UNIVERSITY OF CALIFORNIA
RIVERSIDE

Investigations into the Role of TAF1-mediated Phosphorylation in Gene Regulation

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Cell, Molecular and Developmental Biology

by

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December 2012

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Acknowledgments

I am thankful to Dr. Liu for her patience and support over the last eight years. I am deeply indebted to my committee members, Dr. Frank Sauer and Dr. Frances Sladek for the insightful comments on my research and this dissertation. Thanks goes out to CMDB, especially Dr. Bachant, Dr. Springer and Kathy Redd for their support.

Thanks to all the members of the Liu lab both past and present. A very special thanks to the members of the Sauer lab, including Silvia, Stephane, David, Matt, Stephen, Ninuo, Toby, Josh, Alice, Alex and Flora. You have made all the years here fly by and made them so enjoyable. From the Sladek lab I want to thank Eugene, John, Linh and Karthi.

Special thanks go out to all the friends I've made over the years here. Chris, Amber, Stephane and David, thank you so much for feeding me, encouraging me and keeping me sane.

Thanks to the brothers for all your encouragement and prayers.

To any I haven't mentioned by name, I promise I haven't forgotten all you've done for me during my graduate years.

Dedication

To my parents and my family for their unwavering support. You'll never know how much its meant.

ABSTRACT OF THE DISSERTATION

Investigations into the Role of TAF1-mediated Phosphorylation in Gene Regulation

by

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Doctor of Philosophy, Graduate Program in Cell, Molecular and Developmental Biology
University of California, Riverside, December 2012
Xuan Liu, Chairperson

In response to cellular stresses, the transcription factor p53, a tumor suppressor, controls cell cycle arrest, DNA repair, or senescence by modulating the expression of target genes. Phosphorylation of p53 at threonine-residue 55 (p53-Thr55) by the TBP-associated factor 1 (TAF1), a subunit of the general transcription factor TFIID, suppresses the interaction of p53 with target genes and promotes nuclear export of p53, resulting in degradation of p53 and G1 cell cycle progression. TAF1-mediated p53-Thr55 phosphorylation in response to UV damage or high glucose (HG) treatment terminates the transcription of p21, an important regulator of G1 progression.

In Chapter 2, as a first step to identify additional p53 target genes regulated by TAF1-mediated p53-Thr55 phosphorylation through genome-wide transcription and protein-DNA interaction assays, I recapitulated the HG-induced, TAF1-, and p53-dependent repression of p21 and confirmed the conditions under which maximal repression of p21 can be observed. Furthermore, my preliminary results suggest that HG can alter p53 protein levels in response to UV damage and provide a model for how HG increases the cancer risk of diabetics.

In Chapter 3, I collected lists of p53- and TAF1- target genes, and HG-regulated genes via bioinformatics. I identified a set of genes commonly activated in response to different stress conditions: 12 p53-activated and 11 p53-repressed TAF1 target genes differentially expressed by HG, suggesting that these genes are regulated via TAF1-mediated p53-Thr55 phosphorylation. Furthermore, I describe a number of transcription factors representing putative targets for regulation via TAF-mediated phosphorylation.

In Chapter 4, I describe tools to further investigate the relationship between TAF1 and p53. A 2x3'BS p21-LUC construct shall allow discernment of the role of different p53 DNA binding sites in the differential recruitment of TAF1 by DNA bound p53. Two TAF1 kinase mutant expression constructs shall facilitate the future elucidation of the TAF1 kinase domain responsible for p53-Thr55 phosphorylation *in vivo*. Finally, my preliminary results suggest that the p53 target gene *NOXA* is regulated via TAF1-mediated p53-Thr55 phosphorylation in response to UV, indicating that TAF1-mediated phosphorylation of p53-Thr55 may be involved in regulating the expression of a subset of p53 target genes.

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Chapter 1

Introduction

1.1 Tumor Suppressor p53

The p53 protein is an important tumor suppressor protein. At the molecular level, p53 functions as a transcription factor that can activate and repress target gene expression. p53 is present at low levels in healthy cells. In response to cell stress such as DNA damage the transcriptional activity and level of p53 are increased through mechanisms involving post-translational modification and protein stabilization, respectively. Active p53 [1] can initiate a proper response to cellular stress: transient cell cycle arrest and DNA repair following moderate stress or permanent cell cycle arrest (senescence) or pre-programmed cell death (apoptosis) in cases of more severe insult. Activation of p53 occurs in response to a variety of cell stresses including DNA damage, hypoxia, nutrient deprivation, mitochondrial biogenesis stress, ribosomal biogenesis, spindle poisons, heat or cold shock, protein unfolding, and oncogene activation (Figure 1.1) [164]. In addition to DNA repair, cell cycle

and apoptosis, p53 also participates in auto-regulation, development, differentiation, angiogenesis, migration, and metabolism [173, 214, 260]. Thus, the p53 protein serves as a central sensor of a variety of cell stresses in order to ultimately influence the integrity of the genome and the ability of the cell to divide. The tumor suppressive activity of p53 is readily apparent; p53 is mutated or inactive in more than half of all human cancer [102, 199, 332]. Understanding the mechanisms by which p53 functions as a tumor suppressor has been the focus of intense study over the last thirty years since its discovery. While known to function primarily as a transcription factor, the precise mechanisms by which p53 activity is regulated and how p53 regulates transcription of specific target genes to effect a specific cellular response remains to be fully elucidated.

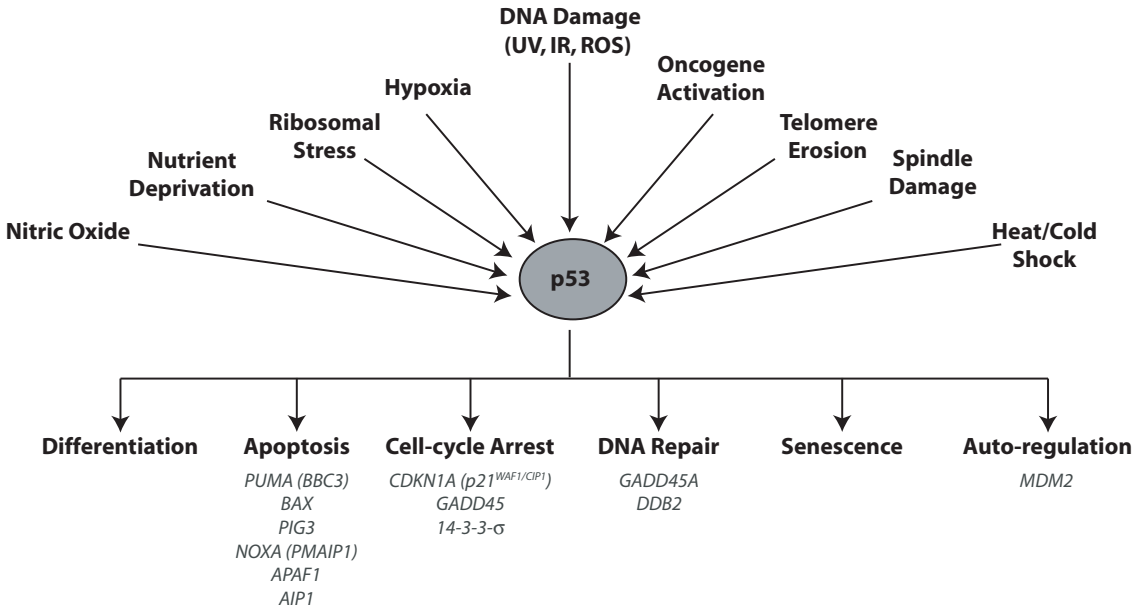

Figure 1.1. Overview of p53 Pathways

[1]Hereafter, I will refer to p53 which has been rendered functionally competent by stress-induced post-translational modification and protein stabilization as "active p53".

1.1.1 The p53 gene

The p53 tumor suppressor gene *TP53* (Gene ID:7157) is located on the short arm of chromosome 17 (17p.13.1). The gene spans 19,198 base pairs containing 11 exons which results in a 2.2 kilobase mRNA encoding the 393 amino acid protein p53. *TP53* is a member of the p53 family which includes *TP63* and *TP73* (encoding p63 and p73, respectively) and is highly conserved across vertebrates [17, 304]. The human p53 gene contains two transcriptional start sites, one in intron-1 and a second in intron-4. In addition, the p53 mRNA is subjected to alternative splicing; both intron-2 and intron-9 can be alternatively spliced resulting in nine different isoforms of human p53 [149]. These p53 isoforms are expressed in humans in a tissue-specific manner, are differentially expressed in various cancers, and can modulate the transcriptional activity of full-length p53 [28].

1.1.2 Structure of the p53 protein

The p53 protein consists of three functional regions with distinct domains: the NH_2-terminal transactivation domain (TAD), the central DNA binding domain (DBD) and the COOH-terminal region (CTR) (Figure 1.2). The first 42 amino acids of the TAD constitute the transactivation domain 1 (TAD1) and include a nuclear export signal (NES, 11-24). This domain is important for the transcriptional activation activity of p53 by contact with coactivators and general transcription factors . The negative regulator of p53, MDM2, binds to the TAD1 to inhibit p53 transcriptional activity and promote ubiquitinatation of residues in the central DNA binding domain and COOH-terminal regulatory domain (CTD) [246]. A second transactivation domain, TAD2, spans residues 43 - 97 and encompasses a proline-

rich region containing 5 repeats of the PXXP motif (residues 61-94) [336]. This proline rich-region is involved in protein-protein interactions [24]. The sequence specific DNA-binding domain (DBD) consists of residues 101-292. The DBD is required for p53 DNA binding and p53 regulation of specific target genes. The functional importance of this domain is underscored by the fact that 86% of p53 mutations cluster within the DNA binding domain [245]. The CTR contains multiple functional regions. Residues 324-355 make up the tetramerization domain responsible for oligomerization of p53 into an active DNA binding tetramer (dimer of dimers) [24]. The CTD domain includes residues 356-393 and is important for p53 protein stability and non-specific DNA binding [246]. Three nuclear localization signals (NLS) are present throughout the CTR at residues 316-324, 370-376, and 380-386. A second nuclear export signal (NES) resides within residues 340-351 [307].

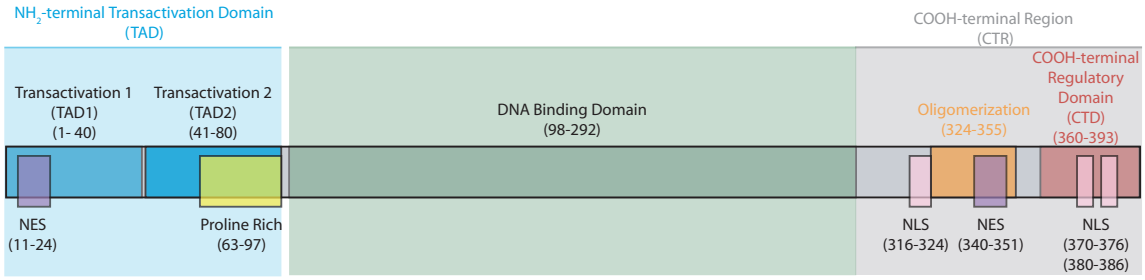

Figure 1.2. The Functional Domains of the p53 Protein
Domains of the p53 protein are shown and numbered by amino acid residue. NLS, nuclear localization signal; NES, nuclear export signal.

1.1.3 Discovery of p53, a historical perspective

Initial Discovery

While now widely known as a tumor suppressor, the initial discovery and characterization of p53 led it to be classified first as a cellular oncogene. First described by several groups in 1979, the p53 protein was identified as a host cellular protein that co-immunoprecipitated with the large T-antigen in SV40-transformed cells [155, 165, 184, 213, 300]. Moreover, p53 was detected using antisera from mice and hamsters with either SV40-induced tumors or inoculated with chemically or spontaneously transformed cell lines [52, 184]. Interestingly, the levels of the p53 were shown to be readily abundant in SV40-transformed cells, mouse embryonic carcinoma cell lines, chemically and spontaneously transformed cells from various species but not their non-transformed counterparts [19, 52, 184, 266]. Cells containing a temperature-sensitive SV40 T-antigen mutant regulated p53 levels and displayed a transformed phenotype in a temperature dependent manner [185]. The level of this newly discovered cellular tumor antigen was shown to be post-translationally regulated; the half-life in transformed cells is significantly longer than in non-transformed cells [247, 263]. Like the large T-antigen of SV40, the E1b-55kDa viral oncogene of adenovirus was also found to complex with p53 leading to higher p53 levels in virally transformed cells [276]. The high levels of p53 in many transformed cell types and its interaction with factors required for virally induced cellular transformation suggested that p53 could be involved in the transformation process and perhaps even function as an oncogene. This hypothesis was supported by experiments showing that exogenous p53 could immortalize cells alone and in conjunction with oncogenic ras [67, 136, 249].

p53 the oncogene: a case of mistaken identity

By the late 1980's several lines of evidence had begun to suggest that the characterization of p53 as an oncogene may be incorrect. First, several retroviral transformed cell types lacked functional expression of p53 due to insertion of viral genetic information into the p53 gene [18, 222, 354]. At that time it remained unknown that these insertion events per se promoted tumorigenesis. It was not until 1988 when several p53 cDNA clones were found to be unable to cooperate with activated ras to transform cells that the argument for p53 a tumor suppressor began to take shape. Mutation of these new cDNA clones rendered them capable of transforming cells in culture suggesting that the original cloned sequences of the p53 gene may not have been the wild-type sequences [80]. The original p53 clones capable of transforming in concert with ras were actually mutants; they contained a single point mutation where alanine 135 was changed to valine [66]. High levels of the mutant form of p53 are required for p53 to cooperate with ras for transformation [114]. As mutant p53 was known to complex with endogenous p53 this suggested that mutant p53 might be responsible for the transformed phenotype observed and might act in a dominant-negative fashion to inactivate the endogenous wild-type p53. Indeed, co-expression of wild-type p53 with mutant p53 or activated ras and E1A was shown to inhibit transformation [79]. This was the first such evidence to directly demonstrate the ability of p53 to act as a tumor suppressor.

Tumor suppressor p53, "Guardian of the Genome"

Further genetic evidence supporting the role of p53 as a tumor suppressor rather than an oncogene quickly followed. Li-Fraumeni syndrome (LFS; OMIM #151623) is a rare autosomal dominant familial cancer syndrome with high penetrance. LFS is characterized by variety of sporadic tumors including soft tissue sarcomas, osteosarcomas, breast cancer, brain cancer, leukemia and adrenocortical carinomas before the age of 45. A study of five such affected families revealed that all inherited germline mutations in the p53 gene [199]. Most often these are missense mutations resulting in a single amino acid exchange in highly conserved regions. As a result p53 adopts a mutant conformation and, unlike wild-type p53, fails to induce growth inhibition [84, 274]. Rearrangements of the p53 gene were found in both osteosarcoma tumors and cell lines and the vast majority of colorectal cancer sampled show deletions in the 17p13.1 region where the p53 gene resides [6, 206]. Closer analysis of colorectal and other tumor types revealed that p53 is initially mutated followed by loss of the second allele during the transition to a malignant state [7, 153, 233]. Upon examination of *TP53* in a spectrum of human tumors, it became evident that mutation or deletion of the p53 gene is a common event in carcinogenesis further suggesting the importance of p53 as a tumor suppressor [233]. This hypothesis was supported by experiments showing that p53 knockout mice developed spontaneous tumors showing that active p53 does not function as an oncoprotein but rather suppresses tumor formation [57].

This new evidence strongly suggested that p53 acted as a tumor suppressor; however, the function and precise biochemical mechanisms by which p53 functions remained elusive. Breakthroughs in understanding the p53 function came in the early 1990's when a

clear link between p53 and the cell cycle control was established. Experiments showed that: (1) p53 is a target of cell cycle regulatory kinase cdc2 and its phosphorylation peaks during mitosis; (2) p53 subcellular localization varies during the cell cycle accumulating in the cytoplasm during G1 and translocating to the nucleus during S-phase; (3) damage to cells by UV, γ-irradiation, radiomimetic or chemotherapeutic drugs results in post-translational stabilization of endogenous wild-type p53 protein and subsequently induce G1 or G2 arrest [22, 143, 200, 286, 309]. Overexpression of wild-type p53 in transformed cells causes G1 arrest or in some cases apoptosis [7, 287, 365]. In summary, these experiments suggested that p53 can respond to various stimuli and induce cell cycle arrest; however, the biochemical mechanisms by which p53 exerted this affect were still unclear.

Finally, biochemical evidence emerged demonstrating that p53 can act as a potent transcription factor. Wild-type p53 was found to bind DNA, a property often lost in mutant forms of the protein [10, 147, 306, 351]. p53 DNA binding was shown to be sequence specific and a p53 consensus DNA binding site (p53-DBS) was defined [64, 87]. When fused to the DNA binding domain of the yeast transcription factor Gal4, the p53 TAD activated the expression of Gal4-dependent targets genes [78, 262]. Wild-type p53 was also shown to activate transcription of genes containing its own DBS site both *in vivo* and *in vitro*, a property lost by mutant p53 proteins [71, 87, 148, 277, 367]. At the same time it was discovered that rat and human homologues of mouse double minute 2, MDM2, an oncogene amplified in a variety of human sarcomas, bound and inhibited p53 suggesting MDM2 binds and inactivates p53 in a manner similar to the T-antigen and other viral oncogenes [220, 242].

A model for p53 function emerged: p53 levels in the cell are normally low but after stimulus by DNA damage p53 protein levels are stabilized and able to activate transcrip-

tion of target genes whose products are involved in cell cycle arrest, perhaps allowing time for DNA repair or inducing apoptosis [163, 331]. Thus, in response to DNA damage, p53 might serve as "guardian of the genome" to ensure that cells with mutated genetic information are unable to propagate [163]. Several direct p53 target genes were soon discovered that began to substantiate and build upon this model. Growth arrest and DNA-damage-inducible gene *GADD45*, was found to be a direct p53 target gene activated following γ-irradiation [144]. The ability of p53 to induce G1 arrest was found to be attributable to a newly discovered target gene, $p21^{WAF1/CIP1}$ (*CDKN1A*), whose product tightly binds and inhibits cyclin A-Cdk2, cyclin E-Cdk2, cyclin D1-Cdk4, and cyclin D2-Cdk4 complexes [65, 105]. Previous findings that wild-type p53 was able to induce apoptosis in some transformed cell types was further substantiated when it was found that p53 can directly induce transcription of the pro-apoptotic gene *BAX* and down-regulate the anti-apoptotic gene *BCL2* [217, 218, 283]. Furthermore, MDM2 was found to be a p53 target gene raising the possibility of a negative regulatory loop to keep tight regulatory control of p53 protein levels to ensure only proper levels of p53 activity [9, 140, 358]. By the mid 1990's the biochemical function and mechanism by which p53 acts as a tumor suppressor seemed clear. Since then, the p53 field has continued to expand and discover new cellular processes to which p53 responds and regulates the cellular response to ensure the ultimate integrity of the genome.

1.1.4 The Function of the p53 protein

The p53 protein acts in both a transcription-dependent and transcription-independent fashion to implement cell-cycle arrest, DNA-repair or apoptosis in response to specific cellular

stresses like DNA damage as well as a variety of other processes autophagy, angiogenesis, metabolism, protection from reactive oxygen species (ROS), immune response, differentiation, cell migration, cell-cell communication and even embryo implantation [214, 333]. The transcriptional activity of p53 is paramount for its tumor suppressive activity. p53 has been found to be bound at several thousand DNA binding sites throughout the human genome and regulate the expression of several hundred target genes [26, 135, 298, 299, 349]. More than 80% of p53 mutations occur within the DNA binding domain and abrogate the DNA binding ability of p53, highlighting the pivotal role DNA binding in the p53 tumor suppressive activity [244]. Interestingly, several transcription-independent functions of p53 have been found to contribute to the p53 response as well [305].

Transcription Factor Properties

p53 is able to bind DNA in both a nonspecific and sequence-specific fashion. The DNA binding domain of p53 binds in a sequence-specific manner to DNA binding sites consisting of two copies of the decamer motif RRRCWWGYYY (where R=A,G; W=A,T; Y=C,T) separated by a spacer of 0-13bp [64, 87]. Each decamer, or half-site, can be visualized by the graphical representation of each quarter-site, RRRCW as $\longrightarrow$, and WGYYY as $\longleftarrow$. Thus the p53 consensus DNA binding sequence consists of two head-to-head quarter-sites and can be visualized as $\longrightarrow \longleftarrow$ spacer $\longrightarrow \longleftarrow$ [264]. Even in unstressed cells, p53 is capable of binding DNA and can be found bound at the promoters of target genes [69, 142]. The COOH-terminus of p53 mediates non-specific DNA binding and facilitates linear diffusion along the DNA while the DNA binding domain frequently dissociates and reassociates with DNA allowing p53 to rapidly search for target binding

sites [1, 210, 313]. Stress-induced activation of p53 by posttranslational modifications weakens the p53 COOH-terminal/DNA interaction and thereby promotes binding by the core DNA binding domain [85, 121, 313]. p53 binds DNA in a cooperative manner as a dimer of dimers, a process dependent upon protein-protein interactions and DNA bending [8, 152, 227–229, 342, 350].

The ability of p53 to activate transcription is dependent on its ability to interact with a variety of factors to ensure a permissible chromatin state and to recruit members of the general transcriptional machinery to promote pre-initiation complex (PIC) formation at the promoter [16]. DNA-bound p53 can promote an open chromatin state in the surrounding regions by recruiting histone acetyl transferases (HATs), and chromatin remodeling factors. p53 can recruit several HATs including p300/CBP [11, 98, 183, 280], PCAF [11, 280], TIP60 [94, 314] which promote histone acetylation at target gnes. In the case of the p21 gene, p53-mediated histone acetylation can lead to eviction of H2A.Z, a repressive histone variant, from the promoter and thereby relieving repression of the promoter [94]. Ordered cooperative modification of histones by the arginine methyltransferases PRMT1 and CARM1 and the HAT p300 result in synergetic activation of p53 target gene *GADD45A* [3]. p53 recruitment of SWI/SNF remodeling complexes to promoters also results in p53-dependent apoptosis and cell growth arrest [168]. p53 can promote PIC formation through interactions with the Mediator complex [97, 369], key PIC factors such as TBP [72, 188, 203], several TBP-associated factors (including TAF3, TAF6, TAF9, TAF1) and the general transcription factors (GTFs) TFIIH and TFIIA [21, 174, 177, 191, 320, 359, 360]. The interaction of the NH_2-terminal transactivation domain of p53 with TAF1, TAF5, and TAF6

within TFIID may stabilize a "closed" TFIID conformation upon binding at the A/C lobe interface [187]. How this conformation regulates transcription is not yet understood.

Interestingly, p53 directly represses a significant number of its target genes [337]. Down regulation by p53 is mediated by three mechanisms [16, 166, 264]. First, p53 can bind its own DNA binding site and recruit corepressors. For example, p53 can recruit the histone deacetylase HDAC1 to the promoter of *MAP4*the Map4 gene via interaction with the corepressor mSIN3A [224, 372]. Second, when present at high levels, p53 can outcompete DNA binding by other transcription factors at overlapping DNA binding sites, thereby precluding transactivator binding and resulting in target gene repression. p53 uses this mechanism to repress the expression of the anti-apoptotic gene *BCL2* by blocking binding of the transactivator Brn-3a [33]. Finally, p53 can repress target gene expression by protein-protein interactions which prevent other factors from binding, a process known as squelching. For instance, p53 binds the transactivator Sp1 to prevent Sp1-mediated transcriptional activation of Cyclin B [125].

The p53 programs

Cell-cycle Arrest

The tumor suppressive activity of p53 is attributed to its ability to induce transient cell growth arrest and DNA repair to maintain genomic stability or to ensure that cells with genomic instability can no longer proliferate by inducing cell senescence or apoptosis. These cellular outcomes primarily depend on the transactivation and transrepression properties of p53. Furthermore, a variety of p53 transcription-independent activities have been described

which may work in concert with the p53 transcriptional programs to affect specific stress response.

p53 is able to halt cell-cycle progression at the G1/S checkpoint primarily through transactivation of *CDKN1A* encoding for the CDK inhibitor protein p21 [65, 105]. p21 is required for arrest at the G1/S transition of the cell cycle [32, 53, 59, 335]. p53 can directly bind and inhibit E2F and as well as cyclinE/Cdk2 and cyclinD/Cdk4 complexes thereby preventing cyclin/Cdk phosphorylation of Rb and the subsequent release of E2F to activate genes necessary for S-phase progression [56, 59, 105, 106]. In response to DNA damage p53 also mediates G2 arrest in part through p21, 14-3-3σ (SFN), and GADD45A, which serve to reduce the levels of active cyclin B/Cdk2 complexes that control mitotic entry [138]. p21 indirectly suppresses cyclin B and cdc2 expression through Rb (as in G1 arrest) and directly inhibits Cdk2 kinase activity [81, 124, 252]. Expression of 14-3-3σ is activated by p53 and is required for successful maintenance of G2 arrest by sequestering cyclin B/Cdk2 complexes in the cytoplasm [38, 111]. GADD45A is able to interact with and reduce Cdk2 kinase activity, further suppressing overall levels of active cyclin B/cdc2 complexes necessary for G2 progression [368].

Cell Senescence

Interestingly, senescence may provide an alternative mechanism by which p53 prevents the rise of transformed cells. Ras transformation of primary human cells results in permanent G1 arrest indistinguishable from senescence and can be prevented by loss of p53 [284]. Furthermore, p53 has been shown to be important for mediating cell senescence in

several murine model tumor types, suggesting this oncogene induced-senescence pathway is relevant *in vivo* [46, 285, 330, 362].

DNA Repair

Genotoxic stress includes a variety of types of DNA damage including cyclobutane pyrimidine dimers, 6-4 photoproducts, DNA oxidation and alkylation, and DNA strand breakage. p53 is able to maintain genomic stability by regulating several DNA repair mechanisms including nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), homologous recombination (HR), and non-homologous end joining (NHEJ) [89]. p53 contributes to the global genomic repair (GGR) mechanisms of nucleotide excision repair (NER) by activating of *GADD45A*, *DDB2* and *XPC* expression. GADD45A can interact with proliferating cell nuclear antigen (PCNA) to stimulate NER. It may also facilitate DNA repair by altering chromatin accessibility in DNA damaged cells, and can stimulate promoter demethylation and repair [12, 35, 301]. XPC binds DNA lesions and is important for UV-induced cyclobutane pyrimidine dimer lesion recognition by DDB2 [89]. There is some evidence that p53 may directly participate in NER by interacting with three subunits of TFIIH involved in transcription coupled repair (TCR): p62, XPB and XPD [172, 341, 359]. p53 can directly stimulate BER by binding DNA polymerase β binding [371]. Furthermore, APEX, a central player in BER can modulate p53 DNA binding perhaps serving as a link between DNA damage and the p53 response [88, 134]. p53 can promote MMR by the activating the expression of key MMR-related genes including *RRM2B*, *MSH2*, (*MLH1*) and *PMS2* [43, 99, 231, 278, 344]. p53 is able to able to suppress HR through interaction with Rad51 and can recognize and inhibit the migration of Holliday-like

junctions created by stalled replication forks in order to promoter error free double-strand break repair [89]. Interestingly, p21 can interact with PCNA, also upregulated by p53 in response to DNA damage. This interaction can inhibit PCNA-dependent DNA synthesis without hindering the ability of PCNA to participate in NER [181, 292, 361]. GADD45A can also interact with PCNA and stimulate NER [301]. Thus, p53 may utilize targets such as p21 and *GADD45A* to coordinate the cell-cycle with DNA repair following genotoxic damage.

Apoptosis

Should DNA damage to the cell be of sufficient intensity or duration p53 can induce apoptosis to prevent the rise of genetically unstable cells. There are two main pathways by which cells induce apoptosis, the intrinsic and extrinsic pathways. The intrinsic pathway is irrevocably activated upon mitochondrial outer membrane permeabilization (MOMPMitochondrial Outer Membrane Permeabilization (MMOP) by pro-apoptotic factors including Bax and Bak, which facilitate release of cytochrome C from the mitochondria leading to activation of APAF-1 and the executioner caspase 3 [104, 305]. p53 can upregulate expression of *NOXA*, *PUMA* and *TP53AIP1* whose protein products alleviate negative inhibition of Bax and Bak, allowing the two pro-apoptotic proteins to oligomerize and insert into the mitochondrial membrane to induce MMOP [209, 232, 236, 237]. p53 can activate expression of *APAF1* and *BAX* to further activate the intrinsic apoptotic pathway, while inhibiting transcription of key anti-apoptotic factors *BCL2* and *BCL2L1* [82, 216, 218, 310]. The extrinsic apoptotic pathway relies on receptor-mediated caspase activation. p53 can directly upregulate several such "death receptors" including *FAS* (and its ligand FasL) and

TNFRSF10B (*KILLER*) [86, 248, 357]. Non-transcriptional activity of p53 also contributes to apoptotic induction. Stress-induced accumulation of cytoplasmic and mitochondrial p53 bind Bax, Bak and Bcl-xL and subsequently alleviate pro-apoptotic factor inhibition and induce MMOP [305].

Role of Non-coding RNAs in the p53 Program Response

In addition to targeting protein coding genes, several classes of non-coding RNAs (ncRNAs) have been shown to be p53 targets and tomodulate p53 stress response. MicroRNA (miRNA) genes are expressed similar to coding genes and the resultant transcripts are processed into 22-23nt RNAs. These small RNAs then target mRNAs for degradation or translational repression in a sequence-specific manner [13]. p53 can directly activate transcription of several microRNAs including miR-22 [325], miR34-a/b/c [25, 40, 109, 251, 261, 316], miR-107 [363], mir-145 [268], miR-192 and miR-215 [29, 93]. p53-regulated miRNAs target the mRNAs of genes important in cell-cycle arrest, apoptosis, DNA-repair, angiogenesis, and hypoxia. In some cases the outcome directed by the expression of a particular p53 target microRNA is dependent on the cellular context. For example, miR-34a can promote cell growth arrest or apoptosis depending on the cell type in which it is expressed [77, 110]. Thus, these microRNAs may serve as a mechanism by which p53 can fine-tune target gene expression and the stress response [14, 171]. Recently, evolutionarily conserved p53 response elements were discovered to be enriched in the promoters of more than thirty long intergenic non-coding RNAs (linc-RNAs) [100, 119]. One such linc-RNA, linc-p21, was shown to be a direct p53 target and mediate repression of genes by recruitment of heterogeneous nuclear ribonucleoprotein K (hnRNP-K), a member of repressive

complexes, to gene promoters [119]. Thus, ncRNAs add another layer of regulatory complexity to the p53 network.

Role of p53 in Metabolism

The widespread ability of cancer cells to reprogram their cellular metabolic program has come into focus recently as one of the emerging hallmarks of cancer [103]. Under aerobic conditions cells typically break down glucose via glycolysis and shuttle pyruvate into the oxidative phosphorylation pathway in the mitochondria for the generation of adenosine triphosphate (ATP). Under anaerobic conditions cells switch and generate their energy primarily from glycolysis alone. However, cancer cells can preferentially make this switch even in the presence of oxygen, opting to increase their glucose intake and excrete most of the partially oxidized carbon source as lactate. This preference for aerobic glycolysis is known as the "Warburg effect." In most cancer cells this switch is not due to a loss of mitochondrial function but rather to an active metabolic reprogramming. It seems most likely that cancer cells need to adjust their metabolic program in order to provide sufficient energy and material for efficient proliferation. Aerobic glycolysis allows sufficient carbon resources (derived from glucose and glutamine) to be devoted to the generation of nucleotides, amino acids, lipids, and NADPH. Aerobic glycolysis can also protect cells from damage by ROS by reducing mitochondrial production of ROS from oxidative phosphorylation. Diverting glucose into the pentose phosphate pathway (PPP) provides NADPH to sustain production of oxidized glutathione, a primary cellular antioxidant. Thus, metabolic reprogramming in cancer cells provides energy, biomass, and protects from environmental stress. The switch to aerobic glycolysis appears to be driven by the signals from onco-

genes and associated proteins. For example, Akt and Myc can directly promote glycolysis. Not surprisingly then, several tumor suppressors have been shown to regulate these metabolic pathways to counter metabolic reprogramming including PTEN (an inhibitor of Akt), TSC1 and TSC2 (inhibitors of mTOR) and LKB1 (an activator of AMPK) and p53 [50, 95, 127, 327, 334].

p53 can regulate glycolysis at several steps in order to promote oxidative phosphorylation and oppose aerobic glycolysis. p53 can reduce the influx of glucose by inhibiting the expression of glucose transporters GLUT1 and GLUT4 [279]. p53 can also indirectly repress expression of GLUT3 by modulating the activity of nuclear factor-κB (NF-κB) [145]. Other steps along the glycolytic pathway are also targets for p53 regulation. Phosphoglycerate mutase (PGM) protein levels be downregulated by p53, further reducing the flux of glucose through glycolysis [154]. Interestingly, the PGM gene is p53 inducible; and p53 can also induce expression of hexokinase, suggesting that p53 may positively regulate glycolysis under certain conditions [207, 267]. Induction of p53 target gene *TIGAR* inhibits phosphofructokinase. As a result glucose, is redirected away from glycolysis and towards the pentose phosphate pathway promoting NADPH production [20].

While inhibiting aerobic glycolysis, p53 also promotes mitochondrial function to stimulate oxidative phosphorylation. Cells expressing p53 undergo more oxidative phosphorylation than those lacking p53, which is in part due to decreased expression of cytochrome *c* oxidase (SCO2) [196]. SCO2 is a key regulator of the cytochrome oxidase complex (COX), an important consumer of oxygen in the mitochondria [208]. Other p53 target genes likely to play a role in mitochondrial maintenance include subunit I of cytochrome c oxidase, and p52R2 [27, 241].

Recently p53 was found to directly modulate cellular metabolism to promote aerobic glycolysis or to oxidative phosphorylation. Pyruvate dehydrogenase kinase isoenzyme-2 (Pdk2) plays a key role in controlling the conversion of pyruvate into acetyl-CoA. The pyruvate dehydrogenase complex (Pdc) converts pyruvate into acetyl-CoA for input into the citric acid cycle and oxidative phosphorylation. Pdk2 phosphorylates and thereby inhibits the activity of Pdc thereby favoring lactate formation and aerobic glycolysis. p53 downregulates transcription of Pdk2 and thereby can directly inhibit the metabolic switch to aerobic glycolysis by directing the cellular utilization of pyruvate for oxidative phosphorylation [48].

Reactive oxygen species are an unavoidable byproduct of cellar metabolism and environmental exposure. p53 to responds to changes in the levels of ROS differently depending on the intensity of the stress. Under basal or low levels of ROS stress p53 activates transcription of sestrins, TIGAR, TP53INP1, glutathione peroxidase, aldehyde dehydrogenase and can indirectly activate expression of Nrf2 to promote anti-oxidant functions and cell survival. Alternatively, under conditions of high stress p53 may induce cellular senescence or pro-oxidant genes such as BAX, PUMA and PIG3 to activate apoptosis. Thus, depending on the cellular context and the stress p53 may exhibit pro-survival or pro-death responses [95, 334].

p53 is also sensitive to signals from metabolic stress such as hypoxia, nutrient starvation, or changes in cellular energy levels. Under hypoxic conditions MDM2 levels drop thereby stabilizing p53 protein levels and activating the p53 response. AMP-activated protein kinase (AMPK) is sensitive to the AMP/ATP levels in the cell. Nutrient depletion or starvation leads to a high AMP/ATP ratio and activation of AMPK. AMPK can both in-

crease expression of p53 and increase p53 activity by phosphorylation of p53 at Ser46. In turn both AMPK and p53 increase TSC1 and TSC2 to inactivate mTOR to repress protein synthesis and promote autophagy. Autophagy is a homeostatic process in which cells shuttle cytoplasmic components to the lysosome for degradation and recycling. This response provides a limited supply of ATP during times of starvation [76, 95, 334].

1.1.5 Regulation of p53

Function of p53 relies upon rapid protein stabilization, activation by posttranslational modifications, and protein-protein interactions. These events regulate the stability, subcellular localization, and DNA binding activity of p53 and consequently the p53 dependent outcome in response to stress [212, 225]. The complexity of p53 regulation is illustrated by the heterogeneous, p53 response to DNA damage *in vivo* [225]. Tissues can be grouped into three categories based on their p53 response to stress [197]. This particular study examined mice treated with γ-irradiation for apoptosis. First, in group 1 tissues, p53 levels were elevated and exhibited a p53 response. IR induced a group 1 response in the spleen, thymus, haemopoietic bone marrow, intestine, and ependyma. Group 2 displayed elevated p53 levels but no p53-dependent response; IR induced a group 2 response in the salivary gland, choroid plexus, myocardium, adrenal, lung parenchyma, osteocytes of bone, and kidney. The group 3 demonstrated neither elevated p53 protein levels nor a stress response; IR induced a group 3 response in skeletal muscle, brain, and liver. Even within a particular tissue the response could be heterogeneous; different cell types within a tissue displayed dissimilar p53 responses. These differences reflect activation of different transcriptional programs by p53 in different tissues and cell types [73]. This differential programming

likely reflects the cell-type specific context of modifiers and co-factors that are proposed to shape the p53 response [225]. Thus, a complex "barcode" of factors are able to determine the nature of the p53 response and outcome to stress [68, 225].

Regulation by MDM2 and MDM4

Basal expression of the p53 gene is constitutive however the p53 protein is tightly regulated and kept at low levels under normal growth conditions. MDM2 is one of the primary negative regulators of p53 and controls p53 protein levels and activity. The contribution of MDM2 to p53 regulation is dramatically apparent in knockout studies with mice; embryonic lethality in *Mdm2* knock-out mice can be rescued by concurrent knock-out of *Tp53* [139, 221]. MDM2 regulates p53 by several mechanisms. First, MDM2 is an E3 ubiquitin ligase thereby promoting p53 degradation via the 26S proteasome (discussed in more detail under Ubiquitination) [70, 107, 116, 159]. MDM2 induced degradation is one of the primary methods by which the cell keeps p53 protein levels low in unstressed cells [30]. Interestingly, low levels of MDM2 results in monoubiquitination of p53, a mark that leads to nuclear export of p53 thereby sequestering p53 away from target genes [179]. MDM2 also binds the NH_2-terminus of p53 thereby occluding the transactivation domain and preventing the interaction of p53 with coactivators [243, 321]. Following DNA damage post-translational modification of p53 destabilizes the p53-MDM2 interaction in order to elevate the levels of p53 in the cell (discussed more under Phosphorylation and Acetylation). p53 binds to and activates expression of the MDM2 gene, thereby forming a negative regulatory loop that controls p53 activity even after stress [358]. The interaction between MDM2 and p53 can be disrupted in response to oncogene activation. Following oncogenic stress the

levels of ARF rise dramatically. ARF can then sequester MDM2 to the nucleolus, promote MDM2 degradation and block MDM2-mediated ubiquitination of p53 [117, 348, 370].

Release of p53 from its negative regulator MDM2 by small molecules could provide a method to re-activate p53. Toward that aim there has been a broad search for small molecules capable of activating p53. Several small molecules have been found that are able to activate p53 either by stabilizing and activating mutant and wild-type p53 [205, 340]. Perhaps most promising is the discovery of a family of small molecules known as nutlins which activate p53 by targeted disruption of the MDM2/p53 binding. Nutlin3 tightly binds to the p53 binding pocket of MDM2 thereby occluding p53 binding and MDM2-mediated ubiquitination. The p53 protein is thereby free to accumulate to levels high enough to activate cell-cycle arrest and apoptosis [328]. Nutlin3 has been proposed as a treatment for cancers commonly containing wild-type p53 (such as haemological malignancies and retinoblastoma) in the hopes of inducing p53 activity in tumor cells and boosting the response to chemotherapy or ionizing radiation treatment [123, 239, 269]. The *in vitro* success of nutlin3 activation of p53 has spawned several clinical trials for nutlin3 to treat various cancers [281].

Another critical regulator of p53 activity is the MDM2 related protein, MDM4 [201, 255]. As in the case for *Mdm2*, *Mdm4* knockout mice can be rescued by concurrent knockout of *Tp53* [250]. While highly homologous, MDM4 lacks the ubiquitin ligase activity of MDM2 [132, 294]. Whereas MDM2 serves primarily to regulate p53 protein stability, MDM4 functions as a major inhibitor of p53 transcriptional activity [83, 294, 323]. MDM4 binds to the NH_2-terminus of p53 similarly to MDM2 thereby inhibiting activity

by occlusion of the transactivation domains and by preventing p300/CBP mediated acetylation [202].

Following IR-induced DNA damage ATM stimulates phosphorylation of MDM2 and MDM4. This event promotes MDM2 auto-ubiquitination and degradation [308]. ATM-stimulated phosphorylation of MDM4 promotes its ubiquitiation by MDM2 and subsequent degradation [45, 240, 254]. Thus, following DNA damage both primary negative regulators of p53 stability and transcriptional activity are themselves destabilized and degraded thereby further facilitating p53 protein stability and transcriptional activity [211, 324].

Regulation by Posttranslational Modification

Posttranslational modification of p53 plays an important role in the regulation of p53 activity. The p53 protein can be modified by a variety of posttranslational modifications including ubiquitination, phosphorylation, acetylation, methylation, sumoylation, neddylation, glycosylation, ADP-ribosylation (Figure 1.3) [4, 24, 157, 158, 212]. Modification of p53 occurs under both basal and stressed conditions and contributes to the stabilization, activation, sub-cellular localization, and DNA binding of the p53 protein [4]. The best studied and most well understood of these modifications are ubiquitination, phosphorylation and acetylation.

Regulation by Ubiquitination

Ubiquitin is a 76 amino acid polypeptide that can be covalently attached to proteins at lysine residues. This process is catalyzed by the sequential action of E1, E2, E3 and in some cases E4 ligases [118, 257]. It is an important, reversible, protein modification

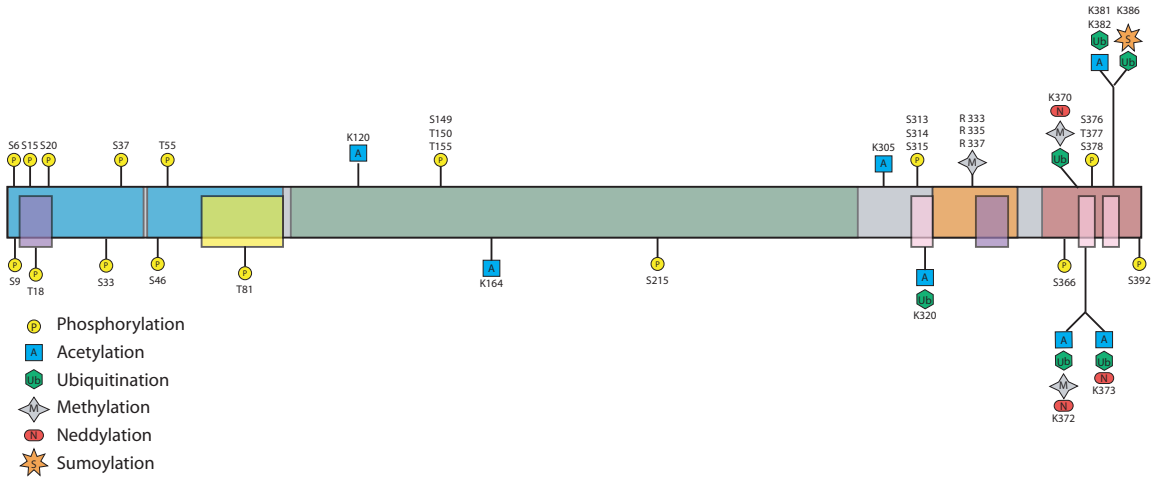

Figure 1.3. Posttranslational Modifications of p53
p53 is modified by a variety of posttranslational modifications including phosphorylation (P), acetylation (A), Ubiquitination (Ub), Methylation (Me), Neddylation(N), and sumoylation (S) in both basal and stressed conditions.

that can regulate diverse cellular processes [101, 112]. Proteins can be monoubiquitinated or polyubiquitinated (a chain of ubiquitin molecules successively attached to a protein by linkage to the previously attached ubiquitin molecule). Polyubiquitination promotes degradation of the marked protein via the 26S proteosome pathway [112]. In unstressed cells p53 protein levels are kept low primarily by the action the E3 ligase MDM2 which is responsible for p53 degradation [30, 70, 160, 201]. MDM2 binds the NH_2-terminus of p53 and predominantly ubiquitinatinates lysines within the COOH-terminus (Lys370, Lys372, Lys373, Lys381, Lys382, and Lys386) [265]. Several lysine residues within the DNA binding domain are also ubiquitinated by MDM2 [39, 190]. E4 ligase activity is the promotion of polyubiquitination required for some, but not all substrates of the ubiquitin system [118]. p300 and CBP both harbor E3 and E4 ligase activity and are required for polyubiquitination and p53 turnover in the cell [96, 290]. Interestingly, the activity is found only in the

cytoplasmic pool of p300/CBP [290]. MDM2 concentration can affect the level of p53 ubitination. High levels of MDM2 promote polyubiquitination while lower levels support only monoubiquitination. Monoubiquitinated p53 is exported from the nucleus [179]. Thus, ubiquitin can serve to modulate p53 localization.

Several other E3-ligases for p53 have been found including Pirh2, COP1, Arf-BP1, E6-AP, TOPORS, TRIM24, and MKRN1. These may play more specific roles in p53 activity and responses [30, 31, 212, 324]. For example, Arf-BP1 can ubiquitinate p53 and this is inhibited by ARF, providing an additional mechanism by which ARF can stabilize p53 in response to oncogenic activation [42]. However, MDM2 plays the primary role in cellular turnover of p53.

Ubiquitination is a reversible modification and two de-ubquitifying enzymes have been identified for p53, namely Herpes-specific ubiquitin specific protease (HAUSP) and USP10 [180, 366]. HAUSP can directly de-ubiquitinate and thereby stabilize the p53 protein [180]. Interestingly, HAUSP can also destabilize p53; after DNA damage HAUSP de-ubiquitinates MDM2 and MDM4 facilitating their stabilization [178, 215]. USP10 specifically counteracts MDM2 by translocating to the nucleus where it de-ubiquitinates and stabilizes p53. Thus, ubiquitin plays a dynamic role in the regulation of the p53 stability and localization.

Regulation by Phosphorylation

The p53 protein is phosphorylated by a variety of kinases in both basal and stressed conditions. The role of phosphorylation in regulating the levels, activity, sub-cellular localization of p53 has been intensely studied. Serine and threonine residues throughout the

protein are targets for phosphorylation. Most of the phosphorylated residues reside within the NH_2-terminus. p53 is rapidly phosphorylated in response to DNA damage such as UV or IR and this phosphorylation is important for the stabilization of the p53 protein levels. The NH_2-terminus of p53 is highly modified: Ser6, 9, 15, 20, 33, 36, 37, 46, and Thr18 and 81 are all know to be phosphorylated following DNA damage. IR-induced DNA damage induces the activation of kinases involved in the DNA damage response including ATM, ATR and Chk2 which mediate phosphorylation of p53 at Ser15 and Ser20 [291]. UV damage induces ATR-mediated phosphorylation of Ser15 and 37 [322]. Serine 15 phosphorylation is critical for ensuring further step-wise modification of p53. Ser15 phosphorylation is required before several NH_2-terminal residues (Ser9, Ser20, and Thr18) can be phosphorylated and subsequently COOH-terminal residues acetylated [62, 270–273]. Many sites of modification on p53 are targeted by multiple enzymes. Furthermore, modification of several residues requires prior modification of others, suggesting that proper control of p53 is controlled by multiple pathways in a redundant and ordered modification process [212].

Phosphorylation is proposed to stabilize and activate p53 by acting as a switch that disrupts the p53/MDM2 interaction and promotes interaction of p53 with p300/CBP [212]. Phosphorylation of Ser18 and Ser20 specifically inhibit MDM2 binding and promote p53 stability [41, 49, 61, 131, 273, 317, 326]. At the same time phosphorylation of these two residues, as well as Ser33, 37, 46, and Thr55, enhances the binding of p300/CBP [58, 60, 74, 137, 162, 167, 259, 317, 318]. This recruitment is important for acetylation of the p53 COOH-terminus which further boosts p53 stability and activity (discussed in Acetylation). Phosphorylation of the NH_2-terminus also stimulates p53 activity by exposing the transactivation domain for interaction with members of the general transcription

machinery. For instance, Ser18 phosphorylation disrupts MDM2 binding and simultaneously promotes the interaction of the TAD with TAF9 [131]. Phosphorylation of Ser46 and Thr55 enhances p53 binding to the p62 subunit of TFIIH [54].

Several residues within the COOH-terminus of p53 are also targets for phosphorylation. Ser392 is phosphorylated following UV damage and promotes p53 activity at least in part by enhancing sequence specific DNA binding [121, 146]. UV damage induces phosphorylation at Ser315 to stimulate transcriptional activation by p53 [23].

Phosphorylation of p53 in unstressed cells also serves to regulate p53. Under these conditions p53 is phosphorylated at Thr55, Ser149, Thr150, Thr155, Ser376, and Ser378. Basal COP9 signalosome CSN kinase-specific phosphorylation of Ser149, Thr150 and Thr155 within the DNA-binding domain of p53 promotes ubiquitination and 26S-dependent degradation [15]. In the absence of DNA damage p53 is phosphorylated at Thr55 by TAF1 [177]. This phosphorylation promotes nuclear export and degradation of the p53 protein and promotes G1 progression [34, 177].

Phosphorylation is a reversible modification and p53 can be regulated by the action of several phosphatases. Damage by IR results in Ser376 dephosphorylation thereby creating a 14-3-3 consensus binding site. The 14-3-3/p53 interaction promotes sequence-specific DNA binding by p53 [347]. Following DNA damage p53 Ser15 phosphorylation by ATM promotes phosphatase B56γ-specific PP2A association with p53 and subsequent dephosphorylation of Thr55. This mechanism is involved in p53-mediated activation of p21 transcription and subsequent G1 arrest [176, 293]. Interestingly, Ser15 phosphorylation can be reversed following DNA damage by the p53 target WIP1, which may serve as another negative regulatory feedback loop regulating p53 activity [192].

