2 also neddylates itself, just as it is able to self-ubiquitinate. The result of neddylation of p53 and MDM2 is that both proteins are inactivated. In this way, neddylation serves to negatively regulate both p53 and MDM2 (Xirodimas et al., 2004). Neddylation of p53 and MDM2 is another way in which the balance in expression of each protein is carefully controlled.

Methylation

Chuikov et al. (2004) demonstrated the first example of methylation as a post-transcriptional modification of p53. Set9, a histone methyltransferase, can methylate K372 in the basic C-terminal domain of p53. A Set9 consensus binding motif was identified by comparing the sites of methylation in histones and p53. Methylation of p53 is observed both *in vitro* and by immunoprecipitation of endogenous p53 from cells (Chuikov et al., 2004). An increase in methylated p53 can be detected in cells treated with adriamycin (ADR). As observed with phosphorylation, acetylation, and sumoylation, methylation at K372 in the C-terminus of p53 results in an increase in p53 transcriptional activity. In addition, overexpression of Set9 in U2OS cells results in increased apoptosis, both basally and following ADR treatment (Chuikov et al., 2004). Interestingly, K372 can be acetylated, ubiquitinated, neddylated, and methylated as determined by these

studies of post-translational modifications of p53. This is another illustration of the complexities of post-translational modifications. Therein lies the potential for distinct cellular outcomes mediated by differential regulation of the many post-transcriptional modifications of p53.

Transcriptional Regulation of Target Genes by p53

In response to cellular stress, p53 regulates a complex signal transduction pathway of genes and proteins that results in a cellular outcome that appropriately deals with the stress. Important to the ability of p53 to suppress tumorigenesis is transcriptional regulation of target genes. The p53 pathway is activated by many different types of cellular stress including (but not limited to) DNA damage, oncogene activation, hypoxia, heat shock, and glucose starvation (Pluquet and Hainaut, 2001; Levine et al., 2006). As described earlier, in the absence of stress, p53 levels are maintained at low steady-state levels through the interaction with MDM2, the E3-ubiquitin ligase of p53 (Honda et al., 1997). MDM2 ubiquitinates the C-terminus of p53, resulting in rapid turnover of the protein by way of degradation by the 26S proteasome. In the event of cellular stress, p53 is post-translationally modified and activated as described in the previous section, resulting in increased levels of p53 protein. Activated p53 binds in a sequence-specific manner to regions of DNA containing p53 consensus binding sites (Kern et al., 1991; El-Deiry et al., 1992). In the majority of p53-regulated genes identified to date, a p53 consensus binding site is present

in the promoter region or the first or second intron of the gene (El-Deiry et al., 1992).

p53 transcriptionally activates downstream target genes corresponding to the type and extent of the cellular stress that activated p53. The protein products of the target genes activated by p53 span a wide range of functions, and result in several distinct cellular outcomes. The most well-known and understood pathways related to p53-mediated tumor suppression include cell cycle arrest, apoptosis, and DNA repair (Levine et al., 2006). p53 target genes have been identified and play distinct roles in these processes. Key target genes involved in cell cycle arrest, apoptosis, and DNA repair will be described in more detail.

Cell Cycle Arrest

Key players in p53-mediated cell cycle arrest include p21 and 14-3-3σ. p21, also known as cyclin-dependent kinase (Cdk)-interacting protein (Cip1) or wild-type p53-activated fragment 1 (WAF1), was first identified using two different strategies. Harper et al. (1993) used a yeast two-hybrid screen to identify Cdk-interacting proteins (Cips), whereas El-Deiry et al. (1993) used subtractive hybridization to detect genes induced by wild-type p53, but not mutant. In these early studies, p21 was shown to interact with Cdk2 and inhibit its kinase activity. In addition, it inhibited the phosphorylation of retinoblastoma protein (Rb) by cyclin-Cdk complexes involved in the G1-S transition of the cell cycle (Harper et al., 1993). Simultaneously, p21 was induced by p53 in multiple cell lines and is

conserved across species. Upon expression of p21 in cancer cells, cell growth was inhibited (El-Deiry et al., 1993). The combined data from these studies implicated p21 in p53-mediated G1-arrest.

Another p53 target gene that plays a role in cell cycle arrest is 14-3-3σ. 14-3-3σ was isolated using serial analysis of gene expression (SAGE) to compare genes upregulated in colorectal cancer cells in response to ionizing radiation (IR), in a p53-dependent manner. When overexpressed, 14-3-3σ causes a G2/M arrest in multiple cell lines (Hermeking et al., 1997). The mechanism by which 14-3-3σ mediates a G2/M arrest is by sequestering cell division cycle 2 (Cdc2)-cyclin B1 complexes in the cytoplasm in response to DNA damage (Chan et al., 1999). Cdc2-cyclin B1 must translocate to the nucleus to allow entry into mitosis (Jin et al., 1998).

Apoptosis

p53 target genes have been implicated in both extrinsic and intrinsic pathways of apoptosis. The extrinsic apoptotic pathway involves binding of death ligands to death receptors and a resulting cell death cascade. Fas/APO-1 (also known as CD95) is a member of the tumor necrosis factor receptor (TNFR) superfamily. Upon binding of Fas ligand to the Fas receptor, the receptor homotrimerizes. The trimerized cytoplasmic domain recruits an adaptor, Fas-associating factor with death domain (FADD), which in turn recruits caspase-8. Oligomerization of caspase-8 results in activation by self-cleavage, followed by

activation of a caspase cascade that modulates apoptosis (Ashkenazi and Dixit, 1998). In cancer cell lines treated with chemotherapeutic agents, an increase in apoptosis was observed. A corresponding increase in the cell-surface expression of the Fas/APO-1 receptor occurred in the cells, but only in the presence of wild-type p53. Further investigation revealed a p53-consensus binding site in the first intron of the Fas/APO-1 gene to which p53 is able to activate transcription in luciferase assays (Muller et al., 1998).

Another p53 target gene involved in the extrinsic pathway of apoptosis is KILLER/DR5 (also called APO-2), a member of the TNF-related apoptosis inducing ligand (TRAIL) family of death receptors. Little is known about the apoptotic signaling cascade that occurs when TRAIL binds to KILLER/DR5 except that it does require caspase activation (Ashkenazi and Dixit, 1998). KILLER/DR5 was a novel gene identified in a subtractive hybridization screen for genes upregulated in cancer cells that have chemosensitivity to ADR compared to cancer cells chemoresistant to ADR. KILLER/DR5 shows sequence similarity to previously identified death receptors. Due to nature of the screen in which it was identified (induced by ADR), KILLER/DR5 was hypothesized to be a potential p53 target gene, in that the p53 pathway is strongly induced by ADR. In agreement with this hypothesis, KILLER/DR5 was only expressed in response to DNA damaging agents in the presence of wild-type p53. In addition, expression of p53 by adenovirus in cancer cell lines lacking p53 results in upregulation of KILLER/DR5 (Wu et al., 1997). Later studies revealed the presence of a p53

consensus binding site in the first intron of the KILLER/DR5 gene and subsequent analysis of this site validated KILLER/DR5 as a direct target of p53 (Takimoto and El-Deiry, 2000).

p53 also regulates genes involved in the intrinsic pathway of apoptosis, which involves disruption of the mitochondrial membrane potential. Bax is an example of a p53 target gene that is involved in intrinsic apoptosis. Bax is a pro-apoptotic member of the Bcl-2 family. Bax forms heterodimers with Bcl-2 and Bcl-X_L, both anti-apoptotic proteins, and these interactions serve to maintain a balance favoring the anti-apoptotic factors (Adams and Cory, 1998). The Bax gene contains a p53 consensus binding site in its promoter, through which p53 can upregulate its expression. Upon upregulation of Bax by p53, the ratio of Bcl-2 and Bcl-X_L to Bax is lowered, and eventually Bax levels reach a point when they can promote the release of cytochrome *c* from the mitochondria and initiate signaling cascades leading to apoptosis (Miyashita and Reed, 1995).

p53-upregulated modulator of apoptosis (PUMA) and Noxa are also pro-apoptotic Bcl-2 family members that participate in the intrinsic pathway of apoptosis. PUMA was identified by two distinct methodologies. Yu et al. (2001) performed SAGE on DLD-1 colorectal cancer cells inducibly-expressing p53 to identify genes upregulated by p53 that mediate apoptosis of these cells upon p53 expression. Nakano and Vousden (2001) originally identified PUMA in a microarray comparing changes in gene expression in cells with or without p53. They performed 5'-rapid amplification of cDNA ends (RACE) to isolate the PUMA

full-length cDNA. Though the results of both groups are not completely concordant, the PUMA gene can be alternately spliced to produce several proteins. In addition, a p53 consensus binding site to which p53 can bind and upregulate expression was identified in the PUMA gene (Yu et al., 2001; Nakano and Vousden, 2001). Noxa was identified as a transcript upregulated in mouse cells treated with x-ray irradiation in a p53-dependent manner (Oda et al., 2000). Regulation of Noxa is achieved by p53 binding a p53 consensus binding site in the Noxa promoter and activating its transcription. PUMA and Noxa are BH3-only members of the Bcl-2 family that are able to bind and inhibit anti-apoptotic Bcl-2 members in the mitochondria. This inhibition results in the release of cytochrome *c* and activation of apoptosis (Yu et al., 2001; Nakano and Vousden, 2001; Oda et al., 2000).

DNA Repair

Examples of p53 target genes involved in DNA repair are p48 (DDB2) and p53-inducible ribonucleotide reductase small subunit 2 homologue (p53R2). p48 plays a role in nucleotide excision repair (NER) and aids in the removal of lesions in DNA caused by UV. People affected with xeroderma pigmentosum (XP) have defects in nucleotide excision repair. XP complementation group E (XPE) consists of people with a mutation in the p48 gene. p48 is one of two subunits in a UV-damaged DNA binding protein (UV-DDB). Previous observations indicated that p48 was upregulated in response to UV in cells containing wild-type p53, but

not in cells deficient for p53. A p53 consensus binding site was identified in the 5' untranslated region (UTR) of the p48 gene. p53 is able to bind to this site *in vitro* and activate transcription from this site in luciferase assays (Tan and Chu, 2002).

p53R2 was identified using differential display to compare genes upregulated upon inducible expression of p53 in SW490 cells to genes upregulated upon inducible expression of mutant p53. The p53R2 protein has 80% identity to the small subunit (R2) of ribonucleoside reductase (Tanaka et al., 2000). Ribonucleotide reductase converts ribonucleotide diphosphates to deoxyribonucleotides (dNTPs) to be used in synthesis of DNA. A p53 consensus binding site is located in the first intron of the p53R2 gene. p53 exhibits *in vitro* binding to this site and activates transcription in luciferase assays. Increased expression of p53R2 is observed in MCF7 breast cancer cells after DNA damage (Tanaka et al., 2000). p53R2 is a p53-inducible subunit induced in response to DNA damage. Upregulation of p53R2 enables a dNTP supply to be available for DNA repair in response to damage.

The p53-MDM2 interaction was discussed earlier as the mechanism by which levels of p53 are kept at low steady-state levels in the absence of stress. When stress occurs, p53 and MDM2 dissociate and allow for the transcriptional activation of target genes by p53. One p53 target gene that is induced in response to stress is the MDM2 gene. The MDM2 gene contains two adjacent binding sites in intron 1 (Zauberman et al., 1995). The upregulation of MDM2 by

p53 in response to damage demonstrates another way in which feedback regulation is maintaining the balance between p53 and MDM2. The multiple levels of feedback control illustrate the importance of the MDM2/p53 interaction, and therefore its stringent regulation. The p53 targets described above exemplify the diverse pathways by which p53 is able to mediate tumor suppression.

p53 Family Members

p63 and p73 were identified to be homologues of p53 (Yang et al., 1998; Kaghad et al., 1997). Using knockout studies in mice, p63 and p73 were found to play very different roles than p53 during development and in adult tissue. p63 is essential for the proper development of skin and epithelial structures during embryogenesis (Mills et al., 1999; Yang et al., 1999). In contrast, p73 is involved in the development of neural structures, such as the hippocampus, as well as proper pheromonal signaling, and regulating fluid dynamics of the cerebrospinal fluid (Yang et al., 2000).

Both the p63 and p73 genes contain two transcriptional start sites and make use of alternative splicing to produce at least six p63 or p73 proteins (Yang and McKeon, 2000). Importantly, all p63 and p73 proteins contain a DNA binding domain having 60% sequence identity to the DNA binding domain of p53 (Yang and McKeon, 2000). At least three of each of the p63 and p73 proteins also contain a transactivation domain with 25% sequence homology to that of p53, and are referred to as TAp63 and TAp73 (Yang and McKeon, 2000). Other p63

and p73 proteins lack the transactivation domain and are designated ΔNp63 and ΔNp73 (Yang and McKeon, 2000). It is thought that ΔNp63 and ΔNp73 can act in a dominant negative fashion to inhibit the function of not only the TAp63 and TAp73 proteins, but also p53 (Yang and McKeon, 2000). In the case of ΔNp63α, which is the most abundantly expressed p63 protein in many different squamous epithelial cells and glandular tissues (Westfall and Pietenpol, 2004), the protein exhibits transcriptional repressor activity and represses transcription at various p53 target gene promoters *in vitro* (Westfall et al., 2003). A role for p63 and p73 in p53-dependent apoptosis was proposed from results generated using E1A-expressing mouse embryonic fibroblasts (MEFs) derived from animals lacking the expression of p53, p63, p73, or combinations of the family members (Flores et al., 2002). E1A MEFs lacking p53 and p63 or p53 and p73 are more resistant to apoptosis induced by treatment with DNA damaging agents than E1A MEFs lacking p53 alone, suggesting that p63 and p73 are necessary for p53 to elicit a full apoptotic response in this model system (Flores et al., 2002). However, in a model system utilizing T lymphocytes lacking p53, p63, p73, or p63 and p73, p63 and p73 are not required for p53-mediated apoptosis in response to ionizing radiation (Senoo et al., 2004). Clearly, further investigation is required to understand the roles of p63 and p73 in p53-mediated signaling and was a goal of the current study.

A major source of debate exists over whether, like p53, p63 and p73 act as tumor suppressors, and their precise roles in the development of cancer in

humans. Very few human cancers exhibit mutations in p63 or p73 (Yang et al., 2002). Examination of mice heterozygous null for p53, p63, and p73 or combinations of the three family members revealed the spontaneous generation of tumors in mice of all genotypes. The p53, p63, and p73 heterozygous null mice displayed tumor spectra unique for each genotype. Mice heterozygous null for both p53 and p63 or p53 and p73 exhibited higher tumor burden and tumors with greater metastatic potential than mice heterozygous null for p53, p63, or p73 alone (Flores et al., 2005). Though these recent data suggest a role for p63 and p73 as tumor suppressors, the opposite results were observed for p63. Mice heterozygous null for p63 did not form spontaneous tumors (Keyes et al., 2006). The conflicting results could be due to differences in the mouse models used for the studies. Additional investigation will be required to determine what role these family members play in human tumorigenesis.

Selectivity of p53 Response

p53 regulates the transcription of many genes that play roles in p53-mediated cell cycle arrest, DNA repair, and apoptosis (El-Deiry, 1998; Levine et al., 2006). In response to cell stress, p53 binds to DNA in a sequence-specific manner (Kern et al., 1991) to a consensus site in a regulatory region of the gene (promoter or intron) (El-Deiry et al., 1992). The stresses that activate p53 signaling are many (Levine et al., 2006) and how p53 mediates a response should be tailored to the type and extent of damage, as well as the overall

outcome that is appropriate under the circumstances. However, the mechanism by which p53 selectively regulates its many target genes is still not well defined.

Many factors are believed to contribute to the ability of p53 to discriminately regulate target genes. One such factor is the differential affinity of p53 for consensus DNA binding sites. *In vitro*, p53 has higher affinity for consensus binding sites of genes involved in cell cycle arrest and DNA repair than the binding sites of genes implicated in apoptosis (Szak et al., 2001; Kaeser and Iggo, 2002; Weinberg et al., 2005). Reporter-based transcriptional assays in yeast and mammalian cells revealed that p53 has higher transcriptional activity when activity was assayed on consensus binding sites from cell cycle arrest, DNA repair, and death receptor apoptotic target genes versus sites from genes involved in mitochondrial apoptosis (Qian et al., 2002). The "match" of a target gene consensus binding site to the ideal consensus binding site may also affect the affinity of p53 to that site. Using chromatin immunoprecipitation, Kaeser and Iggo (2004) observed a two-fold higher occupancy of p21 site 1 (distal site), which has an 18/20 bp match to the consensus, than p21 site 2 (proximal site), which has a 12/20 bp match, by p53 transfected into H1299 cells.

Post-translational modifications of p53 are also thought to play a role in the ability of p53 to selectively regulate target genes. Phosphorylation and acetylation of p53 enhance p53 protein stability in part through disruption of the p53-MDM2 interaction (Bode and Dong, 2004; Appella and Anderson, 2001). Of note, post-translational modifications of MDM2 also contribute to the disruption of

the p53-MDM2 interaction (Brooks and Gu, 2003). Post-translational modifications of p53 also increase the ability of p53 to bind to DNA and facilitate interactions with other chromatin-associated proteins (Bode and Dong, 2004; Appella and Anderson, 2001). For example, phosphorylation of p53 on S15 results in enhanced binding of p53 to CBP/p300, a histone acetyltransferase (Lambert et al., 1998). CBP/p300 acetylates p53 at multiple C-terminal lysine residues *in vitro* and in cells, which then serves to enhance the ability of p53 to bind DNA (Gu and Roeder, 1997; Luo et al., 2004). In addition, p53 phosphorylated on S46 was shown to have a higher affinity for apoptotic target gene promoters compared to non-apoptotic gene promoters (Mayo, et al., 2005). The many forms of stress that challenge a cell lead to differential post-translational modifications of p53 (Bode and Dong, 2004; Appella and Anderson, 2001), resulting in distinctly modified forms of the p53 protein. Depending on the post-translational modifications, p53 exhibits distinct properties of stability, DNA binding ability or affinity, interactions with other proteins, and chromatin access. All of these factors likely contribute to the ability of p53 to differentiate between target genes.

