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Amino-Terminal Transactivation Subdomains of p53 Contribute Equally to p53-Induced Gene Expression

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This thesis is submitted to the
Faculty of Graduate and Postdoctoral Studies
as a partial fulfillment of the
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ABSTRACT

The p53 tumour suppressor is mutated in over 50% of sporadic cancers. Its tumour suppressive functions arise in large part from transcriptional activation of target genes. Two adjacent regions within the transactivation domain of p53 are sufficient to support sequence-specific transactivation when fused to a heterologous DNA binding domain. It has been hypothesized that these two subdomains of p53 may contribute to the expression of distinct p53-responsive genes and regulate distinct biological pathways.

In order to examine the subdomain requirements during transactivation, we used oligonucleotide microarrays to identify transcripts induced by variants of p53 with point mutations within subdomains 1, 2, or both (QS1, QS2, and QS1/QS2, respectively). Expression of wild type p53 ($p53^{WT}$) statistically induced 254 transcripts and apoptosis transcripts (GO#0006915) were the only statistically over represented group. Most of these transcripts were poorly induced by the p53 variants and there was a corresponding decrease in the ability of these variants to induce apoptosis. Strikingly, several well known p53-regulated transcripts including TNFRSF10B, BTG2, and POLH were increased to wild type levels by $p53^{QS1}$ and $p53^{QS2}$ but not $p53^{QS1/QS2}$, indicating that either subdomain1 or 2 is sufficient for p53-dependent expression of a small subset of p53-responsive genes. Unexpectedly, there was no evidence for $p53^{QS1}$- or $p53^{QS2}$-specific gene expression. Taken together, these results demonstrated heterogeneity in the requirement for transactivation subdomains 1 and 2 of p53 without subdomain-specific contributions to p53-induced gene expression.

The *MafB* transcript was identified as a $p53^{WT}$-induced transcript that was not statistically increased in response to either $p53^{QS1}$ or $p53^{QS2}$ expression. Nonetheless,

MafB expression increased more in response to the QS2 compared to the QS1 variant of p53 so *MafB* was studied further as a potential p53 target gene exhibiting a QS2-specific pattern of expression. Expression of $p53^{WT}$ increased MafB mRNA and protein levels and bound to a putative response element 2400bp upstream of the transcription start site. Expression of $p53^{QS2}$ consistently increased *MafB* expression more than $p53^{QS1}$, however this level did not approach the expression of *MafB* induced by $p53^{WT}$. Therefore, *MafB* represents a novel p53-regulated transcript that is poorly induced by both $p53^{QS1}$ and $p53^{QS2}$.

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ABREVIATIONS

aa	amino acid
AD	activation domain
Adp53	adenovirus expressing p53
ADR	adriamycin
AMP	adenosine monophosphate
APAF1	apoptotic activating factor 1
ASPP2	(p53BP2)
β-gal	β-galactosidase
BAX	BCL2-associated X protein
BBC3	BCL2 binding component 3 (PUMA)
Bcl-2	B-cell CLL/lymphoma 2
BER	base excision repair
BH3	Bcl-2-homology domain 3
BPDE	benzo(a)-pyrene diol epoxide
BRCA1	breast cancer 1
BTG2	B-cell translocation gene family, member 2
CARM1	coactivator-associated arginine methyltransferase 1
Caspases	cysteine-aspartic acid proteases
CAT	chloramphenicol acetyltransferases
CBP/p300	cAMP response element binding protein-binding protein /E1A binding protein p300
CDKN1A	cyclin-dependent kinase inhibitor 1A

ChIP	Chromatin immunoprecipitation
CMV	Cytomegalovirus
DAPI	4',6-Diamidino-2-Phenylindole
DBD	DNA binding domain
DISC	death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
EMSA	electrophoretic mobility shift assay
E6	Human papillomavirus type 16 E6 transforming protein
GO	gene ontology
HDAC	histone deacetylase
HEK293	human embryonic kidney 293 cell line
HPV	Human papillomavirus
HRP	horseradish peroxidase
IR	ionizing radiation
IgG	immunoglobulin G
MafB	musculoaponeurotic fibrosarcoma oncogene homolog B
MALDI-TOF MS/MS	matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry
MDM2	mouse double minute 2 p53 binding protein homolog
MEF	mouse embryonic fibroblast
MMR	mismatch repair
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid

mtHSP70	mitochondrial heat shock protein 70
NER	nucleotide excision repair
NHEJ	non-homologous end joining
NOXA	phorbol-12-myristate-13-acetate-induced protein 1
p53RE	p53 response element
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PFU	plaque forming unit
PIC	preinitiation complex
PIG	p53 induced gene
PMSF	phenylmethylsulphonyl fluoride
POLH	polymerase (DNA directed), eta
PUMA	p53-upregulated modulator of apoptosis (aka BBC3)
PRD	proline rich domain
PRMT1	protein arginine methyltransferase 1
QS1	p53Leu22Gln/Trp23Ser
QS2	p53Trp53Gln/Phe54Ser
RE	response element
RIPA	radioimmuno precipitation assay
RNAP II	RNA polymerase II
RPM	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
SAGE	serial analysis of gene expression

SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
Sp1	specificity protein 1 transcription factor
SV40	Simian virus 40
TAD	transcriptional activation domain
TAF1	TBP-associated factor 1
TBP	TATA-binding protein
TBST	Tris-Buffered Saline with Tween-20
TLS	translesion synthesis
TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b
TP53	tumor protein p53
TP53I3	tumor protein p53 inducible protein 3 (aka PIG3)
TUNEL	transferase dUTP nick end labeling
UTR	untranslated region
UV	ultraviolet
WAF1	wild-type p53 activated fragment 1

1 Introduction

1.1 P53 DISCOVERY

In the late 1970s, molecular cancer research was in its infancy. The chance simultaneous observation in two separate laboratories that a relationship existed between the SV40 antigen, known for its carcinogenic properties, and a protein of approximately 53 KDa brought to light one of the most intensely studied proteins to date (Lane and Crawford, 1980; Linzer and Levine, 1979). Although initial observations led researchers to suggest an oncogenic role for p53, within the next two decades it was clearly established as a key tumour suppressor. Subsequent to the cloning of p53, not only was it demonstrated that p53 did not transform cells (Hinds et al., 1989), it was found to actively inhibit cellular transformation by oncogenes (Eliyahu et al., 1989; Finlay et al., 1989) and that murine erytholeukemia cells contained virally disrupted p53 (Ben David et al., 1988; Munroe et al., 1988). Clinical relevance for p53 was established when the sequencing of three colorectal carcinoma cell lines demonstrated mutations in one allele and homozygous reduction or loss of the other (Baker et al., 1989; Nigro et al., 1989). It was also subsequently noted that p53 could negatively regulate cell cycle progression (Baker et al., 1990; Diller et al., 1990; Martinez et al., 1991), induce apoptosis (Shaw et al., 1992; Yonish-Rouach et al., 1991) and transcriptionally regulate genes (Fields and Jang, 1990; O'Rourke et al., 1990; Raycroft et al., 1990). The function of p53 as a tumour suppressor was further supported with the observation that p53 nullizygous mice were cancer prone (Donehower et al., 1992). Research on this protein has since grown at a phenomenal rate and there currently exceeds 46 000 peer-reviewed publications discussing p53.

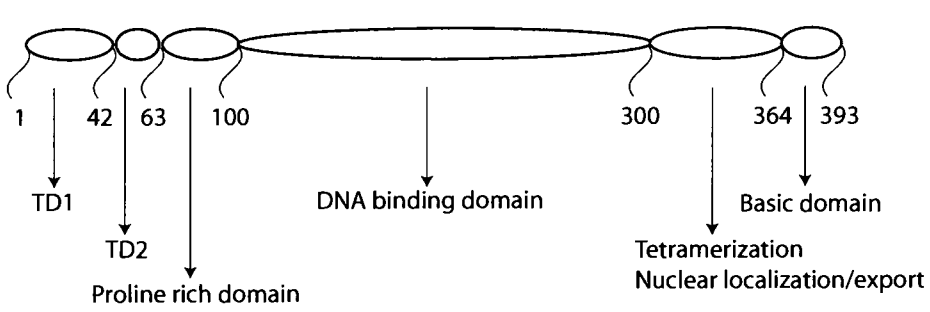
1.2 P53 STRUCTURE

The p53 protein is a modular 393 amino acid protein with several well-characterized functional domains (Figure 1a). It is composed of an amino-terminal transcriptional activation domain (further divided into two sub-domains), a proline rich domain, a core DNA binding domain, a tetramerization domain and a carboxy-terminal regulatory domain.

Amino acids 1 to 63 are acidic and essential for p53 to function as a transcriptional activator (Fields and Jang, 1990; Unger et al., 1992; Zhu et al., 1998). Numerous protein interactions occur within this transcriptional activation domain (TAD) and include binding TATA binding protein (TBP) (Farmer et al., 1996; Liu et al., 1993; Martin et al., 1993; Seto et al., 1992; Truant et al., 1993), p300 (Avantaggiati et al., 1997; Gu et al., 1997), and the negative regulator MDM2 (Chen et al., 1993; Picksley et al., 1994). These interactions are important for transcriptional activation as they alter the chromatin environment or induce conformational changes in transcription-associated proteins, thus promoting the transcription of the target gene (Ko and Prives, 1996; Xing et al., 2001).

The interaction between MDM2 and p53 is critical for the regulation of p53 protein levels and forms an auto-regulatory loop. The *MDM2* gene is a transcriptional target of p53 and the MDM2 protein binds to and suppresses p53 transcriptional activation of genes by masking the TAD and subsequently promotes polyubiquitination and ultimately results in proteosomal degradation of p53 (Chen et al., 1994; Chen et al., 1995; Chen et al., 1996a; Grossman et al., 2003; Haupt et al., 1996; Haupt et al., 1997; Kubbutat et al., 1998; Momand et al., 2000; Murphy et al., 1999). Therefore as p53 induces MDM2, MDM2 in turn promotes the degradation of p53 resulting in oscillation of their protein

a.


1
42
63
100
300
364
393
TD1
TD2
Proline rich domain
DNA binding domain
Tetramerization
Nuclear localization/export
Basic domain

b.

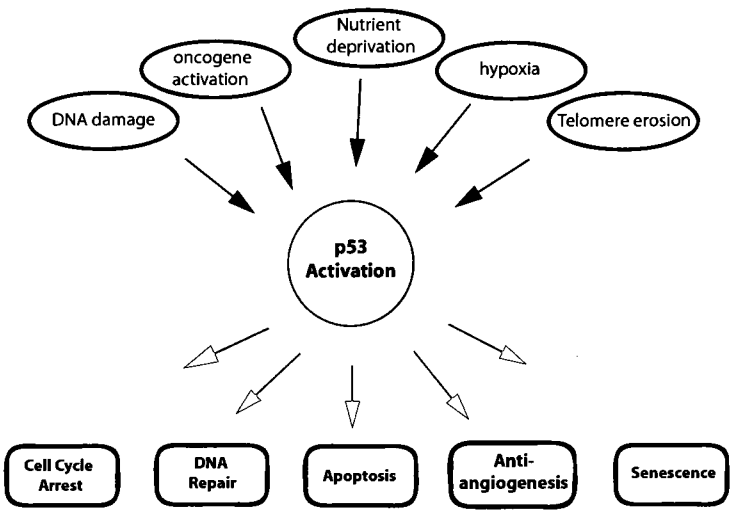

Nutrient deprivation
oncogene activation
hypoxia
DNA damage
Telomere erosion
p53 Activation
Cell Cycle Arrest
DNA Repair
Apoptosis
Anti-angiogenesis
Senescence

Figure 1. p53 structure and induction.

(a) Schematic representation of the structure of p53. The transcriptional activation do main 1 (TD1), transcriptional activation domain 2 (TD2) and other domains are indicated. The amino acids at each of the domain boundaries are indicated. (b) p53 induction results from multiple stresses and subsequently modulates numerous pathways.

levels (Lahav et al., 2004; Lev Bar-Or et al., 2000). MDM2 can also mediate monoubiquitination of p53 for intracellular trafficking (Li et al., 2003; Marchenko et al., 2007; Shmueli and Oren, 2004). Discovery of the TADs, their regulation and activities are discussed in more detail in section 1.8.

The proline rich domain (PRD) is located between residues 60-90 and contains five copies of the "PXXP" motif. The PRD mediates protein interactions with various factors including Pin1 (Wulf et al., 2002), ASPP2 (p53BP2) (Rotem et al., 2007), mSin3a (Zilfou et al., 2001), WOX1 (Chang et al., 2001) and nuclear matrix components including F-actin (Jiang et al., 2001; Okorokov et al., 2002). Such interactions within the PRD are necessary for its proper function during the apoptotic process (Baptiste et al., 2002; Ruaro et al., 1997; Sakamuro et al., 1997; Venot et al., 1998; Walker and Levine, 1996; Zhu et al., 1999; Zilfou et al., 2001). Furthermore, it has been hypothesized that the PRD affects the protein folding kinetics by functioning as a folding barrier between the TAD and DBD owing to the conformational-constraint inherent to proline-rich backbones (Stavridi et al., 2007).

The central portion of p53 contains the sequence-specific DNA binding domain (DBD) required for p53 to function as a transcriptional activator (El Deiry et al., 1992). This region represents the most evolutionarily conserved portion of the protein, both between species and between the different p53 family members p63 and p73 (Yang et al., 2002). p53 binds DNA as a tetramer to the highly degenerate response element containing two copies of the 10 base pair motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 0-13 base pairs (El Deiry et al., 1992). In order to circumvent the steric clashes expected from the binding of four DBDs to DNA (Nagaich et al., 1997), p53 induces bends in the

DNA (Balagurumoorthy et al., 1995; Cherny et al., 1999; Nagaich et al., 1999; Pan and Nussinov, 2007). Further high resolution crystal structures and protein analysis has revealed that the four p53 molecules bind together as a "dimer of dimers" to the two RE half-sites (Kitayner et al., 2006; Weinberg et al., 2004). A key component for this interaction is the oligomerization domain which resides within residues 320-360 (Pavletich et al., 1993). This domain enables p53-p53 interactions during the formation of tetramers. It also contains a nuclear export signal (Boyd et al., 2000; Geyer et al., 2000; Stommel et al., 1999) and overlaps with the dominant nuclear localization signal (Shaulsky et al., 1990).

The C-terminus of p53, located between amino acids 356-393, is highly basic and is involved in sequence-independent DNA binding (Foord et al., 1991; Wang et al., 1993), RNA binding (Riley and Maher, III, 2007) and is central in the regulation of p53 activity (Ahn and Prives, 2001). The C-terminus can recognize and bind to a wide variety of DNA structures (including short single strands, irradiated DNA, four-way junctions, and insertion/deletions) (Ahn and Prives, 2001). The modulation of p53 function by this region is due in large part to several post-translation modifications (Appella and Anderson, 2000; Brooks and Gu, 2003; Xu, 2003). Such modifications are especially important during the promotion of transcriptional activation (Barlev et al., 2001; Espinosa and Emerson, 2001; Wang et al., 2001b). However, p53 can function as a transcription factor in the absence of this region (Candau et al., 1997; Unger et al., 1993). Moreover, several groups have demonstrated that, upon truncation or antibody binding of the C-terminal domain (PAb421), DNA-binding and transcriptional activation by p53 can be

enhanced in certain situations (Abarzua et al., 1995; Ayed et al., 2001; Hupp et al., 1992; Hupp et al., 1993; Hupp et al., 1995; Muller-Tiemann et al., 1998).

1.3 P53 MUTATIONS IN HUMAN CANCER

More than half of all human cancers are associated with alterations in p53 (Harris and Levine, 2005). A study of over 10 000 different human tumours revealed 45-50% contain inactivating mutations of p53 (Landis et al., 1999). Mutations of p53 are predominantly missense substitutions (75%) but also include frameshift insertions and deletions (9%), nonsense mutations (7%), silent mutations (5%) and other less frequent mutations (Olivier et al., 2002). Of the missense mutations, 30% correspond to nucleotide substitutions essential for the contact between the p53 protein and specific DNA sequences (p53 response elements) (Hainaut and Hollstein, 2000; Hollstein et al., 1991) resulting in a drastic decrease in DNA binding capacity and transcriptional activation (Ory et al., 1994). Analysis of the entire tumour-derived spectrum of mutations reveals that 80-85% fall within the DNA binding domain (Figure 2) (Landis et al., 1999; Lowe, 1995). This observation suggests a critical role for the p53-DNA interaction during events that lead to tumour suppression. Additionally, the most frequent of these mutants are capable of interacting with oncogenes for cellular transformation (Hinds et al., 1990). Although the amino-terminal transactivation domain of p53 is essential for target gene upregulation, very few tumourigenic mutations are observed within this region. This suggests that decreased activity within this domain is insufficient to promote neoplastic development (Bullock and Fersht, 2001).

Germ line TP53 mutations result in Li Fraumeni syndrome (Malkin et al., 1990). Individuals with this genotype are faced with early disease onset, high probability of mul-

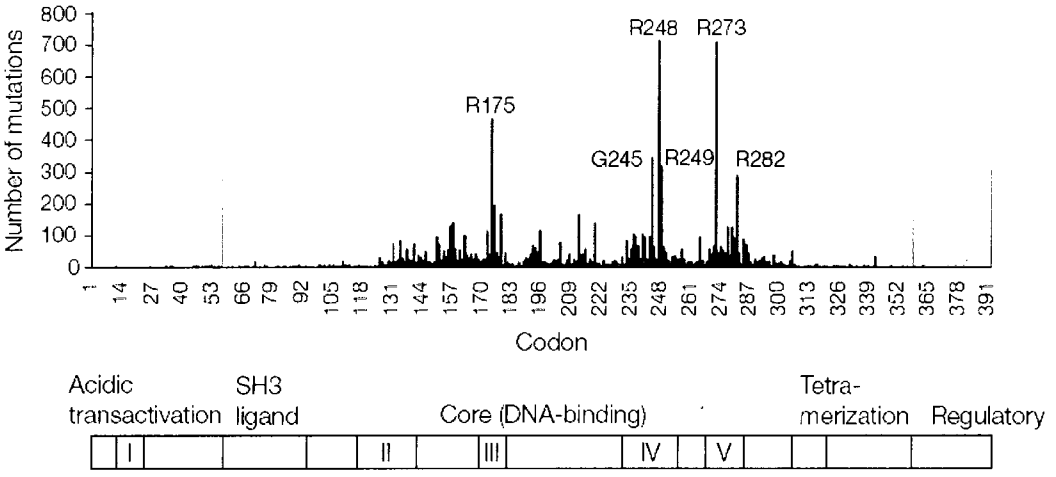

Number of mutations
800
700
600
500
400
300
200
100
0
R248
R273
R175
G245
R249
R282
1
14
27
40
53
66
79
92
105
118
131
144
157
170
183
196
209
222
235
248
261
274
287
300
313
326
339
352
365
378
391
Codon
Acidic transactivation
SH3 ligand
Core (DNA-binding)
Tetra-merization
Regulatory
I
II
III
IV
V

Figure 2. p53 mutation spectrum in human cancers.

Schematic representation of p53 depicting the acidic transactivation, SH3 ligand, Core, Tetramerization, and Regulatory domains. Five boxes showing the regions of greatest sequence conservation are shown; four map to the core domain. A histogram of p53 missense mutations shows that 95% of mutations occur in the core domain; six labeled residues are hot spots for mutation (reproduced with permission from Bullock and Fersht, 2001)

tiple primary tumours, diverse potential tumour types (including melanomas, sarcomas, breast cancer, leukemia, colorectal cancer, adrenal cortical tumours, gonadal germ cell, lung, pancreatic and prostate carcinomas) and high mortality rates (Li and Fraumeni, Jr., 1969; Malkin et al., 1990; Varley et al., 1997). Breast cancer, bone and soft tissue sarcomas, brain tumours and adrenocortical carcinomas are most commonly associated with TP53 mutations (Wong et al., 2006). A genotype-phenotype association is observed with TP53 mutations. Increased prevalence of brain tumours and early age onset of breast cancers are observed as a result of missense mutations located within DNA-binding loops. Adrenocortical carcinomas arise more frequently due to missense mutations outside the DNA-binding loops (Olivier et al., 2003).

Mutagenesis is a critical determinant of TP53 somatic mutational patterns. For example, mutations found in lung cancer diverge from the classical p53 hotspots. Instead, lung cancer-specific hotspots are observed containing G>T transversions resulting from benzo(a)-pyrene diol epoxide (BPDE) (a metbolite arising from combustion products found at high concentrations in tobacco smoke) adducts on guanines (Denissenko et al., 1996; Ruggeri et al., 1993). The p53 molecules produced from these mutations are impaired in transcriptional activation (under 20% wild type activity) (Petitjean et al., 2007). Similarily, non-melanoma skin cancer has a specific spectra of p53 mutations C>T and CC>TT at dipyrimidine sites resulting from ultraviolet radiation exposure (Brash et al., 1991; Moles et al., 1993; Ziegler et al., 1993). A recent review of the incidence of p53 mutations in human skin cancers revealed that five of the eight most commonly mutated sites in p53 (codons 196, 213, 245, 248, and 282) contain mutated dipyrimidines in the sequence context 5'-CCG or 5'-TCG (Pfeifer et al., 2005).

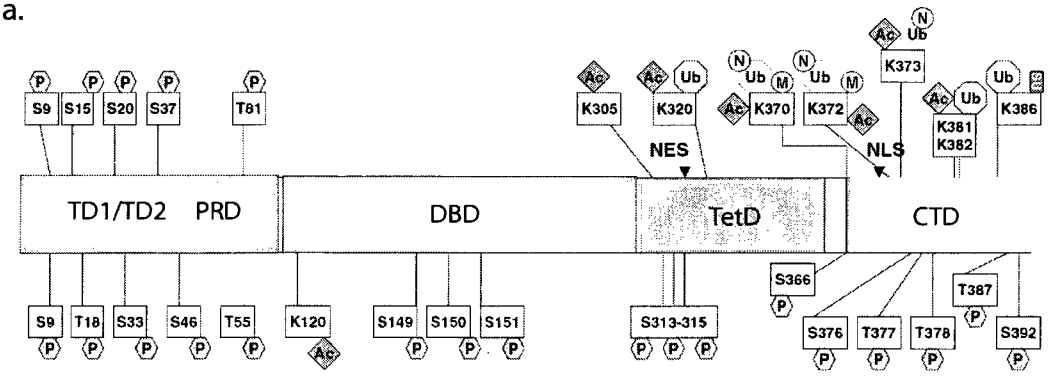
In addition to TP53 mutations, single nucleotide polymorphisms alter biological functions of the protein. The most intensely studied SNP is an Arg72Pro variation in exon 4 (G/C variation at the second position of codon 72). This residue is located within the proline-rich region and results in a protein with altered structure and electrophoretic mobility (Harris et al., 1986; Matlashewski et al., 1987). The Arg72 variant induces apoptosis more efficiently than Pro72 (Bonafe et al., 2002; Dumont et al., 2003) primarily due to an increased capacity to interact with MDM2, resulting in facilitated nuclear export and mitochondrial targeting (Dumont et al., 2003). In contrast, the Pro72 variant is a more efficient inducer of cell cycle arrest and DNA repair (Pim and Banks, 2004; Siddique and Sabapathy, 2006). Further differences exist in either variants ability to bind components of the transcriptional machinery (including TFIID-associated factors TA-$F_{II}32$ and $TAF_{II}70$), activate transcription and repress primary cell transformation (Thomas et al., 1999). Considering these critical variations in biological activity, significant resources have been expended in order to determine the clinical relevance of the Arg/Pro polymorphism. Unfortunately, these studies yield conflicting conclusions likely due to the presence of various TP53 mutations rendering observations difficult to interpret and obscuring phenotypes of the polymorphism. Results from such studies generated divergent conclusions that include; no difference (Klug et al., 2001; Koushik et al., 2004a; Makni et al., 2000), an increased risk for various cancers with the R72 allele (Bastiaens et al., 2001; Bonafe et al., 2003; Buyru et al., 2003; de Oliveira et al., 2004; Dokianakis et al., 2000; Henner et al., 2001; Kawaguchi et al., 2000; Langerod et al., 2002; Ohayon et al., 2005; Pegoraro et al., 2002; Shen et al., 2003; Shen et al., 2004; Soulitzis et al., 2002; Sourvinos et al., 2001; Wu et al., 2002) or an increased risk with

Pro72 (Chen et al., 2003; Fan et al., 2000; Granja et al., 2004; Kuroda et al., 2003; Tiwawech et al., 2003; Tsai et al., 2002a; Tsai et al., 2002b; Wang et al., 1999). Furthermore, an elevated risk of cervical cancer might be expected in individuals with Arg72 as this polymorphic variant is more efficiently targeted for degradation by the E6 protein of human papilloma virus (HPV)16 (Storey et al., 1998). Numerous population-based studies have been performed to address such a correlation yet this hypothesis remains unproven (Govan et al., 2007; Helland et al., 1998; Klug et al., 2001; Koushik et al., 2004b; Malcolm et al., 2000; Oliveira et al., 2008; Storey et al., 1998). Interestingly, although an association between these polymorphic variants and the occurrence of cancer are murky, there is a seemingly undisputed relationship with cancer progression where patients homozygous for the Pro72 allele are diagnosed at a significantly earlier disease stage (presumably owing to its decreased apoptotic potential) (Gottschlich et al., 2000; Jones et al., 2004; Shen et al., 2003).

1.4 POST-TRANSLATIONAL MODIFICATIONS OF P53

P53 post-translational modification is a dynamic and transient process. The most frequent p53 modifications include phosphorylation, acetylation, methylation, ubiquitylation, and sumoylation (Figure 3a) (Bode and Dong, 2004; Olsson et al., 2007). Less frequently, glycosylation (Shaw et al., 1996), ribosylation (Wesierska-Gadek et al., 1996a; Wesierska-Gadek et al., 1996b), and neddylation (Abida et al., 2007; Xirodimas et al., 2004) are also observed. In normal unstressed cells, specific post-translational modifications occur cyclically in conjunction with progression through different stages of the cell cycle (Buschmann et al., 2000). In these cells, the p53 protein has a half-life in the range of 6-20 minutes. Upon cellular stress, the half life of p53 is drastically lengthened and

a.



b.

Kinase	Residue(s) phosphorylated
ataxia telangectasia mutated (ATM)	15
ataxia telangectasia and Rad3-related protein (ATR)	15,37
aurora kinase A (AURKA)	315
cyclin-dependent kinase (CDK)	315
checkpoint kinases 1 and 2 (CHK1/CHK2)	20
casein kinase 1(CK1)	6,9
casein kinase 2 (CK2)	18
cop-9 signalosome (CSN)-associated kinase complex	149,150,155
DNA-dependent protein kinase (DNA-PK)	15,37
dual-specificity tyrosinephosphorylation-regulated kinase 2 (DYRK2)	46
extracellular signal-regulated kinases (ERKs)	15,55
GSK3b, glycogen synthase kinase 3 b (GSK3b)	315,376
homeodomaininteracting protein kinase2 (HIPK2)	46
c-Jun NH2-terminal kinase (JNK)	20,81
mitogen-activated protein kinase-activated protein kinase2 (MAPKAPK2)	20
p38 kinase (p38)	15,33,392
prolyl isomerase (Pin1)	33,81,315
protein kinase C (PKC)	376,378
PKR, double stranded RNA-activated kinase (PKR)	392
TATA-binding protein-associated factor1 (TAF1)	55

Figure 3. Post-translational modifications of p53.

(a) Schematic representation of p53 and post-translational modifications. Post-translational modifications are depicted in the individual domains of p53: P (phosphorylation), Ac (acetylation), Ub (ubiquitination), M (methylation), N (neddylation) and SU (sumoylation) (Olsson et al., 2007, reproduced with permission). (b) Table of kinases known to phosphorylate p53 and their associated phosphorylation sites (modified from Olsson *et al.*, 2007).

protein levels increase (Maki et al., 1996; Maltzman and Czyzyk, 1984; Price and Calderwood, 1993). This increase occurs subsequent to genotoxic and non-genotoxic stresses (Huang et al., 1996; Kastan et al., 1991; Maltzman and Czyzyk, 1984) and is the result of tightly orchestrated post-translational modifications (mainly acetylations and phosphorylations) culminating in decreased interaction with MDM2.

Post-translational modifications can be found throughout p53. Several kinases have been implicated in site specific phosphorylation (Figure 3b). Phosphorylation is seen at serines 6, 9, 15, 20, 33, 37, 46, 149, 315, 376, 378, and 392 and threonines 18, 55, 81, 150, 155 (Attardi and Donehower, 2005; Bode and Dong, 2004; Olsson et al., 2007; Toledo and Wahl, 2006). A stress-specific pattern of residue modification results in elevated phosphorylation of serines 33, 37, 46, and 392 following UV or adriamycin (ADR) treatment compared to IR and increased phosphorylation of threonine 18 is observed following IR or ADR compared to UV light (Saito et al., 2003). C-terminal phosphorylation of Ser315 and Ser392 occurs following UV or ADR treatment and Ser315 is further phosphorylated after IR treatment (Kapoor and Lozano, 1998; Lu et al., 1998). Ser376 and Ser378 are constitutively phosphorylated and dephosphorylation of Ser376 is observed following IR (Waterman et al., 1998).

Subsequent to DNA damage, a post-translational modification dependent signalling cascade involving the acetyltransferases and transcriptional coactivators p300/CBP and PCAF enables the acetylation of p53 (Gu and Roeder, 1997; Prives and Manley, 2001; Sakaguchi et al., 1997; Sakaguchi et al., 1998). The principal site at which p53 is acetylated in response to different kinds of stress is at Lysine 382 (Appella and Anderson, 2001). Phosphoryation at Ser15 enhances the interaction between p53 (Dumaz and Meek,

1999; Lambert et al., 1998) and p300/CBP resulting in acetylation of lysines 372, 373, 381 and 382. Lysine 305 is also acetylated following exposure to actinomycin D, IR, UV and H_2O_2 (Wang et al., 2003). Additionally, acetylation of C-terminal lysines may contribute to stability as they are targets for ubiquitination (Rodriguez et al., 2000).

The p53 protein can be physically altered through post-translational modifications as a result of the specific physiological stimuli encountered by a cell. Post-exposure to IR or UV, phosphorylation of serines 15, 20, and 37 stabilize p53 (Chehab et al., 1999; Shieh et al., 1997). Oligomerization (Sakaguchi et al., 1997) and sequence specific DNA-binding of p53 is influenced by modifications of serines 315 and 392 (Hao et al., 1996; Hupp et al., 1992; Wang and Prives, 1995). Further, in addition to the implication of phosphorylation of the p53 N-terminus in stability, it also modulates the ability of p53 to transcriptionally regulate target genes (Chao et al., 2003; Oda et al., 2000b). Importantly, phosphorylation of Ser15 and Ser20 contribute to p53 activation (Chehab et al., 1999; Shieh et al., 1997; Siliciano et al., 1997). Furthermore, phosphorylation at Ser15 increases the interaction with p300/CBP and transcriptional regulation.(Dumaz and Meek, 1999; Lambert et al., 1998). The murine equivalent of Ser15, Ser18, is has also been demonstrated as an important component of transcriptional activation of target genes and c-terminus acetylation subsequent to DNA damage (Chao et al., 2003). Phosphorylation of Ser46 has also been found to affect transcriptional regulation and induction of apoptosis (Oda et al., 2000b).

Although complex, the post-translation modifications found on p53 are tightly regulated. Aberrant modifications can correlate with cancer development. Some generalized modifications of p53 that are implicated in tumorigenesis include hyper-

phosphorylation in both the human glioblastoma line T98G (Ullrich et al., 1993) and human pulmonary epithelial type II cells (A549) (Higashimoto et al., 2000), and hyperacetylation subsequent to exposure to hepatitis C virus core protein (Kao et al., 2004).

As post-translational modifications are important mediators of p53 stability, it is important to also note that additional methods of increasing p53 levels subsequent to stress also exist. In human colorectal carcinoma cells (RKO), UVC irradiation results in an increase in p53 translation (Mazan-Mamczarz et al., 2003). This increase is observed in multiple cell lines and mediated *in vivo* and *in vitro* by interaction of the RNA binding protein HuR and the 3' UTR of p53 (Mazan-Mamczarz et al., 2003; Zou et al., 2006).

1.5 BIOLOGICAL PATHWAYS REGULATED BY P53

P53 is most often associated with tumour suppression. Although this may be the ultimate outcome of functional p53 in the cellular context, there is great diversity in the pathways regulated by p53. For example, p53 regulatory activity is implicated in pathways indirectly associated with tumour suppression such as glycolysis (Bensaad et al., 2006; Matoba et al., 2006), autophagy (Crighton et al., 2006; Crighton et al., 2007), differentiation (Murray-Zmijewski et al., 2006) and bone remodeling (Wang et al., 2006). These are in addition to the adeptly characterized tumour suppressive pathways including genotoxic damage repair (Gatz and Wiesmuller, 2006), cell survival and regulation of oxidative stress (Bensaad and Vousden, 2005), invasion and motility (Roger et al., 2006), cellular senescence (Kortlever et al., 2006), angiogenesis (Teodoro et al., 2006), cell cycle progression and apoptosis (discussed in detail below) (Figure 1b).

Most notably, p53 has been studied for its role in the developmental program of organisms. Although deletion of p53 has only a modest effect on development

(Armstrong et al., 1995; Choi and Donehower, 1999; Sah et al., 1995), deletion of its family members p63 and p73 has a much more profound phenotype (Celli et al., 1999; Mills et al., 1999; Pozniak et al., 2000; Yang et al., 2000). The primary function of p53 during development involves monitoring the developmental process, destroying aberrant cells or the embryo in its entirety if extreme defects are present (Hall and Lane, 1997). Interestingly, p53 also contributes to the regulation of glucose metabolism. Metabolic stress, such as depletion of nutrients, can activate p53 through an adenosine monophosphate (AMP) kinase pathway resulting in short-term survival during temporary starvation (Jones et al., 2005). Loss of this starvation-induced arrest can potentially contribute to sustained cellular proliferation during nutrient-poor conditions.

1.6 THE P53 MEDIATED CELLULAR STRESS RESPONSE TO GENOTOXIC STRESS

The basis for p53's tumour suppressive function is its ability to orchestrate the activities of multiple pathways in the presence of genotoxic stress. p53 is activated in response to DNA-damage (Kastan et al., 1991; Lu and Lane, 1993; Maltzman and Czyzyk, 1984), hypoxia (Graeber et al., 1994), serum deprivation (Aoki et al., 1997), activation of oncogenes (Serrano et al., 1997), or perturbations of cell cycle regulation (Debbas and White, 1993; Lowe and Ruley, 1993; Symonds et al., 1994). This activation of p53 subsequent to, for example, the induction of DNA-double strand breaks by exposure to ionizing radiation or genotoxic chemotherapy induces rapid phosphorylation of the amino terminus of p53 (Shieh et al., 1997; Siliciano et al., 1997). Consequently, p53 promotes the induction of apoptosis (Clarke et al., 1993; Lowe et al., 1993; Yonish-Rouach et al., 1991), cell cycle arrest (Diller et al., 1990; Kuerbitz et al., 1992; Martinez et al., 1991),

and DNA-repair (Ford and Hanawalt, 1995; Ford and Hanawalt, 1997; Smith et al., 1995; Wang et al., 1995).

A principle mechanism underlying pathway regulation by p53 requires the regulation of specific sets of genes. Sequence-specific p53 transcriptional activation induces genes involved in apoptosis, cell cycle arrest, DNA-repair, angiogenesis and hypoxia, among others (Riley et al., 2008). The pattern of gene induction (type of gene, strength and kinetics of induction) is altered based on p53 levels and type of stress in a tissue-specific fashion (Zhao et al., 2000). Thus, a wide array of responses to p53 activation is possible and is dictated by the specific physiological situation. Zhao *et al.* (2000) performed genome wide analyses in order to examine the temporal response of p53 target genes to p53 activation. They demonstrated that, based on the pattern of mRNA levels subsequent to p53 activation, several characteristic response profiles exist (Figure 4). These profiles clearly demonstrate significant diversity in mRNA response patterns and include gradual versus rapid increases to a plateau, sharp increases followed by sharp decreases, as well as similar patterns of decreased mRNA expression. Demonstrating the complexity of the p53 response, this observation also underlines the presence of secondary methods of transcript regulation during the p53 response (Zhao et al., 2000).

1.6.1 Apoptosis

Apoptosis, or programmed cell death, is essential for the destruction of a variety of abnormal cells and occurs through two main pathways (Figure 5a) (Elmore, 2007; Fridman and Lowe, 2003; Haupt et al., 2003). Cysteine-aspartic acid proteases (caspases) are critical components of both pathways. The extrinsic apoptotic pathway is initiated at the cell surface by the ligation of tumour necrosis factor family members, subsequent as

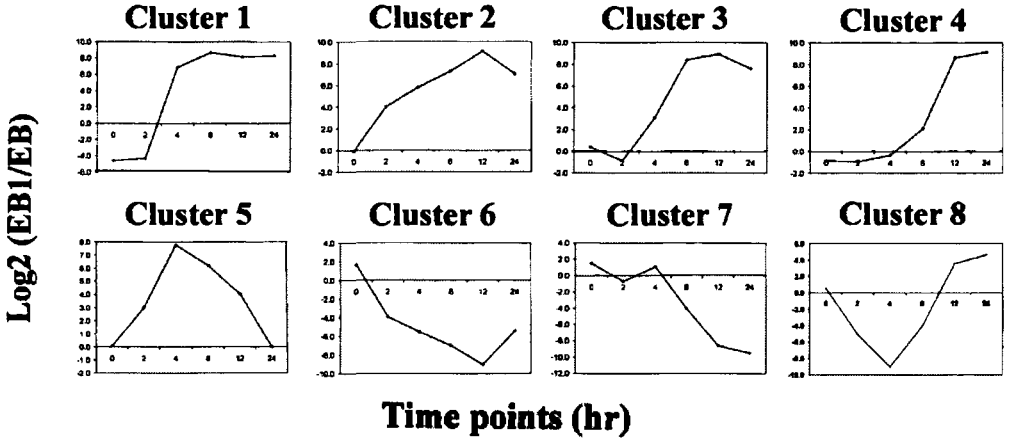

Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8
Log2 (EB1/EB)
Time points (hr)

Figure 4. Temporal Expression of p53 transcriptional targets.

The expression profiles of gene clusters following p53-induction. Eight clusters were derived from the cluster analysis, showing different induction kinetics (reproduced with permission from Zhao *et al.*, 2000).

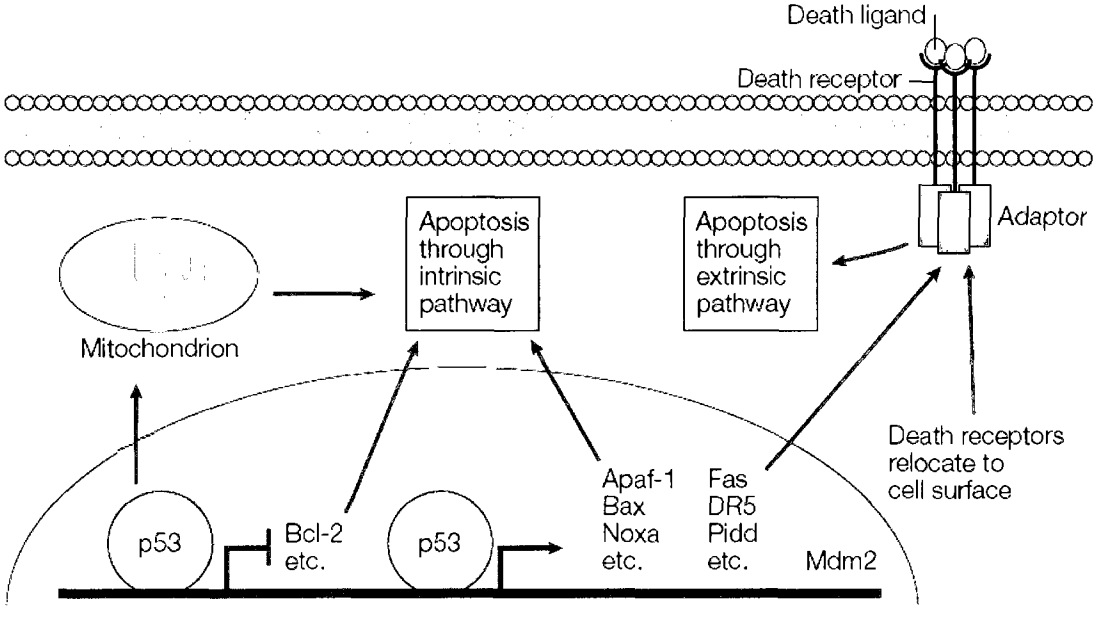

Death ligand
Death receptor
Adaptor
Apoptosis through intrinsic pathway
Apoptosis through extrinsic pathway
Mitochondrion
Death receptors relocate to cell surface
p53
Bcl-2 etc.
p53
Apaf-1 Bax Noxa etc.
Fas DR5 Pidd etc.
Mdm2

Figure 5. p53 and apoptosis.

p53 induces the expression of proteins that target both the mitochondrial- and the death-receptor-induced apoptotic pathways, and specifically represses transcription from several death-inhibiting genes. Further activities of p53 that are entirely independent of transcriptional regulation have been proposed. They include the ability of p53 to drive relo calization of death receptors such as Fas/CD95 from the Golgi to the cell surface and to directly associate with mitochondria. P53 can also directly target the mitochondria and promote mitochondrial membrane permeabilization. Bcl-2, B-cell lymphoma 2; Apaf, Apoptotic protease-activating-factor; Bax, Bcl-2 associated X protein; DR5, death receptor 5; Pidd, p53 protein induced, with death domain (modified with permission to reproduce from Jesenberger and Jentsch, 2002).

sembly of a death-inducing signaling complex (DISC), activation of caspase 8 and caspase 3 thereby resulting in apoptosis. The intrinsic pathway is triggered by DNA damage and is dependent on B-cell CLL/lymphoma 2 (Bcl-2) family members where mitochondrial permeabilization and release of cytochrome c results due to the activation of BAX and BAK. The formation of the apoptosome (composed of cytochrome c, apoptotic activating factor 1 (APAF-1) and procaspase 9) ensues thereby activating capase 9 and subsequently caspases-3, -6, and -7. Classified based on structural similarity, members of the Bcl-2 family play pivotal roles in the regulation of the intrinsic pathway and include both pro- and anti-apoptotic members. Pro-apoptotic Bcl-2 family members include Bax, Bak and Bcl-2-homology domain 3 only (BH3-only). Anti-apoptotic members heterodimerize with and antagonize the pro-apoptotic members.

p53 can modulate multiple components of both the intrinsic and extrinsic pathway (Figure 5b) (Fridman and Lowe, 2003; Haupt et al., 2003). Knock-out and mutational studies targeting specific pro-apoptotic p53-regulated genes demonstrated that, although compromised, deficiency in any one gene did not abolish p53-mediated apoptosis. However, several p53-induced genes appear responsible for a large proportion of the apoptosis. P53 transcriptionally activates the BH3-only member of the Bcl-2 family p53-upregulated mediator of apoptosis gene (*PUMA*) (Nakano and Vousden, 2001). *PUMA* expresses two pro-apoptotic proteins that localize to the mitochondria where they directly interact with BCL2 resulting in membrane permeability, cytochrome c release and apoptosis (Nakano and Vousden, 2001). Deletion or decrease of PUMA severely impedes the induction of apoptosis and can promote tumour formation (Hemann et al., 2004; Jeffers et al., 2003; Nakano and Vousden, 2001; Villunger et al., 2003; Yu et al., 2001). Another

important BH3-only member of the BCL2 family transcriptionally regulated by p53 is phorbol-12-myristate-13-acetate-induced protein 1 (*NOXA*) (Oda et al., 2000a). Noxa also localizes to the mitochondria and interacts with anti-apoptotic BCL2 family members thereby promoting apoptosis (Oda et al., 2000a). Similar to PUMA, decreases in NOXA result in suppressed apoptosis (Oda et al., 2000a; Shibue et al., 2003; Villunger et al., 2003). Although the transcriptional activation of these genes promotes apoptosis, they are not quintessential as apoptosis is not completely abolished.

The absolute requirement for transcriptional activation during p53-mediated induction of apoptosis has been an ongoing issue of debate. Interestingly, in certain systems, transcriptional activation is a dispensable component of p53-induced apoptosis (Caelles et al., 1994; Ding et al., 2000; Haupt et al., 1995; Kokontis et al., 2001; McKay et al., 2000; Roemer and Mueller-Lantzsch, 1996; Wagner et al., 1994). The first evidence that p53 could induce apoptosis in the absence of transcriptional activation was reported by Caelles and coworkers using a temperature sensitive variant of p53 in immortalized GHFT1 cells (1994). At the permissive temperature, p53 induced apoptosis in the presence or absence of actinomycin D or cyclohexamide that blocked RNA or protein synthesis respectively (Caelles et al., 1994). This ability of p53 to induce apoptosis in the absence of *de novo* transcription and translation was further confirmed in other laboratories (Mihara and Moll, 2003; Sansome et al., 2001; Zou et al., 1999). Under certain conditions, even an increase in apoptosis levels is observed when mRNA and protein synthesis are inhibited (Kokontis et al., 2001; McKay et al., 2000).

Further evidence supporting p53 induced apoptosis in the absence of transcriptional activation is observed from murine $p53^{\Delta 214}$ which contains only the first 214 amino

acid residues (lacking nearly half of the DNA binding domain and the tetramerization domain). $p53^{\Delta 214}$ is thus unable to induce sequence specific transcriptional activation (Haupt et al., 1995). However, sub-G1 content analysis, terminal transferase dUTP nick end labeling (TUNEL) and 4',6-Diamidino-2-Phenylindole (DAPI) staining all indicated that this mutant retains its ability to induce apoptosis regardless of its loss of transcriptional activity.

Interestingly, the c-terminus of p53 binds to the XPB and XPB subunits of TFIIH helicase and inhibits its activity (Wang et al., 1994; Wang et al., 1995; Xiao et al., 1994). Human fibroblast cell lines deficient in the expression of XPB and XPD are impaired in their ability to induce apoptosis compared to normal human fibroblast cells (Wang et al., 1996). The deficiency in apoptosis seen in the XPB and XPD cell lines was rescued when XPB and XPD were reintroduced into the respective deficient cell lines. The expression of $p21^{WAF1}$ was similar to normal fibroblasts regardless of the cell line examined suggesting that transcriptional activation was functional. Further examination of several cancer derived cell lines with decreased apoptotic activity revealed these cell lines retained normal transactivation activity. Further, a cell line deficient in transactivation retained the ability to partially induce apoptosis. Taken together, the experiments by Wang *et al.* (1996) suggest that the p53 c-terminus is essential for apoptosis but p53 transcriptional activation is not.