Regulation by Acetylation

Following DNA damage or other forms of cell stress p53 is acetylated at a variety of residues; this modification promotes stabilization of p53, activation and sequence specific DNA binding. Tetramerization of p53 promotes its subsequent acetylation [128, 175]. Residues within the DNA binding domain (Lys164) and the COOH-terminus (Lys305, Lys370, Lys372, Lys373, Lys381, Lys382, and Lys386) of p53 are acetylated by p300/CBP [98, 186, 272, 315, 343]. PCAF acetylates p53 within the COOH-terminus at Lys320 and TIP60/hMOF acetylates Lys120 within the DNA binding domain [186, 272, 312, 314]. The recruitment of p300/CBP for acetylation of p53 is known to occur by several mechanisms. Following DNA damage, phosphorylation at the NH_2-terminus promotes MDM2 dissociation and p300/CBP interaction and facilitates acetylation of the COOH-terminal lysine residues [60, 272]. Furthermore, PTEN can bind p300 to stimulate and maintain a high level of acetylation following DNA damage [175]. Also, after DNA damage the phosphorylated NH_2-terminus of p53 is bound by hAda3 to stimulate p300-mediated acetylation of p53 [226, 339]. ARF activation induces hAda3-stimulated p300 acetylation of p53 as well [282]. Finally, oncogenic ras can induce PML-p53-p300/CBP complex formation in PML nuclear bodies to stimulate p53 acetylation [253].

Acetylation of p53 increases protein stability. Ubiquitination and acetylation both target lysine residues and thus can compete for the same target residue. The COOH-terminal lysines (Lys370, Lys372, Lys373, Lys381, Lys382, and Lys386) are the primary targets for MDM2 ubiquitintion [265]. Acetylation of these residues blocks subsequent ubquitination and promotes p53 stability [130, 180, 230]. Acetylation also stimulates sequence specific DNA binding by p53 [98, 186, 193, 272]. Recent work supports two-state models for p53

response element discovery and binding. Acetylation of the COOH-terminus reduces non-specific p53 DNA binding and shifts p53 from a rapid, linear "scanning" state to a DNA binding domain specific "recognition" state to promote sequence specific binding at p53 DNA binding sites [313].

Acetylation of p53 is important for mediating cell cycle arrest and apoptosis [11, 130, 174, 195, 314, 315]. p53 acetylation promotes p300/CBP recruitment to p53 target promoters in order to promote chromatin architecture favorable for transcription [11, 69, 223]. HDAC1 and SIRT1 can both deacetylate p53 and thereby abrogates p53-driven transcription [129, 194, 195, 329]. The microRNA miR-34a, a p53 target, can downregulate SIRT1 thus providing a regulatory loop through which p53 can upregulate its own activity [77].

Tip60-mediated acetylation of p53 at Lys120 is critical for p53-mediated induction of apoptosis but not cell growth arrest, suggesting that specific posttranslational modifications are capable of directing specific target gene activation [312, 314]. Consistent with this hypothesis a Lys120Arg mutant has lost the ability to activate the pro-apoptotic *BAX* and *PUMA* genes but retains the ability to activate the p21 and *MDM2* genes [312, 314]. It is interesting to note that Lys120 makes contact at the p53 DNA binding site and this interaction is altered when the central base pair is changed from C/G to A/T (as are the interactions of Ala276 and Cys277) [152]. Acetylation at this reside may alter the nature of the p53-DNA interaction and could also affect the recruitment of cofactors such as ASSP1/2 which are known to mediate promoter specific binding of p53 [312].

Mice which express a p53 mutant where all COOH-terminal lysine residues have been mutated to arginine are viable and show no increased cancer risk. These p53 mutants display a normal half-life suggesting that the mutated residues are not the only lysines ubiq-

uitinated [75]. MEFs derived from these mice demonstrate apoptotic and cell-cycle arrest phenotypes similar to those with wild-type p53 whereas thymocytes demonstrate promoter-specific differences in the response compared to wild-type p53 [75, 156]. This suggests that the COOH-terminal lysines can be compensated for by other lysines or by coactivators. Tang *et al.* found that the loss of acetylation at each individual site can be compensated by acetylation at the others. However, loss of the ability to acetylate all eight lysine residues (Lys120, 164 and the COOH-terminal lysines) by mutation to arginine completely abolishes p53-mediated p21 induction and the cell-cycle and apoptotic response; However the induction of Mdm2, is not altered [315]. The authors found that MDM2 and MDM4 were recruited in a promoter-specific manner to repress transcription. Repression could be relieved by inactivation of MDM2 and MDM4. They demonstrated that acetylation of p53 destabilizes its interaction with MDM2 and MDM4, and blocks their recruitment to the p21 promoter thereby, derepressing p53 transcriptional activity [315]. Indeed, MDM2 and MDM4 have been found to bind and negatively regulate the transcriptional activation of several promoters [5, 238, 315]. Thus, it has been proposed that anti-repression from MDM2 and MDM4 may serve as a regulatory step in activation of p53 stability and activity [158].

1.2 TATA box binding protein (TBP)-associated factor 1 (TAF1)

1.2.1 The diverse functions of TAF1

TFIID is composed of the TATA binding protein (TBP) and at least 13 TBP associated factors (TAFs) [319]. TFIID is one of the first general transcription factors (GTFs) to bind at the core promoter. TAF1 is the largest subunit of the GTF TFIID, a key component of the basal transcription machinery. Upon binding, TFIID facilitates PIC formation as necessary for transcription of genes by RNA polymerase II (hereafter RNPII). TFIID has three functions to promote RNPII transcription. First, it acts as a coactivator transmitting signals from activators to the basal transcription machinery to promote PIC formation. Second, TFIID recognizes and binds at both TATA-containing and TATA-less promoters. Third, TFIID utilizes enzymatic activities to posttranslationally modify chromatin and other factors to modulate transcriptional activation [319]. TAF1 plays a critical role in each of these TFIID activities. TAF1 interacts with activators and other GTFs, is capable of promoter recognition and has multiple enzymatic activities [345]. TAF1 is a bipartite kinase and has HAT activity and ubiquitin-activating (E1) and conjugating (E2) activity [55, 219, 256].

Role of TAF1 in TFIID architecture

In *Drosophila*,TFIID is composed of the TATA binding protein (TBP) and at least 13 TBP-associated factors (TAFs) [319]. *Drosophila* TAF1 interacts with TBP, TAF2, TAF4, TAF5,

TAF6, TAF11, and TAF12 [44]. Early evidence indicated that TAF1 nucleates the assembly of TFIID [44, 352]. Indeed, *in vitro* TAF1 can serve as a scaffold for assembly of other TAFs [44]. However, recent molecular and genetic evidence suggests that TFIID assembly is more complex [47]. Wright *et al.* demonstrated that knockdown of TAF1 did not destabilize other TAFs and resulted in stable core complex between TAFs 4,5,6,9 and 12 [355]. The authors suggest that TAF1 may act as a peripheral subunit along with TBP, TAF2, TAF7, and TAF11 which may "decorate" the core complex [355]. Several other studies have supported the notion that TAF1 is not required for TFIID assembly and suggest that a subcomplex of TAFs may exist [47]. Singh *et al.* found that yeast TAF1 containing temperature-sensitive TAF1 mutations, in a region required for interaction with TAF4 could abrogate TFIID assembly. However, TAF4 was still found to associate with other TAFs in a complex similar to that observed by Wright and colleagues. Furthermore, the authors still detected a subcomplex consisting of TBP/TAF1/TAF7 [297]. Studies in yeast, *Drosophila* and *in vitro* can detect a core complex lacking TAF1, TAF2, TAF7 and TBP [47]. The function of TAF1 as a peripheral subunit to TFIID may reflect the differential TAF requirement by certain genes. Several studies suggest that TAF1 responsible for regulation of only 14-27% of genes in yeast and only 18% of genes are misregulated by a temperature sensitive mutation in ts13 hamster cells [115, 170, 235, 288]. However, because these studies used temperature-sensitive mutations that are unlikely to abrogate more than one enzymatic activity or the promoter recognition capacity, it seems likely that TAF1 is required for transcription of many more genes. More recent results seem to confirm this: 80-90% of yeast genes are TATA-less promoters regulated by TFIID with and nearly all of these genes require all of the separate domains of TAF1 for proper expression [120, 126].

Genome-wide analysis of TAF1 promoter occupancy and gene expression in human cells parallels this finding [150].

Regulation of TAF1 by activators

TAF1 appears to function as a relay switch to turn transcription on or off in response to signals from activators and other proteins. For instance, several transcriptional activators interact with TAF1 to upregulate transcription including adenovirus E1A, Herpes simplex virus type 1 ICP4, JUN, and tumor suppressor p53 [36, 92, 174, 189]. TAF1 can also be targeted for suppression by the HIV protein Tat and the tumor suppressor retinoblastoma (Rb) [353]. In many cases these activators regulate TAF1 activity. Tat binds and blocks TAF1 HAT activity thereby repressing TAF1-dependent transcription of the major histocompatibility complex class I genes [353]. Rb can bind and repress TAF1 NH_2-terminal kinase phosphorylation of TFIIA which correlates with a decrease in transcription *in vitro* [295, 302]. Cyclin D and E1A can bind TAF1 and prevent Rb-mediated suppression of TAF1 kinase activity [296].

In addition to regulating TAF1 enzymatic activities, activator interactions with TAF1 can recruit TFIID to promoters. This is implied by the results of several studies. Carrozza *et al.* found that the COOH-terminus of ICP4 binds TAF1 and the same region is necessary for transcriptional activation, suggesting recruitment is necessary for activation [36]. Additionally, TAF1 occupancy at specific genes can be induced by activation and this can be reversed by removal of enhancers [161, 182]. Wang *et al.* showed that a TAF1 responsive enhancer could confer TAF1 dependence on a normally TAF1-independent promoter [338]. Finally, Li *et al.* demonstrated that in response to DNA damage p53 is capable of directly

recruiting TAF1 to the p21 promoter via interactions between the acetylated Lys373 and Lys382 of the p53 COOH-terminus and the double bromodomain of TAF1 *in vivo* [174].

Role of TAF1 in promoter recognition

Classical understanding of transcriptional activation has come from *in vitro* studies examining the role of the GTFs in the assembly of the basal transcription machinery. These early studies examined the well characterized TATA promoter element and found that TBP binds to the TATA box in order to direct TFIID to the promoter and nucleate stable basal transcription machinery assembly [319]. This has proved to be a simplified view of the types of *cis*-regulatory sequences at core promoters and mechanisms of PIC assembly. There are more now than eight characterized core promoter motifs including the TATA box, BRE (TFIIB recognition element), Inr (initiator), MTE (motif ten element), DPE (downstream promoter element), DCE (downstream core element), and XCPE1 (Xcore promoter element) none of which are universal. Promoters can be classified as focused or dispersed. Transcription at focused promoters starts within a region of a few nucleotides (generally found in regulated promoters). Dispersed promotes have multiple start sites over a range of 50 to 100 nucleotides (generally found in constitutive promoters). Promoters showing intermediate properties of both focused and distinct promoters are also common [141]. TFIID plays a key role in recognition and initiation of transcription at focused promoters which tend to be enriched for TATA box and INR elements [141, 275, 319].

In addition to the ability of TFIID to activate transcription from TATA-containing promoters it was also found to activate TATA-less promoters containing an INR in a TBP-independent manner suggesting that TAFs may play a role in promoter recognition [204].

In fact, yeast TAF1 promoter recruitment was found to be core promoter sequence responsive [289]. At least in some cases the core promoter sequence determines whether a gene is TAF-dependent or not [51, 289, 356]. TAF1 and TAF2 form a complex that is able to recognize and bind the INR element (YYANWYY) [37]. In addition, photo-crosslinking experiments have shown that TAF1 also binds the DCE promoter element [169]. Genome-wide analysis of promoters reveals that 76% of human genes lack a TATA-like sequence, and at least 46% of human genes contain an INR [364]. Thus, TAF1 likely serves as an important mediator for TFIID promoter recognition at many promoters throughout the genome.

TAF1 regulation of activators and GTFs by posttranslational modifications

TAF1 has kinase, HAT activity and ubiquitin-activating and conjugating activities (Figure 1.4) [55, 219, 256]. Surprisingly, the domains responsible for these enzymatic activities share little homology with proteins harboring similar activity [345].

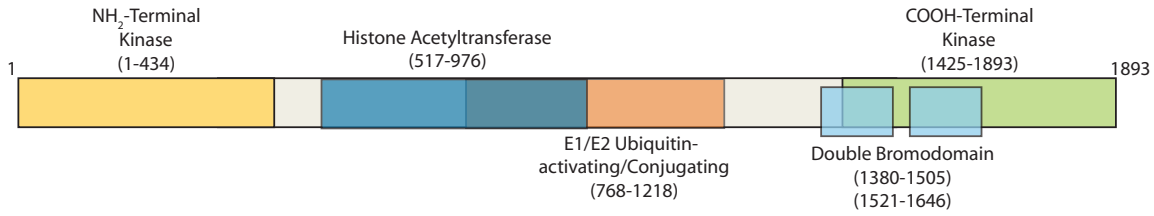

Figure 1.4. Schematic Diagram of the TAF1 Protein
Domains are labled and sizes are numbered by amino acid residue.

Phosphorylation by TAF1

TAF1 is a bipartite kinase; it possesses NH_2-terminal (NTK) and COOH-terminal (CTK)

serine/threonine kinase domains which are independently functional [55, 234]. Several targets for TAF1 phosphorylation have been described including the RAP74 subunit of TFIIF, the β subunit of TFIIA, TAF7, histone H2B, and p53 [55, 91, 177, 198, 302]. Phosphorylation by TAF1 likely plays a role in regulating transcription *in vivo*. Phosphorylation of TFIIA promotes complex formation of TBP, TFIIA and TATA box. It also stimulates transcription *in vitro* while TFIIA mutants that are unable to be phosphorylated hinder transcriptional activation *in vivo* [302, 303]. Phosphatase treatment of RAF74 results in weaker transcription and reduced RNPII elongation *in vitro*. This is like caused by reduced efficiency in DNA/TBP/TFIIB complex formation and subsequent reduction in RNPII association [151]. Maile *et al.* demonstrated that *Drosophila* TAF1 is able to phosphorylate histone H2B at a conserved serine 33 residue. Ser33 phosphorylation *in vivo* coincides with transcription of *string/cdc25* and *giant* genes [198]. Interestingly, this results in G2 arrest whereas in hamster ts13 cells a temperature sensitive mutation that abolishes HAT activity results in G1 arrest [63]. This suggests unique requirements for different enzymatic activities of TAF1 by different genes. This is further supported by the findings of O'Brien *et al.* who found that approximately 18% of genes in ts13 cells required TAF1 HAT activity, only 6% required NTK activity and close to 1% required both HAT and NTK activity for regulation [235].

TAF1 is also able to regulate other transcription factors via phosphorylation. TAF7 binds and inhibits the HAT activity of TAF1 during PIC assembly. Following initiation of transcription TAF7 is released concurrent with phosphorylation of TAF1 and TAF7. It is unclear if TAF1 is the kinase responsible for these phosphorylation events but autophosphorylation of TAF1 *in vitro* is sufficient to drastically inhibit the TAF1-TAF7 interaction

[90, 91]. Our lab has shown that TAF1 can phosphorylate p53 specifically at Thr55 [177]. This event promotes nuclear export, degradation and cell cycle progression [34, 177].

Acetylation by TAF1

TAF1 is also a histone aceyltransferase and can acetylate H3 Lys14 as well as other H3 and H4 lysine residues *in vitro* [219]. In addition, TAF1 acetylates TFIIE β and TFIIF *in vitro* [122]. Hamster ts13 and BN462A cells contain identical glycine to aspartic acid mutations at TAF1 amino acid 690. As a result the TAF1, HAT activity is temperature sensitive and incubation at the non-permissive temperature leads to G1 arest [63, 108]. This arrest appears to be due in part to a loss of TAF1 HAT activity necessary for the transcription of the cyclin A and cyclin D genes [63, 113, 311]. TAF1 may thus play a role in chromatin remodeling via its HAT activity. In addition TAF1 contains two bromodomains which bind to acetylated lysine residues [133]. This domain may serve to target or anchor TAF1 to acetylated histones at promoters. Acetylated activator p53 is able to recruit TAF1 to the p21 promoter through the bromodomain of TAF1; mutation of p53 Lys373 and/or Lys382 to arginine or mutations within the TAF1 bromodomain that abolish acetyl-lysine binding abolish TAF1-p53 interaction and TAF1 recruitment [174].

Ubiquitination by TAF1

TAF1 also possesses ubiquitin activating and conjugating activity [256]. TAF1 E1 activating and E2 conjugating is sufficient for monoubiquitination of the histone linker protein H1 *in vitro* [256]. Point mutations within the putative E1/E2 domain block *in vitro* ubiquitination. Expression of TAF1 carrying these mutations in *Drosophila* embryos reduces

histone linker ubquitiation *in vivo* and disrupts gene expression [256]. The mechanisms by which TAF1 ubquitintation influences gene transcription has yet to be revealed. While it does not appear that TAF1 is directly responsible for ubiquitination of p53, TAF1 downregulates MDM2 autoubiquitination and promotes MDM2-p53 association [2]. In ts13 cells shift to the non-permissive temperature leads to TAF1 inactivation, MDM2 levels rise and p53 is activated leading to G1-arrest [2, 346].

1.3 Examining the Dynamic TAF1/p53 Interplay

Our laboratory studies the p53/TAF1 interaction in the regulation of p53 target gene transcription. In particular we focus on elucidating the mechanisms by which TAF1-mediated Thr55 phosphorylation regulates p53 activity. Recent work in our laboratory has implicated TAF1-mediated p53-Thr55 phosphorylation in the termination of p53-activated transcription. The kinase activity of TAF1 can be modulated by intracellular changes in ATP concentration such as seen in cells following DNA damage or high glucose (HG) treatment (unpublished, X.L. and Y.W.). These increased ATP levels stimulate TAF1-mediated p53-Thr55 phosphorylation which impairs p53 DNA binding and promotes p53 nuclear export and p53 degradation [34, 258]. Consequently, the expression of the p53 target gene p21 is downregulated (unpublished, X.L. and Y.W.). In this work, I investigated the role of TAF1-dependent phosphorylation of p53 and other transcription factors in transcriptional regulation in response stress known to induce fluctuations in intracellular ATP levels. In Chapter 2, I confirm the conditions necessary for genome-wide transcription profiling of

HG-treated cells in order to identify p53 target genes whose expression is regulated by TAF1-mediated p53-Thr55 phosphoryolation and investigate the effects of HG on p53 protein levels in response to UV treatment. As an alternative to transcription profiling, I use a bioinformatics approach to identify genes putatively regulated by TAF1-induced p53-Thr55 phosphorylation in Chapter 3. Furthermore, using bioinformatics I address the hypothesis that other transcription factors besides p53 may be regulated by TAF1-mediated phosphorylation. Lastly, in Chapter 4, I describe a number plasmid constructs generated to further characterize the p53/TAF1 interplay and identify *NOXA* as a possible target of regulation by TAF1-mediated p53-Thr55 phosphorylation following UV damage.

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Chapter 2

High Glucose Induces TAF1-dependent Downregulation of p53 Protein Levels and Activity

2.1 Introduction

Metabolic reprogramming is a hallmark of cancer cells and is required to meet the nutritional needs of rapidly proliferating cancer cells [16]. Metabolic reprogramming increases the rate of energy production (adenosine triphosphate; ATP) and macromolecule biosynthesis, both necessary for rapid cell proliferation, and balances the redox state of the cells in response to increased metabolism [5, 11, 40]. Many cancer cells can produce ATP by glycolysis rather than oxidative phosphorylation, even in the presence of oxygen and functional mitochondria [40]. This metabolic switch is known as the Warburg effect. Glycolysis

generates ATP faster than oxidative phosphorylation and supplies the building blocks for the biosynthesis of nucleotides, amino acids, lipids, proteins and carbohydrates required for cell replication. Furthermore, glycolysis directly and indirectly replenishes the levels of nicotinamide adenine dinucleotide phosphate (NADPH), a key cofactor that provides the cell with the reducing power necessary for macromolecule biosynthesis and protection from the detrimental effects of reactive oxygen species (ROS); its levels are increased as a result of higher metabolic rate [5].

Glucose is a critical source of energy and carbon for cells, in particular proliferating cells [11, 40]. Many cancer cells increase glucose uptake by upregulating the expression of glucose transporters [27]. Phosphatidylinositol 3-kinase (PI3K) signaling promotes glucose uptake, stimulates glycolytic enzymes and activates expression of the mechanistic target of rapamycin (mTOR), a key sensor of growth signals and nutrient levels [19]. Alterations in the PI3K signal transduction pathway are a frequent event in cancer [5]. mTOR can activate the expression of the hypoxia-inducible factor 1 (HIF1) transcription factor, which subsequently activates the expression of glucose transporters and glycolytic enzymes [5]. Because glucose plays an important role in tumor cell growth and proliferation, the factors and mechanisms regulating glucose metabolism play a central role in metabolic reprogramming in cancer cells.

The tumor suppressor p53 acts in response to a variety of cellular stresses to prevent malignant transformation by regulating the expression of genes involved in cell-cycle arrest, DNA repair, and apoptosis or senescence in a context-dependent manner [2]. p53 can abrogate malignant transformation by preventing metabolic reprogramming, an important step in the transformation of healthy into cancerous cells. p53 promotes oxidative

phosphorylation by activating the expression of key factors regulating the function of the electron transport chain such as synthesis of cytochrome *c* oxidase (SCO2) and apoptosis inducing-factor (AIF). SCO2 is critical for the assembly of cytochrome *c* oxidase and AIF is involved in maintaining the respiratory chain complex I activity [6]. In mice, p53 can directly block the metabolic switch from oxidative phosphorylation to glycolysis by down-regulating expression of pyruvate dehydrogenase kinase, isozyme 2 (Pdk2), an inhibitor of the conversion of pyruvate into acetyl-CoA [8]. p53 can directly inhibit glycolysis. by repressing the expression of glucose transporters GLUT1 and GLUT4, p53 reduces the cellular uptake of glucose [42]. Furthermore, by repressing expression of phosphoglycerate mutase (PGM) and by transactivating TIGAR, a fructose 2,6 bisphosphatase, p53 reduces glucose metabolism by glycolysis [6, 15, 42]. Thus, p53 tumor suppressive activity may be linked to the ability of p53 to restrain metabolic reprogramming that is essential to meet the cellular demand for energy and nutrients during malignant transformation and cancerous cell proliferation.

Recent findings in our lab point to another novel link between the energy status of the cell and p53. We previously found that TAF1, the largest subunit of TFIID, phosphorylates p53 at Thr55 (p53-Thr55) under normal growth conditions [23]. Phosphorylation of p53-Thr55 increases the p53/MDM2 interaction, nuclear export of p53, and p53 degradation [4, 23]. Furthermore, p53-Thr55 phosphorylation reduces the interaction of p53 with target DNA *in vitro* and at chromatin *in vivo* (unpublished, X.L. and Y.W.; [32]). Recent work in our lab has demonstrated that high glucose (HG) can increase the level of p53-Thr55 phosphorylation and thereby downregulates p53 activity in a TAF1-dependent manner (Figure 2.1). HG treatment causes an increase in intracellular ATP levels that peak around 6 to 8 hrs

post-treatment. The kinase activity of TAF1 is sensitive to this change in ATP concentration and increasing levels of ATP stimulate TAF1 kinase activity. This correlates with higher p53-Thr55 phosphorylation levels, eviction of p53 from the p21 (*CDKN1A*) promoter, and culminates in repression of p21 transcription. Blocking the HG-induced increase in ATP concentration with the glycolytic inhibitor 2-deoxy-glucose (2DG) prevents an increase in TAF1 kinase activity and p21 downregulation. The observed downregulation of p21 under HG conditions is p53-dependent since this effect is abolished in p53 knockout and p53 RNAi knockdown cells. Similarly, TAF1 RNAi knockdown abolished HG-induced p21 downregulation. Phosphorylation of p53-Thr55 by TAF1 is critical in mediating downregulation of p21: preventing p53-Thr55 phosphorylation by incubating cells with the TAF1 kinase inhibitor apigenin or transfecting cells with p53 containing a single amino acid exchange mutation, threonine 55 into alanine (p53-T55A), inhibits HG-induced dissociation of p53 from the p21 promoter *in vivo* and downregulation of p21 (unpublished, X.L. and Y.W.). These findings suggest that glucose levels can regulate p53 activity. High blood glucose levels in patients with hyperglycemia and diabetes have been correlated with an increased risk in certain types of cancer. The ability of high glucose to modulate the activity of the tumor suppressor p53 may be one molecular mechanism by which high glucose levels increase the cancer risk for those with hyperglycemia or diabetes.

We hypothesize that other p53 target genes may be regulated by HG through TAF1-dependent phosphorylation of p53-Thr55. In order to discover such genes we sought to compare changes in transcription in HG- and mock-treated cells using genome-wide transcription profiling assays using DNA microarray. As a first step towards this goal I recapitulated the down-regulation of p21 by HG in a p53 and TAF1-dependent manner. Based

on the observed functional inactivation of p53 in response to HG, we hypothesized that HG will impair the p53 response to stress such as DNA damage. Indeed, my preliminary results suggest that HG can alter p53 levels in response to UV treatment. The implications of these findings on diabetes and cancer will be discussed.

2.2 Materials and methods

2.2.1 Cell Culture, Glucose Measurements and Treatment

Human osteosarcoma U2OS cells and HCT116 human colorectal carcinoma cells were maintained in McCoy's 5A (Mediatech, 10-050) supplemented with 10% fetal bovine serum (FBS), 100 I.U./ml penicillin and 100 μg/ml streptomycin at 37^{o}C and 5% CO_2. McCoy's 5A media contains 16.7 mM glucose. Cells seeded in McCoy's 5A were treated with glucose 36-48 hrs after seeding. Using a diabetic glucose meter system to monitor glucose levels in the cell culture media we found that 36-48 hrs post seeding the glucose levels in the McCoy's 5A media routinely drop to 7-9 mM. Throughout our experiments I treated cells with 20 or 25 mM glucose in addition to basal media glucose concentrations. Starting and final treatment glucose concentrations for U2OS are given in Table 2.1. Early experiments were performed at 25 mM glucose, but later experiments were carried out at 20 mM glucose in order to mimic blood glucose levels observed in diabetics and maintain strong downregulation of p21 [9, 17, 35]. For Figure 2.2B U2OS cells were maintained in DMEM containing 5.5 mM glucose and supplemented with 10% fetal bovine serum (FBS), 100 I.U./ml penicillin and 100 μg/ml streptomycin for three passages before high glucose

treatment. The glucose concentration in the media for HCT116 cells maintained in McCoy's 5A media was 5-6 mM at the time of treatment 36-48 hrs after seeding. Following treatment of HCT116 cells with 20 mM glucose 36-48 hrs post seeding resulted in a final media glucose concentration of 25-26 mM (450-468 mg/dL). Cell culture glucose concentrations were estimated using the Ascensia Contour Blood Glucose Meter and test strips from Bayer. This meter measures glucose concentrations from 10-600 mg/dL (0.6 - 33.3 mM). In preliminary experiments, mannitol was used as a control for HG-treated cells to account for any change in osmolarity. No change in p21 levels was observed between mannitol and non-HG treated cells and in all subsequent experiments non-HG treatment cells were used as control. UV-damage experiments were performed using a Spectrolinker XL-1000 with 20 J/M^2 at 254 nm (UVC) as previously described [21]. Prior to UV treatment media was removed and was subsequently returned following UV exposure.

2.2.2 Western Blot

Vinculin, TAF1, p53 and p21 protein levels were detected by western blot as described [21]. Briefly, whole-cell lysate was prepared using lysis buffer composed of 20 mM Tris-Cl (pH 7.9), 150 mM NaCl, 0.5% NP-40, 20% glycerol, 2mM EDTA, 0.5mM DTT, 2 mg/ml aprotinin, 2 mg/ml leupeptin, and 0.5mM PMSF. Whole-cell lysate was subject to SDS-PAGE (8% for TAF1, 10% for p53, 12% for p21) and transferred to nitrocellulose. Membranes were blocked in TBST (20mMTris-HCl, pH 7.6, 136mM NaCl, and 0.1% Tween-20) containing 5% fat-free milk. Membranes were incubated with primary antibody [anti-vinculin (VIN-11-5, Sigma), anti-TAF1 (6B3, Santa Cruz), anti-p53 (DO-1, Santa Cruz), or anti-p21 (C-19, Santa Cruz)] overnight at 4^oC. Following primary antibody incubation membranes

were washed in TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1.5 hours at room temperature. Antigens were detected by chemiluminescence using SuperSignal West Pico Substrate (Thermo Scientific Pierce). Western blot signals were quantified using the Kodak Molecular Imaging software.

2.2.3 Reverse-Transcription PCR

Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions and treated using the Turbo DNA-free Kit (Ambion) according to the manufacturer's instructions to remove genomic DNA contamination. Reverse-transcription PCR was performed using the SuperScript One-Step RT-PCR with Platinum Taq kit (Invitrogen). RT-PCR conditions were as follows: 30 minutes at 50^{o}C, 2 minutes at 95^{o}C, followed by 32-36 cycles of: 30 s at 90^{o}C, 30 s at 60^{o}C, 30 s at 72^{o}C followed by a final 10-min extension at 72^{o}C. GAPDH transcript levels were used to normalize input. Primers used are as described in Appendix A, Table A.1.

2.2.4 ATP Measurements

ATP levels were measured in whole-cell lysates, which were prepared as described in the Western Blot section, using the ATP Determination Kit (Invitrogen) according to the manufacturer's protocol. ATP measurements were normalized to total protein levels as measured using the BCA Protein Assay Kit (Thermo Scientific Pierce).

2.2.5 siRNA Transfection

For RNA interference (RNAi) mediated knockdown experiments cells were transfected with 21 nt siRNA duplexes against p53 (Ambion, Cat. 16202) or TAF1 (5'-AGACCCAAACAACCCCGCAT-3' and 5'-AACTACGACTACGCTCCACCA-3') respectively, or with reagent alone (control). Cells were seeded in 60 mm dishes and subsequently transfected at 70% confluency with 175 pmoles or 200 pmoles of p53 or TAF1 siRNA duplexes, respectively, using Lipofectamine 2000 (Invitrogen). For knockdown of p53, cells were treated with siRNA targeting p53 (p53 RNAi knockdown cells) for 24 hrs before HG treatment. For knockdown of TAF1 cells were transfected with siRNA 42 hrs prior to HG treatment. Cells treated with TAF1 siRNA (TAF1 RNAi knockdown cells) were serum starved 6 hrs before HG treatment to increase glucose uptake.

2.3 Results

2.3.1 High Glucose Downregulates Expression of the p53 Target Gene p21

To test whether the observed downregulation of p53 and p21 occurs at glucose concentrations mimicking the blood-glucose concentrations observed in diabetics, a dose course experiment was performed. U2OS cells were maintained in McCoy's 5A media containing 16.7 mM glucose and the glucose concentration in the media was regularly between 7 to 9 mM at the time of treatment (48 hrs post seeding). Cells were treated with glucose concentrations from 5 to 30 mM with no glucose treatment as the control (Figure 2.2A). The

calculated and extrapolated final media glucose concentrations (in mM and mg/dL) upon treatment are given in Table 2.1. Cells were harvested 6 hr after treatment as preliminary experiments suggested that maximal p21 and p53 downregulation occurred at this time-point. p53 and p21 were found to be downregulated under HG conditions Treatment with 20, 25 and 30 mM glucose all induced a 40% drop in p53 levels. 25 and 30 mM glucose induced a 90% downregulation of p21 transcription. There are two ranges of p21 levels observed in response to HG treatment; at 5, 10 and 15 mM glucose p21 levels are reduced 60% as compared to the control (0 hr), whereas at 25 and 30 mM glucose p21 levels are 90% reduced as compared to control cells. In early experiments cells were treated with 25 mM glucose, however, I later chose to use 20 mM as it represents a concentration resembling the glucose level in diabetics. In summary, the results support the notion that that HG can downregulate p53 and p21 over a range of concentrations observed in diabetics.

Next, I tested the effects of HG on cells maintained in media containing lower glucose levels. U2OS cells maintained in DMEM containing 5.5 mM glucose, a concentration resembling the normal fasting plasma glucose levels in humans (100 mg/dL), were treated with 25 mM HG and harvested at 1, 2, 4, 6 or 8 hrs (Figure 2.2B). Treatment of these cells with 25 mM HG resulted in a final glucose concentration in the media of about 30 mM. Both p53 and p21 protein levels were initially increased at 1 hr post treatment and were subsequently downregulated. p53 protein levels were downregulated as much as 80% after 6 hrs and began to recover after 8 hrs. p21 protein levels dropped by 50% after 2 hrs, recovered slightly to 60% after 4 and 6 hrs, and recovered to 80% of the control level after 8 hrs. Under these conditions the p21 level did not drop as much as observed in cells

maintained in McCoy's 5A media containing 16.7 mM glucose (Figure 2.2B). Therefore, I decided to perform future experiments using cells maintained in McCoy's 5A media.

In order to confirm that p21 downregulation occurs at the level of transcription and pinpoint the timepoint of optimal p21 downregulation, p21 mRNA levels were measured by RT-PCR following HG treatment. The identification of the timepoint of optimal p21 downregulation after HG is important in order to generate the large amount of cells (RNA) needed for genome-wide transcription profiling assays. For my experiments, I used human colon carcinoma HCT116$^{p53+/+}$ cells. I choose these cells, as HCT116$^{p53-/-}$ knockout cells are available. Comparison of genome-wide transcription profiles between HCT116$^{p53+/+}$ and HCT116$^{p53-/-}$ cells can be used in the future to assess the specific impact of HG on p53 regulation of its target genes.

HCT116$^{p53+/+}$ cells were maintained in McCoy's 5A media. Cells were treated with 20 mM glucose resulting in a final media glucose concentration of 25-26 mM (450-468 mg/dL) at treatment (see Materials and Methods). RNA pools were generated from cells harvested at 6, 8, 10, 12 and 16 hrs post treatment. HG downregulated p21 in a p53-dependent manner, as seen in Figure 2.2C. p21 mRNA was almost undetectable in cells 6 and 8 hours after HG treatment. At later timepoints p21 mRNA levels recovered and actually increased above the control level. Taken together these results that p21 is downregulated at the mRNA level as previously observed in our laboratory. Furthermore, these results confirmed the choice to use 6 hrs of treatment with 20 mM HG as the condition for preparing RNA samples for microarray analysis.

2.3.2 Downregulation of p21 by High Glucose is p53-dependent

Next, to confirm that HG-induced downregulation of p21 is p53-dependent, I compared HG-mediated downregulation of p21 in HCT116$^{p53+/+}$ and HCT116$^{p53-/-}$ cells, which lack p53. I compared the levels of p53 and p21 protein in mock and HG-treated cells, which were generated as described (Materials and Methods) (Figure 2.3A). Compared to mock, p21 was downregulated 50% and 80% after 6 and 8 hrs, respectively, in HG-treated HCT116$^{p53+/+}$ cells. In addition, in p53 protein levels initially rose 10% after 6 hrs HCT116$^{p53+/+}$ cells, before dropping to 80% of levels observed in mock cells after 10 hrs, indicating that downregulation of p21 coincides with a significant reduction of p53 level. In contrast, p21 expression was not affected in HG-treated HCT116$^{p53-/-}$ cells, suggesting that downregulation of p21 in response to HG is p53-dependent. Our lab previously showed that downregulation of p53-induced p21 expression is due to a HG-induced increase in ATP concentration, which subsequently results in increased TAF1 kinase activity. Thus, to ensure that the differential regulation of p21 in HCT116$^{p53+/+}$ and HCT116$^{p53-/-}$ cells was not due to differences in ATP levels following HG treatment, I measured the ATP levels in HG-treated HCT116$^{p53+/+}$ and HCT116$^{p53-/-}$ cells (Figure 2.3B). As expected, both cell types showed a comparable increase in ATP concentration in response to HG. The ATP concentration peaked 6 hrs after HG treatment, and coincides with the timepoint at which I and others consistently observed the maximal reduction of p21 levels in preliminary experiments. My results support previous findings in our laboratory showing that HG-induced p21 downregulation is p53-dependent, and correlates with increased ATP levels. Furthermore, these results demonstrate that the p53 isogenic cell line HCT116 is appropriate for

future use in genome-wide transcription analysis by DNA microarray to find other p53-dependent, HG-regulated genes.

To further substantiate the role of p53 in HG-induced p21 downregulation, I investigated whether RNAi-mediated destruction of p53 affects p21 transcription in response to HG treatment. U2OS were transfected with or without siRNA against p53 and treated with HG for six hours. RNAi-mediated knockdown of p53 resulted in lower basal levels of p21 protein consistent with its role as a p53 target gene. While control cells show a 40% drop in p21 protein levels following HG treatment, p53 knockdown cells showed a slight increase (Figure 2.3C). My findings confirm the results of prior p53 RNAi-mediated knockdown experiments performed in our lab with scrambled siRNA control (unpublished, X.L. and Y.W.). Taken together these results confirm that HG-induced downregulation of p21 is p53-dependent in U2OS and HCT116 cells.

2.3.3 Downregulation of p21 by High Glucose is TAF1-dependent

In order to confirm that HG-induced downregulation of p21 is TAF1-dependent I tested the effects of HG in TAF1 RNAi knockdown cells. U2OS cells were transfected with or without siRNA against TAF1 and treated with 20 mM HG for 6 hrs. Consistent with published results TAF1 knockdown did not reduce p21 transcription [23]. However, TAF1 knockdown abolished the observed HG-dependent p21 downregulation, suggesting that TAF1 plays a central role in p21 downregulation in response to HG (Figure 2.4). This result confirms that HG-induced downregulation of p21 requires TAF1 as found previously in our lab in TAF1 RNAi-mediated knockdown and scrambled siRNA control experiments (unpublished, X.L. and Y.W.).

2.3.4 High Glucose Alters p53 Protein Levels in Response to UV

DNA damage stabilizes p53 protein levels and this stabilization is critical for the transactivation of p53 target genes. Given that high glucose can downregulate p53 protein levels under basal conditions we reasoned that HG may also impact p53 stability in response to DNA damage. To test this I compared p53 protein levels in UV irradiated cells with or without pre-treatment with 25 mM HG (Figure 2.5A). Cells were pre-treated with HG either 2 or 6 hrs before UV treatment and harvested 2, 4, 6, or 8 hrs post UV treatment. Cells pre-treated with HG 2 hr prior to UV showed an increase in p53 of 2.3 fold at 2 hrs after UV treatment, whereas untreated cells showed p53 levels 1.9 fold above undamaged cells (lanes 2 and 3). However, in cells pre-treated for 6 hrs with HG, the p53 protein levels were slightly reduced 4 hrs post UV treatment (lanes 2 and 4). At 4 hrs post UV treatment, cells pre-treated 2 hrs with HG showed a 4.7 fold increase in p53 levels compared to 7.3 fold in non-HG treated cells (lanes 5 and 6). At 6 hrs post UV, cells pre-treated 2 hrs with HG showed a 7.3 fold increase in p53 levels compared to 8.3 fold in non-HG treated cells (lanes 7 and 8). At 8 hrs post UV HG pre-treated cells showed a higher increase in p53 over non-HG treated cells, 9.6 fold compared to 8.6 (Figure 2.5A, lanes 9 and 10). This increase at later timepoints may reflect stabilization of p53 in response to both DNA damage and HG-induced ROS. Furthermore, pre-treatment with HG 6 hrs prior to UV treatment resulted in a lower p53 accumulation 2 hrs post UV: p53 levels were reduced 1.7 fold compared to 1.9 fold (lanes 2 and 4). Figure 2.5B shows the average fold changes in p53 protein levels for untreated or HG pre-treated cells at 4 and 6 hrs after UV damage from four biological replicates. Consistently, a decrease in p53 protein levels was observed

at 4 hrs post-UV in HG-pre-treated samples. This decrease is not statistically significant (p-value=0.274), however, this decrease was notable in three out of four replicate experiments. These preliminary results suggest that HG can alter p53 protein levels in response to UV damage.

2.4 Discussion

Recent work in our lab demonstrated that high glucose downregulates p21 through TAF1-mediated phosphorylation of p53-Thr55. My results confirm that that HG reduces p53 protein levels and the reduces the expression of the p53 target gene p21 at the level of mRNA and protein as previously observed in our laboratory (Figure 2.2). The reduction of p21 protein levels is likely a direct result of repressed p21 transcription revealed by reduced p21 mRNA levels, as the p21 protein half-life is only 10 minutes in U2OS cells [36]. Furthermore, my results confirm that p21 downregulation is p53 and TAF1-dependent. HG-induced p21 downregulation is abolished in cells lacking p53 through knockout or RNAi (Figure 2.3). Knockdown of TAF1 abolishes HG-mediated p21 downregulation confirming that TAF1 is involved in the HG-induced transcriptional repression of p21 (Figure 2.4).

2.4.1 Influence of ATP Related Factors on p53 Activity

In addition to TAF1, several other factors, which are sensitive to cellular energy levels, may play important roles in regulating the stability of p53 and its DNA binding activity under high glucose conditions. Elevated glucose levels lead to direct modification of p53 by the covalent addition of O-linked N-acetylglucosamine (O-GlcNAc) at Ser149. This mod-

ification decreases Thr155 phosphorylation, thereby stabilizing p53 by inhibiting COP9-mediated ubquitination [38, 43]. In contrast, glycosylation of p53 has also been reported to inhibit its activity [13]. NAD+ can control the sequence-specific DNA binding activity of p53 *in vitro* and *in vivo* in a gene-specific fashion. *In vitro*, NAD+ inhibits binding of p53 to its corresponding DNA binding sites in the *MDM2* promoter but does not affect binding of p53 to the p21 promoter. Treatment of cells with niacinamide increases NAD+ levels and alleviates p53 binding at the *MDM2* and *PIG3* promoters but does not affect binding of p53 to the promoters of p21, *GADD45A*, *PUMA*, or *BAX* genes following IR damage [26].

In vitro, the addition of ATP can directly destabilize p53/DNA interactions leading to the release of p53 from the DNA, whereas ADP stabilizes p53/DNA complexes [30]. The redox state of the cell can directly influence p53 function via oxidation of cysteine residues within the DNA binding domain of p53. A number of enzymes can reverse oxidative modification of these cysteine residues and restore the binding of p53 to DNA [25]. It is notable that in most of these studies p53 binding at the p21 promoter was the least influenced as compared to other genes.

Whether or not these other ATP-related factors contribute to the decrease in p53-mediated transcriptional activation of p21 transcription in response to HG is unknown. However, p53-Thr55 phosphorylation appears to be the major contributor to HG-induced, p53- dependent downregulation of p21. TAF1 knockdown is sufficient to abolish this effect (Figure 2.4) and most importantly, inhibition of Thr55 phosphorylation with apigenin or overexpression of p53-T55A prevents HG-induced downregulation of p53 protein levels, loss of DNA binding and p21 downregulation (unpublished, X.L. and Y.W.). Thus, we have

demonstrated a central role for Thr55 phosphorylation by TAF1 in the regulation of p53 transcriptional activity towards p21.

2.4.2 HG-mediated Abrogation of p53 Function - Beyond p21

On the basis of the results obtained for p21, we speculated that HG-mediated, TAF1-dependent p53-Thr55 phosphorylation can terminate the transcription of additional genes. Several lines of evidence support this hypothesis: (1) p53-Thr55 phosphorylation negatively regulates overall p53 protein levels by promoting nuclear export and degradation [4, 23]; (2) HG causes a global decrease in the levels of chromatin-bound p53 (unpublished, X.L. and Y.W.); (3) Cells with elevated glucose uptake showed attenuated PUMA expression [46]. The precise level of p53 protein is critically important for the ability of p53 to regulate target gene expression. A reduction of overall cellular level of p53 might be reasonably expected to impact the ability of p53 to properly regulate its target genes [45]. It is important to note that p53 is capable of both target gene activation and repression. Thus, a decrease in global levels of p53 by HG might be expected to relieve p53-mediated repression in addition to impairing activation of p53 target gene expression. Future work will be necessary to determine if this is the case.

While TAF1 has been correlated with transcriptional activation, TAF1 apparently represses basal p21 transcription since TAF1 knockdown or disruption of TAF1 activity in ts13 hamster cells increases p21 levels basal or repressed (Figure 2.4, Li et al., 2004 Fig. 4D, Rushton et al., 1997). Indeed, TAF1 was the only factor identified that is selectively recruited to the p21 promoter following UV damage, but not doxorubicin or nutlin-3 treat-

ment, and has been hypothesized to function as a transcriptional repressor under these conditions [12].

The interaction between TAF1 and p53 is dynamic. Under cell growth conditions the association between free p53 and TAF1 in solution is readily detectable [23]. Following DNA damage, free p53 and TAF1 dissociate and the specific interaction between TAF1 and chromatin bound p53 is dramatically enhanced. This recruitment is mediated through the tandem TAF1 bromodomains which bind acetylated lysine residues within the p53 COOH-terminus [21]. This interaction anchors these two proteins on chromatin and increases the probability of p53-Thr55 phosphorylation. Whether or not other genes besides p21 are regulated by the p53/TAF1 interaction remains to be seen. These may represent a subset of genes regulated by TAF1-mediated Thr55 phosphorylation similar to p21.

To fully understand the effect of HG treatment on p53-dependent gene expression and the functional importance of TAF1-mediated p53-Thr55 phosphorylation in p53-dependent gene expression, we decided to perform genome-wide transcription profiling experiments. Towards this aim I have recapitulated HG-induced p21 downregulation and confirmed that treatment with 20 mM glucose for 6-8 hours is optimal for measurement of p21 downregulation at both the protein and mRNA level. The success of genome-wide transcription profiling assays depends largely on the generation of RNA pools from cells, which show the strongest response to an experimental parameter. The conditions described here should provide optimal experimental parameters to generate RNA pools necessary for the HG-induced, p53-dependent changes in gene expression.

Our laboratory recently found that TAF1-mediated p53-Thr55 phosphorylation terminates p21 transcription after UV damage (unpublished, X.L. and Y.W.). Whether TAF1

phosphorylates other transcription factors to repress the transcription remains unknown. The proposed genome-wide transcription profiling experiment would allow us to identify p53-independent changes in gene expression and provide insight into the role of TAF1 kinase activity in gene regulation.

My results, although preliminary, suggest that that HG can alter the protein levels of p53 in response to DNA damage. In particular, cells pre-treated with HG 2 hrs before UV treatment show notably lower p53 protein levels at 4 hrs post UV. My preliminary results also indicate that p21 transcription is downregulated under these conditions and this has since been confirmed in the laboratory (data not shown). Taken together, these findings suggest that HG may attenuate the p53-mediated response to DNA damage. It is interesting to note that despite my preliminary finding suggesting that HG can alter p53 protein levels in response to UV damage, diabetics are not at increased risk for skin cancer. Whether HG can alter p53 protein levels in response to other DNA damage conditions, such as IR, or non-genotoxic stress remains unknown. The dynamics of p53 protein levels may vary based on the type and duration of stress and contribute to differential, p53-dependent cellular response to various stresses [1, 34]. Given that p53 regulates a large number of genes in a temporal fashion, it seems reasonable that HG-mediated modulation of p53 protein levels may delay or even alter the cellular response to various stress factors [28, 45]. Furthermore, p53-Thr55 dephosphorylation following DNA damage is important to the p53-mediated response in UV-treated U2OS cells [22, 23]. Knockdown of PP2A B56γ isoforms responsible for p53-Thr55 dephosphorylation results in a dramatic drop in the number of cells undergoing apoptosis [22]. Based on this preliminary observation, our laboratory has set out to examine HG-induced changes in p53-dependent expression in

response to UV damage. These findings should shed light on the role of TAF1 and p53-Thr55 phosphorylation in p53-mediated transcription.

2.4.3 Implications of HG-mediated abrogation of p53 function in diabetes and cancer

Epidemiological studies have found that diabetes is associated with an increased risk for several types of cancer. Diabetes increases the risk for liver, pancreatic, kidney and endometrial cancers nearly 2-fold, and for colorectal, bladder, and breast cancer nearly 1.5-fold. In addition, diabetes significantly increases mortality of cancer patients [14, 37]. Several known factors have been linked to this increased risk, including hyperinsulinemia, leptin and adiponectin. The best characterized of these factors, hyperinsulinemia, likely contributes directly to cellular proliferation through mitogenic effects of insulin signaling [7]. However, recent studies suggest that hyperinsulinemia does not account for all the different types of cancer associated with diabetes [7, 41]. Thus, other molecular mechanisms remain to be elucidated.

Hyperglycemia has also been implicated as a risk factor for cancer and increased cancer mortality in diabetics [10, 29, 31, 37]. One recent study found that individuals with glucose levels elevated above normal but below diabetic levels are at a moderate increased risk for endometrial and postmenopausal breast cancers [18]. Hyperglycemia has been linked to polyp proliferation and may promote progression to more aggressive adenomas; although the underlying mechanism remains unclear [39]. Confounding variables in epidemiological studies have made it difficult to definitively demonstrate that hyperglycemia alone is a

causal risk factor for cancer. Studies suggest hyperglycemia may contribute to cancer risk by increasing DNA damage and impairing DNA repair. High glucose levels can increase reactive oxygen species (ROS)-induced oxidative damage of DNA resulting in mutagenesis [44]. Diabetics have higher levels of oxidative DNA damage, show increased susceptibility to DNA-damaging agents, and have reduced DNA repair efficacy [3].

Our preliminary experiments showing that p53 and p21 were downregulated in response to HG were performed by treatment of cells with 25 mM glucose, resulting in a final media glucose concentration of approximately 32-34 mM. My results show that p53 and p21 are downregulated, although to a lesser extent as compared to 25mM glucose, at glucose concentrations typically observed by diabetics (11-20.5 mM, 198-369 mg/dL) [9, 33, 35]. Importantly, treatment with 20 mM glucose (resulting in a final glucose concentration in the media of 27-29 mM upon treatment) downregulates p53 and p21, and resembles the glucose levels described for diabetics with hyperosmolar hyperglycemic state (>600 mg/dL, 33.3 mM) [17]. This result further supports our choice to use 20 mM glucose for treated samples to be used in gene expression analysis. The HG-induced downregulation of p53 activity, particularly in response to DNA damage, represents one mechanism, which may contribute to increased cancer risk in diabetics and hyperglycemics (Figure 2.1). Such findings imply that diabetic management of blood glucose levels is important in cancer prevention. This notion is supported by the finding that metformin, an antidiabetic drug that lowers blood glucose levels, can reduce cancer risk [20, 24].