Another aspect that may dictate differential p53-mediated transcription of target genes is the timing of p53 binding to regulatory regions and subsequent recruitment of the basal transcriptional machinery. Espinosa et al. (2003) showed that p53 is constitutively bound to both consensus binding sites in the p21 promoter. In addition, members of the basal transcriptional machinery,

including RNA polymerase II, are constitutively bound to the proximal promoters of p53 target genes involved in cell cycle arrest and DNA repair, but not apoptosis (Espinosa et al., 2003). However, it remains unclear whether there is a direct relationship between the constitutive binding of p53 to a target gene consensus binding sites and the presence of basal transcriptional machinery bound to the proximal promoter of those target genes. Further studies are necessary to clarify the role of p53-mediated recruitment of basal transcriptional machinery in the timing of regulation and selection of target genes.

Finally, occupancy of consensus binding sites of target genes by other p53 family members, p63 and p73, could also play an important role, whether cooperative or antagonistic, in p53-mediated signaling. Again, all p63 and p73 proteins contain a DNA binding domain having 60% sequence identity to the DNA binding domain of p53. In addition, ΔNp63 and ΔNp73 can act in a dominant negative fashion to inhibit the function of not only the TAp63 and TAp73 proteins, but also p53 (Yang and McKeon, 2000), so this may impact p53-selectivity of target genes. My dissertation research aimed to further understand p53 target gene selectivity.

Dissertation Research Goals

The ability of p53 to upregulate target genes in response to genotoxic stress is crucial for suppression of tumorigenesis. The goal of my dissertation research was to elucidate mechanistic details of p53 select target gene

regulation in response to stress with emphasis on the roles of the p53 family members in these processes. To achieve this goal, I determined common and distinct target genes of p53 and p63, including both known and previously unidentified targets. I also examined the binding of transcription factors to regulatory regions in p53 target genes. Using a primary human epidermal keratinocyte (HEK) model system for these studies was important so as to avoid genetic and epigenetic changes that occur in cell lines over time.

My first aim was to identify novel p53 candidate target genes from HEKs treated with a DNA-damaging agent, ADR. To achieve this goal, I used a chromatin immunoprecipitation/yeast screen method developed in our lab. An advantage of this method is that we are only screening fragments of DNA that are directly bound by p53. This ensures that any putative candidate genes are direct targets. Concordantly, I performed microarray analyses to determine the gene expression profile of HEKs infected with an adenovirus expressing p53. Not only did this provide more information about target gene expression in HEKs, it also helped prioritize candidate target genes for validation. Finally, in a parallel microarray experiment, I examined the gene expression profile of HEKs infected with an adenovirus expressing ΔNp63α to identify distinct and overlapping patterns of gene expression compared to p53. The analysis of my library screen and microarrays can be found in Chapter III.

My second aim was to identify factors involved in the selective regulation of target genes by p53, particularly focusing on the involvement of transcription

machinery (RNA polymerase II), p53 family members (ΔNp63α), and p53 post-translational modifications (S15 phosphorylation). Previous studies suggest that one property of p53 target gene selectivity results from the differential types and timing of transcription factor binding (Espinosa et al., 2003). My studies hoped to expand these findings by examining a diverse number of p53 target genes in a primary cell culture system. In the determination in the differences in transcription factor binding, I was interested in both constitutive binding and the binding changes that occurred at target gene regulatory regions in response to stress. Chromatin immunoprecipitation (ChIP) was employed for these experiments and the data are presented in Chapter IV.

Overall, these studies resulted in identification of novel target genes of p53 and ΔNp63α. In addition, a subset of target genes was found to be inversely regulated by p53 and ΔNp63α. Examination of binding of p53 and ΔNp63α to target gene consensus binding sites also revealed an inverse pattern of binding. Finally, my studies indicate that the location of a p53 consensus binding site may dictate the constitutive binding of basal transcription machinery. These conclusions and their implications will be discussed in Chapter V.

CHAPTER II

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment

Primary human epidermal keratinocytes (HEKs) were obtained from the Vanderbilt Skin Disease Research Core. Cells (passages 3 through 5) were cultured in EpiLife M-EPI-500 keratinocyte growth media (Cascade Biologics, Portland, OR) supplemented with human keratinocyte growth supplement #S-001-5 (Cascade Biologics), 0.06 mM $CaCl_2$ #S-013-EPI (Cascade Biologics), and 1% penicillin-streptomycin (v/v). The human colorectal carcinoma HCT116 p53 +/+ and p53 -/- cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (v/v) and 1% penicillin-streptomycin (v/v). All cells were incubated in a humidified incubator at 37°C under 5% CO_2. ADR was obtained from the Vanderbilt Chemotherapy Pharmacy. For the experiments in Chapter III, ADR was used at a final concentration of 0.35 μM. For the experiments in Chapter IV, ADR was used at a final concentration of 0.5 μM. Cells were harvested after treatment for the indicated times. For treatment with UV, cells were rinsed once with phosphate buffered saline (PBS), which was aspirated immediately prior to exposure to 50 J/m^2 UV-C (UV) using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). After UV treatment, conditioned media was replaced, and cells were harvested at the

indicated timepoints. For the microarray experiments, cells were infected with adenoviruses expressing GFP, p53, or ΔNp63α for 30 h.

Immunoblot Analysis

Whole cell lysates were prepared by washing cells with ice-cold PBS, scraping cells in ice-cold radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% Nonidet P-40 [v/v], 0.5% deoxycholic acid [w/v], 0.1% SDS [w/v], 50 mM Tris [pH 8.0], 5 mM EDTA), and frozen immediately at -80˚C. Lysates were supplemented with 50 mM sodium fluoride, 0.2 mM sodium vanadate, and the protease inhibitors chymostatin (10 μg/ml) (Sigma), leupeptin (10 μg/ml) (Sigma), antipain (10 μg/ml) (Sigma), pepstatin A (10 μg/ml) (Sigma), and 4-(2-aminoethyl)-benzenesulfonylfluoride (200 μg/ml) (Calbiochem, San Diego, CA) while thawing on ice. Lysates were clarified at 13,000 x *g* for 15 min at 4˚C. Protein concentration was determined using the Bio-Rad *DC* Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Fifty μg of whole cell lysate were boiled in 1X Laemmli sample buffer. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel, transferred to an Immobilon-P membrane (Millipore, Bedford, MA), and blocked with 5% nonfat dry milk (w/v) in TTBS (100 mM Tris-HCl pH [7.5], 150 mM NaCl, 0.1% Tween-20 [v/v]) for at least 1 h. Primary antibodies included RNA polymerase II N-20 (Santa Cruz Biotechnology, Santa Cruz, CA), p53 DO-1 (Santa Cruz), p63 4A4 (Santa Cruz), phospho-Ser15-p53 (P-S15-p53) catalogue

#9284 (Cell Signaling, Beverly, MA), MDM2 SMP14 (Santa Cruz), $p21^{WAF1}$ Ab-1 (Oncogene Research Products, Boston, MA), and β-actin I-19 (Santa Cruz). Secondary antibodies were isotype-specific horseradish peroxidase conjugates (Sigma). Bands were visualized with enhanced chemiluminescence using the ECL Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ).

Formaldehyde Crosslinking and Chromatin Immunoprecipitation

Prior to crosslinking, growth medium was aspirated and cells were rinsed once with PBS. Cells were crosslinked with a 1.6% formaldehyde solution (v/v) (EMD Chemicals, Inc., Gibbstown, NJ) in PBS for 13 min at room temperature. Crosslinking was stopped by the addition of glycine to a final concentration of 0.125 M and allowed to incubate at room temperature for 2 min. Non-crosslinked cells were treated with identical volumes of PBS. Crosslinked and non-crosslinked monolayers were rinsed twice with PBS. Lysates were harvested by scraping cells in 1 ml of RIPA buffer and frozen immediately at -80˚C. Phosphatase and protease inhibitors were added to lysates on ice as described above. Lysates were sonicated to yield chromatin fragments of approximately 1 kb and clarified by centrifugation at 13,000 x *g* for 15 min at 4˚C. Protein concentration was determined using the Bio-Rad *DC* Protein Assay Kit. Lysates were divided into aliquots and 2 mg of each protein extract were precleared with 10 μg of mouse IgG bound to protein A sepharose (PAS) beads (p53

immunoprecipitation) or 20 μg rabbit IgG bound to PAS beads (p63, pol II, P-S15-p53 immunoprecipitations) for at least 1 h at 4˚C. After centrifugation for 30 s at 13,000 x *g* at 4˚C, supernatants were transferred to new tubes containing a 30-μl bed volume of PAS beads, and 2 μg of the appropriate antibody was added. Antibodies used in the chromatin immunoprecipitations included 1 μg of both Pab 1801 (Santa Cruz) and Ab-1 (Oncogene Research Products) for p53, H-129 (Santa Cruz) for p63, N-20 for RNA polymerase II, and phospho-Ser15-p53 #9284 for P-S15-p53. Formaldehyde-crosslinked lysates were also immunoprecipitated with cyclin B1 GNS1 (Santa Cruz) and Bax N-20 (Santa Cruz) as isotype-specific control antibodies for the p53 and p63/pol II/P-S15-p53 immunoprecipitations, respectively. Immunoprecipitations were performed by rocking overnight at 4˚C.

Immunocomplexes were washed twice with ice-cold RIPA buffer, four times with ice-cold IP wash buffer (100 mM Tris pH [8.5], 500 mM LiCl, 1% Nonidet P-40 [v/v], 1% deoxycholic acid [w/v]), and twice more with ice-cold RIPA buffer. PAS beads were aspirated dry with a 30-gauge needle and 200 μl of Crosslinking Reversal Buffer (125 mM Tris pH [6.8], 10% β-mercaptoethanol [v/v], 4% SDS [w/v]) was added. Samples were heated at 100˚C for 30 min, and then the DNA was isolated from the immunoprecipitated chromatin by phenol-chloroform extraction and ethanol precipitation. DNA was resuspended in 40 μl nuclease-free water (30 μl for the pol II IP) and 2 μl were added to each polymerase chain reaction.

Genomic input DNA was isolated from cells that were formaldehyde-crosslinked, harvested, and sonicated identically to the other experimental plates. After sonication and protein concentration determination, 1 mg of protein extract was incubated in a boiling water bath for 30 min. The boiled lysate was phenol-chloroform extracted and ethanol precipitated to isolate the DNA.

ChIP PCR Amplification

The primer sequences used in the polymerase chain reactions (PCR) are listed in Table 1. PCR conditions for all primer sets listed were optimized using genomic input DNA. The p21 consensus binding site 1, 14-3-3σ consensus binding site 2, p53R2 consensus binding site, Fas/APO1 consensus binding site, MDM2 consensus binding site, p21 proximal promoter, 14-3-3σ proximal promoter, RRAD putative consensus binding site, and MOAP1 putative consensus binding site polymerase chain reactions were performed in a final concentration of 50 mM KCl, 10 mM Tris pH [9.0], 0.1% Triton X-100 [v/v], 0.75 mM $MgCl_2$, 0.25 mM dNTPs, 0.2 μM each primer, and 1.25 U Taq (Promega, Madison, WI). The p48 consensus binding site/proximal promoter, Noxa consensus binding site, p53R2 proximal promoter, Noxa proximal promoter, Fas/APO1 proximal promoter, and MDM2 proximal promoter polymerase chain reactions were performed in a final concentration of 16.6 mM $(NH_4)_2SO_4$, 0.67 mM Tris pH [8.8], 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10% dimethyl sulfoxide [v/v], 1.5 mM dNTPs, 7 ng/μl each primer, and 1.25 U Taq. The p21

consensus binding site 2 and 14-3-3σ consensus binding site 1 polymerase chain reactions were performed using Ready-to-Go PCR beads (Amersham Biosciences) according to manufacturer's instructions with a final primer concentration of 0.4 μM. PCR conditions for p21 consensus binding site 1, p21 consensus binding site 2, 14-3-3σ consensus binding site 2, p53R2 consensus binding site, and Fas/APO1 proximal promoter were 1 cycle of 95˚C, 5 min, 40 cycles of 95˚C, 30 s/ (annealing temperature listed in Table 1), 45 s/ 72˚C, 30 s, followed by 1 cycle of 72˚C, 10 min. PCR conditions for 14-3-3σ consensus binding site 1, p48 consensus binding site/proximal promoter, Noxa consensus binding site, Fas/APO1 consensus binding site, MDM2 consensus binding site, p21 proximal promoter, 14-3-3σ proximal promoter, p53R2 proximal promoter, Noxa proximal promoter, and MDM2 proximal promoter were 1 cycle of 95˚C, 5 min, followed by 40 cycles of 95˚C, 1 min/ (annealing temperature listed in Table 1), 1 min/ 72˚C, 1 min, followed by 1 cycle of 72˚C, 5 min. PCR conditions for RRAD putative consensus binding site and MOAP1 putative consensus binding site were 30 or 35 cycles (respectively) of 94˚C, 45 s/(annealing temperature listed in Table 1), 1 min/72˚C 25 s, followed by 1 cycle of 72˚C, 5 min. To ensure linearity of each polymerase chain reaction, increasing amounts of genomic input were added to separate polymerase chain reactions to be sure corresponding increasing amounts of signal were visualized on 6% polyacrylamide gels (acrylamide-bisacrylamide [19:1]) in 1X Tris acetate-EDTA buffer. Gels were stained with ethidium bromide and destained with water.

Real-time PCR Amplification

For the gene expression analysis in Figures 1, 3 through 8, and 10 total RNA was isolated from cells using the Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA). Reverse transcription of 500 ng of total RNA was performed using the Taqman Reverse Transcription Reagents Kit (Applied Biosystems, Foster City, CA). For the gene expression analysis in Figure 9, cells were trypsinized, pelleted by centrifugation, and resuspended in RNA lysis buffer (10 mM Tris pH [7.5], 100 mM NaCl, 2 mM EDTA, 1% SDS [w/v]). Lysis was completed by passage through a 23-gauge needle eight times. Proteinase K was added to the lysate to a final concentration of 100 μg/ml and incubated at 37°C for 1 h. After digestion by proteinase K, the NaCl concentration was increased to 400 mM. Samples were heated at 65°C for 5 min with constant agitation, followed by immediate cooling in ice water for 30 sec. mRNA was isolated by incubation with oligo-dT cellulose (Ambion Inc., Austin, TX) with rocking at room temperature for at least 2 h. The mRNA-oligo-dT cellulose mixture was washed twice with high-salt buffer (10 mM Tris pH [7.5], 400 mM NaCl, 1 mM EDTA, 0.2% SDS [w/v]) and packed with high-salt buffer on a poly prep chromatography column (Bio-Rad). The oligo-dT cellulose was washed once with high-salt buffer and once with low-salt buffer (10 mM Tris pH [7.5], 100 mM NaCl, 1 mM EDTA, 0.2% SDS [w/v]). The mRNA was eluted from the oligo-dT cellulose with 55°C elution buffer (5 mM Tris [pH 7.5], 1 mM EDTA, 0.2% SDS [w/v]). mRNA was ethanol precipitated by adding two volumes of 95% ethanol,

sodium acetate (pH [5.2]) to a final concentration of 220 mM and incubation overnight at -20°C. After precipitation, mRNA was pelleted by centrifugation at 12,000 x *g* for 30 min and the pellet was rinsed once with 70% EtOH. The mRNA pellet was dried in a centrifugal evaporator (Speed-Vac® type) for 5 min and resuspended in diethylpyrocarbonate (DEPC)-treated sterile H_2O. Reverse transcription of 100 ng of mRNA was performed using the Taqman Reverse Transcription Reagents Kit (Applied Biosystems). Real-time PCR was performed. Each reaction contained reverse transcribed RNA in a final concentration of 1X iQ SYBR-Green Supermix (Bio-Rad) and 0.2 μM of each primer. Real-time polymerase chain reactions were run on an iCycler Thermal Cycler (Bio-Rad). PCR conditions were 1 cycle of 95˚C, 3 min, 40 cycles of 95˚C, 10 s/ (annealing temp.), 45 s. The primers used in real-time PCR were designed using Beacon software (Bio-Rad). Primer sequences and annealing temperatures are listed in Table 2. Gene expression was determined by normalizing each sample to the housekeeping gene glyceraldehyde dehydrogenase (GAPDH), and fold-change was calculated relative to controls.

Microarray Analysis

HEKs were infected with adenoviruses expressing GFP, p53, or ΔNp63α for 30 h. Cells were harvested by trypsinization and pelleted by centrifugation at 1000 rpm for 5 min. mRNA was isolated as described above and submitted to the Vanderbilt Microarray Shared Resource (VMSR). Two independent replicate

experiments were analyzed. Using Agilent's Bioanalyzer microfluidic assay (Agilent Technologies, Palo Alto CA), the VMSR determined the amount of mRNA degradation, as well as protein and DNA contamination. After confirming the mRNA was of high enough quality to use in the analysis, the mRNA was processed using the standard Affymetrix protocol (Affymetrix Inc, Santa Clara, CA). Briefly, mRNA (300 ng) was reverse transcribed to double-stranded (ds) cDNA using an oligo-dT primer coupled to a T7 promoter. T7 polymerase was used to transcribe from the ds cDNA *in vitro* and incorporate biotin-modified CTP and UTP ribonucleotides. The biotinylated cRNA (15 μg) was fragmented and hybridized to the Affymetrix GeneChip U133 Plus 2.0. Following hybridization for 16 h at 45°C, hybridized cRNAs were washed and detected through streptavidin coupled to phycoerythrin using the Affymetrix 450 Fluidics Station and recommended protocols. Results were visualized by laser scanner (Affymetrix GeneChip Scanner 3000) and the image data quantified to generate gene expression values and ratios of gene expression between the hybridized samples. Microarray data analyses were performed using the GeneSpring software platform (Silicon Genetics, Redwood City, CA). Data were normalized on a per chip basis to the 50th percentile, then normalized on a per gene basis to the median signal, and finally normalized with the robust multichip average (RMA) normalization algorithm on a per gene basis to generate ratios of p53- or ΔNp63α-infected cells compared to GFP-infected cells for each replicate.