Haupt and coworkers further studied the requirement for transactivation during apoptosis. Point mutation of critical amino acids within the transcriptional activation domain of p53 Leu22Gln and Trp23Ser ($p53^{QS1}$) severely impedes target gene activation (Lin et al., 1994). Over-expression of $p53^{QS1}$ led to significant levels of apoptosis, albeit

at lower levels than that induced by wild type p53 (Haupt et al., 1995). Therefore, p53 retains its ability to induce apoptosis even when faced with a deficiency in activating transcription. Additional studies with $p53^{QS1}$ further support transactivation-independent apoptosis. The p53 point mutation A135V results in a temperature sensitive form of p53 which is inactivated and cytoplasmic at 37°C but is activated and nuclear at 32°C. HCT116 cells lines were created stably expressing either $p53^{A135V}$ or $p53^{A135V/QS1}$ (Kokontis et al., 2001). Upon switch to the permissive temperature, an elevated level of apoptosis was observed in the $p53^{A135V/QS1}$ cell line compared to $p53^{A135V}$ cell line (as measured by DAPI stained cells).

More recently it was discovered that p53 can shuttle directly to mitochondria. During p53-mediated apoptosis, p53 can translocate to the mitochondria in primary, immortal and transformed cell cultures (Dumont et al., 2003; Marchenko et al., 2000; Mihara et al., 2003; Sansome et al., 2001). MDM2 activity is believed to be a central mediator of the translocation either through direct interaction with the p53 TAD and export (Dumont et al., 2003) or through monoubiquitylation of p53 thereby mediating its export (Marchenko et al., 2007). Functional studies using a p53 fusion protein with the ornithin transcarbamylase mitochondrial import leader, thus artificially directing p53 to the mitochondria, is sufficient for the induction of apoptosis and suppression of colony formation (Mihara et al., 2003). Once localized to the mitochondria, endogenous p53 interacts with anti-apoptotic BH3 family members such as BAK (Leu et al., 2004), Bcl-xL and Bcl2 (Chipuk et al., 2004; Mihara et al., 2003). Furthermore, *in vitro* experiments demonstrate that purified p53 protein induces oligomerization of Bak, permeabilization of the outer mitochondrial membrane and strong promotion of cytochrome C release (Mihara et al.,

2003). Interestingly, the residues critical for the interactions with resident mitochondrial proteins are the most frequently mutated residues in cancer (and also inactivate DNA binding). Thus, the extreme tumourigenicity associated with these mutations arises due to a double-hit to p53 function by disabling both transcriptional activation and mitochondrial targeting (Mihara et al., 2003).

1.6.2 CELL CYCLE ARREST

The induction of cell cycle arrest is another well documented consequence of p53 activation (Figure 6). Transition throughout the cell cycle requires the coordinated interactions of cyclins and cyclin-dependent kinases (Bloom and Cross, 2007). Subsequent to DNA damage, the cell cycle is arrested at multiple points including the G1 or G2 phase transitions (Kastan et al., 1991). p53 mediates the G1 cell cycle arrest through the transcriptional activation of the cyclin-dependent kinase (cdk) inhibitor $p21^{WAF1/Cip1}$ (El Deiry et al., 1993; Li et al., 1994). P21 in turn functions by inactivating several cdks and their complexes (El Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993). Deletion of $p21^{Waf1/Cip1}$ strongly abrogates the ability of p53 to induce a G1 cell-cycle arrest (Brugarolas et al., 1995; Deng et al., 1995). DNA-damage induced arrest at the transition from the G2 to M phase also relies on the activity of p53 where p21 induction inhibits the activity of the cyclin B1-cdc2 complex (Bunz et al., 1998). Furthermore, arrest of cells following DNA-damage at the S-phase checkpoint is pivotal in ensure genomic integrity but is primarily independent of p53 (Gottifredi and Prives, 2005; Stubbert et al., 2007). However, in certain situations, p53-mediated S-phase arrest has been documented including during nucleotide deprivation (Agarwal et al., 2006) and subsequent to exposure of mouse epidermal cells to vanadate (Zhang et al., 2002).

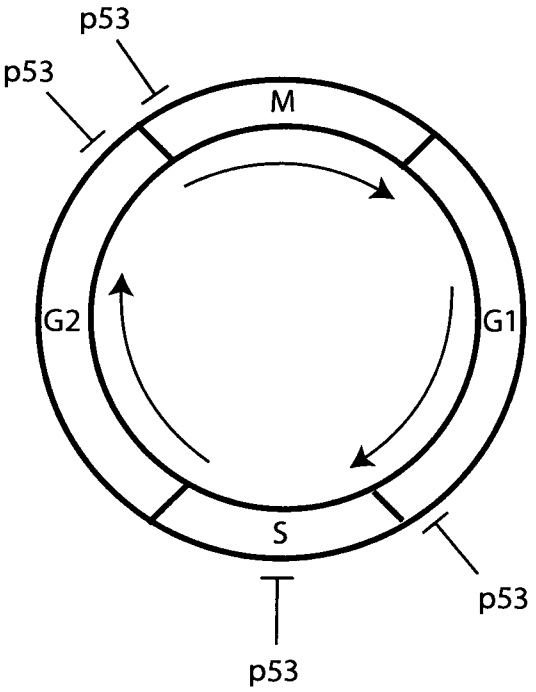

p53
p53
M
G2
G1
S
p53
p53

Figure 6. p53 and the cell cycle.

P53 can modulate the progression through the cell cycle. It can halt the cell cycle, during G1, S, G2 and M phases.

Critical to understanding the cellular response to p53 activation, the relationship between induction of apoptosis and cell cycle arrest has been dissected. In a complimentary series of experiments, Polyak *et al.,* (1996) and Gorospe *et al.*, (1997) both demonstrated that p21 is an important determinant of whether a cell undergoes apoptosis or cell cycle arrest. Using a cell line with the homologous deletion of $p21^{WAF1}$, (and therefore absence of cell cycle arrest) an increased level of apoptosis subsequent to infection with adenovirus expressing p53 (Adp53) was observed compared to wild type cells (Polyak et al., 1996). Conversely, over-expression of p21 (and increased level of cell cycle arrest) blocked the induction of p53-mediated apoptosis (Gorospe et al., 1997). Therefore, it was suggested that the two pathways may function in a mutually exclusive fashion.

Experiments examining the temperature sensitive $p53^{A135V}$ and $p53^{A135V/QS1}$ have been performed in the HCT116-$p21^{-/-}$ cell line. In the absence of p21 it was noted that $p53^{wt}$ could induce apoptosis in the previously apoptosis resistant cell line. However, $p53^{A135V/QS1}$ was again a more potent inducer of apoptosis than $p53^{A135V}$ (Kokontis et al., 2001). Taken together this series of experiments suggests the inhibition of cell cycle arrest results in increased p53-mediated apoptosis and that the two pathways are therefore working in a mutually exclusive fashion.

In order for a cell to resume the cell cycle, it must be able to recover from the arrest. As discussed below, p53 is an important mediator of DNA-repair. A study of $p53^{+/+}$ and $p53^{-/-}$ mouse fibroblast cells revealed that cells with p53 underwent a transient cell cycle arrest following damage and where subsequently able to resume growth (Haapajarvi et al., 1999). However, cells with homozygous deletion of p53 underwent a persistent arrest and apoptosis but did not resume growth. This requirement for p53 dur-

ing the recovery from DNA damage has subsequently been demonstrated in various human cell lines (Cistulli and Kaufmann, 1998; McKay et al., 2000; Mirzayans et al., 1996; Stubbert et al., 2007). This recovery is mediated through nucleotide excision repair as discussed below (Stubbert et al., 2007).

1.6.3 DNA-REPAIR

The implication of p53 in DNA-repair originally arose from linking the arrest of the cell cycle in G1 with an increased level of DNA repair (Kastan et al., 1991). It has since been demonstrated that p53 can directly and indirectly influence DNA repair (Cline and Hanawalt, 2003; Smith and Seo, 2002) and bind to damaged DNA (Yamane et al., 2001). p53 plays an important role in the repair of base damage (through base excision repair (BER), nucleotide excision repair (NER)), and potentially mismatch repair (MMR) and double strand breaks (through non-homologous end joining (NHEJ) and translesion synthesis (TLS)).

Implication of p53 in the BER pathway stems from a requirement of p53 as evidenced by a BER deficiency in p53-null cells (Offer et al., 2001; Zhou et al., 2001). The requirement for p53 during BER is at least partially due to its interaction with DNA polymerase β and direct stimulation of the repair pathway (Zhou et al., 2001). Implications for p53 in nucleotide excision repair (NER) are attributed to regulation of critical gene products such as p48XPE, XPC, and Gadd45 (Amundson et al., 2000; Carrier et al., 1999; Hwang et al., 1999). However, attempts at directly implicating physical interactions between p53 and NER repair components have proven inconclusive. p53 is furthermore a critical regulator of translesion synthesis (TLS) (also known as error-prone repair) (Avkin et al., 2006; Stubbert et al., 2007).

1.7 TRANSCRIPTIONAL ACTIVATION BY P53

Transcriptional activation is undoubtedly the best characterized function of p53. It requires sequence specific DNA binding and subsequent recruitment of chromatin modifying factors and transcriptional machinery (Figure 7). The identification of a specific recognition element for p53 (p53RE) arose independently in two laboratories (El Deiry et al., 1992; Funk et al., 1992). The p53RE is degenerate and composed of two half-sites of the palindromic sequence 5'-RRRCWWGYYY-3' (where R is a purine, Y is a pyrimidine, W is either adenine or thymine, G is guanine, and C is cytosine) separated by a spacer of 0-13 base pairs (El Deiry et al., 1992). Frequently, multiple half sites exist in proximity to each other resulting in cluster sites. An increase in the number of half sites within a cluster site yields a direct correlation with an increase in transcriptional activity (Bourdon et al., 1997; Kern et al., 1992; Tan and Chu, 2002). Additionally, several genes (including CDKN1A) contain multiple p53-binding sites capable of independently contributing to transcriptional activation (El Deiry et al., 1995; Espinosa and Emerson, 2001; Saramaki et al., 2006).

Over the years, several different methods have been employed in order to identify both novel p53 target genes and their binding sites. Identification of regulated transcripts has been achieved through gene-expression techniques including microarray and serial analysis of gene expression (SAGE) in conjunction with bioinformatics tools (Kannan et al., 2001; Kho et al., 2004; Wei et al., 2006; Yu et al., 1999; Zhao et al., 2000). The determination of direct binding sites has primarily been achieved through examination of gene specific promoter regions (Chen and Sadowski, 2005; El Deiry et al., 1992; Yin et al., 2003; Yoon et al., 2002) and chromatin immunoprecipitation (ChIP) alone or in com-

bination with oligonucleotide arrays (Cawley et al., 2004; Mirza et al., 2003). Recent methodological advances have enabled the coupling of chromatin immunoprecipitation with paired-end ditag (PET) sequencing, thus providing a genome wide map of 542 p53 binding sites (Wei et al., 2006). This research has further enabled the examination of the sequences bound by p53 and thus contributed to a refinement of the binding site requirements.

Although several mechanisms have been proposed for the initiation of p53 transcriptional activation, an overwhelming consensus in the literature supports a model whereby transcription requires the remodeling of chromatin near the p53 consensus sequence (Figure 7) (Laptenko and Prives, 2006). During remodeling RE-bound p53 recruits the histone acetyltransferases (Avantaggiati et al., 1997; Barlev et al., 2001; Lill et al., 1997), methyltransferases (An et al., 2004) or chromatin remodeling factors such as SWI/SNF (Bochar et al., 2000; Lee et al., 2002). In 1997 multiple groups demonstrated that p53 directly interacts with the acetyltransferase p300/CBP and found this interaction important for the ability of p53 to activate gene expression (Avantaggiati et al., 1997; Barlev et al., 2001; Espinosa and Emerson, 2001; Gu and Roeder, 1997; Hsu et al., 1995; Lill et al., 1997). The physical interaction with p300/CBP occurs within the amino-terminal region of p53 (Gu and Roeder, 1997; Scolnick et al., 1997) where the phosphorylation of Serine15 increases interaction affinity (Lambert et al., 1998). p300/CBP can acetylate both p53 and histone tails and the implications of the acetylation target may be gene dependent (Barlev et al., 2001; Espinosa and Emerson, 2001).

The role of histone methylation during chromatin remodeling and transcriptional regulation is dependent on PRMT1 and CARM1 (Stallcup, 2001). Although they contri-

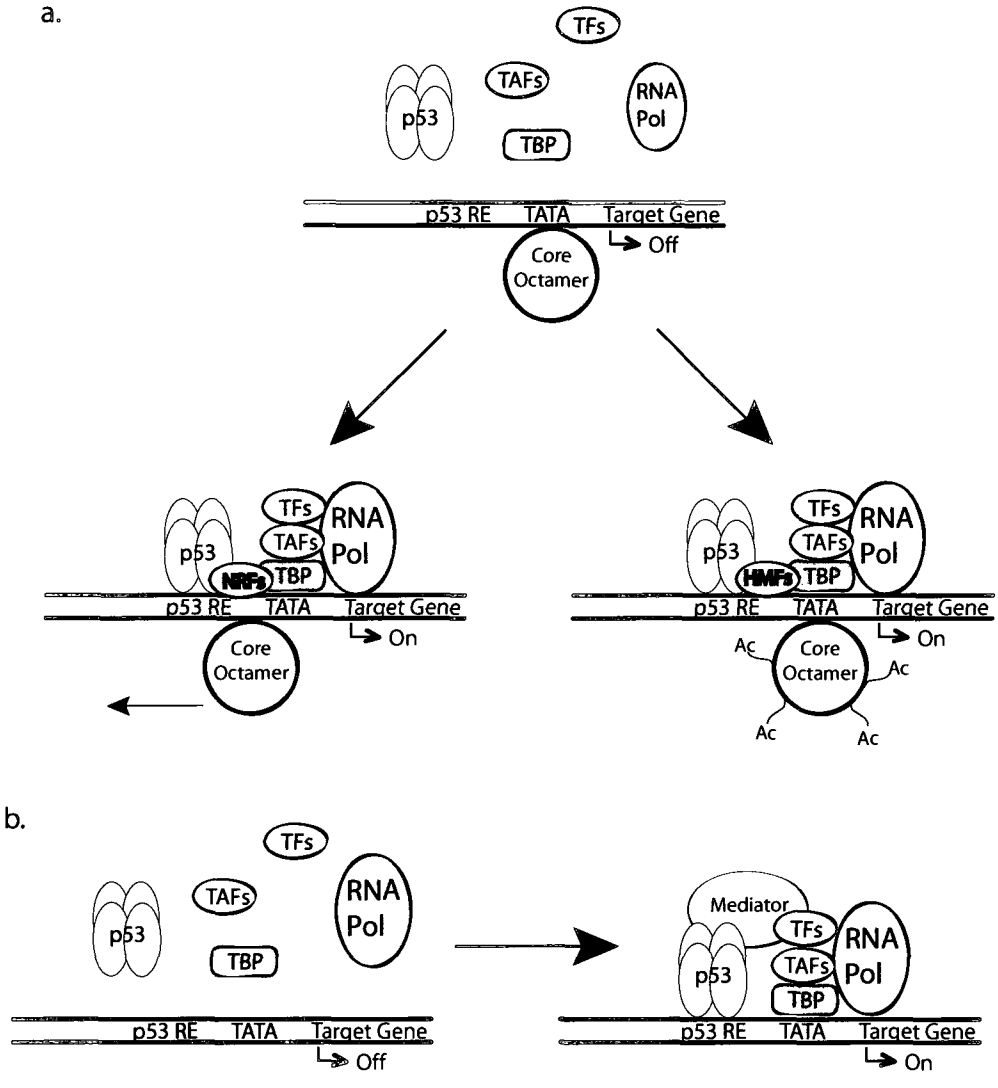

a.
TFs
TAFs
RNA Pol
p53
TBP
p53 RE
TATA
Target Gene
Off
Core Octamer
TFs
TAFs
RNA Pol
p53
NRFs
TBP
p53 RE
TATA
Target Gene
On
Core Octamer
TFs
TAFs
RNA Pol
p53
HMFs
TBP
p53 RE
TATA
Target Gene
On
Ac
Core Octamer
Ac
Ac
Ac
b.
TFs
TAFs
RNA Pol
p53
TBP
p53 RE
TATA
Target Gene
Off
Mediator
TFs
RNA Pol
p53
TAFs
TBP
p53 RE
TATA
Target Gene
On

Figure 7. Transcriptional activation by p53.

Schematic representation of p53-dependent transcriptional activation models (a) the recruitment of histone modifying factors or nucleosome remodeling factors or (b) interaction of mediator complex. Abbreviations : Ac, acetate; TATA, TATA box; TBP, TATA-binding protein; TAFs, transcriptionassociated factors; TFs, other transcription factors; RNA-Pol, RNA Polymerase; HMFs, histone modifying factors; NRFs, nucleosome remodelling factors (adapted with permission to reproduce from Laptenko and Prives, 2006).

bute to activation *in vitro,* the mechanistic requirement for methylation during p53 transcriptional activation is elusive (An et al., 2004). In reconstituted chromatin templates, an ordered cooperative function between PRMT1, CARM1, and p300 exists. Premethylation by PRMT1 stimulates p300 acetylation and conversely, pre-acetylation by p300 represses methylation by PRMT1 (Wang et al., 2001a). However, the implication of these interactions during p53 transcriptional activation has yet to be determined. Large differences in the type and extent of histone modification on various p53 regulated promoters exist (Kaeser and Iggo, 2004).

A further requirement for transcriptional activation is the recruitment of transcriptional machinery and RNA polymerase II. As such, transcriptional initiation requires the assembly of TFII-A, –B, -D, -F, and –H (Orphanides et al., 1996). p53 is known to interact with numerous components of the multi-subunit transcription factor TFIID including TATA-binding protein (TBP) (Farmer et al., 1996; Martin et al., 1993; Truant et al., 1993), TBP-associated factor 1(TAF1) (Li et al., 2004), $TAF_{II}31$(Buschmann et al., 2001; Lu and Levine, 1995a), and $TAF_{II}70$ ($TAF_{II}40$ and $TAF_{II}60$ in Drosophila (Thut et al., 1995)). The interaction with and affinity of p53 for the components of the transcriptional machinery varies according to several factors including stress source and promoter type (Espinosa et al., 2003).

1.8 P53 TRANSCRIPTIONAL ACTIVATION DOMAINS

The N-terminal 73 amino acids of p53 expressed as a fusion protein with the DNA binding domain of the yeast GAL4 protein functions as an activator of GAL4-dependent gene expression (Fields and Jang, 1990). Refinement of this domain determined amino-acids 1-42 composed a major transcriptional activation region (TD1)

(Unger et al., 1992; Unger et al., 1993). In their studies, Unger and coworkers used fusion proteins between the GAL4 DNA binding domain and either full length or truncated p53 (amino acids 1-42) to delineate the segment of p53 required for transcriptional activation. Critical hydrophobic amino-acids for transcriptional activation (Leu-22 and Trp-23) were identified within this region (Candau et al., 1997; Chang et al., 1995; Lin et al., 1994). GAL4 DNA binding assays using the amino-terminal 53 amino acids of p53 demonstrated nearly complete abrogation of CAT activity using the p53 version containing Leu22Gln and Trp23Ser mutations ($p53^{QS1}$) (Lin et al., 1994). Protein interactions primarily occurring within TD1 include TBP, TFIIH subunits $TAF_{II}40$ and $TAF_{II}60$ (Thut et al., 1995), and human $TAF_{II}31$ (Chang et al., 1995; Lu and Levine, 1995b).

The $p53^{QS1}$ variant and the murine equivalent ($p53^{L25Q/W26S}$) are commonly used as transactivation deficient versions of p53 (Attardi et al., 1996; Cregan et al., 2004; Haupt et al., 1995; Jimenez et al., 2000; Johnson et al., 2005b). The $p53^{QS1}$ variant reportedly retains some p53 activity despite a profound transactivation defect (Chen et al., 1996b; Haupt et al., 1995; Zhu et al., 1998; Zhu et al., 2000). Specifically, the $p53^{QS1}$ variant retains the ability to induce apoptosis in some cellular contexts but is unable to induce G1 arrest (Haupt et al., 1995; Johnson et al., 2005b; Zhu et al., 1998; Zhu et al., 2000). Furthermore, the homozygous $p53^{QS1}$ knock-in mice undergo embryonic lethality although p53 has a limited requirement during embryonic development (Johnson et al., 2005b; Johnson and Attardi, 2005). Therefore, the $p53^{QS1}$ variant is not equivalent to the complete loss of p53.

A second functional transactivation sub-domain in the N-terminus of p53 has been identified through a similar strategy. Amino acids 43 to 73 of p53 fused to the DNA

binding domain of GAL4 are able to drive Gal4-dependent reporter gene expression and two critical hydrophobic amino acids (Trp-53 and Phe-54) are critical for this activity (Candau et al., 1997; Chang et al., 1995; Zhu et al., 2000). Protein interactions occurring within TD2 include replication protein A (Bochkareva et al., 2005) and the TFIIH subunit p62/Tfb1 (Di et al., 2006; Xiao et al., 1994). Like the $p53^{QS1}$ variant of p53, the $p53^{W53Q/F54S}$ variant ($p53^{QS2}$) is defective in sequence-specific transactivation, when the expression of a small number of well-characterized p53 target genes is assessed (Chen et al., 1996b; Cregan et al., 2004; Zhu et al., 1998; Zhu et al., 2000). The $p53^{QS2}$ variant is reported to retain the ability to induce p53-dependent G1 arrest but not p53-dependent apoptosis (Zhu et al., 2000). Transcriptional activation of $p53^{QS1}$ and $p53^{QS2}$ was studied in human osteosarcoma (Saos-2) cells using a secreted alkaline phosphatase reporter plasmid driven by a p53-dependent site. Using this model system, the two transactivation domains differentially contribute to p53s ability to transcriptionally activate genes where $p53^{QS2}$ retains more transcriptional activity than $p53^{QS1}$. Importantly, both domains are drastically compromised retaining less than 50% of wild type p53 activity (Candau et al., 1997). A quadruple mutant $p53^{QS1/QS2}$ has also been studied and is considered transcriptionally inactive (Cregan et al., 2004; Venot et al., 1999; Zhu et al., 1998).

Murine based studies of the induction of BH3-only proteins, PUMA and NOXA, by $p53^{QS1}$ or $p53^{QS2}$ revealed that PUMA is primarily controlled by TD1 whereas induction of NOXA is controlled by both TD1 and TD2 (Cregan et al., 2004). Therefore, despite the fact that the $p53^{QS1}$ and $p53^{QS2}$ variants have defects in sequence-specific transactivation, they exhibit some distinct biologic activities. This has led several laboratories to hypothesize that these domains function independently in regulating distinct subsets of

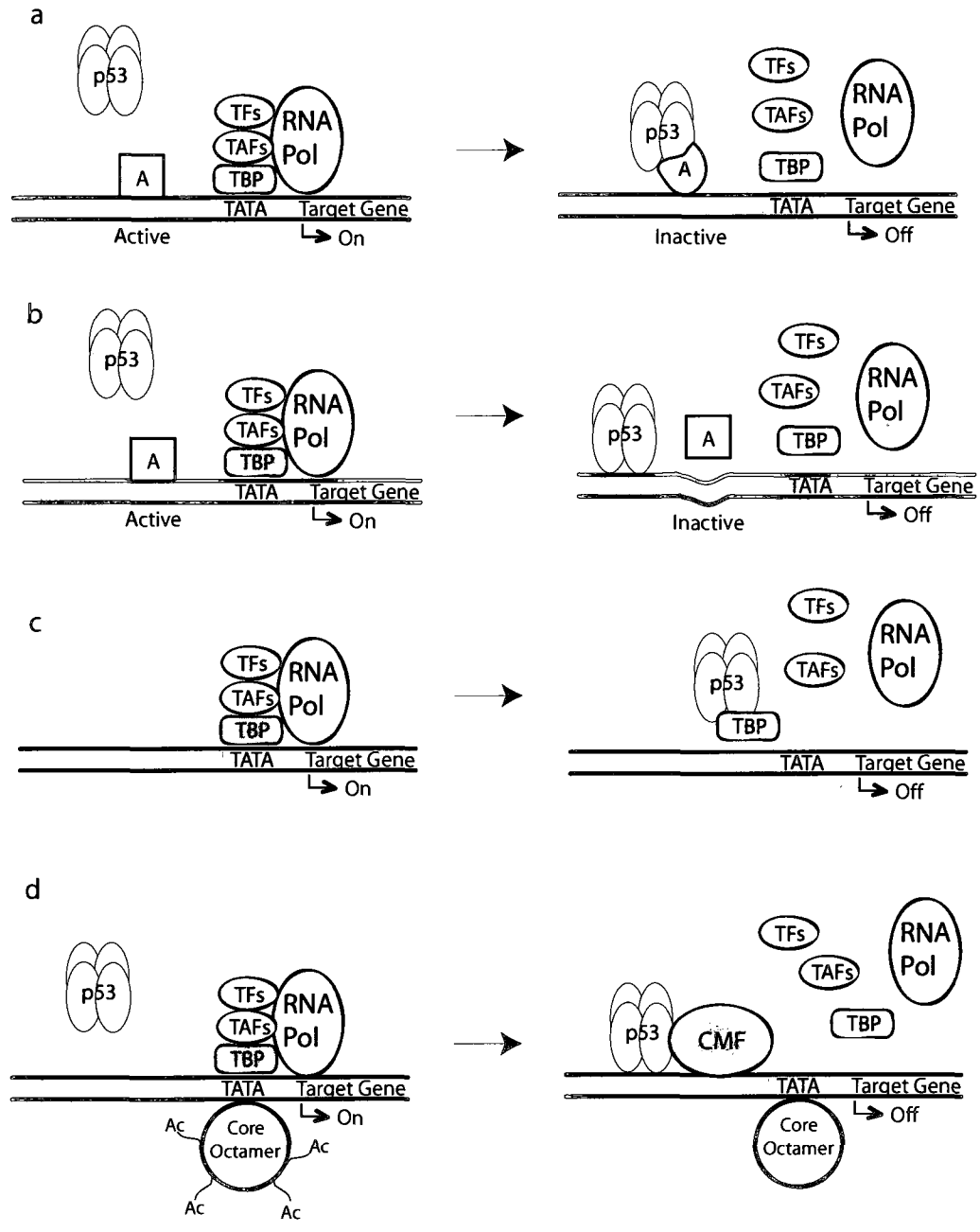
p53 target genes (Cregan et al., 2004; Johnson et al., 2005b; Johnson and Attardi, 2005; Zhu et al., 1998; Zhu et al., 2000).

The N-terminus is primarily unstructured but does contain short structured regions (Lee et al., 2000). Two such structured regions are contained within amino acids 18 to 26 and from amino acids 40 to 44 and 48 to 53. The protein interactions occurring within this region are believed to rely on the presence of these structural elements. The $p53^{QS1}$ and $p53^{QS2}$ variants contain alterations of this structure and interaction defects with several binding partners including MDM2 and p300 are detected as a result (Scolnick et al., 1997; Teufel et al., 2007; Venot et al., 1999).

1.9 TRANSCRIPTIONAL REPRESSION BY P53

p53 is not only recognized as a transcriptional activator but also as a repressor of specific genes (Figure 8). Unlike transcriptional activation, p53-mediated repression can be achieved through several different mechanisms. Transcriptional repression is an important tumour suppressive function as it modulates the levels of genes in several different biological pathways such as c-fos (Kley et al., 1992), topoisomerase IIα (Wang et al., 1997), RECQ4 (Sengupta et al., 2005), Werner Helicase gene (WRN) (Yamabe et al., 1998), POLD1 (Li and Lee, 2001), survivin (Hoffman et al., 2002), interleukin 6 (Santhanam et al., 1991), Map4 (Murphy et al., 1996; Murphy et al., 1999), hTERT (Kanaya et al., 2000; Xu et al., 2000), VEGF (Mukhopadhyay et al., 1995; Pal et al., 2001; Zhang et al., 2000), protein kinase Cα (PKCα) (Zhan et al., 2005) and stathmin (Ahn et al., 1999; Murphy et al., 1999) among others.

Transcriptional repression by p53 can generally be divided into three categories (Figure 8). The first category is frequently described by the recruitment inhibition model


a
p53
TFs
TAFs
TBP
RNA Pol
A
TATA
Target Gene
Active
On
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Target Gene
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Target Gene
On
Ac
Core Octamer
Ac
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Ac
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CMF
TFs
TAFs
TBP
RNA Pol
TATA
Target Gene
Off
Core Octamer

Figure 8. Transcriptional repression by p53.

Different modes of p53-dependent transcriptional repression. Abbreviations: A, site-specific transcriptional activator; CMF, chromatin-modifying factor; the other abbreviations are as in Figure 7. (a) p53 inhibits transcription by physical interaction and inactivation of the specific activator or (b) by displacing it from the adjusting or overlapping binding sites within the promoter. (c) Transcriptional repression by affecting the assembly of transcription machinery though the direct interaction with basal transcription factor(s). (d) Transcriptional repression via recruiting CMF(s) (for example, HDAC) followed by subsequent modification of core histones and promoter closing (adapted with permission to reproduce from Laptenko and Prives, 2006).

(Ho and Benchimol, 2003). In this model p53 interacts and forms a complex with transcriptional activators thereby prohibiting their activity (Borellini and Glazer, 1993). This can occur through competitive displacement where p53 binds to a DNA region overlapping with other transcription factors. Such a phenomena is seen in several genes including alpha-fetoprotein (Crowe et al., 1999; Lee et al., 1999; Ogden et al., 2001), hepatitis B virus (Ori et al., 1998), and polymerase δ catalytic subunit gene (Li and Lee, 2001). Additionally, non-competitive DNA binding can promote repression when the interaction of p53 with DNA alters the activity of nearby transcription factors. This mechanism has been observed during the regulation of IEX-1 (Im et al., 2002) and Bcl-2 (Budhram-Mahadeo et al., 1999; Wu et al., 2001).

p53 is also able to promote transcriptional repression by sequestering several critical transcriptional factors including TBP and Sp1 (Borellini and Glazer, 1993; Farmer et al., 1996; Yamabe et al., 1998). Early experiments reveal a role for the interaction of p53 with TBP during gene repression (Seto et al., 1992). Using CAT assays in the p53 null SAOS-2 cell line, Seto *et al.* demonstrated that transient expression of p53 activated genes that contain a p53 response element and inhibited genes lacking a p53 response element. The role of TBP during this repression was examined by first selectively heat inactivating the TFIID complex leading to an overall decrease in gene transcription. Addition of recombinant TBP resulted in a partial rescue of transcription. However, the ability of TBP to restore transcriptional activity was abrogated if TBP was first incubated with p53, thereby suggesting a repressive role for the interaction of TBP and p53. Finally, a physical interaction between p53 and TBP was demonstrated using purified proteins (Seto et al., 1992). Interference with the activity of SP1 has been implicated in the repres-

sion of telomerase reverse transcriptase (Kanaya et al., 2000), insulin receptor (Webster et al., 1996), insulin-like growth factor-I receptor (Ohlsson et al., 1998), VEGF (Zhang et al., 2000), and POLD (Li and Lee, 2001).

P53-DNA binding is a critical step in the repression of many genes. The promoter of the DNA polymerase catalytic subunit gene (POLD1) contains a p53 RE which is bound by p53 (Li and Lee, 2001). This transrepression was lost when mutants impaired for p53-consensus binding were assayed, thus demonstrating a requirement for site specific DNA binding. Furthermore, the p53 RE bound by p53 surrounds a Sp1 binding site. Therefore, during p53 binding and transrepression, the activity from the POLD1 promoter is further repressed by the inhibition of Sp1 interaction. This provides a dual mechanism for p53 transrepression.

Histone deacetylases also have a pertinent role during p53-mediated transcriptional-repression. The involvement of histone deacetylases (HDACs) was documented in experiments examining the p53-mediated repression of the *Map4* and *stathmin* genes (Murphy et al., 1999). In order to examine p53-mediated repression, the Val5 murine cell line which harbors a temperature-sensitive mutant of p53, was used as well as MCF7 and A2780 cells treated with adriamycin to induce endogenous p53. In conjunction with p53 activation, they examined *Map4* and *stathmin* repression levels in the presence or absence of the histone deacetylase inhibitor trichostatin A (TSA). They noted that in the presence of TSA there were significantly lower levels of repression. Subsequently, using co-immunoprecipitation assays they determined that p53 interacted with HDACs through the mSin3a co-repressor and that both p53 and mSin3a bind at the promoter of Map4 during its deacetylation (Murphy et al., 1999).

The role of mSin3a and HDACs during p53-mediated gene repression was further studied in hematopoietic cells during the repression of bcl-2 (Wu et al., 2001). Wu *et al.* (2001) observed that p53 and TBP bound to the TATA sequence and that co-immunoprecipitation of a complex containing p53, TBP, histone-deacetylase-1 and mSin3a was possible. Also, as previously described for *Map4* and *stathmin*, treatment with TSA resulted in the increased activity of the promoter (Wu et al., 2001). Taken together, these experiments demonstrate that repression by p53 can occur *via* interactions in the promoter region at the TATA site with TBP, mSin3a, and histone deacetylases. However, p53 has also been shown to repress TATA-less promoters (Dugimont et al., 1998; Gopalkrishnan et al., 1998; Iotsova et al., 1996; Lee et al., 1997).

Furthermore, the interplay of multiple pathways during transcriptional repression is highlighted with studies of the RECQ4 promoter. Transcriptional repression of the RECQ4 promoter requires p53-DNA binding at the promoter which also contains two SP1 sites. This inhibition is critically impaired using two tumour derived p53 mutants with defective DNA-binding capabilities: R175H and R248W. The SP1 sites are bound specifically by SP1 and are essential for p53-promoter repression (Sengupta et al., 2005). Chromatin immunoprecipitation assays demonstrate that p53 and SP1 bind to the promoter during non-repressive conditions. However, during repressive conditions, both p53 and SP1 are dissociated from the promoter and a p53-SP1 complex can be coimmunoprecipitated. This coimmunoprecipitation can be inhibited by TSA therefore suggesting a requirement for HDAC-mediated events such as protein acetylation (Sengupta et al., 2005). A similar mechanism is observed at the VEGF promoter during hypoxic conditions, where p53 binds SP1 thereby inhibiting its activity (Pal et al., 2001).

1.10 COOPERATIVE TRANSCRIPTIONAL REGULATION

Several coregulators modulate p53's activity at a subset of promoters or following specific stresses. For example, BRCA1 is a well characterized modulator of p53 transcriptional activation. Through interactions in the N- and C-terminal regions of BRCA1 (Chai et al., 1999; Ouchi et al., 1998; Zhang et al., 1998), it stabilizes p53 and functions as a selective coregulator of transcriptional targets (Chai et al., 1999; Ouchi et al., 1998; Somasundaram et al., 1999; Zhang et al., 1998). BRCA1 preferentially enhances the transactivation of DNA-repair and cell cycle arrest genes but not apoptosis genes (MacLachlan et al., 2002; Ongusaha et al., 2003). Another coregulator is CDK8 which exhibits a stress-specific effect on p53 transactivation (Donner et al., 2007). The presence of CDK8 positively influences the activation of $p21^{WAF1}$ and *Hdm2*.

The family members p63 and p73 share three well conserved regions with p53: N-terminal transactivation domain, the DNA binding domain and the oligomerization domain (OD). p63 and p73 can form hetero-tertamers *via* interaction of the oligomerization domain with each other, but not p53 (Davison et al., 1999). However, select p53 mutants do interact with p63 and p73 through a region distinct from the OD (Bergamaschi et al., 2003; Di Como et al., 1999; Gaiddon et al., 2001; Irwin et al., 2003; Strano et al., 2000; Strano et al., 2002). Although the functions of p63 and p73 are primarily developmental, they may have implications in tumorigenesis. Mouse studies of p63/p73 double knock-outs demonstrate that either p63 or p73 is required during the transactivation of a subset of p53 target genes (Flores et al., 2002). These double knock-out cells are unable to properly induce *Bax, Noxa* and *PERP* but $p21^{WAF1}$ induction remains at wild type levels.

Thus, p63 and p73 are important modulators of p53 activity through an as yet unclear mechanism.

The activity of p53 at the CDKN1A promoter is not limited to binding of the p53 RE for the induction of transcription. This promoter is also equipped with six binding sites for Sp1 and Sp1 family members are implicated in its activation (Gartel et al., 2000). HDAC inhibitors are capable of transcriptionally activating CDKN1A and this activation requires the Sp1 sites (Xiao et al., 1999; Xiao et al., 2000). Furthermore, p53 can directly interact with Sp1 at the CDKN1A promoter and compete with HDAC1 for binding to the C terminus of Sp1 resulting in histone acetylation and concomitant expression of p21 (Lagger et al., 2003).

1.11 P53 CANCER THERAPEUTICS

Given the overwhelming tumour suppressive function of p53 and its aberrant expression at such a high frequency in cancers, treatments involving reintroduction of functional p53 are seemingly a good prospect for treatment. As such, several strategies have been employed to restore or increase p53 in tumours, some of which have even reached clinical trials.

Over-expression of p53 has been extensively examined as a potential therapeutic. Infection of tumour cells, with various p53 genotypes, with replication deficient p53-expressing adenovirus (Ad-p53) induces cell cycle arrest and apoptosis *in vitro* and *in vivo* (Cirielli et al., 1995; Clayman et al., 1995; Katayose et al., 1995; Liu et al., 1995; Mujoo et al., 1996; Seth et al., 1996; Yang et al., 1995; Zeng et al., 1997; Zhang et al., 1994). Although Ad-p53 has never seen significant clinical use, the selective replicating oncolytic adenovirus ONYX-015 (Bischoff et al., 1996) has been extensively studied for

its anti-neoplastic properties (Chiocca et al., 2004; Ganly et al., 2000; Habib et al., 2002; Hamid et al., 2003; Hecht et al., 2003; Khuri et al., 2000; Lamont et al., 2000; Mulvihill et al., 2001; Nemunaitis et al., 2000; Nemunaitis et al., 2001; Reid et al., 2001; Reid et al., 2002; Rudin et al., 2003; Vasey et al., 2002).

As mutant p53 is frequently present at high levels, another possible therapeutic avenue involves the restoration of wild-type structure and function to p53 or targeting of p53 inhibitory proteins. Exploitation of this feature has resulted in the development of two such small molecules CP-31398 (Foster et al., 1999) and PRIMA (Bykov et al., 2002) which are able to restore wild type p53 function and structure to different degrees.

Partial or complete inhibition of p53 function accounts for the majority of tumours unrelated to p53 mutation (Soussi, 2000; Woods and Vousden, 2001) and results from over-expression of cellular MDM2/MDMX (Michael and Oren, 2002), viral HPV E6 (Tommasino et al., 2003) or downstream components of the p53 pathway such as Arf (Sherr and Weber, 2000). Targeting of MDM2/MDMX, HVP E6 and restoration of downstream components in these situations are promising prospective therapeutic avenues. Additionally, the p53-MDM2 interaction has been extensively studied resulting in the rational design of numerous compounds (Chene et al., 2002; Chene, 2003; Duncan et al., 2001; Garcia-Echeverria et al., 2000; Issaeva et al., 2004; Kanovsky et al., 2001; Stoll et al., 2001; Vassilev et al., 2004). Human papilloma virus protein E6, physically interacts with p53 and promotes its degradation by cellular E3-ubiquitin-ligase E6AP (Huibregtse et al., 1993). Therefore, similar attempts to inhibit its targeting of p53 have been conducted and specific molecules have been designed to achieve this end. Two primary methods of negating the effects of HPV E6 include inhibition of its transcription

using a variety of techniques (Abdulkarim et al., 2002; Alvarez-Salas et al., 1998; Goodwin and DiMaio, 2000; Hamada et al., 1996; Jiang and Milner, 2002; Maehama et al., 1998; von Knebel et al., 1992) and interfering with HPV function (Beerheide et al., 1999; Butz et al., 2000).

Confounding the use of p53 as a therapeutic, the ectopic expression of p53 in humans can pose additional risks to the patient. p53 may, in some normal tissues, function as a sensitivity determinant to genotoxic stress (Komarov et al., 1999; Komarova et al., 1997; Komarova and Gudkov, 2001). Therefore, p53 suppression has been examined as an approach for the treatment of side effects such as tissue injury (Gudkov and Komarova, 2003; Komarova and Gudkov, 1998; Komarova and Gudkov, 2001).

The cellular decision to undergo cell cycle arrest or apoptosis when faced with genotoxic stress is complex and has been modeled in three general ways (reviewed Gudkov, Komarova, 2003). Firstly, the amount of p53 induced in response to the insult can determine whether the cell undergoes DNA repair and survives or undergoes apoptosis where elevated levels of p53 are predictive of apoptosis(Vousden, 2000). Secondly, the spectrum of available p53-responsive genes for a quick response (those in active chromatin) can vary between cell lineage and be a determinant of outcome (Schmitt et al., 2000). Thirdly, co-factor availability can alter cell fate by influencing the ability of p53 to bind DNA and transactivate specific genes (Oda et al., 2000b; Samuels-Lev et al., 2001; Wahl and Carr, 2001).

1.12 HYPOTHESIS AND OBJECTIVE

Hypothesis: TD1 and TD2 differentially contribute to p53 transcriptional activation of target genes.

Corollary: Expression of p53 with point mutations of critical amino acids within TD1 and TD2 will give rise to different patterns of target gene activation.

Objective: Evaluate the contributions of TD1 and TD2 during p53 mediated transcriptional regulation.

1.13 IMPORTANCE OF THE CHOSEN PROBLEM

Transcriptional regulation by p53 is arguably its most crucial biological function. Previous studies based on specific subsets of human genes and in murine models have suggested that the two transactivation sub-domains may regulate genes involved in different pathways (notably cell cycle arrest or apoptosis). If specific subsets of genes are differentially regulated by the transactivation domains, understanding this pattern could prove groundbreaking, not only in furthering our understanding of p53, but could also potentially have a great impact in cancer therapeutics. The pursuit of a clear understanding of p53 biological functions will be significantly advanced by a genome wide analysis of the contribution of its TADs.

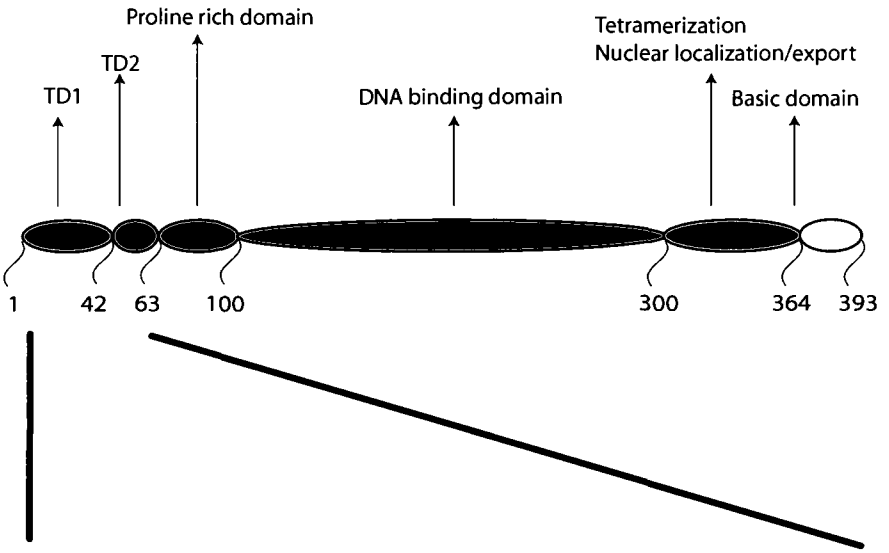
2. MATERIALS AND METHODS

2.1 CELL AND ADENOVIRUS CULTURE

The HCT116 $p53^{-/-}$ cell line was kindly provided by Dr. Bert Vogelstein (John's Hopkins University) and HCT116 cell line was obtained from American Type Tissue Collection (ATCC) (Rockville, MD). Cells were maintained in McCoy's 5A media supplemented with 10% fetal bovine serum (Wisent, St. Bruno, Quebec, Canada) in an incubator at 37°C with 5% CO_2. A549 lung carcinoma cells were obtained from ATCC. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Wisent, St. Bruno, QC). GM38 fibroblasts were obtained from the National Institute of General Medical Sciences Mutant Cell Repository (Camden, NJ). Cells were maintained in DMEM supplemented with 15% fetal bovine serum (Wisent, St. Bruno, QC).

Cell lines were routinely tested for mycoplasma contamination. For mycoplasma testing, cells were grown overnight on a cover slip in antibiotic-free growth media, washed with PBS and fixed with 100% cold methanol (two times for 5 minutes each). Cells were rewashed with PBS and allowed to air dry. The cells were then covered with Hoechst stain and placed in the dark for 15 minutes. The stain was then aspirated and cells were washed with PBS (twice for 5 minutes each in the dark). Cover slips were then mounted onto a slide and examined by microscopy.

Adenovirus constructs expressing wild type p53 ($p53^{wt}$), $p53^{L22Q/W23S}$ ($p53^{QS1}$), $p53^{W53Q/F54S}$ ($p53^{QS2}$), and $p53^{L22Q/W23S/W53Q/F54S}$ ($p53^{QS1/QS2}$) were kindly provided by Dr. Ruth Slack (Figure 9) (University of Ottawa, Ontario, Canada). The adenovirus Ad-$BHG^{\Delta E1\Delta E3}$ containing deletions of the E1 and E3 regions (Ad-empty) was generously



p53wt **meepqsdpsveppisqetfsdiwkllpennvispipsqamddlmlspddieqwftedpgpdea**

QS1 **meepqsdpsveppisqetfsdqsklipennvispipsqamddlmlspddieqwftedpgpdea**

QS2 **meepqsdpsveppisqetfsdiwkllpennvispipsqamddlmlspddieqqstedpgpdea**

QS1/QS2 **meepqsdpsveppisqetfsdqsklipennvispipsqamddlmlspddieqqstedpgpdea**

Figure 9. Recombinant adenovirus constructs.