In summary, the results of my work support the earlier finding in the laboratory that high glucose can reduce p53-dependent transcription. Furthermore, my preliminary results suggest that HG may reduce the level of p53 protein in response to UV. Collectively, our

findings suggest that HG-induced abrogation of the level and activity of the tumor suppressor p53 may be one mechanism responsible for the increased risk for cancer in diabetics.

2.5 Figures and Tables

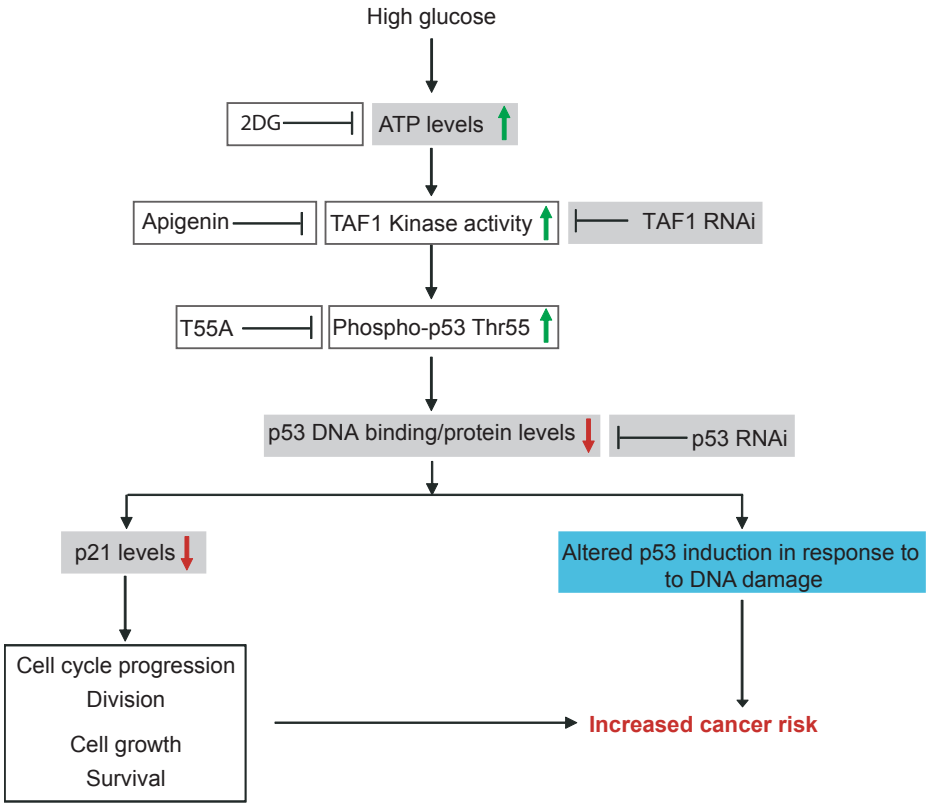

Figure 2.1. A Working Model for p21 Downregulation by HG
Culture in high glucose leads to increased intracellular ATP levels. TAF1 kinase activity, sensitive to ATP concentration, is boosted upon HG-induced increase in ATP levels. Increased TAF1 kinase activity results in higher levels of p53 Thr55 phosphorylation, lower p53 p53 DNA binding and reduced p53 protein levels. Subsequently, p53-dependent p21 expression is downregulated at the transcriptional level. Downregulation of p21 may contribute to cell-cycle progression and increased cell proliferation. This may be one mechanism by which hyperglycemia contributes to cancer risk. HG-induced p21 downregulation is ATP dependent as co-treatment with glycolytic inhibitor 2-deoxy-glucose (2DG) blocks the increase in ATP levels and subsequent increased TAF1 kinase activity, Thr55 phosphorylation, drop in p53 activity and p21 downregulation. Similarly, p21 downregulation is dependent on TAF1 kinase activity; TAF1 knockdown or inhibition of TAF1 kinase activity with the flavonoid apigenin prevents Thr55 phosphorylation, loss of p53 activity and p21 downregulation. Furthermore, HG-induced p21 downregulation is p53 dependent as p21 downregulation is abolished in cells lacking and p53 knockdown cells. Inhibiting p53 Thr55 phosphorylation via apigenin or over expression of the p53-T55A mutant prevents HG-induced p21 downregulation emphasizing the role of Thr55 phosphorylation in mediating the effects of HG. In addition, HG levels alter the levels of p53 induction in response to UV damage. This may alter the p53 response and further contribute to cancer risk. Grey boxes indicate experiments confirmed in this study in support of this model. Open boxes indicate work by others in our lab our laboratory. Blue box indicates new preliminary findings reported in this study.

Table 2.1. Glucose Treatment Concentrations
Glucose concentrations are given for the media at time of treatment, the concentrations used for treatment and the total media glucose concentration following treatment of U2OS cells. Note that, in humans, the criteria for diagnosis of diabetes is a fasting plasma glucose concentration ≥7 mM (126 mg/dL) [33].

Media Glucose Concentration at Time of Treatment	**Glucose Concentration Added at Treatment**	**Total Final Media Glucose Concentration Range at Treatment**	
mM	**mM**	**mM**	**mg/dL**
7-9	0	7-9	126-162
7-9	5	12-14	216-252
7-9	10	17-19	306-342
7-9	15	22-24	396-432
7-9	20	27-29	486-523
7-9	25	32-34	577-613
7-9	30	37-39	667-703

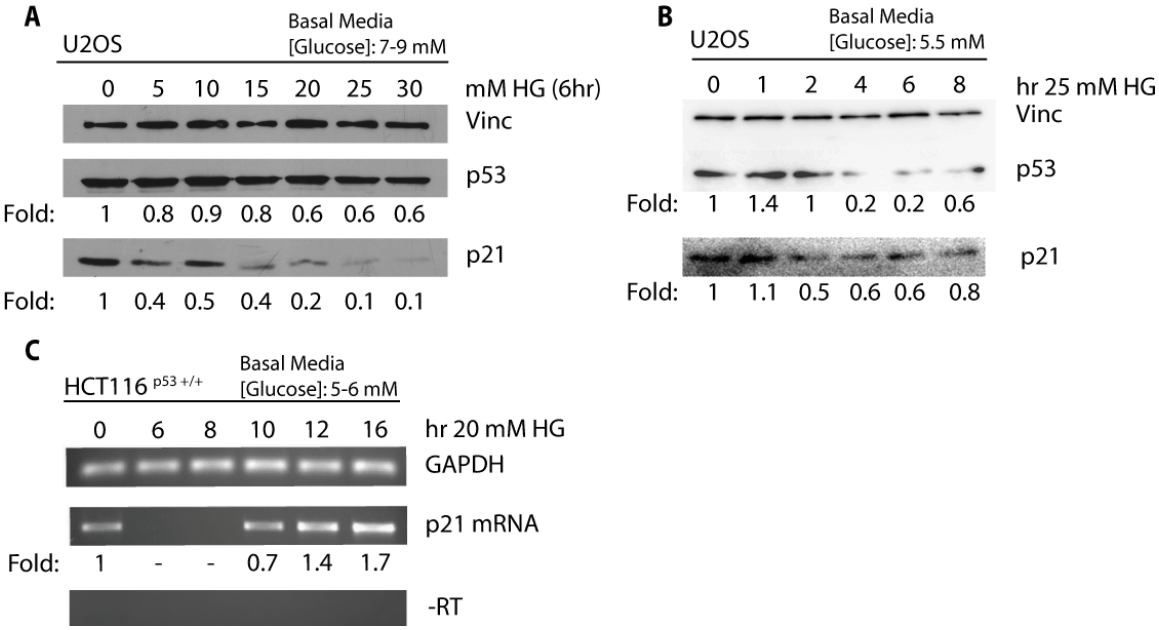

Figure 2.2. HG Downregulates p21 Expression
A. High glucose downregulates p21 and p53 protein levels in a dose dependent manner. A single experiment was performed in which U2OS were treated with increasing concentrations of glucose as indicated for 6 hrs. The basal media glucose concentration was between 7-9 mM at the time of treatment. The final glucose concentration in mM and mg/dL following treatment are given in Table 2.1. **B.** High glucose downregulates p21 and p53 protein levels in cells grown at a physiological glucose concentration. A single experiment was performed in which U2OS cells grown in media containing 5.5 mM (100 mg/dL) glucose were treated with 25 mM glucose for the indicated times. Treatment with 25 mM glucose resulted in a final media glucose concentration range of 25.5-26.5 mM (459-477 mg/dL). **C.** High glucose downregulates p21 at the RNA level. Shown are p21 mRNA levels in HCT116$^{p53+/+}$ cells treated with 20 mM glucose for the indicated times. p21 levels were normalized to GAPDH for fold comparisons. Basal media glucose concentration was between 5-6 mM (90-108 mg/dL) at the time of treatment resulting in final treatment glucose concentration of 25-26 mM (450-468 mg/dL). **A. and B.** Whole-cell lysate was immunoblotted for vinculin, p53 and p21 protein levels using the indicated antibodies.HG: High Glucose.

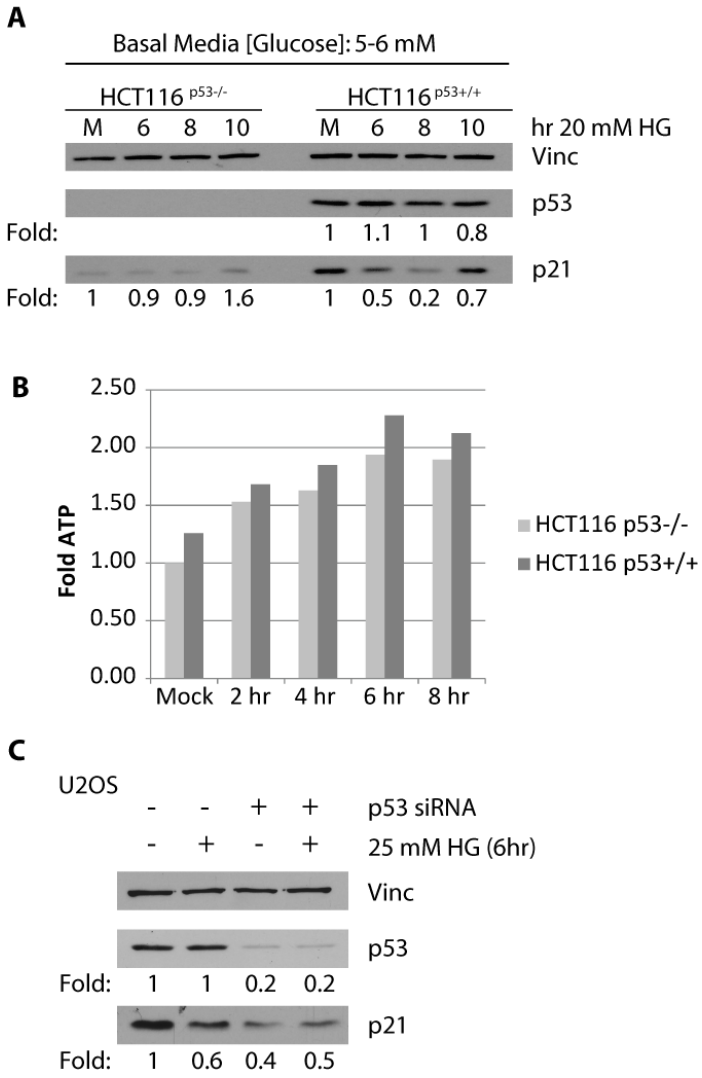

Figure 2.3. HG-mediated Downregulation of p21 is p53-dependent
A. HG downregulates p21 levels in HCT116$^{p53+/+}$ but not HCT116$^{p53-/-}$ cells. HCT116 cells with or without p53 were treated with 20 mM HG for the indicated times. The basal media glucose concentration at the time of treatment was between 5-6 mM (90-108 mg/dL) at the time of treatment resulting in final treatment glucose concentration of 25-26 mM (450-468 mg/dL). Shown are the results of a representative experiment. **B.** ATP levels in HCT116$^{p53+/+}$ and HCT116$^{p53-/-}$ are similar following HG treatment. Shown are relative ATP levels in HCT116 cells treated with HG for the indicated times from a single experiment. **C.** RNAi-mediated p53 knock-down abolishes HG downregulation of p21. A single experiment was performed in which U2OS cells were seeded in 60 mm dishes and transfected with or without siRNA directed against p53 for 24 hours. Subsequently, cells were treated with 25 mM glucose six hours before harvest. **A. and C.** Whole-cell lysate was immunoblotted for vinculin, p53 and p21 protein levels using the indicated antibodies. M: Mock; HG: High Glucose.

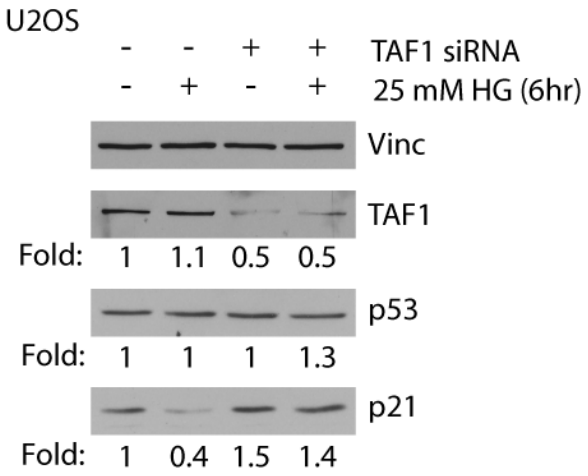

Figure 2.4. HG-mediated Downregulation of p21 is TAF1-dependent
A single experiment was performed in which U2OS cells seeded in 60 mm dishes were transfected at 70% confluence with or without siRNA directred against TAF1 for 42 hrs. Subsequently, cells were treated with 25 mM glucose 6 hrs before harvesting 48 hrs after transfection. Cells were serum starved 6 hrs prior to glucose treatment. Whole-cell lysates were immunoblotted for vinculin, TAF1, p53 and p21 levels. HG: High Glucose.

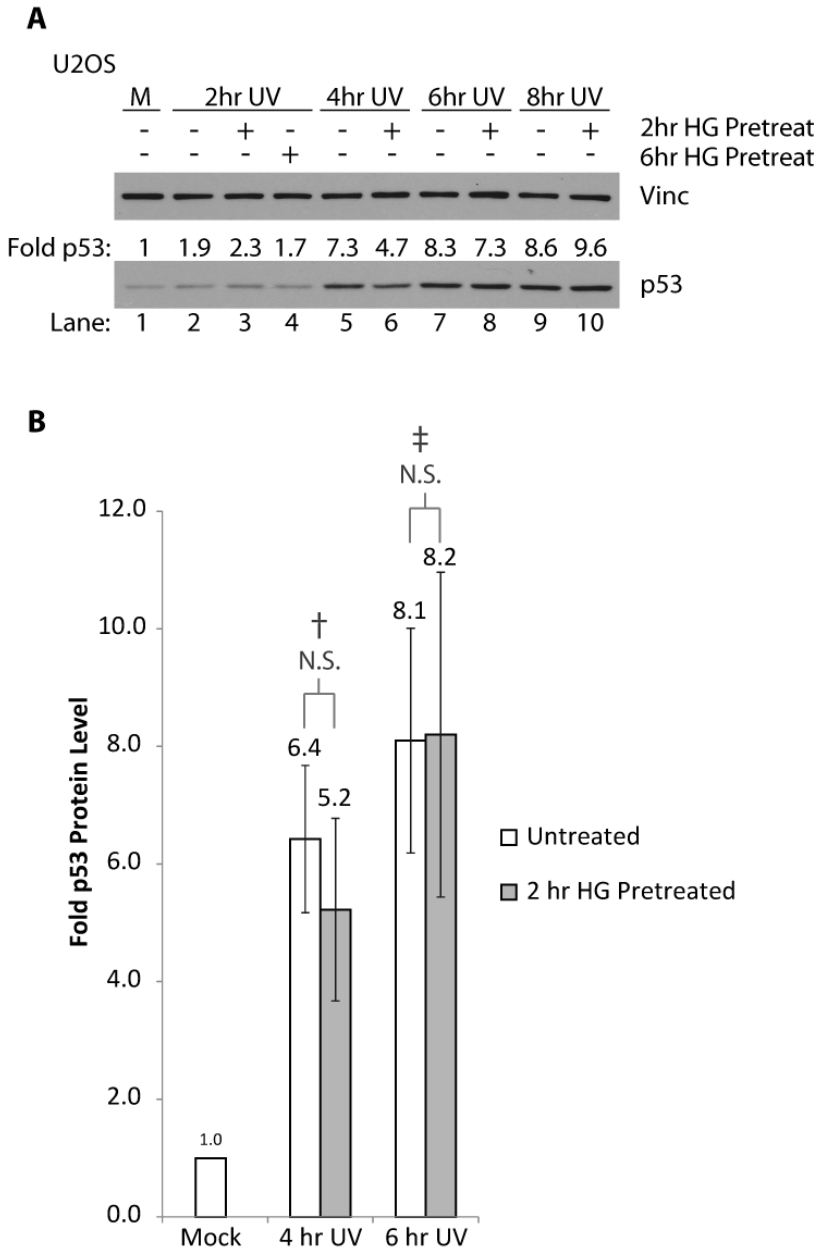

Figure 2.5. HG Alters p53 Stability in UV-treated Cells
A. U2OS cells were pre-treated with 25 mM glucose subsequently treated with UV for the indicated times. Whole-cell lysates were immunoblotted for vinculin and p53. **B.** p53 protein levels from four independent experiments as assayed by immunoblotting and quantified using the Kodak Molecular Imaging software. Error bars represent one standard deviation. †: student t-test, p-value = 0.274; ‡: student t-test, p-value = 0.954; N.S., not significant; HG: High Glucose.

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Chapter 3

Bioinformatic Identification of Putative High glucose-induced TAF1 Regulatory Targets

3.1 Introduction

Transcriptional activation requires the coordinated interaction between transcription factors and components of the general transcription machinery (GTM). TAF1 is the largest subunit of the general transcription factor TFIID and has kinase-, histone acetyltransferase activity (HAT)-, and E1/E2-activities [89]. TAF1 contains both a $NH_2$2-terminal and COOH-terminal kinase domains (referred to as NTK and CTK, respectively; see Figure 1.4). The two TAF1 kinase activities activate the transcription of distinct sets of TAF1 target genes. The NTK plays an important role in cell-cycle progression by regulating the expression of

the cell cycle regulators cyclin D, cyclin A, and cdc2. The NTK is involved in the transcription of 6% of all genes in hamster cells carrying the ts13 temperature sensitive mutation of TAF1 [60, 61].

Several substrates for the NTK and CTK have been identified. TAF1 phosphorylation of TFIIA promotes the formation of a TFIIA-TBP-TATA complex [74]. Autophosphorylation by TAF1 and transphosphorylation of TAF7 are involved in a pre-initiation complex (PIC) assembly check point at MHC class I promoters [26]. In addition, TAF1-mediated phosphorylation of TAF7 is necessary to relieve inhibition of TAF1 HAT and contributes to efficient transcription of cyclin A and D1 [45]. TAF1 phosphorylation of the RAP74 subunit of TFIIH contributes to transcriptional elongation [18, 89]. In *Drosophila*, the CTK has been shown to phosphorylate Ser33 of histone H2B to promote transcription of the cdc25 homolog *string* [53].

In contrast to the established role of TAF1 in transcriptional activation, recent evidence in our laboratory implicates TAF1 kinase activity in termination of p53-mediated transcriptional activation (Figure 3.1). TAF1 can phosphorylate the transcription factor p53, an important tumor suppressor, at threonine-55 (p53-Thr55) [25]. This modification reduces the interaction of p53 with DNA *in vitro* and *in vivo*, and increases the interaction of p53 with its E3 ubiquitin ligase MDM2, resulting in p53 nuclear export, degradation of p53 and G1 cell cycle progression [13, 47, 63]. Recently, our laboratory found that the intracellular ATP concentration regulates TAF1 kinase activity. An increase in ATP levels following ultraviolet (UV) damage stimulates TAF1 kinase activity resulting in increased p53-Thr55 phosphorylation (unpublished, X.L. and Y.W.). Increased p53-Thr55 phosphorylation results in the eviction of p53 from the p21 (*CDKN1A*) promoter and culminates in termi-

nation of p53-mediated p21 transcriptional activation (unpublished, X.L. and Y.W.). Our laboratory has shown that high glucose (HG) cell culture conditions elevate intracellular ATP levels, increase TAF1 kinase activity, p53-Thr55 phosphorylation, and downregulate p53-mediated transactivation of p21 (Chapter 2).

I asked whether HG-induced, TAF1-mediated p53-Thr55 phosphorylation is involved in the transcriptional termination of p53 target genes other than p21. In addition to activating target gene transcription, p53 can repress target genes by (i) directly recruiting corepressors, (ii) competitive binding with transcriptional activators to overlapping DNA target sites and (iii) squelching [8]. Because p53-Thr55 phosphorylation can result in eviction of p53 from target genes and even from the nucleus, it is possible that HG-induced, TAF1-mediated p53-Thr55 phosphorylation may interfere with the activating as well as the repressive activity of p53.

As described in Chapter 2, I planned to use genome-wide transcription profiling of HG-treated cells to identify genes whose transcription is regulated by HG, TAF1, and p53. Because I was unable to perform the transcription profiling assays in the time course of my dissertation, I used an alternative bioinformatics approach to identify genes whose expression might be controlled by HG in a TAF1-p53-dependent manner. Using published data, I defined three sets of genes: i) direct p53 targets, (ii) TAF1 promoter-bound genes (hereafter referred to as TAF1 target genes) and iii) genes regulated in response to HG. The comparison of these three data sets identified 41 genes, whose expression was dependent upon HG, TAF1, and p53. The comparison of p53-regulated genes in response to five different stress conditions revealed that each stress induces the transcriptional changes in a specific subset of p53 target genes and resulted in the identification of a common pool of p53 target genes,

whose expression is regulated by all five stress conditions. My results support the model that different stress conditions trigger a common as well as stress-specific p53-dependent transcriptional response. Whether TAF1 can terminate transcriptional activation by phosphorylating transcription factors other than p53 remains unknown. As a first step towards addressing this possibility, I used bioinformatics to identify p53-independent TAF1 target genes, whose transcription is repressed in response to HG. I identified putative targets of TAF1 and consequently TAF1 kinase activity by searching for transcription factor binding sites (TFBS) enriched within the putative cis-regulatory regions of these p53-independent TAF1 target genes downregulated in response to HG. Using this approach I identified 54 TFBS. The activity of the transcription factors interacting with these TFBS is potentially regulated by TAF1 phosphorylation.

3.2 Materials and Methods

A summary of the studies used to identify target genes of p53, TAF1 and HG is given in Table 3.1. These gene sets were to used identify i) HG regulated, TAF1-bound p53 target genes (Figure 3.2, grey region), and 2) p53-independent, HG-regulated and TAF1-bound genes (Figure 3.2, blue region) for further analysis.

3.2.1 Defining Direct Human p53 Target Genes

To define direct human p53 target genes I used several approaches (Figure 3.2). First, I collected validated p53 target genes from the literature [12, 66, 86]. Second, I collected direct p53 target genes previously identified by correlating global gene expression studies

with genome-wide chromatin-immunoprecipitation coupled to paired-end ditag sequencing (ChIP-PET) or next-generation sequencing (ChIP-seq) assays that detect p53 DNA binding in response to 5-fluorouracil (5-FU), Etoposide (Etopo), or Actinomycin D (ActD) [72, 90]. Third, I defined direct p53 target genes in response to UV and ionizing radiation (IR). To do this, I identified genes with p53-dependent expression in response to UV and IR [4, 39, 54, 100] and then determined which of these genes bound by p53 under other stress conditions in genome-wide p53-DNA binding studies (flow chart in Figure 3.3; summarized in Table 3.2). My bioinformatics approach is described in the following sections.

Validated p53 Target Genes - The p53 Target Literature List

I collected a list of 172 validated human p53 target genes from three different studies [12, 66, 86]. These studies compiled lists of genes previously validated as *bona fide* p53 target genes. These genes met several criteria to be considered direct p53 targets. Generally, they have an identifiable p53 DNA binding capable of directing p53-dependent expression and for which p53 binding is observed *in vivo* using chromatin immunoprecipitation or *in vitro* by gel-shift [66]. Riley et al. compiled their list in a review of known human p53 target genes and p53 mechanisms for regulating gene expression [66]. Wang et al. studied p53 DNA binding sites of human p53 target genes in order to classify sites as activating or repressing based on DNA sequence [86]. Botcheva collected known human p53 target genes and binding sites for comparison with their p53 ChIP-seq results [12]. The p53-dependent expression of the genes in my final list is regulated in response to a variety of stress types including but not limited to DNA damage. This list of genes, the p53 Target Literature List, is provided in Appendix B, Table B.1.

p53 Target Genes Regulated in Response to 5-FU

Using ChIP-PET technology Wei et al. (2006) mapped genome-wide p53 binding in 5-FU treated HCT116 human colon carcinoma cells [90]. Briefly, immunoprecipitation of chromatin cross-linked p53 allowed isolation of the DNA regions with which p53 associated. Subsequently, p53-bound and isolated DNA was used to generate a paired-end (PET) library, which was sequenced to determine 18 bp sequence of either end of isolated DNA fragments. These PETs were then mapped to the genome and ranked by the number of overlapping tags. PET clusters of two or more (PET2+) overlapping tags that cover a genomic sequence represent p53 ChIP enrichment but have significant noise as well. Overlapping clusters of three or more tags (PET3+ clusters) were determined to be significantly and specifically enriched in p53 signals. The authors identified 323 PET3+ clusters and used them as the basis for the development of a novel consensus p53 DNA binding site prediction model, p53PET. This new model was shown to be more sensitive than prior prediction models such as p53MH [34]. Using this new prediction model PET2+ clusters were scanned and 219 of these were predicted to contain p53 DNA binding sites. Based on the presence of a predicted p53 binding site within these PET2+ clusters and the statistically significant p53 enrichment in PET3+ clusters, Wei et al. considered these 542 PET clusters (323 PET3+ and 219 PET2+) to represent high-confidence p53-binding locations. The authors also compared gene expression in HCT116$^{p53+/+}$ and HCT116$^{p53-/-}$ similarly treated with 5-FU to identify genes whose transcription requires p53. Wei et al. then defined p53 target genes by mapping the 542 high-confidence PET clusters to the nearest genes within 100 kb which demonstrated p53-dependent gene expression in response to 5-FU. The 122

p53 target genes identified in this study, their up or downregulation by p53 in response to 5-FU, and the genomic coordinates of the 542 high-confidence PET clusters were collected from Supplemental Table 4 of the published study [44]. Gene symbols were updated by hand using NCBI Gene database (http://www.ncbi.nlm.nih.gov/gene).

p53 Target Genes Regulated in Response to Actinomycin D and Etoposide

Using ChIP-seq Smeenk et al. (2011) defined 1,320 genes bound by p53 in U2OS cells treated with ActD and 1,710 genes bound by p53 in U2OS cells treated with Etopo [72]. The target sites for p53 resided within 25 kb up- or downstream of a gene or within the body of a gene. Using genome-wide transcription profiling assays with DNA microarrays they assayed changes in gene expression following ActD or Etopo treatment for 24 hours. They found that of the 1,320 genes bound by p53 in response to ActD, 185 also showed greater than 1.7-fold change in expression. Of the 1,710 genes bound by p53 in response to Etopo, 177 showed greater than 1.7-fold change in expression. Since these genes are both p53 bound and show differential expression I consider them to be direct p53 targets. I gathered the gene symbols, expression data and associated p53 ChIP-seq peak genomic coordinates for these 362 differentially expressed and p53 bound target genes from Supplementary Tables 1 and 2 of the published work (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3048874/). Gene symbols were reported in association with Affymetrix Human Exon 1.0 ST Array transcript cluster IDs. Transcript cluster IDs with multiple gene symbols associated were filtered by hand for p53 binding proximity by mapping p53 ChIP-seq peaks in the UCSC Genome Browser [43]. Gene symbols were updated by hand using NCBI Gene database (http://www.ncbi.nlm.nih.gov/gene).

Identification of Direct p53 Target Genes Regulated in Response to IR and UV

Identification of Genes withp53-dependent Expression To my knowledge, no genome-wide p53-DNA binding studies in response to UV or IR have been published. I therefore used a bioinformatics approach to identify direct p53 target genes in response to UV and IR by correlating transcriptional changes of known p53 target genes in response to UV and IR with published p53-DNA interactions identified under other stress conditions. I first surveyed the literature for studies that examined p53-dependent gene expression in response to UV or IR. Three studies were chosen based on the analysis of p53-dependent expression and availability of reported data. The list of genes with p53-dependent expression in response to UV damage was collected from a study by McKay et al. in 2004 [54]. Genes demonstrating p53-dependent expression in response to IR were collected from two studies: Zschenker et al. in 2006 and Amundson et al. in 2008 [4, 100].

Using HT29-ts colon carcinoma cells harboring a temperature sensitive p53 allele McKay et al. (2004) characterized genes with p53-dependent expression in response to UV doses of 0, 10, and 30 J/M^2 [54]. RNA was harvested 6 hours after treatment. Gene expression was measured using Affymetrix U95A microarrays. I collected a total of 88 genes with p53-dependent expression in response to UV from Supplementary Tables 3 and 4 of the published study [54].

Zschenker et al. (2006) utilized lymphoblastoid cell lines to characterize gene expression in response to IR (2 Gy, 3 hr) [100]. Here, p53-depedent gene expression was determined by comparing gene expression in TK6 cells carrying wt-p53 and TK6E6 cells following IR treatment; TK6E6 cells are a stable cell line expressing the human papilloma

protein E6, which binds to p53 and leads to rapid degradation of p53 protein. The authors performed gene expression profiling using Affymetrix HG U133A microarrays. The authors found 11 p53 target genes, whose expression is upregulated in response to IR, and 9 target genes, whose expression is downregulated in response to IR.

Amundson et al. (2005) performed a large study of the gene expression changes across the 60 cell lines utilized in the National Cancer Institute Anticancer Drug Screen in response to IR (8 Gy, 4 hr) [3]. These cell lines represent a large variety of cancer cell types, which are well characterized. Specifically, they examined p53-status of each of the cell lines and their gene expression profile in response to IR using custom DNA microarrays. They found 25 genes, which showed a p53-dependent response to IR.

The genes from these studies represented p53-dependent, UV- and IR-responsive genes. I examined whether these genes represent direct p53 target genes by correlating stress-induced transcriptional response with differential binding of p53 to the identified genes as described in the following sections (Summarized in Figure 3.3).

Identifying p53-bound Target Genes At the time of my analysis three published studies described genome-wide p53-DNA interactions in response to stress in human cells [12, 72, 90]. The studies by Wei et al. (2006) and Smeenk et al. (2011) were described above. In 2011, Botcheva et al. published the ChIP-seq analysis of genome-wide p53 binding in IMR90 normal human fibroblast cells in response to 5-FU treatment and found 743 p53 DNA binding sites [12].

These three studies reported only those p53 DNA binding events associated with differential transcription of putative target genes. All three studies detected a large number of

p53 DNA binding events that were not correlated with differential target gene transcription. These binding events were not discussed in the publications, but may represent functional DNA binding sites in a different experimental context.

Thus, in order to obtain a comprehensive list of p53-bound genes under various stress conditions, I collected and annotated all high-confidence p53 binding sites from all three studies [12, 72, 90]. The genomic coordinates for all 542 high-confidence PET clusters were obtained from Supplementary Table 4 published in Wei et al. 2006 [90]. p53 ChIP-seq peaks identified by Smeenk et al. (2011) were downloaded from the NCBI GEO database (Smeenk et al., 2011, accession GSE21939) [72]. p53 ChIP-seq peaks identified by Botcheva et al. (2011) were collected from their Supplementary Table 2 [12]. In addition, I also included a the p53 Target Literature List of 172 genes with known p53 DNA binding sites compiled from the literature [12, 66, 86].

All three studies annotated p53 DNA binding sites (peaks) identified by ChIP-seq or ChIP-PET) by assigning them to nearby genes. However, there is no consistent rule for the distance used for peak-to-gene annotation and each group used different criteria. Wei et al. (2006) assigned the nearest RefSeq genes up- and downstream within 100 kb to their ChIP-PET clusters. Smeenk et al. (2011) chose to assign RefSeq genes within 25 kb of identified peaks. Botcheva et al. (2011) annotated RefSeq genes within 20 kb up- or downstream identified peak and genes where the identified peak lay within the gene body. For consistency and because enhancers are known to act over long ranges, up to 100 kb, I chose to annotate genes within 100 kb up- or downstream of a peak and as well as those for which the peak lay within the body of the gene [52]. While the selected parameters are extremely broad and certainly will result in the identification of a number of false positives,

it serves as a useful filter for assigning p53-dependent genes as potential direct p53 target genes.

The genomic coordinates of identified putative p53 DNA binding sites were uploaded to Galaxy and overlapping coordinates merged using the Operate on Genomic Intervals, Merge tool (http://usegalaxy.org/). These merged p53 DNA binding site coordinates (intervals) were used to generate a new BED file containing the coordinates encompassing 100 kb up- and downstream of the start and end point of each interval. RefSeq gene information in the form of a refFlat (March2006 NCBI Build 36/HG18) file was uploaded to Galaxy using the UCSC Table Browser data retrieval tool [41]. All genes that fall within 100 kb of a p53 binding site were identified using the Operate on Genomic Intervals, Join tool. Intervals were also annotated using CisGenome to assign all genes within 100 kb. CisGenome also provided the location of each binding site relative to the gene (TSS_upstream, TES_downstream, intron, exon, 5'UTR or 3'UTR). Because CisGenome uses the center of the interval for this mapping the resulting annotation contained most but not all of the genes annotated using Galaxy. For the genes annotated by Galaxy but not by CisGenome the location of the interval relative to the annotated gene was determined manually using the UCSC Genome Browser. Annotation of binding sites from each study individually gives the same combined results as merging all peaks together before annotation. A total of 7,955 genes bound by p53 were identified from the three studies (Table 3.3). To this list I added 69 known p53 target genes gathered in the p53 Target Literature List that were not already identified by my annotation. Thus, using genome-wide binding studies and literature compiled direct p53 targets I identified 8,024 p53-bound genes (Figure 3.3).

Additional IR Responsive, Direct p53 Target Genes An additional set of direct p53 target genes in response to IR was compiled from a study by Jen and colleagues in 2005 [39]. The authors performed ChIP-chip to examine p53 binding to the promoters of 489 previously defined IR responsive genes. The authors defined promoters as 2 kb upstream and 1 kb downstream of the TSS. Cells were treated with 10 Gy IR for 4 or 24 hrs before ChIP-chip. They found that p53 bound to the promoter of 38 IR responsive genes. My bioinformatics approach confirmed that p53 directly binds 11 of the 38 genes. In contrast, 27 of the 38 genes were not identified as p53-bound in my bioinformatics approach. Of these 27, 14 are known p53 targets. I therefore decided to include all 38 genes in my list of IR responsive p53 target genes.

3.2.2 Annotation of TAF1 Promoter-bound Genes

I used bioinformatics to identify TAF1 target genes. I defined TAF1 target genes as those containing TAF1 bound to DNA +/- 2.5 kb of the transcriptional start site (TSS). TAF1 DNA binding coordinates were available from a genome-wide ChIP-on-chip study [44]. In order to characterize and map active promoters in IMR90 cells Kim et al. (2005) performed ChIP-on-chip assays to detect the genome-wide distribution of TAF1, RNA polymerase II (RNAPII), acetylated histone H3 (AcH3), and dimethylated lysine 4 on histone H3 (MeH3K4) in chromatin isolated from IMR90 cells. The authors performed ChIP-on-chip using a series of DNA microarrays consisting of a total of approximately 14.5 million 50 bp oligonucleotides spaced every 100 bp throughout the non-repetitive regions of the human genome. TAF1 was used as a marker for TFIID, a key member of the GTM. ChIP-on-chip assays using immunoprecipitated TAF1 identified 9,666 potential TAF1 tar-

get regions throughout the genome. A second ChIP-on-chip array was designed using approximately 380,000 oligonucleotides covering these 9,666 potential TAF1 target regions. ChIP-on-chip for TAF1 using the second array revealed that TAF1 binds 8,597 out of the 9666 regions. Within these regions 12,150 distinct TAF1 binding sites were identified using a peak-finding algorithm. Of these, 10,553 TAF1 binding sites were found within 2.5 kb of transcriptional start sites of known transcripts from the DBTSS, RefSeq, GenBank and Acembly databases. This was reduced to a non-redundant set of 9,328 transcript matched TAF1 DNA binding regions, i.e. promoters. Of these, 8,960 corresponded to 6,763 known EnsEMBL genes.

I re-annotated all TAF1 binding sites identified by Kim et al. (2005) to ensure an up-to-date annotation of TAF1 target genes. A total of 11,181 unique TAF1 binding sites within promoters (1,239 of which classified as putative promoters) were gathered from the supplementary materials and downloaded from the authors' website (http://licr-renlab.ucsd.edu/download.html). Of the 11,181 TAF1 binding site coordinates, 11,170 were successfully converted to the NCBI 36/HG18 build using the Batch Coordinate Conversion (liftOver) tool from the UCSC Genome Bioinformatics Group. RefSeq gene information was obtained by downloading the refFlat file (March2006 NCBI Build 36/HG18) using the UCSC Table Browser data retrieval tool in February 2012 [41]. This was used to generate new BED file containing the coordinates for DNA regions encompassing 2.5 kb up and downstream of all RefSeq transcriptional start sites (TSS). TAF1 binding coordinates and Refseq TSS +/- 2.5 kb coordinates were uploaded into the Galaxy public server for analysis (http://usegalaxy.org/) [10, 27, 29]. Refseq genes with TAF1 binding sites within +/-2.5 kb their TSS were identified in Galaxy using the Operate on Genomic Intervals, Join tool.

Of the Kim et al. non-redundant 9,328 transcript matched TAF1 binding sites (Kim et al. Supplemental Table 1) 8,927 (95.7%) were mapped to gene promoters using my bioinformatics strategy. In addition, to the 1,329 putative promoter TAF1 sites (Kim et al., Supplemental Table 2) 131 were matched to an additional 131 known RefSeq genes. An additional 548 TAF1 promoter sites from the supplementary data downloaded from the authors' website were matched to 630 known RefSeq genes. Using this approach 9,606 TAF1 binding sites were mapped to the promoters of 14,139 transcripts representing 8,850 RefSeq genes.

3.2.3 Identification of High Glucose Regulated Genes

To identify genes differentially regulated in response to HG, I searched the literature and found a single set of published data, which specifically described the effects of HG culture conditions on gene expression [2]. In this study, HEK293 cells were grown in DMEM containing 10% FCS and 100 mg/dl glucose [5.6 mM; low glucose (LG)] or 450 mg/dl glucose [25 mM; high glucose (HG)]. After seven days RNA was isolated, converted to cDNA and hybridized to Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays. Two arrays were used per glucose condition; however it is unclear if the arrays represent biological or technical replicates. Arrays were scanned using Affymetrix GeneChip Scanner 7G and signal intensities were normalized and background corrected using the Affymetrix GeneChip Operating Software v1.4.

I downloaded the raw and processed data from the NCBI GEO database and analyzed the data as follows (Alla et al. 2009, accession GSE15575) [7, 21]. GEO2R was used to generate a boxplot of signal intensities for all four array datasets deposited in GEO. A box

plot of raw probe intensities was generated in R using the Affy package. The comparison of the raw and processed data box plots confirmed that the processed signal intensities deposited in GEO is preprocessed and median-centered.

The preprocessed data sets were subsequently filtered in Microsoft Excel to select for probe sets of sufficient quality. Briefly, probe sets were filtered to retain only those with a detection call P-value <0.01. Probe sets with intensities below the intensity of the lowest detectable spike control BioB probe set intensity for a given array were removed from analysis from all four arrays. This resulted in 12,118 probe sets for which the signal was considered reliable. Finally, probe sets were filtered for those showing a greater than 2-fold change in expression.

As a quality control for my analysis I compared my analysis with that of the original study. I found the same fold change in gene expression for 10 of the 14 genes reported by the authors (Table 3.5). Two genes had similar but not identical changes in gene expression. Two genes had probe sets that were filtered out during my analysis due to the stringent cutoff for probe signal quality during my analysis. Thus, my analysis yields nearly identical values in fold changes in gene expression as original data.

3.2.4 Determination of Overlapping Gene Sets

Gene lists of HG regulated genes, TAF1 target genes and direct p53 target genes were maintained in MS Excel and examined for overlap using Venny and MS Access [62].

3.2.5 Gene Ontology, KEGG Pathway, and TFBS Enrichment Analysis

Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment and transcription factor binding site (TFBS) enrichment analysis was conducted using DAVID v6.7 [36, 37]. For the analysis of TAF1 target genes and HG-dependent genes the list of known p53 targets was first subtracted from the pool of TAF1 and HG target genes. Subsequently TAF1 targets -upregulated or-downregulated by HG were uploaded separately for analysis in using DAVID.

3.2.6 Consensus p53 DNA Binding Site Analysis

The ChIP-PET and ChIP-seq peak coordinates for p53 from the studies of Wei et al. (2006), and Smeenk et al. (2011) were uploaded into Galaxy and sequences for these regions were retrieved in the FASTA format. These sequences were then scanned for consensus p53 DNA binding sites using the p53scan algorithm on default settings (www.ncmls.nl/bioinfo/p53scan/) [73].

3.3 Results

3.3.1 Identification of Direct p53 Target Genes

In order to search for potential p53 target genes, which are regulated in a HG- and TAF1-dependent fashion, I first compiled a comprehensive list of direct p53 target genes. Two basic criteria have been established for defining a direct target gene for a given transcrip-

tion factor - direct binding to a promoter or enhancer DNA sequence and regulation of transcription. Therefore, for p53 targets, I considered the proximity of a p53 DNA binding site to the gene body, evidence of p53 binding to the DNA target sites *in vivo* and/or *in vitro* and evidence of transcriptional control by p53. I generated a list of direct p53 target genes by two methods. First, I collected a list of 172 genes with validated p53 DNA binding sites. This p53 Target Genes Literature List will hereafter be referred to as the Literature List. More details are given in Materials and Methods and the entire list is presented in Appendix B in Table B.1. Second, I collected p53 target genes identified in studies of global gene expression and genome-wide chromatin binding by p53 in response to p53 simulating stress. This p53 Target Genes Experimental List will hereafter be referred to as the Experimental List (overview given in Figure 3.2).

p53 Targets Identified by Correlative Gene Expression and DNA-binding Studies - The Experimental List

Several hundred p53 target genes have been identified using genome-wide studies of p53-DNA binding and estimates suggest there may be several thousand p53 DNA target genes in the human genome [14, 72, 73, 90]. In order to build an extended database of p53 target genes I gathered a list of p53 target genes from two studies which examined genome-wide p53 binding and global gene expression in response to 5-FU, ActD or Etopo [72, 90].

In 2006, Wei et al. used ChIP-PET and genome-wide transcription profiling of p53-containing and p53-deficient HCT116 cells treated with 5-FU in order to identify p53 target genes. By correlating p53 DNA binding with p53-dependent gene expression the authors identified 122 direct p53 target genes. Of these, 65 were upregulated and 57 downregu-

lated in response to 5-FU. I included these 122 p53 target genes in the Experimental List (Summarized in Table 3.2).

In 2011 Smeenk et al. used genome-wide transcription profiling and ChIP-seq to identify p53 target genes in U2OS osteosarcoma cells treated with either ActD or Etopo. Based on their data I classified 191 genes in ActD treated and 186 genes in Etopo treated cells as direct p53 target genes; these genes demonstrated correlation between p53 DNA binding and differential gene expression upon treatment. These p53 target genes were also included in the Experimental List (Summarized in Table 3.2).

Identifying Direct p53 Targets in Response to UV and IR.

The p53 target genes that are regulated in response to different stress conditions can vary significantly. 5-FU, ActD and Etoposide are commonly used chemotherapeutic agents known to activate p53. The p53 response to UV and IR induced DNA damage has been widely studied in part due to natural exposure and therapeutic usage. I searched the literature for genome-wide binding and expression studies that would provide a reliable list of direct p53 target genes specifically activated by UV or IR. To my best knowledge, no genome-wide analysis of DNA binding in response to UV or IR has been published to date. I gathered a list of genes with p53-dependent gene expression in response to UV or IR. I assembled a list of 88 genes with p53-dependent expression in response to UV and 40 genes with p53-dependent expression in response to IR from three published works (see Materials and Methods) [4, 54, 100].

To verify that the UV- and IR-responsive genes are direct targets for p53, I assessed whether p53 binds directly to the identified UV- and IR-responsive genes. First, a list of

8,024 p53-bound genes was compiled from two sources: (1) 7,955 p53 bound genes from three studies which used ChIP-PET or ChIP-seq to detect genome-wide p53 binding in response to 5-FU, ActD or Etopo treatment (Summarized in Table 3.3), and (2) the 172 validated p53 target genes from the compiled Literature List.

Next, using this list of p53-bound genes, I filtered the list of UV- and IR-responsive p53-dependent targets to retain only known p53-bound genes (Figure 3.3). I found that p53 binds and regulates the expression of 46 genes in response to UV and 30 genes in response to IR, of which 22 UV- and 11 IR-responsive genes were not otherwise identified in the studies of p53 in response to 5-FU, ActD or Etopo (summarized by study in Table 3.2). An additional set of 27 p53 target genes was obtained from a study that used ChIP-chip to examine p53 binding at the promoters of 489 IR responsive genes (Table 3.2). In total, 46 UV-responsive and 57 IR-responsive genes were added to the Experimental List.

Once these UV- and IR-responsive, p53-bound genes were included, the Experimental List contained 446 unique direct p53 targets (summarized in Table 3.2); genes listed in Appendix B, Table B.2). To this list I added the 172 known p53 targets compiled from the literature (the Literature List) to define, in total, 563 stress-responsive, direct p53 target genes, hereafter referred to as Direct p53 Target Genes (summarized in Table 3.2).

3.3.2 Comparison of Literature and Experimental Lists of Direct p53 Target Genes

Of the 446 p53 target genes differentially expressed in response to 5-FU, ActD, Etopo, UV or IR (the Experimental List), 55 are previously known direct p53 targets and present in

the Literature List (Figure 3.4A). For the purposes of this study I considered the remaining 391 (Figure 3.4A, 46 + 345) genes as direct p53 targets due to the correlation between target gene expression and p53 binding. This characterization is bolstered by the presence of the p53 consensus DNA binding site in the p53 ChIP-PET or ChIP-seq peaks associated with 345 of these genes as found using the p53scan algorithm (Figure 3.4A). Overall, 88% (391/446) of p53 target genes in the Experimental List are not present in the Literature List, suggesting that a significant number of direct p53 target genes have yet to be characterized. The presence of consensus p53 DNA binding sites associated with these genes implies that these genes are direct p53 target genes and warrant further, detailed *in vivo* investigations of these genes.

Of the 172 Literature List p53 target genes, 68% (117/172) were not found amongst the Experimental List of p53 target genes. These differences likely reflect differences in the p53-inducing stress. The stress conditions used in the studies to generate the Experimental list of p53 targets include 5-FU, ActD, Etopo, UV and IR, which all induce DNA damage whereas the genes compiled in the Literature List are derived from p53 studies in the context of a wider variety of cellular stress conditions. In addition, differences in the intensity and duration of the stress, difference in cell and tissue type and technical differences between the studies may also account for the differences in these two lists. In addition, genome-wide studies may identify enhancer sites located at distances further away than the classically defined proximal promoter regulatory regions.

p53 functions as both a transcriptional activator and repressor. On average, ~64% (359/563) of all direct p53 target genes identified are exclusively activated and ~34% (192/563) are exclusively repressed (Figure 3.4B). Thus, of the 563 p53 target genes identi-

fied, nearly twice as many are activated by p53 as are repressed. Just 2% (12/563) of direct p53 target genes are activated or repressed depending on the stress condition (Figure 3.4B, listed at right). These genes are involved in cell adhesion, cytoskeleton, cell cycle checkpoints and anti-apoptosis. The finding that these genes can be both activated and repressed suggests that regulation of these genes is stress and context dependent. The p53 regulation of three of these genes, *FANCC*, *IER3*, and *MLH1*, is classified differently between the Experimental and Literature Lists (Figure3.4B, right panel, dark grey).

Figure 3.4C shows the breakdown of activated and repressed genes in the Experimental List compared to the Literature List of p53 target genes. The Experimental List contains an additional 222 p53-activated genes not identified in the Literature List, nearly 3.8 times as many as the two lists have in common (222 vs 49) and nearly 1.5 times as many as the number of currently validated p53 target genes (222 vs 149). Similarly, the Experimental list contains an additional 177 p53-repressed genes not present in the Literature List; only three p53-repressed genes are shared by both lists. This expands the number of p53-repressed targets 6.5-fold (177 vs 27). In addition, the proportion of p53-activated and p53-repressed genes in the Literature List does not reflect what is observed in genome-wide studies of the p53 response to 5-FU, ActD and Etopo 3.4E. Only 16% (27/172) of the p53 target genes found in the Literature List are repressed. However, the average percentage of repressed genes found in the genome-wide studies in response to 5-FU, ActD and Etopo is 40%. Taken together, these observations suggest that p53 repressed target genes are underrepresented in the literature compiled list and that a large number of both p53-activated and p53-repressed genes remain to be validated.

3.3.3 p53 Regulates Stress-specific and Common Target Gene Sets

To gain insight into the p53 transcriptional network I examined the overlap in p53 target gene expression in response to 5-FU, ActD, Etopo, UV and IR (Figure 3.5). Of the 446 p53 target genes, 334 (75%) are stress-specific. Only 80 (18%) are regulated by just two stress conditions, 22 (5%) are regulated by just three stress conditions, and 10 (2%) are regulated by four or more conditions. Two genes, p21 (*CDKN1A*) and *DDB2*, are common to 5-FU, ActD, Etopo, UV and IR (Figure 3.5A, Venn region AE). Subsequent to my bioinformatics analysis Nikulenkov et al. (2012) described a set of p53 target genes bound and regulated by p53 in response to nutlin3a [58]. Of the 32 p53 target genes differentially regulated in three or more stress conditions (5-FU, ActD, Etopo, UV or IR), 22 are also regulated in response to nutlin3a (Figure 3.5C). Notably all of these genes are p53-activate target genes.