Generation and Screening of HEK ChIP Library

HEKs were treated with ADR for 6 h. Crosslinking, sonication, and protein concentration determination was performed as described above. Thirty-two mg of lysate were precleared and immunoprecipitation with anti-p53 antibodies was performed as described above. Immunoprecipitations were washed as described, followed by protein degradation in digestion buffer (120 μg/ml Proteinase K, 10 mM Tris pH [7.5], 5 mM EDTA, and 0.5% SDS [w/v]) at 56°C overnight, and then 65°C for 30 min. DNA was isolated by phenol-chloroform extraction and ethanol precipitation. The p53-immunoprecipitated DNA was processed using a PCR Polishing Kit (*Pfu*-based; Stratagene, La Jolla, CA) according to the manufacturer's instructions. pBM947 (a HIS3 reporter plasmid generously provided by M. Johnston at Washington University in St. Louis, MO) was blunted at the BamHI restriction enzyme sites. A blunt ligation was performed to insert the polished DNA fragments into the blunted vector and the ligated vector was amplified by growth in TransforMax EPI300 Electrocompetent *Escherichia coli* (Epicentre, Madison, WI). A total of 100 μg of pBM947-based library DNA were transformed into *Saccharomyces cerevisiae* yeast strain YPH681 containing the pRS314SN vector. Transformation competent yeast cells (50 μl aliquots) were centrifuged at 6000 rpm for 15 s and the supernatant was removed. To the cell pellet (in the following order): 33% polyethylene glycol (PEG; 50% [w/v]) (Sigma), 100 mM LiAc (Sigma), 278 μg/ml boiled salmon-sperm DNA (Sigma), 5.0% DMSO (v/v) (Sigma), and 1 μg library DNA were

added, brought to a final volume of 360 μl with sterile H_2O. The transformation reactions were mixed vigorously using a vortex and incubated at 30°C for 30 min, followed by a 42°C incubation for 30 min. The yeast were pelleted by centrifugation at 6000 rpm for 15 s, washed once, and resuspended in sterile H_2O. The transformed yeast were plated onto selection media containing galactose/lacking tryptophan, uracil, and histidine, followed by incubation at 30°C for 6 days. Colonies were replica plated onto synthetic drop out media containing dextrose/lacking tryptophan, uracil, and histidine for 6 days to screen for false positives. Yeast colony PCR was used to amplify the pBM947-based library DNA fragments from the yeast that grew in a p53-dependent manner. An approximate 0.25-μl scrape of yeast cells were added to each polymerase chain reaction containing 10 mM Tris pH [9.0], 50 mM KCl, 0.1% Triton X-100 [v/v], 0.5 mM $MgCl_2$, 0.25 mM nucleotides, 20 pmol of each primer, and 1.25 U Taq. PCR conditions were 1 cycle of 95˚C, 4 min, 50 cycles of 95˚C, 1 min/ 56˚C, 1 min/ 72˚C, 1.5 min, followed by 1 cycle of 72˚C for 10 min. PCR products were resolved in a 1% [w/v] agarose gel, stained with ethidium bromide, gel purified, and sequenced.

Table 1. Primer Sequences and Annealing Temperatures Used in ChIP PCR.

gene	forward primer	reverse primer	annealing temp.
p21 CBS 1	5'-GCTTGGGCAGCAGGCTG-3'	5'-AGCCCTGTCGCAAGGATC-3'	57°C
p21 CBS 2	5'-GCAGTGGGGCTTAGAGTGGGG-3'	5'-CAGGCTTGGAGCAGCTACAATTAC-3'	70°C
14-3-3σ CBS 1	5'-CATTTAGGCAGTCTGATTCC-3'	5'-GCTCACGCCTGTCATCTC-3'	61°C
14-3-3σ CBS 2	5'-CACTACCTCAAGATACCC-3'	5'-CACAGGCCTGTGTCTCCC-3'	52°C
p48 CBS/PP	5'-TTTGCTCCAGGGAGGTCAGC-3'	5'-GAGGATTGAGAGGCCTCTGG-3'	64°C
p53R2 CBS	5'-CAAAGAAACGGAAGTGGTGG-3'	5'-AGTTTTTTGGGGGACACAGG-3'	56°C
Noxa CBS	5'-ACGATGTTCTTTCTGGCTGG-3'	5'-GCTTTGACCATCTGCAAACG-3'	62°C
Fas/APO1 CBS	5'-GAATTGAAGCGGAAGTCTGG-3'	5'-TGAGCAATATCTGTTCTGAAGG-3'	57°C
p21 PP	5'-GCGGGGCGGTTGTATATC-3'	5'-CGCTCTCTCACCTCCTCTG-3'	57°C
14-3-3σ PP	5'-CTGTTCTGATGATTCACCCC-3'	5'-TCATGAAGGCTGCCATGTCC-3'	64°C
p53R2 PP	5'-GACAGGGATAATCCCTTAGG-3'	5'-TGTCCATTGAGTGACTCTCC-3'	54°C
Noxa PP	5'-TCTAGTTTCCCTACGTCACC-3'	5'-GAGCTGAACACGAACAGTCC-3'	54°C
Fas/APO1 PP	5'-TCACCTGAAGTGAGCATGCC-3'	5'-AGGAGGATGGGAAGGAAGC-3'	62°C
MDM2 CBS	5'-TGATCGCAGGTGCCTGTC-3'	5'-CCTCCAATCGCCACTGAAC-3'	59°C
MDM2 PP	5'-GGCTAAAGGAGTGTCACAGC-3'	5'-GCTTCTTGCTCCATCTTTCC-3'	62°C
RRAD CBS	5'-TCTGGCTCTGCTGTTTCTGG-3'	5'-GCCTCTGCTGTGTTCTAAGG-3'	58°C
MOAP1 CBS	5'-ATTTAGCCATTACCAAGAGG-3'	5'-CAGAAATTTTTCCTTTGGGG-3'	58°C

CBS = consensus binding site; PP = proximal promoter

Table 2. Primer Sequences and Annealing Temperatures Used in Real-Time PCR.

gene	forward primer	reverse primer	annealing temp.
GAPDH	5'-CATGTTCCAATATGATTCCAC-3'	5'-CCTGGAAGATGGTGATG-3'	55°C
p21	5'-AGCGGAACAAGGAGTCAG-3'	5'-CGTTAGTGCCAGGAAAGAC-3'	58°C
14-3-3 σ	5'-CCAGGACCAGGCTACTTCTC-3'	5'-GTCCAGTTCTCAGCCACAAG-3'	56°C
p48	5'-GAAACTCAGGGAAGATGATGTG-3'	5'-TGGCTCCAGATGAGAATGTG-3'	56°C
p53R2	5'-TGAAGAGCCACTCCTAAGAAAG-3'	5'-TGTTCCAGTGAGGGAGATCC-3'	58°C
Noxa	5'-CCGTGTGTAGTTGGCATCTC-3'	5'-CCCACTCAGCGACAGAGC-3'	60°C
Fas/APO1	5'-GAGCTTTGCCACCTCTCC-3'	5'-AGTATCTCCAAACCAGGCTAG-3'	60°C
MDM2	5'-CCCCTTCCATCACATTGC-3'	5'-TTACAATCAGGAACATCAAAGC-3'	53°C
RRAD	5'-TTCATTGAGACATCAGCGGC-3'	5'-GTGGCAGGACTTGGATTTGG-3'	55°C

CHAPTER III

IDENTIFICATION OF P53 TARGET GENES USING A CHROMATIN IMMUNOPRECIPITATION/YEAST SCREEN AND MICROARRAY ANALYSES

Introduction

p53 is a tumor suppressor protein known to be mutated in approximately half of all human cancers and exhibits its tumor suppressive functions through its ability to mediate cell cycle arrest, DNA repair, and apoptosis in response to genotoxic stress (Stewart and Pietenpol, 2001). A major biochemical activity of p53 is its ability to bind DNA and regulate transcription (Kern et al., 1991; Kern et al., 1992). p53 is a sequence specific transcription factor (El-Deiry et al., 1992) that controls the transcription of many genes, the majority of which are transcriptionally activated, though repressed genes have also been identified (El-Deiry, 1998; Tokino and Nakamura, 2000). p53 activates different subsets of target genes in response to a variety of cellular stresses, often in a cell type specific manner (Yu et al., 1999). To better understand p53 signaling pathways, considerable research has been done to identify target genes of p53. Through these efforts, over 100 genes directly regulated by p53 have been identified and many play key roles in p53-mediated tumor suppression and regulation of biological pathways (El-Deiry, 1998; Harms et al., 2004; Nakamura, 2004). However, as seen from the results of knock-out mouse studies, the target genes identified to date still do not account for all aspects of p53 signaling pathways. In

other words, the mechanistic details of p53-mediated cell cycle arrest or apoptosis, among other pathways, are not completely explained using only the current target gene information. In an unbiased screen of all the non-redundant sequences in chromosomes 21 and 22, it is estimated that there are ~1600 p53 consensus binding sites in the human genome. Of note, only 22% of these fragments are located at the 5' end of a gene, and 36% are found 3' of a gene and significantly correlated with noncoding RNAs (Cawley et al., 2004). The vast majority of the estimated sites will likely not be functional, but the screen is still suggestive of many unidentified targets. In addition, the role of p63, namely the ΔNp63α protein, in p53-mediated signaling and regulation of targets is still unclear.

The goal of the research presented in this chapter was to further our understanding of p53-regulated signaling pathways. To achieve this goal, we first identified novel candidate target genes using a chromatin immunoprecipitation-based screen developed in our laboratory (Hearnes et al., 2005). This type of screening would identify candidate p53-regulated target genes in primary human epidermal keratinocytes (HEKs) on the basis that p53 could bind to and transactivate a consensus binding site in the vicinity of the candidate. Furthermore, we used microarray analyses on HEKs expressing adenoviral-p53 to provide us with additional data to aid in the selection of likely candidate target genes. We performed side-by-side microarray analyses of HEKs expressing adenoviral-ΔNp63α to compare transcriptional profiles

regulated by the two of three p53 family members that are expressed in HEKs. Using these methods, we identified many candidate p53- and ΔNp63α-regulated target genes containing p53 consensus binding sites that align with the canonical p53 consensus sequence. Of particular interest were Ras-related associated with diabetes (RRAD), modulator of apoptosis 1 (MOAP-1), and zinc finger protein 90 (ZFP90). In addition to novel targets, we also identified known target genes of p53 thus proving the validity of our screening methods. Interestingly, we found an overlapping subset of both candidate and known target genes that are inversely regulated by p53 and ΔNp63α. Determining the mechanisms of coordinate regulation of target genes by p53 and ΔNp63α will allow for a better understanding of the roles of p53 family members in p53-mediated tumor suppression.

Results

HEK Library Generation/Yeast Screen Analysis

Primary HEKs were used for library generation to minimize the genetic and epigenetic changes that occur when a cell line is subjected to many rounds of passaging. HEKs were treated with ADR (0.35 μM) for 6 h, then formaldehyde-crosslinked. Crosslinked lysates were generated, sonicated to shear the chromatin, and p53-containing immunocomplexes were precipitated using anti-p53 antibodies. DNA fragments from the immunoprecipitated

chromatin were isolated and cloned into the pBM947 yeast vector upstream of gene essential for histidine biosynthesis. The library was transformed into the *S. cerevisiae* yeast strain YPH681 harboring the pRS314SN vector containing the p53 gene under the control of a galactose-inducible promoter. In the presence of galactose, p53 protein was expressed in the yeast. If p53 could bind to the upstream library sequence and activate expression of the histidine biosynthesis gene, a colony would grow on an agar plate containing growth media deficient in histidine. False positives were identified by replica plating the colonies on histidine-deficient plates containing glucose, in which p53 expression is repressed. Any colonies that grew in the presence of glucose had the histidine gene activated by another factor independent of p53, and were eliminated from further screening.

Out of the HEK p53 library, approximately 1 x 10^6 yeast transformants were screened and 50 grew in a p53-dependent manner. The library fragment from each p53-dependent yeast transformant was PCR-amplified and sequenced. On the NCBI website, the Human Genome Resources database was queried using the sequenced clones in the BLAST search function and 34 non-redundant clones were identified. The cloned fragments were analyzed using the p53MH algorithm (Hoh et al., 2002) to determine if they contained putative p53 consensus binding sites. Of the 34 non-redundant fragments, 97% contained a p53 consensus binding site. El-Deiry et al. (1992) defined the p53 consensus site as two repeats of the sequence RRRC(A/T)(T/A)YYY, where R =

A or G and Y = C or T. The two 10 bp repeats can be separated by a 0-13 bp spacer of random sequence. Of the 34 fragments, 24 (70%) contained binding sites matching the ideal p53 consensus sequence at 17 out of 20 bp or better. Of note, 7 of the 34 (20%) total fragments isolated in the library, matched to a region in the genome that did not contain any known or predicted genes within 20 kb of the fragment. A representative list of DNA fragments identified in the library screen is shown in Table 3, as well as where the fragments align to the human genome, the candidate target genes that are located within 20 kb of the fragment, and the corresponding putative p53 consensus binding site for each fragment.

p53 Microarray Analyses

We used microarray technology to provide us with another criterion, ability to be transcriptionally regulated by p53, for prioritizing the likely candidate target genes. HEKs were infected with an adenovirus expressing p53 and mRNA was isolated 30 h post-infection. This time point was chosen because 30 h was when we observed the most robust expression of a majority of known p53 targets. Comparison of the gene expression profiles from HEKs infected with p53-expressing adenovirus to control HEKs infected with GFP-expressing adenovirus identified 866 genes upregulated and 120 genes downregulated at least 2-fold upon p53 expression. Among the regulated genes were several known p53

Table 3. HEK Library p53 Candidate Target Gene Characteristics

RRRC(A/T)(T/A)GYYY--0-13bp--RRRC(A/T)(T/A)GYYY

candidate gene	definition	function	p53 binding site	match
RRAD	ras-related associated with diabetes	GTPase	GAACATGCCC---0bp--GGCCATGTCT	19/20
CGI-128	N/A	unknown		
CES2	carboxylesterase 2	hydrolizes ester- and amide-bonds, thioesters		
CDH16	cadherin 16	cell adhesion		
NRXN1	neurexin 1	cell adhesion	AGGCTGTGCCC---0bp--TAACATGTCC	18/20
FARSL	phenylalanine-tRNA synthetase-like	ligate tRNA with phenylalanine	GGCCAAGATC--14bp--CTCCAAGCCT	15/20
RAD23A	RAD23 homolog A	nucleotide excision repair		
PLINP-1	papilloma virus L2 interacting nuclear protein 1	unknown		
LOC376527	N/A	similar to Erf	GGGCATGTCA---7bp--GAACATGTCA	18/20
LOC374909	N/A	unknown		
TEAD1	TEA-domain family member 1	SV40 transcriptional enhancer factor	TATCTTGTCT--13bp--AGGCAGGCTG	16/20
LOC342346	N/A	unknown	TAACTTGCCC---0bp--GGGCACCGCCC	17/20
FLJ32440	N/A	unknown	AGGCATGTCT--10bp--AGGCAAGTTT	20/20
TLE1	transducin-like enhancer of split	transcriptional corepressor	GAACATGTCC---0bp--CAACATGTTT	19/20
chr.9, no genes	N/A	N/A	AAACATGTCC---0bp--AGGCATGTGC	19/20
chr.12, no genes	N/A	N/A	GGACCAGAAC--13bp--CAGCTTGAAT	14/20
chr.18, no genes	N/A	N/A	TGGCTTGCCT---0bp--GGGCATGCCC	19/20
chr.13, no genes	N/A	N/A	CTGCATGTCT---0bp--GGGCACGCCC	17/20
chr.1, no genes	N/A	N/A	AGGCATGCCC---0bp--GGGCATGTCT	20/20

targets, thereby confirming the activation of p53-signaling pathways by ectopic expression of adenoviral-p53.

Putative Regulation of RRAD by p53

Of the candidate genes identified with our library screen and microarray studies, one putative p53 target we further validated and studied was RRAD, which was first discovered using subtractive library screening to identify genes involved in insulin-resistance in patients with Type II diabetes (Reynet and Kahn, 1993). The RRAD protein has GTPase activity (Zhu et al., 1995) and when overexpressed, is able to negatively regulate insulin-stimulated glucose uptake in myocyte and adipocyte cell lines (Moyers et al., 1996). Interestingly, the GTPase-activating protein (GAP) for RRAD is nm23, a putative tumor metastasis suppressor. The RRAD-nm23 interaction is bi-directional in that each protein could modulate the activity of the other (Zhu et al., 1999).