Schematic representation of p53 with amino acid sequence of wild type p53 expressed from adenovirus constructs and amino acid sequences of p53 variant constructs. Transactivation domain one is represented light brown. Transactivation domain two is represented in dark brown. Point mutations are represented in bold.

provided by Dr. Frank Graham (McMaster University, Canada). Viruses were propagated using human embryonic kidney (HEK293) cells and cesium chloride (CsCl) gradient purification as previously described (Ng and Graham, 2002). Briefly, 30 80-90% confluent 150mm dishes were infected with adenovirus. Upon evidence of near complete cytopathic effect, cells were collected by scraping into the medium, transferred to a centrifuge bottle and spun at 750 xg for 10 minutes. Cells were resuspended in 15 mL of 0.1 M Tris-HCl, pH 8.0 and stored at -70°C until purification. Cells were thawed, 1.5 mL of 5% Na deoxycholate was added per 15 mL of cell lysate, and incubated at room temperature for 30 minutes. For each 15 mL of lysate, 150 µL of 2 M $MgCl_2$ and 75 µL DNase I solution was added, mixed, incubated at 37°C for 60 minutes with mixing every 10 minutes, and spun at 3000 xg for 15 minutes at 5°C. Samples were then applied over CsCl gradients in SW 41 ultraclear tubes (0.5 mL of 1.5 g/mL CsCl overlayed with 1.35 g/mL CsCl and overlayed again with 1.25 g/mL CsCl) and spun at 35 000 rpm in a Beckman SW 41 rotor (151 000 xg) at 10°C for one hour. Virus band was collected from 1.25/1.35 g/mL interface and placed in a Beckman SW 50.1 ultraclear tube (topped up with 1.35 g/mL CsCl), centrifuged in a SW50.1 rotor at 35 000 rpm (115 000 xg) for 16 to 20 hours at 4°C. Virus bands were collected and transferred to Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific, Rockford, Illinois) and dialyzed against three changes of 500 mL Tris-HCL, pH8.0 over at least 24 hours. Glycerol was added to a final concentration of 10% and purified virus was stored in small aliquots at -70°C. Virus titers were determined in HEK293 cells by standard methods (Ng and Graham, 2002) and titers are expressed as plaque-forming units per milliliter (pfu/ml). Briefly, serial dilutions of purified virus were prepared and 60mm dishes of HEK293 cells were infected for 1 hour at 37°C in 0.5 ml of

serum free media. Infected cells were then overlayed with 5 mL of overlay media (1x MEM, 0.5 % agarose, 5% horse serum, Gentamycin, 2.5% (w/v) yeast extract). Plaques were counted and titers determined approximately 10 to14 days post-infection.

2.2 RNA ISOLATION AND QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

HCT116 $p53^{-/-}$ cells at 70% to 80% confluence were infected with 25 pfu/mL of indicated adenovirus in serum-free media for 1 hour. Growth medium containing 10% fetal bovine serum was replaced and cells were returned to the incubator for the indicated time. Infected cells were collected and total RNA was isolated using the RNeasy RNA isolation kit (Qiagen, Valencia, California, USA) according to manufacturer's specifications. Five micrograms of total RNA was reverse-transcribed using a first-strand cDNA synthesis kit (MBI Fermentas, Burlington, ON, Canada). Quantitative RT-PCR was performed using the SYBR Green Fluorescent DNA stain (Invitrogen, Burlington, ON, Canada), a LightCycler 2 quantitative PCR machine (Roche Diagnostics, Mannheim, Germany), and LightCycler software version 3 (Roche Diagnostics, Mannheim, Germany). Samples without the addition of reverse-transcriptase were used as controls. The primers used were:

ACTB (GGGCATGGGTCAGAAGGAT and GTGGCCATCTCTTGCTCGA),

APAF1 (CAACGGGAGATGACAATG and CTGGAGAAAAGCAAAGGTC),

BAK1 (GCCATCAGCAAGGAACAGGAG and ACACCCAGAACCACCAGCAC),

BTG2 (CACAGAGCACTACAAACACC and ACAAGACGCAGATGGAGC),

CASP6 (GCTTTGTGTGTGTCTTCC and CTCAGTTATGTTGGTGTCC),

CDKN1A (CCTCAAATCGTCCAGCGACCTT and CATTGTGGGAGGAGCTGTGAAA),

TNFRSF6 (CTCATCTTAATGGCCTAATGCA and GCTTCAGTTTATAACTATCTTCAC), TNFRSF10B (GGCATCATCATAGGAGTCAC and GTCAAAGGGCACCAAGTC), TP53I3 (TCTCTATGGTCTGATGGG and TTGCCTATGTTCTTGTTG), and MafB (TGCTGAGAGAGAGAACCGAGAG and CACCACCAAGAACTCTTCCTAC), for forward and reverse priming respectively. Alternatively, quantitative RT-PCR was performed using TaqMan Universal PCR Master mix and an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, California, USA) according to manufacturer's instructions. The results were analyzed using the 7500 Fast Real-Time PCR Systems software. Taqman primers and probe sets used were: p53 (Hs99999147_m1), β-actin (4326315E), MafB (Hs00534343_s1), and CDKN1A (Hs00355782_m1). Use of either technique yielded comparable results during our experiments.

2.3 OLIGONUCLEOTIDE MICROARRAY ANALYSIS

HCT116p53$^{-/-}$ cells were infected with 25 pfu/cell of indicated virus (Ad-BHG$^{\Delta E1\Delta E3}$, Adp53^{wt}, Adp53^{QS1}, Adp53^{QS2}, or Adp53$^{QS1/QS2}$) for one hour in serum media in the incubator and then supplemented with fresh media overnight. Total RNA was collected from HCT116p53$^{-/-}$ cells infected for 16 hours using the RNeasy Mini kit (Qiagen, Valencia, California, USA). RNA quality was assessed using an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, California, USA). Human Genome U133plus2.0 oligonucleotide arrays (Affymetrix, Santa Clara, California, USA) were used for expression analysis. Experimental procedures were performed according to the manufacturer specifications at the Ottawa Genomics Innovation Centre Affymetrix Gene- Chip Facility (Ottawa, Ontario, Canada). Affymetrix Microarray Suite 5.0 (MAS5.0) software was used to

analyze the microarray data. MAS6.0 software uses a nonparametric Wilcoxon signed rank test to determine whether statistically meaningful differences in probe cell intensities were detected between samples (change calls were determined using γ_{1H} and γ_{1L} values of 0.0025). Genes were considered to be induced if and only if they were statistically ($P \leq .0025$) increased in all experiments compared to Ad-control infected controls by an average of two-fold. Genes were considered to be repressed if and only if they were statistically ($P \leq .0025$) decreased in all experiments compared to Ad-control infected controls by an average of half.

2.4 WESTERN BLOT ANALYSIS

Cells were washed with cold PBS and collected by centrifugation at 1000 rpm for 5 minutes. Total protein was extracted from cells using 1% sodium dodecyl sulfate or RadioImmunoPrecipitation Assay (RIPA) buffer supplemented with Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Laval, Quebec) and sonicated at 35% with a micro tip (Fisher 300 Sonic Dismembranator, Fisher Scientific, Ottawa, ON). Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories Inc., Hercules, California, USA). The protein samples were mixed with 5X SDS sample buffer supplemented with DTT for each sample. Proteins were separated by SDS-PAGE (4% to 12% Bis–Tris acrylamide), transferred to a nitrocellulose membrane (Hybond-C; Amersham Biosciences, Piscataway, New Jersey, USA) and blocked with 5% skim milk and TBST (10mM Tris pH7.6, 150nM NaCl, 0.05% Tween-20). Membranes were sequentially incubated with primary antibody diluted in 5% skim milk for one hour at room temperature and a horseradish peroxidase (HRP)-conjugated secondary antibody for one hour at room temperature. Monoclonal antibodies raised against p53 were DO-1

(Ab6; Calbiochem, San Diego, California, USA), Pab1801 (Ab2; Calbiochem, San Diego, California, USA), and Pab421 (Ab1; Calbiochem, San Diego, California, USA). Additional antibodies were raised against $p21^{WAF1}$ (Ab1; Calbiochem, San Diego, California, USA), PUMA (Ab1; Calbiochem, San Diego, California, USA), MDM2 (SMP14; Santa Cruz Biotechnology, Santa Cruz, California, USA) and MafB (P-20; Santa Cruz Biotechnology, California, USA). Each incubation was followed by five 5 minute washes with TBST. Protein bands were detected by applying the chemiluminescent substrate SuperSignal WestPico Chemiluminescent Substrate kit (Pierce, Rockford, Illinois, USA) for one minute followed by multiple exposures of varying durations to X-OMAT X-ray film (Kodak, Rochester, New York, USA).

2.5 IMMUNOPRECIPITATION AND MASS SPECTROMETRY

HCT116 $p53^{-/-}$ cells were infected with 25 pfu/cell of $Adp53^{wt}$ for one hour in serum-free media in the incubator and subsequently supplemented with fresh media overnight. Twenty-four hours post-infection cells were washed twice with PBS and scraped into PBS on ice. Cells were then treated as per manufacturer's instructions for the use of Protein A–agarose beads (Roche Molecular Biochemicals, Laval, Quebec). Protein lysates were immunoprecipitated with p53 antibody Pab42. Precipitated protein extracts were run on a 4% to 12% Bis–Tris polyacrylamide gel and subsequently treated with GelCode Blue Stain Reagent according to manufacturer's specifications (Pierce). Bands of interest were excised for analysis. Trypsin digestion and Matrix Assisted Laser Desorption/Ionization Time-of-Flight tandem mass spectrometry (MALDI-TOF MS/MS) was performed at the Ontario Genomics Innovation Centre Proteomics Facility at the Ot-

tawa Health Research Institute (Ottawa, Ontario, Canada). Peptides were identified using Mascot (Perkins et al., 1999).

2.6 CHROMATIN IMMUNOPRECIPITATION ASSAYS

HCT116p53$^{-/-}$ cells were infected with 5 pfu/cell of indicated virus for 24 hours. Chromatin immunoprecipitation (ChIP) assays were performed as previously described with only minor changes (McKay et al., 2004). Briefly, 1x10^6 cells were collected and washed with ice cold PBS, proteins and DNA were crosslinked with 1% formaldehyde in growth medium. Isolated chromatin was sonicated to an average length of 700 bp with a microtip sonifier (Branson Sonifier) and cleared of debris by centrifugation. The fragmented chromatin samples were frozen in liquid nitrogen and stored at -80°C. Prior to immunoprecipitation, the chromatin was diluted 5-fold and precleared with protein A-agarose (Roche Molecular Biochemicals, Laval, Quebec) for 50 min at 4°C. Twenty percent of each pre-cleared sample was retained as a positive control for PCR amplification and the remainder of the solution was divided into equal aliquots and incubated overnight at 4°C, either without antibody or with 5 μg of p53 antibody (Ab-6 antibody, Calbiochem, San Diego, California, USA). Immune complexes were collected overnight at 4°C with the addition of 60μl of protein A-agarose (Roche Molecular Biochemicals, Laval, Quebec). Crosslinks were reversed by addition of NaCl to a final concentration of 0.3 M with RNase A and incubated at 65°C for 4–5 h. DNA was precipitated in 70% ethanol, collected by centrifugation, and proteinase K treated for 2 h at 45°C. DNA was purified by using QiaQuick spin columns (Qiagen, Valencia, California, USA) and was eluted in 10 mM Tris, pH 8.0. Semiquantitative RT-PCRs were performed on the enriched DNA

fragments using a GenAmp PCR System 9700 (Applied Biosystems, Foster City, California, US) and the following primers:

MafB RE (CGTCTACAACCCTCTTCG and GTGATCTGGAAACGAGTG),

Maspin (GAGAGGATTGCCGTACGCATGT and ACATCCAGGTCTTTGTGCTCC) and GAPDH (GTATTCCCCCAGGTTTACAT and TTCTGTCTTCCACTCACTCC).

2.7 MITOCHONDRIAL ISOLATION ASSAYS

HCT116p53$^{-/-}$ cells were infected with 25 pfu/ml of indicated virus for one hour in serum free media and then returned to the incubator with fresh media overnight. Cells were washed with PBS and collected by gentle scraping, spun at 2000 RPM at 4°C for 5 minutes and the pellet was frozen in liquid Nitrogen and stored at -80°C. The pellet was then resuspended in TD buffer (25 mM Tris pH 7.5, 137 mM NaCl, 10 mM KCl, 0.7 mM Na_2HPO_4) and spun at 2000 RPM at 4°C for 5 minutes. The pellet was resuspended in CaRSB buffer (10 mM NaCl, 1.5 mM CaCl2, and 10 mM Tris-HCl, pH 7.5) and incubated for 10min on ice. Suspension was then dounce homogenized with a type B pestel and transferred to a clean tube and spun at 2000 RPM at 4°C for 5 minutes. Centrifugation was performed on three successive supernatants. The supernatant from the final spin was subsequently applied to a discontinuous sucrose gradient (1M and 1.5M). Gradients were centrifuged in SW-41 swinging rotor at 26000 RPM for 35 minutes at 4°C. Concentrated mitochondria were collected from the interface of the 1M and 1.5M sucrose gradient with an 18G needle and aliquoted. Mitochondria were then spun at 16 000g for 15 minutes at 4°C. Supernatant was again aspirated and pellets were pooled and washed once with MS buffer. Mitochondria pellets were resuspended in MS buffer (210 mM

mannitol, 70 mM sucrose, 5 mM Tris pH 7.6, 5 mM EDTA), aliquoted and frozen in liquid Nitrogen and stored at -80°C.

Immunoblot analysis of whole cell extract and mitochondria fractions were performed as described above. An antibody raised against mitochondrial heat shock protein 70 (mtHSP70) was used as a mitochondrial marker (MA3-028, Affinity BioReagents Inc., Golden, Colorado, USA). An antibody raised against proliferating cell nuclear antigen (PCNA) was used as a nuclear marker (MAB424, Chemicon International Inc., Temecula, California, USA). P53 protein was assessed using p53 Ab-1 antibody (Calbiochem, San Diego, California, USA).

2.8 RNA INTERFERENCE

Transient transfections were performed in indicated cell lines grown in antibiotic-free media for a minimum of 48 hours prior to transfection. Duplex siRNA, targeting p53 (D-003329-07) or control non-targeting duplex (Dharmacon Inc., Lafayette, Colorado, USA) at 50 nM final concentration was transfected using Oligofectamine Reagent in OptiMEM (Invitrogen, Burlington, Ontario) according to manufacturer's instructions into cells at 50–80% confluence in 10 cm culture dishes. Cells were sub-cultured into two dishes, 24 hours following transfection. When required, cells were treated with 0 or 20 $j \cdot m^{-2}$ of UV light and collected at the indicated times for either immunoblot or RT-PCR analysis.

2.9 ELECTROPHORETIC MOBILITY SHIFT ASSAYS

HCT116 $p53^{-/-}$ cells were infected with 25 pfu/ml of Ad-$p53^{wt}$ for one hour in serum free media and then returned to the incubator with fresh media overnight. Cells were harvested and washed twice with cold PBS, resuspended in lysis buffer (100 mM Hepes,

5 mM $MgCl_2$, 2.5mM EDTA, 0.5M KCL, 20% glycerol, 1% Nonidet P-40, 0.5mM PMSF and Complete protease Inhibitor Cocktail (Roche Molecular Biochemicals, Laval, Quebec)), incubated on ice for 15 minutes with frequent gentle agitation, and spun 5 minutes at 13 000 rpm and 4°C. The protein concentration of the supernatant was determined using the Bradford assay. The supernatant was aliquoted into pre-chilled tubes and stored at -80°C.

^{32}P-labeled probes were created by mixing: single-stranded oligonucleotide, optikinase, optikinase buffer, ^{32}P-γ-ATP. After brief centrifugation, mixture was incubated at 37°C for 30-60 minutes. Excess ^{32}P was removed using a G-50 column. Probes were heated at 95°C for 3 minutes and the complimentary oligonucleotide was then added, mixed, and spun briefly. The strands were annealed by reheating to 95°C for 3 minutes and then allowed to cool in a beaker filled with boiling water for several hours. Probes were then stored at -20°C. Cold probes (without ^{32}P) were prepared by annealing the DNA strands with 3 minutes of heating followed by cooling as described above. The oligonuclotides used are described in Table 1.

Samples were incubated with binding buffer (100mM Hepes pH 7.4, 250mM KCL, 5mg.ml acetylated BSA, 0.5% Triton-X, 40% glycerol), 0.1M DTT, 1mg/ml salmon sperm DNA and anti-p53 Pab421 (and cold competitors when applicable) at room temperature for 15 minutes. The anti-p53Ab2 antibody was then added to appropriate samples, mixed and incubated at room temperature for 20 minutes. Loading dye was added and samples were run in 5% Native PAGE gels at 275V. Gels were dried on a gel dryer and exposed overnight on X-OMAT Kodak film.

Table 1. Oligonucleotides used during electrophoretic mobility shift assays

	Oligonucleotide	Reverse complement olignucleotide
MafBp53RE1	GGTTGGGGACTCGCACCTTGTAAATGTCCT	AGGACATTTACAAGGTGCGAGTCCCCAACC
MafBp53RE2	CTTGTAAATGTCCTCGTCTTGTTTGAACGC	GCGTTCAAACAAGACGAGGACATTTACAAG
MafBp53RE3	TCGTCTTGTTTGAACGCAGTGAGAGCACACTCGT	ACGAGTGTGCTCTCACTGCGTTCAAACAAGACGA
MafBp53RE4	GGGACTCGCACCTTGTAAATGTCCTCGTCTTGTTTGAACGCAGT GAGAGCACACTCG	CGAGTGTGCTCTCACTGCGTTCAAACAAGACGAGGACATTTACAAGG TGCGAGTCCC
MafBp53REmut1	GGTTGGGGAATCACACCTTGTAAATATCCT	AGGATATTTACAAGGTGTGATTCCCCAACC
MafBp53REmut2	CTTGTAAATATCCTCGTATTATTTGAACGC	GCGTTCAAATAATACGAGGATATTTACAAG
MafBp53REmut3	TCGTATTATTTGAACGCAGTGAGAGAACACTCGT	ACGAGTGTTCTCTCACTGCGTTCAAATAATACGA
MafBp53REmut4	GGGAATCACACCTTGTAAATATCCTCGTATTATTTGAACGCAGT GAGAGAACACTCG	CGAGTGTTCTCTCACTGCGTTCAAATAATACGAGGATATTTACAAGGT GTGATTCCC
p21bshuman	CGTCAGGAACATGTCCCAACATGTTGAGCTCT	AGAGCTCAACATGTTGGGACATGTTCCTGACG
p21bsh-mut	CGTCAGGAATATATCCCAACTTATTGAGCTCT	AGAGCTCAATAAGTTGGGATATATTCCTGACG

2.10 FLOW CYTOMETRY

Cells were infected with an MOI of 50 of indicated Ad-virus for 48 hours and washed once with PBS. Cells were subsequently fixed in 70% ethanol overnight at -20oC and stained with propidium iodide at 4oC for a minimum of one hour. PI stained cells were analyzed via flow cytometry using either a Becton Dickinson LSR FACS station or a Beckman Coulter FACS station. Apoptosis was assessed by quantifying sub-G1 DNA content using FCS Express 3 software (DeNovo Software, Thornhill, ON).

2.11 CELLULAR VIABILITY ASSAYS

Cellular membrane permeability was assessed by PI exclusion. Cells were infected with an MOI of 50 of indicated Ad-virus for 48 hours, washed with PBS, collected using trypsin and immediately incubated with PI on ice briefly. PI stained cells were immediately analyzed using either a Becton Dickinson LSR FACS station or a Beckman Coulter FACS station. Quantification of cellular viability was performed using FCS Express 3 software (DeNovo Software, Thornhill, ON). Cells excluding PI were differentiate from cells stained with PI and a ratio between live and dead cells was determined.

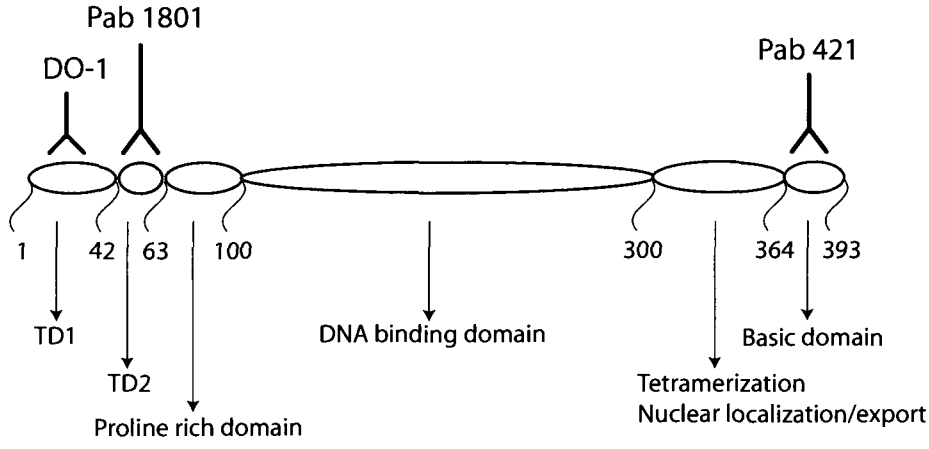
3. RESULTS

3.1 OPTIMIZATION OF P53 ADENOVIRUS-EXPRESSION EXPERIMENTAL MODEL

HCT116 cells in which p53 had been inactivated by gene targeting (HCT116 $p53^{-/-}$) (Bunz et al., 1998) were used to express wild type and transactivation domain variants of p53 from recombinant adenovirus vectors. HCT116$p53^{-/-}$ cells were infected with 25 pfu/cell of $Adp53^{wt}$, $Adp53^{QS1}$, $Adp53^{QS2}$, and $Adp53^{QS1/QS2}$. p53 variant expression was queried using a panel of anti–p53 antibodies. The binding site of the chosen antibodies overlaps with the p53 mutations in $Adp53^{QS1}$ and $Adp53^{QS2}$ thus allowing differentiation between the variant forms of p53 (Figure 10). Immunoblot analysis using any of the p53 antibodies in our panel revealed the presence of two immunoreactive bands that migrated at approximately 47 and 53 kDa following expression of $p53^{wt}$. These bands were both identified to correspond to p53 as described below. The Pab421 (amino acids 371-380) antibody was raised against a c-terminal portion of p53 and detects the three variant forms of p53. The DO-1 (amino acids 11-25) antibody recognizes an epitope within TD1 of p53 and therefore did not detect $p53^{QS1}$ or $p53^{QS1/QS2}$. Similarily, the Pab 1801 antibody (amino acids 46-55) recognizes a region within TD2 of p53 and did not detect $p53^{QS2}$ or $p53^{QS1/QS2}$.

In order to establish the identity of the distinct bands, wildtype p53 was immunoprecipitated with Pab421 and separated by SDS-PAGE (Figure 11a). The bands were gel excised and analyzed by MALDI-ToF mass spectrometry. In both bands, seven peptides were identified, each located within the DNA binding domain of p53 (Figure 11b).

a.


Pab 1801
DO-1
Pab 421
1
42
63
100
300
364
393
TD1
TD2
Proline rich domain
DNA binding domain
Basic domain
Tetramerization
Nuclear localization/export

b.

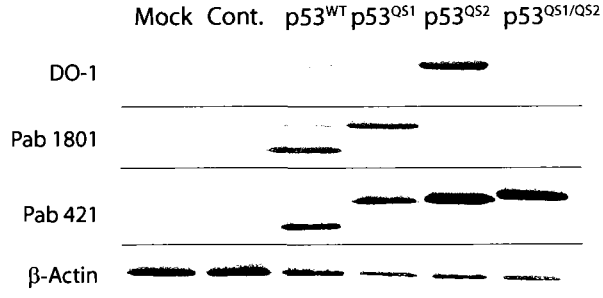
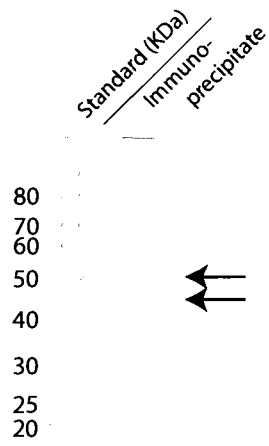

Mock
Cont.
p53WT
p53QS1
p53QS2
p53QS1/QS2
DO-1
Pab 1801
Pab 421
β-Actin

Figure 10. Antibody recognition sites and p53 Western blots.

(a) Schematic representation of epitopes recognized by the indicated monoclonal antibodies (DO-1, Pab1801, and Pab421) and corresponding p53 functional domains. (b) Immunoblot analysis of p53 expression 16 hours following infection of HCT116p53-/- cells with the indicated recombinant adenovirus. The analysis was performed using three different anti-p53 monoclonal antibodies.

a.



b.

Peptide	Band (kDa)		Position
	47	53	
1	----- TYQGSYGFR -----		102-110
2	---KPLDGEYFTLQIR---		321-333
3	--KKPLDGEYFTLQIR--		320-333
4	TCPVQLWVDSTPPPGTR		140-156
5	N/A	ERFEMFR	336-342
6	N/A	QSQHMTEVVR	165-174
7	N/A	VEYLDDRNTFR	203-213
8	N/A	CSDSDGLAPPQHLIR	182-196

Figure 11. MALDI-ToF mass spectrometry.

(a) HCT116p53-/- cells infected with p53wt were used to perform immunoprecipitation assays with anti-p53 (Pab421). MALDI -ToF MS/MS analysis was performed and several peaks corresponding to p53 were identified from the 45 kDa and 50 kDa protein isolates, several were common to both to p53 identified peaks are listed. The identified fragments and their corresponding positions within the p53 protein are listed in (b).

The epitopes recognized by this panel of antibodies span amino acids 11 through 380. Taken together, immunoblot and mass spectrometry analysis strongly suggest that both immunoreactive bands represent full length p53. We surmise that the two forms likely correspond to differentially modified forms of p53. However, the N- and C-termini of p53, containing the known modification sites, were not represented among the identified peptides and thus the specific modifications were not ascertained.

An increase in the expression of variants protein levels relative to wild-type p53 was observed (Figure 10b). An increase was expected as a reflection of the decreased transcriptional activation of MDM2 by the variants and decreased affinity of MDM2 with the QS1 variants. MDM2 is the ubiquitin ligase responsible for the rapid turnover of wild-type p53 and is unable to efficiently bind within the TAD of QS1 variants (Dai et al., 2006).

In order to determine the earliest time point at which maximal p53 protein levels were achieved, total cell lysates were collected for immunoblot analysis at 8, 12, 16, and 24 hours following infection (Figure 12). These time-course experiments demonstrated that maximal protein expression can be seen by 16 hours post-infection.

3.2 GENE REGULATION FOLLOWING ADP53^{WT} INFECTION

HCT116 p53$^{-/-}$ cells were infected with 25 pfu/cell of recombinant adenoviruses expressing; Adp53^{wt}, Adp53^{QS1}, Adp53^{QS2}, and Adp53$^{QS1/QS2}$. Total RNA was collected 16 hours post-infection. Purified RNA was subjected to oligonucleotide microarray analysis as described in materials and methods. Genes considered induced were significantly increased in all experiments and expressed at least two-fold higher than in the control samples (as described in Materials and Methods). A significant increase in the level of

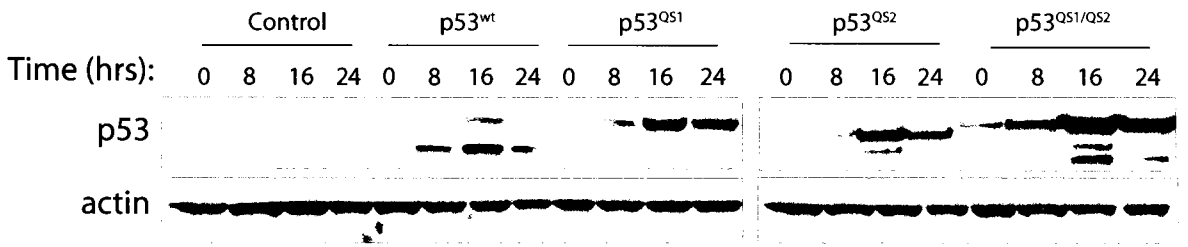

Control
p53wt
p53QS1
p53QS2
p53QS1/QS2
Time (hrs):
0 8 16 24 0 8 16 24 0 8 16 24 0 8 16 24 0 8 16 24
p53
actin

Figure 12. Adenovirus mediated p53 expression time course.

HCT116p53$^{-/-}$ cells infected with a MOI of 25 of adenovirus expressing the indicated variant of p53 were collected and examined for p53 protein content using immunoblot analysis at 0, 8, 16, and 24 hours post-infection.

254 transcripts was observed following infection with the Adp53^{wt} virus compared to control virus infection (Table 2). Of these, twenty six well characterized p53 target genes were represented including APAF1, BTG2, CDKN1A, MDM2, and TP53I3 (indicated in Table 2).

3.3 THE QS1 AND QS2 VARIANTS OF P53 ARE IMPAIRED IN P53-DEPENDENT GENE EXPRESSION

A significant increase in several transcripts was observed following infection with all adenovirus p53 variants. However, the number of transcripts and fold induction varied drastically from p53^{wt}. Of the 254 genes induced by Adp53^{wt}, only 28, 23, and 1 were induced by Adp53^{QS1}, Adp53^{QS2}, and Adp53$^{QS1/QS2}$, respectively (Figure 13 and Table 2). The mean induction level of the Adp53^{wt}-induced transcripts was significantly higher than the increase resulting from the expression of any of the QS variants. Using dot plots to examine the correlation between the expression levels of individual genes, it is noted that very few of the Adp53^{wt}-induced genes appeared to be induced to wild-type levels by either the p53^{QS1} or p53^{QS2} variants (Figure 14). Although, many p53^{wt}-regulated transcripts were induced by a given QS variant according to our definition, rarely was this expression comparable to the induction observed following Adp53^{wt} infection. Therefore, the majority of WT-, QS1-, and QS2-regulated genes were poorly induced by the QS variants. This suggests that both QS1 and QS2 are required for the transactivation of most p53 target genes.

Table 2. Genes Induced Following $p53^{wt}$ infection.

Probe Set(s)*	Gene Symbol†	Induced
218559_s_at	MAFB	5.8‡
205249_at	EGR2	4.7
224646_x_at, 224997_x_at	H19	4.5
1554340_a_at	C1orf187	4.0§
210609_s_at	TP53I3♦	3.9
211421_s_at	RET	3.8
225912_at	TP53INP1♦	3.8
205569_at	LAMP3	3.7
232165_at, 232164_s_at, 208156_x_at	EPPK1	3.5
212942_s_at	KIAA1199	3.1
210090_at	ARC	3.0
201235_s_at, 201236_s_at	BTG2♦	2.9
219583_s_at	PATA7	2.8
226913_s_at	SOX8	2.8
204859_s_at, 211554_s_at	APAF1♦	2.8
215785_s_at, 220999_s_at	CYFIP2	2.8
231115_at	GTPBP2	2.6
212070_at	GPR56	2.6
214890_s_at	DKFZP564J102	2.6
213268_at, 1555370_a_at	CAMTA1Δ	2.5
242517_at	GPR54	2.5
232289_at	FLJ14167	2.5
204780_s_at, 215719_x_at, 216252_x_at, 204781_s_at	TNFRSF6	2.4
202181_at	KIAA0247	2.4
212907_at, 228181_at	SLC30A1	2.4
222546_s_at, 218180_s_at	EPS8L2	2.4
227306_at	FLJ21245	2.4
202284_s_at	CDKN1A♦Δ	2.4
205286_at, 205287_s_at	TFAP2C	2.3
206153_at	CYP4F11	2.3
205493_s_at, 205492_s_at	DPYSL4	2.3
206832_s_at	SEMA3F	2.3
1557701_s_at	POLH	2.2
201578_at	PODXL	2.2
213469_at	PGAP1	2.2
206277_at	P2RY2	2.2
231928_at	HES2	2.2
204855_at	SERPINB5♦	2.2
203865_s_at	ADARB1	2.2
203045_a	NINJ1	2.1
235205_at	LOC346887	2.1
224724_at	SULF2	2.1
221577_x_at	GDF15♦	2.1

Probe Set(s)*	Gene Symbol†	Induced
210367_s_at	PTGES	2.1
238542_at, 221291_at	ULBP2	2.0
228315_at	N/A	2.0
218346_s_at	SESN1♦	2.0
204379_s_at, 204380_s_at	FGFR3	2.0
230356_at	Transcribed locus	2.0
203722_at	ALDH4A1	2.0
202307_s_at	TAP1♦	1.9
219099_at	C12orf5	1.9
224793_s_at	TGFBR1	1.9
219936_s_at	GPR87	1.9
212496_s_at, 212492_s_at	JMJD2B	1.9
203946_s_at	ARG2	1.9
39248_at	AQP3	1.9
205278_at	GAD1	1.8
207813_s_at	FDXR♦	1.8
203570_at	LOXL1	1.8
235230_at	PLCXD2	1.8
209050_s_at, 209051_s_at	RALGDS	1.8
219597_s_at	DUOX1	1.8
213568_at	OSR2	1.8
218032_at	SNN	1.8
227964_at	FKSG44	1.8
209712_at, 209711_at	SLC35D1	1.7
225160_x_at, 229711_s_at	MGC5370	1.7
238335_at	DNAJA5	1.7
235467_s_at	KCNC4	1.7
215411_s_at	TRAF3IP2	1.7
203310_at	STXBP3	1.7
56256_at	SIDT2	1.7
228115_at	N/A	1.7
201032_at	BLCAP	1.7
208978_at	CRIP2	1.7
209693_at	ASTN2 Δ	1.7
227247_at	PLEKHA8	1.7
227295_at	IKIP	1.7
209790_s_at	CASP6♦	1.7
213716_s_at	SECTM1	1.7
210138_at	RGS20	1.7
205483_s_at	G1P2	1.7
31846_at, 209885_at	RHOD	1.6
201412_at	LRP10	1.6
227522_at	LOC134147	1.6
219322_s_at, 236381_s_at	WDR8	1.6
201963_at	ACSL1	1.6

Probe Set(s)*	Gene Symbol†	Induced
235434_at	N/A	1.6
223754_at	MGC13057	1.6
1556194_a_at	N/A	1.6
241348_at, 219239_s_at	ZNF654	1.6
212510_at	GPD1L	1.6
336_at	TBXA2R	1.6
202627_s_at, 202628_s_at	SERPINE1♦	1.5
55081_at, 221779_at	MICAL-L1	1.5
212992_at	C14orf78	1.5
221215_s_at, 234730_s_at	RIPK4	1.5
233550_s_at, 223748_at	SLC4A11	1.5
219358_s_at	CENTA2	1.5
1555609_a_at	WIG1 Δ	1.5
227728_at, 231370_at, 203966_s_at	PPM1A	1.5
209295_at, 210405_x_at, 209294_x_at	TNFRSF10B♦Δ	1.5
204061_at	PRKX	1.5
232946_s_at	NADSYN1	1.5
212800_at, 1552618_at, 212799_at	STX6	1.5
227420_at	MGC17791	1.5
1560228_at	SNAI3	1.5
202023_at	EFNA1	1.4
201834_at, 201835_s_at	PRKAB1♦	1.4
229616_s_at	LOC196996	1.4
203068_at	KLHL21	1.4
212812_at	N/A	1.4
227221_at	N/A	1.4
225864_at	NSE2	1.4
202587_s_at	AK1	1.4
226022_at	SASH1	1.4
212558_at	SPRY1	1.4
201302_at, 201301_s_at	ANXA4	1.4
208258_s_at, 209729_at, 31874_at	GAS2L1	1.4
218007_s_at, 238935_at, 222487_s_at	RPS27L♦Δ	1.4
211833_s_at	BAX♦Δ	1.4
231269_at	ASCC3	1.4
220161_s_at	EPB41L4B	1.4
222451_s_at	ZDHHC9	1.4
226604_at, 226600_at	SMILE	1.4
242705_x_at	LRPAP1	1.4
201117_s_at	CPE	1.4
242463_x_at	ZNF600	1.4
203695_s_at	DFNA5	1.4
205386_s_at	MDM2♦	1.4
203132_at	RB1♦	1.3
204060_s_at	PRKX, PRKY	1.3

Probe Set(s)*	Gene Symbol†	Induced
210962_s_at	AKAP9	1.3
219938_s_at	PSTPIP2 [Δ]	1.3
223342_at	RRM2B [♦] [Δ]	1.3
227134_at	SYTL1	1.3
205109_s_at	ARHGEF4	1.3
224690_at	C20orf108	1.3
213271_s_at	KIAA1117	1.3
221840_at	PTPRE [Δ]	1.3
226782_at	SLC25A30	1.3
225049_at	BLOC1S2	1.3
224862_at	GNAQ [Δ]	1.3
202071_at	SDC4	1.3
224901_at	SCD5	1.3
225734_at	FBXO22	1.3
226805_at	C20orf142	1.3
211675_s_at	MDFIC	1.3
220520_s_at	FLJ20130	1.3
204547_at	RAB40B	1.3
202409_at	LOC492304	1.3
203224_at, 203225_s_at	RFK	1.2
218168_s_at	CABC1	1.2
238480_at	C18orf17	1.2
227204_at	PARD6G	1.2
211272_s_at	DGKA	1.2
226302_at	ATP8B1	1.2
204160_s_at	ENPP4	1.2
224733_at	CKLFSF3	1.2
212099_at	RHOB	1.2
201565_s_at	ID2	1.2
228937_at	FLJ38725	1.2
221012_s_at, 223132_s_at	TRIM8	1.2
215646_s_at, 221731_x_at, 211571_s_at	CSPG2	1.2
218527_at	APTX	1.2
226483_at	FLJ32370	1.2
203115_at	FECH	1.2
202351_at	ITGAV	1.2
209075_s_at	NIFUN	1.2
222874_s_at	CLN8	1.2
222820_at	TNRC6C	1.2
225848_at	FLJ31413	1.2
209513_s_at	HSDL2	1.2
221640_s_at	LRDD [♦]	1.2
213038_at, 36564_at	IBRDC3	1.2
224618_at	N/A	1.2
244467_at	LOC440829	1.2

Probe Set(s)*	Gene Symbol†	Induced
226580_at	BRMS1L	1.2
224461_s_at	AMID	1.2
212968_at	RFNG	1.2
202546_at	N/A	1.2
202392_s_at	PISD	1.2
209260_at	SFN♦	1.2
218373_at	FTS	1.2
218251_at	MID1IP1	1.2
202672_s_at	ATF3♦Δ	1.2
217297_s_at	MYO9B	1.2
217889_s_at	CYBRD1	1.2
225223_at, 225219_at	SMAD5	1.1
212637_s_at, 212638_s_at	WWP1	1.1
218415_at	VPS33B	1.1
227776_at	N/A	1.1
221732_at	CANT1	1.1
226214_at	MIR16	1.1
225473_at	FLJ44670	1.1
209286_at	CDC42EP3 Δ	1.1
210026_s_at	CARD10	1.1
209584_x_at	APOBEC3C	1.1
218627_at	FLJ11259 Δ	1.1
204493_at	BID♦	1.1
216080_s_at	FADS3	1.1
228347_at	SIX1	1.1
44040_at	FBXO41	1.1
235119_at	TAF3	1.1
209558_s_at	HIP1R	1.1
223195_s_at, 223196_s_at	SESN2	1.1
212124_at	RAI17	1.1
203216_s_at, 203215_s_at, 210480_s_at	MYO6	1.1
210260_s_at, 208296_x_at	TNFAIP8 Δ	1.1
220007_at	FLJ13984 Δ	1.1
230563_at	RASGEF1A	1.1
202794_at	INPP1	1.1
200704_at	LITAF	1.1
225334_at	C10orf32	1.1
34206_at	CENTD2	1.1
223474_at	C14orf4	1.1
228098_s_at	MYLIP	1.1
227013_at	LATS2 Δ	1.1
221843_s_at	KIAA1609	1.1
214434_at	HSPA12A Δ	1.1
228220_at	FCHO2	1.1
36711_at	MAFF	1.1

Probe Set(s)*	Gene Symbol†	Induced
213587_s_at	C7orf32	1.1
225604_s_at	C9orf19	1.1
203409_at	DDB2 $^{\Delta}$	1.1
218066_at	SLC12A7	1.1
218764_at	PRKCH	1.1
203499_at	EPHA2$^{\blacklozenge}$	1.1
235252_at	KSR	1.1
201473_at	JUNB	1.1
214435_x_at, 224880_at	RALA	1.0
205442_at	N/A	1.0
222687_s_at	PHCA	1.0
224617_at	N/A	1.0
203537_at	PRPSAP2	1.0
203728_at	BAK1	1.0
212983_at	HRAS$^{\blacklozenge}$	1.0
225319_s_at	FLJ14775	1.0
203367_at	DUSP14	1.0
229746_x_at, 231819_at	CEBPZ	1.0
225347_at	ARL10B	1.0
218288_s_at	MDS025	1.0
223385_at	CYP2S1	1.0
202755_s_at	GPC1	1.0
212966_at	HIC2	1.0
216041_x_at	GRN	1.0
235688_s_at	TRAF4	1.0
35160_at	LDB1	1.0
201494_at	PRCP	1.0
227357_at	TAB3	1.0
207076_s_at	ASS	1.0
202286_s_at	TACSTD2	1.0
209184_s_at	IRS2	1.0

* Probe sets used to identify features on the Affymetrix microarrays. Multiple probe sets are listed if individual genes were represented more than once on the array.

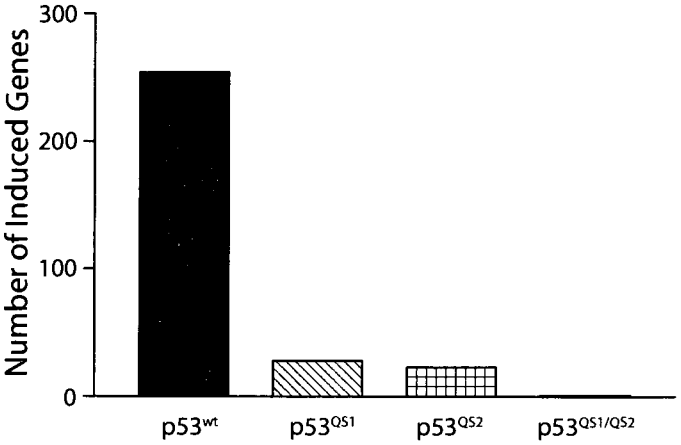
† Official gene symbols were determined using the Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez).

§ The values highlighted in blue were increased in response to one or more variants of p53 and details are provided in Table 3.

♦ Previously characterized p53-responsive genes (Riley *et al.*, 2008).

Δ Identified p53 binding loci (Wei *et al.*, 2006).

a.


Number of Induced Genes
300
200
100
0
p53wt
p53QS1
p53QS2
p53QS1/QS2

b.

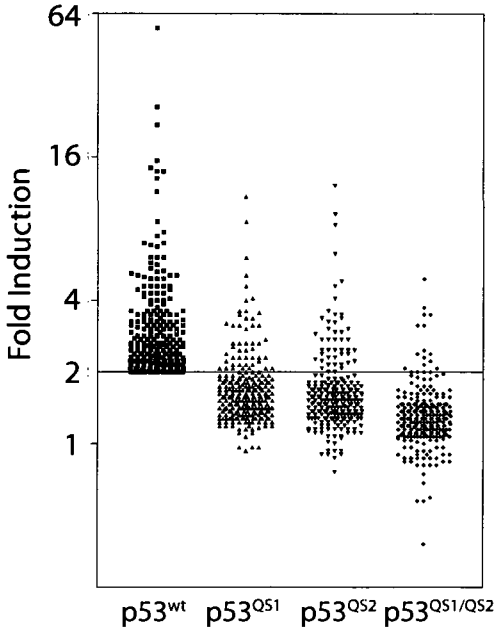

Fold Induction
64
16
4
2
1
p53wt
p53QS1
p53QS2
p53QS1/QS2

Figure 13. Transcript induction following over-expression of p53.

(a) Two hundred and fifty-four genes were induced by Adp53wt. Of these, only 28, 23, and 1 were induced by $p53^{QS1}$, $p53^{QS2}$, and $p53^{QS1/QS2}$ respectively. (b) The fold increase in expression of these 254 genes was determined following infection of cells with adenovirus expressing Adp53wt or the indicated variant of p53. The fold increase in expression following infection with $Adp53^{QS1}$, $Adp53^{QS2}$, $Adp53^{QS1/QS2}$ was less than the fold increase in response to $Adp53^{wt}$ infection (one-way analysis of variance followed by a Tukey's Multiple Comparison's test, $P < 0.001$).

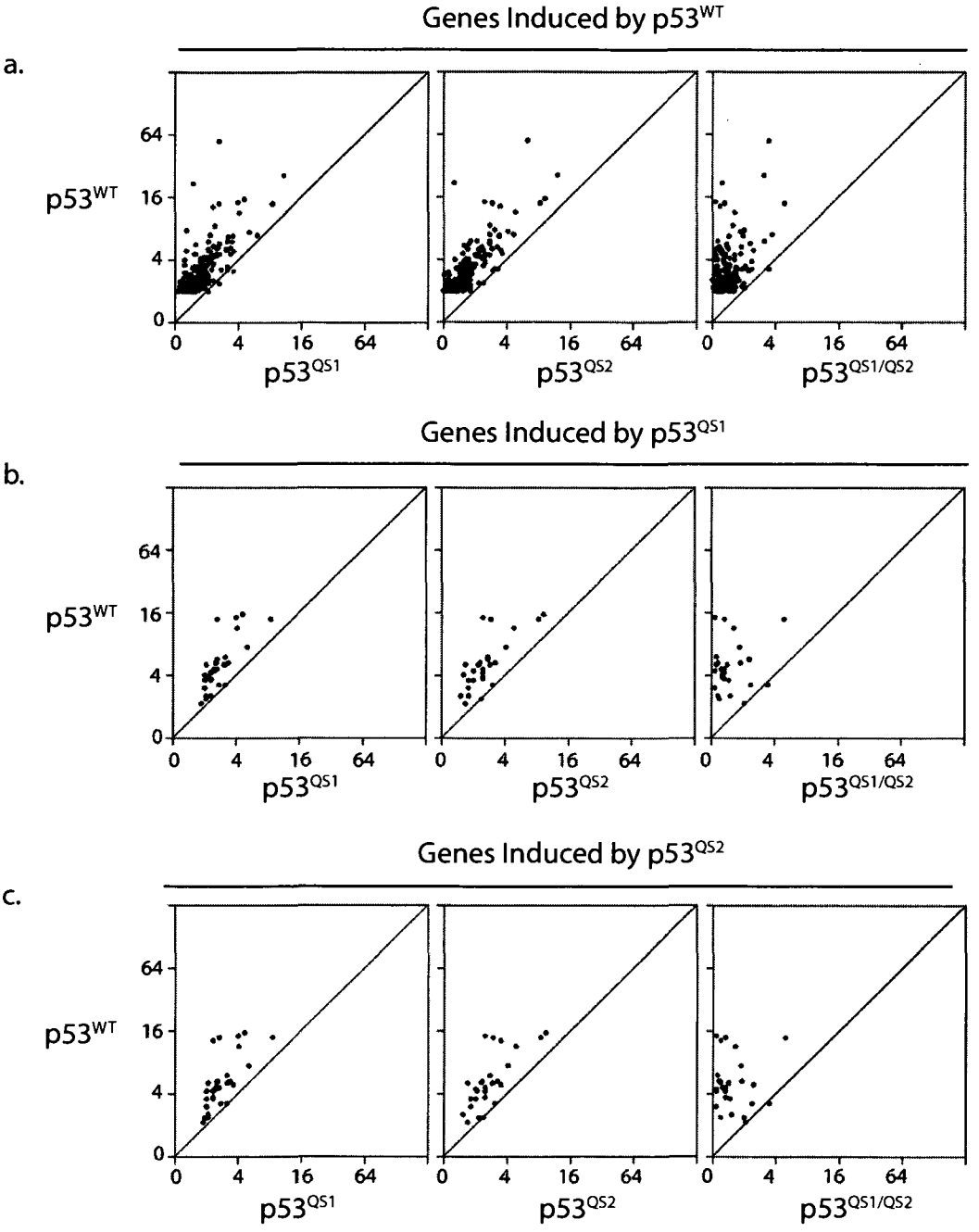

a.
Genes Induced by p53WT
p53WT
64
16
4
0
0
4
16
64
p53QS1
p53QS2
p53QS1/QS2
b.
Genes Induced by p53QS1
c.
Genes Induced by p53QS2

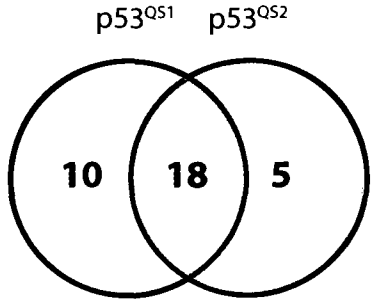
Figure 14. The p53 QS variants are impaired at transcriptional activation.