The 5-FU, ActD and Etopo responsive p53 target genes were identified using directly correlated genome-wide transcription profiling and p53-DNA binding assays from the same study, while the UV and IR p53 target genes stem from my more selective bioinformatics characterization. As such, I examined the regulation of p53 target genes in response to 5-FU, ActD and Etopo in more detail. 5-FU, ActD, and Etopo all regulate the expression of 14 direct p53 target genes (Figure 3.5A, blue shaded regions). Six of these 14 p53 target genes (*RPS27L*, *RRM2B*, *ATF3*, *TP53TG1*, *CDKN1A* and *DDB2*) are also regulated in response to nutlin3a (Figure 3.5C, bold). Interestingly, all of these target genes are p53-activated. Nikulenkov et al. (2012) have recently reported a "default p53 program" of p53 target genes bound in response to 5-FU, nutlin3a and RITA [58]. My finding extends the observation of a "default p53 program" beyond p53-DNA binding alone to show that a

subset of p53 target genes are both bound and activated in response to multiple stress types, supporting the notion of a "default p53 *transcriptional* program".

The six p53 target genes of this "default p53 transcriptional program" program are associated with regulation of p53, cell-cycle arrest, DNA repair and apoptosis. RPS27L is a ribosomal protein, which competes with p53 for MDM2 binding, thereby stabilizing p53 by inhibiting MDM2-mediated degradation [93]. Ribonucleoside-diphosphate reductase (RRM2B) supplies nucleotides for DNA repair in arrested cells [79, 95]. The stress-sensor transcription factor ATF3 binds to 40% of p53 target genes and serves as a cofactor to activate a subset of pro-apoptotic genes [78, 80]. ATF3 has been shown to stabilize p53 by blocking MDM2-mediated degradation citepYan2005. ATF3 can function in a negative feedback loop with p53 through downregulation of TP53 expression [42]. DDB2 complexes with CUL4A, RBX1, and DDB1 to facilitate nucleotide excision repair of UV-induced photolesions [75]. *CDKN1A*, which encodes for the cyclin dependent kinase inhibitor p21, is critical to p53-mediated cell cycle arrest and can regulate transcription, apoptosis and DNA repair [15]. Very little is known about *TP53TG1*. It is induced in a p53-dependent manner following several types of genotoxic damage and overexpression of TP53TG1 alone is unable to suppress cell growth [77]. No known protein product of the TP53TG1 transcript has been identified and its function remains unknown.

3.3.4 Identification of TAF1 Promoter-bound Genes

In order to identify direct TAF1 target genes I examined the dataset from Kim et al. (2005) [44]. This study provides a comprehensive examination of genome-wide TAF1 binding. Kim et al. mapped active promoters in IMR90 cells (primary human fibroblasts) using a

ChIP-on-chip approach in which TAF1 served as a marker for TFIID binding. To provide an up-to-date annotation of TAF1 bound genes all TAF1 binding sites were re-annotated. Briefly, the genomic coordinates of all TAF1 binding sites that were defined as promoter bound were downloaded from the authors' website and collected from the supplementary files of the published paper. I then compared TAF1 binding sites to the location of all RefSeq gene transcriptional start sites (TSS) as described in the Materials and Methods [65]. Of 10,553 TAF1 sites found within 2.5 kb of a known mRNA 5' end, Kim et al. defined a non-redundant set of 9,328 promoters of which 8,960 matched 6,763 known EnsEMBL genes at the time of the study. Using updated RefSeq annotation information I found 9,606 TAF1 binding sites that map within +/-2.5 kb of a RefSeq transcript TSS (Table 3.4). By associating the 9,606 TAF1 binding sites with known genes, I identified 8,050 direct TAF1 target genes.

3.3.5 Identification and Characterization of HG Regulated Genes

In order to identify genes that are differentially regulated under HG culture conditions I searched the literature and public data bases for HG-dependent gene expression studies (*e.g.*, Gene Expression Omnibus [GEO] and ArrayExpress). I found a single study, which specifically examined the effects of HG culture conditions on gene expression [2]. In this study human embryonic kidney epithelial HEK293 cells were grown in media containing HG (25 mM) or low glucose (5.6 mM) for seven days. RNA was collected and analyzed by genome-wide transcription profiling using DNA microarrays. I re-analyzed their published data as described in the Materials and Methods and confirmed that my analysis yielded identical fold change measurements (Table 3.5). My analysis of their results yielded a total

of 950 probe sets covering 794 RefSeq genes, whose expression changes greater than 2-fold change in response to HG. I refer to these 794 genes as HG-dependent genes for the remainder of my analysis. Among the HG-dependent genes, 393 were upregulated and 399 downregulated by HG. Two genes were both up and downregulated in response to HG in different probe sets. I included these genes in both the activated and repressed groups throughout my analysis.

Next, I checked the HG-dependent changes in gene expression for *TP53*, *MDM2*, *MDM4*, and *TAF1* (Table 3.6). p53 function is primarily regulated by mechanisms controlling the activity and cellular level of p53 protein; however the levels of p53 protein were not examined in the original study [2]. My bioinformatics analyses revealed that HG increased *TP53* expression 1.12 and 1.21 fold as measured by two separate probe sets; the expression of *MDM2*, the primary regulator of p53 protein levels in cells, increased 1.21 and 1.3; the expression of *MDM4*, an important negative regulator of p53 transactivation activity, was downregulated 1.17 fold. Thus, HG may enhance the expression of p53 and its regulators. *TAF1* transcription remains apparently unchanged in response to HG (-1.08 fold change). Thus, changes in gene expression for TAF1 target genes are likely not attributed to changes in the TAF1 expression. Because this study represents the only study examining the effects of HG on gene expression for cells cultured *in vitro*, I proceeded to use these data despite the detectable changes in gene expression for *TP53*, *MDM2* and *MDM4*.

Characterization of HG dependent gene expression

Next, I characterized the genes regulated by HG. In agreement with our prior biochemical observations I found that p21 is downregulated in HG-treated HEK293 cells (-3.87 fold).

This suggested that this data set was appropriate for further examination of HG on p53 target genes. I used DAVID to perform gene ontology (GO) analysis of all 794 genes regulated by HG more than 2-fold [36, 37]. The top twenty statistically enriched GO terms for biological processes are shown in Table 3.7. These include translation, RNA processing, apoptosis, cell cycle, and metabolism. GO terms generically identified protein and macromolecule metabolic and catabolic processes and more specifically identified terms that include processes involved in metabolism of RNA, various amino acids, vitamins, and cholesterol.

I searched for enriched signaling pathways among HG dependent genes using DAVID and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Table 3.8). Interestingly, the p53 pathway is enriched amongst HG-dependent genes. A detailed examination of the nine HD-dependent p53 signaling pathway genes reveals seven are direct p53 targets, including p21 (Figure 3.6). HG downregulated the expression of six of these seven direct p53 target genes. These findings support the hypothesis that HG can, in addition to the p21 gene, downregulate a number of p53-activated target genes and further substantiates my bioinformatics approach to identify genes, whose expression is controlled in a fashion similar to p21.

3.3.6 Putative p53 Targets Regulated by HG

In order to identify putative p53 target genes regulated by TAF1 kinase activity I examined the overlap of the three data sets: HG-dependent genes (greater than 2-fold), direct TAF1 target genes, and direct p53 target genes.

Common p53 and HG Target Genes Of the 563 identified direct p53 target genes, 8.7% (49/563) are regulated more than 2-fold in response to HG in HEK293 cells (Figure 3.7A). These 49 genes are listed in Figure 3.8 along with their regulation by p53 in response to various stresses, regulation by HG and TAF1 promoter-binding status.

It should be noted that of the 49 p53 target genes regulated by HG, 16 are solely from the Literature List, 23 are from Experimental List, and 10 are from both lists (Figure 3.8). Genes identified by my analysis of genome-wide binding and gene expression studies are considered to be direct p53 targets, as their expression coincides with the presence of p53 at the gene. The Literature List of 172 validated p53 target genes represents the most reliable set of known direct p53 targets in my overall list of 563 target genes. About two-thirds (21 of 30) of the p53 upregulated targets regulated by HG are from the Literature list. On the other hand nearly three-quarters (14 of 19) of p53-repressed targets are derived from the Experimental List. This difference may reflect the selective nature of the Literature List as nearly 85% are classified as p53-activated target genes. Thus, I have identified a number of p53-activated and p53-repressed genes, which may be regulated by HG and TAF1. Nearly half will need to be further characterized to confirm that they are direct p53 targets.

Common p53, TAF1 and HG Target Genes Having identified genes that are regulated by HG, p53 and TAF1 I sought to determine which p53-activated genes are common to these data sets. Of the 49 p53 target genes regulated by HG, 84% (41/49) are also direct TAF1 target genes in IMR90 cells under basal growth conditions. In contrast, only 66% (495/745) of p53-independent genes differentially regulated genes under HG conditions are direct TAF1 target genes (Figure 3.7C). The proportion of HG-

upregulated, p53-independent genes bound by TAF1 is 19% (76% vs 57%) higher compared to HG-downregulated ones (Figure 3.7D). However, p53-dependent genes show similar levels of TAF1 binding regardless of HG-dependent expression; the percentage of TAF1 bound genes is 3% (85% vs 82%) higher among HG-upregulated genes compared to HG-downregulated ones (Figure 3.7D). TAF1 binding at p53 target genes downregulated by HG supports the model that transcriptional regulation of these genes may involve TAF1-dependent phosphorylation of p53 as previously described for p21 (Chapter 2). Despite the prevalence of TAF1 binding at HG-dependent p53 target genes, the percentage of TAF1 target genes activated or repressed in response to HG is nearly identical between p53-independent and p53-dependent targets (Figure 3.7E). This suggests that TAF1 binding may not preferentially dictate up or downregulation of genes in response to HG or that the influence of other HG-regulated transcription factors mask the influence of TAF1. However, these results demonstrate that TAF1 is present at both p53-dependent and p53-independent genes. The enrichment of TAF1 binding at p53 target genes suggests that TAF1 may play a role in conveying p53-dependent changes in gene expression in response to HG.

p53-activated and repressed genes can be influenced by HG-induced TAF1 kinase activity by two mechanisms. First, HG-induced p53-Thr55 phosphorylation should result in nuclear export and degradation of p53, reducing the level of active p53 protein in the nucleus. Second, HG induces TAF1-mediated phosphorylation of promoter-bound p53 resulting in eviction of p53 from target genes (unpublished, X.L. and Y.W.). In both scenarios, inactivation or degradation of p53 may result in repression or derepression of p53 target genes.

Identification of Putative p53-activated Targets Downregulated by HG

Based on the results obtained for p21 (Chapter 2; unpublished, X.L and Y.W.) p53-activated genes whose expression is downregulated by HG in the presence of promoter-bound TAF1 represent genes most likely downregulated by HG through TAF1 dependent p53-Thr55 phosphorylation. I identified 25 p53-activated target genes, whose expression is HG- and TAF1-dependent, (Figure 3.9A, left panel). Of these 25, 12 are downregulated by HG (Figure 3.9A, right panel). Several of these genes are involved in processes associated with the p53 response such as cell-cycle arrest, DNA repair and apoptosis. Both GADD45A and p21, important mediators of the p53 response, are critical for specifying cell-growth arrest or apoptosis[1]. DDB2 is critical member of the NER surveillance and repair machinery [70]. Cyclin G2 (CCNG2) is capable of both up and downregulating p53. It also regulates the cell cycle through its interactions with PP2A and the centrosome and is able to induce p53-dependent cell-cycle arrest [46]. NDRG is necessary but not sufficient for p53-mediated caspase activation. DDIT4 is an inhibitor of MTOR and plays a role in p53-mediated protection from ROS. NOTCH1, a transmembrane signaling protein, can act as both a tumor suppressor and oncogene depending on the cellular context. For instance, following UV damage NOTCH1 has been shown to mediate p53-dependent survival in keratinocytes but is also important for cellular differentiation [20]. Several p53-activated genes involved in processes other than these classical categories of p53 response are also downregulated by HG. Interferon regulatory factor 2 binding protein (IRF2BP2) functions as a co-repressor for Interferon Regulatory Factor 2 (IRF2). EEF1A1 is subunit of Eukaryotic elongation factor-1. TRIM5 is an E3 ubiquitin ligase, which is thought to protect from retroviral in-

fection. Proyl 4-hydroxylase, alpha peptide II (P4HA2) is a subunit of 4-hydoxylase, an enzyme important for collegen synthesis. MAN2B1 plays a role in turnover of glycoproteins. SARS is an aminoacyl tRNA synthetase which links serine to tRNA and is thought to play a role in angiogenesis. Together these results show that HG can negatively regulate a select subset of p53-activated genes in HEK293 cells.

Identification of Putative p53-repressed Targets Upregulated by HG

While p53 has been typically associated with transcriptional activation, p53 can also function as a repressor of transcription. Genome-wide studies of p53 binding and global gene expression in both human and mouse suggest that p53 represses approximately 30-50% of its target genes [49, 72, 90]. Of the 49 genes regulated by both p53 and HG, 21 are repressed by p53 (Figure 3.9B, left panel). Of these, 11 are upregulated in response to HG (Figure 3.9B, right panel).

These genes are involved in multiple cellular processes. For example, SRSF3 is a member of the mRNA splicing machinery. HSPE1 is a heat shock protein that serves as a chaperonin protein. RHOBTB3 is a GTPase family ATPase that functions in transport from endosomes to the golgi. DHRS2 binds to MDM2 and inhibits MDM2 mediated p53 degradation. HIGD1A regulates the γ-secretase complex and is important for maintaining mitochondrial function [30]. USP9X is a deubiquintase; its activity stabilizes the anti-apoptotic protein MCL1 and promotes survival of tumor cells [69]. It has also been shown to regulate the association of Survivin with centromeres during mitosis [85]. CRIM1 regulates the processing and maturation of BMP proteins and has been implicated in drug-resistance and

[1] Unless otherwise cited, gene functions were gathered from EntrezGene and/or UniProt databases [1, 51].

angiogenesis [28, 64, 92]. Malic enzyme (ME1) links glycolysis and the TCA by catalyzing the the interconversion of malate and pyruvate in an NADP-dependent manner. ACTN1 is a cytoskeleton protein involved in actin anchoring. CRYZ is a crystalline protein which functions as a quinone reductase. LASP1 has been shown to play a role in cell migration. It is overexpressed in a variety of cancer types and promotes cancer growth and metastasis. These results show that a select subset of p53-repressed genes are upregulated by HG in HEK293 cells. Given that HG-induced p53-Thr55 phosphorylation reduces p53 protein levels and DNA binding, it appears possible that HG contributes to the observed depression of p53 target genes under HG conditions.

3.3.7 Identification and Characterization of p53-independent TAF1 Promoter-Bound Genes Regulated by HG and Putative Substrates for TAF1 Kinase Activity

I hypothesized that the HG-induced, TAF1-mediated phosphorylation of transcription factors is a common mechanism of transcriptional regulation. As described for p53 in Chapter 2, phosphorylation of transcription factors by TAF1 could lead to their inactivation, culminating in altered expression of the corresponding target genes. To identify additional transcription factors potentially regulated by TAF1 and TAF1 kinase activity, I characterized all the gene promoters (p53-independent) bound by TAF1 and differentially regulated by HG.

TAF1 and HG Regulate a Diverse Set of Genes

A total of 495 genes are p53-independent, direct TAF1 target genes are differentially regulated more than 2-fold by HG (Figure 3.7A, purple region). Of these, 213 are downregulated and 284 are upregulated by HG (Figure 3.7B). Two genes were found to be both up- and downregulated by HG. Using DAVID, I performed Gene Ontology (GO) and KEGG pathway analysis separately for TAF1 bound genes up- or downregulated by HG (Figures 3.10 and 3.11). The top ten statistically enriched (P-value <0.001) biological processes among TAF1 target genes, which are downregulated by HG, are translation, endoplasmic reticulum stress, starvation, metabolism, negative regulation of microtubule depolymerization and antigen processing and presentation (see Appendix B, Table B.3 for full GO analysis). KEGG pathway analysis revealed that TAF1 bound HG-downregulated genes are enriched (P-value <0.05) in ribosome, lysosome and antigen processing and presentation pathways. These results are consistent with prior reports that TAF1 regulates transcription of MHC class I genes and RNA pol I transcription of ribosomal genes [26, 50]. In agreement with previous reports, I found that TAF1 bound genes are involved in the regulation of metabolic processes and intracellular localization [82]. These findings suggest a role for TAF1 in the regulation of ER stress and lysosome function.

I found that TAF1 target genes, which are upregulated by HG, are enriched in biological processes related to RNA processing, cell cycle, nuclear transport, translation, and protein modification (Figure 3.10). These genes are particularly enriched in the proteasome and focal adhesion pathways (Figure 3.11). The cell cycle pathway was also enriched, although to a lesser level. TAF1 was originally identified as a factor required for G1 progression

which is due in part to its role in the activation of cyclin D1 and cyclin A [76, 87]. Indeed, cyclin D1 is one of the HG-upregulated, TAF1 target genes identified here. The enrichment of cell cycle regulators among HG-upregulated, TAF1 target genes suggests that TAF1 may contribute to G1 progression through the regulation of additional cell cycle related genes. This suggests that TAF1-mediated G1 progression could be sensitive to the glucose available to the cell. My findings also suggest a role for TAF1 in the regulation of the proteasome and focal adhesion pathways, RNA processing and protein modification. These findings also suggest that TAF1-mediated phosphorylation may play a role in the regulation of these genes.

Identification of putative transcription factor targets of TAF1 kinase activity

Since the vast majority of TAF1-bound, HG-regulated genes were not identified in this analysis as p53 targets, I sought to identify additional transcription factors that may be targets of TAF1 kinase activity in response to glucose. Using DAVID, HG up- or downregulated TAF1 target genes were analyzed for enriched TFBS within their regulatory regions (10 kb upstream of the transcriptional start site, 3 kb downstream of the transcriptional end site or within the body of the gene). HG up- and downregulated genes showed distinct sets of TFBS with little overlap (Figure 3.12). This supports the notion that TAF1 may act together with a specific subset of transcription factors in downregulation of HG-dependent target gene expression. Table 3.9 shows the list of 54 TFBS along with the associated transcription factors enriched in the regulatory regions of TAF1 target genes, whose expression is repressed by HG. These transcription factors represent putative targets for the TAF1 kinase activity and subsequent inactivation through TAF1-dependent phosphorylation. The

identified transcription factors are involved in a number of pathways including cell cycle, apoptosis, oxidative stress response, MAPK signaling, AR signal transduction, TGF-beta signaling, and Jak-STAT signaling. Additional experimentation is needed to determine whether these TFs are phosphorylated by TAF1 in response to high glucose, as is p53.

TAF1 Regulation of Antigen Presentation Related Genes Antigen processing and presentation is one of the top enriched GO terms and KEGG enriched pathways amongst my list of TAF1 promoter-bound and HG regulated genes (Figures 3.10 and 3.11). TAF1 kinase activity has been shown to be important for regulating transcriptional activation in response to PIC formation at an MHC class I promoter [26]. Of the six human MHC class I genes, five are downregulated by HG in HEK293 cells and of these, four are TAF1 target genes (Figure 3.13). Furthermore, expression of the TAF1 target gene *CALR* (calreticulin) is downregulated by HG. Calrecticulin is important in mediating proper antigen presentation [24]. Downregulation of these genes under HG conditions implicate HG induced TAF1 kinase activity in the regulation of MHC class I antigen presentation.

3.4 Discussion

TAF1 phosphorylates components of the GTM (TFIIA, TFIIH, TAF7) and the transcription factor p53, implying that TAF1 kinase activity plays a key role in transcriptional regulation. Increased cellular ATP levels in response HG treatment results in increased TAF1 kinase activity, downregulation of p21 in a p53-dependent manner, and may alter p53 protein levels in response UV (Chapter 2). Thus, I hypothesized that other genes besides p21 are

regulated by HG-induced TAF1 kinase activity in both p53-dependent and independent manners. Here, I used bioinformatics to identify p53 target genes potentially regulated through HG-induced p53-Thr55 phosphorylation. My analysis uncovered p53 target genes regulated in response to different stress factors and a common set of p53 target genes, whose expression is regulated in response to multiple stress types. I identified target genes for p53, HG, and TAF1. By comparing these gene pools, I identified p53 target genes whose expression is regulated by TAF1 and HG. These genes are potentially regulated by TAF1 kinase activity. In addition, I examined the promoter regions of p53-indepdendent, HG-downregulated genes for enriched TFBS and describe a number transcription factors that are candidates for TAF1 phosphorylation.

3.4.1 Evaluation of the Bioinformatics Analysis

The bioinformatics approach used in this chapter resulted in the identification of the p21 gene (*CDKN1A*), previously shown to be downregulated by TAF1-phosphorylation of p53-Thr55 in response to increased ATP levels (Chapter 2). This suggests that the approach may be valid and of use in identifying additional HG-regulated, TAF1 targets. This hypothesis is further bolstered by the finding that MHC class I genes and cyclin D1, known to be regulated by TAF1 kinase activity, were found to be TAF1 targets regulated by HG in my analysis [35, 45].

Nonetheless, there are several caveats that must be mentioned. One, my approach was limited to the available data sets. I used data from only a single study to examine the role of HG in global gene expression. The authors treated their cells (HEK293) with 25 mM glucose, a concentration at which others and I observed downregulation of p21 (Chapter 2).

However, the duration of HG exposure in the published study is different from our study. Our laboratory found that HG inhibits the expression of p21 six hours post treatment. In contrast, Alla et al. (2009) cultured their cells in HG conditions for seven days. In addition, the authors did not measure glucose and ATP levels making it impossible to correlate their concentrations with changes in gene expression. The level or activity of p53 following HG treatment were not measured in the Alla et al. study. I observed (minor) expression changes for p53 and its regulators MDM2 and MDM4 in HG treated cells after seven days. The functional consequences of these changes on HG-induced gene expression remain unclear (Table 3.6). Two, I used a 2-fold change in gene expression as a cutoff for classifying genes as differentially regulated. As a result, my list of genes lacks with changes in expression less than 2-fold but which nonetheless could be functionally relevant.

Three, bioinformatics analysis is not always consistent with experimental results. The bioinformatics analysis showed that the p53 target gene *GPX1* is upregulated in HG treated HEK293 cells. However, our laboratory has recently shown that, similar to p21, *GPX1* is downregulated by HG-induced TAF1 kinase activity in a p53-dependent manner (unpublished, X.L. and Y.W.). Thus, the bioinformatics approach likely misses a number of p53-dependent and -independent transcriptional events which are regulated by TAF1 and HG.

Four, the different cell types were used in the studies utilized for the bioinformatics approach. TAF1 target genes were identified by ChIP-chip in IMR90 normal human fibroblasts, while the transcriptional response to HG was studied in hypotriploid HEK293 human embryonic kidney endothelial cells using DNA microarrays. It is very likely that there are differences in TAF1 binding and HG induced gene expression between these two

cell types as a consequence of their tissue of origin, genetic makeup and the culture conditions. It remains unclear whether the TAF1 target repertoire in IMR90 cells is the same as in HEK293 cells, and that both cell types show an identical response to HG treatment. Despite these caveats, the analysis performed in this chapter provides, for the first time, a global comparison of direct p53 target genes with TAF1 binding and HG-dependent expression. It may give further insight into the role of TAF1-mediated phosphorylation in gene regulation and generate new hypotheses.

3.4.2 The p53 Transcriptional Program

As a part of this study I have compiled a list of 563 direct p53 target genes. It is worth noting that the results of genome-wide studies of p53 targets to date suggest that the actual number of p53 regulated genes is much higher than the currently 172 validated p53 target genes. p53-repressed targets appear to be particularly underrepresented. It should be noted that my own list of p53 targets is not exhaustive. Different stresses induce different p53 target genes and I have only examined a few known p53-activating stress conditions in this study. Furthermore, a recent study published after completion of my bioinformatics analysis describes an additional 231 genes bound and regulated by p53 in response to nutlin3a which are not included in my list of 563 direct p53 target genes [58].

I determined p53 target genes, whose expression is regulated in response to 5-FU, ActD, Etopo, UV and IR. The comparison of my results with the number of p53 target genes known to be regulated by these stress conditions revealed that my lists for stress-specific p53 target gene lists are not as complete as expected. For instance, *MDM2*, known to be induced in response to 5-FU, was not identified as a 5-FU-dependent target gene by Wei

et al. (2006), likely because the damage response to 5-FU is slower than the immediate damage caused by irradiation. For example, *MDM2* induction may occur later than the 6 hr time point at which expression and binding were measured by Wei et al. (2006). Furthermore, my lists of p53 target genes in response to UV and IR may lack important target genes due to the selective nature of the filtering steps in my bioinformatics approach.

The lists of genes and analysis performed here is not exhaustive, however, several genes were consistently identified in several stress conditions, highlighting their importance. Two p53 target genes, p21 (*CDKN1A*) and *DDB2*, are common targets shared by all examined stress conditions: 5-FU, ActD, Etopo, UV and IR. Importantly, six p53 target genes are common targets in response to 5-FU, ActD and Etopo, and nutlin3a. 5-FU, ActD and Etopo all induce DNA damage. 5-FU, a pyrimidine analog, is metabolized into several antimetabolites, which can become incorporated into the DNA and can inhibit thymidylate synthase (TS). Inhibition of TS causes depletion of dTTP thereby disrupting DNA synthesis and repair [48, 99]. ActD intercalates into DNA and stabilizes a covalent complex of topoisomerase I and DNA. As a result, RNA polymerse progression is blocked, leading to global transcriptional inhibition and double-strand DNA breaks [9]. Etoposide stabilizes a covalent complex between DNA and topoisomerase II leading to double and single-strand DNA breaks [56]. UV radiation generates reactive oxygen species (ROS) and DNA lesions such as pyrimidine dimers and 6,4 photoproducts and leads to exposure of ssDNA [59]. IR induces ROS, ROS related DNA lesions and double-strand DNA breaks. The genotoxic action of these drugs represents the primary mechanism of p53 induction and, consistent with my observation here, a common signature of p53-dependent gene expression in response to DNA damage has been previously described [3].

In contrast, nutlin3a activates p53 in a non-genotoxic manner by blocking MDM2 binding to p53, thereby preventing MDM2-mediated ubiquitination and degradation of p53 [84]. That p53 activates the expression of a common set of target genes in response to both genotoxic and non-genotoxic stress supports the proposed existence of a "default p53 program". According to the default p53 program hypothesis, nutiln3a, RITA and 5-FU stimuli all trigger the binding of a common set of p53 target genes, whose activity is vital to stress responses in general, as well as the expression of p53 target genes specific to each stress condition [58]. My findings that two p53 target genes, p21 (*CDKN1A*) and *DDB2*, are shown to be activated in response to all of the stress stimuli investigated (5-FU, ActD, Etopo, UV, IR, nutlin3a) strongly supports this hypothesis. Furthermore, the existence of a core group of six genes bound and activated in response to 5-FU, ActD, Etopo, and nutlin3a suggests that a subset of p53 target genes are part of a "default p53 transcriptional program" which are activated in response to both genotoxic and non-genotoxic stress. Interestingly three of the six p53 target genes common to 5-FU, ActD, Etopo, and nutlin3a response are downregulated by HG: *CDKN1A*, *DDB2* and *RPS27L*. p53-Thr55 phosphorylation plays a role in regulating the expression of one of these default p53 target genes (p21) and may be involved in the regulation of others.

Because p53 triggers various biological processes in response to distinct stimuli, it seems logical that there would also be stress-specific target genes of p53. Consistent with this notion, I observed that the majority of p53 target genes are regulated in a stress-specific manner. Selective activation of p53 target genes is thought to occur due to the influence of DNA binding sites, co-factors and specific posttranslational modifications [22]. It will be of interest to examine how these mechanisms contribute to selective, stimulus-specific p53

target gene activation and the contribution of the large number of recently identified p53 target genes to the p53-mediated cell fate in response to different stress conditions.

3.4.3 Predicting p53 Targets Regulated by HG

Overall, a larger proportion of p53 target genes regulated by HG show are also TAF1 targets compared to p53-independent, HG-regulated genes. This suggests that TAF1 may have a role in the HG-induced response at p53 target genes. The anticipated TAF1-p53 interaction at these promoters should increase p53-Thr55 phosphorylation, as demonstrated for p21 (Chapter 2). I have identified 12 p53 target genes, whose expression is activated by TAF1 and p53 in HEK293 cells and becomes downregulated in response to HG (Figure 3.9A). Among these, only the p21 gene has previously identified by biochemical assays. Preliminary results in our laboratory show similar expression profiles for *NOXA* (*PMAIP1*) and the p21 gene following UV damage, suggesting that termination of p53-mediated transcriptional activation of *NOXA* may, like the p21 gene, be regulated by ATP-induced, TAF1-mediated p53-Thr55 phosphorylation (Chapter 4). In support of this notion, *NOXA* expression in HG-treated HEK293 cells was downregulated as much as 1.42-fold and was identified a TAF1 target gene.

The functions of these 12 p53- and TAF1- target genes downregulated by HG include classical p53 responses such cell cycle arrest, DNA repair and apoptosis as well as other processes such as glycoprotein turnover and translation. These findings implicate TAF1 kinase activity in the regulation of broad p53-induced cellular processes and suggest that HG can impact multiple aspects of the p53 transcriptional program.

I am aware of only one other example in the literature describing the downregulation of a p53-activated gene to the context of glucose metabolism. Zhao et al. (2008) showed that cells with increased glucose uptake express lower levels of pro-apoptotic gene *PUMA* (*BBC3*). However, these experiments were performed in murine cells and murine p53 lacks the Thr55 residue. It remains unclear if murine p53 possesses a residue functionally equivalent to human p53-Thr55. BBC3 was not among my list of HG-dependent p53 and TAF1 target genes. I examined the HG raw data and found that *PUMA* was eliminated from my analysis due to a high P-value for one of the four arrays. An analysis of the remaining quality probe sets suggests that *PUMA* is downregulated by HG nearly 3-fold. However, in IMR90 cells TAF1 does not bind the *PUMA* promoter. Thus, *PUMA* fails to meet my criteria for p53-activated genes, which are regulated by HG-induced TAF1 kinase activity in a p53-dependent manner.

My bioinformatics analysis revealed that the expression of the p53 target and pro-apoptotic gene, *TNFRSF10B* (*DR5*, *KILLER*) does not involve TAF1 but is downregulated by HG. It is possible that TAF1 is not bound to pro-apoptotic genes (e.g. *PUMA* and *TNFRSF10B*) under basal growth conditions in IMR90 cells but rather binds to the promoter of both genes in response to DNA damage. Alternatively, their transcription may be TAF1 independent. It remains to be seen whether or not these genes are regulated by HG through TAF1 and p53 similarly to p21.

HG Mediated Abrogation of p53 Repression

DNA-bound p53 can repress target gene expression. The expression of a number of p53 target genes is upregulated by HG implying that HG can impair p53 mediated repression or

activate other transcription factors which override p53-mediated repression. Interestingly, only one of the 11 p53-repressed, TAF1 targets regulated by HG is also a target for the glucose responsive transcription factor ChREBP [40]. p53 mediates repression through both DNA-binding dependent and independent mechanisms. DNA-bound p53 can displace coactivators and can recruit the mSin3A repressive complex to genes [11]. p53 can inhibit transcription in a DNA-binding independent manner by binding to TBP and can squelch transcription factors such as TBP, TFIIB and TFIID [11]. p53 can also downregulate the activity of other transcription factors by competing for binding with the co-activator p300 [68]. Alternatively, HG may impact these mechanisms in a p53-independent manner to relieve p53-mediated repression.

p53 can also repress transcription indirectly by increasing the expression of a number of its direct target genes. For instance, p53 downregulates the expression of E2F targets by inducing expression of the cyclin-dependent kinase inhibitor *CDKN1A* (p21) [19]. Indeed, HG mediated p21 downregulation may be partly responsible for the increased levels of genes like Cyclin D and CDC25A. Recently, the long non-coding RNA lincRNA-p21 has been identified as a p53 target which mediates p53 repression of a large number of genes in mice [38]. Furthermore, p53 activates a number of microRNAs (short, non-coding RNAs that repress gene expression by causing degradation of mRNA transcripts or by inhibiting translation) which can mediate p53-depedent repression of gene expression [23]. The HGU133 Plus 2.0 microarray used in the HG study does not include linRNA-p21 and includes only a handful of microRNA transcript precursors limiting my ability to investigate the impact of HG on their expression. Given the large number of genes that can be regulated by these types of non-coding RNAs it will be extremely interesting to deter-

mine whether HG-induced TAF1 kinase activity also downregulates their p53-dependent expression in future studies.

Global reduction in p53 protein levels and/or functional inactivation of p53 could potentially alleviate p53-mediated repression. In vitro p53-Thr55 phosphorylated p53 has reduced DNA binding ability; however how p53-Thr55 phosphorylation alters p53 DNA binding in vivo is unknown [63]. It possible that HG-induced p53-Thr55 phosphorylation *in vivo* can reduce p53 DNA binding and thereby alleviate p53-mediated repression. Thus, p53-repressed genes upregulated by HG are putative candidates of HG-induced derepression via p53-Thr55 phosphorylation. I found 11 p53 target genes, whose expression is upregulated by HG (Figure 3.9B). Interestingly, all but one TAF1 target genes. It is unclear by which mechanisms p53 represses these particular genes and whether TAF1 and p53 interact at repressed promoters. However, HG-induced derepression could be due in part to the observed depletion of the nuclear pool of active p53 in response to HG.

Repressed p53 target genes are not uniformly de-repressed upon HG treatment. It is known that p53-mediated repression of *MAP4*, *MYC*, *CDKN2A*, *PDK1* and *PLK1* involves HDAC recruitment [33, 55, 57, 83, 97]. HG leads to derepression of *MYC* and *PLK1* (1.3 and 1.84 fold respectively) but *MAP4*, *CDKN2A* and *PDK1* are repressed (-1.87, -1.2 and -1.13 fold respectively). I have described 11 genes potentially derepressed by HG-induced p53-Thr55 phosphorylation. Only two, *LASP1* and *CRYZ* are validated direct p53 target genes. Future work is needed to confirm, whether these genes are direct p53 target genes whether HG can indeed alleviate p53-mediated repression of these genes, and the role of derepression of these genes in the cellular response to these genes.

Metabolic Regulation by TAF1 and HG

Our results implicate TAF1 in the regulation of metabolic genes in HG treated cells. Functional annotation revealed that biological processes related to sterol synthesis and metabolism are preferentially downregulated by HG. For example, genes involved in the import and synthesis of cholesterol are downregulated including LDLR, the receptor for low-density lipoproteins and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), the rate limiting enzyme for cholesterol synthesis. Several enzymes involved in the metabolic flux of the cell are downregulated including PGM3, PGLS and GALT. PGM3, one of several isoforms of phosphoglucomutase, functions in glycogen metabolism. PGLS catalyzes the second step in the pentose phosphate pathway. GALT is a key enzyme in the catabolism of galactose. Two enzymes involved in glycolysis, PGK1 and ENO2, are up and downregulated respectively. PGK1 catalyzes the first substrate-level phosphorylation step of glycolysis and its increased expression suggests increased glycolytic flux in response to culturing HEK293 in HG conditions. Given the influence of intracellular ATP levels on TAF1 kinase activity, it will be of interest to know whether the kinase activity of TAF1 is involved in the regulation of metabolism related genes and thus whether TAF1 may act as a sensor to regulate cellular energy homeostasis.

3.4.4 Putative Regulation of Transcription Factors by TAF1 Kinase Activity

The bioinformatics analysis in this chapter suggests that TAF1 can phosphorylate and regulate additional transcription factors in a manner similar to TAF1-mediated p53-Thr55 phos-

phorylation. By examining the promoters of HG-downregulated genes I identified 54 enriched TFBS. The transcription factors associated with these TFBS may cooperate with TAF1 in transcription and represent putative substrates for phosphorylation by TAF1. Of the putative transcription factors identified for TAF1 phosphorylation only Myc has been previously reported to interact with TAF1 [6]. This interaction was observed in yeast two hybrid assays and the significance of this interaction is unknown. Phosphorylation of Myc can promote its ubiquitination and degradation [71, 94]. This supports the notion that TAF1-mediated phosphorylation of Myc could contribute to transcriptional termination as observed with p53. Given the reported interaction between TAF1 and Myc and the importance of both of these two genes to G1 progression it will be of particular interest to examine their relationship in the future.

TAF1 and Cell Cycle

Consistent with its identity as a cell-cycle regulatory protein TAF1 controls the expression of a number of genes involved in cell cycle. Gene ontology and KEGG pathway analysis showed that HG-upregulated genes are particularly enriched in cell cycle related genes. These genes are involved in different aspects of the cellular proliferation. The kinase activity of TAF1 is critical for Cyclin D1 expression during G1 progression [45]. Cyclin D1 (*CCND1*) is upregulated by HG, as is one of the cyclin-dependent kinase it regulates, *CDK6*. MALAT1, a ncRNA involved in the relocation of growth-control regulated genes from Poly- comb bodies to interchromatin granules, is upregulated nearly 15-fold by HG and is its promoter is -bound by TAF1. There MALAT1 promotes sumoylation of E2F1 and subsequent activation of growth-control genes for G1 to S progression [96]. CKS2 and

SKP2 are also TAF1-bound and upregulated by HG. CKS2 binds and promotes the function of cyclin dependent kinases. SKP2 is important for the degradation of the cyclin-dependent kinase inhibitor 1B to promote G1 progression and binds to Cyclin A-CDK2 complexes thereby promoting their activity and S phase progression [67, 98]. TAF1-bound and HG-upregulated genes also contribute to S and M phases. For example, MCM8 is essential for DNA replication. HAUS2 and SPC25 are necessary for the proper function of the mitotic spindle. CETN3, SGO2 and CENPE are important to centrosome and kinetocore function. NEK6 is a kinase required for metaphase. It appears that TAF1 may contribute to cell cycle progression in response to HG through the regulation of these genes.

Interestingly, nearly a third of the transcription factors associated with enriched TFBS at HG-downregulated genes are associated with the regulation of genes involved in cell cycle and cellular proliferation. This includes transcription factors such as Myc, NF-Y, E2F, Elk-1, Sp1 and ATF. In ts13 hamster cells containing a temperature sensitive mutation in TAF1, ATF proteins induce cyclin A transcription in a TAF1 dependent manner which is abrogated at the non-permissible temperature [88]. Importantly, NTK mutant TAF1 is unable to rescue cyclin A transcription at the non-permissive temperature suggesting that TAF1 kinase activity is important to cyclin A transcriptional activation. Indeed, TAF1 phosphorylation of TAF7 is critical to activating cyclin A transcription [45]. These findings suggest a functional relationship between TAF1 and ATF and thus is seems reasonable that it may also serve as a substrate for TAF1 kinase activity. Sp1 plays a role in TAF1-dependent transcription of both cyclin D1 and cyclin A [32, 87]. Phosphorylation of TAF7 by TAF1 dissociates TAF7 from TAF1, relieves inhibition of TAF1 HAT activity and is thereby thought to make chromatin more accessible for Sp1 binding [45]. Phosphorylation can promote Sp1 activity

in some cases and decrease its DNA binding in others [16, 17]. Thus, it is conceivable that TAF1-mediated phosphorylation could serve to terminate transcription by phosphorylating Sp1 in a manner similar to p53. HG-induced differential expression of TAF1 targets and the potential TAF1-mediated transcription factor phosphorylation may represent mechanisms which contribute to cell cycle regulation in response to HG.

TAF1, Antigen Presentation and Immune Function

I observed significant downregulation of MHC class I genes in HG treated HEK293 cells (Fig. 13). All but one of these genes are TAF1 target genes. This suggests that HG-induced TAF1 kinase activity may be involved in regulating the expression of these genes. Consistent with this notion TAF1 is required for basal transcription from MHC class I gene promoters and functions as part of a TAF7-mediated PIC assembly checkpoint [26, 35, 91]. This finding further suggests that my investigation of changes in gene expression by HG may indeed provide insight into the role of TAF1 kinase activity in gene regulation.

In addition to downregulation of MHC class I genes, HG treatment resulted in the downregulation of XBP1, a transcription factor important for the regulation of MHC class II genes. This gene is TAF1-bound, downregulated by HG and is one of the transcription factors for which TFBS were found to be enriched at HG downregulated TAF1-bound genes. In addition, NF-κB and CUX1 transcription factor binding sites were enriched in HG-downregulated TAF1 target genes; both factors are known to play a role the immune response. NF-κB is important in B-cell and T-cell maturation and lymphoid organogenesis [31]. It also plays a role in the innate immune response through selective gene activation in response to the presence of pathogens [31]. TAF1 mediated phosphorylation of these

transcription factors could play a role in their normal function. Interestingly, diabetics are at higher risk of infection and hyperglycemia has been associated with allograft rejection [5, 81]. Given my findings, it is possible that HG-induced increases in TAF1 kinase activity may misregulate genes associated with immune function and thereby contribute to these complications.

Currently our laboratory is addressing the impact of HG on p53 function in response to UV using RNA-seq and ChIP-seq technology. It will be interesting to see if these experiments confirm at least a part of the results obtained in my bioinformatics approach. In particular, it will be of interest to determine if TAF1 can regulate the transcription of genes other than p21 by phosphorylation of p53 and/or other transcription factors identified in this analysis.

3.5 Figures and Tables

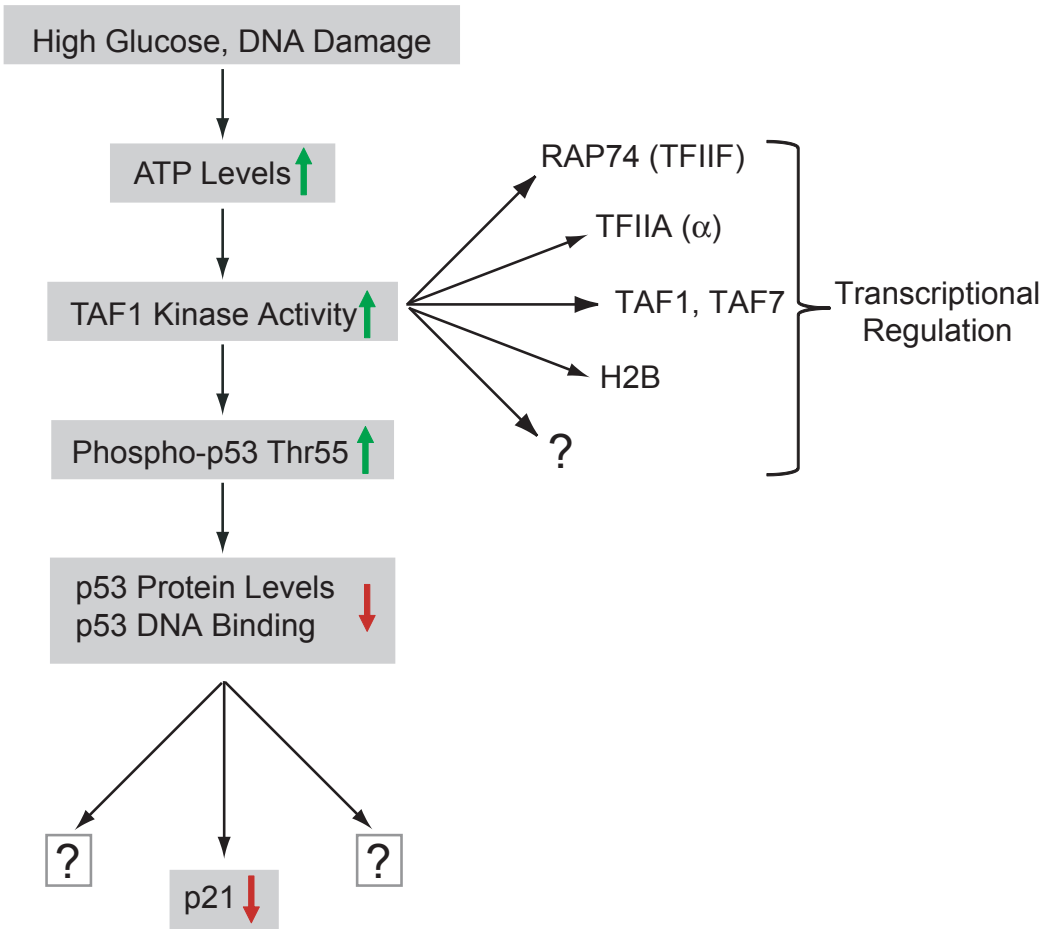


Figure 3.1. Model for TAF1-mediated Regulation of Transcription via p53-Thr55 Phosphorylation
High glucose increases intracellular ATP levels and subsequently TAF1 kinase activity (unpublished). At late stages following DNA damage ATP levels in the cell spike, similarly upregulated TAF1 kinase activity (unpublished). The resulting rise in p53 Thr55 phosphorylation levels inhibits p53 DNA binding, and increases p53 nuclear export and degradation. As a consequence transactivation of CDKN1A (p21) is reduced (unpublished, Chapter 2). High glucose-induced TAF1 kinase activity may also regulate other yet identified transcription factors. Other p53 target genes may also be regulated by TAF1 kinase activity.

Table 3.1. Summary of Studies Used for Bioinformatic Analysis
Listed are the studies used for the bioinformatic analysis in this work. If applicable, the type of DNA binding assay used is indicated by checkmark and the protein assayed for DNA binding indicated in parenthesis. *Jen et al. (2005) measured p53 DNA binding only for genes who expression was previously determined to be UV or IR responsive by DNA microarray. ‡ Literature derived p53 target genes were previously validated as direct p53 targets by meeting at least three of the four following criteria: (1) proximity of p53 DNA binding site to the gene; 2) demonstration of p53-dependent expression; (3) p53 driven expression of a reporter downstream of the identified p53 DNA binding site; (4) p53 binding at the identified p53 DNA binding site *in vivo* or *in vitro* [66]. 5-fluorouracil; ActD, Actinomycin D; Etopo, Etoposide; UV, ultraviolet; IR, ionizing radiation; HG, High Glucose; N/A, not applicable.

Experimentally Derived Gene Sets							
				DNA Binding Assay			**Gene Expression**
Study	**Ref.**	**Cell Type**	**Stress**	**ChIP-on-Chip**	**ChIP-PET**	**ChIP-seq**	**DNA Microarray**
Wei et al., 2006	[90]	HCT116$^{p53+/+}$, HCT116$^{p53-/-}$	5-FU		✓ (p53)		✓
Smeenk et al., 2011	[72]	U2OS	ActD			✓ (p53)	✓
Smeenk et al., 2011	[72]	U2OS	Etopo			✓ (p53)	✓
McKay et al., 2004	[54]	HT29-ts	UV				✓
Amundson et al., 2008	[4]	NCI60	IR				✓
Zschenker et al., 2006	[100]	TK6, TK6E6	IR				✓
Nikulenkov et al., 2012	[58]	MCF7	nutlin3a			✓ (p53)	✓
Jen and Cheung, 2005	[39]	Lymphoblastoid	IR	✓ (p53)			*
Alla et al., 2009	[2]	HEK293	HG				✓
Kim et al., 2005	[44]	IMR90	N/A	✓ (TAF1)			✓

Literature Derived p53 Target Genes‡			
Study	**Ref.**	**Cell Type**	**Stress**
Riley et al., 2008	[66]	Various	Various
Wang et al., 2009	[86]	Various	Various
Botcheva et a., 2011	[12]	Various	Various

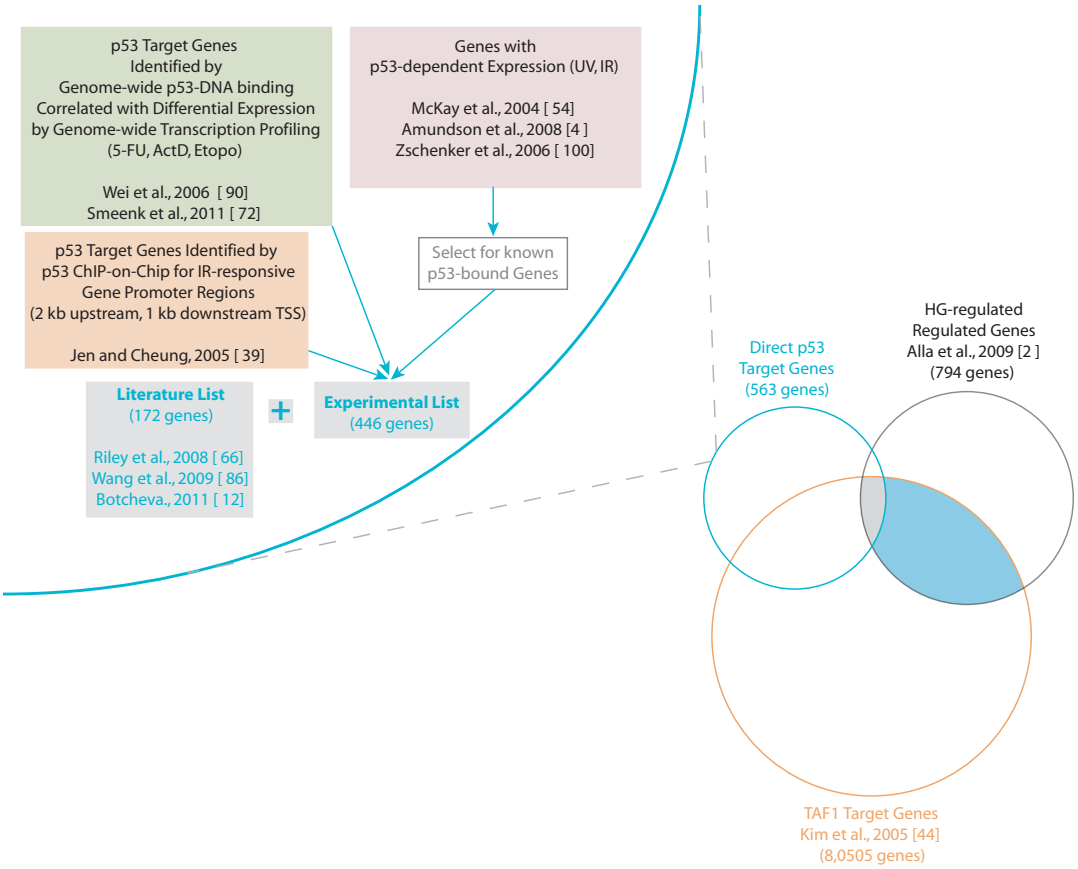


Figure 3.2. Overview of Bioinformatic Analysis
Overview of the identification and comparison of p53-, TAF1- and HG-regulated genes in this study. 5-FU, 5-fluorouracil; Etopo, Etoposide; ActD, Actinomycin D; UV, ultraviolet light; IR, ionizing radiation; HG, high glucose.