We identified a DNA fragment in the HEK library screen that aligned 2.4-kb upstream of the RRAD gene, therefore fitting our criteria for a target potentially regulated by p53. Additionally, RRAD transcript levels were upregulated 2-fold by p53 in our HEK p53 microarray. Since the RRAD gene was identified in two independent assays, this increased the likelihood that the RRAD gene was a bona fide p53 target. In order to further validate this putative target gene, we performed chromatin immunoprecipitation in the colorectal cancer cell line, HCT116, containing wild-type (functional) p53. Our goal was to

determine p53 occupancy at the putative consensus binding site in the RRAD promoter in response to treatment with ADR. HCT116 cells were treated with ADR and formaldehyde-crosslinked at 0, 2, and 8 h after treatment. ChIP analysis revealed that p53 was bound constitutively to the putative consensus binding site in the RRAD promoter prior to ADR treatment (Figure 1A). Increased p53 binding was observed at the RRAD putative consensus binding site after 2 and 8 h of treatment with ADR. These results were confirmed in HEKs treated with ADR, also (Deb Mays, unpublished data). Using real-time PCR, a corresponding increase in RRAD mRNA was observed after ADR treatment, also, but only in HCT116 cells with wild-type p53 (Figure 1B). Isogenic HCT116 p53 -/- cells did not upregulate RRAD mRNA after treatment with ADR. The HCT116 ChIP and real-time PCR data provide further evidence that RRAD is regulated by p53. Further validation of the regulation of RRAD by p53 and the potential role of RRAD in p53-signaling pathways is currently under investigation in our laboratory.

ΔNp63α Microarray Analyses

Another goal of my dissertation research was to analyze target gene regulation by the p53 family member p63, namely the ΔNp63α protein, and compare the gene expression profiles to those obtained from p53-expressing HEKs. This goal was achieved using parallel microarray analyses to those described above. Cultures of HEKs identical to those infected with GFP- and

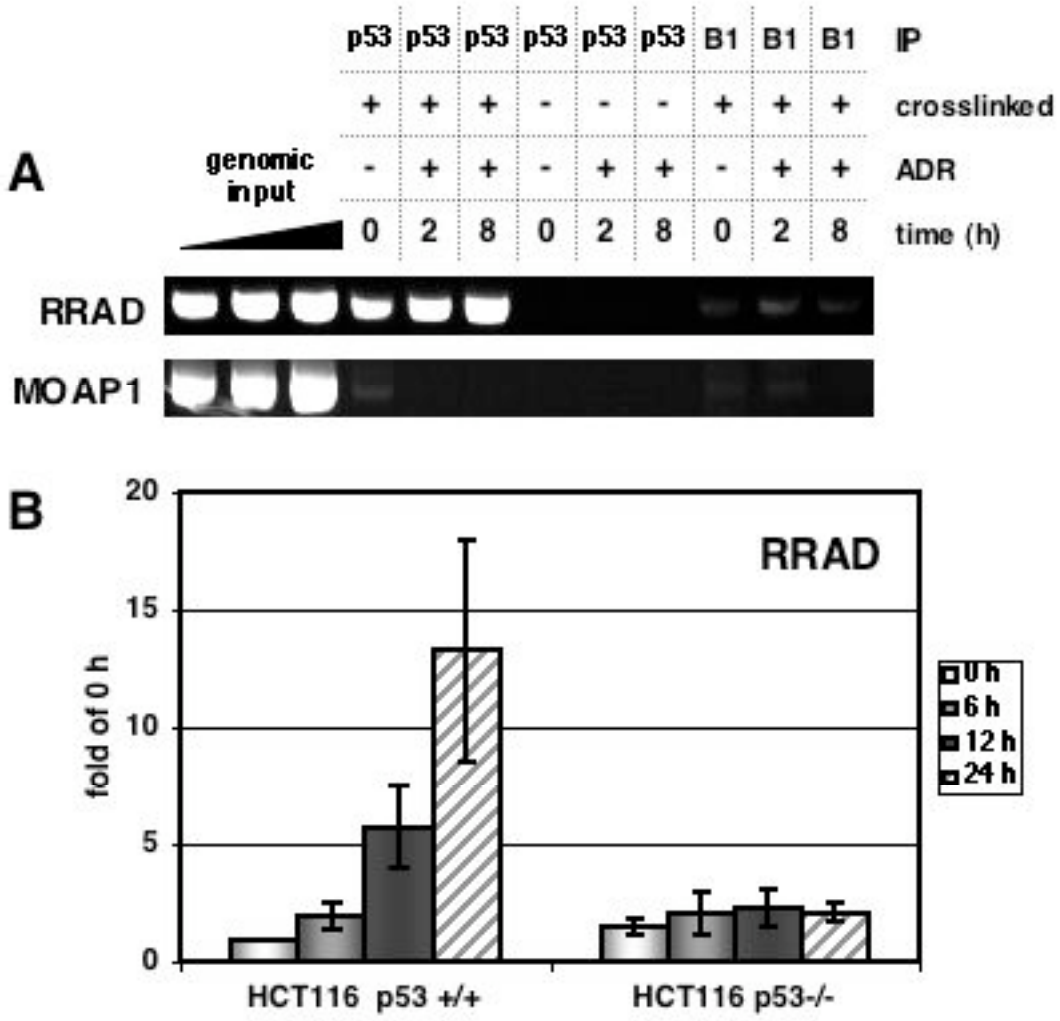

Figure 1. Candidate target gene ChIP and qRT-PCR analyses. A. Isogenic HCT116 p53 +/+ and p53 -/- cells were treated with ADR for 0, 2, or 8 h. p53 was immunoprecipitated from formaldehyde-crosslinked or non-crosslinked HCT116 +/+ or -/- lysates. Lysates were immunoprecipitated with an isotype-matched antibody, B1 (cyclin B1) as a negative control. The regions of RRAD and MOAP1 containing putative p53 consensus binding sites were analyzed. Increasing amounts of genomic input demonstrate that the PCR reactions were performed in the linear range (left-most panels). The ethidium bromide-stained gel most representative of the average of six independent experiments is shown. **B.** Isogenic HCT116 p53 +/+ and p53 -/- cells were treated with ADR for 0, 6, 12, and 24 h. Real-time PCR was used to quantify the expression levels of RRAD mRNA. Each sample was normalized to GAPDH expression, followed by fold-change determination as compared to gene expression at 0 h.

p53-expressing adenovirus were infected with an adenovirus expressing ΔNp63α. Microarray analysis was performed to obtain gene expression profiles.

Again, comparison to HEKs infected with GFP-expressing adenovirus resulted in the identification of 145 genes upregulated and 620 genes downregulated by ΔNp63α. Of great interest to us was the identification of a subset of genes that were inversely regulated by p53 and p63, and are listed in Table 4. The majority of these have not been previously identified as targets of either transcription factor. Initial validation was performed on two candidate targets in this list, MOAP-1 and ZFP90. MOAP-1 was identified as a Bax-interacting protein in a yeast two-hybrid screen (Tan et al., 2001). Recent work has shown that the tumor suppressor Ras association domain family protein 1A (RASSF1A) interacts with MOAP-1 and utilizes this interaction to activate Bax (Vos et al., 2006). To date, functional characteristics of ZFP90 remain to be elucidated. The promoter and intronic regions of MOAP-1 and ZFP90 were analyzed using the p53MH algorithm (Hoh, et al., 2002). Potential consensus binding sites were identified (Table 4) and ChIP analysis was first attempted to determine p53 occupancy of the potential sites. The DNA fragments isolated using ChIP in HCT116 cells as described above were PCR amplified using primers specific for each putative consensus site. For several of these sites, we encountered difficulties in obtaining PCR conditions providing a specific product on genomic input DNA. However, PCR on one of the potential sites in the MOAP1 promoter yielded specific and robust signal using genomic input.

Table 4. Microarray Candidate Targets Exhibiting Inverse Regulation by p53 and ΔNp63α

candidate	p53 binding site	location	match
BRD1	GAGCTAGaTC--5bp--AAGCATGCTT	promoter	19/20
EIF2AK4	AGGCTTGCTC--10bp-cAcCAAGCTg	promoter	17/20
MOAP1	AGACcAGCCT--3bp--cAACATGCTg	promoter	17/20
NCOA6IP	GGACTTGTCT--1bp--cAACcTGTCT	intron	18/20
ORC4L	ttACTTGTTC--0bp--AGACAAGTTa	promoter	17/20
TNFRSF6	GGACAAGCCC--0bp--tGACAAGCCa	intron	18/20
UBE2N	GGtCTTGCCC--14bp-GAGCAAGaCg	promoter	17/20
ZFP90	GGACTTGTTC--12bp-tAACTcGTTC	intron	18/20

BRD1 = bromodomain containing 1
EIF2AK4 = eukaryotic translation initiation factor 2 alpha kinase 4
MOAP1 = modulator of apoptosis 1
NCOA6IP = nuclear receptor coactivator 6 interacting protein
ORC4LL = origin recognition complex, subunit 4-like (yeast)
TNFRSF6 = tumor necrosis factor receptor superfamily, member 6 (Fas/APO1)
UBE2N = ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast)
ZFP90 = zinc finger protein 90

However, binding of p53 at this site could not be detected above background levels at any of the time points (Figure 1A). More experimentation is necessary before conclusions can be made regarding MOAP1 and ZFP90 as candidate p53 or ΔNp63α target genes.

Discussion

The HEK ChIP library/yeast screen described herein was undertaken to identify candidate p53 target genes. Of the fragments pulled out of the library, the majority contained a putative p53 consensus binding site matching the canonical consensus site at 17 out of 20 bp or better. The "match" of a target gene consensus binding site to the ideal consensus binding site may affect the affinity of p53 to that site. Using chromatin immunoprecipitation, Kaeser and Iggo (2004) observed a two-fold higher occupancy of the distal p21 consensus binding site 1 (18/20 bp match to the consensus), than the proximal p21 consensus binding site 2 (12/20 bp match) by p53 transfected into H1299 cells. The fact that the library fragments match well to the canonical consensus binding site suggests that the library is identifying sites to which p53 has substantial affinity. Also, *in vitro* studies showed that p53 has higher affinity for consensus binding sites of genes involved in cell cycle arrest and DNA repair than the binding sites of genes implicated in apoptosis (Szak et al., 2001; Kaeser and Iggo, 2002; Weinberg et al., 2005). Reporter-based transcriptional assays in yeast and mammalian cells revealed that p53 has higher transcriptional activity

when activity was assayed on consensus binding sites from cell cycle arrest, DNA repair, and death receptor apoptotic target genes versus sites from genes involved in mitochondrial apoptosis (Qian et al., 2002). The DNA fragments in our ChIP library were generated from HEKs treated with ADR for 6 h. In other studies with HEKs, we observed that cells underwent cell cycle arrest in response to ADR treatment at 6 h and no signs of apoptosis were observed (see Chapter IV). Therefore, we would expect that the majority of the fragments identified in our library would correspond to target genes that are involved in cell cycle arrest or DNA repair.

Another interesting finding from our HEK library screen was that 20% of fragments matched to the genome in areas of chromosomes where there were no known or predicted genes within 20 kb of the fragment. This suggests that as the human genome continues to be sequenced and annotated more thoroughly, perhaps genes will be discovered in these regions, and lead to the identification of additional target genes of p53. Another possibility is that p53 may be regulating microRNAs in these regions of the genome. MicroRNAs are a group of regulatory RNAs that control gene expression by decreasing the stability and translation of the messenger RNA of the target of the microRNA (Kent and Mendell, 2006). Current estimates predict that microRNAs account for approximately 3% of all human genes, though many remain to be validated (Cummins and Velculescu, 2006). Regulation of microRNAs may be another

mechanism by which p53 exerts its tumor suppressive functions (Kent and Mendell, 2006).

To complement our library screening, we performed microarray analyses as a way to identify genes that are direct targets of p53 regulation. One caveat to microarray studies is that the observed changes in gene expression in response to p53 expression are not limited to primary targets of p53. However, the identification of secondary targets can be valuable in the identification and understanding of pathways regulated by p53. In addition, comparison to published microarray data would help to eliminate some of the secondary targets.

One candidate target gene of particular interest is RRAD. RRAD is an example of a candidate target identified in our library screen that was also upregulated by p53 in our microarray analyses. One study found that RRAD expression is often lost in invasive carcinoma of the breast and yet in a subset of tumors that retain expression of RRAD there was a correlation with characteristics of poor prognosis: increased tumor size, higher grade, and greater nodal involvement (Tseng et al., 2001). When RRAD was overexpressed in the MDA-MB435 breast cancer cell line, it resulted in increased colony formation in soft agar and increased tumor size when injected into nude mice (Tseng et al., 2001). Strikingly, when nm23 was co-expressed with RRAD in the same experiments, the increased tumorigenicity was abrogated in both model systems. Recent studies have identified the RRAD locus to be aberrantly methylated in malignant mesotheliomas (Suzuki et al., 2005), invasive cervical

carcinomas (Sova et al., 2006), and prostate cancers (Suzuki et al., 2006). As these findings suggest conflicting roles of RRAD, further experimentation is necessary to define and understand the role of RRAD in tumorigenesis.

In HCT116 colorectal cancer cells, p53 occupied the candidate consensus binding site in the RRAD promoter constitutively in the absence of genotoxic stress. p53 occupancy at this candidate binding site increased after 2 and 8 h of ADR treatment. Our data are strongly suggestive that RRAD is a direct target gene of p53. In support of this notion, RRAD has also been identified in a recent screen for novel p53 targets from HCT116 cells treated with 5-fluorouracil (5-FU) utilizing ChIP coupled with paired-end ditag (PET) sequencing (Wei et al., 2006). RRAD is also upregulated in H1299 lung carcinoma cells ectopically expressing TAp73γ (Jennifer Rosenbluth, unpublished data). p73 is a p53 family member thought to have overlapping functions with p53 in the response to DNA damage. p73 is able to activate target genes involved in cell cycle arrest, DNA repair, and apoptosis that have been previously identified as p53 targets, or that function in a similar manner to p53 targets (Harms et al., 2004). Further investigation will be necessary to determine if both p53 and p73 can regulate transcription of RRAD as well as the functional implications of the regulation. Interestingly, p73 is overexpressed in breast cancer (Zaika et al., 1999; Dominguez et al., 2001; Garcia et al., 2004). For this reason, it would seem that RRAD is more likely a p73 target gene that can be modulated by p53 in certain circumstances. Given

the low levels of p73 in HEKs, perhaps the regulation of RRAD by p53 is more evident.

Many questions remain regarding the role of p63, another p53 family member, in p53-mediated signaling. Microarray analyses on HEKs ectopically expressing ΔNp63α were performed in parallel with those on GFP- and p53-expressing HEKs. Of note, the number of genes upregulated by p53 (866) far exceeded the number of genes downregulated by p53 (120). In contrast, the number of genes downregulated by ΔNp63α (620) far exceeded the number upregulated by ΔNp63α (145). These results are likely indicative of the prevalent function of p53 and ΔNp63α as a transcriptional activator and repressor, respectively. Of particular interest was a subset of genes that were inversely regulated by p53 and ΔNp63α. Validation of two of these genes, MOAP-1 and ZFP90, is currently in progress. MOAP-1 is of interest because it plays a role in apoptosis (Tan et al., 2001). An important tumor suppressive function of p53 is its ability to induce apoptosis, though many questions remain regarding the mechanisms by which p53 initiates and potentiates this process (Fridman and Lowe, 2003). The role of ΔNp63α in p53-regulated apoptosis also remains to be elucidated, though it can repress pro-apoptotic genes (Barbieri et al., 2005). Identification of additional apoptotic target genes regulated by p53 or ΔNp63α, such as candidate target gene MOAP-1, would help in the understanding of p53-mediated apoptotic pathways. Though very little information is known about ZFP90 specifically, it remains an interesting candidate target gene due to the

presence of zinc-finger domains in the protein through which ZFP90 has the potential ability to bind DNA or RNA. Initial attempts using ChIP to determine p53 occupancy of potential consensus binding sites in the MOAP-1 and ZFP90 genes have not been successful. However, efforts at validating MOAP-1 and ZFP90 as potential p53 or ΔNp63α candidate genes will continue in future endeavors. There are multiple potential p53 binding sites in both the MOAP-1 and ZFP90 genes. ChIP will be utilized to determine p53 binding at other potential sites. Also, the PCR conditions for some of the potential binding sites need to be troubleshot. In general, ChIP library/yeast screens and microarray studies have been useful in identifying candidate p53 consensus binding sites and target genes, which will ultimately provide information in an attempt to complete our understanding of p53-mediated tumor suppression.

CHAPTER IV

P53 AND ΔNP63α DIFFERENTIALLY BIND AND REGULATE TARGET GENES INVOLVED IN CELL CYCLE ARREST, DNA REPAIR, AND APOPTOSIS

Introduction

p53 is a sequence specific transcription factor (El-Deiry et al., 1992) that controls the transcription of many genes (El-Deiry, 1998; Tokino & Nakamura, 2000) in response to a variety of cellular stresses, often in a cell type specific manner (Yu et al., 1999). However, the mechanism by which p53 selectively regulates its many target genes is still not well defined. Many factors are believed to contribute to the ability of p53 to differentially regulate target genes. One such factor is the differential affinity of p53 for consensus DNA binding sites. *In vitro*, p53 has a higher affinity for consensus binding sites of genes involved in cell cycle arrest and DNA repair than the binding sites of genes implicated in apoptosis (Kaeser & Iggo, 2002; Qian et al., 2002; Szak et al., 2001; Weinberg et al., 2005). The "match" of a target gene consensus binding site to the canonical consensus binding site may affect the affinity of p53 to that site (Kaeser & Iggo, 2004). Post-translational modifications of p53 are also thought to play a role in the ability of p53 to selectively regulate target genes (Appella & Anderson, 2001; Bode & Dong, 2004). Another aspect that may dictate differential p53-mediated transcription of target genes is the timing of p53 binding to regulatory regions and

subsequent recruitment of the basal transcriptional machinery. Espinosa *et al.* recently showed that p53 is constitutively bound to both consensus binding sites in the p21 promoter (Espinosa et al., 2003). In addition, members of the basal transcriptional machinery, including RNA polymerase II, have been shown to be constitutively bound to the proximal promoters of p53 target genes involved in cell cycle arrest and DNA repair, but not apoptosis (Espinosa et al., 2003). The generality of these findings is not established nor whether there is a direct relationship between the constitutive binding of p53 to target gene consensus binding sites and the presence of basal transcriptional machinery bound to the proximal promoters of those target genes.