We examined the increase in transcripts using scatter plots for each of the p53 variants induced transcripts. The fold increase in expression resulting from $Adp53^{wt}$ infection was compared to the fold increase in expression resulting from infection with $Adp53^{QS1}$ (a), $Adp53^{QS2}$ (b), or $Adp53^{QS1/QS2}$ (c). The 254, 28 and 23 genes induced by $Adp53^{wt}$, $Adp53^{QS1}$ and $Adp53^{QS2}$ are listed in Tables 2 and 3.

3.4 CORRELATION BETWEEN GENES INDUCED BY THE $P53^{QS1}$ AND $P53^{QS2}$ VARIANTS

Having established that most $Adp53^{wt}$-induced genes were poorly induced by the variants, we sought to determine whether distinct subgroups of p53 target genes were preferentially responsive to one of the QS variants of p53. Of the 28 and 23 genes induced by $p53^{QS1}$ and $p53^{QS2}$, 18 were in common to both (Figure 15a). Ten and 5 wild-type p53-induced transcripts appeared to be increased preferentially in response to $p53^{QS1}$ or $p53^{QS2}$, respectively when considering statistically upregulated transcripts (Figure 15a). To visualize the relationship between QS1- and QS2-regulated gene expression, the fold change in p53 target gene expression in response to $Adp53^{QS1}$ infection was plotted with respect to $Adp53^{QS2}$ infection (Figure 15b). A very striking linear correlation was observed between the fold change in expression induced by the QS1 and QS2 variants of p53 regardless of whether the expression of $Adp53^{wt}$-, $Adp53^{QS1}$-, or $Adp53^{QS2}$-induced genes were considered (Figure 15b; R^2 values were 0.73, 0.64, and 0.61, respectively). Therefore, the $Adp53^{QS1}$- and $Adp53^{QS2}$-induced genes were induced to a similar extent by both variants (Figure 15 and Table 3). These results indicate that the disruption of either subdomain of p53 similarly affected the overall pattern of p53 transcriptional activation. We interpret these results to indicate that the contribution of transactivation subdomains 1 and 2 to p53-mediated gene expression was heterogeneous but not subdomain-specific.

a.


p53QS1
p53QS2
10
18
5

b.

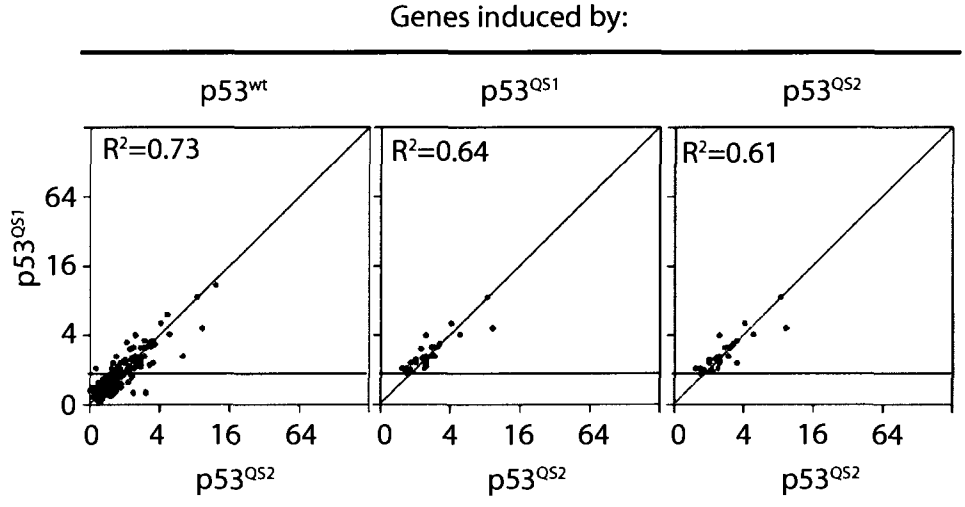

Genes induced by:
p53wt
p53QS1
p53QS2
R2=0.73
R2=0.64
R2=0.61
p53QS1
64
16
4
0
0
4
16
64
p53QS2

Figure 15. Correlation between $p53^{QS1}$ and $p53^{QS2}$ induced genes.

(a) A Venn diagram is used to represent the overlap between $Adp53^{QS1}$ and $Adp53^{QS2}$ induced genes. (b) The effect of $Adp53^{QS1}$ and $Adp53^{QS2}$ infection on the expression of the 254 $Adp53^{WT}$, 28 $Adp53^{QS1}$ and 23 $Adp53^{QS2}$ induced genes was determined. A very tight correlation (R^2 values are inset) between $Adp53^{QS1}$ and $Adp53^{QS2}$ induced gene expression was observed within the subset of target genes.

Table 3. Genes Induced in Response to expression of Ad-p53^{QS1} and/or Ad-p53^{QS2}.

Gene Symbol	Variant of p53			
	WT	QS1	QS2	QS1/QS2
QS1 and QS2				
C1orf187 *	15.5[†]	4.6	9.2	0.6[‡]
TP53I3[§]	14.4	4.0	2.5	1.1
RET	13.9	8.6	8.3	4.9
TP53INP1	13.9	2.6	2.9	1.3
EPPK1	11.4	4.1	4.8	1.6
BTG2	7.5	5.1	4.1	1.9
GPR56	6.1	3.1	2.7	1.1
FAS	5.3	3.4	3.2	1.9
KIAA0247	5.3	2.5	2.3	1.1
EPS8L2	5.1	3.1	2.9	1.4
PODXL	4.6	2.6	2.5	1.2
PGAP1	4.6	2.5	2.5	1.3
SERPINB5	4.4	2.5	2.0	1.1
NINJ1	4.3	2.3	2.5	1.3
TAP1	3.7	2.3	2.5	1.3
C12orf5	3.6	2.3	2.0	1.5
DNAJA5	3.2	3.1	3.0	2.4
PTPRE	2.4	2.1	2.4	1.2
QS1 not QS2				
CAMTA1	5.8	2.6	2.7	2.3
FLJ21245	5.1	3.0	2.2	1.1
CDKN1A	5.1	2.1	1.7	0.6
ULBP2	4.1	2.0	1.6	1.3
Transcribed locus	3.9	2.1	2.5	1.3
TGFBR1	3.6	2.0	1.8	1.5
KCNC4	3.2	2.7	0.9	3.5
LRP10	3.0	2.0	1.8	1.1
BAX	2.5	2.3	1.8	1.1
ASCC3	2.5	2.1	1.5	1.5
QS2 not QS1				
LAMP3	13.0	2.3	3.5	1.2
SLC30A1	5.2	2.5	2.3	1.2
CYP4F11	4.9	3.6	3.5	2.5
LOC346887	4.3	2.0	2.1	1.1
SLC25A30	2.4	1.9	2.1	2.0

Genes induced by one or both variants of p53 are grouped together.

*Official gene symbols were determined using the Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez).

[†]Fold increase in expression following infection with adenoviruses expressing the indicated variant of p53. The values are ordered by decreasing fold change in expression following $Adp53^{wt}$ infection.

[‡]The values highlighted in blue were not considered to be induced because they did not meet statistical criteria described in the Materials and Methods.

[§]The fold induction of the gene highlighted in green in response to $Adp53^{QS1}$ and $Adp53^{QS2}$ infection was reduced more than two-fold compared to the level induced in response to $Adp53^{wt}$ infection.

3.5 REAL-TIME REVERSE TRANSCRIPTASE PCR CONFIRMS QS VARIANT EXPRESSION PATTERNS

To determine whether these apparently $p53^{QS1}$- and $p53^{QS2}$-specific genes were in fact specifically and preferentially upregulated by one of the variants, the pattern of allele-specific gene expression was confirmed by quantitative RT-PCR for 11 different transcripts at several different times following viral infection. In accordance with our microarray results (Table 4), the quantitative RT-PCR demonstrated that many p53 target genes were poorly induced by the $p53^{QS1}$ and $p53^{QS2}$ variants (Figure 16a). However, several p53 target genes were significantly induced by the QS variants but to a lesser extent than wild-type p53 (Figure 16b). Lastly, a few target genes were induced to near wild-type levels by $p53^{QS1}$ and $p53^{QS2}$ but not $p53^{QS1/QS2}$ (Figure 16c). The quantitative RT-PCR data correlated well with the microarray analysis and none of the p53-upregulated transcripts examined displayed a subdomain-specific pattern of gene expression. Therefore, only modest differences are observed between the induction of genes by $p53^{QS1}$ and $p53^{QS2}$.

The expression of several p53-regulated proteins was assessed by immunoblot in independent cell lines (HCT116 and HeLa). Protein induction of PUMA, MDM2, and $p21^{WAF1}$ were examined. As expected, p53 expression resulted in an observable increase in these protein levels (Figure17). Expression of the QS variant resulted in either a limited induction or no induction in the examined proteins. There were no significant differences in protein expression when comparing the induction of $p53^{QS1}$ and $p53^{QS2}$. This is quite interesting in light of the potential differential regulation of the CDKN1A transcript previously observed (Table 3). However, the expression of $p53^{QS1/QS2}$ did not result

Table 4. Correlation between Microarray and RT-PCR Results.

Transcript	Variant of p53							
	WT		QS1		QS2		QS1/QS2	
	M*	RT	M	RT	M	RT	M	RT
TP53IP3	+++†	+++	+	+	+	+	-	-
MAFB	+++	+++	-	-	-	-	-	-
APAF1	++	++	-	+	-	+	-	-
CDKN1A	++	++	+	+/-	-	+/-	-	-
SERPINB5	++	++	+	+	+	+	-	-
MDM2	+	+	-	_	-	-	-	-
DDB2	+	+	-	_	-	-	-	-
TNFRSF6	++	++	++	++	++	++	-	-
BTG2	+	+	+	+	+	+	-	-
TNFRSF10B	+	+	+/-	+	+/-	+	-	-
BAK1	+	+	-	+	-	+	-	-
CASP6	+	+	-	+/-	-	+/-	-	-

*M and RT denote microarray and RT-PCR expression data, respectively.

†The number of + symbols indicates the relative increase in transcript level, - indicates that the transcript was not increased by the variant, and +/- indicates that the transcript is marginally increased.

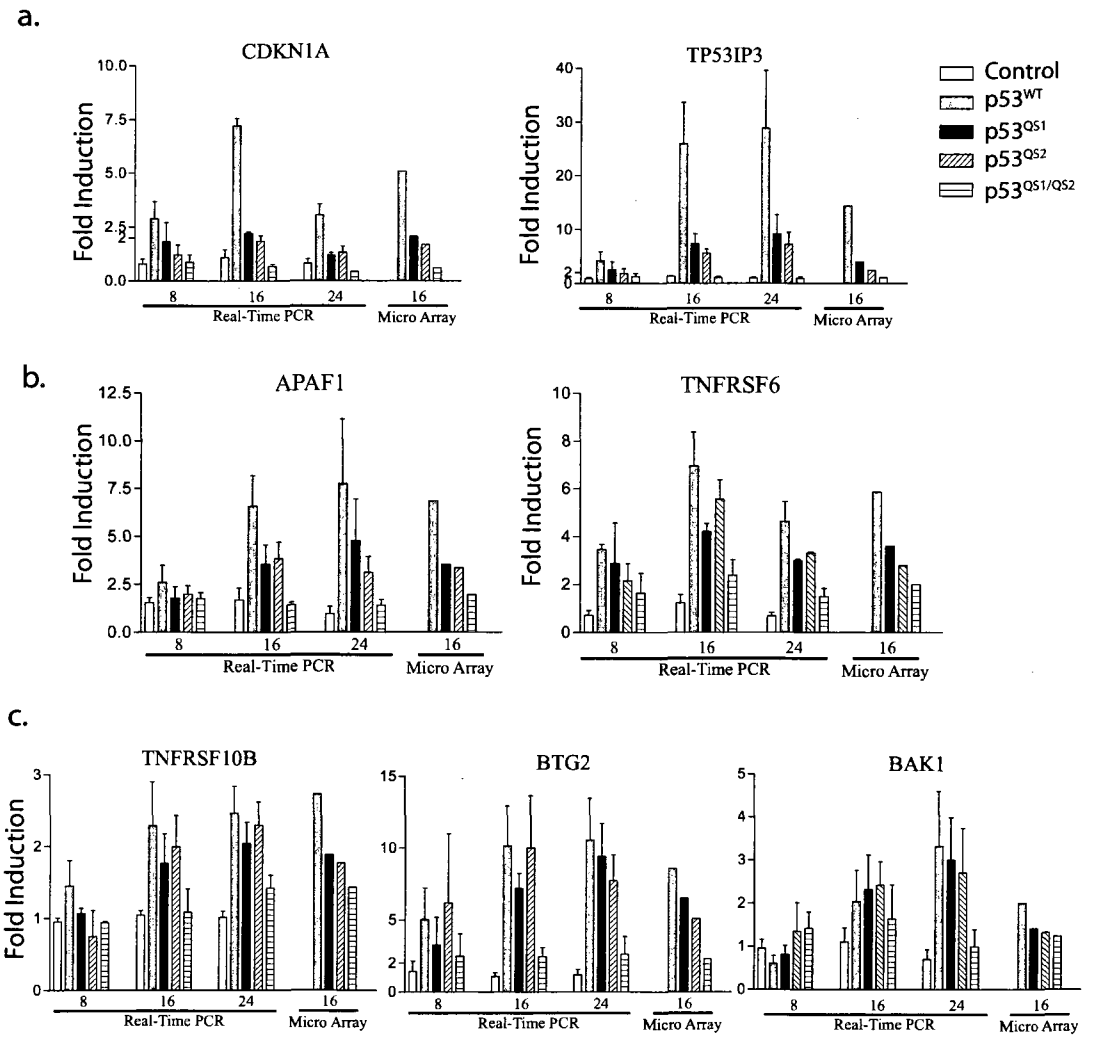
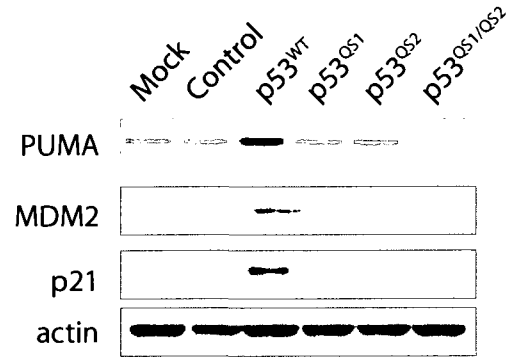

a.
CDKN1A
TP53IP3
Fold Induction
Control
p53WT
p53QS1
p53QS2
p53QS1/QS2
8
16
24
16
Real-Time PCR
Micro Array
b.
APAF1
TNFRSF6
c.
TNFRSF10B
BTG2
BAK1

Figure 16. Quantitative RT-PCR analysis.

Representative transcripts induced by wild-type p53. (a-c) Expression of the indicated transcript was determined by real-time RT-PCR using samples collected at the indicated time following virus infection (8, 16, or 24 hours). Expression of β-actin was used to normalize all RT-PCR results. Open, black, grey, hatched, and crosshatched bars represent control, $Adp53^{WT}$-, $Adp53^{QS1}$-, $Adp53^{QS2}$-, and $Adp53^{QS1/QS2}$-infected samples. Each value represents the mean fold increase in expression (± SEM) determined from a minimum of three independent experiments.

a.


Mock
Control
p53WT
p53QS1
p53QS2
p53QS1/QS2
PUMA
MDM2
p21
actin

b.

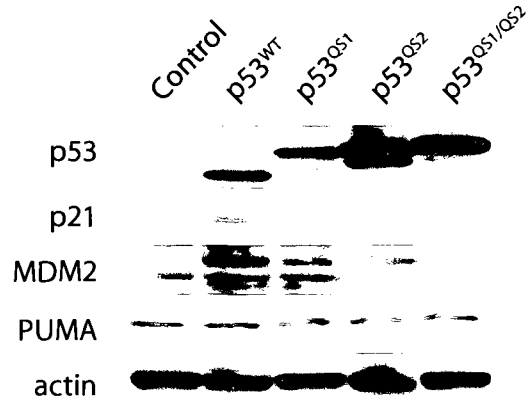

Control
p53WT
p53QS1
p53QS2
p53QS1/QS2
p53
p21
MDM2
PUMA
actin

Figure 17. Western blot analysis of p53 target genes.

HCT116 (a) and HeLa (b) cells were infected with an MOI of 25 for 16 hours of indicated Adenovirus construct. Protein levels of PUMA, MDM2, p21, and actin were determined by immunoblot analysis. P53 levels were also assessed in the HeLa cell line using Pab421 antibody.

in an observable change in protein levels (Figure 17). Collectively, we interpret our results to indicate that the apparently $p53^{QS1}$- and $p53^{QS2}$-specific targets, represent only modest differences in mRNA regulation, and these changes did not approach wild type levels. Therefore, the response of p53-induced transcripts to the QS variants was heterogeneous but not subdomain-specific.

3.6 QS VARIANT INDUCED GENES DISPLAY NO PATHWAYS SPECIFICITY

The 254 Adp53wt-induced genes were subjected to gene ontology (GO) analysis (http://www.geneontology.org/). Several of these transcriptional targets were associated with the GO terms apoptosis (GO:0006915), cell cycle (GO:0007049), and DNA repair (GO:0006281) (Table 5), consistent with known p53 biology (Vogelstein et al., 2000). Using the web-based GOstat software (http://gostat.wehi.edu.au/) only apoptosis was identified as statistically over-represented ($P < .01$). There were 14 apoptosis related genes induced by $p53^{WT}$. Of these, 2 were induced by both $p53^{QS1}$ and $p53^{QS2}$. Only one transcript (corresponding to BAX) appeared to be differentially upregulated by a QS variant. *BAX* was induced 2.3 fold by $p53^{QS1}$ and 1.8 fold by $p53^{QS2}$. We analyzed the protein expression of BAX and did not detect a significant induction by $p53^{WT}$ or the variants above control levels (data not shown). Therefore, $p53^{QS1}$ and $p53^{QS2}$ were equally impaired at regulating apoptosis related genes.

Cell cycle–annotated genes were also poorly induced by all variants (Table 5). Eleven transcripts were induced by $p53^{WT}$. Only one transcript, CDKN1A, was induced by any of the variants. Although CDKN1A was modestly more induced by $p53^{QS1}$ than $p53^{QS2}$, there was no difference in protein expression. There were no cell-cycle annotated

Table 5. Adp53^{wt} -Induced Genes Involved in Apoptosis, Cell Cycle, and/or DNA Repair.

GO Term*	Locus	Variant of p53			
		WT	QS1	QS2	QS1/QS2
Apoptosis (GO:0006915)	TP53I3	3.9†	2.0	1.3	0.1
	TP53INP1	3.8	1.4	1.5	0.4
	APAF1	2.8	1.8	1.8	1.0
	TNFRSF6	2.4	1.8	1.7	0.9
	CASP6	1.7	0.8	0.7	0.4
	TNFRSF10B	1.5	0.9	0.8	0.5
	MDM2	1.4	0.3	0.1	0.0
	BAX	1.4	1.2	0.9	0.2
	AKTIP	1.2	0.3	0.4	0.4
	AMID	1.2	0.4	0.2	0.2
	BID	1.1	0.4	0.3	0.3
	CARD10	1.1	0.5	0.4	0.4
	BAK1	1.0	0.5	0.4	0.3
	TRAF4	1.0	0.4	-0.1	-0.2
Cell Cycle (GO:0007049)	CDKN1A	2.4	1.1	0.8	-0.8
	SESN1	2.0	0.9	0.8	0.1
	GAS2L1	1.4	0.8	0.6	0.4
	MDM2	1.4	0.3	0.1	0.0
	RB1	1.3	0.8	0.6	0.8
	RHOB	1.2	0.4	0.3	0.5
	PARD6G	1.2	0.7	0.6	0.4
	SFN	1.2	0.4	0.3	-0.3
	SESN2	1.1	0.3	0.2	0.0
	LATS2	1.1	0.5	0.4	0.4
	HRAS	1.0	0.5	0.4	0.2
DNA Repair (GO:0006281)	BTG2	2.9	2.4	2.0	0.9
	POLH	2.2	1.8	1.9	0.9
	DDB2	1.1	0.4	0.3	-0.3

*Genes involved in apoptosis, cell cycle and DNA repair were identified using the Gene Ontology database (http://geneontology.org/). Of these GO terms, only apoptosis (GO:0006915) and related GO terms were significantly over-represented among the Adp53^{wt}-induced genes based on GOstat analysis (http://gostat.wehi.edu.au/).

[†]The mean fold increase in expression (log2) determined from microarray experiments, as described in the Materials and Methods section.

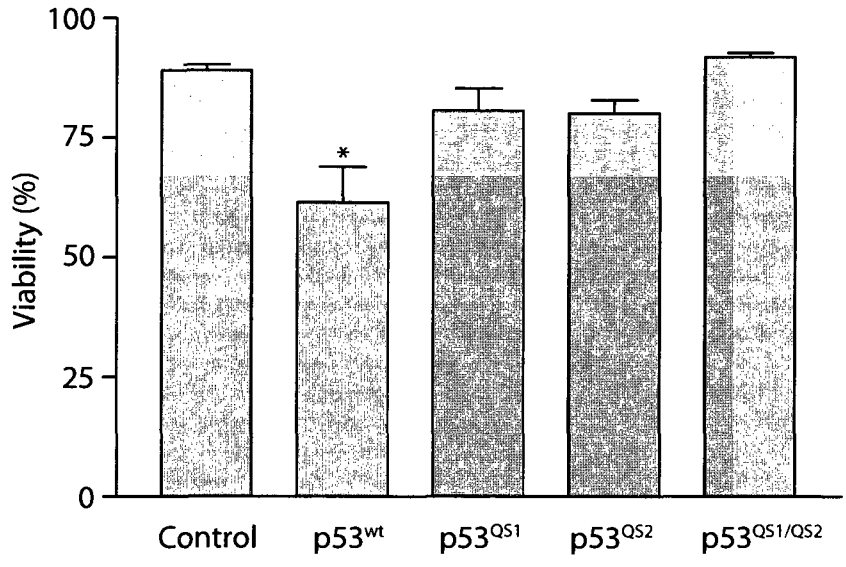
Values highlighted in blue represent transcripts that are statistically induced according to our definition in materials and methods.

genes increased by either $p53^{QS2}$ or $p53^{QS1/QS2}$. Interestingly, two of the three genes associated with DNA repair (BTG2 and POLH) induced by $p53^{WT}$ were also induced to near wild-type levels by $p53^{QS1}$ and $p53^{QS2}$ but not $p53^{QS1/QS2}$ (Table 5 and Figure 16c). Due to the limited number of repair-related genes, the significance of this specific observation remains unclear. Overall, our results suggest that there is substantial heterogeneity in the contribution of subdomains 1 and 2 to p53-mediated gene expression but there is no subdomain-specific effects.

3.7 THE P53QS VARIANTS ARE DEFICIENT IN THEIR ABILITY TO INDUCE APOPTOSIS

The requirement for transcriptional activation during p53-mediated apoptosis has been the subject of significant debate. In our study, we noted that there was a significant reduction in the ability of all QS variants to induced apoptosis related genes (as with genes involved in other pathways examined). We studied the effects of this reduction on the ability of the p53 variants to influence (1) cellular viability using a Trypan blue dye exclusion assay and (2) apoptosis levels using sub-G1 DNA content analysis. Cellular viability as assessed by Trypan blue dye exclusion 48 hours post-infection demonstrated a significant decrease following infection with $p53^{WT}$ (Figure 18a). Infection with $p53^{QS1}$ and $p53^{QS2}$ variants also resulted in decreased cellular viability. However, this decrease was significantly less than that observed following $p53^{WT}$ infection. Cells infected with the $p53^{QS1/QS2}$ variant did not demonstrate a reduction in cellular viability compared to control treated cells. Similarily, flow-cytometric sub-G1 DNA analysis using PI staining demonstrated a significant increase in apoptotic cells 48 hours post-infection with $Adp53^{WT}$ (Figure 18b). Cells infected with either $p53^{QS1}$ or $p53^{QS2}$ resulted in sub-G1

a.


100
75
50
25
0
Viability (%)
*
Control
p53wt
p53QS1
p53QS2
p53QS1/QS2

b.

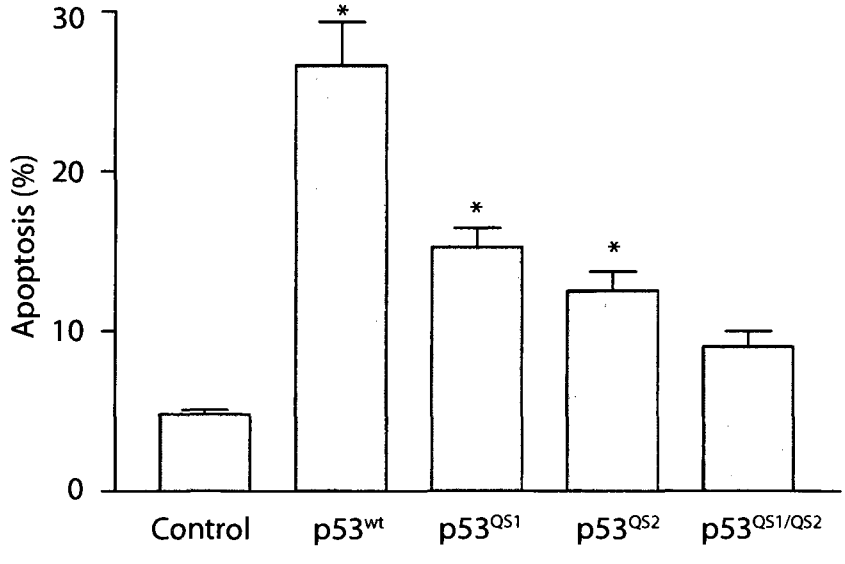

30
20
10
0
Apoptosis (%)
*
*
*
Control
p53wt
p53QS1
p53QS2
p53QS1/QS2

Figure 18. The p53 QS are impaired at inducing cell death.

Apoptosis (a) and cell viability (b) assessed 48 hours following infection with either control adenovirus or adenoviruses expressing the indicated variants of p53. Apoptosis was assessed by subdiploid DNA content and viability was assessed by Trypan blue exclusion. Each bar represents the mean (+/-SEM) determined from three independent experiments. $Adp53^{wt}$ induced more apoptosis than the variants (one-way analysis of variance followed by Tukey's Multiple Comparisons test, $P \leq .01$ for apoptosis and $P \leq .05$ for viability). No significant difference in viability or apoptosis was observed when comparing QS1 and QS2 variants.

DNA content of 15% and 13% respectively. Infection with $p53^{QS1/QS2}$ only resulted in the induction of sub-G1 DNA content in 9% of cells. Therefore, our results demonstrate a correlation between the integrity of the TADs and the induction of apoptosis in HCT116 cells. These results support a role for transactivation of p53 target genes in apoptosis in these cells. Our results further indicate that QS1 and QS2 are similarly impaired.

3.8 THE P53QS VARIANTS RETAIN ABILITY TO TRANSLOCATE TO MITOCHONDRIA

p53 can induce apoptosis by translocating to the mitochondria and interacting with Bcl2 and Bcl-X_L resulting in BAX homodimerization, outermembrane permibilization, cytochrome c release and apoptosis (Dumont et al., 2003; Marchenko et al., 2000; Mihara et al., 2003; Sansome et al., 2001). Considering the limited induction of apoptotic genes by the QS1 and QS2 variants, we wanted to determine if the resultant apoptosis could arise from direct mitochondrial targeting by the variants. Defects in the ability of the TD variants to target mitochondria are expected as a result of their decreased ability to interact with MDM2 (Dumont et al., 2003; Marchenko et al., 2007). Confounding the potential quantification of mitochondrial targeting is the differential stability of the p53 variant proteins. The decreased interaction with MDM2 favors protein stability and yields elevated protein levels of the p53 QS variants. We used cellular fractionation in order to isolate the mitochondrion and immunoblot analysis to examine the presence of p53. Mitochodrial fractions were confirmed using immunoblot analysis of mtHSP70 (Figure 19). Immunoblot analysis of PCNA was used to assess nuclear contamination (Figure 19). All of the p53 variants were detected at the mitochondria (Figure 19). Therefore, p53 QS variants retain the ability to translocate to mitochondria.

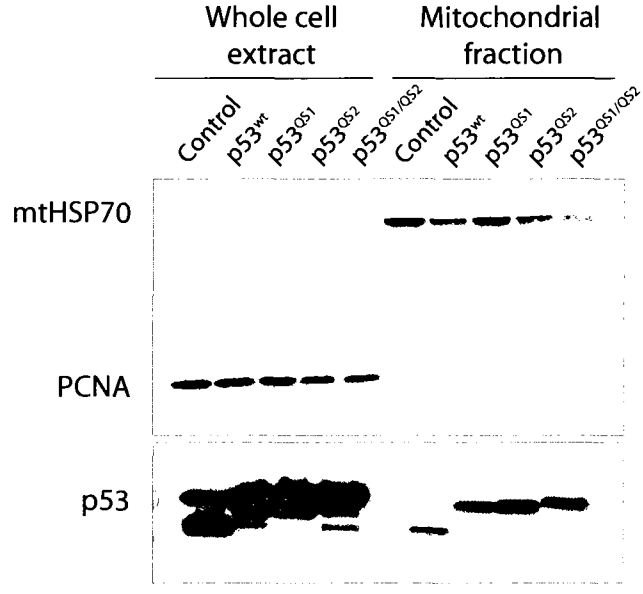

Whole cell extract
Mitochondrial fraction
Control
p53wt
p53QS1
p53QS2
p53QS1/QS2
Control
p53wt
p53QS1
p53QS2
p53QS1/QS2
mtHSP70
PCNA
p53

Figure 19. The p53 QS variants are proficient at targeting the mitochondria.

HCT116p53$^{-/-}$ cells infected with indicated Adenovirus construct for 16 hours. Immunoblot analysis was performed on whole cell extracts and mitochondrial fractions. The antibodies used were: PCNA as a marker of nuclear content, mtHSP mitochondria marker and p53 Pab421 was used to detect the presence of p53.

3.9 GENES INDUCED BY QS VARIANTS BY NOT $p53^{WT}$

The above-mentioned correlations stem from the examination of all $p53^{wt}$ induced transcripts. Eighteen transcripts however were induced by at least one of the QS variants and not by $p53^{wt}$ (Table 6). This small pool of transcripts includes 4 induced by $p53^{QS1}$, 4 induced by $p53^{QS2}$ and 11 induced by $p53^{QS1/QS2}$. Interestingly, of these transcripts, only one is induced by multiple QS variants. These groups of genes have mean induction levels of 2.08, 2.01, and 2.07 following expression of $p53^{QS1}$, $p53^{QS2}$ and $p53^{QS1/QS2}$ respectively. These represent mean inductions that are much lower than that seen by $p53^{wt}$ induction.

3.10 THE P53 QS VARIANTS HAVE LIMITED EFFECT ON GENE REPRESSION

Unlike p53-transcriptional activation that requires sequence specific DNA binding and interactions of the transactivation domain (as discussed in sections 1.7 and 1.8), gene repression occurs through several mechanisms often independent of sequence-specific DNA binding. However, several repressive mechanisms may require an intact tertiary structure, potentially altered by the QS mutants. Therefore, a change in the overall structure of the p53 protein may further impact transcriptional repression. A study of the pattern of gene repression following 16 hours of $p53^{wt}$ over-expression identified very few repressed genes. Only 3 $p53^{WT}$ repressed genes and 2 others by each of $p53^{QS1}$, $p53^{QS2}$ and $p53^{QS1/QS2}$ (Table 7) were identified (using the criteria described in materials and methods). Interestingly, one p53-induced gene was repressed by $p53^{QS1/QS2}$. However, the small number of transcripts makes interpretation of this observation difficult.

Table 6. Genes induced by QS variants that are unregulated by $p53^{WT}$.

Gene Symbol†	$P53^{WT}$	$P53^{QS1}$	$P53^{QS2}$	$P53^{QS1/QS2}$
METRNL	0.8‡	1.05	1	0.3
MAP3K12	2.5	1.8	1.85	0.9
MYLIP	1.15	1.35	-0.15	1.1
PANK1	1.8	1.15	0.55	-0.2
EGR1	0.65	0.45	1.05	0.7
FGF18	1.9	2.6	2.6	0.2
EGR1	0.8	0.55	1.05	0.75
PCNX	0.8	0.95	0.6	1.2
SRI	0.85	0.8	0.55	1
RP11-529I10.4	0.75	0.75	0.6	1.05
ISOC1	0.85	0.6	0.45	1.05
ACAP2	0.6	0.5	0.25	1.05
GABRA2	2.7	3.2	3.75	2.65
NUDT12	0.6	0.5	0.45	1.4
TTC33	0.95	0.4	0.55	1.05
PSMG1	2.8	1.25	2	2.6
RNASE4	0.5	0.75	0.45	1.1
SLC10A4	0.65	0.4	0.55	1.05

†Official gene symbols were determined using the Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez).

‡Log2 (fold increase in expression) resulting from infection with adenoviruses expressing the indicated variant of p53.

Values highlighted in pink were statistically induced (as described in Materials and Me thods).

Values in the $p53^{wt}$ column highlighted in yellow were called "increased" in at least one of the experiments.

Gene symbols in the $p53^{wt}$ column highlighted in blue were displayed a value of $\log_2$ 1.0 or higher.

Gene symbols in the $p53^{wt}$ column highlighted in green were both, called "increased" in at least one of the experiments and displayed a value of $\log_2$ 1.0 or higher.

Table 7. Repressed genes by $p53^{wt}$ and QS variants.

Gene Symbol	$P53^{WT}$	$P53^{QS1}$	$P53^{QS2}$	$P53^{QS1/QS2}$
NRP1	-1.15	-1.05	-1.25*	-0.3
NKIRAS2	-1.1	-1.25	-0.9	-0.85
CCDC85B	-1.1	-0.55	-0.55	0.15
HDGF	-1	-0.95	-0.95	-0.7
SOCS2	-0.9	-0.65	-1.1	-0.15
GM2A	-0.85	-1.4	-1.05	-1.3
C9orf114	-0.85	-0.8	-0.75	-1
RARA	-0.7	-0.05	-1.65	-0.6
GM2A	-0.65	-1.1	-0.75	-0.5
COMMD1	-0.65	-1	-0.85	-0.7
GDF15	2.1	-0.1	-0.2	-1.4

†Official gene symbols were determined using the Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez).

‡Log2 (fold decrease in expression) resulting from infection with adenoviruses expressing the indicated variant of p53.

*Transcripts that were determined to be significantly repressed (as defined in materials and methods) are highlighted in blue.

3.11 CHARACTERIZATION OF THE NOVEL P53 TRANSCRIPTIONALLY REGULATED GENE *MAFB*

Although the vast majority of p53-regulated genes are convincingly regulated to a similar extent by $p53^{QS1}$ and $p53^{QS2}$, it could be argued that there is deviation from this trend by a small subset (Figure 20a). Although not statistically induced in the oligonucliotide microarrays (the control sample levels were too low to be considered present), subsequent quantitative RT-PCR analysis determined that the *MafB* transcript is significantly upregulated by $p53^{WT}$, $p53^{QS1}$ and $p53^{QS2}$ (Figure 20b and c). MafB protein was also induced following infection with $p53^{WT}$ (Figure 20d). A significant increase in *MafB* mRNA is not observed following infection with $p53^{QS1/QS2}$. *MafB* would appear to be a transcript preferentially regulated by $p53^{QS2}$. We can reconcile this issue by examining the induction levels, not in absolute terms defining induction (*i.e.* a 2.0 fold increase cut off) but instead relative to $p53^{wt}$ induction levels. Although the induction of *MafB* by $p53^{QS1}$ or $p53^{QS2}$ appears different, both inductions were less than 30% of $p53^{wt}$ induction and both are statistically decreased compared to $p53^{wt}$ (student's *t*-test, $p<0.05$). Therefore induction of *MafB* by both $p53^{QS1}$ and $p53^{QS2}$ is impaired. In light of this significant deficiency in induction compared to $p53^{WT}$, the biological relevance of the differential induction is likely limited.

3.12 *MAFB* IS A NOVEL TRANSCRIPTIONAL TARGET GENE OF P53

We identified *MafB* as a gene potentially differentially regulated by the $p53^{QS1}$ and $p53^{QS2}$ variants. *MafB* is not a *bona fide* p53 transcriptional target gene but was significantly upregulated by p53 and the two single domain variants in our studies. We there

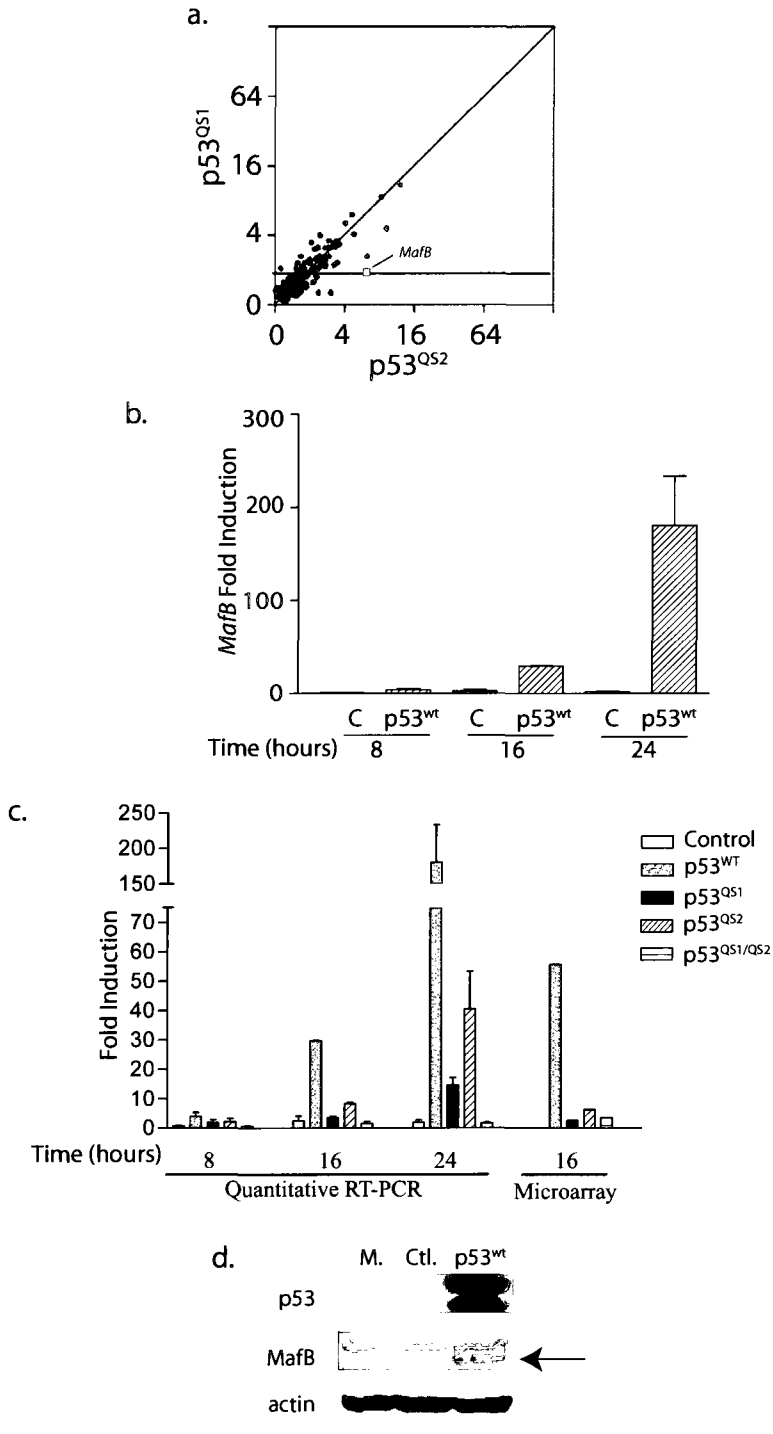

a.
64
16
4
0
p53QS1
MafB
0
4
16
64
p53QS2
b.
300
200
100
0
MafB Fold Induction
C
p53wt
C
p53wt
C
p53wt
Time (hours)
8
16
24
c.
250
200
150
70
60
50
40
30
20
10
0
Fold Induction
Control
p53WT
p53QS1
p53QS2
p53QS1/QS2
Time (hours)
8
16
24
16
Quantitative RT-PCR
Microarray
d.
M.
Ctl.
p53wt
p53
MafB
actin

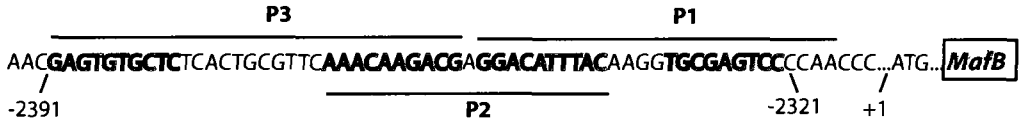
Figure 20. MafB mRNA and protein are induced following Adp53 infection.

(a) The effect of $Adp53^{QS1}$ and $Adp53^{QS2}$ infection on the expression of the 254 $Adp53^{WT}$, induced genes indicating the location of MafB. (b) Quantitative RT-PCR analysis of total RNA collected at indicated times following infection with $Adp53^{WT}$. Samples were normalized to beta-actin. Each bar represents the mean of a minimum of three independent experiments (+/-SEM). (c) Quantitative reverse transcripatse PCR analysis and microarray results for the expression of *MafB*. *MafB* expression for quantitative RT-PCR was normalized to beta-actin. (d) Immunoblot analysis of nuclear extracts from HCT116$p53^{-/-}$ cells infected with a MOI of 50 of $Adp53^{WT}$ for twenty four hours. Antibodies used were p53 Pab421, MafB and beta-actin as a loading control. A non-specific band is present in all lanes when probing with MafB antibody. MafB is the lower molecular weight band. Lanes are mock (M), control adeno-virus infected (Ctl.), and Adp53wt infected.

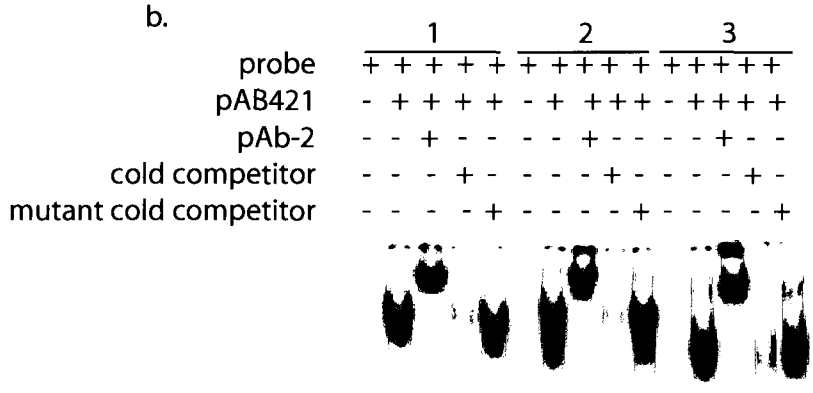
fore examined whether it was indeed a *bona fide* p53 target gene. Sequence-specific DNA binding by p53 requires the presence of a highly degenerate response element (RE) made up of two half-sites separated by 0-13bp (El Deiry et al., 1992). A search up-stream of the *MafB* promoter unveiled a series of four potential half sites separated by short spacers (Figure 21a). We constructed a panel of oligos corresponding to the potential REs comprised of any two sequential half sites (Table 1) and examined the ability of these regions to interact with p53 *in vitro* using an electrophoretic mobility shift assay (EMSA) (Figure 21a). Using the panel of oligos corresponding to these putative REs (S1, S2 and S3 in Figure 21a), we found that p53 bound to all of the potential half sites (Figure 21b). This interaction was not observed following the insertion of mutations at essential residues for binding within the oligos or in the presence of cold competitor. Therefore, p53 can bind to the putative REs upstream of the *MafB* promoter *in vitro*.

We next tested the ability of p53 to bind to the *MafB* RE *in vivo* using a chromatin immunoprecipitation (ChIP) assay. HCT116p53$^{-/-}$ cells were infected with Adp53^{wt} for 24 hours and cell lysates were prepared. Semi-quantitative PCR was performed using primers spanning 178bp encompassing the putative RE. Using the *Maspin* RE as a positive control and *GAPDH* promoter as a negative control, a specific interaction between p53 and the putative RE *in vivo* using ChIP was observed (Figure 21c). Taken together, the EMSA and ChIP assays demonstrate that p53 can specifically bind to the putative p53 RE upstream of MafB *in vitro* and *in vivo*.

a.


P3
P1
AACGAGTGTGCTCTCACTGCGTTCAAACAAGACGAGGACATTTACAAGGTGCGAGTCCCCAACCC...ATG..MafB
-2391
P2
-2321
+1

b.


1
2
3
probe + + + + + + + + + + + + + + +
pAB421 - + + + + - + + + + - + + + +
pAb-2 - - + - - - - + - - - - + - -
cold competitor - - - + - - - - + - - - - + -
mutant cold competitor - - - - + - - - - + - - - - +

c.

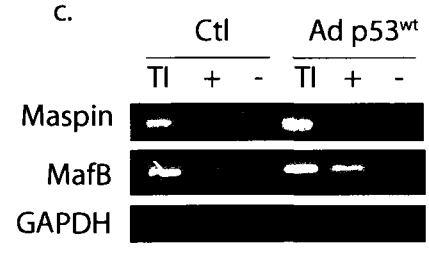

Ctl
Ad p53wt
Tl + - Tl + -
Maspin
MafB
GAPDH

Figure 21. p53 binds to a putative RE upstream of *MafB in vivo* and *in vitro*.