Table 3.2. Summary of Sources and Numbers of Direct p53 Target Genes
The number of direct p53 target genes compiled for the Experimental List from each study is indicated. The cell line, stress type and duration of stress before measuring p53 binding and gene expression are shown. Genes marked with the asterisk have been filtered for p53 binding using the list of annotated p53 ChIP-PET and ChIP-seq binding sites (see Fig. 3.3). The Literature List of known p53 direct targets was compiled from three studies [12, 66, 86]. DE: Differentially Expressed.

Experimental List				
Study	**Ref.**	**Cell Line**	**Stress, Duration**	**Number of DE Genes with correlated p53 Binding Sites (p53 Targets)**
Wei et al., 2006	[90]	HCT116 ($p53^{+\backslash+}$ vs. $p53^{-\backslash-}$)	5-FU (375 μM), 6 hrs	122
Smeenk et al., 2011	[72]	U2OS	ActD (5 nm), 24 hrs	191
Smeenk et al., 2011	[72]	U2OS	Etopo(10 μM) , 24 hrs	186
McKay et al., 2004	[54]	HT29-ts	UV (10, 30 J/M^2), 6 hrs	46*
Amundson et al., 2008	[4]	NCI60	IR (8 Gy), 4 hrs	26*
Zschenker et al., 2006	[100]	TK6 vs. TK6E6	IR (2 Gy), 3 hrs	20*
Jen and Cheung, 2005	[39]	Lymphoblastoid	IR (10 Gy), 4 and 24 hrs	38
Experimental List Unique Subtotal				446
Literature List				172
Unique Total Direct p53 Target Genes				563

Table 3.3. Defining p53-bound Genes from Genome-wide Binding Studies
For four studies, the cell type, stress, total number of p53 DNA binding sites, number of annotated p53 binding sites and number of annotated genes are listed. The "number of p53 DNA binding sites annotated" refers to the number of DNA binding sites with genes within 100 kb. The "number of p53 bound genes" refers to the total number of genes within 100 kb of p53 DNA binding sites. ND: Not Determined

Study	Cell Line	Stress	Ref.	Total Number of p53 DNA Binding Sites	Number of p53 DNA Binding Sites Annotated	Number of p53 Bound Genes
Wei et al., 2006	HCT116	5-FU	[90]	542 ChIP-PET Clusters	450	1,302
Smeenk et al., 2011	U2OS	ActD	[72]	2,132 ChIP-seq Peaks	1,732	4,690
Smeenk et al., 2011	U2OS	Etopo	[72]	2,920 ChIP-seq Peaks	2,346	5,821
Botcheva et al., 2011	IMR90	5-FU	[12]	743 ChIP-seq Peaks	698	2,656
Unique Total				**ND**	**3,197**	**7,955**

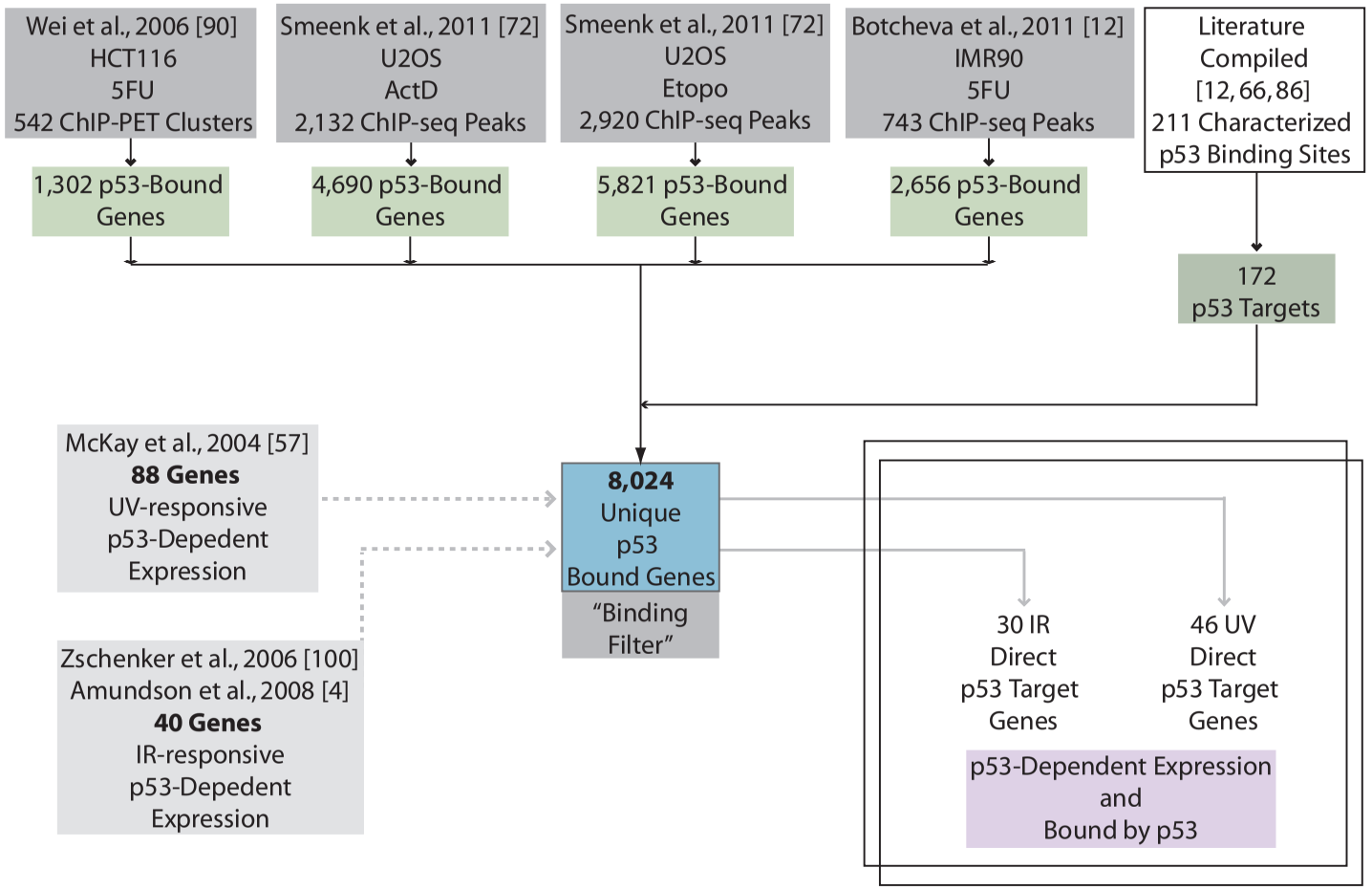


Figure 3.3. Workflow to Identify UV and IR responsive, p53-dependent Genes Bound by p53
p53 binding events identified by ChIP-PET and ChIP-seq (dark grey boxes) were annotated with all genes within 100 kb as potential p53 bound target genes (light green boxes). These were added to the 172 known p53 target genes compiled from the literature (dark green box) to give a total of 8,024 p53 bound genes (blue box). All 8,024 bound p53 genes were used as a filter to select only UV and IR responsive p53-dependent genes (light grey boxes) which also were found to be p53-bound. These are considered direct p53 targets in response to UV and IR.

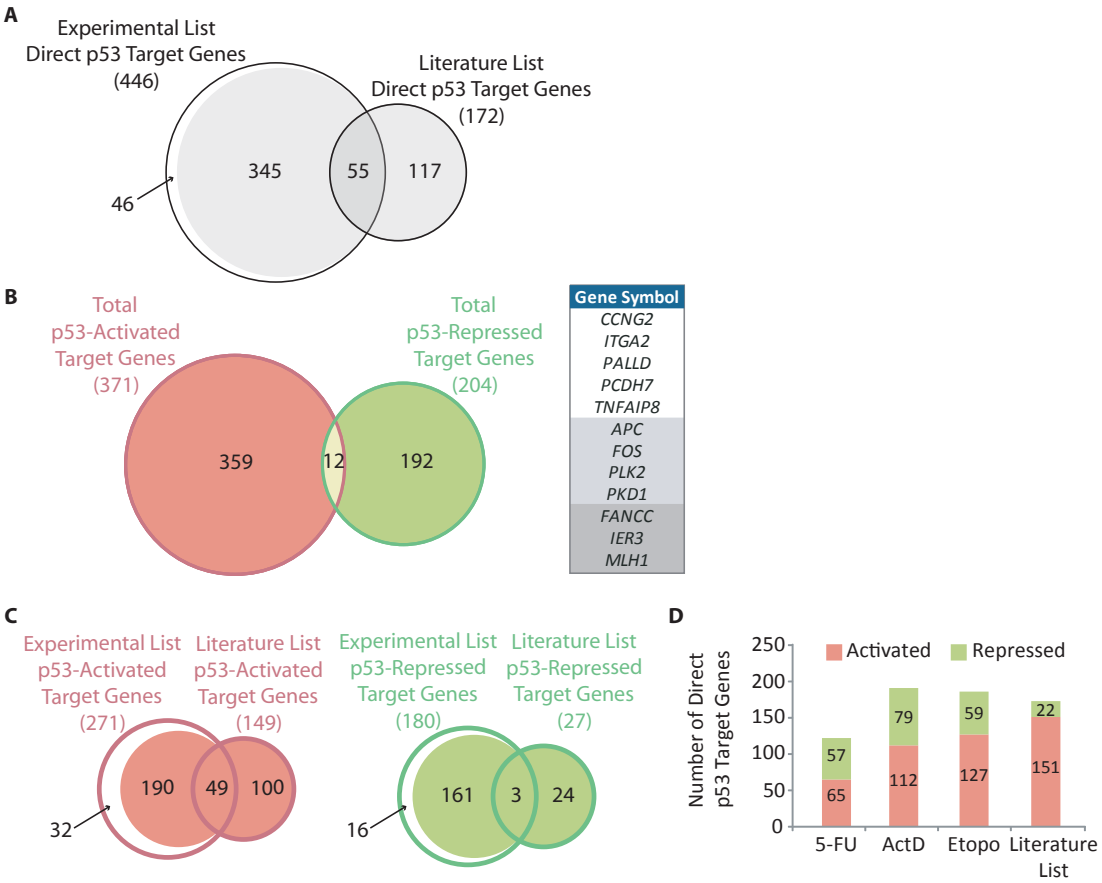
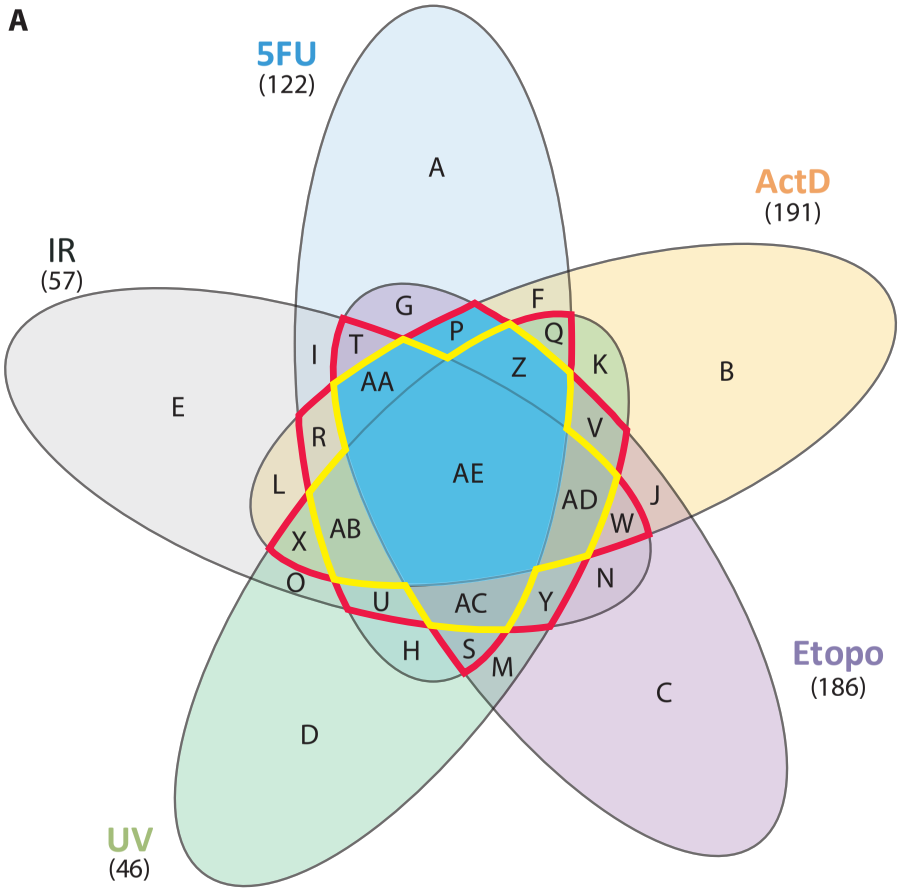


Figure 3.4. Comparison of p53 Target Genes Experimental and Literature Lists
A. A Venn diagram shows the number of common and unique direct p53 target genes between the validated Literature List and our Experimental List. Regions shaded in grey represent number of p53 targets with p53 motifs characterized from the literature or found in corresponding p53 bound ChIP-PET or ChIP-seq regions using the p53scan algorithm. The unshaded region corresponds to genes for which no p53 motif was identified. **B.** Left Panel: A Venn diagram shows the number of p53-activated and repressed genes among all 563 direct p53 target genes (Experimental and Literature Lists). Right Panel: Genes both activated and repressed by p53. These genes are classified both p53-activated and p53-repressed in the Literature List (unshaded) or in the Experimental List (grey). Genes in dark grey are classified p53-activation or p53-repressed in opposite manners between the Literature and Experimental Lists. **C.** Comparison of the number of activated (red, left) or repressed (green, right), p53 target genes shared and unique between the p53 Experimental and Literature lists. A total of 52 are found common since three genes are not classified the same in both lists (see B, right panel, dark grey). Shaded regions represent p53 targets with p53 DNA binding sites characterized in the literature or found in corresponding p53 bound ChIP-PET or ChIP-seq regions using the p53scan algorithm. The unshaded region corresponds to the number of genes for which no p53 DNA binding site was identified. **D.** The number of p53 target genes activated or repressed from the Literature List and as determined in genome-wide p53 binding and global gene expression in response to 5-FU [90] or ActD and Etopo [72].



Venn Region	Stress Condition(s)	Number Shared
A	5FU Only	95
B	ActD Only	96
C	Etopo Only	93
D	UV Only	24
E	IR Only	26
F	5FU, ActD	4
G	5FU, Etopo	2
H	5FU, UV	1
I	5FU, IR	1
J	ActD, Etopo	58
K	ActD, UV	1
L	ActD, IR	2
M	Etopo, UV	3
N	Etopo, IR	2
O	UV, IR	6
P	5FU, ActD, Etopo	10
Q	5FU, ActD, UV	1
R	5FU , ActD, IR	0
S	5FU, Etopo, UV	0
T	5FU, Etopo, IR	2
U	5FU, UV, IR	0
V	ActD, Etopo, UV	1
W	ActD, Etopo, IR	7
X	ActD, UV, IR	1
Y	Etopo, UV, IR	0
Z	5FU, ActD, Etopo, UV	0
AA	5FU, ActD, Etopo, IR	2
AB	5FU, ActD, UV, IR	2
AC	5FU, Etopo, UV, IR	0
AD	ActD, Etopo, UV, IR	4
AE	5FU, ActD, Etopo, UV, IR	2

Figure 3.5. p53 Regulates Both Stress-specific and Common Target Genes in Response to Multiple Stresses
A. A Venn diagram shows the common and unique 446 p53 target genes differentially regulated in response to 5FU, ActD, Etopo, UV or IR (left panel; Experimental List summarized in Table 3.2). Regions encompassed by the red line are common to three or more stress conditions. Intersect regions are labeled by letter. Regions encompassed by the yellow line are common to four or more stress conditions. The dark blue shaded area is common to 5-FU, ActD and Etopo. The corresponding stress conditions(s) and number of genes for each Venn intersect region is given (right panel). Genes are exclusively regulated by the stress conditions of the Venn intersection region to which they belong.

B

P	Q	T	V	W	X
5-FU, ActD, Etopo	5-FU, ActD, UV	5-FU, Etopo, IR	ActD, Etopo, UV	ActD, Etopo, IR	ActD, UV, IR
CCNG2	CDC42EP3	***XPC***	***TRIAP1***	BTG1	***CCNG1***
KITLG		***ZMAT3***		***C12orf5***	
PGPEP1				FUCA1	
PSTPIP2				***MDM2***	
PTPRM				***PLK3***	
RPS27L				***PPM1D***	
RRM2B				***SESN1***	
TGFA					
TRIM22					
USP34					

AA	AB	AD	AE
5-FU, ActD, Etopo, IR	5-FU, ActD, UV, IR	ActD, Etopo, UV, IR	5-FU, ActD, Etopo, UV, IR
ATF3	***GADD45A***	***BTG2***	***CDKN1A***
TP53TG1	***TNFRSF10B***	***FAS***	***DDB2***
		GDF15	
		NINJ1	

Figure 3.5. p53 Regulates Both Stress-specific and Common Target Genes in Response to Multiple Stresses

B. Genes common to three or more stress conditions are listed by Venn intersect regions from A. Gene names are colored according to regulation. Red, activated; Green, repressed; Green and Red, activated and repressed in different stress conditions. Target genes also regulated by p53 in response to nutlin3a are in bold [58].

C

p53-activating Stimuli	Gene Symbol	Gene Name	Cellular Processes
nutlin3a, 5-FU, ActD, Etopo	*PSTPIP2*	proline-serine-threonine phosphatase interacting protein 2	Cytoskeleton, Membrane
nutlin3a, 5-FU, ActD, Etopo	***RPS27L***	ribosomal protein S27-like	Cell cycle arrest
nutlin3a, 5-FU, ActD, Etopo	***RRM2B***	ribonucleotide reductase M2 B (TP53 inducible)	DNA repair
nutlin3a, 5-FU, Etopo, IR	*XPC*	xeroderma pigmentosum, complementation group C	DNA repair
nutlin3a, 5-FU, Etopo, IR	*ZMAT3*	zinc finger, matrin-type 3	Apoptosis
nutlin3a, ActD, Etopo, UV	*TRIAP1*	TP53 regulated inhibitor of apoptosis 1	Cell cycle arrest, Anti-apoptosis
nutlin3a, ActD, Etopo, IR	*C12orf5* (*TIGAR*)	chromosome 12 open reading frame 5	Anti-apoptosis
nutlin3a, ActD, Etopo, IR	*MDM2*	Mdm2, p53 E3 ubiquitin protein ligase homolog (mouse)	Anti-apoptosis, Cell cycle
nutlin3a, ActD, Etopo, IR	*PLK3*	polo-like kinase 3	Cell cycle arrest, Apoptosis
nutlin3a, ActD, Etopo, IR	*PPM1D* (*WIP1*)	PPM1D protein phosphatase, Mg2+/Mn2+ dependent, 1D	G2/M transition, Anti-apoptosis
nutlin3a, ActD, Etopo, IR	*SESN1*	sestrin 1	Cell cycle arrest,
nutlin3a, ActD, UV, IR	*CCNG1*	cyclin G1	Anti-apoptosis, Cell cycle
nutlin3a, 5-FU, ActD, Etopo, IR	***ATF3***	activating transcription factor 3	Apoptosis
nutlin3a, 5-FU, ActD, Etopo, IR	***TP53TG1***	TP53 target 1 (non-protein coding)	Unknown
nutlin3a, 5-FU, ActD, UV, IR	*GADD45A*	growth arrest and DNA-damage-inducible, alpha	Cell cycle arrest, DNA Repair
nutlin3a, 5-FU, ActD, UV, IR	*TNFRSF10B* (*DR5/KILLER*)	tumor necrosis factor receptor superfamily, member 10b	Apoptosis
nutlin3a, ActD, Etopo, UV, IR	*BTG2*	BTG family, member 2	Cell cycle arrest
nutlin3a, ActD, Etopo, UV, IR	*FAS*	Fas (TNF receptor superfamily, member 6)	Apoptosis
nutlin3a, ActD, Etopo, UV, IR	*GDF15*	growth differentiation factor 15	Growth inhibition
nutlin3a, ActD, Etopo, UV, IR	*NINJ1*	ninjurin 1	Cell Adhesion, Cell cycle arrest
nutlin3a, 5-FU, ActD, Etopo, UV, IR	***CDKN1A* (*p21*)**	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	Cell cycle arrest
nutlin3a, 5-FU, ActD, Etopo, UV, IR	***DDB2***	damage-specific DNA binding protein 2, 48kDa	DNA repair

Figure 3.5. p53 Regulates Both Stress-specific and Common Target Genes in Response to Multiple Stresses
C. Common 5-FU, ActD, Etopo, UV and IR p53 target genes also regulated in tesponse to nutlin3a. Genes regulated by p53 in response to nutlin3a (as found by genome-wide transcription profiling and p53 ChIP-seq) were compared to those regulated by three or more stress conditions in response to 5-FU, ActD, Etopo, UV, and IR (A) [58]. Light grey: genes regulated in response to four stimuli. Medium grey: genes regulated in response to five stimuli . Dark grey: genes regulated in response to six stimuli. All genes listed are p53-activated targets. Genes in bold are targets found in common to genome-wide transcription profiling and p53-DNA binding studies in response to nutlin3a [58], 5-FU [90], ActD [72] and Etopo [72].

Table 3.4. Comparison of TAF1 Promoter-bound Gene Annotation by Kim et al. (2005) and Current Study

Shown are the number of TAF1 binding sites annotated by Kim et al. (2005) and in our re-annotation of their TAF1 binding data using the NCBI/hg18 build of the human genome [44]. Genes with TAF1 binding sites within 2.5 kb of the TSS were annotated as TAF1 promoter-bound.

Annotation of TAF1Binding Sites	TAF1 Sites Mapped to Known Genes	EnsEMBL Genes Annotated	RefSeq Genes Annotated
Kim et al., 2005 [44]	8,960	6,763	6,848 - 7,139[1]
Re-annotation	9,606	N/A[2]	8,050

[1]Estimate from number of gene and transcript identifiers listed in Kim et al. (2005) supplementary materials.

[2]For this study only the RefSeq database was used.

Table 3.5. Comparison of HG Microarray Analysis Results
A comparison of my analysis of the HG microarray data from the original study with that of the authors' [2]. The authors' reported the fold change (FC) for genes of interest in Figure 3A of the original study. For genes with multiple probe sets, the FC for each probe set calculated in my analysis is reported (Calculated FC). Several of the authors' reported FC values appear to be the average of the FC for multiple probe sets. For comparison, both separate (Calculated FC) and averaged probe set values (Calculated FC Avg) from my analysis are reported for genes with multiple probe sets. For two genes the probe sets for two of the genes compared were filtered out in my analysis due to high p-values for the Present or Absent call in our analysis.

Gene	Alla et al., 2009 Reported FC [1]	Calculated FC	Calculated FC Avg
DNAJB9	6.36	5.33	—
HYOU1	5.68	5.68	—
HSPA5	4.97	Probe Set(s) Filtered Out	—
PPP1R15A	4.96	3.94, 5.98	4.96
PDIA4	3.85	2.88, 4.83	3.86
DNAJB5	3.47	Probe Set(s) Filtered Out	—
ARMET	3.34	3.35	—
SEL1L	3.16	3.78	—
FKBP2	2.79	2.79	—
OS-9	2.74	2.75	—
SDF2L	2.68	2.68	—
HSEC61	2.63	2.63	—
CALR	2.50	2.50	—
CANX	2.50	2.50	—

[1] Alla et al. only reported probe sets with greater than 2.5 fold change. Consequently, my results are similarly reported.

Table 3.6. Change in Gene Expression for *TP53*, *TAF1*, *CDKN1A*, *MDM2* and *MDM4* in HG-treated HEK293 Cells
Reported values derived from Alla et al., 2009 [2].

Gene Symbol	Affymetrix Probe ID	Fold Change
MDM2	225160_x_at	1.21
	229711_s_at	1.31
MDM4		
	236814_at	-1.17
	225740_x_at	-1.25
	235589_s_at	1.27
p21(*CDKN1A*)	202284_s_at	-3.87
TAF1	227205_at	1.08
TP53	211300_s_at	1.13
	201746_at	1.21

Table 3.7. The Top 20 Statistically Enriched Biological Process GO Terms for 794 HG-regulated Genes in HEK293 Cells
Derived from analysis of Alla et al., 2009 [2].

GO Accession	Go Term Biological Processes	P-Value
GO:0006412	translation	2.76E-09
GO:0006396	RNA processing	1.76E-06
GO:0051789	response to protein stimulus	2.76E-06
GO:0006457	protein folding	6.81E-06
GO:0034613	cellular protein localization	1.59E-05
GO:0070727	cellular macromolecule localization	1.86E-05
GO:0006446	regulation of translational initiation	4.64E-05
GO:0046907	intracellular transport	5.30E-05
GO:0065003	macromolecular complex assembly	7.20E-05
GO:0051726	regulation of cell cycle	8.86E-05
GO:0010608	posttranscriptional regulation of gene expression	1.05E-04
GO:0051248	negative regulation of protein metabolic process	1.51E-04
GO:0006886	intracellular protein transport	1.53E-04
GO:0006986	response to unfolded protein	1.99E-04
GO:0042981	regulation of apoptosis	2.07E-04
GO:0010605	negative regulation of macromolecule metabolic process	2.29E-04
GO:0034622	cellular macromolecular complex assembly	2.45E-04
GO:0032269	negative regulation of cellular protein metabolic process	2.60E-04
GO:0043067	regulation of programmed cell death	2.61E-04
GO:0007049	cell cycle	2.70E-04

Table 3.8. Enriched KEGG Pathways Amongst 794 HG-regulated Genes in HEK293 Cells
Derived from analysis of Alla et al., 2009 [2].

KEGG Pathway ID	KEGG Pathway	P-Value
hsa03010	Ribosome	3.61E-04
hsa04115	p53 signaling pathway	0.009203
hsa04612	Antigen processing and presentation	0.00994
hsa00750	Vitamin B6 metabolism	0.025553
hsa04510	Focal adhesion	0.029107
hsa03420	Nucleotide excision repair	0.041913
hsa04142	Lysosome	0.070135
hsa04150	mTOR signaling pathway	0.075989
hsa05416	Viral myocarditis	0.089748
hsa05212	Pancreatic cancer	0.09452

Gene Symbol	p53 Regulation	HG Fold Change	Direct p53 Target
CDKN1A (*p21*)	1	-3.9	Y
SESN2	1	-3.0	Y
GADD45A	1	-2.2	Y
TNFRSF10B (*DR5/KILLER*)	1	-2.0	Y
CCNG2 (*Cyclin G2*)	1	-2.0	Y
DDB2	1	-2.0	Y
SFN	1	2.0	Y
CDK6	-1	2.3	N
CCND1 (*Cyclin D*)	-1	3.8	N

Figure 3.6. KEGG p53 Signaling Pathway Genes
Genes in the p53 signaling pathway as defined by KEGG pathway enrichment amongst HG-regulated genes in HEK293 cells. Red (1) signifies upregulation. Green (-1) signifies downregulation. Shown is the HG-induced fold change in gene expression. Whether these genes are known direct p53 targets is indicated by Y (yes) or N (no).

Figure 3.7. A Subset of p53 Target genes are TAF1 Targets Regulated by HG
A. A Venn diagram shows the overlap between direct p53 target genes, HG-regulated genes and TAF1 target genes. Blue shaded region shows p53 target genes which are also TAF1 target genes and that are regulated in response to HG. The shaded purple shaded region shows p53-independent TAF1 target genes that are regulated in response to HG. **B.** Venn diagrams showing the overlap between direct p53 target genes, TAF1 target genes and either HG up- or downregulated genes. Two p53-independent, TAF1 target genes were found to be both up- and downregulated by HG and were included in both HG-upregulated (213 genes) and HG-downregulated (284 genes) lists for analysis. HG, High Glucose.

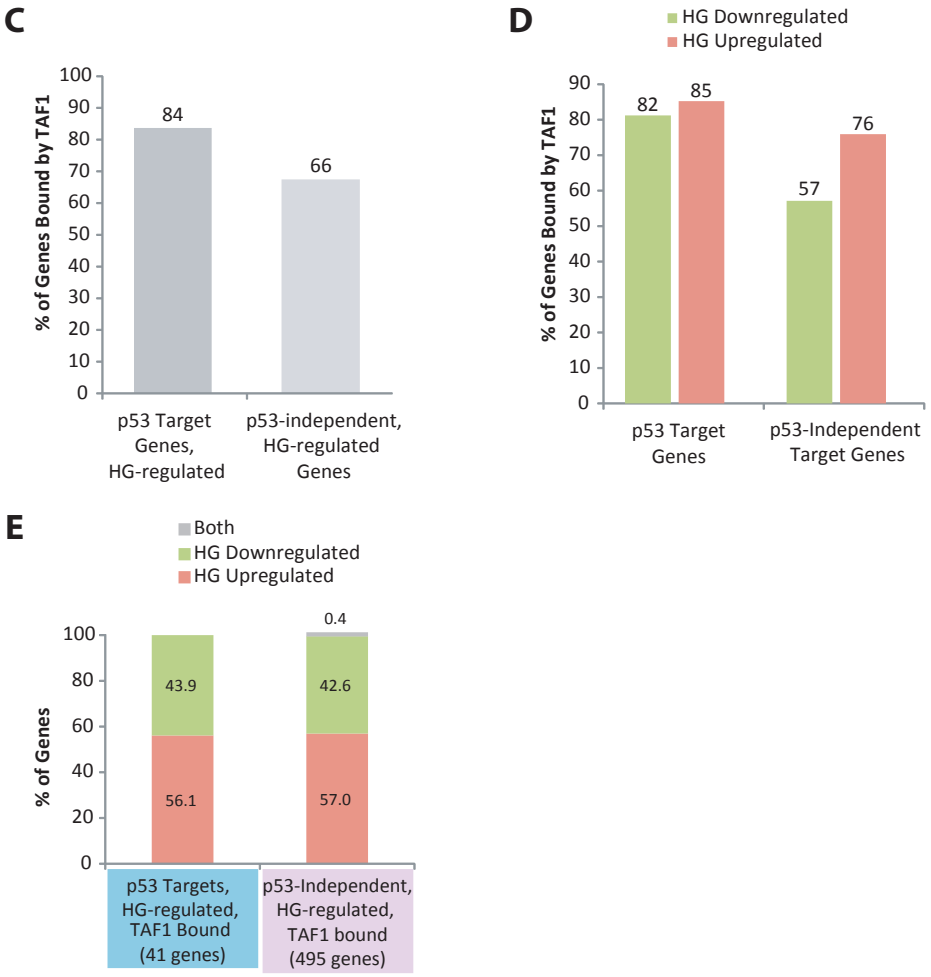


Figure 3.7. A Subset of p53 Target genes are TAF1 Targets Differentially Regulated by HG
C. A comparison of TAF1 binding at 49 HG-regulated p53 target genes and TAF1 binding at 745 p53-independent HG-regulated genes. **D.** A comparison of TAF1 binding among HG up- and downregulated p53 target genes and p53-independent genes. **E.** Breakdown of the regulation of HG-regulated, TAF1 bound p53 targets (blue shaded region in A) and TAF1 bound HG-regulated genes (p53-independent; purple region in A). HG, High Glucose.

GeneSymbol	5-FU_Reg	ActD_Reg	Etopo_Reg	UV_Reg	IR_Reg	p53ConsensusReg	HG_Reg	TAF1 Bound	Source
CDKN1A (p21)	1	1	1	1	1	1	-1	X	Both
DDB2	1	1	1	1	1	1	-1	X	Both
TNFRSF10B (*DR5, KILLER*)	1	1		1	1	1	-1		Both
GADD45A	1	1		1		1	-1	X	Both
TRIM5		1	1			1	-1	X	Exp
CCNG2	-1	1	1			-1/1	-1	X	Exp
DDIT4	1					1	-1	X	Both
NOTCH1	1					1	-1	X	Both
P4HA2				1		1	-1		Exp
SARS					1	1	-1	X	Exp
MAN2B1					1	1	-1	X	Exp
IRF2BP2						1	-1	X	Lit
NDRG1						1	-1	X	Lit
EEF1A1						1	-1	X	Lit
RPS27L	1	1	1			1	1		Both
C12orf5 (TIGAR)		1	1		1	1	1	X	Both
CALD1	1					1	1	X	Exp
FAM162A		1				1	1	X	Exp
CBR4		1				1	1	X	Exp
CYR61				1		1	1	X	Exp
DUSP1						1	1	X	Lit
SFN (14-3-3 σ)						1	1	X	Lit
GPX1						1	1	X	Both
AIFM2						1	1	X	Lit
EGFR						1	1	X	Lit
CAV1						1	1	X	Lit
COL18A1		1				1	1		Lit
EPHA2		1				1	1	X	Lit
KRT8		1				1	1		Lit
SLC38A2		-1				-1	-1	X	Lit
SCD						-1	-1	X	Lit
POLD1						-1	-1		Lit
EIF2AK3	-1					-1	-1	X	Exp
STARD4	-1					-1	-1	X	Exp
C15orf42		-1				-1	-1		Exp
PSAT1		-1				-1	-1	X	Exp
HNRPDL		-1				-1	-1	X	Exp
IER3						-1/1	1	X	Both
CRYZ						-1	1	X	Lit
LASP1						-1	1	X	Lit
HIGD1A	-1					-1	1	X	Exp
USP9X	-1					-1	1	X	Exp
HSPE1		-1				-1	1	X	Exp
ACTN1		-1				-1	1	X	Exp
RHOBTB3			-1			-1	1	X	Exp
CRIM1			-1			-1	1	X	Exp
ME1				-1		-1	1	X	Exp
DHRS2		-1	-1			-1	1		Exp
SRSF3		-1	-1			-1	1	X	Exp

Figure 3.8. p53, TAF1 and HG Regulated Genes
Listed are 49 p53 direct target genes also differentially regulated by high glucose more than two-fold. Regulation by p53 following treatment with 5-FU, Etopo, ActD, UV or IR is indicated. p53 Consensus Reg: indicates whether the gene is generally known to be up or downregulated by p53. HG Reg: regulation by high glucose in HEK293 cells. Red (1) signifies upregulation. Green (1) signifies downregulation. Genes promoter-bound by TAF1 are indicated by the orange X. Source: p53 target genes from the Literature List are labeled as Lit, genes from the Experimental List are labeled as Exp. Genes found in both lists are labeled as Both. Sources summarized in Tables 3.1 and 3.2.

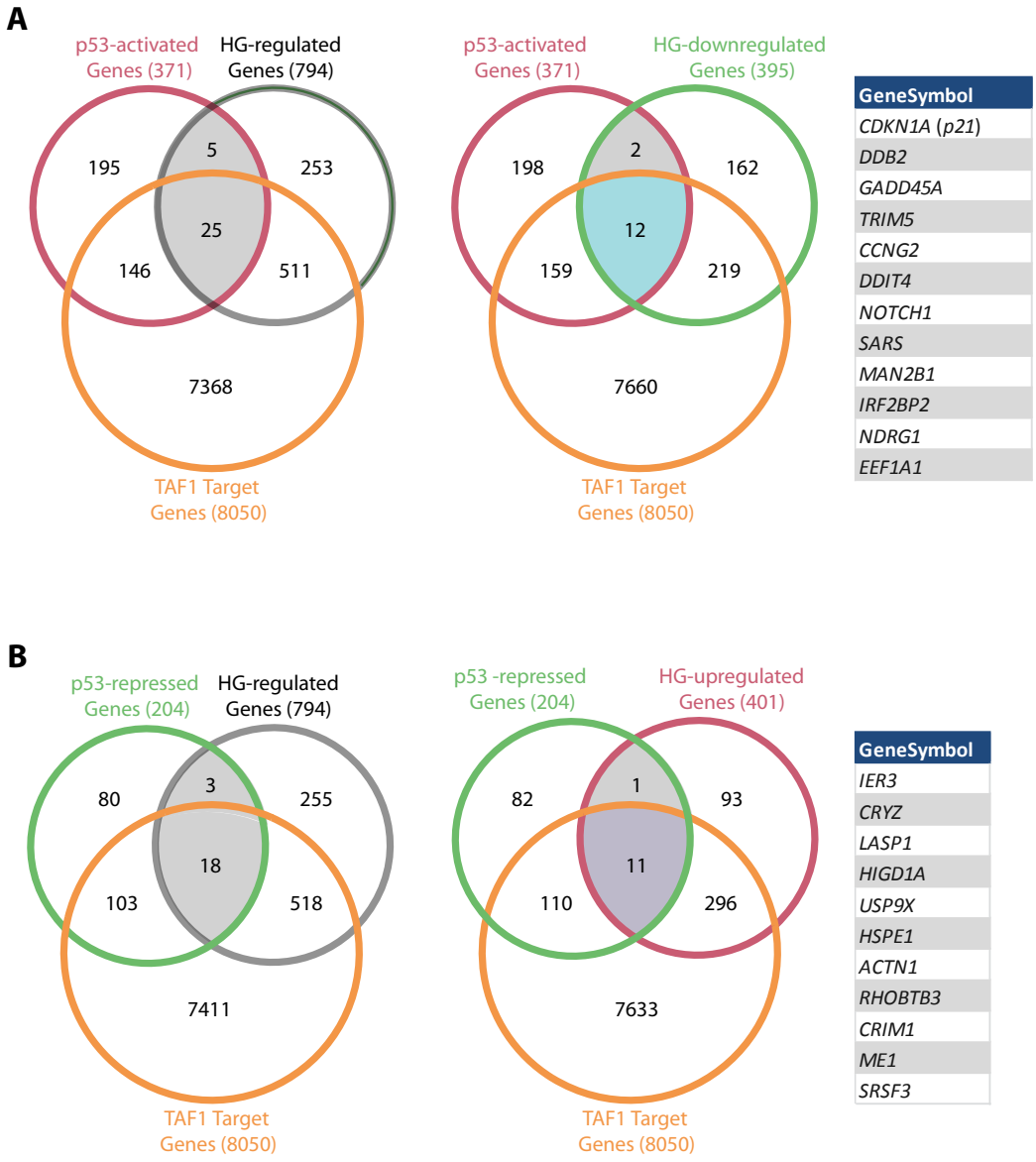


Figure 3.9. A subset of TAF1-bound p53 Target Genes are Inversely Regulated by HG
A. Venn diagrams comparing all TAF1 target genes, p53-activated genes and HG-regulated genes (at left) or HG-downregulated genes (at center). HG-downregulated and TAF1-bound p53-activated targets are listed at right. **B.** Venn diagrams comparing all TAF1 target genes, p53-repressed genes and HG-regulated genes (at left) or HG-upregulated genes (at center). HG-upregulated and TAF1-bound p53-repressed targets are listed at right. Note that there were two genes which are both p53-activated and p53-repressed targets and which were included in both lists for analysis. Thus the total number of p53-regulated genes is [(5+25+3+18)-2]=49. HG: High Glucose.

Figure 3.10. Gene Ontology Analysis of p53-independent, HG-regulated TAF1 Target Genes
The top ten statistically significant (P-Value/EASE Score of <0.001) GO Biological Processes terms are listed for 495 p53-independent TAF1 target genes differentially regulated by HG (Figure 3.7A, purple region). Up- and downregulated gene sets were analyzed separately using DAVID.

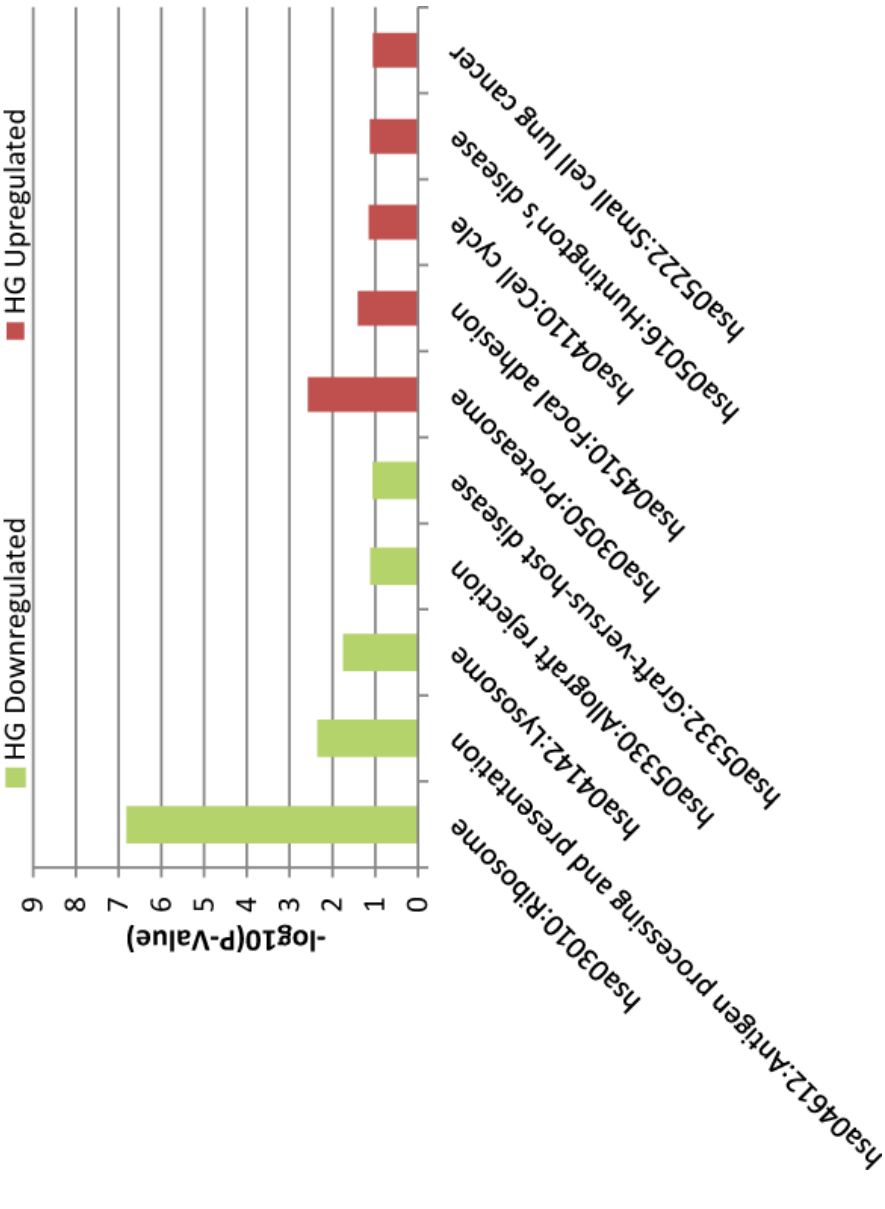


Figure 3.11. KEGG Analysis of p53-independent, TAF1 Target Genes Regulated by HG
KEGG enriched pathways amongst 495 p53-independent, TAF1 target genes regulated by HG (Figure 3.7A, purple region). Up- and downregulated gene sets were analyzed separately using DAVID.

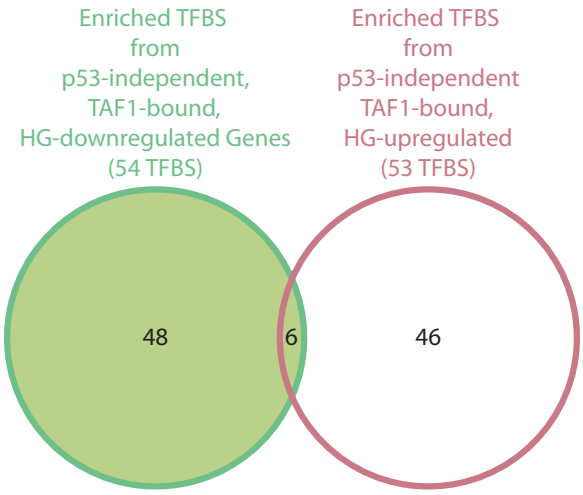


Figure 3.12. Transcription Factor Binding Sites are Differentially Enriched Between p53-independent, TAF1 Target Genes Up- or Downregulated by HG
The 213 p53-independent TAF1 promoter-bound and HG-downregulated gene set (Figure 3.7B, left panel, hatched region) and the 284 p53-independent, TAF1 promoter-bound and HG-upregulated gene set (Figure 3.7B, right panel, hatched region) were analyzed for TFBS enrichment separately using DAVID. Up- and downregulated gene sets were analyzed separately using DAVID.

Table 3.9. Transcription Factor Binding Sites Enriched at HG-downregulated, p53-independent, TAF1 Target Genes
DAVID was used to search for enriched transcription factor binding sites (TFBS) within the regulatory regions for 213 p53-independent, TAF1 target genes downregulated in response to HG (Figure 3.7B, left panel, hatched region). This includes TFBS 10 kb upstream of the transcriptional start site, 3 kb downstream of the transcriptional end site or within the body of the gene. Gene count and % are out of the 213 genes considered. Expression of transcription factors in bold was downregulated more than 2-fold by HG.

UCSC_TFBS	Transcription Factor(s)	Gene Count	%	P-Value
SP1	Sp1	72	34.8	8.27798E-07
ARNT	Arnt (HIF-1β)	112	54.1	1.4136E-05
NRF2	Nrf2	78	37.7	1.50073E-05
MZF1	MZF-1	111	53.6	8.23391E-05
ATF6	**ATF6**	106	51.2	8.25605E-05
GATA2	GATA-2	82	39.6	0.000325575
USF	USF1	127	61.4	0.000360503
XBP1	**XBP-1**	110	53.1	0.000384155
NFY	NF-Y	108	52.2	0.000459011
STAT1	STAT1	91	44.0	0.000553924
AP2GAMMA	AP-2γ	33	15.9	0.000595906
MYCMAX	Myc, Max	133	64.3	0.000600962
MAX	Max	47	22.7	0.000712535
MYOGNF1	Myf-4, NF-1	104	50.2	0.000970469
CREB	CREB	95	45.9	0.001259996
PAX5	Pax-5	120	58.0	0.001558559
TAXCREB	Tax, CREB	115	55.6	0.002014406
NMYC	N-Myc	72	34.8	0.002973825
AP4	AP-4	132	63.8	0.004339881
AHR	AhR	74	35.7	0.0068059

Continued on next page

Table 3.9 – *Continued from previous page*

UCSC_TFBS	Transcription Factor(s)	Gene Count	%	P-Value
GATA1	GATA-1	160	77.3	0.007982372
ZID	ZID	99	47.8	0.009586575
COMP1	COMP1	103	49.8	0.01009266
AHRARNT	AhR, Arnt (HIF-1β)	108	52.2	0.011038768
E47	E47	123	59.4	0.013119018
SPZ1	Spz1	69	33.3	0.015925722
SREBP1	SREBP-1	121	58.5	0.017543113
RFX1	**RFX1**	123	59.4	0.018280456
BACH1	Bach1	102	49.3	0.019831721
ZIC1	ZIC1	34	16.4	0.021582863
PAX2	Pax-2	117	56.5	0.021697085
ELK1	Elk-1	101	48.8	0.022501769
E2F	E2F	114	55.1	0.022534586
P300	p300	78	37.7	0.025631646
MRF2	MRF-2	102	49.3	0.027179807
NRSF	NRSF	107	51.7	0.029892073
CDPCR3HD	Cux1	90	43.5	0.037644221
CMYB	c-Myb	86	41.5	0.03768485
BACH2	Bach2	87	42.0	0.040617002
NF1	NF-1	76	36.7	0.043578569
AREB6	ZEB	162	78.3	0.047417685
NFKB	NF-κB	98	47.3	0.054095452
NGFIC	Egr-4	61	29.5	0.056294555
EGR1	Egr-1	20	9.7	0.056309286
ROAZ	Roaz	90	43.5	0.06106183

Continued on next page

Table 3.9 – *Continued from previous page*

UCSC_TFBS	Transcription Factor(s)	Gene Count	%	P-Value
HMX1	HMX1	90	43.5	0.064451452
AML1	AML1	148	71.5	0.066881323
CETS1P54	c-Ets-1	52	25.1	0.069069353
ATF	ATF	64	30.9	0.072439216
HEN1	HEN1	113	54.6	0.075051765
LUN1	LUN-1	97	46.9	0.076716533
OLF1	Olf-1	88	42.5	0.09071808
CP2	CP2	77	37.2	0.093110257
ZIC2	ZIC2	48	23.2	0.09733855

Gene Symbol	HG Fold Change	TAF1 Bound
HLA-A	-2.27	
HLA-B	-3.51	
HLA-C	-3.19	
HLA-E	-2.16	
HLA-F	-2.15	
HLA-G	N/A	

Figure 3.13. MHC Class I Genes are TAF1 Targets Downregulated by HG
Fold change induced by high glucose is indicated. Green signifies downregulation. Orange signifies TAF1 promoter binding in IMR90 cells.

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Chapter 4

Tools for Analysis of TAF1 and p53

4.1 Introduction

Elucidating the molecular mechanisms underpinning transcriptional regulation in response to intra- and extracellular stress factors is fundamental for understanding how cells maintain homeostasis and stay healthy and alive. The transcription factor p53 is an important tumor suppressor protein, which can mediate both transcriptional activation and repression of target genes in response to cellular stress to control cell cycle arrest, DNA repair, and apoptosis [1]. p53 can interact with various coactivators and members of the basal transcription machinery to activate transcription of target genes in a stress-dependent fashion. For instance, p53 can recruit the HATs p300/CBP, PCAF, and TIP60, as well as the SWI/SNF remodeling complexes for transcriptional activation [24]. In addition, p53 can promote pre-initiation (PIC) formation through interactions with the Mediator complex,

subunits of TFIID (TBP, TAF1, TAF3, TAF6, TAF9), TFIIA, and TFIIH [1, 24]. Our laboratory studies the role of the interaction of p53 with TAF1 in transcriptional regulation.

Elucidating the Mechanism of p53-mediated Recruitment of TAF1 to the 3' but Not 5' p53 DNA Binding Site of the p21 Promoter

DNA has been shown to act as an allosteric regulator for a number of transcription factors. Changes in the estrogen DNA binding site (DBS) sequence can induce conformational changes leading to altered ER coactivator recruitment and transcriptional activity [13, 21]. Changes in the sequence of the Glucocorticoid Receptor (GR) binding site can influence coactivator recruitment or activity [23]. A single nucleotide exchange in the DBS sequence for NFκB can alter coactivator recruitment [18]. Differences in the sequence of the DBS of p53 (p53-DBS) can affect the transcriptional regulatory activity of p53 [14, 17, 32]. Simply altering the sequence of the half-site dinucleotide WW motif can change an activating DBS into a repressing one [32]. Molecular modeling of p53-DNA interactions reveal that changes in DBS sequence can change the orientation of the p53 DNA binding domain [26]. Pan et al. (2010) propose that conformational changes in the DNA binding domain propagate to the p53 NH_2 and COOH- terminal domains and render them accessible for specific cofactors, suggesting that the sequence of the DBS may play a role in p53 coactivator selectivity.