Occupancy of p53 consensus binding sites in target genes by other p53 family members, p63 and p73, may also play an important role, whether cooperative or antagonistic, in p53-mediated signaling. ΔNp63α is the most abundant, if not only, expressed p63 isoform at the protein level in many different squamous epithelial cells and glandular tissues (Westfall & Pietenpol, 2004). The ΔNp63α protein exhibits transcriptional repressor activity and can repress transcription at various p53 target gene promoters (Westfall et al., 2003). However, the relative occupancy of select target genes by each of the family members after various forms of genotoxic stress in relation to other key transcription factors, such as RNA polymerase II, has not been examined and was a goal of the current study.

Using chromatin immunoprecipitation, we examined the binding of p53 family members and an essential component of the basal transcriptional machinery (RNA polymerase II) to p53 target genes involved in either cell cycle arrest, DNA repair, or apoptosis in primary human epidermal keratinocytes (HEKs). We found that in general, p53 occupancy at consensus binding sites in target genes increased after treatment with ADR and UV, and ΔNp63α occupancy decreased under these conditions. Further, we observed an inverse regulation of a panel of previously identified p53 target genes by p53 and ΔNp63α, consistent with the theory that ΔNp63α and p53 can play antagonistic roles at various target genes. These data provide insight to a potential role of ΔNp63α in the p53-mediated response to genotoxic stress.

Results

Divergent Binding of p53 Family Members To Target Genes Involved In Cell Cycle Arrest and DNA Repair

To investigate the role of p53 family members in the regulation of select target genes, we sought to examine the occupancy of p53, p63, and p73 at consensus binding sites in target gene regulatory regions using chromatin immunoprecipitation (ChIP). We used primary HEKs as our model system to minimize genetic and epigenetic alterations frequently observed in established cell lines. Prior to performing ChIP, we verified expression levels of the proteins under analysis and assessed the cellular response of HEKs to genotoxic agents.

HEKs were treated with either ADR (0.5 mM) or UV (50 J/m^2) for 2, 6, 12, and 24 h. Western analysis was performed to determine pol II, p53, phospho-S15-p53 (P-S15-p53), ΔNp63α, and p73 protein levels. (Figure 2). Control, untreated HEKs constitutively expressed low levels of p53. A continual increase in p53 levels was observed over the 2 to 24 h exposure to ADR. Levels of p53 increased at 2 h after exposure to UV and remained elevated for the duration of the timecourse. Accumulation of p53 protein after ADR and UV treatment was accompanied by a corresponding increase in p53 phosphorylation at S15. Conversely, there was a decrement in the levels of the only detectable isoform of p63 expressed in the primary cultures of the HEKs, ΔNp63α, after both ADR and UV treatment. Over the same time course, pol II protein remained unchanged after either genotoxic treatment. Of note, we were unable to detect expression of any of the p73 protein isoforms in control or treated HEKs that were assayed in parallel Western analyses with a p73-specific antibody. This same antibody generated a strong signal on protein lysates from H1299 cells ectopically expressing the TAp73α or ΔNp73β isoforms (data not shown). Further, we could not detect appreciable levels of p73 protein after immunoprecipitation of 2 mg of cell lysate followed by Western analysis (data not shown). Since p73 protein was not readily detectable in control or treated HEKs, we focused on p53 and ΔNp63α.

We also examined p53 downstream signaling events by determining the levels of p21 and MDM2 proteins, two key p53 targets (Figure 2). Levels of p21

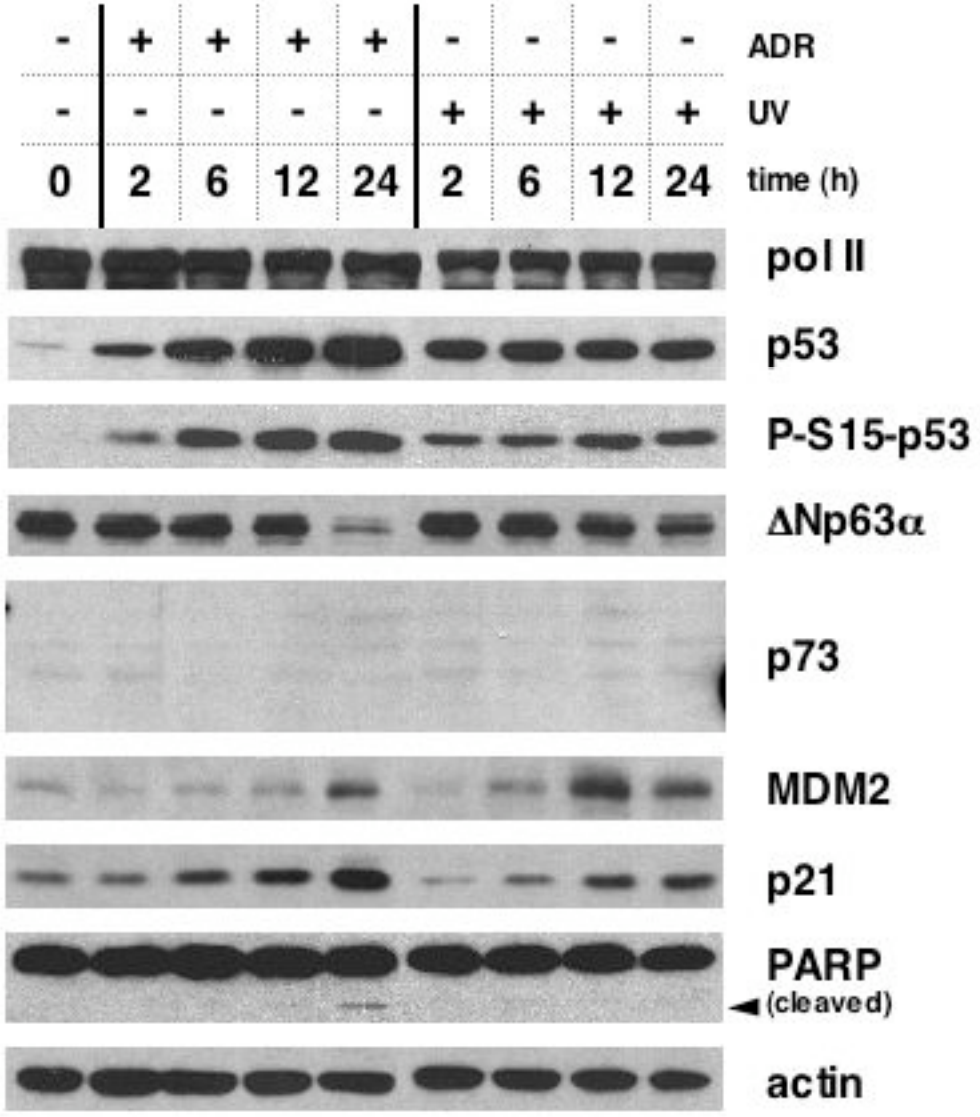

Figure 2. Western analysis of protein expression in HEKs. Untreated HEKs (0 h) were treated with 0.5 μM ADR or 50 J/m^2 UV and harvested at 2, 6, 12, and 24 h. Western blots were incubated with antibodies specific for pol II, p53, P-S15-p53, p63, p73, MDM2, p21, and PARP. Equal loading was confirmed by β-actin analysis. Results are representative of at least three independent experiments.

and MDM2 increased in response to ADR and UV. After treatment of the primary HEKs with either ADR or UV radiation, we did not observe any gross changes in viability of the cultures (data not shown). To assess HEK viability after ADR or UV radiation, we performed Western analyses for poly-ADP-ribose polymerase (PARP) and determined if there was any PARP cleavage present in the treated cells. There were very low levels of PARP cleavage at 24 h of ADR treatment. Thus, in these primary, untransformed cells, ADR and UV radiation stimulated the p53 signaling pathway as evidenced by elevation of p21 and MDM2 without concomitant induction of robust apoptosis. Further, the observed inverse regulation of p53 and ΔNp63α is relevant for interpretation of the following ChIP analyses.

To analyze p53 and ΔNp63α occupancy at select target gene consensus sites, rapidly growing HEKs were either untreated, or treated with ADR or UV as described above. After 2, 6, 12, or 24 h, the cells were formaldehyde-crosslinked, and protein lysates were harvested. To generate control templates for PCR, identical plates of HEKs were harvested without formaldehyde-crosslinking. Crosslinked and non-crosslinked lysates were sonicated to shear the chromatin and subjected to immunoprecipitation using p53 or p63 antibodies. To control for non-specific binding, crosslinked lysates were also immunoprecipitated using isotype-matched antibodies. Initially we examined p53 target genes involved in growth arrest (p21 and 14-3-3σ) and DNA repair (p48 and p53R2). Using the DNA fragments immunoprecipitated with p53- or p63-

specific antibodies, PCR was employed to amplify regions of the p21 (El-Deiry et al., 1993) and 14-3-3σ (Hermeking et al., 1997) promoters, p48 5'-UTR (Tan & Chu, 2002), and p53R2 intron 1 (Tanaka et al., 2000) that contain p53 consensus binding sites. As a negative control, a region of the GAPDH promoter was amplified from each set of immunoprecipitated DNA fragments and detectable binding of p53 or ΔNp63α was not observed (data not shown). Due to our inability to readily detect p73 protein, we did not perform p73 ChIP analyses.

The ChIP analyses of p21, 14-3-3σ, p48, and p53R2 showed that both p53 and ΔNp63α were bound constitutively, in the absence of genotoxic stress, to the consensus binding sites of all genes examined (Figures 3, 4, 5, and 6, "p53", "ΔNp63α"). After treatment with the genotoxic agents, an increase in p53 binding to all target gene consensus binding sites was observed. We observed a decrease in ΔNp63α binding accompanying the increase in p53 binding.

Post-translational modifications such as phosphorylation are known to play important roles in p53 protein stability, DNA binding, and chromatin access (Appella & Anderson, 2001; Bode & Dong, 2004). Phosphorylation of p53 at S15 is one modification that occurs after genotoxic stress and is able to stimulate p53 transactivation (Dumaz and Meek, 1999) primarily by conferring enhanced binding of p53 to CBP/p300 (Lambert et al., 1998). We examined if the p53 bound, either constitutively or after genotoxic stress, was phosphorylated at S15. We performed parallel ChIP experiments using a phospho-S15-p53 antibody to immunoprecipitate P-S15-p53 out of crosslinked and non-crosslinked

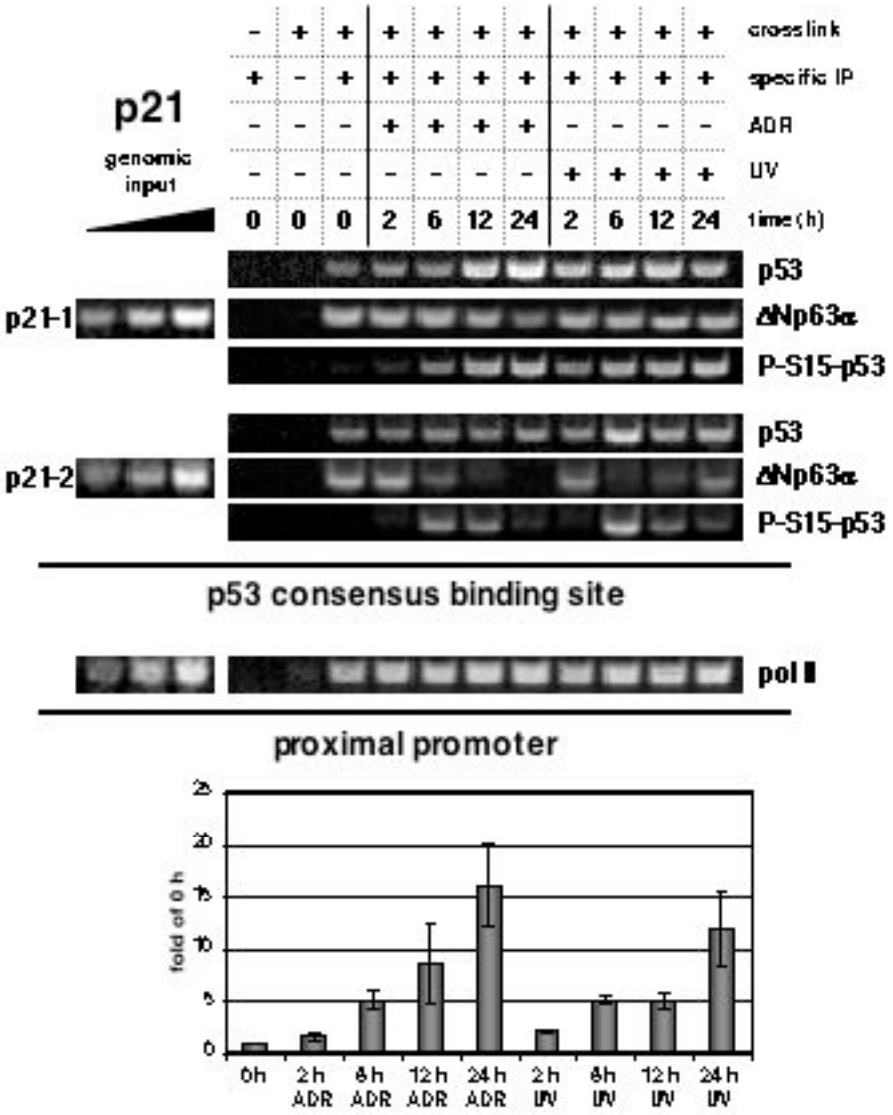

Figure 3. p21 ChIP and qRT-PCR analyses. p53, ΔNp63α, P-S15-p53, and pol II were immunoprecipitated from formaldehyde-crosslinked HEK lysates harvested 0, 2, 6, 12, and 24 h after ADR or UV treatment. Identical immunoprecipitations from non-crosslinked lysates and crosslinked lysates immunoprecipitated with an isotype-matched antibody ("-" specific IP) were used as controls. The regions of the p21 promoter (2 sites) containing previously identified p53 consensus binding sites and the proximal promoter region of the gene were analyzed. Increasing amounts of genomic input demonstrate that the PCR reactions were performed in the linear range (left-most panels). The ethidium bromide-stained gel most representative of the average of six independent experiments is shown. Bottom panel: real-time PCR was used to quantify the expression levels of p21 mRNA. Each sample was normalized to GAPDH expression, followed by fold-change determination as compared to gene expression at 0 h.

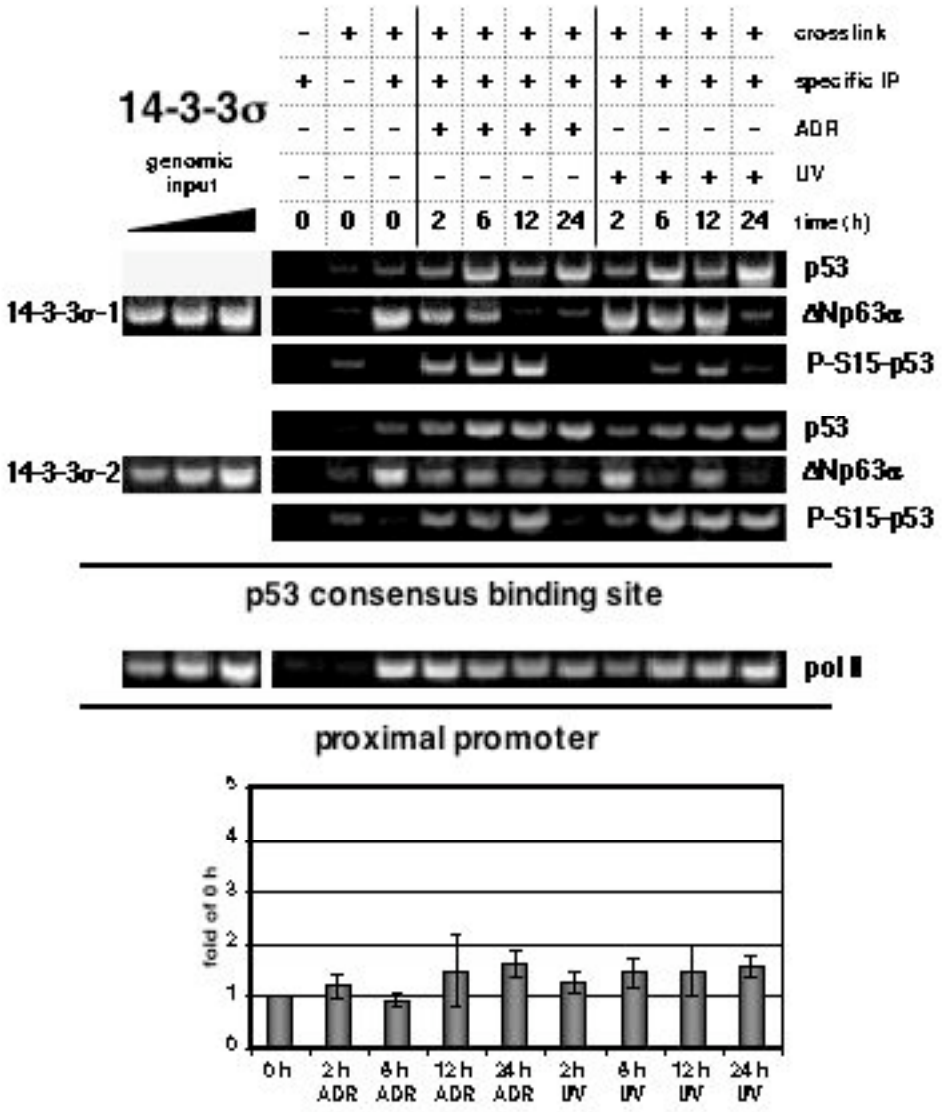

Figure 4. 14-3-3σ ChIP and qRT-PCR analyses. p53, ΔNp63α, P-S15-p53, and pol II were immunoprecipitated from formaldehyde-crosslinked HEK lysates harvested 0, 2, 6, 12, and 24 h after ADR or UV treatment. Identical immunoprecipitations from non-crosslinked lysates and crosslinked lysates immunoprecipitated with an isotype-matched antibody ("-" specific IP) were used as controls. The regions of the 14-3-3σ promoter (2 sites) containing previously identified p53 consensus binding sites and the proximal promoter region of the gene were analyzed. Increasing amounts of genomic input demonstrate that the PCR reactions were performed in the linear range (left-most panels). The ethidium bromide-stained gel most representative of the average of six independent experiments is shown. Bottom panel: real-time PCR was used to quantify the expression levels of 14-3-3σ mRNA. Each sample was normalized to GAPDH expression, followed by fold-change determination as compared to gene expression at 0 h.