(a) DNA sequence upstream of MafB and indicating the putative p53 response element sites (in red). The probes used for in vitro analysis are indicated (P1, P2, P3). (b) EMSA on extracts of HCT116p53$^{-/-}$ cells infected with Adp53^{WT} for 16 hours using indicated probes and antibodies. (c) ChIP assay in HCT116p53$^{-/-}$ cells infected with Adp53^{WT} for 16 hours. Ctl, TI, +, and - represent control sample, total input, plus antibody, and with out antibody respectively.

3.13 *MafB* IS UPREGULATED FOLLOWING UVC AND X-RAY TREATMENTS

Transcriptional activation of target genes by p53 commonly occurs as a result of its activation following exposure to cellular stresses such as ultraviolet and ionizing radiation (Horn and Vousden, 2007; Murray-Zmijewski et al., 2008). A study of alveolar macrophage from the lungs of smokers revealed that *MafB* is substantially upregulated in smokers. This elevation is believed to contribute a protective phenotype against apoptosis in these cells (Machiya et al., 2006). Additionally, MafB promotes proliferation, inhibits apoptosis (Van Stralen et al., 2009) and promotes transformation of certain cell types (Pouponnot et al., 2006). Taken together, these reports strongly suggest a role for MafB during the cellular stress response. We therefore sought to determine if *MafB* could be regulated by other genotoxic stresses such as UVC and X-ray treatment in a p53-dependent manner.

We examined *MafB* levels in tumour derived HCT116 and A549 cells and the normal fibroblast line (GM38) twenty four hours after treatment with 20 $J \cdot m^{-2}$ UVC. In all three cell lines UVC treatment resulted in a significant induction of *MafB* mRNA levels as determined by real-time RT-PCR (Figure 22). This induction was either similar to or stronger than the induction of the well-characterized p53-regulated gene CDKN1A. Similarily, *MafB* levels were upregulated in HCT116 cells following X-irradiation with 10Gy (Figure 23).

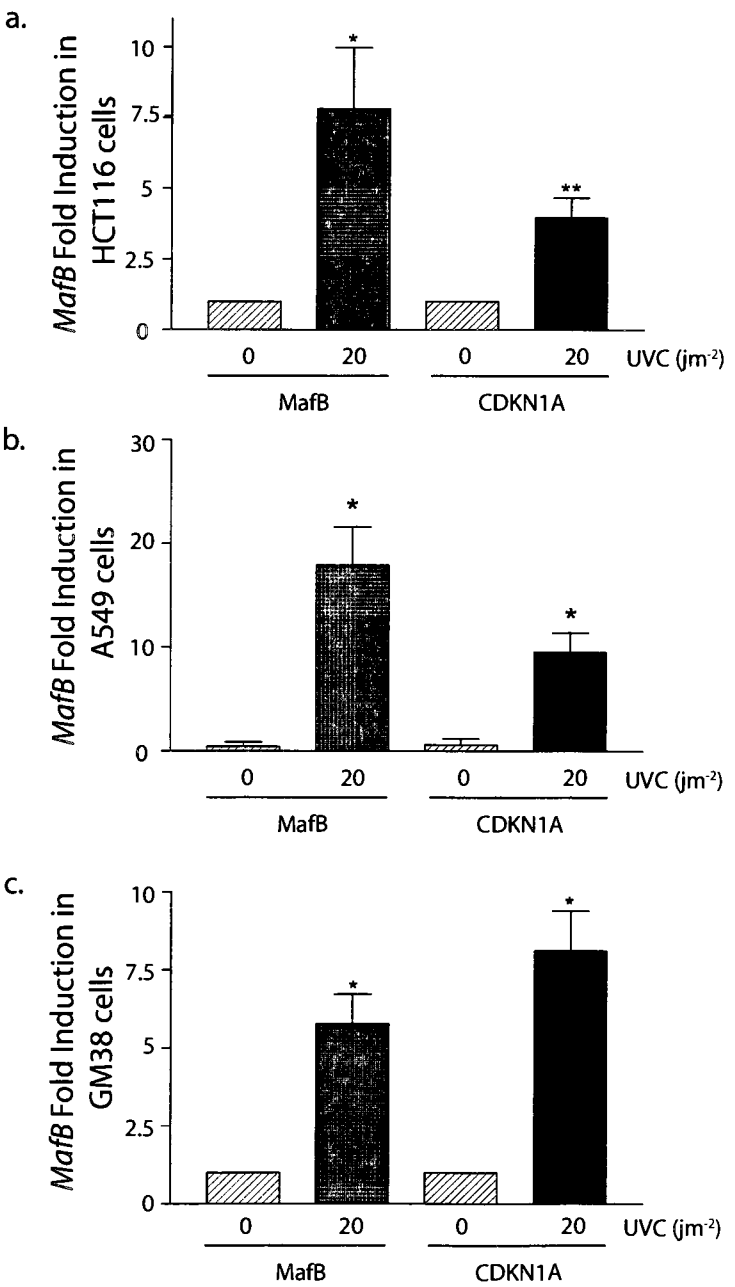
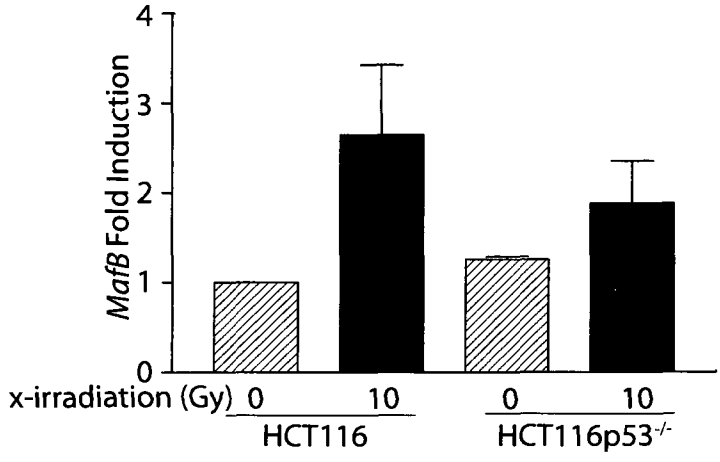

a.
MafB Fold Induction in HCT116 cells
10
7.5
5
2.5
0
*
**
0
20
0
20
UVC (jm-2)
MafB
CDKN1A
b.
MafB Fold Induction in A549 cells
30
20
10
0
*
*
0
20
0
20
UVC (jm-2)
MafB
CDKN1A
c.
MafB Fold Induction in GM38 cells
10
7.5
5
2.5
0
*
*
0
20
0
20
UVC (jm-2)
MafB
CDKN1A

Figure 22. *MafB* is induced following treatment with UVC in various cell lines.

Quantitative RT-PCR analysis of *MafB* and *CDKN1A* transcript levels twenty four hours following mock treatment or treatment with 20 jm^{-2} UVC in HCT116 cells (n=7) (a), A549 cells (n=3) (b), and GM38 (n=4) (c) (+/-SEM). All samples are normalized to β-actin. *MafB* and *CDKN1A* are induced following UVC treatment (analyzed by students *t*-test * $p < 0.05$, **$p<0.01$).

a.


4
3
2
1
0
MafB Fold Induction
x-irradiation (Gy) 0 10 0 10
HCT116
HCT116p53-/-

b.

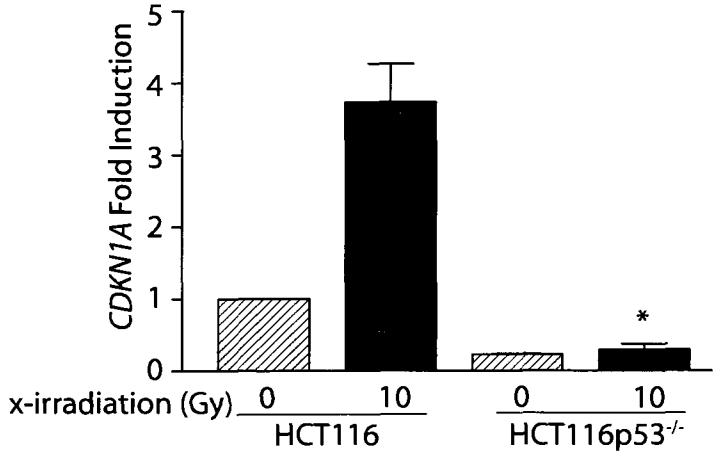

5
4
3
2
1
0
CDKN1A Fold Induction
*
x-irradiation (Gy) 0 10 0 10
HCT116
HCT116p53-/-

Figure 23. *MafB* is induced following treatment with x-irradiation.

HCT116 and HCT116p53$^{-/-}$ cells were mock x-irradiated or x-irradiated with 10 Gy. Total RNA was collected twenty four hours later and transcript levels of *MafB* (a) and *CDKN1A* (b) were analyzed by quantitative RT-PCR. Each bar represents a mean (+/- SEM) determined from a minimum of three independent experiments. *MafB* and *CDKN1A* are induced in HCT116 cells following exposure to 10 Gy x-irradiation. *CDKN1A* induction is deficient in HCT116p53$^{-/-}$ cells compared to HCT116 cells (Student's *t*-test, $P \leq 0.05$).

3.14 STRESS MEDIATED *MAFB* UPREGULATION IS INDEPENDENT OF P53 STATUS

Thus far we have demonstrated that *MafB* is a transcriptional target of p53 and that it is transcriptionally upregulated following exposure to UVC and X-irradiation. We next wanted to examine the requirement for p53 during the UVC and X-ray induction of *MafB*. We examined the induction of MafB following 20 $J \cdot m^{-2}$ UVC in isogenic HCT116 and HCT116p53$^{-/-}$ cell lines and found that a significant induction occurred in the absence and presence of p53 (Figure 24).

We further examined A549 cells using RNAi treatment to disable p53 activity. A549 cells were mock treated, treated with non-targeting siRNA (si-NT), or p53-targeting (si-p53) siRNA. Exposure of these differentially treated A549 cells to 20 $J \cdot m^{-2}$ UVC did not impair the induction of *MafB* in si-p53 treated cells (Figure 25). Again, *MafB* levels were induced to at least the same levels in p53 deficient cells as in cells with intact levels of p53.

We further performed time-course experiments using the HCT116 and HCT116p53$^{-/-}$ cell lines to study any potential delays in transcriptional activation (Figure 26). We examined *MafB* levels at 0, 4, 8, and 24 hours post-treatment and found that the absence of p53 did not abrogate or delay the induction of *MafB* mRNA. *MafB* was induced to at least the same levels in p53 deficient cells as in cells with unaltered levels of p53.

Together, this panel of experiments demonstrates that although p53 can induce *MafB* and exposure to X-irradiation and UVC can induce MafB, p53 is not essential for

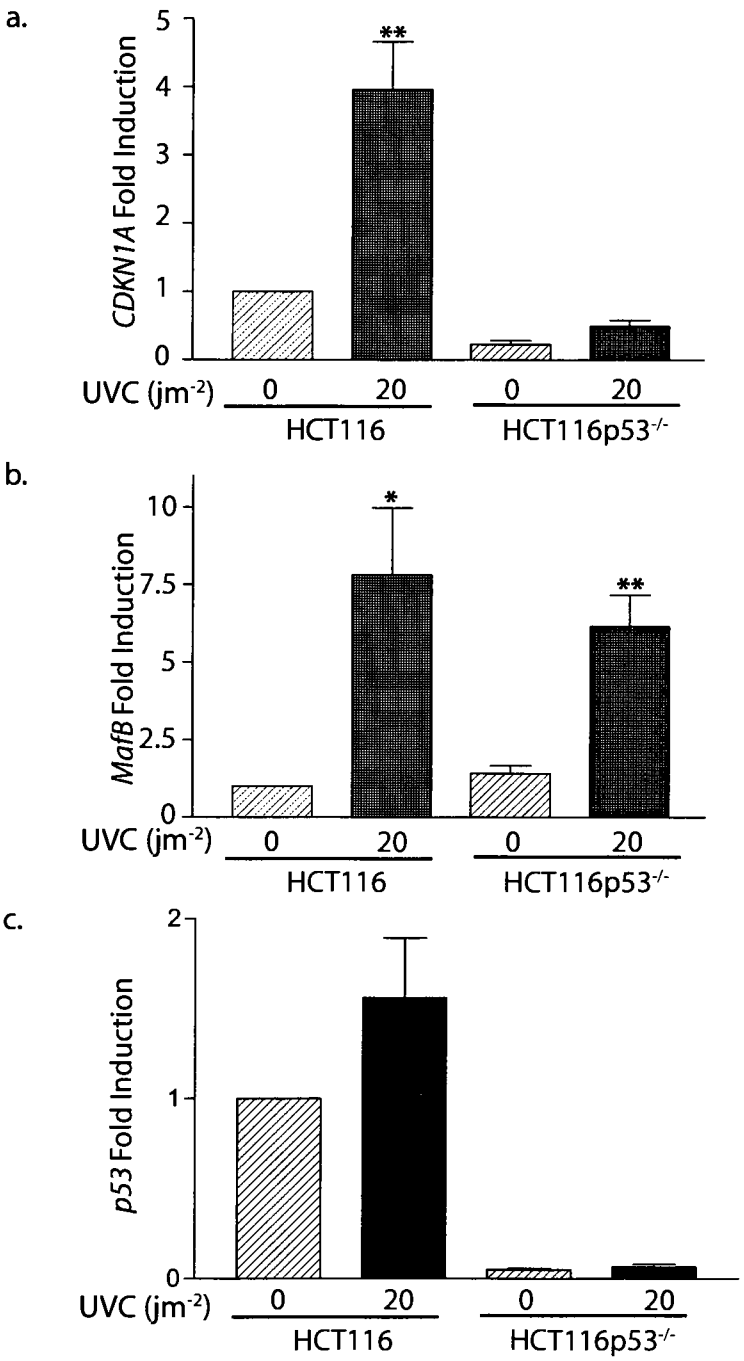

a.
CDKN1A Fold Induction
5
4
3
2
1
0
**
UVC (jm-2)
0
20
0
20
HCT116
HCT116p53-/-
b.
MafB Fold Induction
10
7.5
5
2.5
0
*
**
UVC (jm-2)
0
20
0
20
HCT116
HCT116p53-/-
c.
p53 Fold Induction
2
1
0
UVC (jm-2)
0
20
0
20
HCT116
HCT116p53-/-

Figure 24. Induction of *MafB* following exposure to UVC in HCT116 cells is p53 independent.

HCT116 and HCT116p53$^{-/-}$ cells were mock treated or treated with 20 jm^{-2} UVC and analyzed by quantitative RT-PCR for transcript levels of *CDKN1A* (a), *MafB* (b), and *p53* (c) twenty four hours post-treatment. Each bar represents a minimum of three independent experiments and error bars represent standard error. Differences in induction were analyzed using a paired student's *t*-test against mock treated samples * $p<0.05$, ** $p<0.01$. Samples were compared to HCT116 untreated samples.

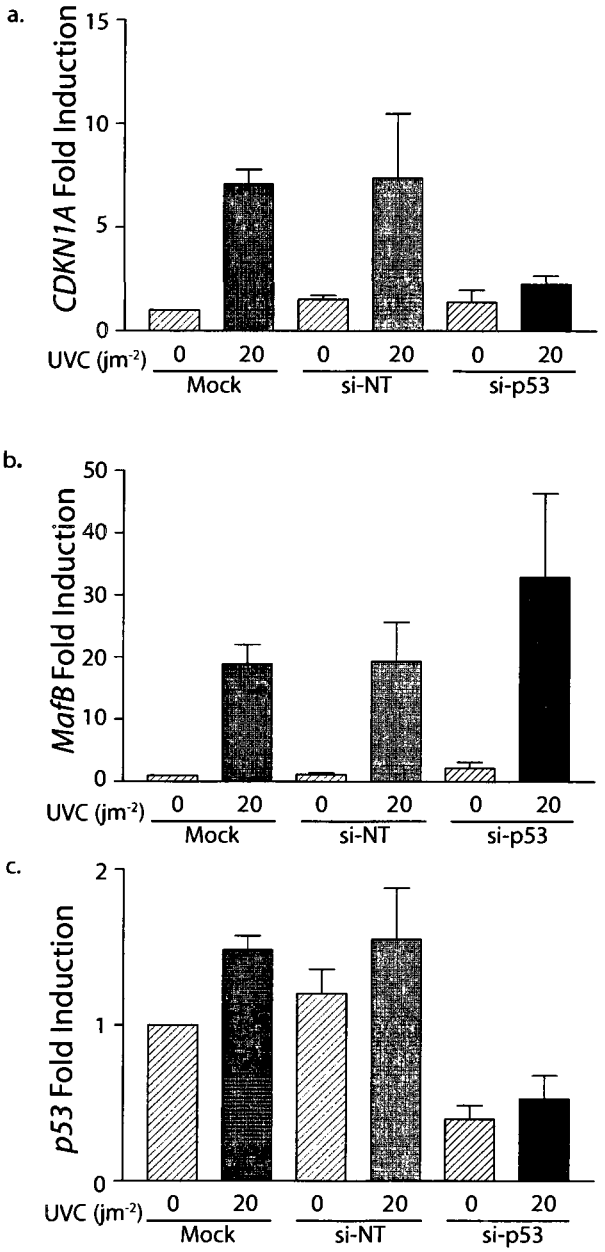
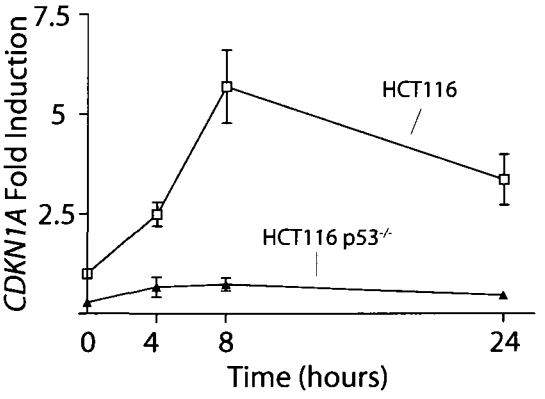

a.
CDKN1A Fold Induction
15
10
5
0
UVC (jm-2)
0
20
0
20
0
20
Mock
si-NT
si-p53
b.
MafB Fold Induction
50
40
30
20
10
0
UVC (jm-2)
0
20
0
20
0
20
Mock
si-NT
si-p53
c.
p53 Fold Induction
2
1
0
UVC (jm-2)
0
20
0
20
0
20
Mock
si-NT
si-p53

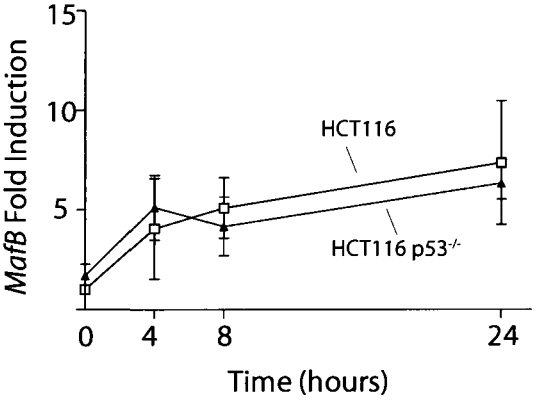
Figure 25. Induction of *MafB* following exposure to UVC in A549 cells is p53 independent.

A549 cells were mock treated, treated with non-targeting siRNA (si-NT) or siRNA targeting p53 (si-p53) and subsequently treated with 0 or 20 jm^{-2} UVC. Twenty four hours post-UVC treatment total RNA was collected and quantitative RT-PCR was performed to analyzed the transcripts of *CDKN1* (a), *MafB* (b), *p53* (c).

a.


7.5
5
2.5
CDKN1A Fold Induction
HCT116
HCT116 p53-/-
0
4
8
24
Time (hours)

b.


15
10
5
MafB Fold Induction
HCT116
HCT116 p53-/-
0
4
8
24
Time (hours)

c.

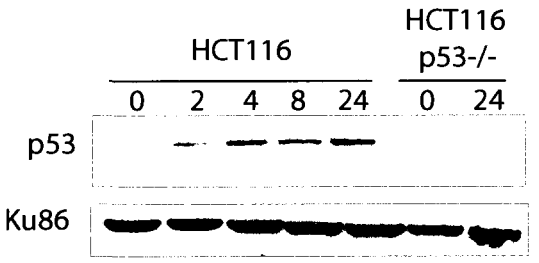

HCT116
HCT116 p53-/-
0 2 4 8 24 0 24
p53
Ku86

Figure 26. Time course of *MafB* induction following treatment with UVC and in the presence or absence of p53.

Quantitative RT-PCR analysis of *CDKN1A* (a) and *MafB* (b) transcript levels at indicated time points following treatment with 20 jm^{-2} UVC. Squares represent HCT116 cells and triangles represent HCT116$p53^{-/-}$ cells. (c) Immunoblot analysis of p53 levels in HCT116 and HCT116 $p53^{-/-}$ cells at indicated time points following treatment with 20 jm^{-2} UVC. Ku86 is used as a loading control.

the stress-mediated induction of *MafB*. Thus, multiple pathways are likely involved during endogenous transcriptional activation of the *MafB* transcript.

4. DISCUSSSION

4.1 SEQUENCE SPECIFIC TRANSACTIVATION BY P53

Transcriptional regulation, both positive and negative, contributes significantly to p53's function as a tumour suppressor. During transcriptional activation, p53 binds in a sequence specific manner (Kern et al., 1991) to its response element (two half sites of 5′-RRRCWWGY YY-3′ separated by a short spacer of 0-13 nucleotides in length) (El Deiry et al., 1992; Funk et al., 1992). These can be located in promoters, enhancer regions, introns, or the 5' untranslated regions of these genes (Harris and Levine, 2005). P53 contains an amino-terminal transcriptional activation domain (Fields and Jang, 1990; Raycroft et al., 1990) that subsequently recruits histone modifiers including acetyl transferases CBP/p300 (Avantaggiati et al., 1997; Barlev et al., 2001; Espinosa and Emerson, 2001; Lill et al., 1997), methyltransferases including PRMT1 (An et al., 2004), chromatin remodeling complexes such as SWI/SNF complexes (Bochar et al., 2000; Lee et al., 2002). Collectively, these proteins modify chromatin structure in the proximal promoter allowing preinitiation complex (PIC) assembly and the engagement of general transcription to transcribe RNA polymerase II target genes (An et al., 2004; Buschmann et al., 2001; Farmer et al., 1996; Gomes et al., 2006; Leveillard et al., 1996; Liu et al., 1993; Martin et al., 1993; Thut et al., 1995).

Over the years, transcriptional activation by p53 has been studied using a variety of techniques. Once it was established that p53 could function as a transcriptional activator (Farmer et al., 1992; Funk et al., 1992; Kern et al., 1992; Scharer and Iggo, 1992; Zambetti et al., 1992), a search for the transcriptional targets began. Among the first identified targets were muscle creatine kinase (Weintraub et al., 1991; Zambetti et al., 1992),

GADD45 (Kastan et al., 1992), *MDM2* (Barak et al., 1993; Wu et al., 1993), a *GLN* retroviral element (Zauberman et al., 1993) and *CDKN1A* (El Deiry et al., 1993). Subsequently, numerous genes have been identified using a variety of techniques. Although many of the targets have been identified a single gene at a time, more global approaches using oligonucleotide microarrays (Kannan et al., 2000; Kannan et al., 2001; Kho et al., 2004; Spurgers et al., 2006; Zhao et al., 2000), ChIP-on-ChIP (Ceribelli et al., 2006; Jen and Cheung, 2005; Krieg et al., 2006) and other high throughput technologies have also been employed (Sbisa et al., 2007; Wei et al., 2006). A recent review has attempted to consolidate our current knowledge of *bona fide* p53 transcriptional target genes. Riley *et al.* considered a gene to be p53 regulated if it met a minimum of three out of the following four criteria (1) a p53 response element was identified within or in proximity of the gene (2) the gene was up- or down-regulated at the RNA or protein levels by activated wild-type p53 protein and not by mutant p53 (3) the putative response element was functional in a reporter gene assay (4) p53 protein was identified on the gene's RE site in the DNA by chromatin immunoprecipitation with a p53 specific antibody (Riley et al., 2008). Their analysis identified 129 p53 target genes. This list of confirmed transcriptional targets is significantly lower than the projected 542 target genes based on the identification of p53 REs using a ChIP based strategy (SAGE) by Wei *et al.* (2006). Wei *et al.* examined 66 p53 target genes and found that 41 of these were bound by p53 in their analysis. However, establishing clear and concise lists of p53 target genes and REs is complicated due to stress and cell type specific patterns of the p53 response (Feng et al., 2007; Zhao et al., 2000).

In this study, adenovirus-mediated delivery of wild type p53 led to an increase in the expression of 254 transcripts (Table 2). Recent literature analysis of p53 transcriptional targets (Riley et al., 2008) confirms that 26 of these transcripts correspond to well characterized p53 target genes (identified in Table 2). A similar comparison with the potential p53 REs identified by SAGE revealed an additional 12 transcripts in our analysis that contain a potential p53 RE (Table 2) (Wei et al., 2006). Although much overlap exists in the p53 target genes analyzed in the Riley and Wei studies, there are many targets unique to each. The context of the p53 studies therefore greatly alters the pattern of gene expression and is especially apparent when we consider the different p53-target gene responses arising from alternate stressors (Feng et al., 2007; Zhao et al., 2000).

4.2 THE QS VARIANTS AS MODELS OF TRANSACTIVATION DEFICIENT P53

The transcriptional activation domain of p53 was initially identified to be contained within the first 73 acidic amino-acids at the amino-terminus of p53 (Fields and Jang, 1990). The transcriptional activation domain of p53 was subsequently determined to contain two independent subdomains (TD1 and TD2). Amino acids 1 to 42 (TD1) were found to function as a minimal transcriptional activation domain when fused with the GAL4 DNA binding domain (Fields and Jang, 1990; Unger et al., 1992). It was similarly shown that the second subdomain (amino acids 42-73) was capable of supporting sequence-specific transactivation when expressed as a fusion protein with a heterologous DNA binding domain (Candau et al., 1997; Chang et al., 1995).

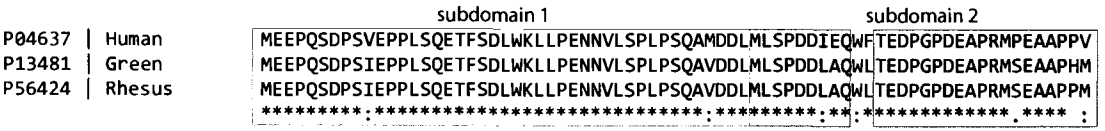
Like many other transactivation domains, the N-terminus of p53 is not highly conserved overall at the level of amino acid sequence between species; however, there is

a high level of sequence conservation among rodents and primates within sub-domain 1 (34% identity; see Figure 27). The region of highest homology includes amino acids 13 through 26 (93% identity), a region of p53 termed box 1 (Liu et al., 2001). Subdomain 2, within the transactivation domain of p53, is well conserved among primate species but is poorly conserved when the sequence comparison is extended to rodent versions of p53 (Figure 27). Unlike QS1, the limited homology between mouse and human p53 makes the QS2 variant difficult to model in mice.

The N-terminus of p53 is rich in acidic residues characteristic of acidic ADs and is mostly unstructured under physiological conditions (Lee et al., 2000). Regions of limited secondary structure within mostly unstructured ADs are common among transcriptional activators (Chi et al., 2005; Lin et al., 1994; Mapp and Ansari, 2007; Teufel et al., 2007). Box 1 contains a number of hydrophobic residues and nuclear magnetic resonance studies indicate that amino acids 18 to 26 within box 1 form a helix within the context of the larger disordered transactivation domain (Lee et al., 2000). Mutation of Leu-22 and Trp-23 within the helical region in the QS1 variant is predicted to disrupt this region of limited secondary structure (Lee et al., 2000) which is critical for interaction with cofactors (Gu et al., 1997; Lee et al., 2000; Liu et al., 2003; Teufel et al., 2007). Much like sub-domain 1, hydrophobic residues within subdomain 2 of human p53 give rise to localized secondary structure within the mostly unstructured acidic AD (nascent turns between Met-40 and Met-44 and between Asp-48 and Trp-53) (Chi et al., 2005; Kaustov et al., 2006; Lee et al., 2000). The second subdomain of p53 reportedly binds to many proteins known to interact with subdomain 1, such as mdm2, RPA, TFIID, TFIIH, and p300

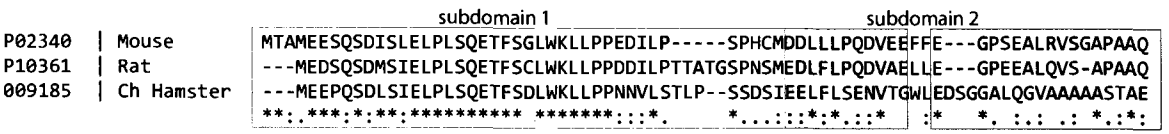
A

Primate



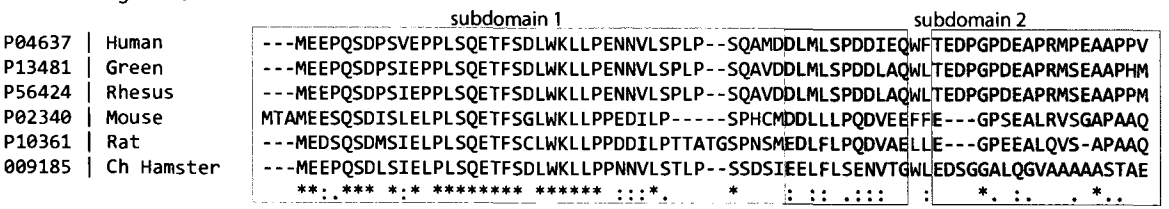
B

Rodent



C

Combined alignment



box 1

"*" denotes sequence identity.
":" denotes conservative substitutions.
"." denotes semiconservative substitutions.
Amino acids highlighted in yellow are mutated in the human QS variants of p53.

Figure 27. p53 evolutionary conservation.

Amino acid sequence alignments of the N-terminal transactivation domains of p53 of rodent and primate origin. Sequence of the first 72 amino acids of p53 from representative primate and rodent species was performed using CLUSTAL W software (Thompson et al., 1994). Sequence comparisons were performed for (a) human (*Homo sapiens*), African green monkey (*Chlorocebus sabaeus*) and rhesus monkey (*Macaca mulatta*) p53, (b) mouse (*Mus musculus*), rat (*Rattus norveigicus*), and Chinese hamster (*Cricetulus griseus*) p53, and (c) all six species listed in a and b.

(An et al., 2004; Avantaggiati et al., 1997; Buschmann et al., 2001; Chang et al., 1995; Chi et al., 2005; Espinosa and Emerson, 2001; Farmer et al., 1996; Leveillard et al., 1996; Lill et al., 1997; Liu et al., 1993; Martin et al., 1993; Teufel et al., 2007; Thut et al., 1995). Analogous to QS1, the QS2 point mutations likely disrupt the localized secondary structure and would be expected to disrupt protein–protein interactions important for transcriptional activation of target genes (Chi et al., 2005; Kaustov et al., 2006; Lee et al., 2000; Lin et al., 1994; Teufel et al., 2007).

Several studies have employed the QS variants of TD1 and TD2 as models for transactivation deficient p53 in order to examine their relative contributions in different biological settings. A few prominent studies, using different approaches, contrasted the properties of transactivation sub-domains 1 and 2. In 1999, Zhu *et al.* sought to determine the p53 domains critical for the induction of apoptosis. In their studies, they used the p53 null H1299 lung carcinoma and p53 wild type MCF7 breast carcinoma cells inducibly expressing $p53^{QS1}$ and $p53^{QS2}$ alone or in conjunction with a series of different domain mutants (Zhu et al., 2000). They noted that $p53^{QS2}$ had a severe deficiency in inducing *Cdkn1a* similar to previous reports for $p53^{QS1}$ (Chen et al., 1996b; Lin et al., 1994; Zhu et al., 1998). Using the QS variants and a panel of deletion and point mutations, they reported that $p53^{QS1}$ was unable to induce G1 arrest but retained the ability to induce apoptosis in these tumour cell lines. In contrast, $p53^{QS2}$ was able to induce cell cycle arrest but was impaired in its ability to induce apoptosis in these same cell lines (Zhu et al., 2000). A biological difference in the requirements between TD1 and TD2 and their regulated genes during these biological processes was inferred from this data.

Knock-in mice expressing the QS1 variant from the endogenous p53 locus have been generated (Johnson et al., 2005b). Homozygous $p53^{QS1}$ expression results in embryonic lethality; however, homozygous $p53^{QS1}$-targeted mouse embryonic fibroblasts (MEFs) were obtained (Johnson et al., 2005b). Although the induction of most genes, including *Cdkn1a*, was decreased to levels between 5 and 30% of $p53^{wt}$ expressing MEFs, *BAX* was induced to levels near those of $p53^{wt}$ following doxorubicin treatment (Johnson et al., 2005b). Johnson *et al.* postulated that distinct target gene-specific mechanisms were involved in the regulation of *BAX* that bound to an area unaffected by the QS1 mutation. Therefore, some transcriptional activity was retained by the QS1 variant.

Cregan *et al.* (2004) studied the consequences of $p53^{QS1}$ and $p53^{QS2}$ on the expression of a subset of p53 target genes in the context of apoptosis in murine neuronal cells. Semiquantitative RT-PCR analysis of known p53 target genes revealed that either the $p53^{QS1}$ or $p53^{QS2}$ subdomain induced *Noxa* expression but neither variant was able to increase *PUMA* expression alone. They also noticed that forced expression of the $p53^{QS2}$ variant in p53 nullizygous neuronal cells led to more apoptosis than the $p53^{QS1}$ variant when these cells were subsequently treated with camptothecin (Cregan et al., 2004). Based on this work, it has been hypothesized that the p53 transactivation sub-domains contribute to the regulation of distinct subsets of p53 target genes, which are linked to the biological activity of these variants. However, the relative contribution of the two distinct transactivation sub-domains to global p53-dependent transcriptional activity had remained untested. In order to evaluate the individual contributions of TD1 and TD2 to genome-wide transcriptional activation, we studied the previously characterized QS variants of p53 ($p53^{QS1}$, $p53^{QS2}$, and $p53^{QS1/QS2}$).

4.3 $P53^{QS1}$ AND $P53^{QS2}$ SHARE SIMILAR ACTIVITY IN TRANSCRIPTIONAL ACTIVATION AND BIOLOGICAL PATHWAYS

4.3.1 *TRANSCRIPTIONAL ACTIVATION BY $P53^{QS1}$, $P53^{QS2}$, AND $P53^{QS1/QS2}$ IS IMPAIRED COMPARED TO $P53^{WT}$*

In these experiments, despite infection with an equivalent viral load, protein expression of the variants was elevated compared to $p53^{wt}$ (Figure 10b). This was expected as the variants are deficient in the transcriptional activation of MDM2 (Figure 16) and the QS1 variants are further impaired in their interaction with MDM2 resulting in decreased proteosomal mediated degradation. Despite increased protein levels, infection of HCT116$p53^{-/-}$ cells with recombinant adenoviruses expressing $p53^{QS1}$, $p53^{QS2}$, and $p53^{QS1/QS2}$ resulted in the induction of far fewer transcripts than $Adp53^{wt}$ infection (Table 2). The majority of p53-regulated transcripts were induced poorly by $p53^{QS1}$, $p53^{QS2}$, and $p53^{QS1/QS2}$, indicating that both sub-activation domains are required to increase the expression of most p53 target genes (Figure 14). The identity and fold increase in expression of the $p53^{QS1}$- and $p53^{QS2}$-upregulated transcripts were strongly correlated, indicating that these sub-domains do not contribute to the expression of distinct subsets of genes (Figure 15).

In accordance with the results of Johnson *et al.* (2005) we have also found that the human QS1 variant was able to upregulate *BAX* expression to near wild-type levels (Figures 17). Unexpectedly, we found that $p53^{QS1}$ and $p53^{QS2}$ similarly induced the expression of *BAX* along with three other known p53 target genes that were poorly induced by the $p53^{QS1/QS2}$ variant (TNFRSF10B, BTG2, and POLH) (Figure 16). Therefore, only one

of the two sub-domains appears to be necessary and sufficient for p53-dependent gene expression of this subset of p53 target genes in these cells. We interpret the heterogeneous requirement for sub-domains 1 and 2 to indicate that the requirement for specific protein–p53 AD interactions must vary in a p53 target gene–specific manner.

4.3.2 *$p53^{QS1}$ AND $p53^{QS2}$ ARE IMPAIRED AT INDUCING TRANSCRIPTS IN THE SAME BIOLOGICAL PATHWAYS AND REGULATING CELL DEATH*

Gene ontology analysis using GoStat (http://gostat.wehi.edu.au/) indicated that apoptosis-associated transcripts were statistically over-represented following $Adp53^{wt}$ infection (Table 4). These apoptosis related transcripts were in general poorly induced by $p53^{QS1}$ and $p53^{QS2}$ (consistent with their ability to regulate cell death discussed below). Similarly, p53-induced transcripts corresponding to cell cycle regulators were poorly induced by both TD variants. Two of the three DNA-repair genes induced by $p53^{wt}$ were induced to near wild type levels by $p53^{QS1}$ and $p53^{QS2}$ but not $p53^{QS1/QS2}$. However, due to the small number of p53-upregulated DNA repair genes, this was not significant.

The requirement of the transcriptional activation domains during the induction of apoptosis is currently unclear. In certain situations TD1 is thought to be essential for the induction of apoptosis (Attardi et al., 1996; Cregan et al., 2004) whereas in others it is not (Chen et al., 1996b; Ding et al., 2000; Haupt et al., 1995). In experiments with QS1 knock-in MEFS, the apoptotic potential following doxorubicin treatment, serum deprivation and hypoxia varied greatly by individual stressors (Johnson et al., 2005b). Specifically, $p53^{QS1}$ was unable to induce apoptosis following treatment with doxorubicin, induced a moderate level of apoptosis following serum deprivation, but maintained significant apoptotic potential following exposure to hypoxia (Johnson et al., 2005b). Their results

suggest stress and gene specific regulation by the TD1 region. The requirement for TD2 during apoptosis has similarly been studied. Zhu *et al.* employed multiple techniques (including the QS2 mutant and domain deletions) in order to study TD2. They found TD2 to be essential for apoptosis (Zhu et al., 1998; Zhu et al., 2000).

From our genome-wide analysis it can be seen that $p53^{QS1}$ and $p53^{QS2}$ retain partial ability to transactivate pro-apoptotic genes but $p53^{QS1/QS2}$ did not (Tables 2 and 4). The p53 variants were assessed for their ability to induce apoptosis (Figure 18). We found that the QS1 and QS2 variants retained a limited apoptotic potential whereas the QS1/QS2 variant did not induce apoptosis. Previous studies in the p53 null H1299 and Saos-2 cell lines demonstrated strikingly similar results (Venot et al., 1999). In this study, Venot *et al.* ectopically expressed p53 and variants and examined growth suppression and apoptosis by colony formation assay and FACS analysis. They observed intermediate growth suppression and induction of apoptosis by QS1 and QS2 whereas QS1/QS2 was completely deficient for both (Venot et al., 1999). Therefore, based on these results, we infer a correlation between the integrity of the transcriptional activation domains and the induction of cell death by p53 in our system.

4.3.3 *IDENTIFICATION OF TWO DISTINCT CLASSES OF P53 REGULATED PROMOTERS*

Taken together, our strategy failed to identify sub-domain specificity for p53 target gene expression. Instead, the present work suggests there are at least two classes of promoters regulated by p53 (Figure 28). The first class requires the function of both TD1 and TD2 and is found in the vast majority of p53 target-genes. The second class of promoters only requires the integrity of either TD1 or TD2 which can function interchangea-

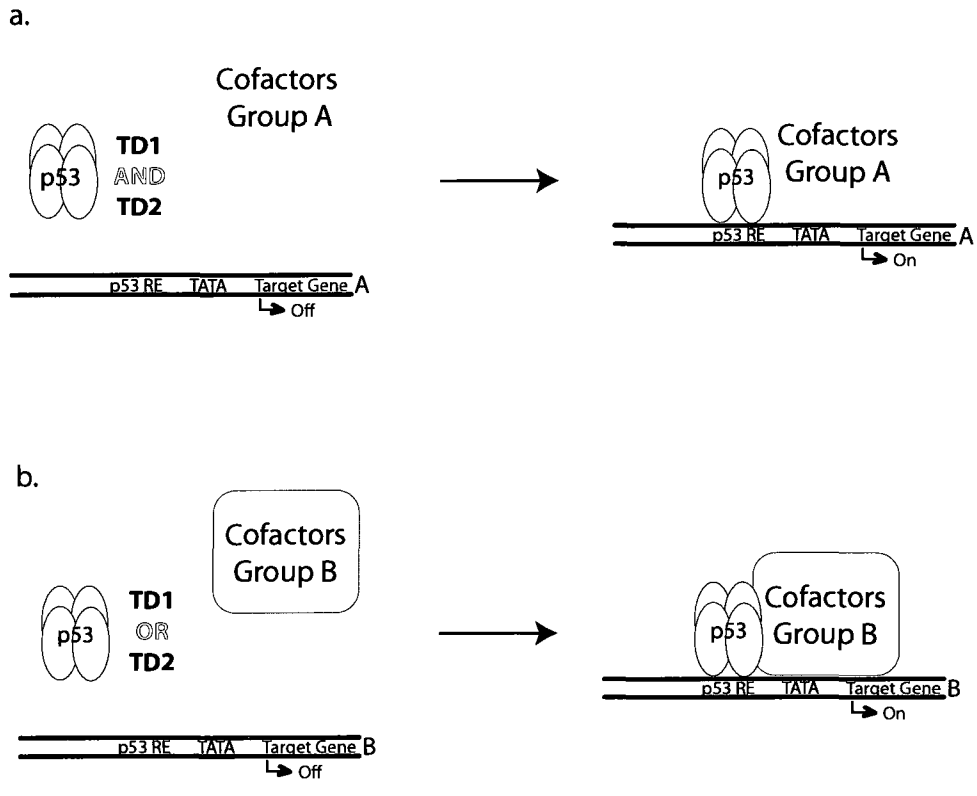

a.
Cofactors
Group A
p53
TD1
AND
TD2
p53 RE
TATA
Target Gene A
Off
Cofactors
Group A
p53
p53 RE
TATA
Target Gene A
On
b.
Cofactors
Group B
p53
TD1
OR
TD2
p53 RE
TATA
Target Gene B
Off
Cofactors
Group B
p53
p53 RE
TATA
Target Gene B
On

Figure 28. Model of the involvement of transactivation domains one and two to p53 transcriptional activation.

Schematic representation of the requirement of TD1 and TD2 during p53 mediated transcriptional activation. (a) p53 transcriptional activation of the majority of target genes requires the activity of both TD1 and TD2. (b) p53 transcriptional activation of a small group of genes requires only the presence of TD1 or TD2.

bly. There is compelling evidence supporting the existence of distinct classes of p53 target genes. For example, expression profiling identified a variety of gene clusters exhibiting distinct expression patterns (Zhao et al., 2000). In addition, p53 regulated genes frequently exhibited cell line-dependent patterns of p53-dependent expression (Yu et al., 1999). These early reports suggested the existence of promoter-specific effects of p53-induced gene expression.

More recently, ChIP experiments indicated that a subset of p53 target genes, exemplified by p21, are transcriptionally poised (Espinosa et al., 2003). Preinitiation complex (PIC) assembly is p53 dependent in unstressed cells. Conversely other p53 target genes such as TNFRSF6 are not transcriptionally poised as they do not preassemble PIC. Activation of p53 leads to distinctly slow kinetics of p53-induced gene expression. Espinosa *et al.* (2003) also examined the assembly of transcriptional machinery on multiple promoters following various stresses. They reported that distinct promoter- and stress-specific activation mechanisms existed. Most notably, they observed a gradient of paused RNA polymerase II (RNAP II) among p53 promoters that correlated with key transcriptional components such as TAFII250 and TFIIB in order to selectively regulate target genes (Espinosa et al., 2003). In a subsequent study, different requirements for the transcription elongation factor (P-TEFB) as well as the phosphorylation of RNAP II were identified (Gomes et al., 2006). A decreased requirement for P-TEFB and phosphorylation of RNAP II are proposed as mechanisms enabling continued transcription of a specific set of target genes in light of a stress-induced decrease in mRNA synthesis. Therefore, these studies support the existence of different classes of p53-regulated promoters.

4.4 DIFFERENTIAL GENE REGULATION BY $P53^{QS1}$ AND $P53^{QS2}$ FROM $p53^{wt}$

We considered the possibility that $p53^{QS1}$, $p53^{QS2}$ or $p53^{QS1/QS2}$ could have altered transcriptional activity and thereby regulate non-p53-regulated genes. In this study, the transcriptional activity of the QS variants was interpreted based on the subgroup of $p53^{wt}$ induced genes. Interestingly, a small group of genes was induced by either $p53^{QS1}$, $p53^{QS2}$ or $p53^{QS1/QS2}$ that was not induced by $p53^{wt}$. The effect of infection with $p53^{wt}$ for this subset of transcripts was also examined. However, most of the transcripts within this analysis were extremely close to our described threshold (see Materials and Methods). In terms of p53-induction, all but one transcript fell into one of the following categories (1) statistically increased in one experiment (2) transcript statistically increased in both experiments but not above 2.0 fold (3) transcript increased above 2.0 fold and statistically increased in only one experiment. Therefore, this group represents transcripts that fall just short of our criteria to be classified as induced so we cannot interpret this pattern of gene expression with confidence.

4.5 REPRESSION BY P53WT AND THE QS VARIANTS

Transcriptional regulation by p53 can be both positive and negative. Transcriptional repression occurs through distinct mechanisms from activation which can be broadly grouped into the following five categories (Laptenko and Prives, 2006): recruitment of HDACs and chromatin modifying factors (Ho et al., 2005; Koumenis et al., 2001; Murphy et al., 1999), repression of transcriptional activators through physical interaction and interference with promoter activation (Gu et al., 2004; Kim et al., 2003; Liu et al., 1999; Maeda et al., 2002; Maiyar et al., 1997; Zhu et al., 2005), displacement of

transcriptional activators from binding sites in the promoter (Lee et al., 1999; Li and Lee, 2001; Ori et al., 1998; St et al., 2004), interference with the assembly of transcription machinery (Ko and Prives, 1996; Subbaramaiah et al., 1999), and novel REs containing unique architecture (Johnson et al., 2001; Johnson et al., 2005a). Currently, there appear to be anywhere from 200 to 1700 p53-repressed genes based on genome wide studies (Burns and El-Deiry, 2003; Mirza et al., 2003; Robinson et al., 2003).To the best of our knowledge, there is only one transcript, *Map4*, that is repressed in a p53 transactivation domain-dependent manner (Murphy et al., 1996). Therefore, it is not entirely surprising that we found very few changes in p53 repressed genes.