The p53 DNA binding site sequence consists of two repeats of the motif RRRCWW-GYYY separated by up to 13 base pairs, where R is C or G, W is A or T, and Y is C or T [8, 10]. Each half-site can be graphically represented by two inverted arrows (Figure 4.1B). The p21 promoter contains two p53 DNA binding sites: a proximal 3' p53-DBS and

a distal 5' p53-DBS (Figure 4.1A). The 5' p53-DBS sequence nearly perfectly matches the consensus motif; it contains two head-to-head half-sites and, important for activation by p53, an AT sequence for the central WW motif of each half-site. The 3' p53-DBS deviates from the consensus p53 motif; it contains two tail-to-tail half-sites and, associated with a repressive p53-DBS sequence, a TG sequence for the central WW motif of each half-site. Interestingly, despite the repressive sequence resemblance, the 3' p53-DBS is critical to p53-mediated activation of the p21 gene [9].

Our laboratory has shown that TAF1 is recruited to the p21 promoter in a p53-dependent manner following UV damage [19]. This recruitment involves the interaction of the TAF1 double bromodomain (DBrD) and the acetylated COOH-terminus of p53. Acetylated p53 is present at both the 5' and 3' p53-DBS, however, p53 recruits TAF1 only to the 3' p53-DBS. This differential recruitment may be a result of the different distances of the 5' and 3' p53-DBS to the proximal promoter, the sequences surrounding the DBS, the sequences of the 3' and 5' p53-DBS themselves, or a combination of these factors. The divergence in the sequence composition of the 5' and 3' p53-DBS suggests an attractive model in which the sequence of the 3' p53-DBS is responsible for the differential recruitment of TAF1 by p53.

Elucidating the TAF1 Kinase Domain Responsible for p53-Thr55 Phosphorylation

TAF1 is a bipartite kinase possessing functional kinase domains at both the NH_2 and COOH-termini, referred to as the NTK and CTK, respectively [7]. A number of substrates for TAF1 kinase activity have been identified including the Rap74 subunit of TFIIF, the β subunit of TFIIA, TAF7, histone H2B and p53 [7, 12, 20, 22, 31]. Our laboratory has

previously shown that TAF1 phosphorylates Threonine 55 of p53 (p53-Thr55) [20]. This modification, which occurs under normal growth conditions, increases the interaction of p53 with its negative regulator MDM2, p53 nuclear export, and p53 degradation, thereby promoting G1 progression [5, 20]. Previous work in our laboratory has shown that baculovirus expressed and purified NTK and CTK domains are both capable of phosphorylating p53, but that the NTK is the major kinase responsible for p53-Thr55 phosphorylation *in vitro*. In addition, in a baculovirus co-infection model, sf21 cells infected with baculoviruses expressing p53 and full length TAF1 or the NTK showed significantly higher levels of p53-Thr55 phosphorylation compared to those co-infected with baculoviruses expressing p53 and the CTK, A2 (a kinase dead NTK mutant), or empty vector [29]. These results suggest that the NTK is the major kinase domain responsible for p53-Thr55 phosphorylation *in vivo*. However, it remains to be definitively shown whether the NTK, CTK or both domains are responsible for p53-Thr55 phosphorylation *in vivo*.

TAF1-mediated p53-Thr55 Phosphorylation May Regulate the Expression of Multiple p53 Target Genes

The mechanisms by which p53-activated transcription may be shut off have not been explored in great detail. Our study results suggest that p53-Thr55 phosphorylation may play a role in the termination of p53-mediated transcriptional activation. First, p53-Thr55 phosphorylation leads to increased interaction with MDM2, nuclear export and degradation of p53 [5, 20]. In addition, our laboratory has found that TAF1 is recruited to the p21 promoter by acetylated p53 both *in vivo* and *in vitro*; promoter bound p53 is phosphorylated by TAF1 *in vitro* and phosphorylated p53-Thr-55 has reduced affinity for DNA *in vitro*

[19, 29]. Thus, it is plausible that p53-Thr55 phosphorylation may cause dissociation of p53 from promoter DNA either directly or in conjunction with MDM2 mediated interaction and subsequent nuclear export.

We have recently demonstrated that fluctuations in intracellular ATP levels can inhibit p53-mediated p21 transcription (Chapter 2). Intracellular ATP levels fluctuate in response to DNA damage. Upon DNA damage, poly (ADP-ribose) polymerase 1 (PARP1) is activated resulting in depletion of NAD+ and exhaustion of ATP levels [3, 16, 33]. A drop in the ATP/ADP ratio activates AMPK thereby stimulating ATP synthesis. Our laboratory recently found that UV damage causes a temporary increase in ATP levels. This spike in ATP concentration correlates with a stimulation of TAF1 kinase activity, phosphorylation of p53-Thr55 and temporary repression of p21 transcription. Blocking PARP1 activity using the specific inhibitor 4-amino-1,8-napthalimide (4-AN) prevented fluctuation in ATP levels, increased Thr55 phosphorylation and prevented a drop in p21 transcription at later time points in response to UV damage. The TAF1 kinase inhibitor apigenin blocked p53-Thr55 phosphorylation and prevented repression of p21 transcription (unpublished, X.L. and Y.W.). Thus, it does indeed appear that phosphorylation of p53-Thr55 plays a role in termination of the p53-mediated transcriptional activation of the p21 gene. Because TAF1 is a subunit of the general transcription factor TFIID, which is involved in the transcription of a large number of genes, it appears likely that TAF1-mediated phosphorylation of transcription factors is involved in the transcriptional regulation of p53 target genes as well as p53-independent genes.

Here, I will discuss tools generated to study three aspects of the relationship between TAF1 and p53. First, I developed a mutant p21 promoter construct to examine the po-

tential role of the 3' and 5' p53-DBS sequences in the differential recruitment of TAF1 at the p21 promoter. Second, I generated two TAF1 kinase mutant expression constructs to identify the kinase domain of TAF1 responsible for p53-Thr55 phosphorylation in vivo. Third, I have profiled the expression of several p53 target genes to determine if their transcriptional program may involve TAF1-mediated phosphorylation of p53. My preliminary results suggest that termination of p53-mediated transcription of *NOXA* is regulated by TAF1-mediated p53-Thr55 phosphorylation similarly to p21.

4.2 Materials and Methods

4.2.1 Reporter Assays

H1299 cells (seeded in 60-mm plates) were transfected by the calcium phosphate method as follows: 1 μg reporter (p21-LUC or 2x3'BS p21-LUC) and 0, 100 or 200 ng pcDNA-p53 and carrier (sonicated salmon sperm DNA to 7.5 μg DNA final total). After 36 hours cells were harvested and luciferase levels assayed using the Luciferase Assay System (Promega) according to the manufacturer's instructions.

4.2.2 Transient Transfection

U2OS cells were seeded in 10-cm plates and transfected at 80% confluence with 2μg of pCMV-HAhTAF1 , pCMV-HAhTAF1 DD, p-LXSN-MT-TAF1N1398 A2/N7 Ala, pcDNA3.1($*$) FLTAF1 A2/N7 Ala, or pCMV-HAhTAF1 N1646 encoding WT TAF1, TAF1 DD, A2/N7

Ala, FL TAF1 A2/N7 Ala or TAF1 N1646 respectively (Figure 4.5). Cells were transfected for 48 hrs using FuGENE according to the manufacturer's instructions.

4.2.3 Western Blot

Vinculin, TAF1, p53, and p21 protein levels was performed by western blot as described [19]. Briefly, whole-cell lysate was prepared using lysis buffer composed of 20 mM Tris-Cl (pH 7.9), 150 mM NaCl, 0.5% NP-40, 20% glycerol, 2mM EDTA, 0.5mM DTT, 2 mg/ml aprotinin, 2 mg/ml leupeptin, and 0.5mM PMSF. Whole-cell lysate was subject to SDS-PAGE (8% for TAF1, 10% for p53, 12% for p21) and transferred to nitrocellulose. Membranes were blocked in TBST (20mMTris-HCl, pH 7.6, 136mM NaCl, and 0.1% Tween-20) containing 5% fat-free milk. Blots were incubated with primary antibody [anti-vinculin (VIN-11-5, Sigma), anti-TAF1 (6B3, Santa Cruz), anti-p53 (DO-1, Santa Cruz), anti-p21 (C-19, Santa Cruz), anti-c-Myc (9E10, Santa Cruz), or anti-HA antibodies] at 4^oC overnight. Following primary antibody incubation membranes were washed in TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1.5 hours. Immunoblot signal was detected by chemiluminescence using SuperSignal West Pico Substrate (Thermo Scientific Pierce)

4.2.4 Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and treated with Turbo DNA-free kit (Ambion) to remove genomic contamination. RNA was reverse transcribed

using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the iQ SYBR Green Supermix (Bio-Rad). The primers used are listed in Appendix C, Table C.2. Quantitative PCR was performed using the Bio-Rad CFX96 system. PCR conditions were as follows: 2 min at 95^{o}C followed by 40 cycles of 95^{o}C for 30 sec, 60^{o}C for 30 sec and 72^{o}C for 30 sec. Non-template control and no RT reactions were run for each primer pair. Gene expression was normalized to GAPDH using Pfaffl method [28].

4.2.5 Endpoint Real Time-Polymerase Chain Reaction

cDNA prepared for RT-qPCR was used for endpoint RT-PCR. The primers used are given in Appendix C, Table C.2. PCR conditions were as follows: 2 min at 95^{o}C followed by 37 cycles of 95^{o}C for 30 sec, 60^{o}C for 30 sec and 72^{o}C for 30 sec. Products were resolved on 2% agarose gels, bands visualized with ethidium bromide and quantified using the Kodak Image Station 4000R Pro.

4.3 Results

4.3.1 Tools and Assays to Dissect the Differential Recruitment of TAF1 to the 3' but not 5' p53 Binding Site of the p21 Promoter

Construction of the 2x3'BS p21-LUC Plasmid

To assess the differential recruitment of TAF1 to the 3' and 5' p53-DBS in the p21 promoter, I developed a mutant p21 promoter construct, which contains a 3' p53-DBS inserted

into the position of the 5' p53-DBS. This construct was generated as follows. A 2,133 bp fragment of the p21 promoter region from Xho I to Pst I was excised from p21-LUC and subcloned into the multiple cloning site of pBluescript SK+ to create p21pBluescript SK+. This construct was then used as a template to replace the 5' p53 DBS sequence with the 3' p53 DBS sequence by PCR. First, p21pBluescript SK+ was used as a template for PCR using the primers BShort5' and PstI3'. All primers used to generate 2x3'BS p21-LUC are described in Appendix C, Figure C.1. The primer BShort5' contains 13 bp of the 3' p53 DBS sequence preceded by 22 bp of the p21 promoter sequence that immediately follows the 5' p53 DBS. This PCR resulted in a 2,087 bp amplicon of the p21 promoter in which the 5' p53 DBS has been replaced with part of the 3' p53 DBS sequence. This 2,087 bp amplicon was subsequently used as template for a second round of PCR using the ALong5' and PstI3' primers. The primer Along5' contains the complete 3' p53 DBS sequence preceded by 30 bp of the p21 promoter immediately upstream of the 5' p53 DBS, and followed by 7 bp of the p21 promoter immediately downstream of the 5' p53 DBS. This second round PCR resulted in a 2,124 bp amplicon of the p21 promoter in which the sequence of the 5' p53 DBS has been completely replaced with that of the 3' p53 DBS. This 2,124 bp amplicon was then used for a third round of PCR using the primers BLong5' and PstI3'. This PCR resulted in a 2,161 bp amplicon of the p21 promoter which contains an Xho I restriction site at the 5' end, and Pst I restriction site at the 3' end. This 2,161 bp PCR product was purified using the Qiagen PCR cleanup kit according to the manufacturer's instructions, digested with Xho I and Pst I and subcloned into Xho I/Pst I digested p21pBluescript SK+ to create 2x3p21pBluescript SK+. Replacement of the 5' p53 DBS sequence with the 3' p53 DBS sequence was confirmed by sequencing. Next, the 2,133 bp p21 promoter

fragment containing two p53 3' binding sites from 2x3p21pBluescript SK+ was subcloned into XhoI/PstI digested p21-LUC backbone to create 2x3'BS p21-LUC. Figure 4.2 shows the plasmid map for 2x3'BS p21-LUC. Figure 4.3A shows a schematic diagram comparing the WT and 2x3'BS p21 promoter reporter constructs.

The Luciferase Reporter Assay

In order to address the functional significance of replacing the 5' p53-DBS with that of the 3' p53-DBS, I planned to use the luciferase reporter assay system. Toward this goal I tested the ability of p53 to drive expression of luciferase in the context of 2x3'BS p21-LUC. Figure 4.4 shows the results of a single representative experiment. Consistently, p53 activated luciferase expression from p21-LUC and 2x3'BS p21-LUC. However, increasing the amount of transfected p53 did not appear to significantly increase luciferase expression for either construct. This is in contrast to prior findings in our laboratory which demonstrated a p53 dose-dependent increase in reporter levels for p21-LUC [19]. Variation in my transfection efficiency between experiments and low reporter activation with both p21-LUC and 2x3'BS p21-LUC precluded any conclusions on the effect of the second 3' p53-DBS on p53-dependent transcription. In summary, these results show that the 2x3'BS p21-LUC construct is p53-responsive.

4.3.2 Tools and Assays for the Identification of the TAF1 Kinase Domain Responsible for p53-Thr55 phosphorylation

Our laboratory has investigated the interactions between p53 and TAF1 using several TAF1 expression constructs (Figure 4.5). Overexpression of WT TAF1 in U2OS cells increases the levels of phosphorylated p53-Thr55 [20]. TAF1 N1398 A2/N7 Ala encodes a TAF1 protein carrying a series of kinase-inactivating mutations in the NTK and is truncated to eliminate the CTK [25]. Overexpression of this mutant (A2/N7 Ala) fails to increase p55-Thr55 phosphorylation [20]. TAF1-DD contains a point mutation in each bromodomain acetyl-lysine binding pocket resulting in the loss of the TAF1 DBrD/acetyl-p53 interaction, lower overall p53 interaction and loss of TAF1 recruitment to the p21 promoter [19]. I sought to determine whether the NTK or CTK of TAF1 is responsible for p53-Thr55 phosphorylation *in vivo*. Toward this aim, I constructed two TAF1 kinase mutant expression constructs to examine the role of each of these domains in p53-Thr55 phosphorylation *in vivo* (Figure 4.5). To preserve the p53/TAF1 interaction and to be able to evaluate the role of the NTK and CTK in p53 phosphorylation, I generated plasmids which express TAF1 proteins that contain an intact DBrD and lack the NTK or CTK. FL TAF1 A2/N7 Ala contains the mutant NTK domain derived from TAF1 N1398 A2/N7 Ala and retains the remainder of the full-length wild-type protein including the CTK. TAF1 N1646 encodes for a truncated TAF1 protein, which contains a functional NTK and retains the DBrD region but has lost the remainder of the CTK.

Construction of the pcDNA3.1(∗) Plasmid

To generate a FL TAF1 A2/N7 Ala construct it was necessary to first modify the multiple cloning site of pcDNA3.1(+) to contain the necessary type and orientation of restriction enzyme sites. A single-stranded DNA oligo containing NheI, ClaI, XbaI, BamHI and PspOMI cut sites and its reverse complement were annealed to create a dsDNA oligo. The multiple cloning site of pcDNA3.1(+) between NheI and PspOMI was replaced with this dsDNA oligo as follows. Both the oligo and pcDNA3.1(+) were digested with NheI and PspOMI. The digested oligonucleotide was ligated into the digested, CIP treated and purified pcDNA3.1(+) vector backbone. The resulting construct, pcDNA3.1(∗), was confirmed by ClaI digestion and sequencing (shown in Figure 4.6)

Construction of the pcDNA3.1(∗)FL TAF1 A2/N7 Ala Plasmid

A ClaI-XbaI fragment containing the Myc tag and partial TAF1 NTK region was excised from p-LXSN-MT-TAF1N1398-A2/N7-Ala [25] and subcloned into ClaI/XbaI digested pcDNA3.1(∗) to generate pcDNA3.1(∗) NTK A2/N7 Ala. This region of the NTK contains a series of alanine mutations in two regions, NT1 and N7, which abrogate the kinase activity of the domain [25]. Subsequently, an XbaI fragment containing the remaining COOH-terminal sequences of wild-type TAF1 from pCMV-HAhTAF1 was subcloned into pcDNA3.1(∗) NTK A2/N7 Ala to make pcDNA3.1(∗) FLTAF1 A2/N7 Ala. The resulting full-length TAF1 construct contains a catalytically inactive NTK and a wild-type CTK domain. The integrity of this construct was confirmed by restriction digest analysis and sequencing. Figure 4.7 shows the plasmid map for pcDNA3.1(∗) FL TAF1 A2/N7 Ala.

Construction of the pCMV-HAhTAF1 N1646 Plasmid

In order to generate a TAF1 construct which retains the double bromodomain but is truncated for the remaining CTK, Quikchange site-directed mutagenesis was performed using pCMV-HAhTAF1 as template and the following primers:
5'-GTACTGCTAAAGAAGCAGCTTAGTAGGAAGCAGAATTAGAAAGC-3'
and
5'-GCTTTCTAATTCTGCTTCCTACTAAGCTGCTTCTTTAGCAGTAC-3'. Mutagenesis of nucleotide 4991 from T to A and nucleotide 4993 from G to T (numbered according to NM_004606) results in two tandem stop codons at amino acids 1647 and 1648 immediately following the double bromodomain. This construct was confirmed by sequencing. Figure 4.8 shows the plasmid map for pCMV-HAhTAF1 N1646.

Overexpression of WT and Mutant TAF1 Constructs

I tested each of the TAF1 constructs for expression in U2OS cells. All of the generated constructs express the corresponding protein in U2OS as detected by Western blot (Figure 4.9). FL TAF1 A2/N7 Ala contains a c-Myc epitope tag and is detectable by Western blot with anti-TAF1 and anti-c-Myc antibodies. TAF1 N1646 contains an HA epitope tag and is detectable by Western blot with anti-TAF1 and anti-HA antibodies. As expected, TAF1-N1646 protein migrates slightly above the shorter truncation construct TAF1 N1398 A2/N7 Ala but below WT TAF1 (Figure 4.9, compare lanes 1, 3 and 5). Furthermore, FL-TAF1-A2/N7 Ala co-migrates with WT TAF1, TAF1-DD and endogenous TAF1. The expression levels of TAF1 N1398 A2/N7 Ala and TAF1 N1646 were regularly lower thanTAF1-WT,

-DD or FL TAF1 A2/N7 Ala. These results suggest that pcDNA3.1(∗)FL TAF1 A2/N7 Ala and pCMV-HAhTAF1 N1646 successfully express the expected FL TAF1 A2/N7 Ala and TAF1 N1646 mutants.

4.3.3 *NOXA* is a Putative Target for Termination of p53-mediated Transcriptional Activation by Thr55 Phosphorylation

Our laboratory has found that TAF1 can phosphorylate promoter-bound p53. Phosphorylation of p53-Thr55 in response to UV damage evicts p53 from the p21 promoter and coincides with termination of p53-mediated transcriptional activation of p21 (unpublished, X.L. and Y.W.). To investigate the possibility that the p53-mediated activation of other p53 target genes may be regulated similarly, I performed RT-qPCR and RT-PCR on RNA collected from UV-irradiated U2OS cells at 0, 2, 4, 6, 8, 12, and 16 hours post treatment. As expected, RT-qPCR shows that p21 is induced in response to UV: both mRNA and hnRNA (unspliced heteronuclear RNA) levels rise in response to UV treatment and appear to peak at 12 hrs post treatment. The levels of hnRNA present a more accurate picture of the rate of transcription by showing the levels of newly synthesized RNA. Consistent with previous findings in our laboratory, p21 hnRNA drops from 12 to 16 hours post UV treatment (Figure 4.10). Levels of p21 mRNA did not decrease as previously observed. The drop in p21 hnRNA suggested that I was able to recapitulate the TAF1-mediated termination of p53-activated transcription and that this sample set was appropriate to examine other p53 target genes for similar expression profiles. Next, utilizing the same RNA samples, I examined the mRNA and hnRNA levels of three well-known p53 target genes: *NOXA* (*PMAIP1*),

PUMA (*BBC3*), and *TIGAR*(*C12orf5*). As expected, *NOXA* mRNA and hnRNA levels rose in response to UV damage, peaking at about 8 hrs. Similar to p21, *NOXA* transcription was reduced following UV treatment; *NOXA* mRNA and hnRNA levels dropped off beginning between 8 to 12 hours after UV-treatment (Figure 4.11). In contrast, *PUMA* mRNA levels decreased slightly and *TIGAR* mRNA levels dramatically dropped in response to UV damage before returning to basal levels at later timepoints. This result is unexpected given that these genes are known targets for activation by p53 [2, 4, 27]. These preliminary results suggest that *NOXA* could be regulated, like p21, by TAF1-mediated termination of p53-activated p21 transcription.

4.4 Discussion

I have generated several tools to allow further dissection of the functional relationship between TAF1 and p53 in transcriptional regulation. Technical difficulties in generating proper control data precluded my ability to generate new data using the 2x3'BS p21-LUC and TAF1 constructs. However, these constructs will allow future study of the p53 and TAF1 interplay. The 2x3'BS p21-LUC construct provides the necessary p21 promoter to test the specific role of the 3' p53 DBS sequence in the p53-mediated TAF1 recruitment. The construction of the TAF1 mutants FL TAF1 A2/N7 Ala and TAF1 N1646 allow future testing the role of the CTK and NTK domains respectively, in Thr55 phosphorylation *in vivo*.

4.4.1 Theoretical Design of Assays to Assess the DBS-specific Recruitment of TAF1 by p53

Recent results suggest that the sequence of the 3' p53-DBS may be responsible for the p53-mediated differential recruitment of TAF1 to the 3' but not 5' p53-DBS at the p21 promoter. I planned to test this hypothesis using three approaches: an immobilized template system, chromatin immnoprecipitation (ChIP), and a reporter assay. Toward this end, I generated the 2x3'BS p21-LUC plasmid. In this p21 promoter reporter construct, the 20 bp sequence of the 5' p53 BS was replaced with that of the 3' site (Figure 4.3A). This promoter construct was to serve as the basis of the following experiments in order to test whether sequence of the 3' DBS is responsible for TAF1 recruitment through p53.

The Immobilized Template System

I planned to use an immobilized template system to examine whether the DNA sequence of the p53-DBS is involved in the differential recruitment of TAF1 by DNA bound p53 by testing whether purified, acetylated p53 is capable of recruiting TAF1 to the 3' p53-DBS but not the 5' p53-DBS. In this system, biotinylated DNA is immobilized to magnetic beads coated with covalently linked streptavidin. Our laboratory has previously used this system to demonstrate that acetylated p53 recruits TAF1 to p21 promoter *in vitro* [19]. For the proposed experiments a 246 bp HindIII-NsiI fragment of the p21 promoter containing the sequence surrounding and including only the distal p53-binding site location from -2281 to -2262 of the p21 promoter from p21-LUC (containing the wild-type promoter) or from 2x3'BS p21-LUC will be isolated and biotinylated (Figure 4.3A). The fragment isolated

from p21-LUC (WT 5' p53-DBS) contains only the 5' p53 DBS sequence while the fragment isolated from 2x3'BS p21-LUC (3' p53-DBS Distal) contains the 3' p53-DBS in the position of the 5' p53-DBS. These templates, WT 5' p53-DBS and 3' p53-DBS-Distal, can then be assayed for recruitment of TAF1 by acetyl-p53 as follows. Templates will be immobilized on the magnetic beads and washed. Next, baculovirus expressed and purified actylated p53 are pre-bound to the templates and any unbound p53 is subsequently washed away. Then, baculovirus expressed and purified TAF1 is incubated with the immobilized template for thirty minutes. Unbound TAF1 is washed away and the bound and supernatant fractions assayed for the presence of TAF1 by SDS-PAGE and immunoblotting. The interaction of TAF1 with p53 when bound to the 3' but not the 5' p53-DBS would support the hypothesis that the sequence of p53-DBS supports the recruitment of TAF1 by DNA-bound p53. In order to control that recruitment is due to template DNA, a control reaction should be utilized, which contains beads, acetylated p53 and TAF1 but no template DNA. To assess whether the recruitment of TAF1 requires acetylated p53, a control reaction containing unacetylated instead of acetylated p53 should be performed. The inability of unacetylated p53 to recruit TAF1, would demonstrate that the TAF1 specifically interacts with acetylated, DNA bound p53.

Chromatin Immunoprecipitation

The role of the p53 DBS sequence in p53-mediated TAF1 recruitment can also be tested *in vivo* using ChIP. To do this U2OS cells will be transfected with either p21-LUC or 2x3'p21-LUC for 24 hrs followed by an UV irradiation time course and ChIP analysis. Treated cells will be harvested at 0 (mock) and 4 hours post UV treatment and cross-linked. Immunopre-

cipitation will be performed for p53 and TAF1, and co-immunoprecipitated DNA subjected to PCR to detect enrichment for DNA at the 3' and 5' p53-DBS as well as the proximal promoter. To evaluate TAF1 recruitment at the distal p53-DBS it is necessary to distinguish the signal from the exogenous and endogenous p21 promoters. To accomplish this I designed a forward primer that anneals to the pGL2 backbone of the p21-LUC and 2x3' p21-LUC plasmids for use in the PCR detection step of ChIP. This new forward primer, when used in conjunction with the standard reverse primer for detection of occupancy at the distal binding site, detects PCR signal from exogenous p21-LUC DNA (Figure 4.3B).

In order to confirm the reliability of ChIP in our transient transfection system, it will be necessary to evaluate p53 binding to the 3' p53-DBS and proximal promoter region of the p21 promoter on these plasmids. This will be achieved by comparing the signal from transfected cells and non-transfected cells. A significant increase in PCR signal for transfected over non-transfected cells would indicate binding of p53 to the exogenous promoter. This result can also be confirmed by comparing the signal to noise ratios obtained for the endogenous and exogenous distal binding site. The use of qPCR would enhance the accuracy of these measurements. Successful detection of TAF1 at the distal p53 DBS in cells transfected with 2x3' p21-LUC would suggest that p53 can recruit TAF1 to this site in a sequence specific manner. A failure to detect TAF1-binding at this site suggests that either the surrounding sequences or the distance to the proximal promoter are responsible for directing TAF1 recruitment of TAF1 by p53.

Reporter Assays

Using the calcium phosphate transfection method, $p53^{-/-}$ H1299 cells will be transfected with the reporter plasmids p21-LUC or 2x3'BS p21-LUC, a constant amount of pcDNA-p53 and increasing levels of pCMV-HA-hTAF1. Alternatively, transfection using Lipofectamine 2000 may increase transfection consistency and efficiency. Using this system our laboratory has previously shown that for constant amount of p53, TAF1 increases transcription from the WT p21 promoter in a dose-dependent manner. This increase is dependent on the interaction of the TAF1 DBrD with acetylated COOH-terminus of p53 [19]. Similarly, if the sequence of the 3' p53 DBS is responsible for directing p53-mediated recruitment of TAF1, we expect that increasing the levels of TAF1 in this system will greatly enhance the expression of the reporter from 2x3'BS p21-LUC, as p53 can bind to two instead of one 3' p53-DBS. I failed to observe enhanced transcription with increasing levels of TAF1 in cells transfected with p21-LUC, precluding any conclusions concerning the effect of the second copy of the 3' p53 DBS or the role of TAF1 on transcription from 2x3'BS p21-LUC. Furthermore, variability in my calcium phosphate transfections prevented any conclusion on the effect of the 5' p53-DBS to 3' p53-DBS sequence swap on p53 driven-transcription.

4.4.2 Experimental Approach to Identify the Kinase Domain Responsible for p53-Thr55 Phosphorylation *In Vivo*

To evaluate the role of the CTK and NTK of TAF1 in p53-Thr55 phosphorylation, it will first be necessary to determine the levels of interaction of each TAF1 construct with p53. This is particularly important for FL TAF1 A2/N7 Ala to ensure that the truncated pro-

tein can still interact with p53 at levels similar to WT TAF1. To test this, U2OS cells overexpressing TAF1 constructs will be used for p53 immunoprecipitation and TAF1 immunoblotting can be used to assess whether or not the mutant proteins associate with p53 in vivo. Once confirmed that the mutant proteins bind p53, Western blot analyses of using cell extracts obtained from cells overexpressing one the mutant TAF1 proteins can be used to assess which of the mutant TAF1 proteins can phosphorylate p53-Thr55. WT TAF1 and A2/N7 Ala serve as positive and negative controls respectively. The comparison of p53-Thr55 phosphorylation in extracts containing FL TAF1 A2/N7 Ala or TAF1 N1646 will reveal whether the NTK, CTK, or both phosphorylate p53-Thr55 in vivo. Testing TAF1-DD will allow us to evaluate the role the DBrD-dependent interaction of TAF1 and p53 in p53-Thr55 phosphorylation. p53-Thr55 phosphorylation will be evaluated using a polycolonal antibody directed against phosphorylated p53-Thr55 designated PAb202 [11]. Following transfection with the appropriate TAF1 WT or mutant construct and MG132 treatment, phosphorylated p53-Thr55 will be immunoprecipitated from transient transfection lysates, using PAb202 followed by immunoblotting with anti-p53 antibody DO-1 to detect p53, or vice versa.

4.4.3 Potential Target Genes of Regulation by TAF1-mediated p53-Thr55 Phosphorylation

It remains unclear if the interaction between TAF1 and p53 on promoter DNA is a widespread phenomenon in p53-dependent transcriptional regulation. My preliminary results here suggest that termination of p53-mediated transcriptional activation of *NOXA* may be

regulated in a fashion similarly to p21. Downregulation of both *NOXA* and p21 transcription occurred coincides with a previously reported increase in ATP level in response to DNA damage; however, it remains unclear if the drop in transcription correlates with increased ATP levels since they were not measured in this experiment. My result is based on a single experiment, however, follow up experiments in the laboratory have since confirmed that *NOXA* transcription is reduced in response to UV at later timepoints. My preliminary results suggest that *PUMA* and *TIGAR* transcription is not repressed in response to UV damage in a fashion similar to p21 and *NOXA*. It should be noted, however, that the expression results I observed are unexpected. *PUMA* expression has been previously shown to increase in response to UV [15]. Expression of *TIGAR* increases in response to DNA damage from Adriamycin, Actinomycin D and IR [2, 4, 27]. In addition, our laboratory has found that p53 binding at the *TIGAR* promoter matches the pattern seen at the p21 promoter (unpublished, X.L. and J.L.). This apparent conflict in p53 promoter binding and expression may reflect the presence of other cofactors at the promoter or may be due to technical limitations or measurement from only a single assay. The techniques to use these tools have previously been applied successfully in our laboratory and provide a basis for future understanding of the dynamic relationship between p53 and TAF1. In addition, ongoing experiments by our laboratory using ChIP-seq and RNA-seq should identify other p53 target genes which are regulated by TAF1-mediated Thr55 phosphorylation.

4.5 Figures and Tables

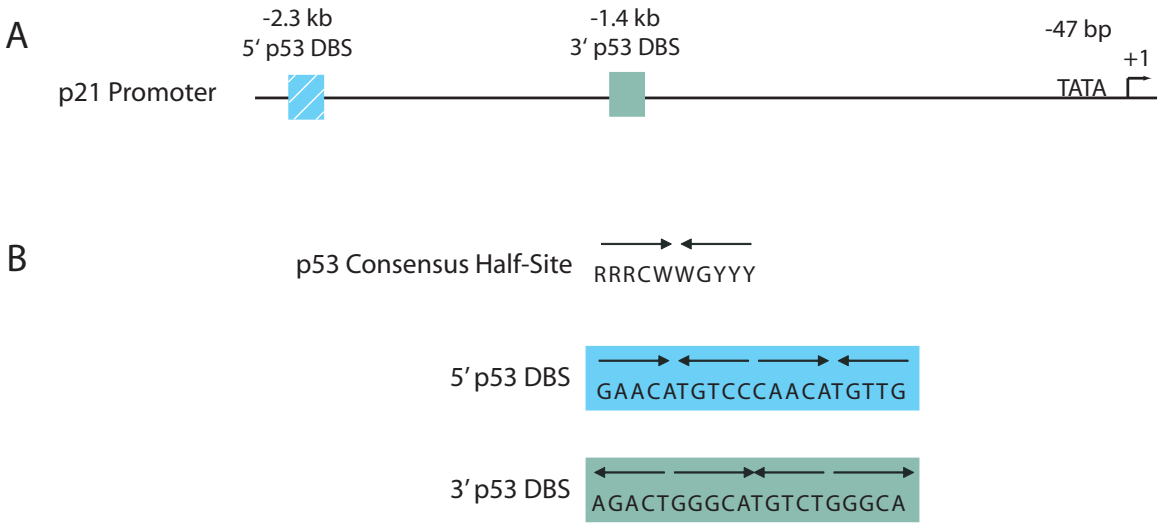


Figure 4.1. The p21 Promoter and p53 DNA Binding Sites
A. Schematic of the p21 (*CDKN1A*) promoter. Locations of the p53 DNA binding sites (DBS) and TATA box are shown relative to +1. **B.** The p53 consensus half-site motif is shown where R=A or G, W=A or T and Y=C or T. The sequence of the 5' and 3' p53 DNA binding sites (DBS) at the p21 promoter are shown. Arrows indicate the orientation of each quarter-site.

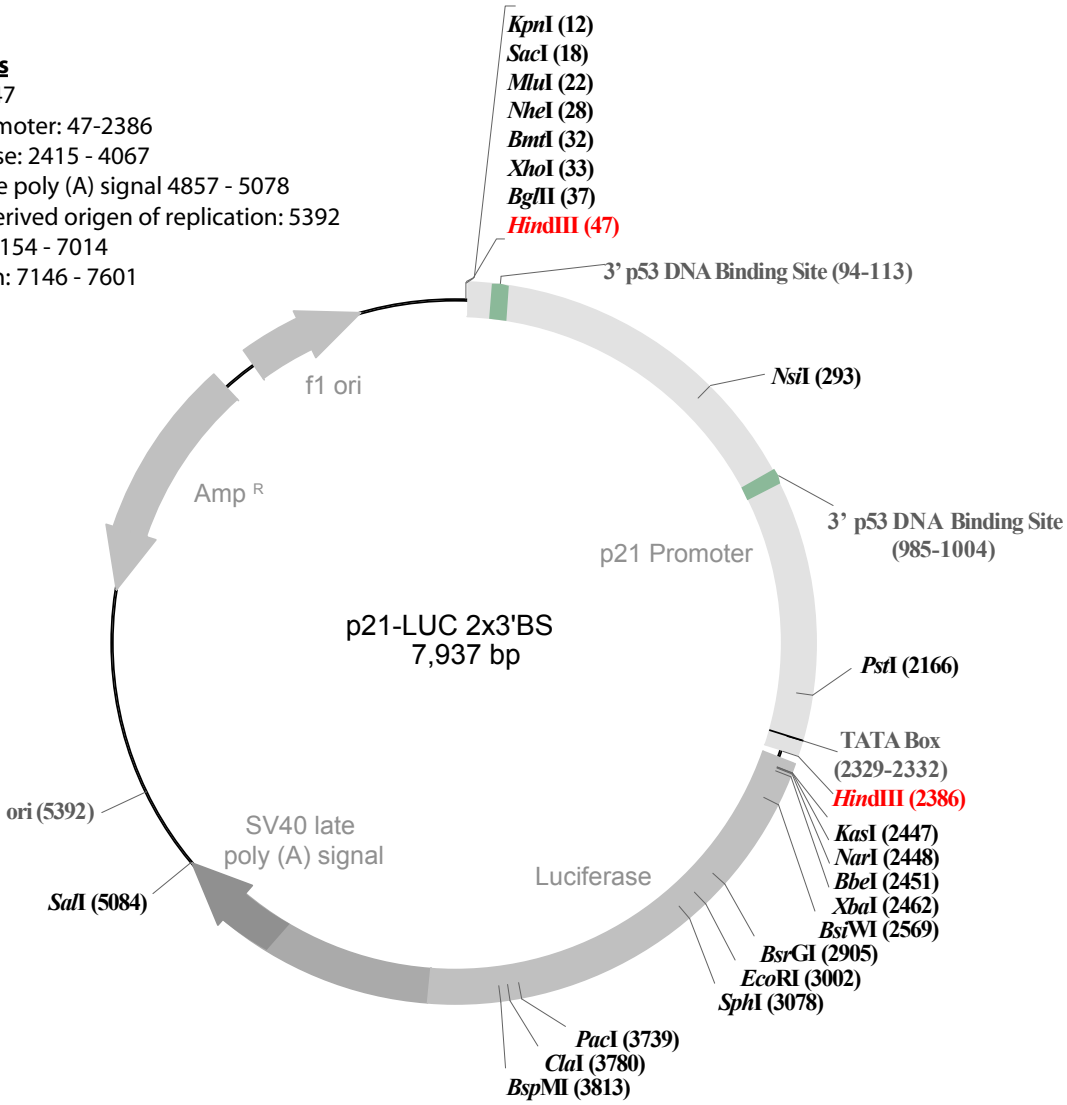


Figure 4.2. Map of the 2x3'BS p21-LUC Plasmid
Unique restriction sites are in black. 2x3'BS p21-LUC was generated from p21-LUC originally generated by the insertion of the 2.4 kb p21 promoter into the pGL2-basic backbone as a HindIII fragment [6]. In 2x3'BS p21-LUC the distal 5' p53 DBS sequence has been replaced with a copy of the proximal 3' p53 DBS sequence: AGACTGGGCATGTCTGGGCA. p21-LUC and 2x3'BS p21-LUC can be differentiated by digestion; replacement of the 5' p53 DBS with the 3' p53 DBS sequence eliminates a Sac I restriction site (which would cut at position 117).

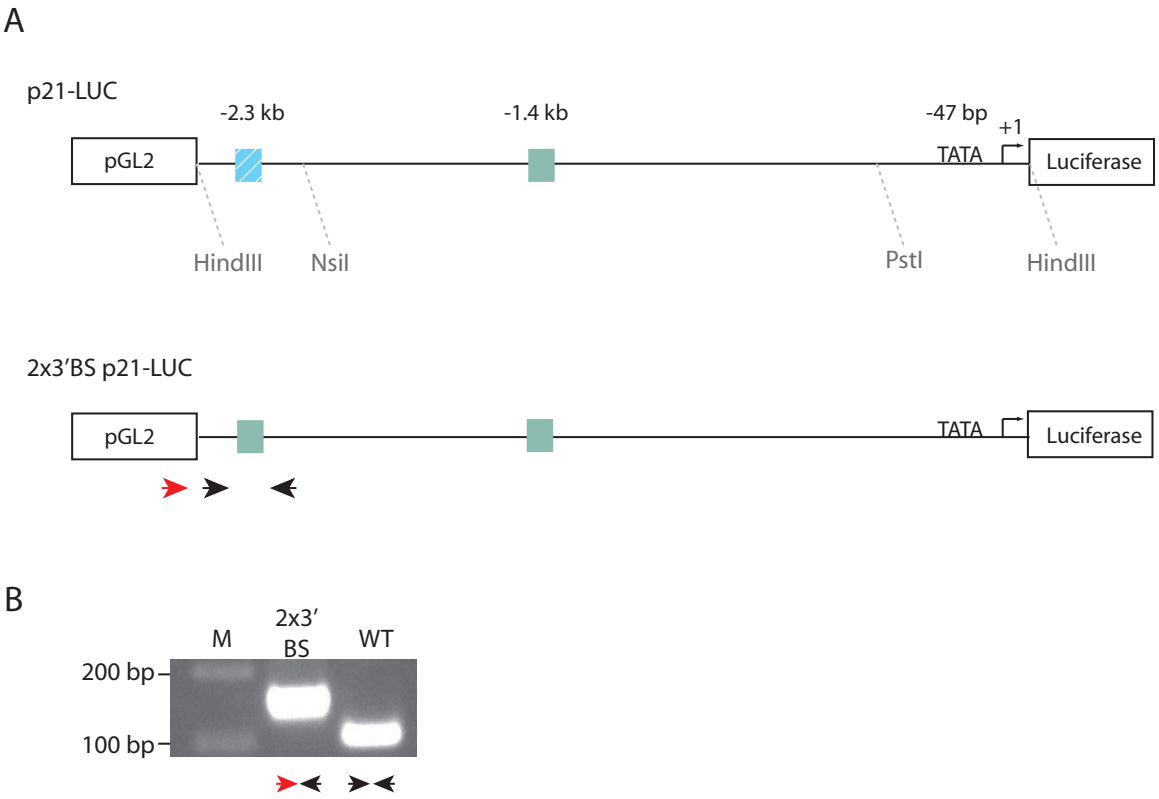


Figure 4.3. Construction of a p21LUC Construct Containing Two 3' p53 DNA Binding Sites
A. Schematic of the p21 promoter within the context of p21-LUC and 2x3'BS p21-LUC reporter constructs. Locations of the 5' p53 DBS (blue, hatched box), 3' p53 DBS (green box) and TATA box are shown relative to +1. Black arrow heads show the relative location of the wild-type (WT) ChIP 5' p53 DBS -forward and -reverse primers which recognize the p21 promoter. The red arrow head shows the pGL2 ChIP p53 DBS -Forward primer specific to the pGL2 plasmid backbone for detection of p53 binding on plasmids transfected into cells (Plasmid ChIP). See Appendix C, Table C.1 for primer information. The locations of unique restriction sites to be used for the isolation of promoter fragments in use with the *in vitro* immobilized template system are indicated in grey. Restriction sites are the same for both plasmids. **B.** Testing primers for plasmid ChIP. Using p21-LUC as a template PCR was performed using the primer pairs indicated in A. WT signal results in an amplicon of 105 bp. 2x3'BS signal results in an amplicon of 156 bp. M: Marker.

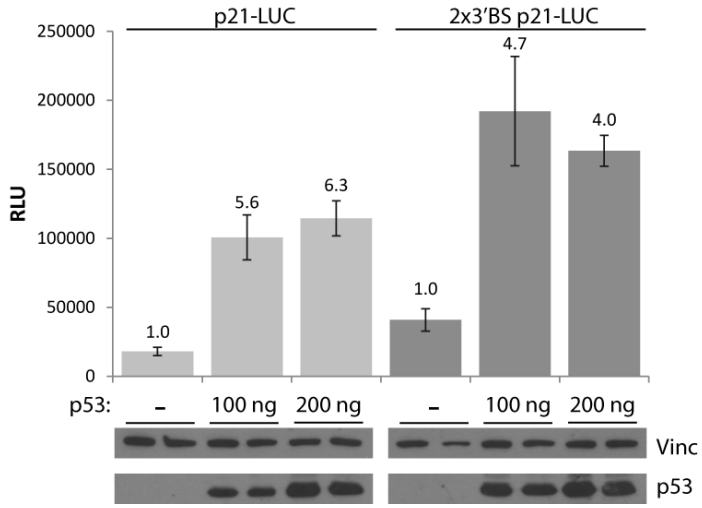


Figure 4.4. p53-driven Luciferase Expression from WT and 2x3'BS p21 Promoters
H1299 cells were transfected with p21-LUC or 2x3'BS p21-LUC and increasing amounts of a p53 expression vector. Shown is a single experiment demonstrating p53-driven expression from both reporters. Error bars represent one standard deviation for duplicate samples assayed. Upper panel: relative luciferase expression. Lower panel: expression of transfected cells as assayed by western blotting.

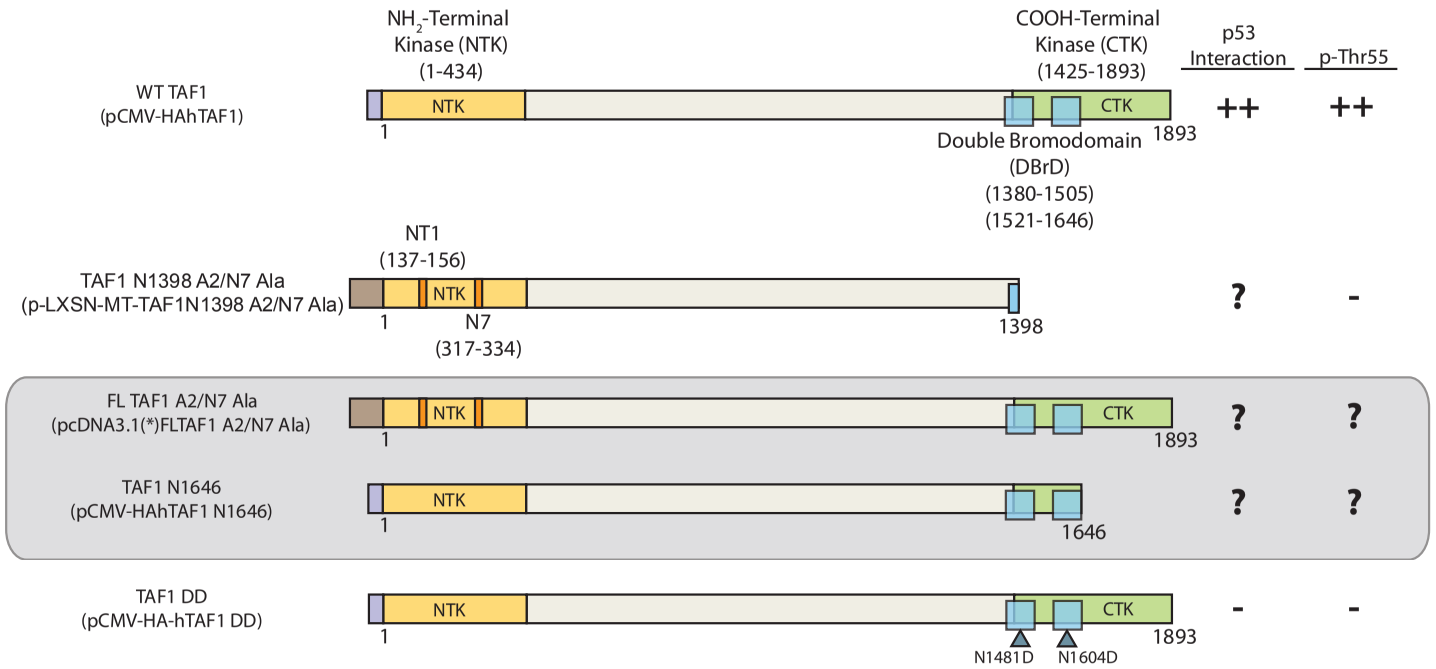
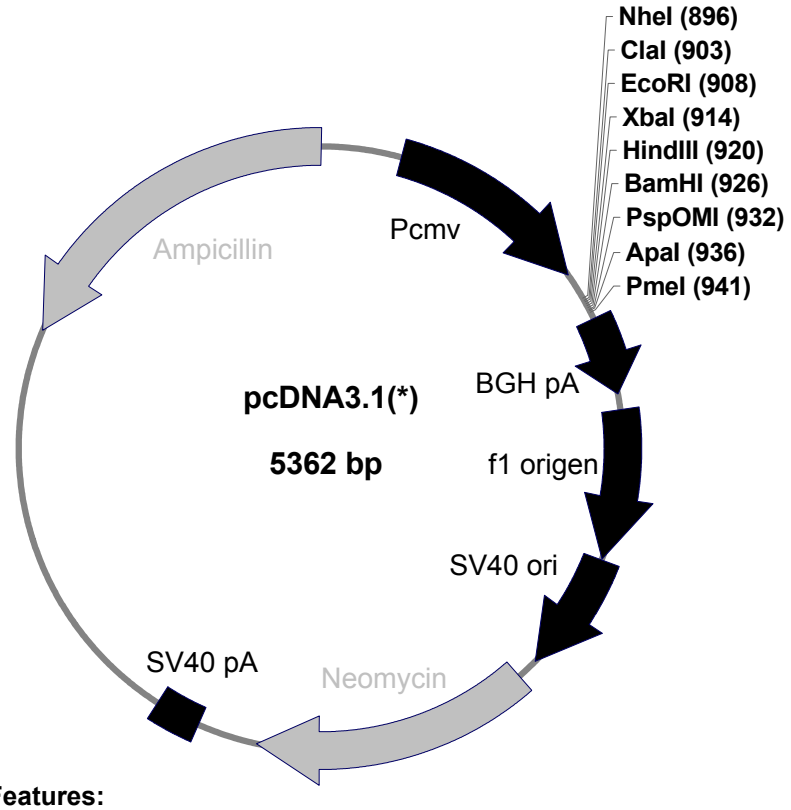


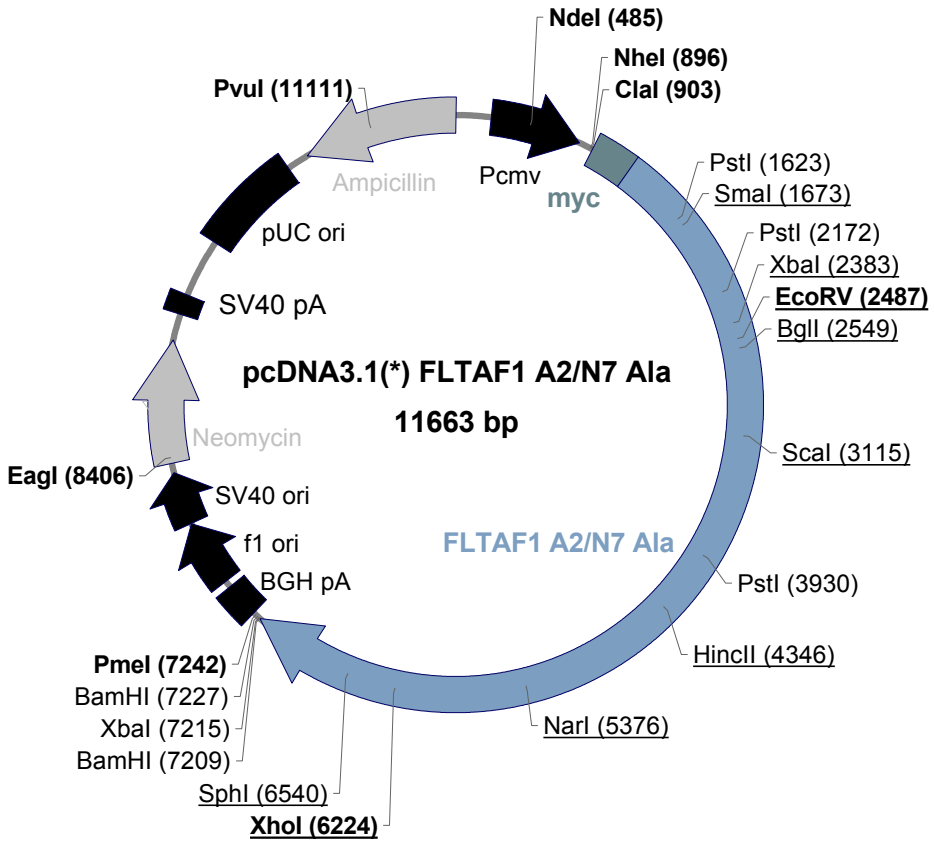
Figure 4.5. Schematic Diagram of WT and Mutant TAF1 Proteins

The total relative length of the expressed proteins, the kinase domains, and bromodomains of each TAF1 construct are shown. Full plasmid construct names are given in parenthesis. HA epitope tag is in purple. Myc eitope tag is in brown. WT TAF1: Wild-type TAF1 [30]. TAF1 N1398 A2/N7 Ala contains a series of alanine mutations in the NT1 and N7 regions (orange boxes) of the NTK rendering it inactive [25]. TAF1 DD contains two single N to D point mutations, one in each bromodomain (blue boxes), rendering it unable to bind to acetylated lysine residues. TAF1 DD has reduced interaction with p53 in solution, and fails to be recruited by p53 to the p21 promoter [19]. The two TAF1 kinase mutants generated in this work are highlighted in grey. FL TAF1 A2/N7 Ala contains the alanine mutations in the NT1 and N7 regions rendering the NTK domain non-functional but retains the wild-type full length CTK. TAF1 N1646 is truncated by the insertion of two stop codons immediately following the second bromodomain. If known, the ability of these TAF1 constructs to interact with p53 or phosphorylate p53 is indicated as follows. ++: strong interaction or phosphoryation. +: weak interaction or phosphorylation. -: No phosphorylation or interaction. ?: interaction or phosphorylation has yet to be determined.