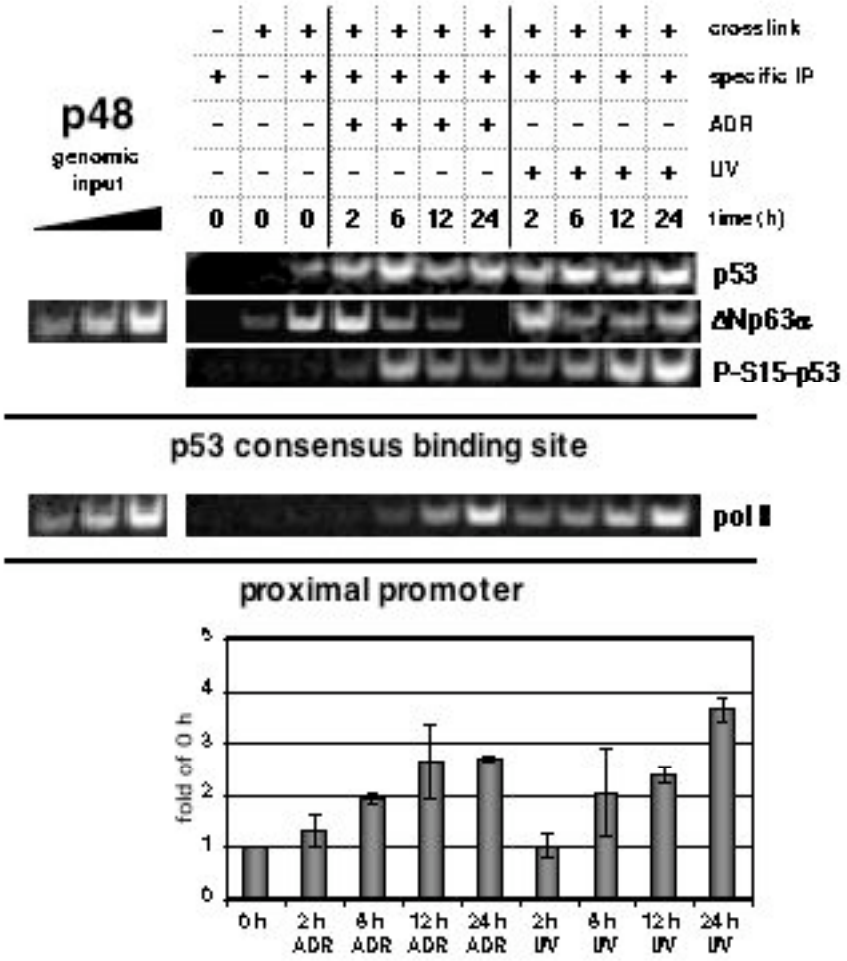

Figure 5. p48 ChIP and qRT-PCR analyses. p53, ΔNp63α, P-S15-p53, and pol II were immunoprecipitated from formaldehyde-crosslinked HEK lysates harvested 0, 2, 6, 12, and 24 h after ADR or UV treatment. Identical immunoprecipitations from non-crosslinked lysates and crosslinked lysates immunoprecipitated with an isotype-matched antibody ("-" specific IP) were used as controls. The region of the p48 5'UTR containing a previously identified p53 consensus binding site and the proximal promoter region of the gene were analyzed. Increasing amounts of genomic input demonstrate that the PCR reactions were performed in the linear range (left-most panels). The ethidium bromide-stained gel most representative of the average of six independent experiments is shown. Bottom panel: real-time PCR was used to quantify the expression levels of p48 mRNA. Each sample was normalized to GAPDH expression, followed by fold-change determination as compared to gene expression at 0 h.

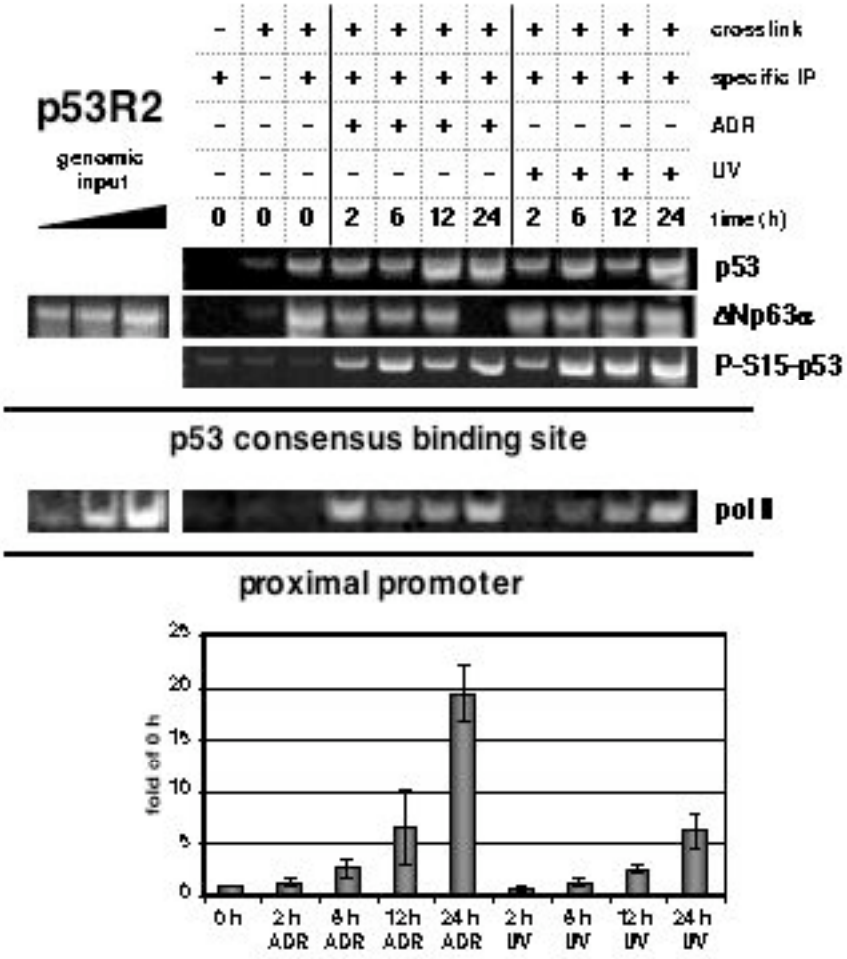

Figure 6. p53R2 ChIP and qRT-PCR analyses. p53, ΔNp63α, P-S15-p53, and pol II were immunoprecipitated from formaldehyde-crosslinked HEK lysates harvested 0, 2, 6, 12, and 24 h after ADR or UV treatment. Identical immunoprecipitations from non-crosslinked lysates and crosslinked lysates immunoprecipitated with an isotype-matched antibody ("-" specific IP) were used as controls. The region of the p53R2 intron 1 containing a previously identified p53 consensus binding site and the proximal promoter region of the gene were analyzed. Increasing amounts of genomic input demonstrate that the PCR reactions were performed in the linear range (left-most panels). The ethidium bromide-stained gel most representative of the average of six independent experiments is shown. Bottom panel: real-time PCR was used to quantify the expression levels of p53R2 mRNA. Each sample was normalized to GAPDH expression, followed by fold-change determination as compared to gene expression at 0 h.

lysates. Under conditions of rapid growth, P-S15-p53 was not detected at any of the target gene consensus binding sites (Figures 3 through 6, "P-S15-p53"). Two to six hours after treatment with ADR or UV, binding of P-S15-p53 was detectable at all binding sites.

In order to determine if there was a relationship between p53 and ΔNp63α binding and the occupancy of select promoters by a component of the basal transcriptional machinery, we examined the binding of pol II to target gene proximal promoters. We performed ChIP on the same templates as described above using a pol II antibody to immunoprecipitate pol II from formaldehyde-crosslinked and non-crosslinked lysates. The resulting DNA fragments were used as template for amplification of the proximal promoter region of each p53 target gene. For these studies, the proximal promoter region was defined as the region 50-100 bp up and downstream of the transcriptional start site. In the absence of genotoxic stress, pol II was constitutively bound to the proximal promoters of p21 and 14-3-3σ, though no constitutive binding of pol II was detected at the proximal promoters of p48 or p53R2 (Figures 3 through 6, "pol II"). After ADR treatment, binding of pol II was elevated, remained relatively constant at the proximal promoters of p21, 14-3-3σ, and p53R2 and increased over time at the p48 proximal promoter. Similarly, after UV treatment, binding of pol II was elevated and remained constant at the p21 and 14-3-3σ proximal promoters; however, binding increased over time at both the p48 and p53R2 proximal promoters. The lack of constitutive binding of pol II at the proximal

promoters of p48 and p53R2 suggests that there is not a direct relationship between constitutive binding of p53 or ΔNp63α and constitutive binding of pol II. Rather, our data suggest there is a relationship between the location of the p53 consensus binding site in the target gene and constitutive binding of pol II. p21 and 14-3-3σ contain consensus binding sites 5' of the transcriptional start site (El-Deiry et al., 1993; Hermeking et al., 1997). Correspondingly, these genes also have robust constitutive pol II binding at their proximal promoters (Figures 3 and 4, "pol II"). In contrast, p48 and p53R2 have consensus binding sites downstream of the transcriptional start site (Tan & Chu, 2002; Tanaka et al., 2000) and we were not able to detect constitutive binding of pol II to their proximal promoters (Figures 5 and 6, "pol II").

To determine the relationship between p53, ΔNp63α, P-S15-p53, and pol II binding to gene regulatory regions and gene expression, we isolated RNA from HEKs in parallel with the ChIP experiments and examined expression of p21, 14-3-3σ, p48, and p53R2 using quantitative real-time PCR. We observed a significant increase in p21 (ADR: 16-fold increase, UV: 12-fold increase), p48 (ADR: 2.7-fold increase, UV: 3.6-fold increase), and p53R2 (ADR: 19-fold increase, UV: 6.1-fold increase) mRNA over the time courses of both ADR and UV treatment (Figures 3, 5, and 6, bottom panels), whereas 14-3-3σ mRNA levels stayed relatively constant (ADR: 1.6-fold increase, UV: 1.6-fold increase) (Figure 4, bottom panel). The general findings that emerged from the ChIP binding and gene expression data were that in response to genotoxic stress,

binding of p53 increased at the consensus binding sites of target genes under study, as did P-S15-p53. In fact the latter appeared to closely correlate with significant increases in transcript levels. Concomitantly, ΔNp63α occupancy at the majority of target gene consensus binding sites decreased after treatment with ADR and UV. Corresponding to these changes in consensus binding site occupancy there was a significant upregulation of mRNA levels of the target gene over the time course of ADR and UV treatment, as observed for p21, p48, and p53R2. The exception was that similar ChIP binding trends were observed at the 14-3-3σ consensus binding sites as at the other target gene consensus binding sites, yet little change in 14-3-3σ mRNA expression occurred during the time course (ADR: 1.6-fold increase, UV: 1.6-fold increase). Of note, qRT-PCR and Western analysis revealed high constitutive levels of 14-3-3σ mRNA and protein, respectively (data not shown). Thus, in primary HEKs, 14-3-3σ is regulated by additional mechanisms that diminish any significant p53-mediated increase after genotoxic stress.

Differential Constitutive Binding of p53 To Target Genes Involved in Apoptosis

To investigate how p53 and its family members regulate target genes involved in apoptosis we amplified regions of the Noxa promoter (Oda et al., 2000) and Fas/APO1 intron 1 (Muller et al., 1998) that contain functional p53 consensus binding sites. Constitutive binding of p53 was observed at the Noxa consensus binding site, but not at the Fas/APO1 consensus binding site (Figures

7 and 8, “p53”). Increased binding of p53 to the binding sites in both genes was observed following ADR and UV treatment. In contrast, ΔNp63α bound to both the Noxa and Fas/APO1 consensus binding sites constitutively; and its levels decreased over the time course of ADR treatment and remained relatively constant following UV treatment (Figures 7 and 8, “ΔNp63α”). Phospho-S15-p53 binding increased at both the Noxa consensus binding site and the Fas/APO1 consensus binding site after treatment with ADR and UV (Figures 7 and 8, “P-S15-p53”). A low level of pol II was constitutively bound to the Noxa proximal promoter, though pol II was not detected at the Fas/APO1 proximal promoter in the absence of genotoxic stress (Figures 7 and 8, “pol II”). Binding of pol II increased at the Noxa and Fas/APO1 proximal promoters after ADR and UV treatment. Again, these data provide further evidence for a relationship between the location of the p53 consensus binding site in the target gene and constitutive binding of pol II as the binding sites of Noxa and Fas/APO1 are upstream and downstream of the transcriptional start site, respectively.

When we examined mRNA levels of Noxa by quantitative real-time PCR, there was a significant increase 2 h after ADR and UV treatment (Figure 7, bottom panel) and the transcript remained elevated for the duration of the time course. A gradual increase in Fas/APO1 mRNA was observed in response to both forms of genotoxic stress (Figure 8, bottom panel). The more moderate increase observed could be due to the absence of constitutively bound pol II. Similar to our observations with the cell cycle arrest and DNA repair target

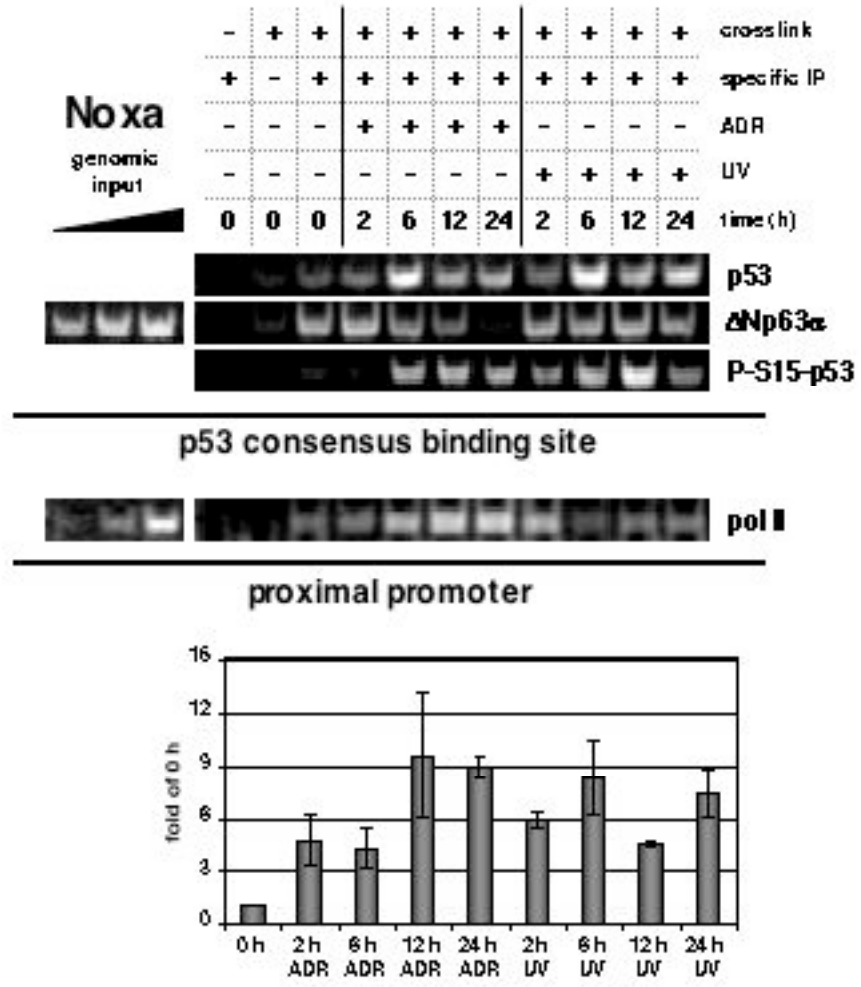

Figure 7. Noxa ChIP and qRT-PCR analyses. p53, ΔNp63α, P-S15-p53, and pol II were immunoprecipitated from formaldehyde-crosslinked HEK lysates harvested 0, 2, 6, 12, and 24 h after ADR or UV treatment. Identical immunoprecipitations from non-crosslinked lysates and crosslinked lysates immunoprecipitated with an isotype-matched antibody ("-" specific IP) were used as controls. The region of the Noxa promoter containing a previously identified p53 consensus binding site and the proximal promoter region of the gene were analyzed. Increasing amounts of genomic input demonstrate that the PCR reactions were performed in the linear range (left-most panels). The ethidium bromide-stained gel most representative of the average of six independent experiments is shown. Bottom panel: real-time PCR was used to quantify the expression levels of Noxa mRNA. Each sample was normalized to GAPDH expression, followed by fold-change determination as compared to gene expression at 0 h.