4.6 THE QS VARIANTS RETAIN MITOCHONDRIA-TARGETTING ACTIVITY

In addition to the regulation of apoptosis-related genes, p53 can translocate to mitochondria (Dumont et al., 2003; Marchenko et al., 2000; Mihara et al., 2003; Sansome et al., 2001). Mitochondrial p53 induces apoptosis by interacting with anti-apoptotic BH3 family members including BAK (Leu et al., 2004), BclXL and Bcl2 (Mihara et al., 2003). Interestingly, a p53 construct fused with the prototypical mitochondrial import leader of ornithin transcarbamylase that is unable to transactivate target genes is capable of inducing apoptosis. As it is reported that the normal targeting of p53 to the mitochondria requires MDM2 activity either by interaction with the TAD (Dumont et al., 2003) or monoubiquitination (Marchenko et al., 2007), we assessed whether the p53 variants retained the ability to localize to the mitochondria. Using cellular fractionation assays and immunoblot assay, we determined that all the variants could be detected in the mitochondrial fraction following adenovirus mediated expression (Figure 19). Therefore, our experi-

ments suggest that the QS mutants possess similar abilities to translocate to the mitochondria.

4.7 POTENTIALL DIFFERENTIAL REGULATION BY $P53^{QS1}$ AND $P53^{QS2}$

An overwhelming pattern of transcriptional activation by the variants demonstrated that the majority of p53 target genes are induced to similar levels by both $p53^{QS1}$ and $p53^{QS2}$. However, a few genes appeared as though they may be preferentially induced by either of the variants (Figure 11 and 20a). One gene that appeared preferentially induced by $p53^{QS2}$ compared to $p53^{QS1}$ was v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (*MafB)*. Although *MafB* was not statistically induced by QS1 and QS2 in the microarrays, it exhibited a QS2- specific pattern of expression in our microarray experiments that was subsequently confirmed using quantitative RT-PCR analysis (Figure 20a). As the most prominent potential domain-specific transcript, we examined the pattern of regulation of this gene by quantitative RT-PCR. Wild type p53 expression led to a large fold increase in expression confirmed at 8, 16, and 24 hours post-infection respectively and this gave rise to an increase in MafB protein (Figure 20b-d). Infection of cells with $Adp53^{QS1}$ and $Adp53^{QS2}$ led to a reproducible increase in the expression of *MafB*. Indeed $p53^{QS2}$ consistently led to a greater increase in *MafB* expression compared to $p53^{QS1}$. However, both variants were reduced in their ability to increase *MafB* expression compared to wild type p53. We interpret this analysis to mean that, although minor differences exist in the induction level following expression of either $p53^{QS1}$ or $p53^{QS2}$, neither variant is able to activate *MafB* to wild type levels. Again we find no clear evidence of domain-specific p53-regulated gene expression.

4.8 EXPLORING MECHANISMS REQUIRING DIFFERENTIAL ACTIVITY OF TD1 AND TD2

Our observations have demonstrated that both TD1 and TD2 contribute to a similar extent during p53-mediated gene regulation. However, as previously noted, there are several reports that propose different requirements for TD1 or TD2 during specific biological processes. Although transcriptional activation by p53 is an important component of its tumour suppressive function, transactivation-independent mechanisms are also important. A recent study examined the activity of the N-terminus of p53 by swapping the p53 transcriptional activation domain with the Herpes Simplex Virus VP16 transcriptional activation domain in the endogenous murine p53 locus (Johnson et al., 2008). Although competent for activation of p53-target genes, this chimeric protein retained the ability to induce senescence but not apoptosis. Therefore an activity within the amino-terminus in addition to transcriptional activation is required during the induction of apoptosis (Johnson et al., 2008).

Further, p53 targeting to the mitochondria is a transactivation independent activity. Mitochondrial p53 interacts with Bcl2 and Bcl-xL through its core DNA-binding domain thereby promoting mitochondria membrane permeabilization. The localization of p53 to the mitochondria reportedly requires the activity of MDM2, likely through monoubiquitination (Dumont et al., 2003; Marchenko et al., 2007). Hence, the ability of MDM2 to interact with its binding region in the amino-terminus may be a critical component during localization to the mitochondria.

Post-translational modifications play a vital role in the regulation of p53 activity. They regulate protein stability, localization, protein-interactions and importantly transcriptional activation patterns.

4.9 *MAFB* IS A PUTATIVE P53 TARGET GENE

According to a recent study of potential p53 binding sites in the genome, there exists approximately 540 potential binding sites, representing several hundred more targets than the current 129 *bona fide* p53 target genes (Riley et al., 2008; Wei et al., 2006). Novel p53 transcriptional target genes are continuously being identified. Here, we observed a significant induction of *MafB* which had not yet been described as a p53 target. MafB is a member of the Maf family of proteins which are characterized by a DNA-binding domain containing both, a basic domain and a leucine zipper motif (bzip) (Chinenov and Kerppola, 2001; Vinson et al., 2002). They interact with Maf recognition elements (MARE), DNA consensus sequences containing an AP-1 core with characteristic 3-bp extensions ($TGC(N)_{6-7}GCA$) (Blank and Andrews, 1997; Motohashi et al., 2002).

Since *MafB* has not previously been characterized as a p53 target gene and we found that over-expression of p53 can induce both its mRNA and protein levels we sought to assess its potential as a *bona fide* p53 target. Examination up-stream of the *MafB* promoter revealed a sequence containing four potential p53 binding half sites (Figure 21a). We found p53 could bind to the putative RE *in vitro* and *in vivo* (Figure 21b and c). These results therefore demonstrate an *in vitro* and *in vivo* interaction between p53 and this sequence up-stream of *MafB*. Therefore, *MafB* appears to be a *bona fide* p53 target gene as it contains a p53 RE and is specifically upregulated by p53 at mRNA and protein levels (and conforms to the definition of a p53 target gene of Riley *et al.*, 2008).

To date, MafB has been implicated in development and differentiation processes. A significant role for MafB has been established in monocytic differentiation (Kelly et al., 2000; Pouponnot et al., 2006) and podocyte differentiation (Sadl et al., 2002). Mice with the Kreisler (Krml1) mutation of MafB are plagued with developmental anomalies affecting the caudal hindbrain and inner ear (Cordes and Barsh, 1994). In mice, homozygous deletion of *MafB* results in fatal central apnea at birth likely due to defective respiratory rhythomogenesis (Blanchi et al., 2003).

Several studies have demonstrated a clear oncogenic role for MafB. In multiple myeloma a translocation between the immunoglobulin heavy chain region resulting in the ectopic expression of MafB is frequently detected (Boersma-Vreugdenhil et al., 2004a; Hanamura et al., 2001a). *In vitro* studies of multiple myeloma derived cell lines demonstrate that MafB promotes proliferation and inhibits apoptosis when exposed to Doxycycline (Van Stralen et al., 2009). Although weaker than other Maf family members such as MafA and c-Maf, MafB can promote transformation in certain cell lines (Pouponnot et al., 2006). Interestingly, a recent study aimed at identifying the cause of prolonged alveolar macrophage survival when exposed to cigarette smoke, determined that both *MafB* levels and MafB DNA binding capacity to MARE sequences are significantly upregulated in these tissues (Machiya et al., 2006). Taken together, the current state of knowledge of MafB activity suggests it can contribute to oncogenesis. However, our understanding of the involvement of p53 during *MafB* regulation is wanting.

The primary roles thus far associated with MafB is as an inducer of monocyte differentiation (Kelly et al., 2000), as a protooncogene during the development of multiple myeloma (Boersma-Vreugdenhil et al., 2004b; Hanamura et al., 2001b) and as an anti-

apoptotic factor in alveolar macrophages of smokers (Machiya et al., 2006). Although seemingly counterproductive to its role as a tumour suppressor, p53 has been shown to regulate anti-apoptotic genes and possess anti-apoptotic properties under certain circumstances. Anti-apoptotic genes upregulated in the presence of activated p53 include WT1 (Maheswaran et al., 1995), TNF-related apoptosis-inducing ligand (TRAIL) decoy receptor (TRUNDD) (Meng et al., 2000), cyclooxygenase 2 (COX-2) (Han et al., 2002), and heparin-binding epidermal growth factor (HB-EGF) (Fang et al., 2001). Furthermore, a protective role for low levels of p53 has been observed in mouse fibroblast when faced with serum deprivation (Lassus et al., 1996) and human normal fibroblasts when exposed to UVC and cisplatin (El-Mahdy et al., 2000; McKay et al., 2001; McKay and Ljungman, 1999; Wani et al., 1999). As p53 mediates DNA-repair, the requirement for p53 during DNA-repair is postulated to result in the survival of cells with intact repair pathways (McKay et al., 2001; McKay and Ljungman, 1999). Additionally, a fine balance exists between the induction of apoptosis and cell cycle arrest. Adenovirus mediated expression of $p21^{WAF1}$ in cells results in a shift towards cell cycle arrest instead of apoptosis following Adp53 expression (Gorospe et al., 1997). Similarly, expression of p53 in cells with a homologous deletion of the $p21^{WAF1}$ gene results in elevated levels of apoptosis compared to cells expressing wild type $p21^{WAF1}$ (Kokontis et al., 2001; Polyak et al., 1996). Therefore the p53-mediated induction of the $p21^{WAF1}$ gene can also be interpreted as anti-apoptotic.

4.10 STRESS MEDIATED REGULATION OF *MAFB*

Subsequent to cellular stress, the transcriptional program of p53 is critical for the proper cellular response. Given the aforementioned association between *MafB* and the

regulation of cellular survival and transformation, we examined the expression of *MafB* in several cell lines following exposure to the well known genotoxic stress, UVC. We found that in HCT116, GM38 and A549 cells *MafB* was significantly up-regulated twenty four hours post-treatment with 20 $J \cdot m^{-2}$ of UVC (Figure 22). Similarly, following exposure to 10 Gy X-irradiation, a moderate increase in *MafB* was observed (Figure 23). *MafB* may therefore be an important part in the response to cellular stress.

Considering that p53 can induce *MafB* and its induction is observed following UVC exposure and X-ray treatment, we examined whether there is a requirement for p53 during this stress-mediated induction. We were unable to detect any requirement for p53 during the activation of MafB subsequent to either UVC or X-ray treatment. Therefore, p53 activity is not essential during the induction of *MafB* following UVC and X-irradiation of HCT116 cells. Although neither of the treatments examined displayed a requirement for p53, these results do not preclude the possible involvement of p53 in *MafB* regulation subsequent to other stress of biological activities.

4.11 POSSIBLE MECHANISMS OF *MAFB* REGULATION

The observation that p53 is not essential for the induction of MafB prompted the reexamination of its promoter. Using PROSCAN Version 1.7, which predicts promoter regions based on scoring homologies with putative eukaryotic Pol II promoter sequences (http://www-bimas.cit.nih.gov/molbio/proscan/), we examined the promoter region of *MafB* (Prestridge, 1995). Interestingly we found that several different transcription factor binding sites are present including twelve Sp1 sites. Cooperation between different transcription factors is a central theme in transcriptional regulation and specific cooperation between p53 and Sp1 has been reported to positively or negatively influence transcription

of different genes (Bond et al., 2004; Innocente and Lee, 2005; Kanaya et al., 2000; Koutsodontis et al., 2005; Kubicka et al., 1999; Li and Lee, 2001; Ohlsson et al., 1998; Sun et al., 2004; Thornborrow and Manfredi, 2001; Zhu et al., 2000). Quite interestingly, the p53 target gene *CDKN1A* can be efficiently induced in $p53^{-/-}$ MEFs following UV-damage (Haapajarvi et al., 1999). The p53-independent induction of CDKN1A was mediated through Sp1 following UV-damage through two Sp1 consensus sites. Therefore, the regulation of *MafB* is likely the product of the interplay between several different transcriptional regulators and not solely p53.

4.12 CONCLUSION

In summary, our results suggest that compound mutations of critical hydrophobic amino acids in either subdomain 1 or 2 decrease the affinity of the AD for cofactors or other components of basal transcription apparatus that are required for p53-dependent gene expression at most p53- induced promoters (Figure 28). There appear to be at least two classes of promoter regulated by p53. The vast majority of p53 regulated genes require both TD1 and TD2 to be intact. The second contains a small subset of p53-responsive genes, including BAX, TNFRSF10B, BTG2, and POLH, which are not limited by mutations in either of the subdomains alone. Therefore, this latter group of genes appears to have a less stringent requirement for as yet unidentified protein–protein interactions. Importantly, we did not find any genes that were preferentially induced by any single TAD variant. Our results support a model in which the TAD subdomains of p53 contribute equally to p53-dependent target gene expression. Further study of the p53TAD will hopefully uncover the mechanisms of sub-domain regulation following the various biological stimuli which trigger p53 activity.

The identification of a new p53-regulated gene *MafB* is clearly of great importance in advancing our understanding of p53 biology. Although we found that a p53 RE is located upstream of *MafB*, that p53 can transcriptionally regulate *MafB* and that *MafB* is induced subsequent to UVC and x-irradiation, p53 is not essential for the DNA damage mediated regulation of MafB. Further studies involving the p53 family members p63 and p73 as well as different p53 activating stimuli will likely aid in uncovering the significance of this finding.

4.13 Future Directions

Our study of the p53 transactivation domains revealed that, in the absence of cellular stress, both transcriptional activation domains of p53 contribute similarity to gene activation. However, several studies have suggested differential patterns of gene regulation for the two domains, pointedly in the presence of cellular stress. Recently, several studies have examined the consequences of post-translational modifications on the affinity of protein-protein interactions involving the p53 amino-terminus. It is well documented that specific cellular stresses give rise to distinct patterns of post-translational modification within p53 including the amino-terminus (Figure 3). Jenkins *et al.* (2009) and Teufel *et al.* (2007) examined the interaction between p53 and the Taz2 domain of p300 and found that both TD1 and TD2 were capable of binding this domain (Jenkins et al., 2009; Teufel et al., 2007). Interestingly, when specific residues are phosphorylated within TD1 there is a significant increase in the affinity of the p53-Taz2 interaction but phosphorylation of residues within TD2 does not alter the interaction affinity. In addition, p300 can interact with p53 in five different regions and the affinity of these regions for p53 also varies with specific phosphorylations in the p53 amino-terminus thereby providing an array of potential configurations with likely differing promoter regulation (Polley et al., 2008). When considering the diverse group of transcriptional co-regulators involved in p53 transcriptional activation that may be affected in a similar manner to the p53-p300 interaction, a diverse network of potential response in promoter regulation emerges. In conjunction with our results, this would suggest that in unstressed cells, TD1 and TD2 have a similar potential to transactivate genes and that the specific modifications that occur subsequent to cellular stress dictate the affinity of specific protein-protein

interactions within the amino-terminus and subsequently the down-stream regulation of specific promoters. Therefore, further study of the relationship between stress mediated post-translational modifications, interaction with transcriptional regulators, and promoter specific regulation is warranted to uncover the nature of this relationship.

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Regulation of ultraviolet light-induced gene expression by gene size

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UV light induces the expression of a wide variety of genes. At present, it is unclear how cells sense the extent of DNA damage and alter the expression of UV-induced genes appropriately. UV light induces DNA damage that blocks transcription, and the probability that a gene sustains transcription-blocking DNA damage is proportional to locus size and dose of UV light. Using colon carcinoma cells that express a temperature-sensitive variant of p53 and undergo p53-dependent apoptosis after UV irradiation, we found that the number of p53-induced genes identified by oligonucleotide microarray analysis decreased in a UV dose-dependent manner. This was associated with a statistically significant shift in the spectrum of p53-induced genes toward compact genes with fewer and smaller introns. Genes encoding proapoptotic proteins involved in the initiation of the mitochondrial apoptotic cascade were prominent among the compact p53 target genes, whereas genes encoding negative regulators of p53 and the mitochondrial apoptotic pathway were significantly larger. We propose that the shift in spectrum of UV-responsive gene expression caused by passive effects of UV lesions on transcription acts as a molecular dosimeter, ensuring the elimination of cells sustaining irreparable transcription-blocking DNA damage.

DNA damage | microarray | p53 | transcription | stress responses

The human genome contains between 26,000 and 39,000 genes distributed throughout ≈3 billion base pairs of DNA (1). The coordinated regulation of this many genes presents a daunting task. Superimposed upon the complexity of transcriptional regulation is the fact that many DNA damaging agents can induce damage that inhibits elongation by RNA polymerase II (2). These transcription-blocking DNA lesions pose an obvious difficulty to the coordinated regulation of gene expression in those cells that have sustained sufficient damage. It is not fortuitous that highly conserved DNA repair pathways coupling transcription to DNA repair have evolved and that mechanisms exist to eliminate cells with excessive transcription-blocking lesions.

Transcription-coupled nucleotide excision repair (TC-NER) preferentially removes transcription-blocking lesions from the transcribed strand of active genes, facilitating the recovery from DNA damage-induced transcriptional arrest (3–5). Much of what is known about TC-NER and DNA damage-induced transcriptional stress stems from studies using the model DNA damaging agent, UV light. Cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs), the most common lesions induced by short-wavelength UV light, block transcription and are substrates for TC-NER (6, 7). After exposure to cytotoxic doses of UV light, cells fail to recover transcription and undergo apoptosis (4, 8), and this is thought to occur primarily through the mitochondrial pathway involving cytochrome *c*, Apaf1, and caspase 9 (9–11). There is a very tight correlation between the sustained inhibition of transcription and UV-induced apoptosis, indicating that cells sense sustained transcriptional stress as an indicator of irreparable DNA damage (4, 8, 12, 13).

UV light activates signaling cascades that ultimately result in the activation of transcription-factors such as AP-1 and the p53 tumor suppressor (14, 15). However, UV-induced DNA damage also inhibits transcription by posing a physical impediment to elongation by RNA polymerases (2). The likelihood that a gene sustains DNA damage after UV irradiation is proportional to gene size because the induction of UV photoproducts is a stochastic event (16). A moderate dose of 10 J/m^2 of UV light that induces apoptosis in <20% of UV-irradiated primary human fibroblasts or most other DNA repair-proficient human cell lines (4, 13, 17–20) yields ≈2.4 lesions per strand of an average gene (Fig. 4, which is published as supporting information on the PNAS web site) (1, 7, 21–25). This high lesion frequency led us to hypothesize that transcription-blocking UV lesions could dramatically effect the expression of UV-induced genes. Here we report that the spectrum of genes induced by UV light or by p53 in the presence of UV-induced DNA damage is significantly altered in a dose-dependent manner. The genes induced at cytotoxic doses of UV light were compact, with fewer and smaller introns, and were enriched for initiators of apoptosis acting through the mitochondrial pathway. We propose that cells use this passive mechanism of gene regulation to ensure that cells sustaining irreparable DNA damage are eliminated by apoptosis.

Materials and Methods

Cell Culture and UV Treatment. The HT29-tsp53 colorectal carcinoma cells expressing a temperature-sensitive variant of p53 and HT29-neo vector control cells (26) were obtained from Mats Ljungman and Jon Maybaum (University of Michigan, Ann Arbor). Cells were grown at the restrictive temperature (38°C) in 10-cm tissue culture dishes in DMEM supplemented with 10% FCS (GIBCO/BRL) and 5 μg/ml gentamicin (Sigma). Growth medium was removed before UV iradiation, and fresh medium was immediately replaced after treatment. Fluence was determined with a UV radiometer (Ultraviolet Products) to deliver ≈1 J/m^2/s from a germicidal bulb (Philips) emitting predominately at 254 nm.

RNA Isolation. Cells were seeded 48 h before treatment. At the time of treatment, cells were irradiated with the indicated doses of UV light and were either returned immediately to the restrictive temperature (38°C) or switched to the permissive temperature (32°C). Cells were harvested 6 h after treatment. Total RNA was isolated by using the RNeasy RNA isolation kit (Qiagen, Valencia, CA) according to manufacturer's specifications. The yield of total cellular RNA was not dramatically affected by UV dose by this time.

Quantitative RT-PCR. Five micrograms of total RNA was reverse-transcribed by using first-strand cDNA synthesis kit (MBI Fer-

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mentas). Quantitative RT-PCR was performed by using the Sybr green fluorescent DNA stain (Molecular Probes), a LightCycler 2 quantitative PCR machine (Roche Diagnostics) and LIGHTCYCLER software version 3 (Roche Diagnostics). The primers used were CRYAB (AGCCGCCTCTTTGACCAGTTCTT and GCGGTGACAGCAGGCTTCTCTTC), p21WAF-1/CDKN1A (CCTCAAATCGTCCAGCGACCTT and CATTGTGGGAGGAGCTGTGAAA), FAS (CTCATCTTAATGGCCTAATGCA and GCTTCAGTTTATAACTATCTTCAC), DDB2 (CCACCTTCATCAAAGGGATTGG and CTCGGATCTCGCTCTTCTGGTC), serpinB5/maspin (CTTTTCTGTGGATGCCGATT and CCTGCCAGGGCTTAACATAA), XP-C (AAGTTCACTCGCCTCGGTTGC and TTCTTTCCTGATTTTAGCCTTTTT), SMAD-3 (AGGCGTGCGGCTCTACTACATC and GGGCGTTTCTGGTTGGACTG), EFNB1 (GGAGGCAGACAACACTGTCA and GAACAATGCCACCTTGGAGT), LOC254531 (CCGAATGTGAGTTTGTAGG and TTTGGTGTCTGGGAGGTG), MAX (CTCTCGTGGTATGTATGGG and AGTAGGAAAGGAAGTGGGATG), MYB (AACTCCTACACCATTCAAAC and CTGCTCCTCCATCTTTCC), PMAIP1/NOXA (TAAAGCAAGAATGGAAGAC and GACCGAAGAAATCAACAC), and ACTIN (GGGCATGGGTCAGAAGGAT and GTGGCCATCTCTTGCTCGA).

Microarray. Twenty micrograms of each RNA sample was labeled by using the Super Script II (Invitrogen) and the Enzo BioArray HighYield RNA transcript labeling kit (Affymetrix). Labeled complementary RNA was hybridized to the Affymetrix U95A microarrays containing 12558 probe sets according to the specifications of the manufacturer (Affymetrix) at the Affymetrix gene expression facility at the Ottawa Hospital Research Institute (Ottawa). Affymetrix MICROARRAY SUITE 5.0 software was used to compare each sample to mock-irradiated controls maintained at the restrictive temperature. A nonparametric Wilcoxon signed rank test was used to determine whether statistically meaningful differences in probe cell intensities were detected between samples (change calls were determined by using γ_1H and γ_1L values of 0.0025). We considered genes to be differentially expressed under a given condition if and only if they were statistically ($P \leq 0.0025$) increased or decreased 2-fold in all experiments compared to samples collected from cells maintained at the restrictive temperature. The NetAffx database was used to determine the identity of differentially expressed genes (27).

The National Centre for Biotechnology Information Locuslink database (www.ncbi.nlm.nih.gov/LocusLink) was used to obtain detailed information regarding the physical makeup of all differentially expressed genes. The size of genes, mRNAs, and introns did not fit a Gaussian distribution, so statistical analysis was performed on log-transformed data by using the PRISM software package (GraphPad, San Diego). However, similar results were obtained with nontransformed data by using a nonparametric Mann–Whitney U test.

Chromatin Immunoprecipitation. The chromatin immunoprecipitation assay was performed as described by Kallesen and Rosen (http://public.bcm.tmc.edu/rosenlab/protocols/ChIP.pdf), which represents a minor modification of the method of Boyd and Farnham (28). Briefly, proteins and DNA were crosslinked with 1% formaldehyde in growth medium. Chromatin was isolated and sonicated to an average length of 700 bp with a microtip sonifier (Branson Sonifier) in 20-sec bursts. Debris was cleared by centrifugation, and the resulting supernatants were frozen in liquid nitrogen and stored at −80°C.

Upon analysis, the chromatin was diluted 5-fold and precleared with protein A-agarose (Roche Diagnostics) for 50 min at 4°C. Twenty percent of the precleared chromatin was retained

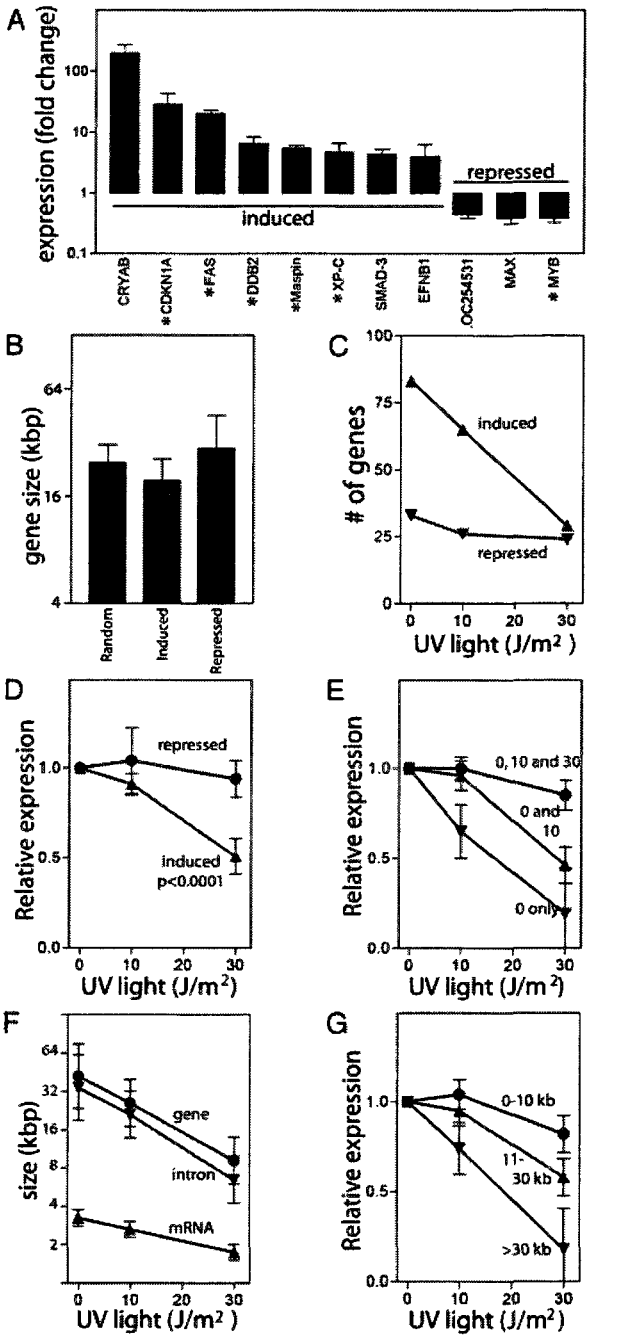

Fig. 1. The effect of UV light on expression of genes induced at the permissive temperature. (*A*) Fold increase in gene expression at the permissive temperature was determined by real-time RT-PCR. Known p53-regulated genes are denoted with asterisks. (*B*) The mean size of genes induced or repressed at the permissive temperature compared to the size of randomly selected genes. The effect of the UV light on the number (*C*) and expression (*D*) of genes induced or repressed at the permissive temperature. Expression in *D* for each gene was determined and normalized to the induced level of expression of that gene at the permissive temperature alone and is expressed as the mean level of expression determined from all genes in the group. (*E*) Genes were grouped by behavior to UV light: induced at 0 (inverted triangle), 0 and 10 (triangle), or 0, 10, and 30 (circle) J/m^2. Relative expression was determined as indicated in *D*. (*F*) The mean size of loci (circles), mean size of mRNA (triangles), and the total intron size (inverted triangles) are indicated for each group in *E*. (*G*) Genes were sorted into three groups by gene size (≤10 kb, 11–30 kb, and >30 kb, n = 28, 26, and 27, respectively) and the mean effect of UV light on gene expression in each group was determined. Error bars in *A* and *D–G* indicate the 95% confidence interval of the indicated mean. The *P* value in *D* was determined by one-way ANOVA.

Table 1. Effect of UV light on the expression of known p53-responsive genes

Expression	UV, J/m²	*n*	Median gene size, bp	mRNA size, $\log_2$ bp	Gene size, $\log_2$ bp	Gene size, bp†	Total intron size, $\log_2$ bp
Random‡	NA	132	29,104	11.1 ± 0.1	14.6 ± 0.2	24,800	14.4 ± 0.2
p53 induced	All	43	13,751	11.1 ± 0.1	14.0 ± 0.2	16,500	13.7 ± 0.3
	0	33	23,036	11.1 ± 0.1	14.1 ± 0.3	17,600	13.8 ± 0.3
	10	32	11,934	11.1 ± 0.1	13.7 ± 0.3*	13,300	13.5 ± 0.3*
	30	18	8,176	10.8 ± 0.2	13.2 ± 0.3**	9,400	12.8 ± 0.3**

Asterisks indicate that the indicated value is significantly different from randomly selected genes using a Student's *t* test at *, $P \leq 0.05$ or **, $P \leq 0.005$. NA, not applicable.

†For clarity, mean gene size is presented in a linear form but was determined from the mean of transformed values by using the following formula: (mean gene size = $2^{\text{meanlog2 gene size}}$).

‡A random number generator was used to generate a random series of Locuslink accession numbers.

as a positive control for PCR amplification. The remainder of the solution was divided into equal aliquots and incubated overnight at 4°C, either without antibody or with 5 μg of p53 antibody (Ab-1 antibody, Oncogene Science, or phospho-Ser-15 antibody, Cell Signaling Technologies). Immune complexes were collected overnight at 4°C with the addition of 60 μl of protein A-agarose (Roche Diagnostics).

Crosslinks were reversed by addition of NaCl to a final concentration of 0.3 M with RNase A and incubated at 65°C for 4–5 h. DNA was precipitated in 70% ethanol, collected by centrifugation, and proteinase K treated for 2 h at 45°C. DNA was purified by using QiaQuick spin columns (Qiagen) and was eluted in 10 mM Tris, pH 8.0. Semiquantitative PCRs were performed by using a GenAmp PCR System 9700 (Applied Biosystems). The binding of p53 to the serbinB5 (maspin), CDKN1A ($p21^{WAF1}$), and FAS p53 response elements was determined by using the following primer pairs: CTTTTCTGTGGATGCCGATT and CCTGCCAGGGCTTAACATAA, GTGGCTCTGATTGGCTTTCTG and CTGAAAACAGGCAGCCCAAG (29), and GGGACCCCGGTTGGAGAG and CTGCTTCGGTGCTGACTTATTTC, respectively. Amplification of upstream sequences of the GAPDH (GTATTCCCCCAGGTTTACAT and TTCTGTCTTCCACTCACTCC; ref. 29), XAB2 (AACGAGCTGGGACCCTCAGT and TATCAGTTTTTGGGGGCCGAGT) and KiSS-1 (CCTGGGGCCCGCACTTAGC and CCCCCGCACCTTCTCCATTTG) promoters and enhancers served as negative controls.

Results and Discussion

To evaluate the inhibitory affect of UV photoproducts on DNA damage-induced gene expression in a well controlled model system, we examined gene expression profiles in a human colorectal carcinoma cell line harboring a temperature sensitive allele of p53 (26). At the restrictive temperature (38°C), this variant of p53 is in a mutant conformation and cytoplasmic, but becomes nuclear and functional at the permissive temperature (32°C) (30). This variant is ideal for these studies because conditional expression of active p53 does not require *de novo* transcription of the transgene nor does it require a DNA damage signal. Statistically significant changes in gene expression were determined for each individual experiment by using the MICROARRAY SUITE 5.0 software (Affymetrix). We found 83 genes were induced at least 2-fold at the permissive temperature in each of four independent experiments. Thirty-three of these genes have previously been reported to be p53-induced (31–38). By similar criteria, 33 genes were repressed at the permissive temperature. The differential expression of several gene products was confirmed by using real-time RT-PCR (Fig. 1*A*). In all instances, RT-PCR data confirmed the differential expression detected by microarray analysis.

We obtained information regarding the physical makeup of all differentially expressed genes (Tables 3–5, which are published as supporting information on the PNAS web site). For statistical purposes, detailed information regarding the physical makeup of 132 randomly selected genes was also obtained (Table 6, which is published as supporting information on the PNAS web site). As expected, the average size of the randomly selected genes closely approximated that reported by the human genome project (1). Overall, the mean size of the genes induced at the permissive temperature was not significantly different from the size of randomly selected genes (Fig. 1*B*).

We determined the effect of UV light on the spectrum and expression of these p53 target genes. Of the 83 genes induced at the permissive temperature, only 29 were still induced after exposure to 30 J/m² (Fig. 1*C*). In contrast, the number of repressed genes was not dramatically altered by UV light (Fig. 1*C*). Whereas the expression of p53-induced genes was also strongly inhibited by UV light ($P < 0.0001$), the expression of the p53-repressed genes remained largely unaffected (Fig. 1*D*). Thus, UV irradiation dramatically altered the spectrum and expression of p53-induced but not p53-repressed genes.

Genes induced at the permissive temperature were grouped by behavior after exposure to UV light (i.e., induced at "0 J/m² only," "0 and 10 J/m²," or "0, 10, and 30 J/m²") (Fig. 1*E*). There was a significant UV dose-dependent decrease in the size of genes induced but not repressed at the permissive temperature (Fig. 1*F*, $P < 0.0001$, ANOVA). This was associated with a decrease in the number and average size of introns, but mRNA size was not significantly different from that of randomly selected genes (P = 0.005, 0.004, and 0.17, respectively). The p53-induced genes were sorted into groups based on gene size, and the effect of UV light on the expression of each group of genes was determined. The expression of large genes was strongly inhibited, whereas the

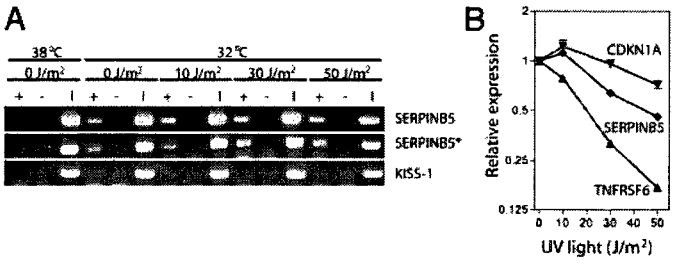

Fig. 2. The effect of UV dose on p53 binding and p53 target gene expression. (*A*) Representative chromatin immunoprecipitation experiments demonstrate an interaction of p53 and ser15 phosphorylated p53 (asterisk) with the SERPINB5 p53-response element at all doses of UV light. KiSS-1 served as a negative control. +, antibody; −, the no-antibody control; I, input DNA. (*B*) The effect of UV light on representative p53 target genes was determined by real-time RT-PCR. Values are expressed relative to the corresponding mean determined for p53 target genes induced at the permissive temperature alone. Values represent the mean ± standard error.

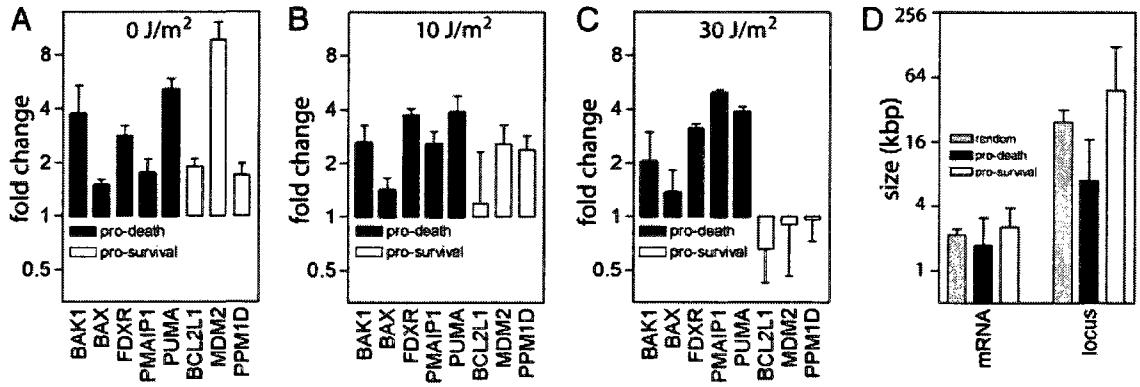

Fig. 3. p53 target genes involved in mitochondrial apoptotic pathways are resistant to inhibition of gene expression by UV light. (*A–C*) The fold change in the expression of genes encoding antiapoptotic (open symbols) and proapoptotic (filled symbols) proteins after exposure to 0 (*A*), 10 (*B*), or 30 (*C*) J/m². (*D*) The mean size of the mRNA and locus of genes encoding p53-regulated proapoptotic proteins or survival promoting proteins (in *A–C*).

expression of small genes was not (Fig. 1*G*). Because many of these putative p53-regulated genes identified through our microarray analysis have not been independently confirmed to be p53-regulated, we performed a similar analysis on the 43 previously reported target genes (31–40) (Table 5). Again, genes induced at higher doses of UV light were significantly smaller (Table 1), and this was associated with a significant decrease in the average number and size of introns ($P < 0.005$, *t* test). Thus, UV light inhibits the p53 response in a dose-dependent manner, resulting in the preferential induction of compact p53 target genes with fewer and smaller introns.

To ensure that p53 was associated with consensus response elements *in vivo* at high doses of UV light, we performed chromatin immunoprecipitation experiments. An interaction between p53 and the SERPINB5 (maspin, Fig. 2*A*), CDKN1A ($p21^{WAF1}$, data not shown), and TNFRSF6 (Fas, data not shown) p53-response elements *in vivo* by chromatin immunoprecipitation was detected even though UV light inhibited the expression of SERPINB5 and TNFRSF6 (Fig. 2*B*). The interactions were apparent for all three promoters using two different p53 antibodies. We interpret these results to indicate that dose-dependent variation in the spectrum of p53-induced genes cannot be explained by a decrease in the binding of p53 to its response elements.

We have previously reported that the HT29-tsp53 cells used here undergo extensive apoptosis at the permissive temperature after exposure to 30 but not after exposure to 0 or 10 J/m² of UV light (20). Of the 43 known p53 target genes differentially expressed in our microarray experiments, 17 have been reported to regulate the induction of apoptosis. Of these, five are inducers of apoptosis through the mitochondrial apoptotic pathway (Fig. 3) important for both p53- and UV-induced apoptosis (9–11, 32, 33, 41–45). All five genes implicated in the p53-mediated mitochondrial pathway were highly resistant to inhibition by UV light (Fig. 3 *A–C*), whereas the expression of genes known to inhibit p53-mediated apoptosis was strongly blocked by UV light (Fig. 3 *A–C*). The proapoptotic genes were significantly smaller than the prosurvival genes (Fig. 3*D*). The large size of MDM2 and PPM1D genes ensures that feedback inhibition is blocked at higher doses, permitting cells to be eliminated by apoptosis. Taken together, UV irradiation resulted in the expression of a restricted set of small p53-responsive genes at the expense of larger p53 targets, and this coincides with the induction of apoptosis in a dose-dependent manner.

To determine whether gene size had an effect on the p53-independent induction of UV-responsive genes, we assessed the effect of UV light on gene expression at the restrictive temperature, as well. We found that the size of genes induced after exposure to high doses of UV light at the restrictive temperature were again smaller ($P = 0.005$ and $P < 0.0001$, respectively, Table 2). Furthermore, we determined the average size of genes reported to be induced by UVC, UVB, and γ radiation in a variety of cell types by several other groups (38, 46–49). In striking support of our results, we found that these previously reported UVB- and UVC-induced genes were also significantly smaller than randomly selected genes (Table 7, which is published as supporting information on the PNAS web site). The compact UV-induced genes included p53-responsive genes, AP-1 responsive immediate early genes, cytokines, chemokines, and histones (38, 46–49). In contrast, exposure of tumor cells to γ radiation did not result in the striking induction of compact genes (Table 7). In fact, UV-induced genes were significantly smaller than genes induced by ionizing radiation ($0.05 < P < 0.0001$, depending on the studies compared). This is consistent with the fact that γ radiation does not strongly inhibit transcription (8). Therefore, gene size is an important determinant of UV-induced but not γ-radiation-induced gene expression.

In summary, we found that the induction of UV-induced gene expression is subject to a strong gene size constraint. The gamut of UV-induced genes was enriched for compact genes with fewer and smaller introns. We propose that this gene size constraint has played a significant role in the evolution of UV-response pathways and that the dose-dependent change in the spectrum of p53-induced and UV-induced genes acts as an elegant molecular dosimeter. Selective gene expression based on gene size represents a gene regulatory mechanism that acts at the level of transcript elongation because of blocked RNA polymerases. We propose that differences in gene expression caused by gene size are used to interpret the extent of irreparable transcription-blocking DNA damage sustained by the cell. This dose-

Table 2. Size of genes induced by UV light at the restrictive temperature

UV	*n*	Median	Gene size*	Mean†	*P*‡
Random§	132	29,104	14.6 ± 0.2	24,800	
10 J/m²	14	10,340	13.1 ± 0.4	8,800	0.005
30 J/m²	17	5,822	12.6 ± 0.3	6,200	<0.0001

*The size of individual genes in bp were $\log_2$ transformed, and the mean (±SEM) of these $\log_2$ transformed values is presented.

†For clarity, mean gene size is presented in a linear form, but was determined from the mean of transformed values by using the formula: mean gene size = $2^{\text{mean gene size (log2)}}$.

‡Each mean was compared to the mean determined from randomly selected genes by using a *t* test.

§Randomly selected genes.

dependent pattern of expression explains in large part the tight association between the UV-induced inhibition of transcription and the induction of apoptosis (4, 8, 12, 13, 20).

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Report

DDB2-Independent Role for p53 in the Recovery from Ultraviolet Light-Induced Replication Arrest

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ABSTRACT

Ultraviolet light (UV light) induces helix distorting DNA lesions that pose a block to replicative DNA polymerases. Recovery from this replication arrest is reportedly impaired in nucleotide excision repair (NER)-deficient xeroderma pigmentosum (XP) fibroblasts and primary fibroblasts lacking functional p53. These independent observations suggested that the involvement of p53 in the recovery from UV-induced replication arrest was related to its role in regulating the global genomic subpathway of NER (GG-NER). Using primary human fibroblasts, we confirm that the recovery from UV-induced replication arrest is impaired in cells lacking functional p53 and in primary XP fibroblasts derived from complementation groups A or C (XP-A and XP-C) that are defective in GG-NER. Surprisingly, DNA synthesis recovered normally in GG-NER-deficient XP complementation group E (XP-E) cells that carry mutations in the p53 regulated DNA repair gene DDB2 and are specifically defective in the repair of cyclobutane pyrimidine dimers (CPD) but not pyrimidine (6-4) pyrimidone photoproducts. Disruption of p53 in these XP-E fibroblasts prevented the recovery from UV-induced replication arrest. Therefore, the roles of p53 and GG-NER in the recovery from UV-induced replication are separable and DDB2-independent. These results further indicate that primary human fibroblasts expressing functional p53 efficiently replicate DNA containing CPD whereas p53-deficient cells do not, consistent with a role for p53 in permitting translesion synthesis of these DNA lesions.

INTRODUCTION

In response to genotoxic agents, several cell cycle checkpoints are activated, blocking cell cycle progression in the G_1, S or G_2 phases of the cell cycle.[1-3] In addition to activated checkpoints, progression of replicative DNA polymerases can be inhibited by UV-induced DNA lesions.[4,5] Immediately after UV exposure, the synthesis of nascent DNA decreases in a dose-dependent manner but nascent DNA synthesis recovers markedly within 6–8 hours following exposure to moderate doses of UV light in normal fibroblasts.[5-11] Recovery from UV-induced replication arrest is thought to require nucleotide excision repair because NER-defective fibroblasts derived from patients with xeroderma pigmentosum (XP) fail to recover nascent DNA synthesis normally.[4,5] Cells lacking functional p53 have a NER defect[10,12,13] and also fail to recover nascent DNA synthesis normally.[9,10] Based on these observations, it was hypothesized that p53 facilitates the recovery from replication arrest by stimulating NER[9,10] but until now, this hypothesis had remained untested.

Here we report that fibroblasts lacking functional p53 and primary XP fibroblasts from complementation groups A and C (XP-A and XP-C) fail to recover from the UV-induced replication arrest. In stark contrast to previous predictions though, nascent DNA synthesis recovers normally in XP complementation group E cells despite the fact that approximately 80% of the CPD remain 24 hours following UV exposure. Furthermore, disruption of p53 in these cells exacerbated their replication arrest and led to a massive accumulation of cells in S phase. These results clearly indicate that the roles of GG-NER and p53 in the recovery from UV-induced replication arrest are separable. Furthermore, these results suggest that the recovery from UV-induced replication arrest involves translesion synthesis in p53 expressing fibroblasts but not in the absence of functional p53.

MATERIALS AND METHODS

Cell culture and UV-irradiation. Normal neonatal foreskin fibroblasts (NF) were obtained from Dr. Mats Ljungman (University of Michigan). Normal fibroblasts (GM38),

XP-A (GM5509), XP-C (GM671), CS-B (GM739) and XP-E (GM1389) fibroblasts were obtained from National Institute of General Medical Sciences Mutant Cell Repository (Camden, NJ). The generation of human papillomavirus-E6-expressing normal fibroblast strains was described previously.[14] Li-Fraumeni syndrome cells (LFS), hemizygous for a frame shift mutation at codon 184 of p53 were obtained from Dr. Michael Tainsky (Wayne State University). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (Wisent, St. Bruno, QC) and 5 μg/ml gentamicin (Sigma-Aldrich Canada Ltd, Oakville, ON).

For UV treatment, growth medium was removed, cells were irradiated immediately at room temperature, fresh prewarmed medium was replaced and cells were returned to the incubator for the indicated period of time. A germicidal bulb (Philips) emitting UV predominately at 254 nm at 1 $J/m^2/s$, as determined with a hand held UV-radiometer (UVX Radiometer, UVP Inc, Uplands, CA) was used to irradiate cells.

Flow cytometry. Control and UV-irradiated cells were collected at various time points following UV and/or drug treatment. Where indicated, colcemid was added to a final concentration of 1 μM. The harvesting, fixation and staining of cells with propidium iodide (PI) has been described previously.[15] To detect the synthesis of nascent DNA, BrdU (30 μM, Sigma-Aldrich Canada Ltd, Oakville, ON) was added to the medium for between 15 minutes and 24 hours, depending on the experimental design. In pulse chase experiments, BrdU was added for 1 hour, washed twice with PBS and returned to the incubator for the indicated period of time. Detection of BrdU incorporation in single stranded DNA using a primary anti-BrdU antibody (1:100, PharMingen, San Diego, CA) and secondary anti-mouse FITC-conjugated antibody (1:15, Sigma-Aldrich Canada Ltd, Oakville, ON) was performed, as described previously.[15] Detection of UV-induced DNA damage in single stranded DNA was performed similarly except that 1 μg of either anti-thymine dimer antibody (clone KTM53, Kamiya Biomedical Company, Seattle, WA) or Anti-(6-4)PP (clone 64M-2, MBL Internationsal, Woburn, MA) was used instead of anti-BrdU antibody. Stained cells were analyzed via flow cytometry using either a Becton Dickinson LSR FACS station or a Beckman Coulter FACS station. Cell cycle distribution was calculated from the DNA histograms using cell cycle analysis software (ModFit, Verity Software House, Topsham, ME) whereas quantification of BrdU incorporation and UV-induced DNA damage was performed using FCS Express 3 software (DeNovo Software, Thornhill, ON).