Features:
CMV promoter: bases 239 - 819
T7 promoter/priming site: bases 863 - 882
Multiple Clone Site(*): bases 895 - 944
pcDNA3.1/BGH reverse priming site: bases 956 - 973
BGH polyadenylation sequence: bases 962 - 1186
f1 origen: bases 1232 - 1660
SV40 early promoter and origen: bases 1665 - 2008
Neomycin resistance gene (ORF): bases 2070 - 2864
SV40 early polyadenylation signal: bases 3038 - 3168
pUC origen: bases 3551 - 4221 (complementary strand)
Ampicillin resistance gene (*bla*): bases 4366 - 5362 (complementary strand)
ORF: bases 4366 - 5226 (complementary strand)
Ribosome binding site: bases 5234 - 5238 (complementary strand)
bla promoter (P3): bases 5261 - 5267 (complementary strand)

Figure 4.6. Map of the pcDNA3.1(∗) Plasmid
Unique restriction in the multiple cloning site (MCS) are shown. This plasmid was generated by replacing the MCS between NheI and PspOMI of pcDNA3.1(+) with an oligonucleotide containing the restriction sites shown.



Features:

CMV promoter: bases 239 - 819
T7 promoter/priming site: bases 863 - 882
pcDNA3.1/BGH reverse priming site: bases 7257 - 7274
BGH polyadenylation sequence: bases 7263 - 7487
f1 origen: bases 7533 - 7901
SV40 early promoter and origen: bases 7966 - 8309
Neomycin resistance gene (ORF): bases 8371 - 9165
SV40 early polyadenylation signal: bases 9339 - 9469
pUC origen: bases 9852 - 10522 (complementary strand)
Ampicillin resistance gene (*bla*): bases 10667 - 11663 (complementary strand)
ORF: bases 10667 - 11527 (complementary strand)
Ribosome binding site: bases 11535 - 11539 (complementary strand)
bla promoter (P3): bases 11562 - 11568 (complementary strand)

Figure 4.7. Map of the pcDNA3.1(∗)FL TAF1 A2/N7 Ala Plasmid
Unique restriction sites shown in bold. Sequencing of the NT1 and N7 regions revealed that the alanine mutations introduced within these regions produced two new PstI restriction sites, one in each region. FL TAF1 A2/N7 Ala expressed from this construct contains a kinase dead NTK but wild-type CTK.

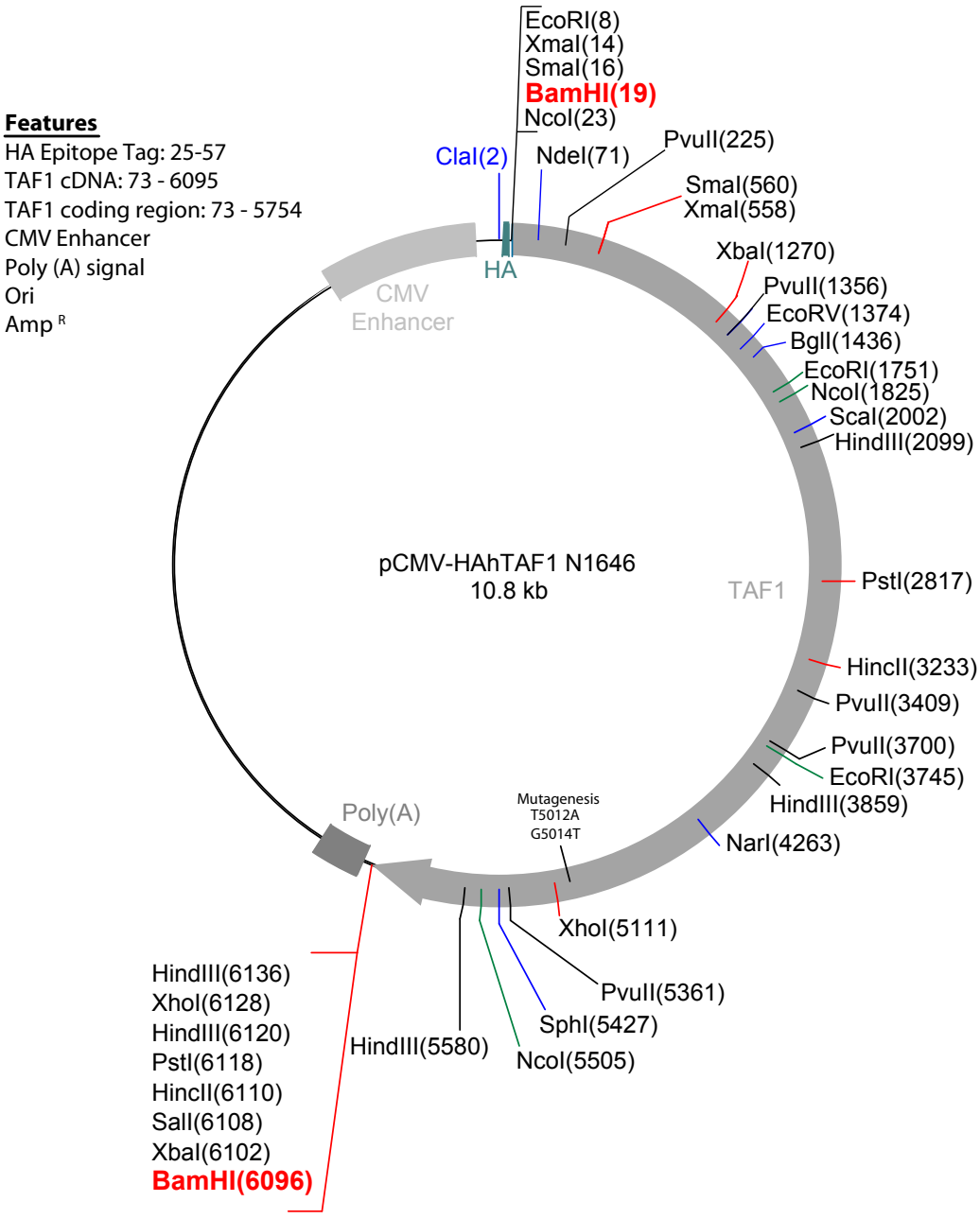


Figure 4.8. Map of the pCMV-HAhTAF1 N1646 Plasmid
Important restriction sites are shown. ClaI is unique to the plasmid. Precise location of the CMV enhancer, poly(A) signal, ori and ampicillin restance gene (*bla*) are unknown. The TAF1 N1646 protein expressed from this plasmid contains a wild-type NTK but is truncated at the CTK immediately following the DBrD.

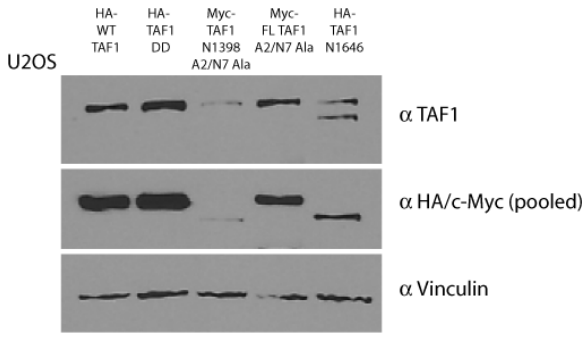


Figure 4.9. Overexpression of WT and Mutant TAF1 Constructs
U2OS cells were transfected with the indicated TAF1 constructs for 48 hours and whole cell extract subjected to Western blot to monitor expression. TAF1 overexpression was monitored using the 6B3 monoclonal antibody or by pooled anti-HA and anti-Myc antibodies.

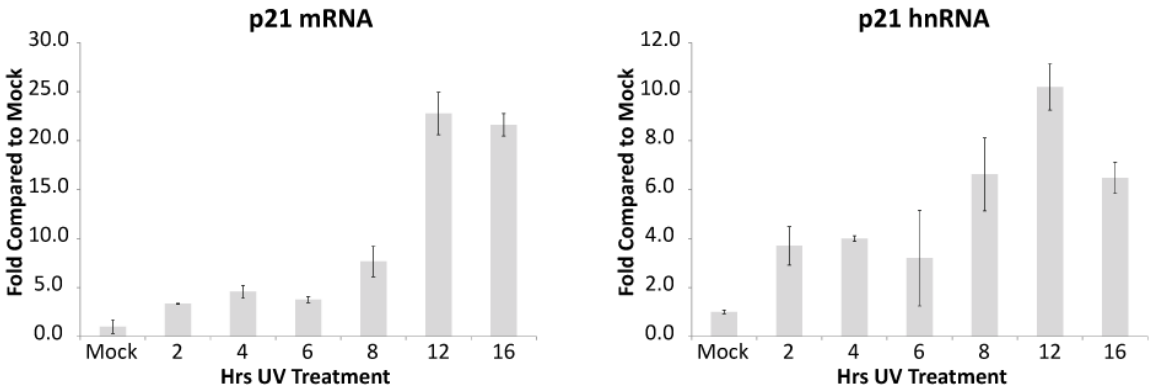


Figure 4.10. p21 is Regulated in a Time Dependent Manner Following UV Damage
U2OS cells were damaged with 20 J/M^2 UVC for the indicated times and p21 mRNA and hnRNA levels measured using RT-qPCR. This experiment was performed once. Error bars represent one standard deviation of replicate measurements. No RT control is shown in Figure 4.11. mRNA: messenger RNA, hnRNA: unspliced heteronuclear RNA.

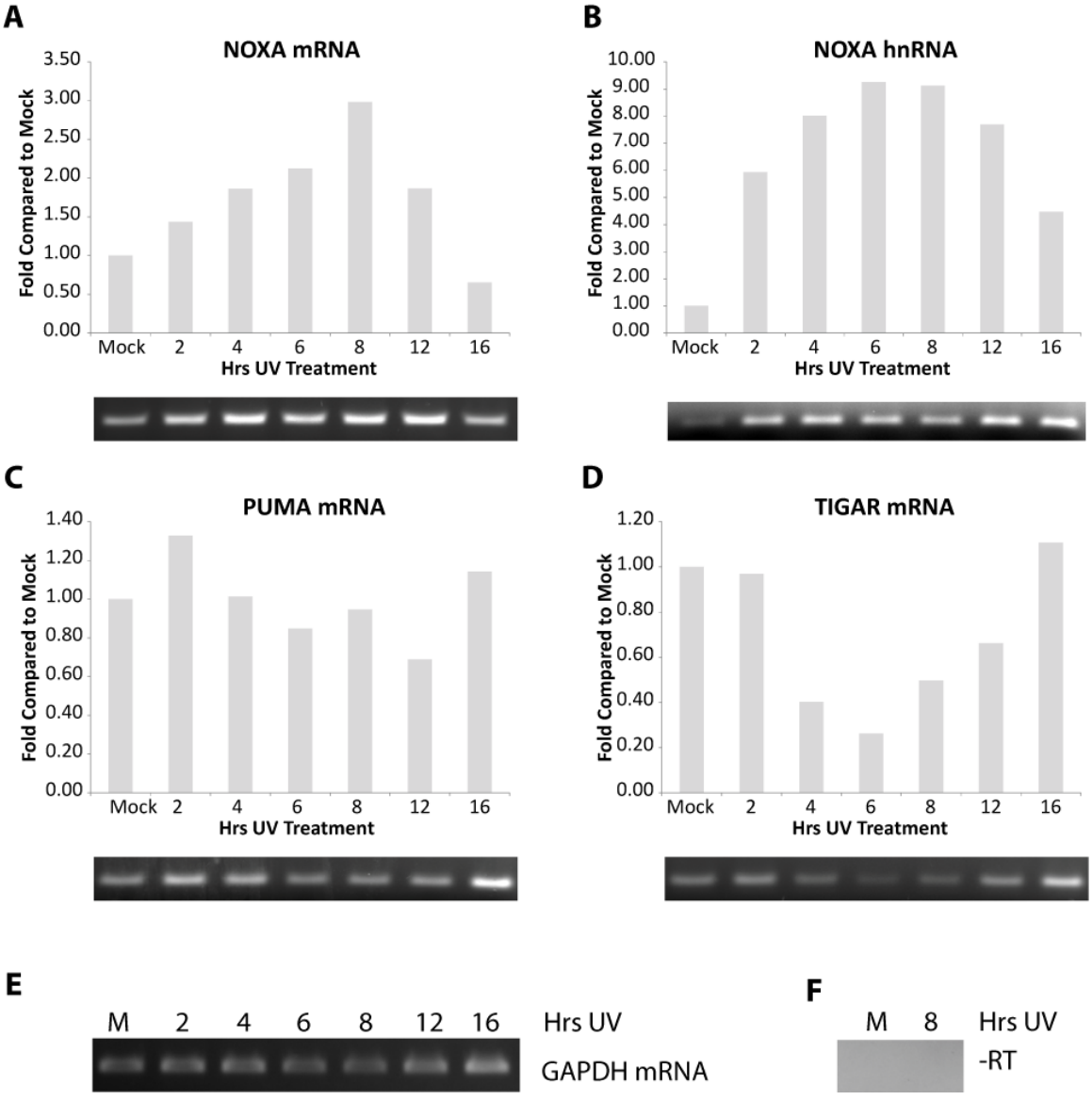


Figure 4.11. ***NOXA*** **May Be Regulated Similarly to p21 Following UV Damage**
U2OS cells were treated with 20 J/M^2 UVC for the indicated times. NOXA (**A** and **B.**), PUMA (**C.**) and TIGAR (**D.**) expression was measured by RT-PCR. **A-D**. Lower panel: ethidium staining of RT-PCR products run on a 2% agarose gel. Upper panel: bar graphs show the relative levels of RNA normalized to GAPDH signal (**E.**). **F.** No RT control. This experiment was performed once. mRNA: messenger RNA, hnRNA: unspliced heteronuclear RNA.

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Chapter 5

Conclusions

The tumor suppressor p53 functions as a transcription factor (TF) that regulates the transcription of numerous target genes in response to cellular stress and damage. p53 controls the expression of genes, whose activities are involved in cell cycle arrest, DNA repair, apoptosis, and senescence [6]. The inactivation of p53 results in tumor formation in mice and mutations in p53 have been associated with the majority of human cancers [26]. The activity of p53 is tightly regulated by mechanisms that govern the level, sub-cellular localization, and DNA binding activity of the TF [4]. Our laboratory has previously described that phosphorylation of threonine residue 55 in p53 (p53-Thr55) plays a pivotal role in regulating the activity of p53. Phosphorylation of p53-Thr55 increases (i) the interaction of p53 with MDM2, the primary E3 ubiquitin ligase responsible for p53 degradation [28], (ii) promotes nuclear export of p53, and (iii) reduces the association of p53 with target genes (unpublished, X.L., Y.W. and J.L.; [9, 36]). Our laboratory recently found that TAF1 can phosphorylate p53-Thr55 and that the kinase activity of TAF1 is regulated by

changes in intracellular ATP levels (unpublished, X.L. and Y.W.). Increased intracellular ATP concentrations following UV damage or treatment with high glucose (HG) lead to increased TAF1 kinase activity, p53-Thr55 phosphorylation, reduced p53 binding at the p21 (*CDKN1A*) promoter in vivo, and culminates in attenuation of p21 transcription (Chapter 2; unpublished, X.L. and Y.W.). These findings raised the questions: Is TAF1-mediated phosphorylation of p53 involved in regulating the transcription of genes other than p21? Does TAF1-mediated phosphorylation control the activity of TFs other than p53? In this work I have sought to answer these questions by using molecular, biochemical and bioinformatics approaches.

Microarray Analysis of High Glucose Induced Changes in Gene Expression

To identify p53 target genes, whose expression may be regulated by TAF1 kinase activity in a HG-dependent fashion, we designed genome-wide transcription profiling assays using DNA microarrays (Affymetrix). I planned to compare the expression profiles of mock-treated with HG-treated cells and HG-treated cells lacking p53 and/or TAF1. Towards this aim, I recapitulated the described HG-induced downregulation of p21 and confirmed the optimal conditions for treatment (Chapter 2). HG-induced p21 downregulation was abolished in TAF1- and p53-RNAi knockdown cells, and in p53 knockout cells, indicating that HG-induced p21 downregulation is TAF1- and p53-dependent. Treatment with 20 mM glucose for 6-8 hrs was confirmed to be the optimal condition for downregulation of p21. In the future, RNA harvested under these conditions should be used for genome-wide transcription profiling assays. I expect that the comparison of the transcription profiles of mock-treated cells with HG-treated cells and HG-treated cells lacking p53 and/or TAF1

will identify genes, whose transcription is regulated by TAF1 and p53 in a HG-dependent fashion. Once these genes have been identified, molecular, cell and biochemical assays can be employed to assess whether, similar to p21, the transcription of the identified genes involves HG-induced phosphorylation of p53-Thr55 by TAF1.

Putative Targets for Regulation by p53-dependent Phosphorylation of p53-Thr55 Regulation

Since the designed genome-wide transcription profiling assays were not completed during the course of this dissertation, I used a bioinformatics approach to identify p53 target genes potentially regulated by TAF1-mediated p53-Thr55 phosphorylation (Chapter3). I compared p53 target genes regulated across a variety of stresses to genes bound by TAF1 and regulated by HG [2, 3, 8, 19, 22, 30, 32, 43, 46, 49].

The data used for this analysis came from a number of different studies. All these studies were performed in human cells. However the cell type and the form, intensity and duration of stress all varied. In addition, my ability to identify genes of interest is limited by the experimental techniques utilized by these studies. Thus, my analysis is inherently based on the correlation and will require cellular and biochemical assays to determine whether any of the genes identified are indeed regulated through TAF1/p53 interaction and p53-Thr55 phosphorylation. In light of this, I identified p53 target genes whose transcription may be TAF1- and HG-dependent. This analysis revealed that the transcription of only a small subset of p53 target genes (approx. 8%) are sensitive to glucose levels or bound by TAF1. These include known, characterized p53 target genes as well as those that have yet to be validated as direct p53 targets.

Notably, I identified 12 p53-activated genes bound by TAF1 and whose transcription is decreased under HG conditions. This is similar to the observed transcriptional decrease of p21 and implies that the transcription of the identified genes may be repressed by TAF1-mediated phosphorylation of p53-Thr55. In agreement with our previous studies, I found p21 among the 12 identified genes. This result reveals that the comparison of genome-wide transcription and protein-DNA interaction assays which detect target genes of p53, TAF1, and HG, can identify target genes of p53 and TAF1 whose activity may be downregulated in response HG. Importantly, my results suggest that *NOXA* is regulated in a similar manner to p21 following DNA damage (Chapter 4). This result is supported by my bioinformatics analysis, which revealed that TAF1 binds the *NOXA* promoter and that *NOXA* transcription is downregulated in response to HG.

Given that p53-Thr55 phosphorylation can promote nuclear export and degradation of p53, it is possible that HG can also lead to derepression of p53 target genes. I identified 11 p53-repressed target genes whose transcription is activated in response to HG. Whether TAF1 interacts with p53 at the promoter of repressed target genes is unknown. My analysis revealed that TAF1 bound only one of the repressed p53 target genes activated by HG and consequently it remains unknown whether TAF1 per se and/or TAF1-dependent p53-Thr55 phosphorylation can alleviate p53-mediated repression. The identified genes represent suitable models to assess the role of TAF1, p53, and HG-dependent phosphorylation of p53-Thr55 in transcriptional regulation of p53 target genes. In summary, the results of others and my work support a model whereby high ATP levels, in response to DNA damage or HG treatment, increases TAF1 kinase activity and subsequently p53-Thr55 phosphorylation,

thereby inhibiting the ability of p53 to transactivate and repress target genes (Discussed in detail in Figure 5.1).

During the course of my bioinformatics study I identified p53 targets which have been found to be regulated in response to multiple stress conditions [3, 19, 30, 32, 43, 46, 49]. I found a core set of six p53 target genes that are activated in response to both genotoxic and non-genotoxic p53 activating stimuli, supporting the recently proposed existence of a default p53 program. [32]. According to the proposed program, p53 binds a select subset of p53 target genes in response to three p53-activating stimuli: RITA, nutlin3a and 5-FU. Three of these genes are TAF1 target genes that are downregulated by HG, suggesting a role for TAF1-mediated p53-Thr55 phosphorylation in the regulation of the general p53 transcriptional response.

HG Impairment of p53 Activity

Recent studies have associated hyperglycemia and diabetes with increased cancer risk [13, 17, 31, 34, 39]. On the basis of the role of p53 as a critical tumor suppressor, and because HG can downregulate p53 under non-stressed conditions, I tested the effects of HG on p53 in response to UV damage (Chapter 2). Generally, in response to UV damage p53 protein is stabilized and p53 levels rise. I treated cells with HG prior to UV and monitored p53 protein levels. Compared to UV only treated cells, p53 protein levels were reduced 4 hrs post UV damage in HG- and UV-treated cells, suggesting that HG prevents an increase in p53 levels in response to UV. Indeed, our preliminary data have shown that the observed decrease in p53 levels coincides with repression of p21 transcription. In addition, *NOXA* expression follows a similar pattern as p21 at late time points following UV

damage, suggesting it may be similarly regulated by p53-Thr55 phosphorylation (Chapter 4). Furthermore, my bioinformatics analysis has identified several other key p53 target genes including *GADD45A* and *DDB2*, whose expression is repressed by HG. My results raise the possibility that by reducing the p53 protein level, HG may cause changes in the p53-mediated response to cellular damage such as UV and provides a possible mechanism contributing to the increased cancer risk in diabetics. However, the ultimate physiological consequences of the observed HG-induced reduction of the cellular p53 level and consequently p53 activity remain to be determined.

Putative Transcription Factor Phosphorylation by TAF1

Given the ability of TAF1 to regulate transcription through p53-Thr55 phosphorylation, the question arises as to whether TAF1 phosphorylates TFs other than p53. The identification of genes whose transcription is HG- and TAF1-dependent but p53-independent provided an opportunity to investigate the possibility that the HG-induced stimulation of TAF1 kinase activity controls gene expression. I hypothesized that TAF1-dependent phosphorylation of TFs may represent a general, rather than p53-specific, fashion to regulate transcription. HG-dependent phosphorylation of p53-Thr55 by TAF1 results in eviction of p53 from the p21 promoter and repression of p21 transcription (Chapter 2; unpublished, X.L. and Y.W.). Thus, TFs, which are overrepresented at genes, whose transcription is downregulated by HG, represent possible substrates for TAF1-dependent phosphorylation. I used a bioinformatics approach that detected enriched TF DNA binding sites in the regulatory regions of HG-downregulated genes, which allowed me to identify TFs that could potentially be involved in regulating the expression of HG- and TAF1-dependent genes.

My approach identified approximately 54 potential TF substrates. Consistent with the role of TAF1 as a regulator of cell cycle progression, a number of these putative substrates play a role in controlling the expression of genes involved in cell cycle progression such as Myc, E2F, and NF-Y.

Several of the identified TFs regulate both Cyclin D1 and Cyclin A. In light of this and recent findings on TAF1 kinase activity I propose the following model for the regulation of Cyclin D1 and Cyclin A transcription (Figure 5.2). Before TAF1-dependent Cyclin D1 and Cyclin A transcription begins, TAF7 binds TAF1 thereby inhibiting TAF1 histone acetyl transferase activity [15, 25]. Upon completion of PIC assembly *in vitro*, TAF1 phosphorylates TAF7 [16]. *In vivo* and *in vitro*, TAF1 phosphorylation of TAF7 is concomitant with TAF7 dissociation and activation of TAF1 HAT activity [16, 25]. The ensuing histone H3 acetylation at the promoter is thought to create a more permissible chromatin environment for Sp1 binding and could presumably promote the binding of other TFs to further upregulate transcription [16, 18, 25]. In the absence of a proliferation signal Rb can bind and inhibit the NH_2-terminal kinase (NTK) domain of TAF1 [40, 41]. Initial transcription of Cyclin D1 can stimulate a positive feedback loop whereby Cyclin D1 suppresses Rb-mediated inhibition of TAF1 kinase activity and further accelerates transcription [42].

ATP levels increase throughout G1 and S phases and drop slightly during G2/M [45]. Increased ATP levels stimulate TAF1 kinase activity (unpublished, X.L. and Y.W.). Progression through G1 and S phases may be partially regulated through ATP-dependent regulation of TAF1 kinase activity. The establishment of precise ATP level provides a new checkpoint for cell cycle progression by ensuring that sufficient energy is available for and through S-phase. In addition, active TAF1 may subsequently phosphorylate a number of

TFs bound at the promoter of their target genes. I have identified several TFs that are putative substrates for phosphorylation by TAF1 and bind the Cyclin D1 and Cylin A promoters (Chapter 3, Figure 5.2). TAF1-mediated phosphorylation of Myc or E2F1 could be one mechanism to relieve transcriptional repression at the Cyclin D1 promoter [24, 35]. To terminate activator-mediated transcriptional activation, TAF1 may also phosphorylate a number of these TFs including Sp1, NFκB, Ets1, c-Myb, CREB, ATF, Myc and NF-Y. Phosphorylation has been shown to negatively regulate DNA binding or stability of Sp1, Ets1, c-Myb, CREB and Myc [10, 14, 20, 38, 44, 47]. Acetylation of NFκB functions as one mechanism to alleviate NFκB-mediated transcription. Because TAF1 has HAT-activity, it is possible that TAF1-mediated acetylation of NFκB could mediate downregulation of NFκB target genes [21]. In summary, the ability of TAF1 to inactivate several TFs through post-translational modification in response to stresses and changing energetic conditions may contribute to the regulation of cell cycle progression.

Interestingly, Myc is among the TFs identified and an interaction between Myc and TAF1 has been previously reported [5]. Myc regulates a number of metabolic genes that can promote aerobic glycolysis or oxidative phosphorylation and promote tumorigenesis [12]. ATP-induced, TAF1-mediated downregulation of Myc could serve as a mechanism to modulate energy homeostasis in the cell. Furthermore, the roles of TAF1 and Myc in G1 progression strongly support the study of their interaction in future studies.

5.1 Future Work

My work has laid the foundation for future experiments to examine the functional importance of the interplay of TAF1 with p53 in regulating the expression of genes, whose activities control cell cycle progression and other biological processes. I identified p53 target genes potentially regulated by HG -induced, TAF1-mediated p53-Thr55 phosphorylation. Based on my preliminary results suggesting that HG can impair p53 in response to UV damage, our laboratory has decided to use genome-wide transcription profiling to measure HG-induced gene expression in UV damaged cells treated with or without HG. This approach will allow us to identify p53 targets affected by HG that contribute to the p53 DNA damage response. It will be of particular interest to see which, if any, of the putative targets identified in my bioinformatics analyses are affected by HG in response to UV.

It would be of interest to assess whether or not HG can lead to the derepression of p53-repressed target genes via TAF1-mediated p53-Thr55 phosphorylation. I have identified HG de-repressed p53 target genes in Chapter 3, which can be used to address this question. To test this, RT-qPCR measurements detecting the transcript levels of the putatively de-repressed genes using RNA pools isolated from untreated, HG-treated or HG- and apigenin-treated cells would provide a rapid answer to this question. If TAF1-mediated phosphorylation of p53-Thr55 is involved in derepression of the identified p53 target genes, HG-induced derepression of these genes should be eliminated in the presence of apigenin, which inhibits TAF1 kinase activity. Any such derepression would most likely rely on HG-induced global drop in p53 levels.

In this work I have also generated tools to further examine the dynamic relationship between TAF1 and p53. p53 binds two DNA binding sites in the p21 promoter. DNA-bound p53 recruits TAF1 to the 3' but not 5' p53 DNA binding site, suggesting that the p53/TAF1 interaction depends on the surrounding context, distance from the core promoter or sequence of the p53 DNA binding site itself. I generated a p21 promoter construct where the 5' p53 DNA binding site sequence has been replaced with that of the 3' p53 DNA binding site. This construct will allow future study of the differential recruitment of TAF1 to the 3' but not the 5' p53 DNA binding site of the p21 promoter. Understanding the mechanisms underlying the DNA binding site dependent recruitment of TAF1 to promoters by p53 may provide further insights into (i) the basic mechanisms underlying p53 transcriptional regulation, (ii) recruitment of TAF1 and TFIID to promoters, and (iii) the role of p53 DNA binding site in promoting TAF1-mediated phosphorylation of p53-Thr55.

I have also generated two TAF1 kinase mutants to identify the kinase domain of TAF1 responsible for p53-Thr55 phosphorylation. FL TAF1-A2/N7-Ala contains alanine mutations which inactivate the NTK, and retains a functional COOH-terminal kinase (CTK) domain. TAF1 N1646 lacks the CTK but contains a wild-type NTK domain. An initial assessment of the role of the NTK and CTK domains in p53-Thr55 phosphorylation can be achieved using these constructs. Following overexpression of each of these TAF1 mutant constructs, p53-Thr55 phosphorylation levels can be evaluated by immunoprecipitation using the phospho-specific Ab202 antibody against p53-Thr55 phosphorylation and subsequent Western blot using the p53 antibody DO-1. In addition, these constructs can be used to study CTK or NTK specific regulatory functions of TAF1. The NTK regulates 6% of all transcription events in ts13 hamster cells [33]. Using these mutant constructs we

can further evaluate the role of TAF1 kinase activity in gene regulation. Others in our laboratory have recently modified these TAF1 kinase mutant constructs to be RNAi resistant. To evaluate the role of TAF1 kinase activity, both the NTK and CTK, in gene regulation, endogenous TAF1 can be knocked down concurrent with overexpression of RNAi resistant TAF1 kinase mutants. This would allow for the isolation of RNA pools for genome-wide transcription profiling to evaluate changes in gene expression due to loss of TAF1 NTK or CTK activity. These experiments can also provide further insight into the p53/TAF1 network by allowing us to identify the p53 target genes misregulated by these TAF1 kinase mutants.

Implications of HG on Cancer and Diabetes

HG-induced downregulation of p53 target genes occurs at glucose concentrations (12-39 mM) associated with hyperglycemia and diabetes [11, 23, 37]. This downregulation of p53 activity may contribute to the increased cancer risk observed in diabetics [13, 31, 34, 39]. In addition, I have shown that glucose at diabetic levels can reduce the activity of the tumor suppressor p53 (Chapter 2). Hyperglycemia has been shown to induce DNA damage and reduce the efficacy of DNA repair [7, 48]. Thus, diabetics may be hit with a one-two punch: hyperglycemia-induced DNA damage and downregulation of p53 activity. In addition, HG may cause misregulation of cell cycle related genes such as Cyclin D1 and Cyclin A through TAF1. This highlights the importance of recent findings showing that lowering blood glucose levels in diabetics can reduce cancer risk [27, 29]. Apigenin, is a naturally occurring flavonoid found in high levels in parsley, celery and rutabagas [1]. Apigenin inhibits the kinase activity of TAF1 and has been shown to sensitize cells to

DNA damage by restoring nuclear localization of cytoplasmic sequestered wild-type p53 in neuroblastoma cells [9]. Regulation of blood glucose levels and dietary supplement with foods rich in apigenin should help to alleviate the potential cancer risk resulting from the aberrant activity of the p53/TAF1 network in diabetics.

My work provides new tools for the dissection of the TAF1/p53 interaction in transcriptional regulation. My bioinformatics findings suggest that TAF1-mediated p53-Thr55 phosphorylation may regulate the transcription of a subset of p53 target genes. In addition, I have identified a number of possible transcription factors that may be regulated in a similar manner and implicate TAF1-mediated phosphorylation in the regulation of cell cycle progression. It will be of great interest to continue to characterize the TAF1 and p53 interplay and the role of TAF1 phosphorylation in transcriptional regulation.

5.2 Figures and Tables

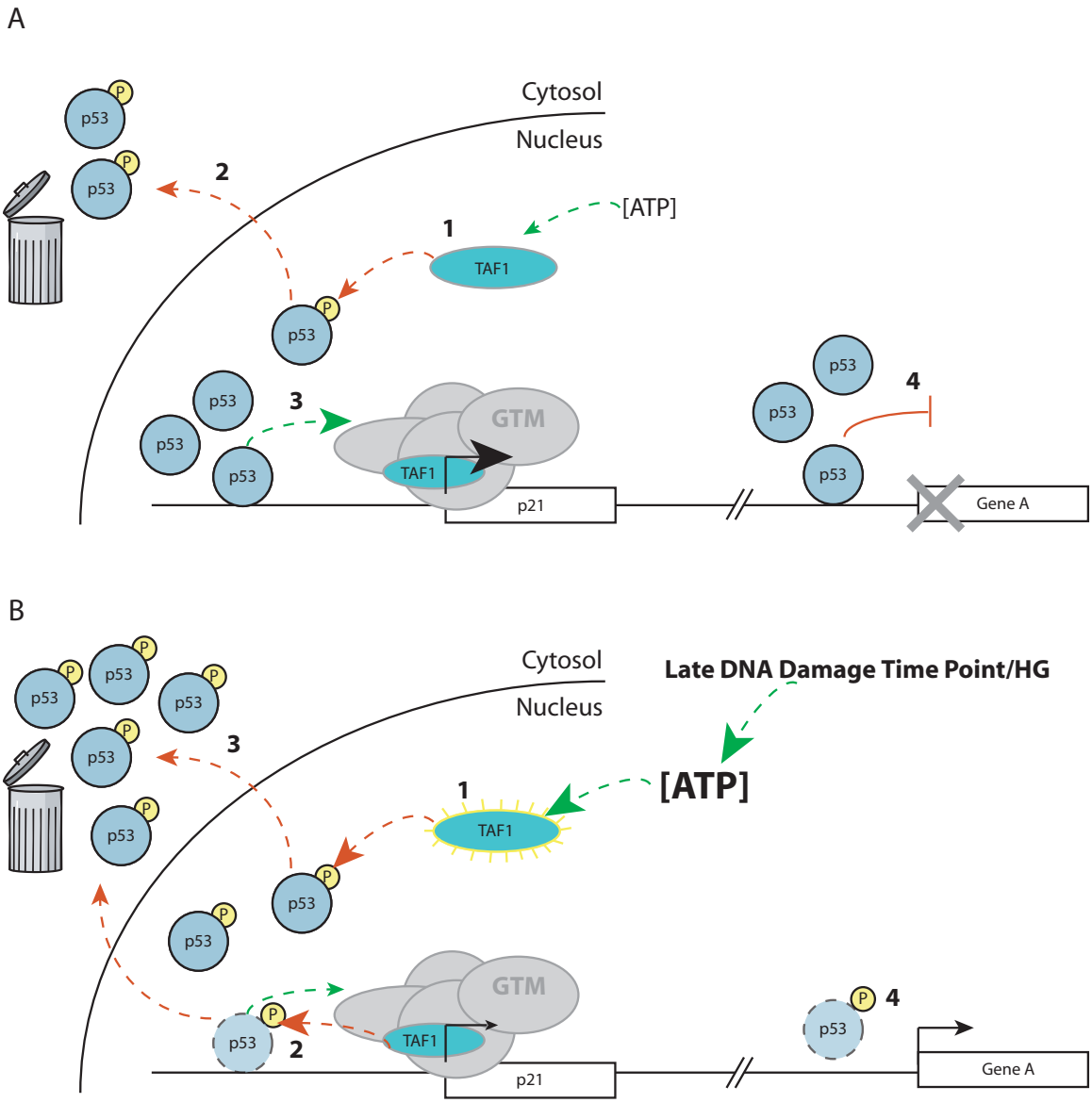

Figure 5.1. A model for the regulation of p53 by TAF1-mediated Thr55 phosphorylation. **A.** Under basal growth conditions TAF1 phosphorylates p53 at Thr55 (1), thereby promoting nuclear export and degradation of p53 (2). Some basal level of p53-dependent p21 expression is observed under normal growth conditions and p53 activation leads to p21 transcription following DNA damage (3). In addition, p53 can repress some of its target genes (4). **B.** Increased levels of intracellular ATP at later stages following DNA damage or following HG treatment levels increases TAF1-mediated phosphorylation of p53 (1,3). This leads to a drop in p53 DNA binding (3,4) and increased nuclear export and degradation (2). As a result p21 transcription is downregulated (3) and p53 repressed targets may be activated (4).

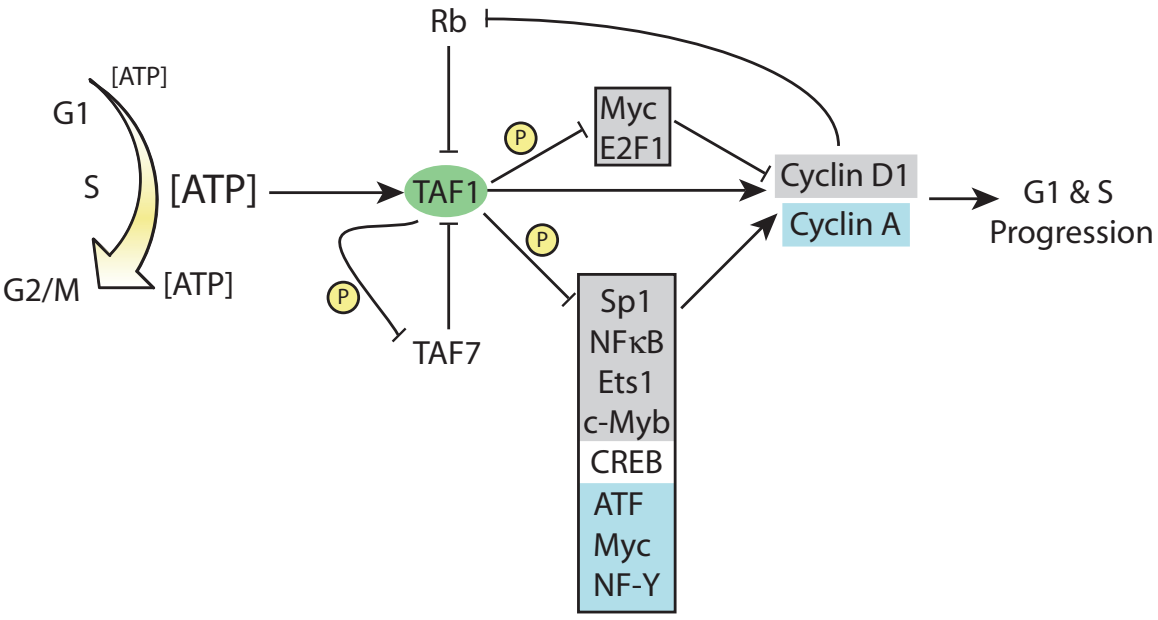

Figure 5.2. A model for TAF1 kinase activity in G1 progression. Prior to transcriptional activation the N-terminal kinase (NTK) domain of TAF1 is bound and inhibited by Rb [40, 41]. TAF7 binds TAF1 and inhibits TAF1 histone acetyl transferase activity (HAT) [15, 25]. During the course of G1 and S phases ATP levels rise before falling toward G2/M [45]. Increased ATP levels increase TAF1 kinase activity (unpublished). TAF1 subsequently phosphorylates TAF7 resulting in dissociation of the two factors, activation of TAF1 HAT activity, and promoter histone H3 acetylation [16, 18, 25]. This is suggested to facilitate Sp1 binding [18]. These events promote efficient Cyclin A and Cyclin D1 transcription [16, 25]. Cyclin D1 acts as part of a positive feedback loop by supressing Rb-mediated inhibition of TAF1 kinase activity [42]. TAF1 may phosphorylate a number of transcription factors associated with these promoters to regulate transcription. Phosphorylation of Myc and E2F1 by TAF1 could relieve Cyclin D1 repression [24, 35]. Phosphorylation of transcriptional activators found at these promoters may also be one mechanism by which TAF1 turns off transcription (Chapter 3). Transcription factors labeled in grey are associated with the Cyclin D1 promoter, those in blue are associated with the Cyclin A promoter. CREB1 is associated with both promoters.

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Appendix A

Table A.1. Primers used for RT-PCR

Gene Symbol	Orientation	Primer Name	Sequence (5'-3')	Amplicon
GAPDH	F	hgapdhrt5	AGGTGAAGGTCGGAGTCAAC	197 bp
	R	hgapdhrt3	GACAAGCTTCCCGTTCTCAG	
CDKN1A	F	p21#2 Forward	CGACTGTGATGCGCTAATGG	371 bp
	R	p21 Reverse Wen et al.	GGCGTTTGGAGTGGTAGAAATC	

Appendix B

Table B.1. The p53 Target Genes Literature List
This table lists the 172 validated direct p53 target genes compiled from three studies [1–3].

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
ABCB1	5243	ATP-binding cassette, sub-family B (MDR/TAP), member 1	Repressed
ACTA2	59	actin, alpha 2, smooth muscle, aorta	Activated
ADARB1	104	adenosine deaminase, RNA-specific, B1 (RED1 homolog rat)	Activated
AEN	64782	apoptosis enhancing nuclease	Activated
AFP	174	alpha-fetoprotein	Activated
AIFM2	84883	apoptosis-inducing factor, mitochondrion-associated, 2	Activated
ANLN	54443	anillin, actin binding protein	Repressed
APAF1	317	apoptotic peptidase activating factor 1	Activated
APC	324	adenomatous polyposis coli	Both
AR	367	androgen receptor	Activated
ARHGEF7	8874	Rho guanine nucleotide exchange factor (GEF) 7	Activated
ARID3A	1820	AT rich interactive domain 3A (BRIGHT-like)	Activated
ATF3	467	activating transcription factor 3	Activated
BAI1	575	brain-specific angiogenesis inhibitor 1	Activated
BAX	581	BCL2-associated X protein	Activated
BBC3	27113	BCL2 binding component 3	Activated
BCL2L14	79370	BCL2-like 14 (apoptosis facilitator)	Activated

Continued on next page

Table B.1 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
BCL6	604	B-cell CLL/lymphoma 6	Activated
BDKRB2	624	bradykinin receptor B2	Activated
BID	637	BH3 interacting domain death agonist	Activated
BIRC5	332	baculoviral IAP repeat-containing 5	Repressed
BNIP3L	665	BCL2/adenovirus E1B 19kDa interacting protein 3-like	Activated
BTG2	7832	BTG family, member 2	Activated
BTG3	10950	BTG family, member 3	Activated
C12orf5	57103	chromosome 12 open reading frame 5	Activated
C13orf15	28984	chromosome 13 open reading frame 15	Activated
C16orf5	29965	chromosome 16 open reading frame 5	Activated
CASP1	834	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	Activated
CASP10	843	caspase 10, apoptosis-related cysteine peptidase	Activated
CASP6	839	caspase 6, apoptosis-related cysteine peptidase	Activated
CAV1	857	caveolin 1, caveolae protein, 22kDa	Activated
CCNG1	900	cyclin G1	Activated
CCNK	8812	cyclin K	Activated
CD82	3732	CD82 molecule	Activated
CDC25C	995	cell division cycle 25 homolog C (S. pombe)	Repressed

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Table B.1 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
CDKN1A	1026	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	Activated
CHMP4C	92421	chromatin modifying protein 4C	Activated
COL18A1	80781	collagen, type XVIII, alpha 1	Activated
CRYZ	1429	crystallin, zeta (quinone reductase)	Repressed
CTSD	1509	cathepsin D	Activated
CX3CL1	6376	chemokine (C-X3-C motif) ligand 1	Activated
CYFIP2	26999	cytoplasmic FMR1 interacting protein 2	Activated
DCC	1630	deleted in colorectal carcinoma	Activated
DDB2	1643	damage-specific DNA binding protein 2, 48kDa	Activated
DDIT4	54541	DNA-damage-inducible transcript 4	Activated
DDR1	780	discoidin domain receptor tyrosine kinase 1	Activated
DKK1	22943	dickkopf homolog 1 (Xenopus laevis)	Activated
DNMT1	1786	DNA (cytosine-5-)-methyltransferase 1	Repressed
DRAM1	55332	DNA-damage regulated autophagy modulator 1	Activated
DSC3	1825	desmocollin 3	Activated
DUSP1	1843	dual specificity phosphatase 1	Activated
DUSP5	1847	dual specificity phosphatase 5	Activated
EDN2	1907	endothelin 2	Activated
EEF1A1	1915	eukaryotic translation elongation factor 1 alpha 1	Activated

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Table B.1 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
EGFR	1956	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	Activated
EOMES	8320	eomesodermin homolog (Xenopus laevis)	Activated
EPHA2	1969	EPH receptor A2	Activated
FANCC	2176	Fanconi anemia, complementation group C	Activated
FAS	355	Fas (TNF receptor superfamily, member 6)	Activated
FDXR	2232	ferredoxin reductase	Activated
FLT1	2321	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	Activated
FOS	2353	FBJ murine osteosarcoma viral oncogene homolog	Both
GADD45A	1647	growth arrest and DNA-damage-inducible, alpha	Activated
GAMT	2593	guanidinoacetate N-methyltransferase	Activated
GDF15	9518	growth differentiation factor 15	Activated
GLS2	27165	glutaminase 2 (liver, mitochondrial)	Activated
GML	2765	glycosylphosphatidylinositol anchored molecule like protein	Activated
GPX1	2876	glutathione peroxidase 1	Activated
GSTP1	2950	glutathione S-transferase pi 1	Activated
HGF	3082	hepatocyte growth factor (hepapoietin A; scatter factor)	Activated
HIC1	3090	hypermethylated in cancer 1	Activated

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Table B.1 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
HRAS	3265	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	Activated
HSP90AB1	3326	heat shock protein 90kDa alpha (cytosolic), class B member 1	Repressed
HSPA8	3312	heat shock 70kDa protein 8	Repressed
HTT	3064	huntingtin	Activated
IER3	8870	immediate early response 3	Repressed
IGFBP3	3486	insulin-like growth factor binding protein 3	Activated
IRF2BP2	359948	interferon regulatory factor 2 binding protein 2	Activated
IRF5	3663	interferon regulatory factor 5	Activated
KRT8	3856	keratin 8	Activated
LASP1	3927	LIM and SH3 protein 1	Repressed
LGALS3	3958	lectin, galactoside-binding, soluble, 3	Repressed
LIF	3976	leukemia inhibitory factor (cholinergic differentiation factor)	Activated
MAD1L1	8379	MAD1 mitotic arrest deficient-like 1 (yeast)	Repressed
MDM2	4193	Mdm2 p53 binding protein homolog (mouse)	Activated
MET	4233	met proto-oncogene (hepatocyte growth factor receptor)	Activated
MLH1	4292	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	Activated
MMP2	4313	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	Activated
MSH2	4436	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)	Activated

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Table B.1 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
NANOG	79923	Nanog homeobox	Repressed
NDRG1	10397	N-myc downstream regulated 1	Activated
NLRC4	58484	NLR family, CARD domain containing 4	Activated
NOS3	4846	nitric oxide synthase 3 (endothelial cell)	Repressed
NOTCH1	4851	Notch homolog 1, translocation-associated (Drosophila)	Activated
ODC1	4953	ornithine decarboxylase 1	Repressed
P2RX6	9127	purinergic receptor P2X, ligand-gated ion channel, 6	Activated
PCBP4	57060	poly(rC) binding protein 4	Activated
PCNA	5111	proliferating cell nuclear antigen	Activated
PDGFC	56034	platelet derived growth factor C	Activated
PERP	64065	PERP, TP53 apoptosis effector	Activated
PHLDA3	23612	pleckstrin homology-like domain, family A, member 3	Activated
PIDD	55367	leucine-rich repeats and death domain containing	Activated
PKD1	5310	polycystic kidney disease 1 (autosomal dominant)	Both
PLAGL1	5325	pleiomorphic adenoma gene-like 1	Activated
PLK2	10769	polo-like kinase 2 (Drosophila)	Both
PLK3	1263	polo-like kinase 3 (Drosophila)	Activated
PMAIP1	5366	phorbol-12-myristate-13-acetate-induced protein 1	Activated
PML	5371	promyelocytic leukemia	Activated

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Table B.1 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
PMS2	5395	PMS2 postmeiotic segregation increased 2 (S. cerevisiae)	Activated
POLD1	5424	polymerase (DNA directed), delta 1, catalytic subunit 125kDa	Repressed
PPM1J	333926	protein phosphatase 1J (PP2C domain containing)	Activated
PRDM1	639	PR domain containing 1, with ZNF domain	Activated
PRKAB1	5564	protein kinase, AMP-activated, beta 1 non-catalytic subunit	Activated
PTEN	5728	phosphatase and tensin homolog	Activated
PTK2	5747	PTK2 protein tyrosine kinase 2	Repressed
PTP4A1	7803	protein tyrosine phosphatase type IVA, member 1	Activated
PYCARD	29108	PYD and CARD domain containing	Activated
RAB6C	84084	RAB6C, member RAS oncogene family	Activated
RABGGTA	5875	Rab geranylgeranyltransferase, alpha subunit	Activated
RAD51	5888	RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)	Repressed
RB1	5925	retinoblastoma 1	Activated
RBM38	55544	RNA binding motif protein 38	Activated
RFWD2	64326	ring finger and WD repeat domain 2	Activated
RNF144B	255488	ring finger protein 144B	Activated
RPS27L	51065	ribosomal protein S27-like	Activated
RRM2B	50484	ribonucleotide reductase M2 B (TP53 inducible)	Activated
S100A2	6273	S100 calcium binding protein A2	Activated

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Table B.1 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
S100A9	6280	S100 calcium binding protein A9	Activated
SCARA3	51435	scavenger receptor class A, member 3	Activated
SCD	6319	stearoyl-CoA desaturase (delta-9-desaturase)	Repressed
SCGB1D2	10647	secretoglobin, family 1D, member 2	Activated
SCN3B	55800	sodium channel, voltage-gated, type III, beta	Activated
SEMA3B	7869	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	Activated
SEMA3F	6405	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F	Activated
SERPINB5	5268	serpin peptidase inhibitor, clade B (ovalbumin), member 5	Activated
SERPINE1	5054	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	Activated
SERTAD1	29950	SERTA domain containing 1	Activated
SESN1	27244	sestrin 1	Activated
SFN	2810	stratifin	Activated
SH2D1A	4068	SH2 domain protein 1A	Activated
SIVA1	10572	SIVA1, apoptosis-inducing factor	Activated
SLC38A2	54407	solute carrier family 38, member 2	Repressed

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Table B.1 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
SLC6A6	6533	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	Repressed
SOD2	6648	superoxide dismutase 2, mitochondrial	Activated
STAG1	10274	stromal antigen 1	Activated
STEAP3	55240	STEAP family member 3	Activated
STX6	10228	syntaxin 6	Activated
TAP1	6890	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	Activated
TGFA	7039	transforming growth factor, alpha	Activated
TNFRSF10A	8797	tumor necrosis factor receptor superfamily, member 10a	Activated
TNFRSF10B	8795	tumor necrosis factor receptor superfamily, member 10b	Activated
TNFRSF10C	8794	tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	Activated
TNFRSF10D	8793	tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	Activated
TNFSF10	8743	tumor necrosis factor (ligand) superfamily, member 10	Activated
TP53	7157	tumor protein p53	Activated
TP53AIP1	63970	tumor protein p53 regulated apoptosis inducing protein 1	Activated
TP53I3	9540	tumor protein p53 inducible protein 3	Activated
TP53INP1	94241	tumor protein p53 inducible nuclear protein 1	Activated

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Table B.1 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
TP63	8626	tumor protein p63	Activated
TP73	7161	tumor protein p73	Activated
TRAF4	9618	TNF receptor-associated factor 4	Activated
TRIAP1	51499	TP53 regulated inhibitor of apoptosis 1	Activated
TRIM22	10346	tripartite motif-containing 22	Activated
TRPM2	7226	transient receptor potential cation channel, subfamily M, member 2	Repressed
TSC2	7249	tuberous sclerosis 2	Activated
TYRP1	7306	tyrosinase-related protein 1	Activated
UBD	10537	ubiquitin D	Repressed
UBTD1	80019	ubiquitin domain containing 1	Activated
VCAN	1462	versican	Activated
VDR	7421	vitamin D (1,25- dihydroxyvitamin D3) receptor	Activated
XRCC5	7520	X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining)	Activated
ZMAT3	64393	zinc finger, matrin type 3	Activated

Table B.2. p53 Target Genes Experimental List
This table lists the 446 identified as p53 direct target genes in response to 5-FU, Actinomycin D, Etoposide, UV and IR. These genes were classified as direct p53 targets based on p53 binding as determined by ChIP-PET, ChIP-chip, or ChIP-Seq and differential expression as determined by microarray. Genes which are validated p53 targets from the p53 Target Genes Literature List are in bold.