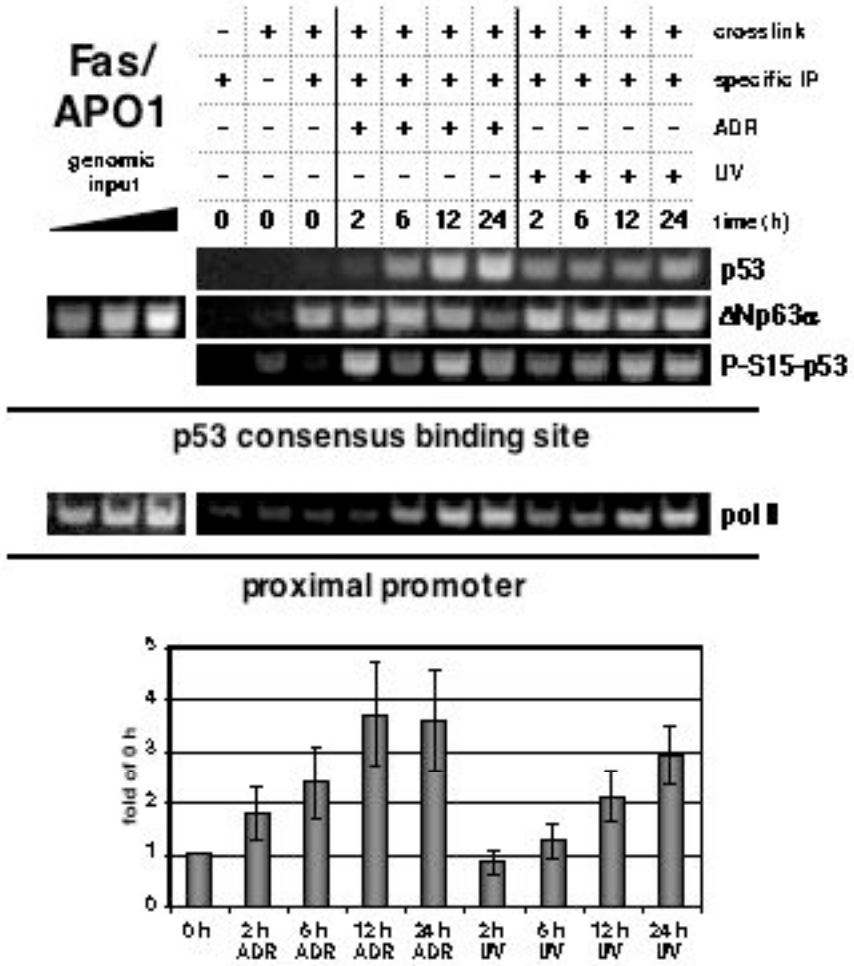

Figure 8. Fas/APO1 ChIP and qRT-PCR analyses. p53, ΔNp63α, P-S15-p53, and pol II were immunoprecipitated from formaldehyde-crosslinked HEK lysates harvested 0, 2, 6, 12, and 24 h after ADR or UV treatment. Identical immunoprecipitations from non-crosslinked lysates and crosslinked lysates immunoprecipitated with an isotype-matched antibody ("-" specific IP) were used as controls. The region of the Fas/APO1 intron 1 containing a previously identified p53 consensus binding site and the proximal promoter region of the gene were analyzed. Increasing amounts of genomic input demonstrate that the PCR reactions were performed in the linear range (left-most panels). The ethidium bromide-stained gel most representative of the average of six independent experiments is shown. Bottom panel: real-time PCR was used to quantify the expression levels of Fas/APO1 mRNA. Each sample was normalized to GAPDH expression, followed by fold-change determination as compared to gene expression at 0 h.

genes, p53 and P-S15-p53 binding increased at both target gene consensus binding sites after ADR and UV treatment. Simultaneously, ΔNp63α occupancy at the consensus binding sites generally decreased following exposure to ADR and UV. In addition, there were other significant differences in transcription factor binding at the two apoptotic target gene regulatory regions that relate to changes in mRNA expression. For example, only a slight decrease in ΔNp63α occupancy at the Fas/APO1 consensus binding site was observed following ADR treatment and pol II constitutive binding was absent at the Fas/APO1 proximal promoter. We did not observe an increase in pol II binding until 2-6 h after treatment with ADR, and Fas/APO1 mRNA levels increased gradually over the time course (3.6-fold increase at 24 h). In contrast, a more striking decrease in ΔNp63α binding to the Noxa consensus binding site was observed after ADR treatment. Constitutive binding of pol II was present at the Noxa proximal promoter, and a sharp increase in Noxa mRNA occurred by 2 h (4.7-fold increase at 2 h) in response to both ADR. As observed for the other set of genes above, increases in P-S15-p53 binding to both the Noxa and Fas/APO1 consensus binding sites correlated with the increases in mRNA for both target genes.

Inverse Regulation of Select Target Genes by p53 and ΔNp63α

In our ChIP analyses, we observed the constitutive binding of ΔNp63α to p53 target genes involved in cell cycle arrest, DNA repair, and apoptosis and a decrease in ΔNp63α occupancy after genotoxic stress at a majority of the sites

examined. These data, along with previous studies showing that ΔNp63α has the ability to act as a transcriptional repressor (Bakkers et al., 2002; Westfall et al., 2003; Barbieri et al., 2005; Barbieri et al., 2006) led us to investigate if p53 and ΔNp63α could coordinately regulate a broader range of gene targets. Accordingly, we infected HEKs with adenoviruses expressing p53 or ΔNp63α. We isolated mRNA 30 h post-infection as this was a time point at which we observed a very robust expression of the majority of known targets. Of note, very little, if any, detectable apoptosis occurred in the HEKs infected with p53 or ΔNp63α-expressing adenoviruses (data not shown). Gene expression profiles were obtained using the Affymetrix GeneChip and the expression levels of a panel of p53 target genes are shown in Figure 9A. Known p53 target genes were upregulated after ectopic expression of p53 (Figure 9A, left side). In contrast, we observed a striking downregulation of a majority of these genes after ectopic expression of ΔNp63α (Figure 9A, right side). Of note, the target genes examined have known roles in a number of p53-mediated signaling pathways, including cell cycle arrest, DNA repair, and apoptosis. The expression levels of a subset of known p53 targets were quantitatively assayed using real-time PCR (Figure 9B). Although fold-changes varied, the inverse regulation of these genes by p53 and ΔNp63α was readily apparent.

Of the genes shown in Figure 9B, the one showing the greatest degree of upregulation by p53 and downregulation by ΔNp63α was MDM2, a negative regulator of p53. To gain some mechanistic insight to this regulation, we

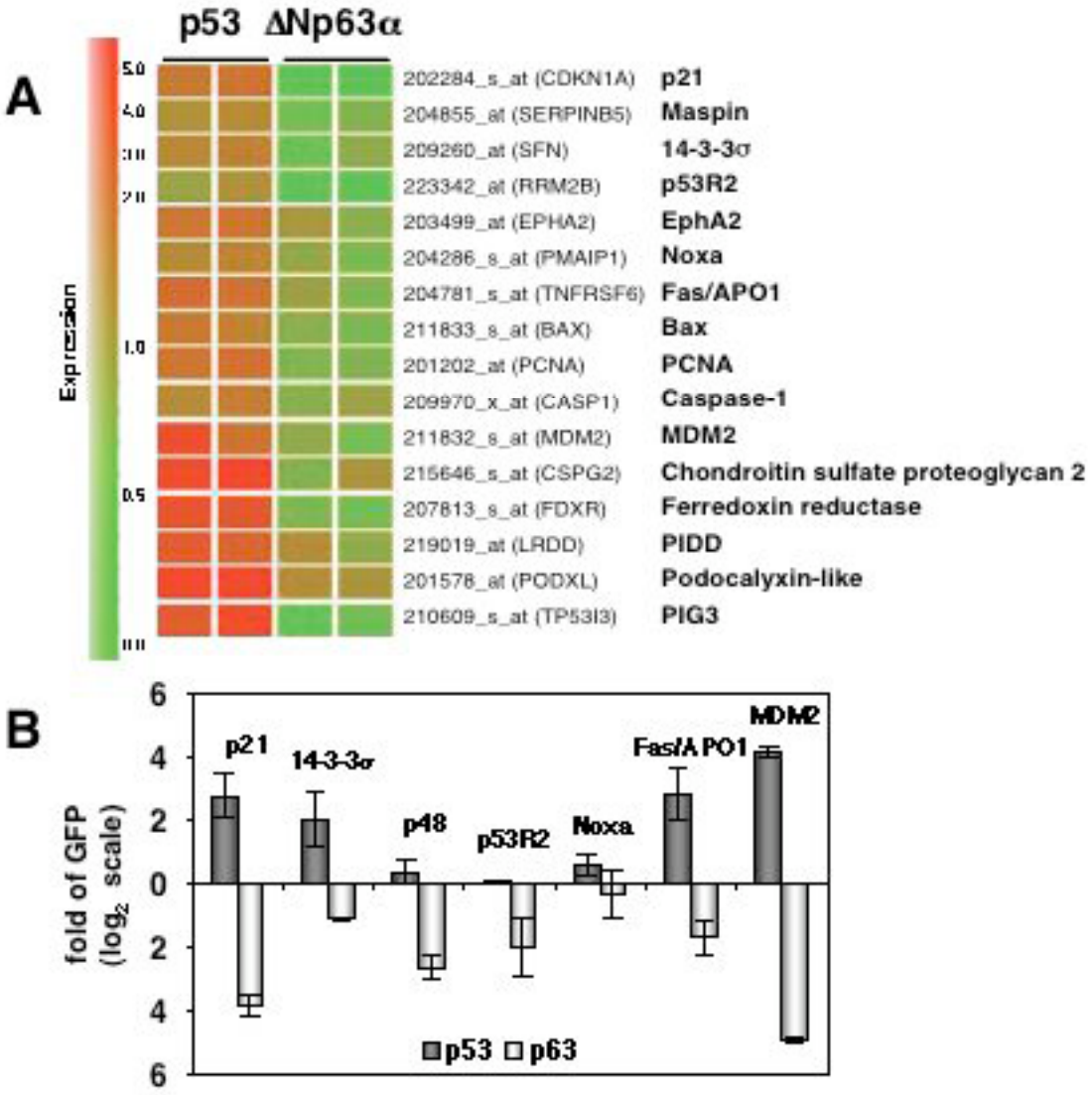

Figure 9. Inverse regulation of target genes by p53 and ΔNp63α. A. Microarray analysis was performed to determine gene expression profiles of HEKs infected with p53- or ΔNp63α-expressing adenoviruses, compared to HEKs infected with a GFP-expressing adenovirus. Expression levels of a panel of known p53 target genes are shown for the duplicate experiments. Increased or decreased expression of a gene is indicated by the colors red and green, respectively. Immediately to the right of the colored boxes are the Affymetrix probe-set ID numbers and the gene abbreviation in parentheses. The definition of each gene abbreviation is shown to the right. **B.** Real-time PCR was used to quantify the expression levels of select p53 target genes. Each sample was normalized to GAPDH expression, followed by fold-change determination as compared to gene expression in HEKs infected with the GFP-expressing adenovirus. Fold change is expressed on a $\log_2$ scale. The error bars are indicative of the standard error of the mean.

repeated the ChIP experiments described above to examine p53, ΔNp63α, and P-S15-p53 binding to the known p53 consensus binding sites in the MDM2 gene (Zauberman et al., 1995). Only ΔNp63α bound constitutively to the MDM2 consensus binding sites (Figure 10). Following ADR and UV treatment, increased binding of p53 and P-S15-p53 was observed, as well as a corresponding decrease in ΔNp63α binding. The ChIP binding patterns observed for p53, ΔNp63α, and P-S15-p53 as well as the 14- and 15-fold elevation in MDM2 mRNA expression in response to ADR and UV respectively, exhibited the same trend as that seen with the cell cycle arrest, DNA repair, and apoptotic target genes. Pol II was not constitutively bound to the MDM2 proximal promoter in the absence of genotoxic stress, though binding increased after both ADR and UV treatment (Figure 10). The p53 consensus binding sites in the MDM2 gene are both located in intron 1 (Zauberman et al., 1995), and thus similar to our observations with p48, p53R2, and Fas/APO1, we found that the intronic location of a consensus binding site correlates with absence of constitutive pol II binding to the proximal promoter region.

Discussion

The goal of this study was to provide further mechanistic insight to p53 family member regulation of target genes in response to genotoxic stress. We used primary HEKs as a model system to avoid genetic abnormalities present in transformed cell lines and employed ChIP to examine occupancy of select target

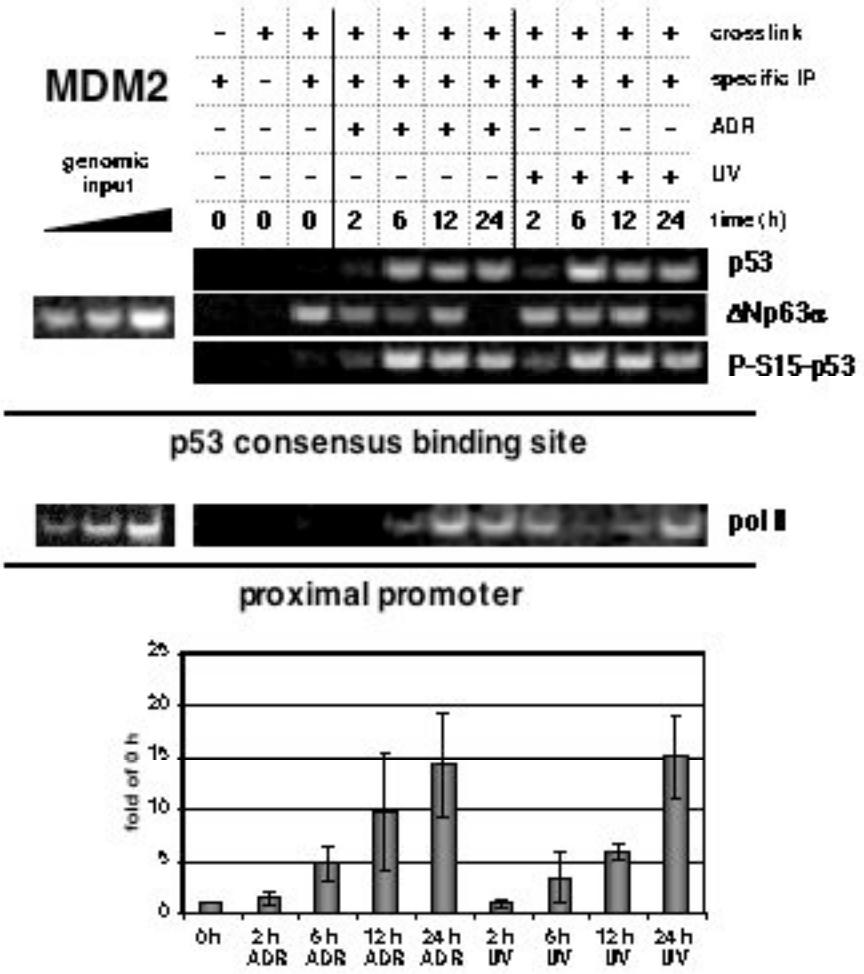

Figure 10. Noxa ChIP and qRT-PCR analyses. p53, ΔNp63α, P-S15-p53, and pol II were immunoprecipitated from formaldehyde-crosslinked HEK lysates harvested 0, 2, 6, 12, and 24 h after ADR or UV treatment. Identical immunoprecipitations from non-crosslinked lysates and crosslinked lysates immunoprecipitated with an isotype-matched antibody ("-" specific IP) were used as controls. The region of the MDM2 intron 1 containing previously identified p53 consensus binding sites and the proximal promoter region of the gene were analyzed. Increasing amounts of genomic input demonstrate that the PCR reactions were performed in the linear range (left-most panels). The ethidium bromide-stained gel most representative of the average of six independent experiments is shown. Bottom panel: real-time PCR was used to quantify the expression levels of MDM2 mRNA. Each sample was normalized to GAPDH expression, followed by fold-change determination as compared to gene expression at 0 h.

gene consensus binding sites by p53 family members. Since p73 protein expression was not readily detectable in control or treated HEKs (Figure 2), we focused on p53 and ΔNp63α.

We observed constitutive binding of ΔNp63α to consensus binding sites in target genes involved in cell cycle arrest, DNA repair, and apoptosis. After treatment with ADR or UV, a decrease in ΔNp63α occupancy was observed at the majority of binding sites examined. p53 was constitutively bound at all target genes consensus binding sites, with the exception of the Fas/APO1 consensus binding site. Binding of p53 increased at all consensus binding sites after treatment with ADR and UV. Given the transcriptional repressor activity of ΔNp63α (Westfall et al., 2003; Barbieri et al., 2005; Barbieri et al., 2006), constitutive binding of this protein to p53 target gene consensus binding sites would allow for repression of genes involved in cell cycle arrest and apoptosis and thus, allow cell viability and proliferation. Coordinate binding of p53 would allow for rapid induction of growth arrest and apoptosis with the onset of regulatory events such as phosphorylation at S15 and decrement of ΔNp63α. The loading of a promoter with a repressor and an activator allows for a rapid switch type control when necessary or a gradual change. Likely the difference depends on the type of stress and the resulting post-translational modifications of p53 and ΔNp63α. ΔNp63α can modulate cell proliferation during development through upregulation of the cyclin-dependent kinase inhibitor $p57^{Kip2}$ (Beretta et al., 2005). It is believed that ΔNp63α exhibits this function of controlling cell

proliferation during development in order to prevent aberrant expression of $p57^{Kip2}$, which can lead to developmental defects (Beretta et al., 2005).