Immunoblotting. Cells were harvested, rinsed with PBS, lysed with 1% SDS, sonicated for 10s using a microtip (Branson Sonifier, VWR International Ltd., Mississauga, ON) and protein concentrations determined using the BioRad Protein Assay (BioRad, Mississauga, ON). Whole cell lysates were prepared using LDS NuPAGE sample buffer (Invitrogen Canada Inc., Burlington, ON). Proteins (20 μg per lane) were separated in 10 or 4–12% Bis-Tris NuPAGE precast gels, transferred to nitrocellulose membranes (Hybond-C Extra, Amersham, Baie d'Urfe, QC) and stained with Ponceau S Red (5 mg/ml in 2% glacial acetic acid) to confirm transfer of proteins. Membranes were blocked in PBSMT-A (PBS, 5% nonfat dry milk powder, 0.05% Tween 20) for 1 hr at room temperature, incubated with primary antibody (p53Ab-6 from Oncogene Research Products or β-actin clone AC-74 from Sigma-Aldrich Canada Ltd) diluted in PBSMT-B (PBS, 0.5% nonfat dry milk powder, 0.05% Tween 20) for 1.5 hr at room temperature. Protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and either Kodak film or a gel documentation system (GeneGnome, Synoptics, Bristol, UK). Membranes were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) in order to visualize additional proteins on the same blot.

DNA replication. Cells were seeded at 1 x 10^5 cells/well in six well dishes. Twenty-four hours later, cells were irradiated with 0 or 10 J/m^2 and 2 mL of fresh medium containing 5 μCi/mL [^{3}H]-thymidine were added to cells. Cells were collected at 0, 6 or 12 hours following UV-irradiation. Cell pellets were snap frozen and stored at -80°C until analyzed. Cell pellets were resuspended in 500 μL 1% SDS at room temperature then placed on ice. One mL of 10% trichloroacetic acid (TCA)/0.1 M sodium pyrophosphate (NaPPi) was added and DNA was precipitated by incubating on ice for 30 min. Precipitated DNA was collected on glass fiber filters and washed three times with 2 mL 5% TCA/0.05M NaPPi, 5 mL ddH_2O, 2 mL 100% EtOH. Filters were allowed to dry on vacuum for 1 minute and transfered to scintillation tubes to be counted.

γH2AX immunofluorescence. Cells were seeded into dual chambered slides and the next day sub-confluent monolayers were irradiated as indicated with UV light. Fresh prewarmed media was added to cells and plates were returned to the incubator for 8 hours. Cells were then rinsed with PBS and fixed in 2% formaldehyde in PBS for 20 min at 4°C. Cells were permeabilized in 1:1 ice-cold acetone:methanol. Monolayers were blocked for one hour in PBS supplemented with 10% FBS at 37°C in a humidified incubator. Monolayers were probed with primary anti-γH2AX antibody (Trevigen, Gathersburg, MD) diluted to 1:250 in PBS supplemented with 1% BSA and 0.5% Tween 20 for two hours at 37°C in a humidified incubator. Monolayers were washed three times for five minutes in PBS supplemented with 1% BSA. Monolayers were incubated with secondary goat-anti-rabbit IgG antibody conjugated to Alexa-488 (Invitrogen Canada Inc., Burlington, ON) diluted to 1:2000 in PBS supplemented with 1% BSA for one hour in the dark at 37°C in a humidified incubator. Monolayers were washed three times for five minutes each in PBS supplemented with 1% BSA. Cells were incubated with 0.05 μg/mL Hoechst stain (Sigma) in PBS supplemented with 1% BSA. Monolayers were washed twice for 5 minutes in PBS supplemented with 1% BSA. Chambers were removed and slides were covered with glass coverslips with a drop of fluormount mounting media and sealed with nail polish. Slides were examined using a Ziess Axioscop 2 microscope using the 63X oil immersion objective. A minimum of 100 cells were counted for each treatment and scored for the presence of increased focal staining of γH2AX within nuclei of cells. Counting was performed by an individual without knowledge of the treatment group.

RNA interference. Cells were maintained in antibiotic-free media at least 48 hours prior to transfection. Three days prior to UV-irradiation, cells at 50–80% confluence in 10 cm culture dishes were transfected with SmartPool siRNA duplexes targeting p53 or control duplex (Dharmacon Inc., Lafayette, CO) at 50 nM final concentration using Oligofectamine Reagent and Opti-MEM I (Invitrogen, Burlington, ON) according to manufacturers instructions. Cells were split 1 to 2, 24 hours following transfection. Cells were treated with the indicated doses of UV light and collected at the indicated times for either immunoblot or cell cycle analysis. We routinely found greater than a 4 fold decrease in p53 protein expression in unirradiated samples and greater than 10 fold decrease in expression following UV-irradiation.

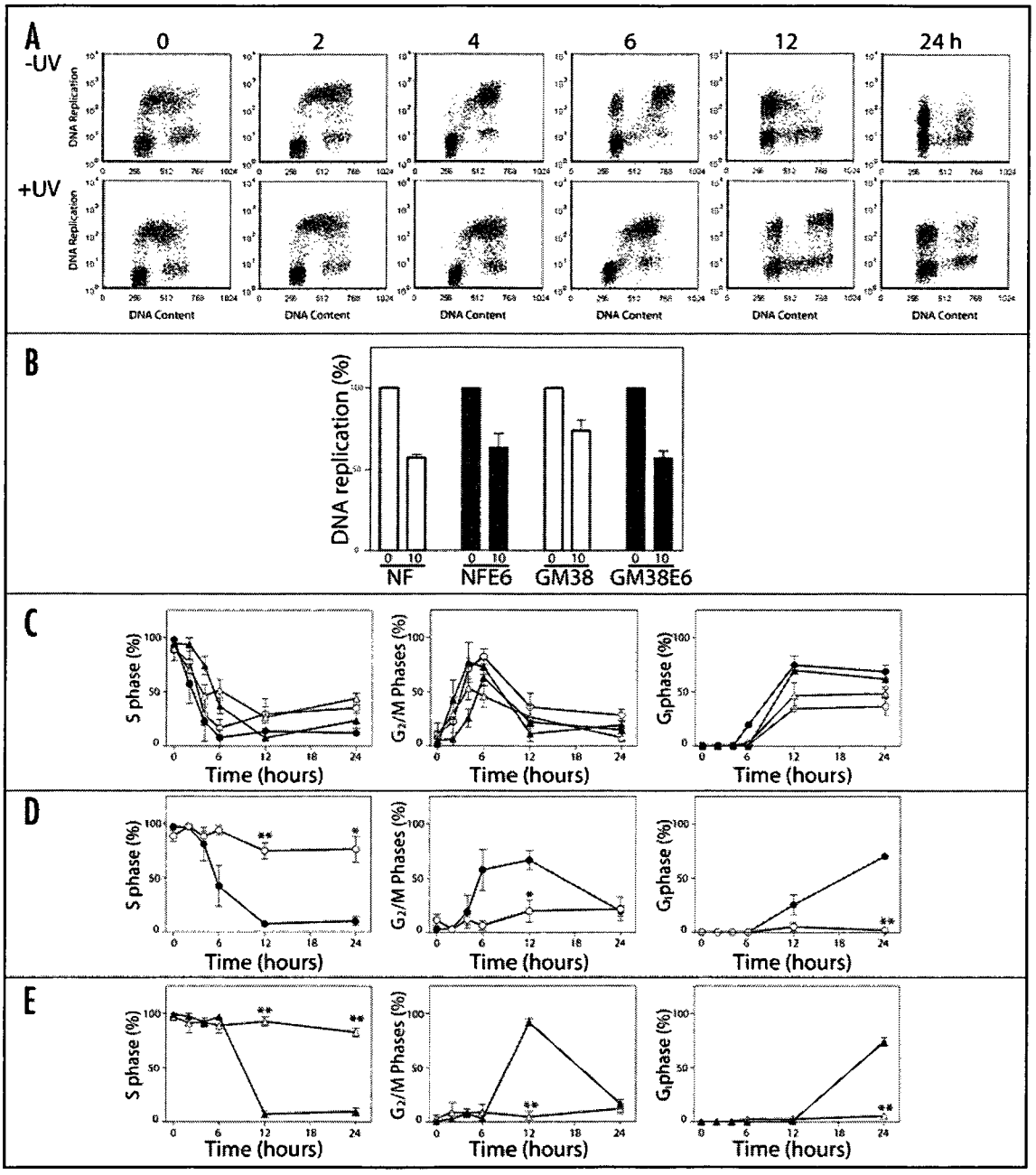

Figure 1. Prolonged ultraviolet light-induced replication arrest in fibroblasts lacking functional p53. Cells were treated with either 0 or 10 J/m^2 of UV light, pulsed with 30 μM BrdU for 1 h, rinsed thoroughly with PBS and returned to the incubator for the indicated time. Two-parameter flow cytometric analysis was performed as described in the materials and methods. (A) Representative dot plots are presented for normal fibroblasts. (B) The relotive amount of BrdU incorporated within the first hour following UV exposure compared to unirradiated controls was determined from dot plots like those presented in (A). (C–E) The BrdU-positive cells in (A) were gated and cell cycle distribution was determined based on DNA content for NF (closed circles in C and D), NF-E6 (open circles in C and D), GM38 (closed triangles in C and E) and GM38E6 (open triangles in C and E). Cell cycle distribution is plotted as a function of time following release from BrdU treatment. Cells were exposed to either 0 J/m^2 (C) or 10 J/m^2 (D and E). Each point represents the mean (± SEM) determined from a minimum of three independent experiments. Statistically significant differences between the indicated value ond its corresponding control collected at the same time are indicated by either * or ** ($p \leq 0.05$ and $p \leq 0.001$, respectively, student t test, two tailed).

RESULTS

Ultraviolet light induced replication arrest is prolonged in fibroblasts lacking functional p53. In order to study the effects of UV light on the inhibition and recovery of DNA synthesis following UV-irradiation, two parameter flow cytometric BrdU pulse chase experiments were performed. This allowed us to label and follow the fate of cells irradiated in S phase. Cells were pulsed with BrdU for 1 hour immediately following mock- or UV-irradiation and were subsequently incubated for the indicated time in fresh medium without BrdU. Anti-BrdU immunofluorescence was used to quantify replication while PI was used to distinguish cell cycle position based on DNA content (Fig. 1A). UV exposure resulted in a two-fold decrease in the incorporation of BrdU throughout

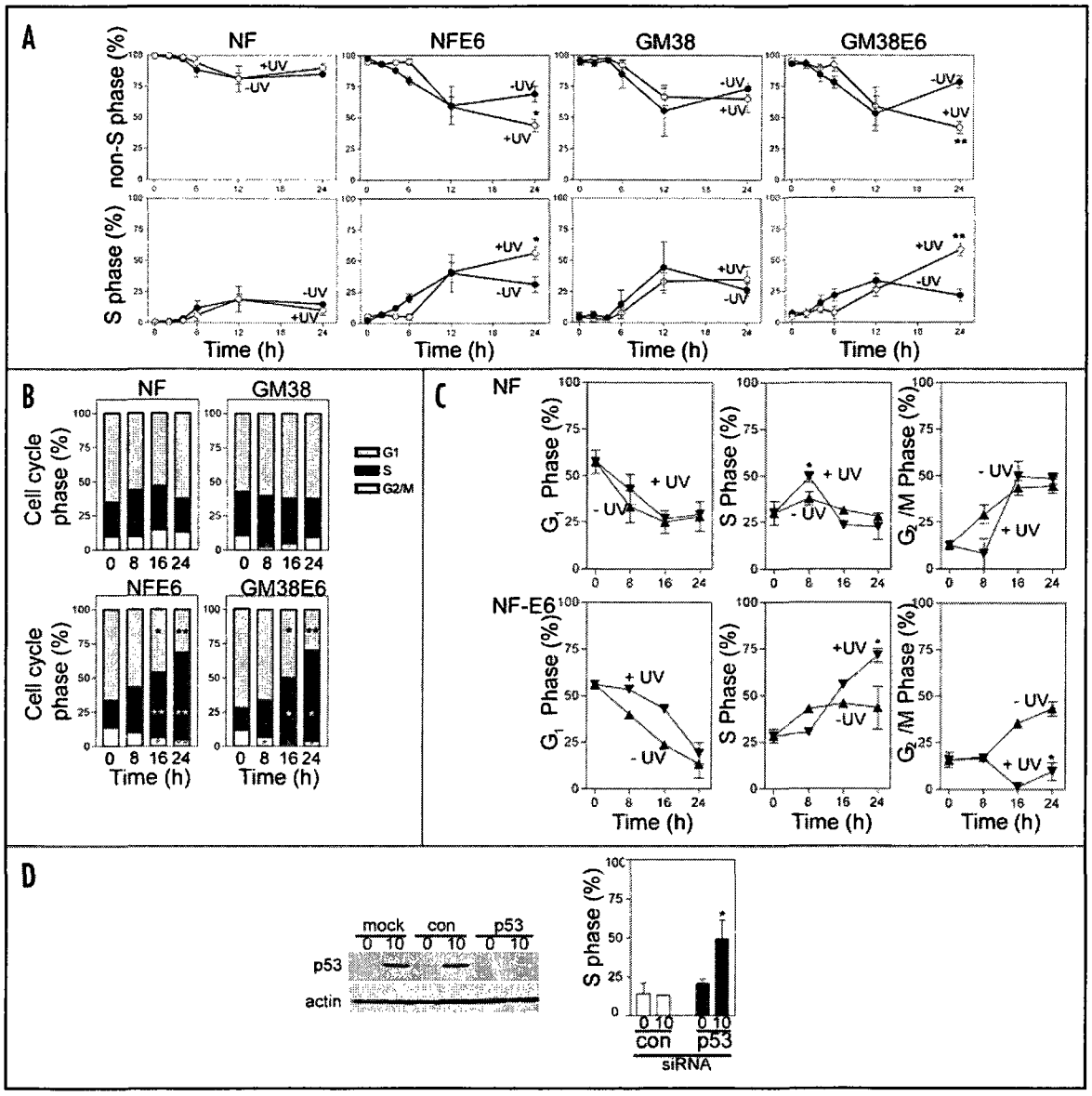

Figure 2. Pronounced S phase arrest in p53-deficient fibroblasts following UV exposure. (A) BrdU pulse chase experiments were performed on the indicated cell line and cell cycle distribution based on DNA content was determined from the BrdU-negative population of cells. (B) One parameter flow cytometric analysis of propidium iodide stained cells was performed and the mean proportion of cells in each phase of the cell cycle was determined from a minimum of three experiments in NF, GM38, NFE6 and GM38E6 fibroblasts. (C) One parameter flow cytometric analysis of propidium iodide stained cells was performed as in (B) except thot colcemid was added at the time of irradiation to block entry of cells into the second cell cycle. The mean proportion of cells in the G_1, S and G_2/M phases of the cell cycle was determined from up to ten experiments. (D) GM38 cells were mock transfected or transfected with control siRNA duplex (con) or p53 siRNA duplex 48 hours prior to exposure to 10 J/m^2 of UV light. Lysates were collected 24 hours following UV exposure and subjected to immunoblot and cell cycle onalysis based on DNA content. Each value in (A, C and D) represents the mean (± SEM) determined from a minimum of 3 independent experiments. Statistically significant differences between the indicated value and its corresponding control in (A–D) are indicated by either * or ** ($p \leq 0.05$ and $p \leq 0.001$, respectively, student t test, two tailed).

S phase within the first hour following UV exposure, consistent with the direct inhibtion of DNA replication (Fig. 1B). Importantly, the BrdU-positive and BrdU-negative populations of cells remained easily distinguishable (Fig. 1A) following UV exposure allowing us to gate BrdU positive cells separately for cell cycle analysis based on DNA content.

The vast majority of BrdU-positive cells had S phase DNA content at the time of irradiation (Fig. 1C). The BrdU-positive population of unirradiated normal fibroblasts completed S phase within six hours, passed synchronously through G_2 and M phases then entered into the G_1 phase of the subsequent cell cycle within 12 hours (Fig. 1C). A second cycle G_1 phase population was prominent by 24 hours (Fig. 1A). The time required for the BrdU-positive population of normal fibroblasts to exit S phase was extended following exposure to 10 J/m^2 of UV light (Fig. 1A, C and D). UV-irradiated normal fibroblasts passed synchronously through G_2, M and into the G_1 phase of the subsequent cell cycle, with a six hour delay (Fig. 1A, D and E). This short S phase delay is consistent with previous measurements of the inhibition and recovery of nascent DNA synthesis using incorporation of ^{3}[H] thymidine as a measure of DNA replication.[5-11]

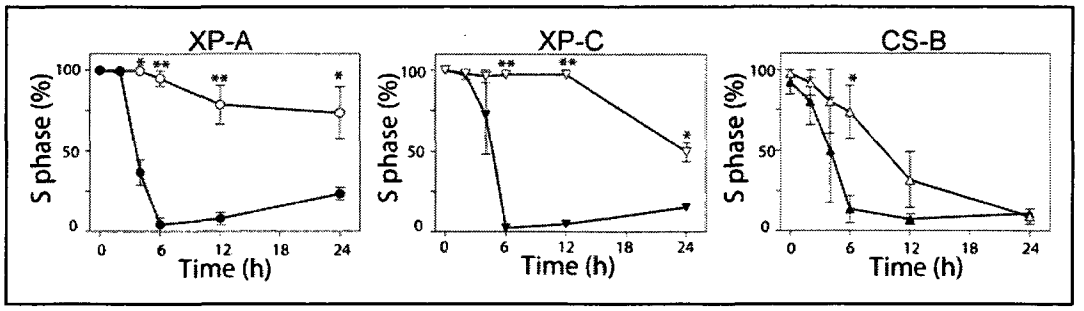

Figure 3. GG-NER contributes to the recovery from UV-induced replication arrest. BrdU pulse chase experiments were performed on XP-A (circles), XP-C (inverted triangles) and CS-B (triangles) fibroblasts and the cell cycle distribution based on DNA content was determined for the BrdU positive population of cells. The proportion of cells in S phase is plotted as a function of time following exposure to either 0 (closed symbols) or 10 J/m^2 (open symbols) of UV light. For representative two parameter flow cytometric data, refer to Supplementary Figures 3 and 4. Each point represents the mean (± SEM) determined from a minimum of three independent experiments. Statistically significant differences between the indicated value and its unirrodiated control are indicated by either * or ** ($p \leq 0.05$ and $p \leq 0.001$, respectively, student t test, two tailed).

The unirradiated BrdU positive population of HPV-E6 expressing derivative fibroblast strains exited S phase and progressed through subsequent phases of the cell cycle as rapidly as the parental strains (Fig. 1C). However, in stark contrast to normal fibroblasts, the BrdU positive population of HPV-E6 expressing derivative fibroblasts failed to complete S phase within 24 hours following UV treatment (Figs. 1D and E and Supplementary Fig. 1). Similarly, LFS fibroblasts lacking functional p53 were significantly delayed in S phase compared to the normal diploid human fibroblast strains (Supplementary Fig. 2). Therefore, the wild type p53 expressing strains resumed DNA synthesis with only a brief delay and passed synchronously through G_2 and M phases, entering the subsequent cell cycle, while the p53-deficient strains remained arrested in S phase for an extended period of time.

p53 facilitates the passage of G_1-irradiated fibroblasts through S phase. The effect of UV light on cells irradiated in other phases of the cell cycle was assessed by determining the cell cycle distribution of the BrdU negative population of cells from the experiments described in Figure 1. The majority of BrdU negative cells were in the G_1 phase of the cell cycle at the time of BrdU treatment (Fig. 1A). Analyzing the cell cycle distribution of BrdU-negative cells permitted us to monitor the effect of UV light on S phase entry and progression of primarily G_1-irradiated fibroblasts. A discernible BrdU-negative S phase population was observed in all unirradiated samples within 6 hours and was maximal by 12 hours following BrdU treatment (Figs. 1A and 2A). UV light did not significantly delay the entry of normal fibroblasts into S phase (Figs. 1A and 2A). Therefore, normal fibroblasts entered S phase readily following UV exposure without a prominent first cycle G_1 arrest.

Two obvious differences in cell cycle distribution were observed in the G_1-irradiated HPV-E6-expressing strains. First, the entry of p53-deficient strains into S phase was delayed compared to mock-irradiated controls (Fig. 2A). Second, the HPV-E6 expressing fibroblasts irradiated in the G_1 phase of the cell cycle accumulated in S phase and the non-S phase fraction was significantly depleted by 24 hours (Fig. 2A). These results suggested that p53-deficient fibroblasts underwent a sustained replication arrest following UV exposure regardless of the cell cycle phase at the time of irradiation.

To confirm the results by independent means, cell cycle distribution was determined by one parameter flow cytometric analysis on cells collected 0, 8, 16 and 24 hours following UV exposure. A small increase in the proportion of normal fibroblasts was detected in S phase, 8 and 16 hours following UV exposure but this returned to unirradiated levels within 24 hours (Fig. 2B). In distinct contrast, approximately 70% of HPV-E6 expressing fibroblasts were in S phase 24 hours after UV exposure (Fig. 2B). Consistent with the BrdU pulse chase experiments, HPV-E6 expressing fibroblasts undergo a prolonged replication arrest following UV exposure.

Given the complexity of analyzing the cell cycle distribution in the first and second cell cycles, similar one parameter flow cytometric experiments were performed in the presence of the mitotic inhibitor colcemid. We were thus able to monitor the fraction of cells exiting the G_1 phase, entering S phase and accumulating at the colcemid block in the presence and absence of UV light. Exposure to colcemid alone led to a depletion of cells in G_1 and a corresponding increase in the proportion of cells with 4N DNA content (Fig. 2C). Following UV-irradiation, normal fibroblasts exited G_1 at a similar rate to unirradiated control cells, again indicating that UV light did not induce a pronounced first cycle G_1 arrest (Fig. 2C). Normal fibroblasts accumulated in S phase within eight hours following UV-irradiation and reached the colcemid block within 16 hours (Fig. 2C). In clear contrast, the HPV-E6 expressing cells exited G_1 phase somewhat more slowly, consistent with a previous report,[16] accumulated in S phase, and failed to reach the colcemid block within 24 hours (Fig. 2C).

To provide further evidence that the cell cycle defect in HPV-E6 expressing cells was associated with p53-deficiency and not due to HPV-E6 expression per se, BrdU pulse chase and one parameter flow cytometric analysis were performed in hemizygous LFS fibroblasts carrying a frameshift mutation at codon 184 resulting in premature termination and an unstable p53 protein.[17] These cells underwent a sustained UV-induced replication arrest (Supplementary Fig. 2). Furthermore, targeting p53 by RNA interference led to a significant increase in the proportion of cells in S phase 24 hours following UV exposure, as assessed by one parameter flow cytometry (Fig. 2D). Therefore, all three flow cytometric methods and inactivation of p53 by three separate means yielded similar results. Taken together, normal fibroblasts recovered from UV-induced replication arrest far more efficiently than p53-deficient strains and this supports previous literature.[9,10,15,18,19]

GG-NER contributes to the recovery from replication arrest following UV exposure. UV-induced DNA damage is repaired through NER.[20] It has been reported that NER-defective XP fibroblasts fail to recover nascent DNA synthesis normally so NER is thought to contribute to the efficient recovery from UV-induced replication arrest.[4,5] Therefore, BrdU pulse chase experiments were performed in fibroblasts with a complete defect in NER (XP-A) or specific defects in either GG-NER (XP-C) or TC-NER (CS-B). Unirradiated BrdU-positive XP-A, XP-C and CS-B fibroblasts progressed through S phase within six hours, passing synchronously

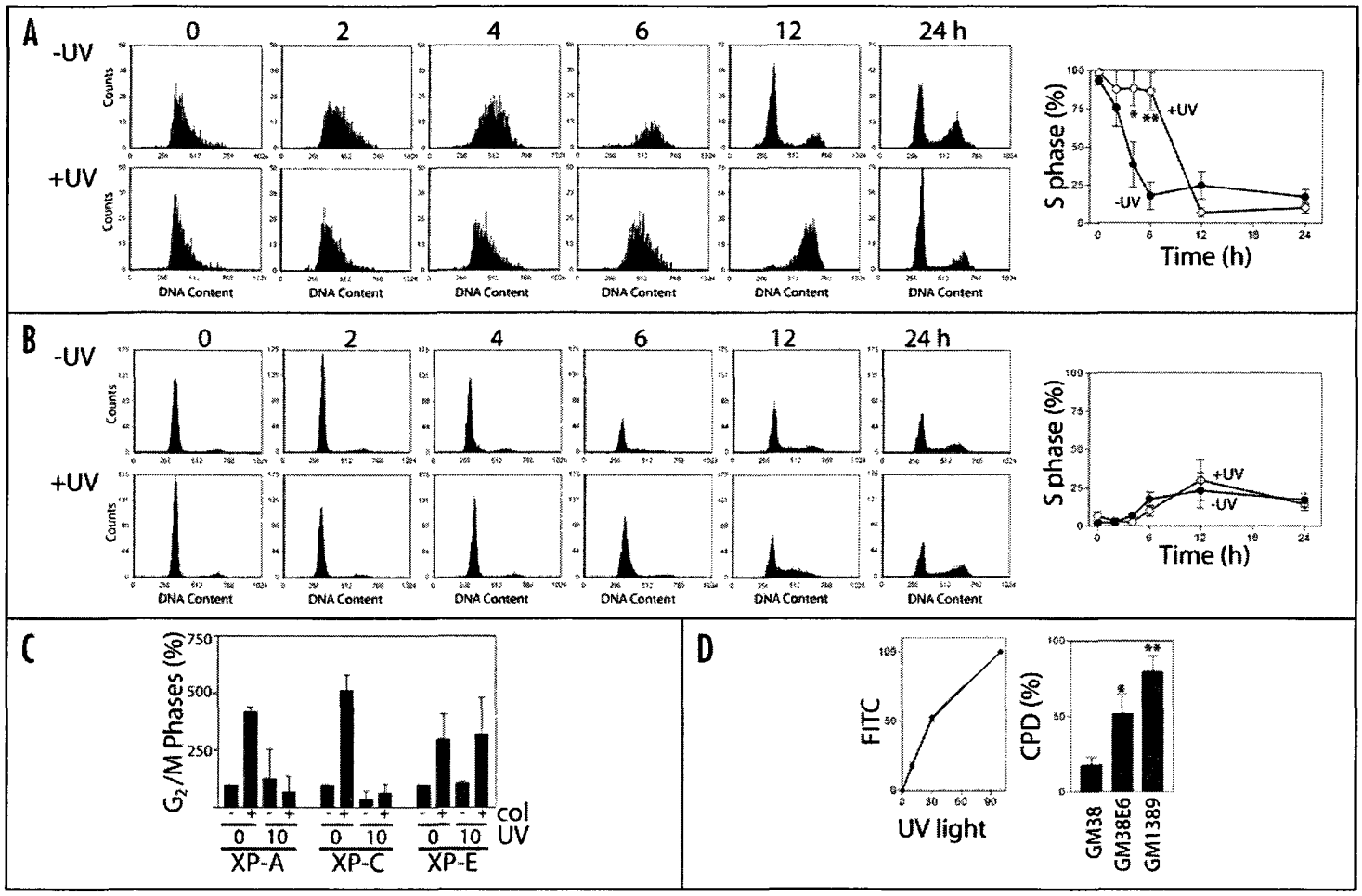

Figure 4. The recovery from UV-induced replication arrest precedes the repair of CPD in XP-E fibroblasts. BrdU pulse chase experiments were performed in XP-E fibroblasts. The BrdU-positive (A) and BrdU-negative population of cells (B) were gated separately and the data are presented as DNA histograms. The proportion of cells in S phase as estimated based on DNA content was determined for the BrdU-positive (right panel in A) and BrdU-negative (right panel in B) from a minimum of four independent experiments. For representative two parameter flow cytometric dot plots, see supplementary figure 5. (C) The proportion of cells with 4N DNA content was assessed 24 hours following mock- or UV-irradiation in the presence or absence of colcemid (col), as indicated. (D) CPD were detected either immediately or 24 hours following exposure of cells to 0, 10, 30 or 100 J/m^2 using an anti-thymine dimer antibody and flow cytometric methods. Fluorescent signal assessed immediately following UV exposure increased in proportion to UV fluence up to 30 J/m^2 (left panel). The proportion of CPD remaining 24 hours following exposure to 10 J/m^2 was determined for GM38, GM38E6 and GM1389 cells (right panel). Each point represents the mean (± SEM) determined from a minimum of 3 independent experiments. Statistically significant differences between the indicated value and its respective control are indicated by either * or ** ($p \leq 0.05$ and $p \leq 0.001$, respectively, student t test, two tailed).

through G_2 and M phases and were able to enter the second cycle G_1 within 12 hours, as observed in normal and HPV-E6 expressing fibroblasts strains (Fig. 3). The effect of UV exposure on the recovery from UV-induced replication arrest varied in a repair-dependent manner. The BrdU-positive population of XP-A and XP-C fibroblasts remained in S phase up to at least 24 hours following UV exposure (Fig. 3, left and centre panels), as expected based on previous literature.[5,7] In contrast, the BrdU positive population of CS-B fibroblasts progressed through S phase following UV exposure (Fig. 3B, right panel). Taken together, these results indicate that it is the GG-NER and not the TC-NER subpathway of NER that contributes to the recovery from UV-induced replication arrest.

Recovery from replication arrest involves p53 but not DDB2 and precedes the repair of cyclobutane pyrimidine dimers. Cells lacking functional p53 have a defect in GG-NER, however this defect is specific for CPD and not pyrimidine (6-4) pyrimidone photoproducts [(6-4) PP], the other major photoproduct induced by UV light.[12,13,21-23] This DNA repair phenotype is identical to that of XP group E cells (XP-E), homozygous for a mutation in the gene encoding DDB2, a p53-regulated DNA repair protein.[10,24] Therefore, to examine how a defect in GG-NER that more closely reflects the defect in p53-deficient cells affects cell cycle progression, BrdU pulse chase experiments were performed using XP-E fibroblasts.

Unirradiated BrdU-positive XP-E fibroblasts were able to complete S phase within 6 hours (Fig. 4A). As with normal fibroblasts, quantitative cell cycle analysis of the BrdU-positive population of XP-E cells indicated that S phase was prolonged by only 6 hours following UV-irradiation (Fig. 4A, right panel). A strikingly normal cell cycle distribution was restored within 24 hours following UV exposure (Fig. 4A, lower right histogram). The progression of XP-E fibroblasts through S phase was readily distinguishable from that of XP-A and XP-C fibroblasts (compare Fig. 3 and 4A, right panel). Similarly, UV light had very little effect on the cell cycle distribution of

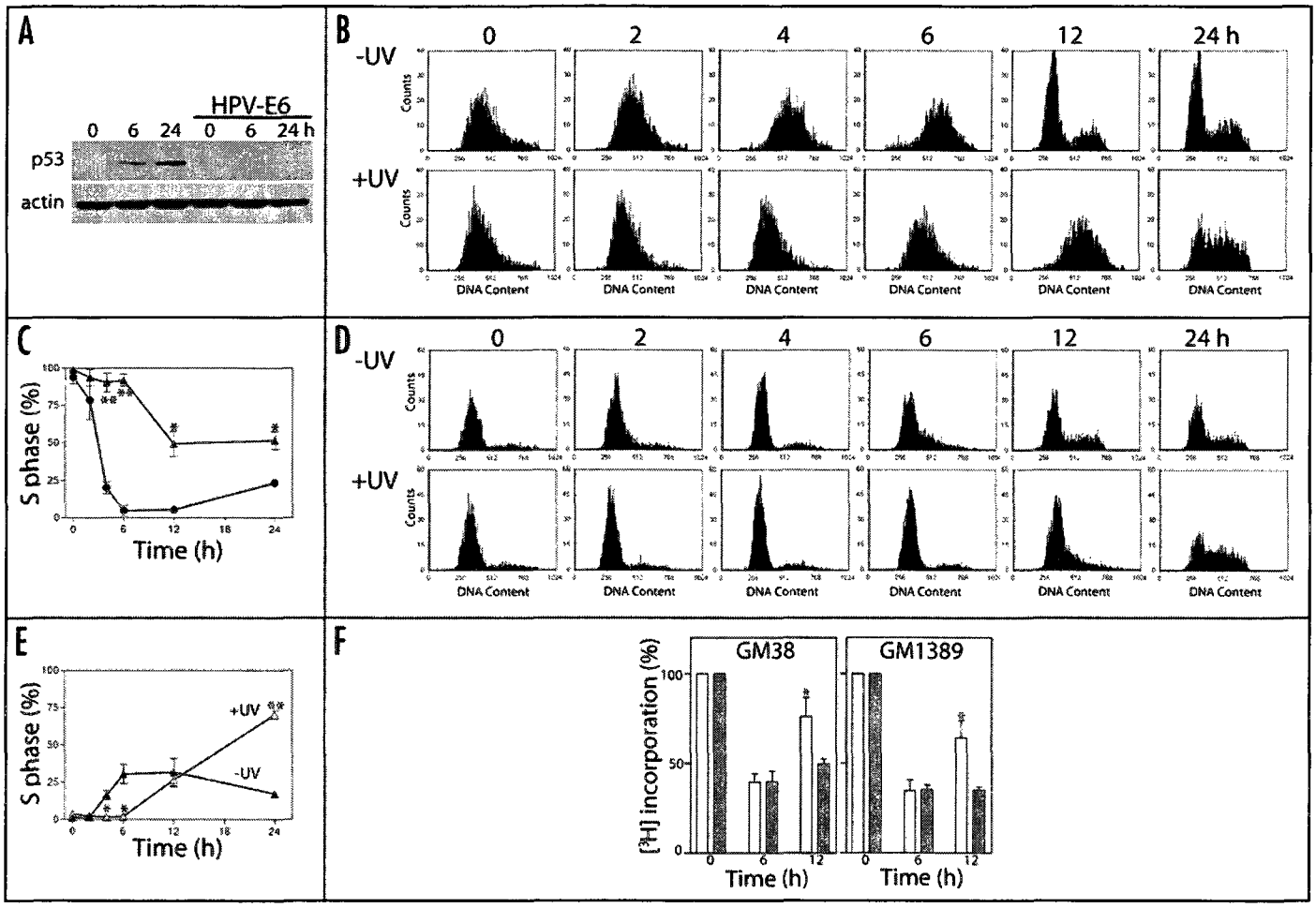

Figure 5. HPV-E6 expression inhibits the recovery from replication arrest in XP-E fibroblasts. (A) Immunoblot analysis comparing the UV-induced accumulation of p53 in XP-E and its isogenic derivative expressing HPV-E6. (B–E) BrdU pulse chase experiments were performed in these HPV-E6 expressing XP-E fibroblasts. The BrdU-positive cells were gated separately and the data are presented as DNA histograms (B) and as the proportion of cells in S phase (C). Similar analysis is presented for the BrdU-negative population of cells (D and E). For representative two parameter flow cytometric data, see supplementary figure 5. (F) The incorporation of [^{3}H] thymidine was assessed over the first 6 and 12 hours following UV exposure. [^{3}H] incorporation is represented as the counts determined from UV treated samples compared to their unirradiated controls collected at the same time. Each point in (C, E and F) represents the mean (± SEM) determined from a minimum of three independent experiments. Statistically significant differences between the indicated value and its corresponding control are indicated by either * or ** ($p \leq 0.05$ and $p \leq 0.001$, respectively, two tailed student t test).

the BrdU-negative population of XP-E cells (Fig. 4B). Therefore, the BrdU negative population of XP-E fibroblasts responded like normal fibroblasts.

To independently assess the effect of UV exposure on S phase progression, the ability of XP-E, XP-A and XP-C fibroblasts to reach G_2/M in the presence of colcemid was assessed, as described in (Fig. 2). Consistent with the BrdU pulse chase experiments, XP-A and XP-C fibroblasts failed to reach the colcemid block following UV-irradiation (Fig. 4C). In contrast, XP-E fibroblasts progressed through S phase and reached the colcemid block following UV-irradiation (Fig. 4C). Therefore, the reported defect in the repair of CPD in XP-E fibroblasts did not prevent these cells from recovering from UV-induced replication arrest.

To ensure that our XP-E fibroblast strain exhibited the reported GG-NER of CPD defect,[24] an anti-CPD monoclonal antibody was used to measure DNA damage and repair in normal, HPV-E6 expressing normal and XP-E fibroblasts using a flow cytometric method. A linear relationship between UV fluence (up to 30 J/m^2 of UV light) and the amount of DNA damage was observed immediately following UV exposure (Fig. 4D). This indicates that fluorescence intensity was proportional to the number of CPDs per cell through this dose range. To measure DNA damage and repair, cells were UV irradiated with either 0 or 10 J/m^2 and samples were collected immediately and 24 hours following UV exposure for flow cytometric analysis of CPDs. Approximately 20, 50 and 80% of CPDs remained in the GM38, GM38-E6 and GM1389 fibroblasts, respectively, twenty-four hours following UV-irradiation (Fig. 4D). This observation is consistent with previous measurements of DNA damage and repair of CPDs in these and similar fibroblast strains.[24] Taken together, the recovery from UV-induced replication arrest occurs normally in GG-NER-deficient XP-E fibroblasts, indicating that this recovery precedes the repair of CPDs.

To further dissociate the roles of p53 and GG-NER in the recovery from UV-induced replication arrest, cell cycle distribution was assessed in HPV-E6 expressing XP-E fibroblasts. As expected, p53 levels increased in the control strain following UV exposure but not in the HPV-E6 expressing XP-E derivative (Fig. 5A). Whereas

the BrdU-positive XP-E fibroblasts were delayed in S phase for approximately 6 hours following UV exposure (recall Fig. 4A), more than 50% of the BrdU-positive XPE-E6 cells remained arrested even 24 hours following UV exposure (Fig. 5B and C). The BrdU-negative population of HPV-E6 expressing XP-E fibroblasts were delayed in their entry into S phase following UV exposure but eventually entered and arrested in S phase (Fig. 5D and E). As another means of visualizing the difference in the recovery between isogenic pairs of fibroblast strains, the incorporation of [^{3}H] thymidine into DNA was used as a quantitative measure of DNA replication over the first 12 hours following UV exposure. The incorporation of [^{3}H] thymidine was reduced in all strains to a similar extent over the first 6 hours following UV exposure (Fig. 5F). However, DNA synthesis recovered significantly within 12 hours in the p53 expressing normal and XP-E strains but not in the p53-deficient strains (Fig. 5F). Therefore, XP-E cells irradiated in S phase resumed DNA synthesis prior to the repair of CPD whereas p53-deficient derivatives arrest in S phase regardless of whether they were irradiated in G_1 or S phase. These results clearly dissociate the roles of p53 and DDB2-dependent GG-NER in the recovery from UV-induced replication arrest.

DISCUSSION

Global genomic nucleotide excision repair and ultraviolet light-induced replication arrest. It has been recognized for over thirty years that UV light inhibits the synthesis of nascent DNA.[4,25-31] In response to low doses of UV light (≤ 1 J/m^2), the firing of replication origins can be inhibited following exposure to UV light.[29,30] In addition, UV lesions induced by higher doses of UV light can stall replication forks directly.[4,5,7,29-31] The extent of the initial block to replication fork progression reflects the probability that replication forks will encounter DNA lesions and is thus dose-dependent. In the present study, we used a dose of UV light (10 J/m^2) that is known to block the progression of replication forks.[4,5,7,29-31] Using BrdU-incorporation as a measure of DNA replication, we found that replication was initially inhibited to a similar extent in all fibroblast strains immediately following UV exposure, regardless of p53 status or NER capacity.

The inhibition and recovery of DNA synthesis following UV exposure has been studied using [^{3}H] thymidine incorporation and velocity sedimentation.[4,5,7] Early work suggested that a single CPD is sufficient to block a replication fork immediately following UV exposure.[8] The recovery from replication arrest is thought to be facilitated by the repair of these replication blocking lesions because UV-irradiation of XP fibroblasts results in a sustained inhibition of [^{3}H] thymidine incorporation.[4,5,7] Our data are again consistent with previous studies since we found that XP-A and XP-C fibroblasts irradiated in S phase remained arrested in that phase of the cell cycle following UV exposure. Importantly, CS-B cells were able to complete S phase, indicating that it is the GG-NER and not the TC-NER subpathway of NER that is required for the recovery from UV-induced replication arrest.

Surprisingly, DDB2-deficient XP-E fibroblasts progressed through S phase as readily as normal cells regardless of whether they were UV-irradiated in the G_1 or S phase of the cell cycle. Consistent with their reported DNA repair defect,[24] we found 80% of the CPD remained 24 hours after UV exposure in these cells. Therefore, XP-E fibroblasts are able to tolerate extensive DNA damage and resume DNA synthesis prior to the repair of CPDs. It is possible that XP-E fibroblasts are able to efficiently synthesize DNA past CPD using one or more of the translesion DNA polymerases.

It seems somewhat paradoxical when considering that GG-NER is required for the recovery from replication arrest yet GG-NER-deficient XP-E fibroblasts appear to tolerate unrepaired CPDs. The severity of the GG-NER defects in XP-A and XP-C fibroblasts is much greater than that described for XP-E. Both XP-A and XP-C fibroblasts are unable to repair the two major UV-induced pyrimidine dimers (CPD and (6-4)PP) from the genome overall while XP-A are further defective in the repair of both predominant UV lesions by transcription-coupled NER.[32-35] In contrast, the GG-NER defect in XP-E fibroblasts is specific to CPD while (6-4)PP are repaired with near normal efficiency[24] (see Supplementary Fig. 6). In general, GG-NER of (6-4)PP is much faster than GG-NER of CPD because (6-4)PP cause a greater structural distortion to the DNA helix and are recognized more readily by DNA damage recognition proteins required for GG-NER.[24,32] Conversely, translesion synthesis (TLS) of CPD is much more efficient than TLS of (6-4)PP[36,37] so (6-4)PP inhibit DNA replication more strongly than CPD.[38] Which is to say that the (6-4)PP pose a greater impediment to translesion polymerases but these lesions are repaired more efficiently by GG-NER. Intriguingly, the short replication arrest in p53 expressing cells corresponds very well temporally with the time required to repair (6-4)PP because the repair of (6-4)PP is essentially complete within 6 hours[24] (see Supplementary Fig. 6) when cells resume DNA synthesis (see Figs. 1 and 4). Taken together, XP-E fibroblasts likely recover from UV-induced replication arrest normally because they retain both the ability to repair (6–4)PP and the ability to replicate DNA templates containing CPD.

p53 and ultraviolet light-induced replication arrest. We found that p53-deficient fibroblasts have a striking defect in the recovery from replication arrest. These results are consistent with previous reports in which nascent DNA synthesis was measured by incubating irradiated cells in medium containing [^{3}H] thymidine for a short period of time, 6-8 hours following UV exposure.[9,10] Under these conditions, p53-deficient strains replicated DNA poorly compared to control strains. Similarly, colorectal cancer cells expressing a temperature sensitive variant of p53 reportedly underwent a pronounced S phase arrest following UV-irradiation at the restrictive temperature but that this S phase arrest could be overcome by restoring functional p53 at the permissive temperature.[18] By monitoring cell cycle position and the recovery from replication arrest simultaneously, the S phase defect in p53-deficient cells following UV-irradiation reported here appears far more pronounced than previously reported. S phase irradiated cells lacking functional p53 remained arrested in S phase even 24 hours following UV exposure. In addition, p53-deficient cells irradiated in G_1 phase entered S phase and underwent a sustained replication arrest. The net effect of the sustained replication arrest in S phase- and G_1 phase-irradiated populations of cells was the massive accumulation of p53-deficient fibroblasts in S phase 24 hours following UV exposure.

Given the GG-NER defect reported in p53 deficient fibroblasts, previous reports assessing the inhibition and recovery of nascent DNA synthesis in p53-deficient fibroblasts suggested that p53 contributed to the recovery of nascent DNA synthesis by regulating NER.[9,10] This was consistent with available data; however the role of p53 in GG-NER was not fully understood at the time. Fibroblasts lacking functional p53 have a repair defect that is virtually indistinguishable from that of XP-E fibroblasts because p53-deficient and XP-E fibroblasts have reduced DDB2 activity.[24,39] Our results indicate that XP-E fibroblasts recovered from the UV-induced replication arrest as

readily as normal fibroblasts despite their repair defect. Furthermore, inactivation of p53 in XP-E cells led to a sustained replication arrest in cells irradiated in either the S or G_1 phase of the cell cycle. Taken together, the S phase defect associated with the absence of p53 in UV treated fibroblasts, is independent of the role of p53 in regulating DDB2 and GG-NER. Perhaps more striking, the recovery from replication arrest appears to involve DNA damage tolerance mechanisms in p53-expressing fibroblasts that are not observed in cells lacking functional p53.

p53 and ultraviolet light-induced G_1 arrest. The p53 protein can induce G_1 arrest by regulating the expression of the cyclin-dependent kinase inhibitor $p21^{WAF1}$.[40-43] A clear p53-dependent G_1 arrest is induced in many cell types in response to agents that induce DNA double strand breaks (DSB).[19,40,41,44,45] This p53-dependent G_1 arrest has been reported in response to UV exposure.[29,46-48] However, there is accumulating evidence that the p53-dependent G_1 checkpoint is inefficient following UV exposure[18,19,44,45,49,50] and that p53-independent G_1 checkpoints also exist.[16,19,45,51,52] Therefore, the role of p53 in mediating a first cycle G_1 arrest in response to UV exposure remains equivocal.

The comprehensive cell cycle analysis presented here clearly indicates that UV treatment of asynchronous repair proficient primary human fibroblasts does not lead to a significant first cycle p53-dependent G_1 arrest, under these conditions. A minor delay in S phase entry was noted but this was more prominent in the HPV-E6 expressing cells, as previously reported.[16] We interpret these results to indicate that the induction of $p21^{WAF1}$ occurs after most cells have passed the restriction point and are either in S phase or irreversibly committed to enter S phase. The integrity of the p53-dependent G_1 arrest in the second cell cycle could not be assessed because cells lacking functional p53 remained arrested in the first cycle S phase. Assessing p53-dependent cell cycle checkpoints at a single time point (18–24 hours) following exposure to UV light could easily be misinterpreted as a bonefide p53-dependent G_1 checkpoint response.

Role of p53 in S phase following UV-irradiation. Resumption of DNA synthesis following replication fork blockage at CPD is thought to involve either translesion synthesis or homologous recombination.[1,3,53-55] DNA polymerase η can readily bypass CPD,[56-58] inserting the correct bases opposite the lesion.[59] Recently, it was reported that p53 regulates the expression of the *POLH* gene encoding DNA polymerase η[60] and DNA polymerase η-deficient XP variant fibroblasts have an S phase defect following UV-irradiation.[61,62] The p53-dependent regulation of bypass polymerases may be more common than currently recognized since the mouse *Dinb* gene encoding DNA polymerase κ also appears to be p53-regulated.[63] Therefore, it is conceivable that p53 expressing cells recover DNA synthesis more efficiently, in part, because of higher levels of TLS polymerases. However, there is presently no evidence that the level of expression of these polymerases is limiting for TLS.