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
ACBD4	79777	acyl-Coenzyme A binding domain containing 4	Activated
ACTG2	72	actin, gamma 2, smooth muscle, enteric	Activated
ACTN1	87	actinin, alpha 1	Repressed
ADK	132	adenosine kinase	Repressed
ADORA2B	136	adenosine A2b receptor	Activated
ADRB1	153	adrenergic, beta-1-, receptor	Activated
AEN	**64782**	**apoptosis enhancing nuclease**	**Activated**
AGAP1	116987	ArfGAP with GTPase domain, ankyrin repeat and PH domain 1	Repressed
ALDH1A3	220	aldehyde dehydrogenase 1 family, member A3	Activated
ALOX5	240	arachidonate 5-lipoxygenase	Activated
AMZ2P1	201283	archaelysin family metallopeptidase 2 pseudogene 1	Activated
ANK1	286	ankyrin 1, erythrocytic	Repressed
ANKRA2	57763	ankyrin repeat, family A (RFXANK-like), 2	Activated
ANKRD1	27063	ankyrin repeat domain 1 (cardiac muscle)	Activated
ANKRD10	55608	ankyrin repeat domain 10	Repressed
ANKRD11	29123	ankyrin repeat domain 11	Repressed

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
ANP32D	23519	acidic (leucine-rich) nuclear phosphoprotein 32 family, member D	Repressed
ANXA4	307	annexin A4	Activated
APBB2	323	amyloid beta (A4) precursor protein-binding, family B, member 2	Repressed
APOBEC3H	164668	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3H	Activated
ARHGAP5	394	Rho GTPase activating protein 5	Repressed
ARHGEF3	50650	Rho guanine nucleotide exchange factor (GEF) 3	Activated
ARL14	80117	ADP-ribosylation factor-like 14	Activated
ARL2BP	23568	ADP-ribosylation factor-like 2 binding protein	Activated
ARVCF	421	armadillo repeat gene deletes in velocardiofacial syndrome	Activated
ASAP1	50807	ArfGAP with SH3 domain, ankyrin repeat and PH domain 1	Repressed
ASCC3	10973	activating signal cointegrator 1 complex subunit 3	Repressed
ASTN2	23245	astrotactin 2	Activated
ATF3	**467**	**activating transcription factor 3**	**Activated**
ATG4A	115201	ATG4 autophagy related 4 homolog A (S. cerevisiae)	Activated
ATXN3	4287	ataxin 3	Activated

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
B4GALT6	9331	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 6	Activated
BAX	**581**	**BCL2-associated X protein**	**Activated**
BAZ1B	9031	bromodomain adjacent to zinc finger domain, 1B	Repressed
BBC3	**27113**	**BCL2 binding component 3**	**Activated**
BBS2	583	Bardet-Biedl syndrome 2	Activated
BBS9	27241	Bardet-Biedl syndrome 9	Activated
BCAS3	54828	breast carcinoma amplified sequence 3	Repressed
BCL2A1	597	BCL2-related protein A1	Repressed
BCL2L1	598	BCL2-like 1	Activated
BFSP1	631	beaded filament structural protein 1, filensin	Activated
BICD2	23299	bicaudal D homolog 2 (Drosophila)	Activated
BLOC1S2	282991	biogenesis of lysosomal organelles complex-1, subunit 2	Activated
BRCA1	672	breast cancer 1, early onset	Activated
BRE	9577	brain and reproductive organ-expressed (TNFRSF1A modulator)	Repressed
BTG1	694	B-cell translocation gene 1, anti-proliferative	Activated
BTG2	**7832**	**BTG family, member 2**	**Activated**
C10orf88	80007	chromosome 10 open reading frame 88	Activated

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
C12orf5	**57103**	**chromosome 12 open reading frame 5**	**Activated**
C12orf62	84987	chromosome 12 open reading frame 62	Activated
C15orf42	90381	chromosome 15 open reading frame 42	Repressed
C1S	716	complement component 1, s subcomponent	Activated
C2orf29	55571	chromosome 2 open reading frame 29	Repressed
C8orf84	157869	chromosome 8 open reading frame 84	Activated
CACNA2D1	781	calcium channel, voltage-dependent, alpha 2/delta subunit 1	Repressed
CALD1	800	caldesmon 1	Activated
CALN1	83698	calneuron 1	Repressed
CAMK2D	817	calcium/calmodulin-dependent protein kinase II delta	Repressed
CAMK4	814	calcium/calmodulin-dependent protein kinase IV	Repressed
CBLC	23624	Cas-Br-M (murine) ecotropic retroviral transforming sequence c	Activated
CBR4	84869	carbonyl reductase 4	Activated
CCBE1	147372	collagen and calcium binding EGF domains 1	Repressed
CCDC144B	284047	coiled-coil domain containing 144B	Repressed
CCDC144NL	339184	coiled-coil domain containing 144 family, N-terminal like	Repressed
CCDC30	728621	coiled-coil domain containing 30	Repressed
CCDC51	79714	coiled-coil domain containing 51	Activated

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
CCNG1	**900**	**cyclin G1**	**Activated**
CCNG2	901	cyclin G2	Both
CD163L1	283316	CD163 molecule-like 1	Activated
CD70	970	CD70 molecule	Activated
CD80	941	CD80 molecule	Activated
CDC25C	**995**	**cell division cycle 25 homolog C (S. pombe)**	**Repressed**
CDC42BPB	9578	CDC42 binding protein kinase beta (DMPK-like)	Repressed
CDC42EP3	10602	CDC42 effector protein (Rho GTPase binding) 3	Activated
CDC6	990	cell division cycle 6 homolog (S. cerevisiae)	Repressed
CDC7	8317	cell division cycle 7 homolog (S. cerevisiae)	Repressed
CDCA7	83879	cell division cycle associated 7	Repressed
CDH13	1012	cadherin 13, H-cadherin (heart)	Repressed
CDK1	983	cyclin-dependent kinase 1	Repressed
CDKAL1	54901	CDK5 regulatory subunit associated protein 1-like 1	Repressed
CDKN1A	**1026**	**cyclin-dependent kinase inhibitor 1A (p21, Cip1)**	**Activated**
CEP68	23177	centrosomal protein 68kDa	Activated
CEP76	79959	centrosomal protein 76kDa	Activated
CERS5	91012	ceramide synthase 5	Activated
CERS6	253782	ceramide synthase 6	Repressed

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
CHD2	1106	chromodomain helicase DNA binding protein 2	Activated
CHEK1	1111	CHK1 checkpoint homolog (S. pombe)	Activated
CHML	1122	choroideremia-like (Rab escort protein 2)	Repressed
CHST12	55501	carbohydrate (chondroitin 4) sulfotransferase 12	Repressed
CLDN1	9076	claudin 1	Activated
COL4A1	1282	collagen, type IV, alpha 1	Activated
CPE	1363	carboxypeptidase E	Activated
CPEB4	80315	cytoplasmic polyadenylation element binding protein 4	Activated
CPN1	1369	carboxypeptidase N, polypeptide 1	Repressed
CRIM1	51232	cysteine rich transmembrane BMP regulator 1 (chordin-like)	Repressed
CROT	54677	carnitine O-octanoyltransferase	Activated
CSMD3	114788	CUB and Sushi multiple domains 3	Activated
CTNNA3	29119	catenin (cadherin-associated protein), alpha 3	Activated
CTSD	**1509**	**cathepsin D**	**Activated**
CYP1B1	1545	cytochrome P450, family 1, subfamily B, polypeptide 1	Activated
CYP4F3	4051	cytochrome P450, family 4, subfamily F, polypeptide 3	Activated
CYR61	3491	cysteine-rich, angiogenic inducer, 61	Activated
DCP1B	196513	DCP1 decapping enzyme homolog B (S. cerevisiae)	Activated
DDB2	**1643**	**damage-specific DNA binding protein 2, 48kDa**	**Activated**

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
DDIT4	**54541**	**DNA-damage-inducible transcript 4**	**Activated**
DDX10	1662	DEAD (Asp-Glu-Ala-Asp) box polypeptide 10	Repressed
DEK	7913	DEK oncogene	Repressed
DENND2D	79961	DENN/MADD domain containing 2D	Activated
DHRS2	10202	dehydrogenase/reductase (SDR family) member 2	Repressed
DHRS3	9249	dehydrogenase/reductase (SDR family) member 3	Activated
DHRS7	51635	dehydrogenase/reductase (SDR family) member 7	Activated
DKK3	27122	dickkopf homolog 3 (Xenopus laevis)	Repressed
DLGAP5	9787	discs, large (Drosophila) homolog-associated protein 5	Repressed
DNAJB2	3300	DnaJ (Hsp40) homolog, subfamily B, member 2	Activated
DRAM1	**55332**	**DNA-damage regulated autophagy modulator 1**	**Activated**
DSCC1	79075	defective in sister chromatid cohesion 1 homolog (S. cerevisiae)	Repressed
DTWD1	56986	DTW domain containing 1	Activated
DUSP11	8446	dual specificity phosphatase 11 (RNA/RNP complex 1-interacting)	Activated
DUSP5	**1847**	**dual specificity phosphatase 5**	**Activated**
DYRK3	8444	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3	Activated
EDA2R	60401	ectodysplasin A2 receptor	Activated

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
EDN2	**1907**	**endothelin 2**	**Activated**
EEA1	8411	early endosome antigen 1	Repressed
EFNA5	1946	ephrin-A5	Repressed
EI24	9538	etoposide induced 2.4 mRNA	Activated
EIF2AK3	9451	eukaryotic translation initiation factor 2-alpha kinase 3	Repressed
ENC1	8507	ectodermal-neural cortex (with BTB-like domain)	Activated
ENPP2	5168	ectonucleotide pyrophosphatase/phosphodiesterase 2	Activated
EPB41L2	2037	erythrocyte membrane protein band 4.1-like 2	Repressed
EPHA4	2043	EPH receptor A4	Activated
EPHB2	2048	EPH receptor B2	Repressed
ERBB4	2066	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	Repressed
EXT1	2131	exostoses (multiple) 1	Repressed
F2R	2149	coagulation factor II (thrombin) receptor	Activated
FAF1	11124	Fas (TNFRSF6) associated factor 1	Repressed
FAM13C	220965	family with sequence similarity 13, member C	Activated
FAM162A	26355	family with sequence similarity 162, member A	Activated
FAM172A	83989	family with sequence similarity 172, member A	Repressed
FAM198B	51313	family with sequence similarity 198, member B	Activated

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
FAM19A5	25817	family with sequence similarity 19 (chemokine (C-C motif)-like), member A5	Repressed
FAM46A	55603	family with sequence similarity 46, member A	Activated
FAM49A	81553	family with sequence similarity 49, member A	Activated
FAM65B	9750	family with sequence similarity 65, member B	Activated
FAM84B	157638	family with sequence similarity 84, member B	Activated
FANCC	**2176**	**Fanconi anemia, complementation group C**	**Repressed**
FAS	**355**	**Fas (TNF receptor superfamily, member 6)**	**Activated**
FAT1	2195	FAT tumor suppressor homolog 1 (Drosophila)	Repressed
FBXO22	26263	F-box protein 22	Activated
FBXO32	114907	F-box protein 32	Activated
FBXW7	55294	F-box and WD repeat domain containing 7	Activated
FDXR	**2232**	**ferredoxin reductase**	**Activated**
FGF2	2247	fibroblast growth factor 2 (basic)	Activated
FLJ26850	400710	FLJ26850 protein	Activated
FOXN3	1112	forkhead box N3	Repressed
FRMD4A	55691	FERM domain containing 4A	Activated
FST	10468	follistatin	Activated
FUCA1	2517	fucosidase, alpha-L- 1, tissue	Activated

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
GADD45A	**1647**	**growth arrest and DNA-damage-inducible, alpha**	**Activated**
GALNT2	2590	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2 (GalNAc-T2)	Repressed
GCC2	9648	GRIP and coiled-coil domain containing 2	Activated
GDF15	**9518**	**growth differentiation factor 15**	**Activated**
GMDS	2762	GDP-mannose 4,6-dehydratase	Repressed
GML	**2765**	**glycosylphosphatidylinositol anchored molecule like protein**	**Activated**
GMNN	51053	geminin, DNA replication inhibitor	Repressed
GNAI1	2770	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	Activated
GNAQ	2776	guanine nucleotide binding protein (G protein), q polypeptide	Repressed
GNG12	55970	guanine nucleotide binding protein (G protein), gamma 12	Repressed
GOLGB1	2804	golgin B1	Activated
GPC1	2817	glypican 1	Activated
GPC3	2719	glypican 3	Repressed
GPC6	10082	glypican 6	Repressed
GPHN	10243	gephyrin	Repressed
GPR39	2863	G protein-coupled receptor 39	Activated
GPR87	53836	G protein-coupled receptor 87	Activated

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
GPX1	**2876**	**glutathione peroxidase 1**	**Activated**
GRAMD1B	57476	GRAM domain containing 1B	Activated
GREB1	9687	growth regulation by estrogen in breast cancer 1	Activated
GSPT1	2935	G1 to S phase transition 1	Repressed
GTF2B	2959	general transcription factor IIB	Activated
HCG11	493812	HLA complex group 11 (non-protein coding)	Activated
HDAC9	9734	histone deacetylase 9	Repressed
HEXIM1	10614	hexamethylene bis-acetamide inducible 1	Activated
HIGD1A	25994	HIG1 hypoxia inducible domain family, member 1A	Repressed
HIST1H2AC	8334	histone cluster 1, H2ac	Activated
HNRPDL	9987	heterogeneous nuclear ribonucleoprotein D-like	Repressed
HRCT1	646962	histidine rich carboxyl terminus 1	Activated
HSD17B3	3293	hydroxysteroid (17-beta) dehydrogenase 3	Activated
HSPE1	3336	heat shock 10kDa protein 1 (chaperonin 10)	Repressed
IER3	**8870**	**immediate early response 3**	**Activated**
IER5	51278	immediate early response 5	Activated
IGDCC4	57722	immunoglobulin superfamily, DCC subclass, member 4	Activated
IGF2BP2	10644	insulin-like growth factor 2 mRNA binding protein 2	Repressed
ITGA2	3673	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	Both

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
ITGAM	3684	integrin, alpha M (complement component 3 receptor 3 subunit)	Activated
JAG1	182	jagged 1 (Alagille syndrome)	Activated
JMJD6	23210	jumonji domain containing 6	Repressed
KAT2B	8850	K(lysine) acetyltransferase 2B	Repressed
KCMF1	56888	potassium channel modulatory factor 1	Repressed
KCNMA1	3778	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	Activated
KCNQ3	3786	potassium voltage-gated channel, KQT-like subfamily, member 3	Activated
KCNQ5	56479	potassium voltage-gated channel, KQT-like subfamily, member 5	Repressed
KIAA0247	9766	KIAA0247	Activated
KIAA0564	23078	KIAA0564	Activated
KIAA1279	26128	KIAA1279	Repressed
KIRREL3	84623	kin of IRRE like 3 (Drosophila)	Repressed
KITLG	4254	KIT ligand	Activated
KRT80	144501	keratin 80	Activated
LAMA1	284217	laminin, alpha 1	Activated
LAMP3	27074	lysosomal-associated membrane protein 3	Activated

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
LAPTM5	7805	lysosomal protein transmembrane 5	Activated
LAT	27040	linker for activation of T cells	Activated
LATS2	26524	LATS, large tumor suppressor, homolog 2 (Drosophila)	Activated
LCE1A	353131	late cornified envelope 1A	Activated
LCE1E	353135	late cornified envelope 1E	Activated
LCE5A	254910	late cornified envelope 5A	Repressed
LINC00518	221718	long intergenic non-protein coding RNA 518	Activated
LOC145474	145474	uncharacterized LOC145474	Repressed
LOC256021	256021	uncharacterized LOC256021	Activated
LSM6	11157	LSM6 homolog, U6 small nuclear RNA associated (S. cerevisiae)	Repressed
LTBP1	4052	latent transforming growth factor beta binding protein 1	Repressed
MAD1L1	**8379**	**MAD1 mitotic arrest deficient-like 1 (yeast)**	**Repressed**
MAN2B1	4125	mannosidase, alpha, class 2B, member 1	Activated
MAP4K4	9448	mitogen-activated protein kinase kinase kinase kinase 4	Repressed
MARS	4141	methionyl-tRNA synthetase	Repressed
MDM2	**4193**	**Mdm2 p53 binding protein homolog (mouse)**	**Activated**
MDM4	4194	Mdm4 p53 binding protein homolog (mouse)	Repressed
ME1	4199	malic enzyme 1, NADP(+)-dependent, cytosolic	Repressed

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
MED18	54797	mediator complex subunit 18	Activated
MELK	9833	maternal embryonic leucine zipper kinase	Repressed
MLH1	**4292**	**mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)**	**Repressed**
MMADHC	27249	methylmalonic aciduria (cobalamin deficiency) cblD type, with homocystinuria	Repressed
MMP2	**4313**	**matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)**	**Activated**
MOXD1	26002	monooxygenase, DBH-like 1	Activated
MSH6	2956	mutS homolog 6 (E. coli)	Repressed
MST1	4485	macrophage stimulating 1 (hepatocyte growth factor-like)	Activated
MTHFD1L	25902	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	Repressed
MYBL1	4603	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	Repressed
MYC	4609	v-myc myelocytomatosis viral oncogene homolog (avian)	Repressed
MYL2	4633	myosin, light chain 2, regulatory, cardiac, slow	Activated
MYO1A	4640	myosin IA	Activated
NAB1	4664	NGFI-A binding protein 1 (EGR1 binding protein 1)	Activated
NAV3	89795	neuron navigator 3	Activated
NCAPD2	9918	non-SMC condensin I complex, subunit D2	Repressed

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
NCAPG2	54892	non-SMC condensin II complex, subunit G2	Repressed
NCK2	8440	NCK adaptor protein 2	Repressed
NDC80	10403	NDC80 homolog, kinetochore complex component (S. cerevisiae)	Repressed
NEAT1	283131	nuclear paraspeckle assembly transcript 1 (non-protein coding)	Activated
NEDD4L	23327	neural precursor cell expressed, developmentally down-regulated 4-like	Repressed
NEFL	4747	neurofilament, light polypeptide	Activated
NEGR1	257194	neuronal growth regulator 1	Repressed
NEO1	4756	neogenin homolog 1 (chicken)	Repressed
NEXN	91624	nexilin (F actin binding protein)	Repressed
NF2	4771	neurofibromin 2 (merlin)	Repressed
NID2	22795	nidogen 2 (osteonidogen)	Activated
NINJ1	4814	ninjurin 1	Activated
NKAIN4	128414	Na+/K+ transporting ATPase interacting 4	Activated
NLGN1	22871	neuroligin 1	Repressed
NME1	4830	non-metastatic cells 1, protein (NM23A) expressed in	Activated
NME2	4831	non-metastatic cells 2, protein (NM23B) expressed in	Activated
NMU	10874	neuromedin U	Repressed

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
NOTCH1	**4851**	**Notch homolog 1, translocation-associated (Drosophila)**	**Activated**
NPIP	9284	nuclear pore complex interacting protein	Activated
NR2F1	7025	nuclear receptor subfamily 2, group F, member 1	Repressed
NR6A1	2649	nuclear receptor subfamily 6, group A, member 1	Activated
NRXN3	9369	neurexin 3	Repressed
NTM	50863	neurotrimin	Repressed
NTPCR	84284	nucleoside-triphosphatase, cancer-related	Activated
NUPR1	26471	nuclear protein, transcriptional regulator, 1	Activated
NYAP2	57624	neuronal tyrosine-phosphorylated phosphoinositide-3-kinase adaptor 2	Activated
OGFR	11054	opioid growth factor receptor	Repressed
ORAI3	93129	ORAI calcium release-activated calcium modulator 3	Activated
OSBP	5007	oxysterol binding protein	Repressed
P4HA2	8974	prolyl 4-hydroxylase, alpha polypeptide II	Activated
PADI4	23569	peptidyl arginine deiminase, type IV	Activated
PADI6	353238	peptidyl arginine deiminase, type VI	Activated
PAG1	55824	phosphoprotein associated with glycosphingolipid microdomains 1	Activated
PALLD	23022	palladin, cytoskeletal associated protein	Both

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
PAPOLG	64895	poly(A) polymerase gamma	Activated
PARD3	56288	par-3 partitioning defective 3 homolog (C. elegans)	Repressed
PCCA	5095	propionyl Coenzyme A carboxylase, alpha polypeptide	Repressed
PCDH7	5099	protocadherin 7	Both
PCMTD1	115294	protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 1	Activated
PCNA	**5111**	**proliferating cell nuclear antigen**	**Activated**
PDP1	54704	pyruvate dehyrogenase phosphatase catalytic subunit 1	Activated
PGF	5228	placental growth factor	Activated
PGPEP1	54858	pyroglutamyl-peptidase I	Activated
PHF14	9678	PHD finger protein 14	Repressed
PHLDA3	**23612**	**pleckstrin homology-like domain, family A, member 3**	**Activated**
PIAS2	9063	protein inhibitor of activated STAT, 2	Repressed
PIDD	**55367**	**p53-induced death domain protein**	**Activated**
PKD1P1	339044	polycystic kidney disease 1 (autosomal dominant) pseudogene 1	Activated
PLAC8	51316	placenta-specific 8	Repressed
PLCB1	23236	phospholipase C, beta 1 (phosphoinositide-specific)	Repressed

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
PLEKHA1	59338	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 1	Activated
PLEKHF1	79156	pleckstrin homology domain containing, family F (with FYVE domain) member 1	Activated
PLK1	5347	polo-like kinase 1 (Drosophila)	Repressed
PLK2	**10769**	**polo-like kinase 2 (Drosophila)**	**Activated**
PLK3	**1263**	**polo-like kinase 3 (Drosophila)**	**Activated**
PLLP	51090	plasma membrane proteolipid (plasmolipin)	Activated
PLXNB1	5364	plexin B1	Activated
PMAIP1	**5366**	**phorbol-12-myristate-13-acetate-induced protein 1**	**Activated**
POLD3	10714	polymerase (DNA-directed), delta 3, accessory subunit	Repressed
POLH	5429	polymerase (DNA directed), eta	Activated
PPFIBP1	8496	PTPRF interacting protein, binding protein 1 (liprin beta 1)	Activated
PPM1D	8493	protein phosphatase 1D magnesium-dependent, delta isoform	Activated
PPP1R3C	5507	protein phosphatase 1, regulatory (inhibitor) subunit 3C	Activated
PPP3CA	5530	protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform	Repressed
PRDM1	**639**	**PR domain containing 1, with ZNF domain**	**Activated**
PRIM2	5558	primase, DNA, polypeptide 2 (58kDa)	Repressed

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
PRKAB1	**5564**	**protein kinase, AMP-activated, beta 1 non-catalytic subunit**	**Activated**
PRKAG2	51422	protein kinase, AMP-activated, gamma 2 non-catalytic subunit	Activated
PRKCE	5581	protein kinase C, epsilon	Repressed
PRKDC	5591	protein kinase, DNA-activated, catalytic polypeptide	Repressed
PRODH	5625	proline dehydrogenase (oxidase) 1	Activated
PRR11	55771	proline rich 11	Repressed
PSAT1	29968	phosphoserine aminotransferase 1	Repressed
PSG10P	653492	pregnancy specific beta-1-glycoprotein 10, pseudogene	Activated
PSG6	5675	pregnancy specific beta-1-glycoprotein 6	Activated
PSG8	440533	pregnancy specific beta-1-glycoprotein 8	Activated
PSG9	5678	pregnancy specific beta-1-glycoprotein 9	Activated
PSTPIP2	9050	proline-serine-threonine phosphatase interacting protein 2	Activated
PTK2	**5747**	**PTK2 protein tyrosine kinase 2**	**Repressed**
PTPN14	5784	protein tyrosine phosphatase, non-receptor type 14	Repressed
PTPRE	5791	protein tyrosine phosphatase, receptor type, E	Activated
PTPRM	5797	protein tyrosine phosphatase, receptor type, M	Repressed
PTPRO	5800	protein tyrosine phosphatase, receptor type, O	Activated
PTPRU	10076	protein tyrosine phosphatase, receptor type, U	Activated
PVRL4	81607	poliovirus receptor-related 4	Activated

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
RABGGTA	**5875**	**Rab geranylgeranyltransferase, alpha subunit**	**Activated**
RAD18	56852	RAD18 homolog (S. cerevisiae)	Repressed
RAD23B	5887	RAD23 homolog B (S. cerevisiae)	Repressed
RAD51C	5889	RAD51 homolog C (S. cerevisiae)	Activated
RAP2B	5912	RAP2B, member of RAS oncogene family	Activated
RBL1	5933	retinoblastoma-like 1 (p107)	Repressed
RBM19	9904	RNA binding motif protein 19	Repressed
RBMS3	27303	RNA binding motif, single stranded interacting protein	Repressed
RFC3	5983	replication factor C (activator 1) 3, 38kDa	Repressed
RGS16	6004	regulator of G-protein signaling 16	Activated
RHOB	388	ras homolog gene family, member B	Activated
RHOBTB3	22836	Rho-related BTB domain containing 3	Repressed
RIN1	9610	Ras and Rab interactor 1	Activated
RNASE7	84659	ribonuclease, RNase A family, 7	Activated
RND3	390	Rho family GTPase 3	Activated
RNF216	54476	ring finger protein 216	Repressed
ROBO1	6091	roundabout, axon guidance receptor, homolog 1 (Drosophila)	Repressed
RORA	6095	RAR-related orphan receptor A	Activated
RPL37A	6168	ribosomal protein L37a	Repressed

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
RPS27L	**51065**	**ribosomal protein S27-like**	**Activated**
RPTOR	57521	regulatory associated protein of MTOR, complex 1	Repressed
RRAD	6236	Ras-related associated with diabetes	Activated
RRM2B	**50484**	**ribonucleotide reductase M2 B (TP53 inducible)**	**Activated**
RXRA	6256	retinoid X receptor, alpha	Repressed
S100A2	**6273**	**S100 calcium binding protein A2**	**Activated**
SARS	6301	seryl-tRNA synthetase	Activated
SBF2	81846	SET binding factor 2	Repressed
SCMH1	22955	sex comb on midleg homolog 1 (Drosophila)	Repressed
SCN2A	6326	sodium channel, voltage-gated, type II, alpha subunit	Activated
SDC4	6385	syndecan 4	Activated
SDK1	221935	sidekick homolog 1, cell adhesion molecule (chicken)	Repressed
SEMA3C	10512	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	Repressed
SERPINB5	**5268**	**serpin peptidase inhibitor, clade B (ovalbumin), member 5**	**Activated**
SERPINE1	**5054**	**serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1**	**Activated**
SERTAD1	**29950**	**SERTA domain containing 1**	**Activated**
SESN1	**27244**	**sestrin 1**	**Activated**

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
SHROOM3	57619	shroom family member 3	Activated
SIPA1L1	26037	signal-induced proliferation-associated 1 like 1	Repressed
SIRPA	140885	signal-regulatory protein alpha	Repressed
SLC12A4	6560	solute carrier family 12 (potassium/chloride transporters), member 4	Activated
SLC25A13	10165	solute carrier family 25, member 13 (citrin)	Repressed
SLC2A5	6518	solute carrier family 2 (facilitated glucose/fructose transporter), member 5	Activated
SLC30A1	7779	solute carrier family 30 (zinc transporter), member 1	Activated
SLC39A14	23516	solute carrier family 39 (zinc transporter), member 14	Repressed
SLC44A5	204962	solute carrier family 44, member 5	Activated
SLC48A1	55652	solute carrier family 48 (heme transporter), member 1	Activated
SLC4A10	57282	solute carrier family 4, sodium bicarbonate transporter, member 10	Repressed
SMAD3	4088	SMAD family member 3	Activated
SMARCB1	6598	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1	Repressed
SNORD56B	319139	small nucleolar RNA, C/D box 56B	Repressed
SNRPD1	6632	small nuclear ribonucleoprotein D1 polypeptide 16kDa	Repressed

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
SNUPN	10073	snurportin 1	Repressed
SNX5	27131	sorting nexin 5	Repressed
SP110	3431	SP110 nuclear body protein	Activated
SPAG9	9043	sperm associated antigen 9	Repressed
SPANXC	64663	SPANX family, member C	Activated
SPANXF1	171490	SPANX family, member F1	Activated
SRSF3	6428	serine/arginine-rich splicing factor 3	Repressed
STARD10	10809	StAR-related lipid transfer (START) domain containing 10	Activated
STARD4	134429	StAR-related lipid transfer (START) domain containing 4	Repressed
STAT3	6774	signal transducer and activator of transcription 3 (acute-phase response factor)	Activated
STAU1	6780	staufen, RNA binding protein, homolog 1 (Drosophila)	Repressed
STK17A	9263	serine/threonine kinase 17a	Activated
STX5	6811	syntaxin 5	Activated
STX6	**10228**	**syntaxin 6**	**Activated**
TBC1D22A	25771	TBC1 domain family, member 22A	Repressed
TCF12	6938	transcription factor 12	Repressed
TCF4	6925	transcription factor 4	Repressed
TCF7L2	6934	transcription factor 7-like 2 (T-cell specific, HMG-box)	Repressed

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
TCP11L1	55346	t-complex 11 (mouse)-like 1	Activated
TEP1	7011	telomerase-associated protein 1	Activated
TEX9	374618	testis expressed 9	Activated
TGDS	23483	TDP-glucose 4,6-dehydratase	Activated
TGFA	**7039**	**transforming growth factor, alpha**	**Activated**
THSD1	55901	thrombospondin, type I, domain containing 1	Activated
TLR3	7098	toll-like receptor 3	Activated
TM7SF3	51768	transmembrane 7 superfamily member 3	Activated
TMEM68	137695	transmembrane protein 68	Activated
TNFAIP8	25816	tumor necrosis factor, alpha-induced protein 8	Both
TNFRSF10B	**8795**	**tumor necrosis factor receptor superfamily, member 10b**	**Activated**
TNFSF10	**8743**	**tumor necrosis factor (ligand) superfamily, member 10**	**Activated**
TNS3	64759	tensin 3	Repressed
TP53I3	**9540**	**tumor protein p53 inducible protein 3**	**Activated**
TP53INP1	**94241**	**tumor protein p53 inducible nuclear protein 1**	**Activated**
TP53TG1	11257	TP53 target 1 (non-protein coding)	Activated
TPO	7173	thyroid peroxidase	Activated
TPTE2P3	220115	transmembrane phosphoinositide 3-phosphatase and tensin homolog 2 pseudogene 3	Activated

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
TRAF4	**9618**	**TNF receptor-associated factor 4**	**Activated**
TRIAP1	**51499**	**TP53 regulated inhibitor of apoptosis 1**	**Activated**
TRIM22	**10346**	**tripartite motif-containing 22**	**Activated**
TRIM24	8805	tripartite motif-containing 24	Repressed
TRIM29	23650	tripartite motif-containing 29	Activated
TRIM32	22954	tripartite motif-containing 32	Activated
TRIM38	10475	tripartite motif-containing 38	Activated
TRIM5	85363	tripartite motif-containing 5	Activated
TRIML2	205860	tripartite motif family-like 2	Activated
TRIP6	7205	thyroid hormone receptor interactor 6	Activated
TRPM1	4308	transient receptor potential cation channel, subfamily M, member 1	Repressed
TSKU	25987	tsukushi small leucine rich proteoglycan homolog (Xenopus laevis)	Activated
TSPAN1	10103	tetraspanin 1	Activated
UBC	7316	ubiquitin C	Repressed
UBE4B	10277	ubiquitination factor E4B (UFD2 homolog, yeast)	Repressed
UBP1	7342	upstream binding protein 1 (LBP-1a)	Repressed
USP34	9736	ubiquitin specific peptidase 34	Repressed

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Table B.2 – *Continued from previous page*

Gene Symbol	Entrez GeneID	Gene Name	Regulation by p53
USP9X	8239	ubiquitin specific peptidase 9, X-linked	Repressed
UTRN	7402	utrophin	Repressed
VAMP2	6844	vesicle-associated membrane protein 2 (synaptobrevin 2)	Activated
VAV2	7410	vav 2 guanine nucleotide exchange factor	Repressed
VIM	7431	vimentin	Repressed
WNK1	65125	WNK lysine deficient protein kinase 1	Repressed
WWC1	23286	WW and C2 domain containing 1	Repressed
XDH	7498	xanthine dehydrogenase	Activated
XPC	7508	xeroderma pigmentosum, complementation group C	Activated
ZAK	51776	sterile alpha motif and leucine zipper containing kinase AZK	Repressed
ZMAT3	**64393**	**zinc finger, matrin type 3**	**Activated**
ZMIZ1	57178	zinc finger, MIZ-type containing 1	Repressed
ZP3	7784	zona pellucida glycoprotein 3 (sperm receptor)	Activated

Table B.3. Statistically significant Gene Ontology terms for 213 genes TAF1-promoter bound in IRM90 cells, downregulated by high glucose in HEK293 cells and are p53-independent

GO Term BP FAT	Count	%	P-Value
GO:0006414 translational elongation	11	5.314009662	1.69E-07
GO:0006412 translation	16	7.729468599	4.04E-06
GO:0034976 response to endoplasmic reticulum stress	6	2.898550725	3.37E-05
GO:0009267 cellular response to starvation	5	2.415458937	4.10E-04
GO:0008203 cholesterol metabolic process	7	3.381642512	5.47E-04
GO:0006984 ER-nuclear signaling pathway	5	2.415458937	5.82E-04
GO:0007026 negative regulation of microtubule depolymerization	4	1.93236715	6.73E-04
GO:0031114 regulation of microtubule depolymerization	4	1.93236715	6.73E-04
GO:0006457 protein folding	9	4.347826087	7.99E-04
GO:0002474 antigen processing and presentation of peptide antigen via MHC class I	4	1.93236715	8.11E-04
GO:0031111 negative regulation of microtubule polymerization or depolymerization	4	1.93236715	8.11E-04
GO:0016125 sterol metabolic process	7	3.381642512	8.97E-04
GO:0042594 response to starvation	5	2.415458937	0.001171493
GO:0051789 response to protein stimulus	7	3.381642512	0.001211406
GO:0031669 cellular response to nutrient levels	5	2.415458937	0.001396672
GO:0033554 cellular response to stress	16	7.729468599	0.001550833
GO:0010033 response to organic substance	18	8.695652174	0.002638296

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Table B.3 – *Continued from previous page*

GO Term BP_FAT	Count	%	P-Value
GO:0048002 antigen processing and presentation of peptide antigen	4	1.93236715	0.003572271
GO:0031110 regulation of microtubule polymerization or depolymerization	4	1.93236715	0.004784166
GO:0051129 negative regulation of cellular component organization	7	3.381642512	0.005011204
GO:0031668 cellular response to extracellular stimulus	5	2.415458937	0.005511168
GO:0046907 intracellular transport	16	7.729468599	0.006282144
GO:0016051 carbohydrate biosynthetic process	6	2.898550725	0.006739551
GO:0016126 sterol biosynthetic process	4	1.93236715	0.006744974
GO:0034637 cellular carbohydrate biosynthetic process	5	2.415458937	0.007186631
GO:0008202 steroid metabolic process	8	3.8647343	0.007292532
GO:0046364 monosaccharide biosynthetic process	4	1.93236715	0.007879523
GO:0006986 response to unfolded protein	5	2.415458937	0.007941292
GO:0043242 negative regulation of protein complex disassembly	4	1.93236715	0.010468279
GO:0070507 regulation of microtubule cytoskeleton organization	4	1.93236715	0.011183416
GO:0046165 alcohol biosynthetic process	4	1.93236715	0.012696573
GO:0010639 negative regulation of organelle organization	5	2.415458937	0.013024003
GO:0009719 response to endogenous stimulus	11	5.314009662	0.014804089
GO:0034613 cellular protein localization	11	5.314009662	0.016246064
GO:0032886 regulation of microtubule-based process	4	1.93236715	0.016969603
GO:0070727 cellular macromolecule localization	11	5.314009662	0.017005115

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Table B.3 – *Continued from previous page*

GO Term BP_FAT	Count	%	P-Value
GO:0043244 regulation of protein complex disassembly	4	1.93236715	0.018877062
GO:0019318 hexose metabolic process	7	3.381642512	0.020122407
GO:0034620 cellular response to unfolded protein	3	1.449275362	0.022342152
GO:0015804 neutral amino acid transport	3	1.449275362	0.022342152
GO:0030968 endoplasmic reticulum unfolded protein response	3	1.449275362	0.022342152
GO:0031667 response to nutrient levels	7	3.381642512	0.022504362
GO:0051494 negative regulation of cytoskeleton organization	4	1.93236715	0.023034409
GO:0006886 intracellular protein transport	10	4.830917874	0.023539184
GO:0046942 carboxylic acid transport	6	2.898550725	0.023923603
GO:0015849 organic acid transport	6	2.898550725	0.024546671
GO:0006006 glucose metabolic process	6	2.898550725	0.027735379
GO:0006695 cholesterol biosynthetic process	3	1.449275362	0.033356885
GO:0009991 response to extracellular stimulus	7	3.381642512	0.035901541
GO:0005996 monosaccharide metabolic process	7	3.381642512	0.037264945
GO:0042149 cellular response to glucose starvation	2	0.966183575	0.043624971
GO:0019319 hexose biosynthetic process	3	1.449275362	0.046049316

Appendix C

Orientation	Primer Name	Sequence (5'-3')
F	BShort5'	GCATGTCTGGGCAAGCTCTGGCATAGAAGAGGCTG
F	ALong5'	TGTGGCTCTGATTGGCTTTCTGGCCATCAGAGACTGGGCATGTCTGGGCAAGCTCTG
F	Blong5'	CCCCCCTCGAGATCTAAGTAAGCTTGGGCAGCAGGCTGTGGCTCTGATTGGCTTTCTG
R	PstI3'	GAACTAGTGGATCCCCCGGG

Figure C.1. Primers for construction of p21-LUC 2x3'BS
Shown are primers used in sequential PCR steps for construction of the p21 promoter containing two copies of the 3' p53 DBS (2x3'BS p21-LUC). The 3' p53 DBS sequences replacing that of the 5' p53 DBS are highlighted in red. Underlined regions in the forward primers represent the regions of annealing. The XhoI restriction site is highlighted in blue. F: Forward, R: Reverse

Table C.1. ChIP Primers
p53 binding at the 5' p53 DNA binding site (DBS) of the WT p21 promoter is detected using WT ChIP 5 p53 DBS - Forward and WT ChIP 5 p53 DBS - Reverse primers. Binding of p53 at the 5' p53 DBS region of exogenous p21 promoter, e.g. 2x3'BS p21-LUC, is detected using pGL2 ChIP p53 DBS -Forward and WT ChIP 5' p53 DBS -Reverse.

Primer	**Sequence (5'-3')**
WT ChIP 5' p53 DBS -Forward	GTGGCTCTGATTGGCTTTCTG
WT ChIP 5' p53 DBS -Reverse	CTGAAAACAGGCAGCCCAAG
pGL2 ChIP 5' p53 DBS -Forward	CGAGCTCTTACGCGTGCTAG

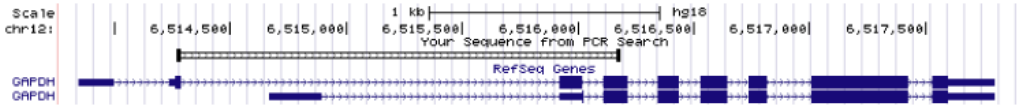
Table C.2. Primers used for RT-PCR and RT-qPCR
F: Forward, R: Reverse, ND: Not Determined.

Target	Primer Name	F/R	Sequence (5'-3')	Primer Pair Efficiency (60^oC annealing)	Amplicon Size
GAPDH mRNA	hgapdhrt5	F	AGGTGAAGGTCGGAGTCAAC	84.3%	197 bp
	hgapdhrt3	R	GACAAGCTTCCCGTTCTCAG		
p21 (*CDKN1A*) mRNA	p21#2 Forward	F	CGACTGTGATGCGCTAATGG	84.6%	371 bp
	p21 Reverse Wen et al.	R	GGCGTTTGGAGTGGTAGAAATC		
p21 (*CDKN1A*) hnRNA	BG p21 hnRNA 5	F	CCGGCCAGGTAACATAGTGTCTAAT	113.4 %	242 bp
	BG p21 hnRNA 3	R	GTGTCTCGGTGACAAAGTCGAAGT		
NOXA (*PMAIP1*) mRNA	NOXA (I)F	F	ACTGTTCGTGTTCAGCTC	ND	182 bp
	NOXA (I)R	R	GTAGCACACTCGACTTCC		
NOXA (*PMAIP1*) hnRNA	NOXA hnRNA I1F	F	TGTCCATGTTTTGCTTTCCT	ND	133 bp
	NOXA hnRNA E2R	R	TCAGGTTCCTGAGCAGAAGA		
PUMA (*BBC3*) mRNA	PUMA (I)F	F	GACCTCAACGCACAGTA	ND	106 bp
	PUMA (II)R	R	GTAAGGGCAGGAGTCCCAT		
TIGAR (*C12orf5*) mRNA	TIGAR (I)F	F	TGATCTCATGAGGACAAAGCA	ND	101 bp
	TIGAR (I)R	R	TCCTTTCCCGAAGTCTTGAG		

GAPDH:

hgapdhrt5: 5′-AGGTGAAGGTCGGAGTCAAC
hgapdhrt3l: 5′-GACAAGCTTCCCGTTCTCAG

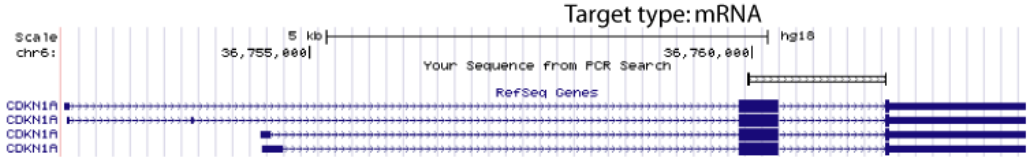
RT-PCR Amplicon: 197bp
Genomic DNA Amplicon: 1,919 bp
Target type: mRNA



CDKN1A (p21):

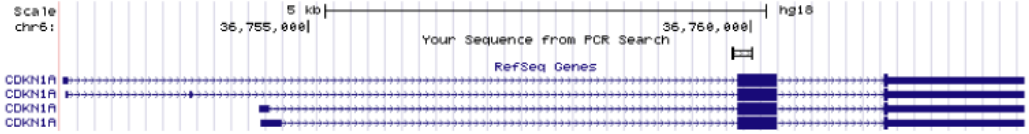
p21#2 Forward: 5′-CGACTGTGATGCGCTAATGG
p21 Reverse Wen et al: 5′-GGCGTTTGGAGTGGTAGAAATC

RT-PCR Amplicon: 371bp
Genomic DNA Amplicon: 1,575bp
Target type: mRNA



BG p21 hnRNA 5′: 5′-CCGGCCAGGTAACATAGTGTCTAAT
BG p21 hnRNA 3′: 5′-GTGTCTCGGTGACAAAGTCGAAGT

RT-PCR and Genomic DNA Amplicon: 242bp
Target type: hnRNA

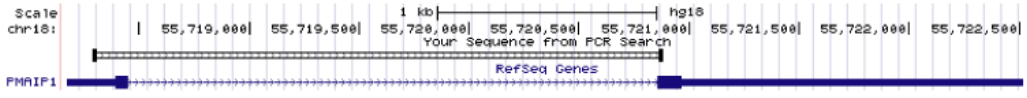


PMAIP1 (NOXA):

NOXA (I) F: 5'-ACTGTTCGTGTTCAGCTC
NOXA (I) R: 5'-GTAGCACACTCGACTTCC

RT-PCR Amplicon: 182bp
Genomic DNA Amplicon: 2,593bp
Target Type: mRNA

Source: p300 regulates p53-dependent apoptosis after DNA damage in colorectal cancer cells by modulation of PUMA/p21 levels. Proc Natl Acad Sci U S A. 2004 May 11;101(19):7386-91. Epub 2004 Apr 27.



NOXA hnRNA I1F: 5′-TGTCCATGTTTTGCTTTCCT
NOXA hnRNA E2R: 5′-TCAGGTTCCTGAGCAGAAGA

RT-PCR and Genomic DNA Amplicon: 133bp
Target Type: hnRNA

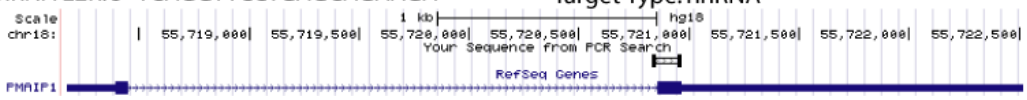
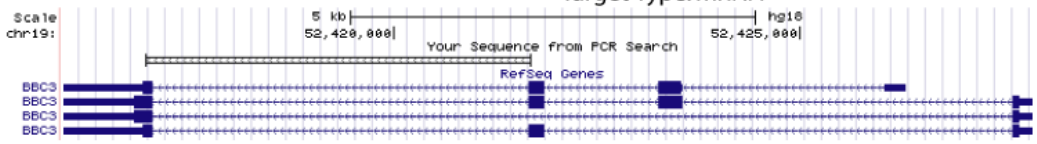


Figure C.2. Visual alignment of RT-PCR Primers

BBC3 (PUMA):

PUMA (I)F: 5′-GACCTCAACGCACAGTA
PUMA (II)R: 5′-GTAAGGGCAGGAGTCCCAT

RT-PCR Amplicon: 106 bp
Genomic DNA Amplicon: 4,751 bp
Target Type: mRNA



C12orf5 (TIGAR):

TIGAR (I)F: TGATCTCATGAGGACAAAGCA
TIGAR (I)R: TCCTTTCCCGAAGTCTTGAG

RT-PCR Amplicon: 101 bp
Genomic DNA Amplicon: 12,759 bp
Target Type: mRNA

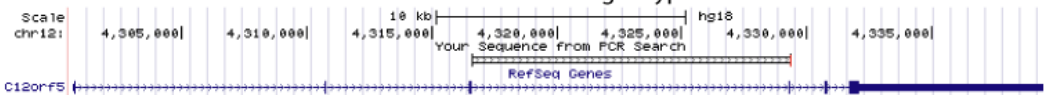


Figure C.2. Visual alignment of RT-PCR Primers
Shown is the visual alignment of RT-PCR primers to target genes as visualized using UCSC Genome Browser. The location of the aligned primers are presented by the black bars at the end of the open track above the Refseq Gene tracks (blue). Exons are represented by thick blocks and UTR regions by thin blocks. Introns are represented by the lines between exons and arrowheads indicate the direction of transcription. mRNA: messenger RNA, hnRNA: unspliced heteronuclear RNA.

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