In our ChIP analyses, we observed increased levels of P-S15-p53 at all consensus binding sites 2 to 6 h following ADR or UV treatment. When p53 is phosphorylated on S15, p53 transactivation is stimulated (Dumaz and Meek, 1999). Phosphorylation at S15 confers enhanced binding of p53 to CBP/p300 (Lambert et al., 1998). Our data combined with previous observations (Espinosa et al., 2003) suggests that this post-translational modification is a key regulatory step in the upregulation of target gene expressions we observed following genotoxic stress. Colocalization of P-S15-p53 and p21 mRNA signal detected by RNA-immuno fluorescence in situ hybridization was observed very early in the response to damage indicating that P-S15-p53 targets p21 immediately in response to stress, such as DNA damage (Espinosa et al., 2003).

We also examined the binding of pol II to the proximal promoters of select p53 target genes to determine the relationship between p53 or ΔNp63α and pol II binding to regulatory regions of target genes. We observed constitutive pol II binding to the p21, 14-3-3σ, and Noxa proximal promoters. We conclude that constitutive binding of p53 or ΔNp63α to target gene consensus binding sites does not dictate the binding of pol II to target gene proximal promoters as previously reported (Espinosa et al., 2003). Rather, our results suggest that there is a relationship between the location of the p53 consensus binding site in the target gene and constitutive binding of pol II. The target genes p21 (El-Deiry

et al., 1993), 14-3-3σ (Hermeking et al., 1997), and Noxa (Oda et al., 2000) all contain p53 consensus binding sites in the promoter region upstream of the transcriptional start site. Robust binding of pol II was observed at these proximal promoter regions before and after treatment with ADR and UV. The p53 consensus binding site for p48 is downstream of the transcriptional start site, in the 5'-UTR region (Tan & Chu, 2002), and constitutive binding of pol II to the p48 proximal promoter was not discernable. p53R2 (Muller et al., 1998), Fas/APO1 (Tanaka et al., 2000), and MDM2 (Zauberman et al., 1995) all contain intronic p53 consensus binding sites; again, constitutive binding of pol II was not detectable at the proximal promoters of these genes (Please see Table 5 for a summary of these results). Recent studies show that the positioning of nucleosomes in eukaryotic genomes is dependent on the distribution of specific sequences of DNA to which histones have higher affinity (Segal et al., 2006; Ioshikhes, et al., 2006). Perhaps the location of the p53 consensus binding site can dictate nucleosome positioning and render chromatin more or less accessible to large complexes of basal transcription factors, including pol II.

We also examined the corresponding mRNA expression of the genes included in our ChIP analyses. We saw consistent trends in the relationship between promoter occupancy and regulation of transcript levels. In response to ADR and UV, increased binding of p53 and P-S15-p53 was observed at consensus binding sites of genes involved in cell cycle arrest (p21, 14-3-3σ), DNA repair (p48, p53R2), apoptosis (Noxa, Fas/APO1), and p53 negative

Table 5. Location of Consensus Binding Site and Constitutive Pol II Binding at Proximal Promoter

gene	binding site	match	location	pol II
p21	GAACATGTCC--0bp--cAACATGTTg	18/20	promoter	yes
	AGACTgGgCa--0bp--tGtCTgGgCa	12/20		
14-3-3σ	AGGCATGTgC--0bp--cAcCATGCCC	17/20	promoter	yes
	GtAGCAttAGCCC--0bp--AGACATGTCC	17/20		
p48	GAACAAGCCC--1bp--GGGCATGTTT	20/20	5' UTR	no
p53R2	tGACATGCCC--0bp--AGGCATGTCT	19/20	intron 1	no
Noxa	GGGCTTGTTT--0bp--AccCAAGTCT	18/20	promoter	yes
Fas/APO1	GGACAAGCCC--0bp--tGACAAGCCa	18/20	intron 1	no
MDM2	GGtCAAGTTC--0bp--AGACAcGTTC	18/20	intron 1	no
	AGttAAGTCC--0bp--tGACTTGTCT	17/20		

pol II: constitutive pol II binding

match: number of nucleotides in the gene consensus site matching the 20 nucleotides in the canonical consensus site

regulation (MDM2). Conversely, binding of ΔNp63α to consensus binding sites of these genes decreased. As these changes in promoter occupancy were occurring, the transcript levels of each of these genes increased over the time course of ADR and UV treatment, with the exception of 14-3-3σ, whose mRNA levels remained constant in the absence and presence of ADR and UV. However, there are constitutively high levels of 14-3-3σ mRNA and protein in HEKs, indicating regulation of 14-3-3σ by factors in addition to p53. The absence of a relationship between the kinetics of p53 binding to target gene regulatory regions and mRNA expression observed with 14-3-3σ is consistent with previous findings (Szak et al., 2001).

Analysis of gene expression in HEKs ectopically-expressing p53 or ΔNp63α revealed an inverse regulation of a panel of known p53 target genes that have roles in cell cycle arrest, DNA repair, and apoptosis. One target gene inversely regulated by p53 and ΔNp63α in the microarray analysis was MDM2, a negative regulator of p53. We performed ChIP to determine the kinetics of binding of p53 and ΔNp63α to the p53 consensus binding sites in MDM2 intron 1. In HEKs, we found that ΔNp63α was bound to the MDM2 consensus binding sites both constitutively in the absence of genotoxic stress and following treatment with ADR and UV (though binding decreased after treatment). In contrast, using E1A-expressing mouse embryonic fibroblasts (MEFs), Flores *et al.* (2002) did not detect constitutively bound p63 at the MDM2 consensus binding sites, they only observed binding of p63 after 12 h of ADR treatment.

This difference in p63 binding can be explained by the use of different model systems in terms of species (mouse vs. human), tissue type (epithelial vs. mesenchymal), and transformation status (primary, non-transformed vs. E1A-expressing). Repression of MDM2 by ΔNp63α adds another level of regulation to the balance of MDM2 and p53 levels in unstressed cells.

Important to the understanding of p53-, p63-, and p73-mediated signaling is deciphering the mechanisms and implications of coordinate regulation of target genes by these transcription factors. Target genes are emerging that are commonly regulated by multiple p53 family members, or exhibit regulation by only a single p53 family member (Harms et al., 2004; Ihrie et al., 2005; Sasaki et al., 2005). The ChIP and microarray analyses reported here support an antagonistic role for ΔNp63α in the regulation of select p53 target genes in HEKs, representing a physiologically relevant model system in which to study p53 and ΔNp63α-mediated signaling. Though its role as a p53-antagonist is supported by results of the current study, as well as studies in primary mouse epidermal keratinocytes in which ΔNp63α is acting as a dominant negative regulator of p53-mediated apoptosis in response to UV-B treatment (Liefer et al., 2000), it is clearly not the only role of p63. For example, p63 has been demonstrated to have an essential role in the development of epithelial structures and maintenance of the epidermis both in humans and zebrafish (Koster and Roop, 2004). Interestingly, in addition to $p57^{Kip2}$ (as mentioned earlier), ΔNp63α can transactivate select p53 target genes (Dohn et al., 2001). The p53 family

members can likely interact with a range of diverse components of the basal transcription machinery and target gene expression is dictated by situation-specific stimuli and availability of specific cofactors. ΔNp63α may serve as a cofactor for p53, and may be important for localization of protein complexes involved in target gene discrimination. Recently it was shown that p53 required TAp63α in order to initiate apoptosis in neurons, exemplifying a setting in which p53 acts as a p63 cofactor (Jacobs et al., 2005). Identification of p63-associated proteins will further clarify the role of p63 in the regulation of target genes. As additional target genes are discovered, it will be important to explore the presence/absence of p53 family members at consensus binding sites under physiologically relevant conditions.

CHAPTER V

CONCLUSIONS

The p53 tumor suppressor protein is mutated in over 50% of all human cancers (Vogelstein, 1990). The majority of mutations in p53 are located in the central core of the protein containing the DNA binding domain (Pavletich et al., 1993). p53 is a sequence-specific DNA binding protein (El-Deiry et al., 1992) and directly activates transcription upon binding to DNA (Farmer et al., 1992). Most importantly, p53 requires the sequence-specific transcriptional activity to function as a tumor suppressor (Pietenpol et al., 1994). Therefore, the proper regulation of target genes involved in such processes as cell cycle arrest, DNA repair, and apoptosis by p53 is of great importance in preventing tumorigenesis. However, questions still remain regarding how p53 coordinately regulates all of its target genes in response to genotoxic stress.

The purpose of the research presented in this dissertation was to provide a greater understanding of the mechanisms of p53 select target gene activation in response to genotoxic stress, specifically focusing on the role of the p53 family member, ΔNp63α. The first goal of my project was to identify novel p53 candidate target genes in primary HEKs. We used ChIP/yeast screen methodology followed by comparison to gene expression profiles generated by microarray analyses of HEKs exogenously expressing p53 or ΔNp63α to

accomplish this goal. The use of primary cells is of great value in that the majority of screens to identify p53 target genes to date have been performed in cancer cell lines. Our library screening and microarray experiments resulted in the identification of many potential p53 candidate target genes. The validation of these candidates will yield knowledge of valuable target genes that both improve our current understanding of p53 signaling pathways, but potentially identify novel pathways in which p53 is involved. Our microarray analyses also identified a subset of target genes that are inversely regulated by p53 and ΔNp63α. Most of these have not been previously identified as p53 or ΔNp63α target genes. This group of genes is of interest to us in that the observation of inverse regulation aligns with our data discussed in Chapter IV.

Rationale for screening for novel p53 target genes is that the more p53 targets identified, the more complete our understanding regarding mechanisms of p53-regulated tumor suppressive pathways. For instance, analysis on a greater number of target gene consensus binding sites may identify sequence-specific patterns that dictate activation by p53. Also, perhaps trends will emerge regarding cell-type and stress-type specific responses mediated by p53. p53 signaling pathways are complex, especially as new p53-mediated functions are elucidated. The continued identification of p53-regulated transcriptional targets provides information with which to understand these pathways.

After mutation of the p53 gene, most often in the DNA binding domain, the p53 protein can no longer engage its multitude of target genes to counteract

genotoxic stress and prevent transformation. Optimistically, each target gene of p53 represents a potential therapeutic target for cancer treatment or prevention due to its participation in p53 tumor suppression. For example, introducing a specific p53 target gene or a target gene peptide-mimetic to cancer cells could potentially induce the outcome that p53 activation would otherwise achieve (i.e. apoptosis) and could be a useful therapeutic strategy. Better technologies continue to be developed to specifically target cancer cells, which is essential for such a strategy to be effective.

The second goal of my project was to identify factors involved in the selective regulation of target genes by p53. Again, using primary HEKs as a model system we determined the constitutive binding status of p53, ΔNp63α, P-S15-p53 and pol II to regulatory regions in p53 target genes involved in cell cycle arrest, DNA repair, and apoptosis. We then ascertained the changes in binding that occurred after treatment with ADR and UV.

Prior to our ChIP experiments, we analyzed the response of HEKs to ADR and UV. We observed an increase in p53 and P-S15-p53, as well as an increase in p53 target genes p21 and MDM2 after both ADR and UV treatment. We observed a decrease in ΔNp63α protein levels after both types of DNA damage, consistent with previous studies in our lab (Westfall et al., 2005). Upon treatment with UV and paclitaxel, ΔNp63α protein levels decrease, accompanied by changes in the phosphorylation status of ΔNp63α and an increase in ubiquitinated ΔNp63α (Westfall et al., 2005). Further experimentation will be

required to determine if the post-translational modifications of p53 and ΔNp63α are coordinately regulated.

In our ChIP studies, we were interested in determining what transcription factors were constitutively bound to regulatory regions in p53 target genes involved in cell cycle arrest, DNA repair, apoptosis, and p53 negative regulation. Though the levels of binding varied, we observed constitutive binding of p53 at all target gene consensus binding sites except the Fas/APO1 consensus binding site. This lack of binding at the Fas/APO1 consensus binding site aligns with previous studies determining that p53 has higher affinity for binding sites in target genes involved in cell cycle arrest and DNA repair compared to binding sites in genes involved in apoptosis (Szak et al., 2001; Kaeser and Iggo, 2002; Weinberg et al., 2005). However, we observe p53 constitutively bound at the Noxa consensus binding site, which does not support of this theory. Many more target gene consensus binding sites need to be examined to definitely prove or disprove this theory.

The “match” to the ideal consensus binding site is also thought to influence the affinity of p53 to a target gene consensus binding site. In our ChIP studies, the match of the consensus binding site to the ideal consensus sequence does not influence affinity as measured by constitutive binding. We observe ample constitutive binding of p53 to both consensus binding site in the p21 promoter (consensus binding site 1 is an 18 out of 20 match; consensus binding site 2 is a 12 out of 20 match). In addition, we do not detect p53

constitutive binding to the Fas/APO1 consensus binding site, which matches the defined consensus at 18 out of 20 basepairs. Again, more consensus binding sites need to be analyzed to know definitely if this is a factor that can dictate p53 selective regulation of its target genes.

Our results show that the binding of p53 increases at all target gene consensus binding sites after treatment with ADR and UV. The level of P-S15-p53 also increases at consensus binding sites in response to ADR and UV treatment. The phosphorylation of p53 S15 contributes to the disruption of the interaction between p53 and MDM2 (Shieh et al., 1997) and also increases p53 binding to p300/CBP (Lambert et al., 1998). Therefore, phosphorylation at p53 S15 results in increased stability and enhanced transcriptional activity of p53. In accordance with these properties, we observe accumulation of p53 (and P-S15-p53) at the protein level after treatment with ADR and UV. p53 (and P-S15-p53) binding of target gene consensus binding sites increases after damage, and we observe upregulation of the majority of target gene mRNA levels.

We were also interested in the binding of ΔNp63α to p53 target gene consensus binding sites before and after genotoxic stress. We detected constitutive binding of ΔNp63α to p53 consensus binding sites of all the genes we examined in the absence of stress. Following treatment with ADR and UV, we observed inverse trends in binding compared to p53: ΔNp63α binding decreased at target gene promoters after genotoxic stress. The binding corresponds to the decrease in ΔNp63α protein levels after damage. Overall, the

following pattern emerges from our data. After treatment with ADR and UV, p53 (and P-S15-p53) binding increases at target gene consensus binding sites, while ΔNp63α binding decreases. Correspondingly, an upregulation of target gene mRNA occurs.

To extend these observations, we examined the transcript levels of a panel of known p53 target genes in our microarrays in which p53 and ΔNp63α were exogenously expressed. Again, a clear trend emerged. Upon expression of p53, mRNA levels of known p53 target genes increase. However, when ΔNp63α is exogenously expressed, mRNA levels of the same target genes decrease. Our ChIP and microarray data provide further support of the role of ΔNp63α as a transcriptional repressor.

Our results also add to the knowledge of the role of ΔNp63α in p53-mediated signaling. ΔNp63α may act as a constitutive repressor at p53 target genes in order to prevent aberrant expression. p53 may be constitutively bound simultaneously, allowing for an expedient response to genotoxic stress. ΔNp63α is overexpressed in several squamous cell carcinomas (Hibi et al., 2000; Sniezek et al., 2004), supporting the view of ΔNp63α as an oncogene. Our data could potentially support such a role for ΔNp63α in that overexpression of this protein would lead to suppression of p53 target genes and likely enable the transformation process (Barbieri et al., 2006).

Examination of the binding of a member of the basal transcription machinery, pol II, to target gene proximal promoters yielded interesting results.

We did not observe differences in constitutive binding of pol II to target gene promoters classified on their involvement in cell cycle arrest, DNA repair, or apoptosis. Rather, the location of the p53 consensus binding site dictated the constitutive binding of pol II. We observed constitutive pol II occupancy of the proximal promoters of p21, 14-3-3σ, and Noxa. These three genes all contain binding sites in their promoters, 5' of the transcription start site. Conversely, constitutive binding of pol II was not observed p48, p53R2, Fas/APO1, or MDM2 proximal promoters. The p53 binding site is located downstream of the transcription start site (5'UTR or intron) for this group of genes. Analysis of the chromatin structure and histone acetylation status surrounding each of these genes may provide insight to the general conformation (opened/closed) in that particular region of the chromosome. Further experimentation is necessary to confirm this trend and understand the functional implications. An important next step will be determine whether pol II binding is observed at the proximal promoter of a target gene with a p53 consensus binding site in the promoter region of the gene in the absence of constitutive binding of p53. We do observe constitutive p53 binding without concurrent pol II constitutive binding, but only in target genes with consensus binding sites 3' of the transcription start site. This experiment will help to determine whether p53 constitutive binding is required for constitutive pol II binding at target genes with binding sites 5' of the transcription start site. It is possible that simply the presence of the p53 consensus binding site at a target

gene promoter renders that region of chromatin more open and flexible to allow pol II binding.

Thus, the dissertation research presented here has resulted in the identification of a number of potential p53 and ΔNp63α candidate target genes. Preliminary validation of RRAD strongly suggests that it is a novel p53-regulated gene. In addition, we have observed inverse regulation of a panel of p53 target genes involved in cell cycle arrest, DNA repair, apoptosis, and p53 negative regulation by p53 and ΔNp63α. This inverse regulation corresponds to inverse binding of p53 and ΔNp63α at p53 consensus binding sites, and upregulation of target gene mRNA. These results provide further understanding of the role of ΔNp63α in p53-mediated signaling. Overall, increased knowledge regarding p53-mediated tumor suppression, whether it is the role of ΔNp63α or the understanding of RRAD as a target gene, ultimately brings us a small step closer to a cure for cancer.

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