Alternatively when repair and replicative bypass fail, persistently stalled replication forks can be converted to DSB.[31,64-66] Other replication stressors such as hydroxyurea also lead to stalled replication forks that can be converted to DSB.[55,67-70] Intriguingly, UV- and hydroxyurea-induced DSB form predominantly in cells lacking functional p53, suggesting that p53 stabilizes and prevents the conversion of stalled replication forks to DSB.[31,54,55,67,71] The formation of γH2AX foci, frequently used as indicators of DSB, form in response to UV light predominantly as a consequence of replicative stress[64,72] and that this occurs preferentially in cells lacking functional p53 following UV exposure[64,66,73] (Supplementary Fig. 7). It is tempting to speculate that the profound S phase defect in p53-deficient cells is the consequence of secondary DNA damage associated with stalled replication forks. By contrast, cells expressing functional p53 are able to utilize DNA damage tolerance pathways to resume DNA synthesis and complete S phase in the presence of CPD.

S phase defect and sensitivity to ultraviolet light. A striking observation in the present work was the duration of the replication arrest in p53-deficient fibroblasts. Maintaining the stability of stalled replication forks for upwards of 24 hours must constitute a significant challenge to the cell. Given the duration of this block to replication, it is not surprising that DSB form in p53-deficient cells following the collapse of replication forks at sites of DNA damage.[31] DSB are highly cytotoxic lesions so the formation of DSB following UV treatment of p53-deficient cells[31] could have profound effects on the viability of UV treated cells. We and others have reported that p53 protects primary human fibroblasts, some tumour cell lines and primary mouse embryonic fibroblasts against UV-induced apoptosis following exposure to moderate doses of UV light.[14,18,23,49,74,75] Furthermore, the commitment to UV-induced apoptosis appears to require the entry of cells into S phase[15,38,75-77] and UV-induced apoptosis was reportedly preceded by the formation of replication associated DSB[77] (see Supplementary Fig. 7). Taken together, a model is emerging that suggests that the formation of replication associated DSB underlies the sensitivity of p53-deficient fibroblasts to UV-induced apoptosis.

In summary, we clearly demonstrate that fibroblasts expressing functional p53 recover from UV-induced replication arrest prior to the repair of CPD whereas p53-deficient cells have a profound defect in the recovery from this arrest. The role of p53 in promoting recovery from replication arrest is separable from its role in regulating DDB2 and GG-NER of CPD. It remains to be determined whether this defect is linked to impaired TLS, replication fork collapse, DSB formation and sensitivity to UV-induced apoptosis. Deciphering these issues will be important to further our understanding of the role of p53 in tumour suppression.

Note

Supplementary material can be found at:
www.landesbioscience.com/supplement/StubbertCC6-14-sup.pdf

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The anti-apoptotic role for p53 following exposure to ultraviolet light does not involve DDB2

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ABSTRACT

The p53 tumour suppressor is a transcription factor that can either activate or repress the expression of specific genes in response to cellular stresses such as exposure to ultraviolet light. The p53 protein can exert both pro- and anti-apoptotic effects depending on cellular context. In primary human fibroblasts, p53 protects cells from UV-induced apoptosis at moderate doses but this is greatly affected by the nucleotide excision repair (NER) capacity of the cells. The damage-specific DNA binding protein 2 (DDB2) is involved in NER and is associated with xeroderma pigmentosum subgroup E (XP-E). Importantly, DDB2 is also positively regulated by the p53 protein. To study the potential interplay between DDB2 and p53 in determining the apoptotic response of primary fibroblasts exposed to UV light, the expression of these proteins was manipulated in primary normal and XP-E fibroblast strains using human papillomavirus E6 protein (HPV-E6), RNA interference and recombinant adenoviruses expressing either p53 or DDB2. Normal and XP-E fibroblast strains were equally sensitive to UV-induced apoptosis over a broad range of doses and disruption of p53 in these strains using HPV-E6 or RNA interference led to a similar increase in apoptosis following exposure to UV light. In contrast, forced expression of p53 or DDB2 did not affect UV-induced apoptosis greatly in these normal or XP-E fibroblast strains. Collectively, these results indicate that p53 is primarily protective against UV-induced apoptosis in primary human fibroblasts and this activity of p53 does not require DDB2.

1. Introduction

Ultraviolet light (UV light) induces DNA damage that poses a block to the progression of both DNA and RNA polymerases [1,2]. These DNA lesions are repaired by two related but genetically separable subpathways of nucleotide excision repair (NER) termed transcription-coupled repair (TCNER) and global genomic repair (GGNER). TCNER is required for the selective removal of transcription-blocking UV lesions from the template strand of active genes [3]. In contrast, GGNER is responsible for the removal of UV lesions from the bulk of the genome and does not require ongoing transcription [3]. Through the concerted action of these repair processes, cells are able to survive genotoxic challenges and restore the integrity of genomic DNA.

The p53 transcription factor is activated by diverse cellular stresses [4,5]. In response to UV light, p53 is activated and accumulates in the nuclei of cells where it either increases or decreases the expression of target genes, including DNA repair genes like DDB2 [4–6]. Also prominent among p53 targets, are genes encoding proteins involved in inducing apoptosis through the intrinsic mitochondrial cell death pathway [7–10]. Apoptosis plays a critical role in suppressing carcinogenesis in sun exposed skin so the tumour suppressive activity of p53 is in part dependent on the regulation of apoptosis [11,12]. TCNER-deficient fibroblasts accumulate p53 and undergo apoptosis in response to lower doses of UV light compared to repair proficient controls so there is a very tight correlation between the induction of p53 and UV-induced apoptosis in primary human fibroblasts [13–18]. Initially this led to the assumption that UV-induced apoptosis was p53-mediated in primary human fibroblasts [13,14,17,18]. However, this view has been challenged because inhibition of p53 activity in primary human fibroblasts did not protect these cells from UV-induced apoptosis [19,20]. In fact, loss of p53 in TCNER-proficient strains increased their sensitivity to moderate doses of UV light [19,21,22]. Therefore,

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p53 has both pro-apoptotic and anti-apoptotic activities depending in large part on the DNA repair capacity of the cells [23,24].

Xeroderma pigmentosum is a UV-sensitive syndrome associated with defects in he repair of UV-induced DNA lesions along with a concomitant increase in risk of sunlight-induced skin cancers [25]. There are eight known XP complementation groups: XP-A through XP-G and the variant form XP-V. Each is caused by mutations in a single protein [25]. Mutations in the damage-specific DNA binding protein 2 (DDB2) are associated with XP-E [26,27]. DDB2 is not required *in vitro* for NER [28] but is required *in vivo* for GGNER of cyclobutane pyrimidine dimers (CPD) [6]. DDB2 is associated with a large cullin 4A (Cul4A)-dependent ubiquitin ligase E3 complex (DDB1-Cul4A-DDB2) [29]. DDB1-Cul4A-DDB2 activity appears to be required for the recruitment of other NER proteins to bind UV lesions [30–34]. There is also considerable evidence suggesting that DDB2 loss could impact various DNA damage responses, largely independent of its role in GG-NER. For example, DDB2 is a transcriptional coactivator of E2F1 [35,36] and DDB2 binds the histone acetyltransferase p300/CBP [37]. Furthermore, it has been reported that XP-E fibroblasts express very little p53 and that this results in decreased susceptibility to UV-induced apoptosis [38].

Here, p53 and DDB2 levels were manipulated in normal and XP-E fibroblasts using HPV-E6 expression, siRNA targeting p53 and recombinant adenoviruses expressing either p53 or DDB2. We found that DDB2 expression had no effect on the sensitivity of primary fibroblasts to UV-induced apoptosis. Similarly, forced expression of p53 had very little effect on the sensitivity of these cells to UV-induced apoptosis. Conversely, disruption of p53 in normal and XP-E fibroblasts led to similar increases in the sensitivity of these strains to UV-induced apoptosis, suggesting that p53 protects fibroblasts from cell death in a DDB2-independent manner. Taken together, we found no evidence to support a role for DDB2 in regulating p53 function in these cells.

2. Materials and methods

2.1. Cell culture and UV-irradiation

Normal neonatal foreskin fibroblasts (NF) were obtained from Dr. Mats Ljungman (University of Michigan). Normal (GM00038 and AG1522) and XP-E (GM01389 and GM02415) fibroblasts were obtained from Coriell Repositories (Camden, NJ). GM01389 carry a missense mutation (L350P) in one allele and a 3 bp in frame deletion (N349Del) in the second allele of DDB2 [26]. GM02415 (XP2RO) is homozygous for a missense mutation (R273H) [26]. Normal and XP-E fibroblasts expressing HPV-E6 were described previously [19,39]. Cells were maintained in DMEM supplemented with either 10 or 15% fetal bovine serum (Wisent, St. Bruno, QC), as recommended by Coriell Repositories. Medium was supplemented with gentamicin (5 μg/ml, Sigma–Aldrich Canada Ltd, Oakville, ON).

To UV irradiate cells, medium was removed and cells were exposed to UV light at a dose rate of 1 J/m^2/s, at room temperature using a germicidal bulb (Philips) emitting UV predominantly at 254 nm. A hand held UV-radiometer was used to estimate fluence prior to each experiment (UVX Radiometer, UVP Inc., Uplands, CA). Following UV exposure, fresh pre-warmed medium was replaced and cells were returned to the incubator for the indicated period of time.

2.2. Adenovirus infections

Virus was propagated and titer was determined using standard methods [40]. Cells were seeded in 10 cm tissue culture dishes in order to obtain cultures approximately 70% confluent at the time of infection. Cells from one plate were collected with trypsin and the number of cells was determined using a cell counter (Vi-Cell XR, Beckman Coulter). Unless otherwise stated, infections were performed at a multiplicity of infection of 25 plaque-forming units (pfu) per cell. To infect cells, DMEM was removed and replaced with 1 ml of serum free DMEM with the required amount of virus. Virus was allowed to adsorb for 1 h at 37 °C in the virus suspension, while dishes were rocked every 15 min to ensure even distribution of virus. Fresh pre-warmed medium was added and cells were returned to the incubator. To prevent cells from reaching confluence, infected cells were split 1:2 6 h following infection and again returned to the incubator for the indicated time.

The adenovirus expressing DDB2 was generated by standard methods using the AdMax vector system (Microbix, Toronto, ON). The coding region of the DDB2 cDNA was subcloned from pBJ5 (kind gift from Dr. Gilbert Chu) into the pDC316 adenoviral shuttle vector. The pDC316-DDB2 plasmid was co-transfected into 293 cells along with pBHGCreloxdelta1,3 along with a Cre recombinase expressing plasmid. The resulting virus (AdDDB2) was plaque purified, expanded and the integrity of the DDB2 cDNA was confirmed by DNA sequencing. Infection of primary fibroblasts with AdDDB2 led to the over-expression of DDB2 protein (Supplementary Fig. 1).

2.3. Apoptosis

Three conceptually different methods were used to assess apoptosis. First, changes in forward light scatter, side light scatter and membrane integrity of unfixed cells were used to discriminate early stages of apoptosis from late apoptosis and secondary necrosis using flow cytometry [41–43]. Briefly, control and UV-irradiated cells were collected at various times following UV treatment, rinsed in PBS and then were resuspended in PBS containing propidium iodide (PI, 18 μg/ml, Sigma–Aldrich Canada Ltd, Oakville, ON). The early apoptotic cells were identified as the population with low forward light scatter, high side light scatter and low red fluorescence [41–43], using a BD LSRII Flow cytometer (Becton Dickenson, Mississauga, ON). Data was analyzed using FCS Express 3.0 (De Novo Software, Los Angeles, CA).

Second, internucleosomal DNA fragmentation that occurs during apoptosis was detected as an increase in the proportion of ethanol fixed cells with sub-diploid DNA content. Briefly, control and UV-irradiated cells were collected 72 h following UV treatment, washed twice with PBS, fixed with 70% ethanol and stored at −20 °C for a minimum of 30 min. The fixed cells were rinsed twice in PBS and then incubated in phosphate buffered saline (PBS, Hyclone) with RNase A (40 μg/ml, Sigma–Aldrich Canada Ltd, Oakville, ON) and PI for a minimum of 30 min. The proportion of sub-diploid cells was identified using a BD LSRII Flow cytometer (Becton Dickenson, Mississauga, ON). Data was analyzed using FCS Express 3.0 (De Novo Software, Los Angeles, CA).

Third, fluorometric immunosorbent enzyme assays for caspase 3 (Roche Canada, Mississauga, ON), 8 and 9 (Clontech, BD Biosciences, Mississauga, ON) activity were performed according to the supplier's instructions using cell lysates collected at various times between 0 and 48 h following UV treatment. Cells were counted at the time of collection using a ViCell XR automated cell counter (Beckman Coulter, Mississauga, ON) and caspase activity is expressed as units of activity per 10^6 cells.

2.4. Cell viability

Viable cell number was assessed by trypan blue dye exclusion. Adherent cells were collected at the indicated times, rinsed and resuspended in PBS. Viable cell number was determined using a ViCell XR automated cell counter (Beckman Coulter) and is expressed relative to the number of viable cells at the time of irradiation.

2.5. Western blotting

Whole cell lysates were collected with either 1% SDS or RIPA buffer. Samples were sonicated for 10 s using a microtip (Branson Sonifier, VWR International Ltd., Mississauga, ON) and protein concentrations determined using the BioRad Protein Assay (BioRad, Mississauga, ON). Proteins (20 μg per lane) in LDS NuPAGE sample buffer were separated using 10 or 12% Bis–Tris NuPAGE pre-cast gel (Invitrogen, Burlington, ON). Proteins were then transferred to Hybond-C Extra nitrocellulose membranes (Amersham, Baie d'Urfe, QC), stained with Ponceau S Red (5 mg/ml Ponceau S Red, 2% glacial acetic acid) and blocked for a minimum of 1 h at room temperature in PBSMT-A (PBS, 5% nonfat dry milk powder, 0.05% Tween 20). Membranes were incubated with primary antibody directed against p53 (Ab-6, 1:250, Calbiochem, Cambridge, MA), p21^{WAF1} (Ab-1, 1:250, Calbiochem), DDB2 (AF3297, 1:1000, R&D Systems, Minneapolis, MN) or β-actin (clone AC-74, 1:15000, Sigma–Aldrich Canada Ltd, Oakville, ON) for 1.5 h at room temperature in PBSMT-B (PBS, 0.5% nonfat dry milk powder, 0.05% Tween 20). SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) in combination with either Kodak film (Rochester, NY) or a gel documentation system (Gene Gnome, Synoptics, Bristol, UK) was used to detect bands. Multiple proteins were detected using the same blots using Restore Western Blot Stripping Buffer (Pierce, Rockford, IL).

2.6. RNA interference

Transfection of siRNAs was performed as previously described [39]. Briefly, cells were maintained in antibiotic-free media at least 48 h prior to transfection. Cells at 50–80% confluence in 10 cm culture dishes were transfected with SmartPool siRNA duplexes targeting p53 or control duplex (Dharmacon Inc., Lafayette, CO) at 50 nM final concentration using Oligofectamine Reagent and Opti-MEM I (Invitrogen, Burlington, ON) according to manufacturers instructions. Cells were split 1:2, 24 h following transfection and treated with the indicated doses of UV light 72 h following transfection.

3. Results

3.1. UV induced apoptosis in normal and XP-E fibroblasts

Normal and XP-E fibroblasts were exposed to 10 J/m^2 of UV light and cell lysates were collected for immunoblot analysis. The p53

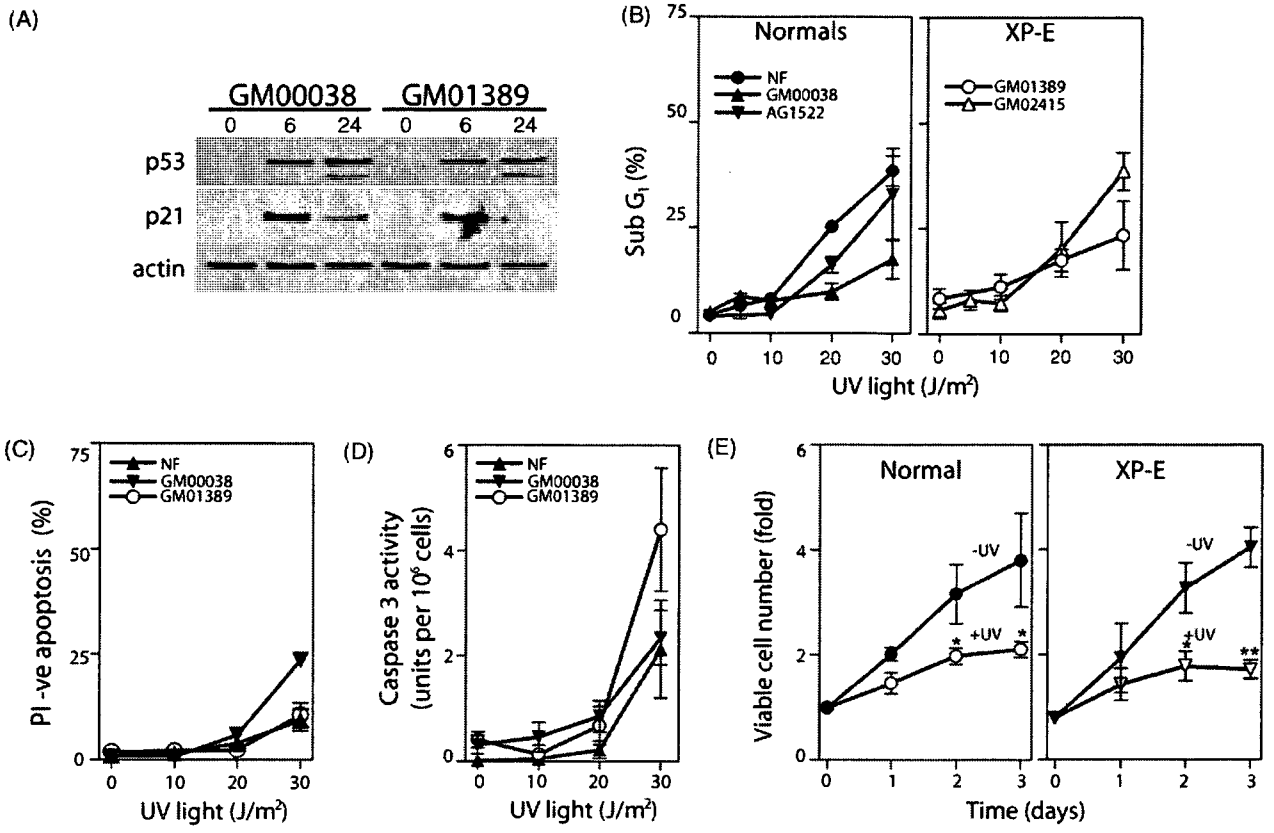

Fig. 1. Ultraviolet light-induced apoptosis in normal and XP-E fibroblasts. (A) Normal and XP-E fibroblasts were exposed to 10 J/m^2 of UV light and cell lysates were collected for immunoblot analysis. (B–E) Normal (NF, GM00038 and AG1522) and XP-E fibroblasts (GM01389 and GM02415) were exposed to the indicated dose of UV light and cell death was assessed 72 h later by subdiploid DNA content (B) and PI-negative early apoptosis (C). Similarly, caspase 3 activity was assessed 48 h following exposure to the indicated dose of UV light (D). Viable cell number was determined by trypan blue exclusion up to 3 days following exposure to 10 J/m^2 of UV light (E). Each value in B, C, D and E represents the mean (±SEM) determined from a minimum of 3 independent experiments. Significant differences ($^*P<0.05$, $^{**}P<0.001$ in E) were determined using Student *t* test.

protein levels were low in untreated cells and p53 was induced in both fibroblast strains following exposure to UV light (Fig. 1). The proportion of cells undergoing apoptosis was assessed using three independent indicators of apoptosis. Apoptosis was not significantly increased in any normal fibroblast strain in response to 10 J/m^2 of UV light (Fig. 1B–D). Despite heterogeneity in the proportion of normal fibroblasts undergoing apoptosis following exposure to high doses, 30 J/m^2 of UV light induced apoptosis in all three normal fibroblast strains (Fig. 1B–D). Both XP-E fibroblast strains were similarly insensitive to the induction of apoptosis following exposure to 10 J/m^2 of UV light but apoptosis was again detectable in response to 30 J/m^2 (Fig. 1B–D). Similar results were obtained when viability was assessed by trypan blue dye exclusion (Fig. 1E). These results support a model in which GGNER capacity does not impact the sensitivity of primary fibroblasts to UV-induced apoptosis [13,14,17,19].

3.2. Disruption of p53 leads to increased sensitivity of XP-E fibroblasts to UV-induced apoptosis

HPV-E6 was used to target p53 for proteasome-mediated degradation in XP-E fibroblasts. The expression of p53 was induced following UV-irradiation in normal and XP-E fibroblasts and HPV-E6 expression prevented the UV-induced accumulation of p53 in normal and HPV-E6 (Fig. 2A). Targeting p53 in this way led to a significant increase in the sensitivity of normal and XP-E fibroblast strains to UV-induced apoptosis (Fig. 2B–D). Furthermore, UV light led to a more substantial decrease in viable cell number in both HPV-E6 expressing strains compared to their respective controls (Fig. 2E). Therefore, these results suggest that XP-E fibroblasts retain anti-apoptotic p53 activity.

Small inhibitory RNAs (siRNA) were used to target p53 as an alternative means of inactivating p53. Transient transfection of these siRNAs significantly decreased p53 protein levels before and after UV treatment (Fig. 3A and B). Consistent with the results obtained in HPV-E6 expressing strains, transient knockdown of p53 increased the sensitivity of normal and XP-E fibroblast strains to UV-induced apoptosis (Fig. 3C and D). Taken together, the expression of wildtype p53 protected primary human fibroblasts against UV-induced apoptosis, in a DDB2-independent manner.

3.3. Caspases 3 and 9 are activated in a p53- and DDB2-independent manner following UV exposure

Caspases are highly specific cysteine proteases involved in the execution of apoptosis [44,45]. The activity of caspases 3, 8 and 9 was assessed at various times following exposure to several doses of UV light because the activity of these caspases is frequently used to discriminate between the intrinsic (caspase 3- and 9-dependent) and extrinsic (caspase 3- and 8-dependent) apoptotic pathways [44,45]. The activity of caspase 3 and caspase 9 increased in a dose-dependent manner between 24 and 48 h following UV exposure and this increase correlated with the induction of apoptosis in these primary human fibroblasts (Figs. 1 and 4A and B). Importantly, the activity of caspase 9 was greater in both HPV-E6 expressing sublines compared to isogenic controls (Fig. 4A and B). In contrast, caspase 8 activity was very low in all fibroblast

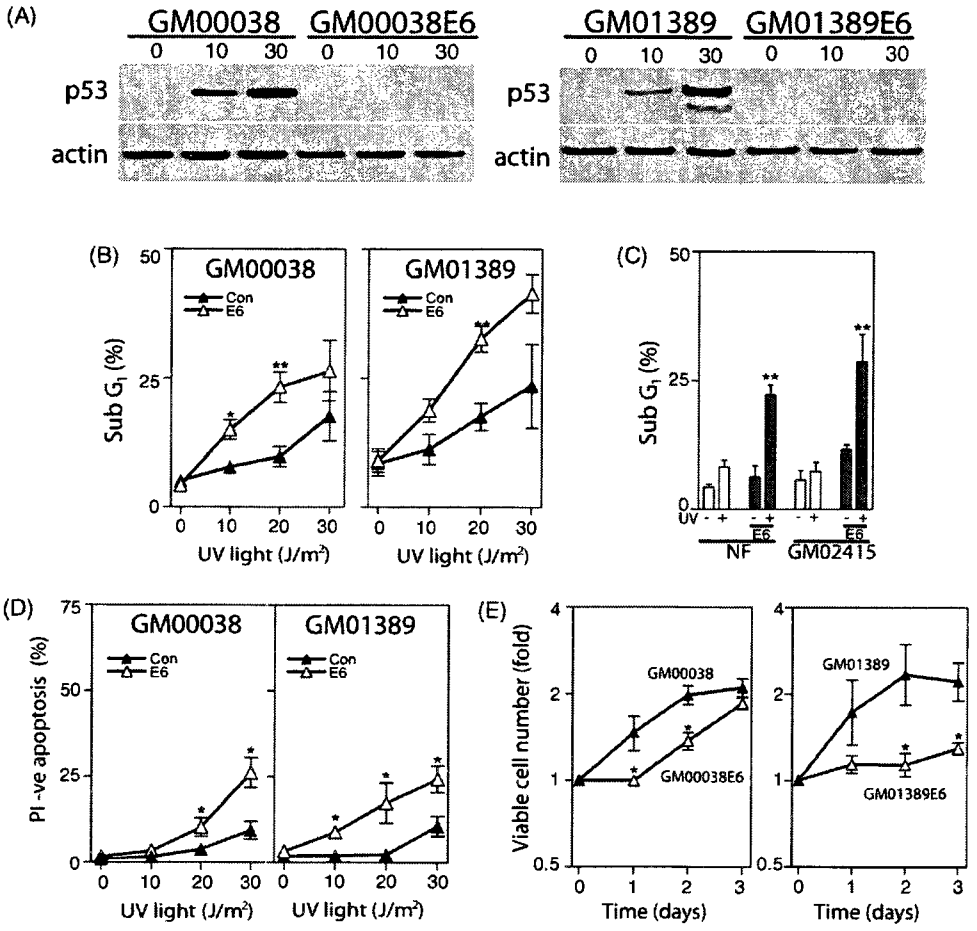

Fig. 2. The effect of HPV-E6 on UV-induced apoptosis in normal and XP-E fibroblasts. (A) Cell lysates were collected from primary human fibroblasts and derivative HPV-E6 expressing sublines 24 h following exposure of cells to either 10 or 30 J/m^2 of UV light, as indicated. (B) Normal (GM00038) and XP-E (GM01389) and their respective HPV-E6 expressing sublines were exposed to the indicated dose of UV light and 72 h later cell death was measured by assessing subdiploid DNA content (B) and PI-negative early apoptosis (D). (C) Other normal (NF) and XP-E (GM2415) fibroblasts and their respective HPV-E6 expressing sublines were exposed to 0 or 10 J/m^2 of UV light and apoptosis was measured 72 h later by assessing subdiploid DNA content. (E) Trypan blue exclusion was determined up to 3 days following exposure to 10 J/m^2 of UV light. Each value in B to E represents the mean (±SEM) determined from a minimum of 3 independent experiments. Significant differences ($^*P<0.05$, $^{**}P<0.001$) between the indicated value and its respective control collected at the same time was determined using a Student *t* test.

strains and changes in caspase 8 activity following UV exposure did not correlate with apoptosis (Fig. 4A and see Supplementary Fig. 2). Therefore, UV-induced apoptosis was associated with increased activity of caspases 3 and 9 but not caspase 8, suggesting that cell death was mediated through the intrinsic mitochondrial cell death pathway.

3.4. The effect of DDB2 and p53 overexpression on UV-induced apoptosis

Primary human fibroblasts carrying mutations in DDB2 exhibited a normal dose–response for the induction of apoptosis following UV exposure (recall Fig. 1). To determine whether overexpression of DDB2 altered the sensitivity of primary fibroblasts to UV-induced apoptosis, AdDDB2 was used to express DDB2 in normal and XP-E fibroblasts. Infection of normal and XP-E cells with AdDDB2 24 h prior to UV-irradiation yielded readily detectable DDB2 protein that peaked 24 h following infection (Fig. 5A and B). Exogenously expressed DDB2 was detectable in both fibroblast strains for several days (Fig. 5A and B and Supplementary Fig. 1). Consistent with the fact that XP-E fibroblasts exhibited a normal UV dose–response relationship, overexpression of DDB2 did not significantly alter the sensitivity of normal or XP-E fibroblasts to UV-induced apoptosis measured through two independent methods (Fig. 5C and D). Our results indicate that neither DDB2 mutations nor over-expression of DDB2 altered the sensitivity of fibroblasts to apoptosis following UV exposure.

In many model systems, p53 contributes strongly to DNA damage-induced apoptosis and the overexpression of p53 can be sufficient to induce apoptosis in many cell types [8,9,24,46]. In primary human fibroblasts, p53 appears to be primarily protective against UV-induced apoptosis because disruption of p53 by mutation [21,22], HPV-E6 ([19] and present study), pharmacological inhibitors of p53 [20] or siRNA (present study) increased the sensitivity of primary fibroblasts to UV-induced apoptosis. To complement these loss of function experiments, a recombinant adenovirus expressing wildtype p53 (Adp53) was used to determine if forced expression of p53 could drive p53-dependent apoptosis alone or in combination with UV light in these cells. Adp53 infection of normal and XP-E fibroblasts led to a large increase in p53 levels that were further increased following UV exposure (Fig. 6A). Surprisingly, Adp53 did not induce significant apoptosis alone. Adp53 did not protect fibroblasts from UV-induced apoptosis but appeared to stimulate cell death marginally (Fig. 6B and C). This only approached statistical significance in XP-E fibroblasts following exposure to 30 J/m^2 of UV light. Unlike the disruption of p53, the overexpression of p53 had very little effect on the sensitivity of primary human fibroblasts to UV-induced apoptosis. We interpret these results to indicate that primary human fibroblasts are relatively insensitive to p53-mediated apoptosis.

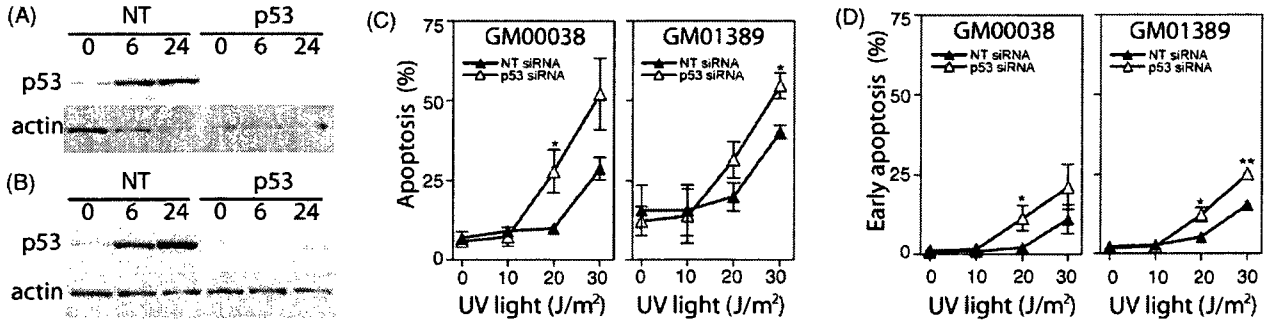

Fig. 3. The effect of targeting p53 by RNA interference on UV-induced apoptosis in normal and XP-E fibroblasts. Normal (GM00038 in A) and XP-E (GM01389 in B) fibroblasts were transfected with control non-targeting (NT) or anti-p53 siRNAs. At 0, 6 or 24 h following exposure to 10 J/m^2 of UV light, cell lysates were subjected to immunoblot analysis. Similarly, normal and XP-E fibroblasts were transfected with siRNAs described in A and B and apoptosis was measured by assessing subdiploid DNA content (C) and PI negative early apoptosis (D). Each value in (C) and (D) represents the mean (+SEM) determined from a minimum of 3 independent experiments. Significant differences ($^*P<0.05$, $^{**}P<0.001$) between the indicated value and its respective control exposed to the same dose of UV light was determined using a Student t test.

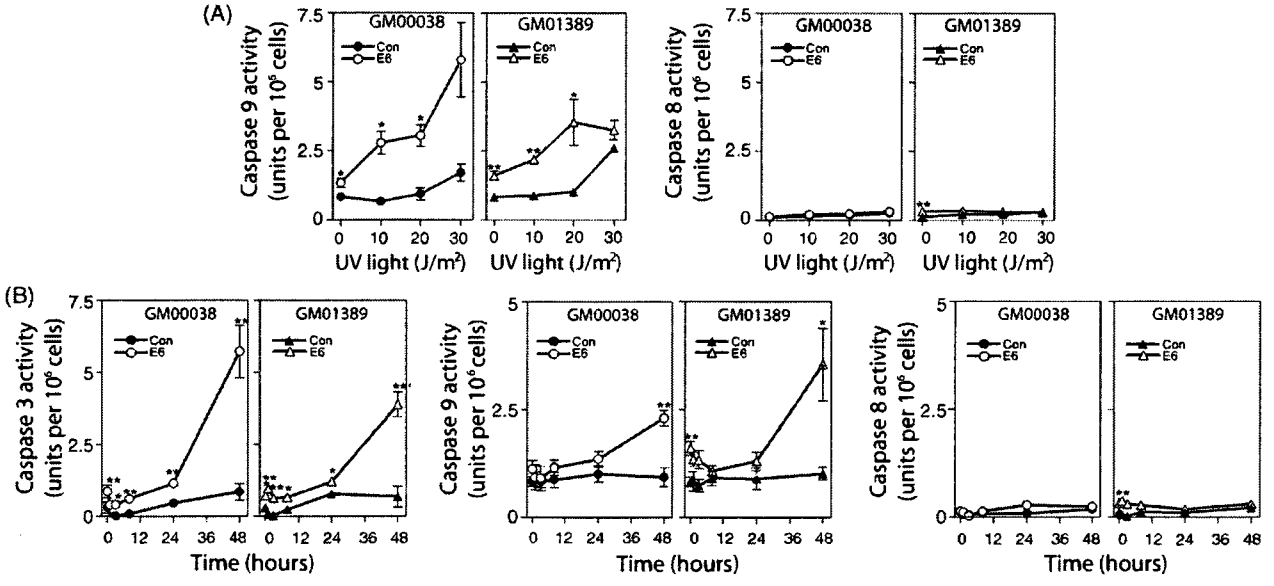

Fig. 4. Ultraviolet light-induced apoptosis is associated with increased activity of caspases 3 and 9 but not caspase 8. (A) GM00038, GM01389 and their respective HPV-E6 expressing sublines were exposed to the indicated dose of UV light and the activity of caspases 8 and 9 was determined 48 h later. (B) The indicated fibroblast strains and their respective HPV-E6 expressing sublines were exposed to 20 J/m^2 of UV light and the activity of caspases 3, 8 and 9 was determined at the indicated time following UV exposure. Each value represents the mean (±SEM) determined from a minimum of 3 independent experiments. Significant differences ($^*P<0.05$, $^{**}P<0.001$) between the indicated value and its respective control exposed to the same dose of UV light were determined with a Student t test.

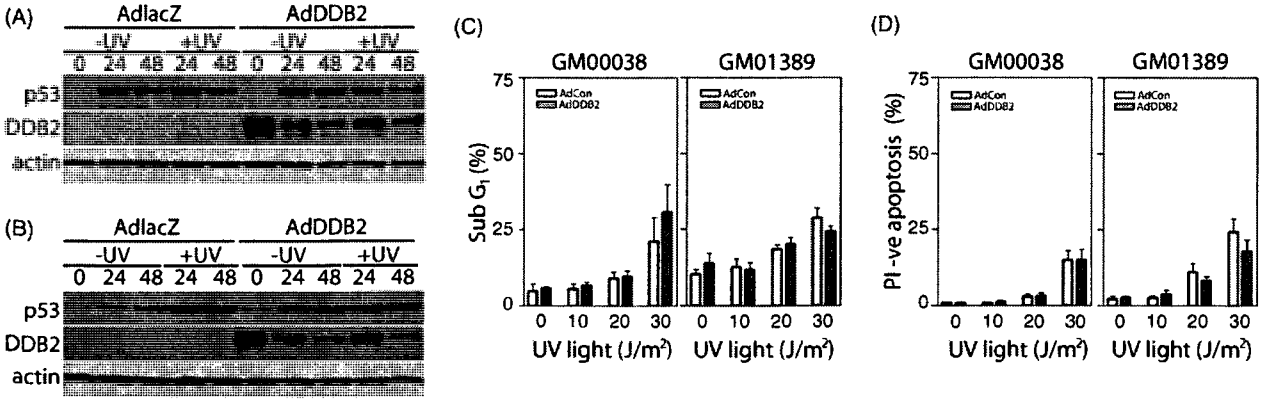

Fig. 5. Forced expression of DDB2 did not alter the sensitivity of normal or XP-E fibroblasts to UV-induced apoptosis. Normal (GM00038) and XP-E (GM01389) fibroblasts were infected with the adenoviruses expressing either lacZ or DDB2, 24 h prior to UV exposure. Cell lysates were collected for immunoblot analysis from normal (A) and XP-E (B) fibroblasts at 0, 24 and 48 h following exposure to 0 or 10 J/m^2 of UV light. Similarly, subdiploid DNA content (C) and by PI-negative early apoptosis (D) were assessed 72 h following exposure to the indicated dose of UV light. Each value in C and D represents the mean (±SEM) determined from a minimum of 3 independent experiments.

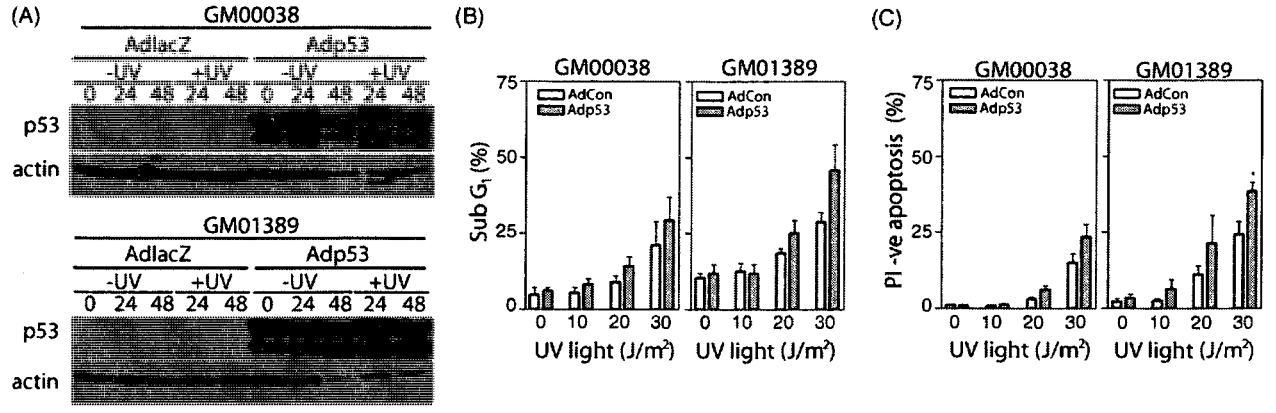

Fig. 6. The effect of p53 overexpression on UV-induced apoptosis in primary human fibroblasts. Normal (GM00038) and XP-E (GM01389) fibroblasts were infected with control or p53 expressing adenoviruses 24 h prior to mock- or UV-irradiation. (A) Cell lysates were collected for p53 immunoblot analysis at the indicated times following exposure to either 0 or 10 J/m^2 of UV light. Subdiploid DNA content (B) and PI negative early apoptosis (C) were measured in normal and XP-E fibroblasts exposed to the indicated dose of UV light infected with the indicated adenovirus. Each value in (B) and (C) represents the mean (±SEM) determined from a minimum of 4 independent experiments. Significant differences ($^*P < 0.05$) between the indicated value and its respective control exposed to the same dose of UV light was determined using a Student *t* test.

4. Discussion

4.1. The relationship between nucleotide excision repair and UV-induced apoptosis

The relationship between DNA repair capacity and apoptosis has important implications for DNA damage responses. We, and others, have reported that TCNER-deficient XP and CS fibroblasts are hypersensitive to the induction of apoptosis following exposure to low doses of UV light [13,14,17,19,47,48]. By contrast, GGNER defects in XP-C strains do not increase the susceptibility of these cells to apoptosis [13,14,17,19,47,48]. There is a very tight inverse correlation between the ability of primary fibroblasts to recover transcription and their sensitivity to UV-induced apoptosis [13,19,21,49]. Collectively these results indicate that the initial amount of DNA damage does not determine the apoptotic response because there is time to recover from damage before commitment to cell death. Furthermore, it is primarily unrepaired lesions in the template strand of active genes (i.e. transcription-blocking DNA lesions) that trigger the apoptotic response following UV exposure. In the present work, XP-E fibroblast strains exhibited normal responses for caspase activation and apoptosis following UV exposure. The response of these GGNER deficient XP-E fibroblasts is similar to that of other GGNER-deficient fibroblast strains (i.e. XP-C cells). The present work reaffirms previous observations indicating that TCNER-deficiency alters the apoptotic response to UV light whereas GGNER-deficiency has little effect [13,14,17,19]. Furthermore, these results imply that the DNA repair phenotype of XP-E fibroblasts contributes more to their apoptotic response than the proposed role of DDB2 in regulating apoptosis through p53 [38,50].

4.2. Anti-apoptotic activity of p53 in primary human fibroblasts

The p53 protein is a central player in the regulation of DNA damage-induced apoptosis in many cell types [46]. However, it is also well-established that p53 has anti-apoptotic activities in some contexts [23,24]. Here we report that normal and XP-E fibroblasts activated caspases 3 and 9 and underwent apoptosis following UV exposure but that expression of HPV-E6 or targeting p53 by RNA interference increased the sensitivity of these fibroblast strains to UV-induced apoptosis. A similarly protective effect provided by p53 was reported in normal [17,19,21,22,51] and TCNER-proficient fibroblasts [19] but not in TCNER-deficient strains [19]. The increased sensitivity to UV-induced apoptosis in the absence of functional p53 was associated with a delay in the recovery of mRNA synthesis following UV exposure [19,21,49]. Taken together, p53 contributes to the recovery from UV-induced transcriptional arrest and this plays a significant role in determining the apoptotic response of primary human fibroblasts.

Although persistently blocked transcription is clearly one important determinant of the sensitivity of fibroblasts to UV-induced apoptosis [5,13,19,49], a sustained blockage of transcription is not sufficient to trigger cell death [48]. There is also a requirement for cells to enter S phase prior to apoptosis [48,52,53]. Briefly, nascent DNA can be detected in fibroblasts undergoing apoptosis following exposure to UV light [48] and blocking the entry of UV treated cells into S phase significantly inhibits apoptosis despite prolonged inhibition of transcription [48,52,53]. Entry of cells in S phase is regulated in large part by the E2F family of transcription under control of pRb. Interestingly, DDB2 binds the transactivation domain of E2F1 and stimulates E2F1-dependent transcription [35]. In addition, forced expression of E2F1 can increase the sensitivity of primary fibroblasts to UV-induced apoptosis [14]. Despite the link among DDB2, E2F1 and UV-induced apoptosis [14,35], XP-E fibroblasts exhibited a normal apoptotic response following UV exposure.

Persistently stalled replication forks can be converted to replication-associated DNA double strand breaks (DSB) and these DNA lesions form predominantly in the absence of functional p53 [39,54,55]. The p53 tumour suppressor prevents DNA DSB formation in response to other replication stressors [56]. We recently reported that loss of p53 in normal and XP-E fibroblasts led to a dramatic UV-induced S phase arrest that was associated with the accumulation of γ-H2AX foci, consistent with DNA DSB formation in p53-deficient cells [39]. These S phase abnormalities may contribute to the increased sensitivity of p53-deficient fibroblasts to UV-induced apoptosis. Taken together, a model is emerging whereby p53-independent UV-induced apoptosis is triggered indirectly by DNA DSB formation, resulting from a combination of transcription and replication abnormalities.

4.3. p53 overexpression in primary human fibroblasts

Overexpression of p53 typically triggers either apoptosis [8,57,58] or G_1 arrest [57,59] in a cell type specific manner. In some model systems, UV light can shift the outcome of p53 overexpression from G_1 cell cycle arrest to apoptosis [24]. Here, the massive overexpression of p53 failed to induce apoptosis in primary human

fibroblasts regardless of UV dose. These results indicate that primary human fibroblasts are relatively unresponsive to apoptosis triggered by p53. This explains, in large part, why pro-survival activities of p53 are readily observed in primary human fibroblasts while they may not be detected in other cell lines or model systems.

4.4. DDB2 and UV-induced apoptosis

Certain aspects of the present work contrast a previous report [38]. Whereas we find that XP-E fibroblasts exhibit a normal sensitivity to UV-induced apoptosis, it was reported that XP-E fibroblasts were significantly more resistant to UV-induced apoptosis than normal fibroblasts due to decreased expression of p53 [38]. Although the precise reason for the disparity is not entirely clear, the control fibroblast strains used in the prior publication underwent apoptosis faster and at lower doses of UV light than observed by other laboratories [13,14,17–19,21,22,47,48,53,60–65]. The precise reason for the disparity remains unclear. Nonetheless, our work indicates that XP-E fibroblasts exhibit normal sensitivity to UV-induced apoptosis.

Two independent laboratories have generated DDB2 targeted mice [50,66]. Disruption of the DDB2 gene was associated with a small decrease in GG-NER while overexpression of DDB2 enhanced repair in UV-treated embryonic fibroblasts and keratinocytes [66,67]. In both mouse models, the resulting DDB2-nullizygous mice were susceptible to UVB-induced skin cancers compared to wildtype litter mates while DDB2 heterozygous mice exhibited an intermediate susceptibility [50,66,67]. Transgenic mice overexpressing DDB2 under control of the keratin 14 promoter were markedly resistant to UVB-induced skin cancer [66]. Both strains clearly indicate that DDB2 is anti-neoplastic in mouse skin exposed to UV light. However one group reported that DDB2−/− mouse embryonic fibroblasts were resistant to UV-induced apoptosis [50] while the other group reported a normal apoptotic response in both embryonic fibroblasts and keratinocytes from DDB2 nullizygous and DDB2 transgenic mice [66]. Our work with primary XP-E fibroblasts and fibroblasts overexpressing DDB2 are fully consistent with this latter report [66]. Therefore, we conclude that DDB2 does not affect the sensitivity of primary human or mouse cells to UV-induced apoptosis.

In summary, DDB2-deficient fibroblasts derived from patients affected with XP group E exhibit a normal sensitivity to UV-induced apoptosis and overexpression of DDB2 had no effect on the sensitivity of these cells to this form of cell death. Furthermore, UV-induced apoptosis was largely p53-independent and occurred through the intrinsic mitochondrial cell death pathway. In fact, p53 was primarily protective against UV-induced apoptosis, even in the absence of functional DDB2 protein. Our results suggest that the susceptibility of XP-E patients to solar carcinogenesis is more likely a consequence of their GG-NER defect and not the consequence of a combined defect in DNA repair and p53-dependent apoptosis. The present work has important implications for understanding the anti-neoplastic role of DDB2 in solar carcinogenesis.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mrfmmm.2009.01.010.

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