MECHANISMS OF P53-MEDIATED APOPTOSIS

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ABSTRACT

The p53 tumor suppressor is the most commonly inactivated gene in human cancers. The ability of p53, a transcription factor, to regulate genes that mediate cell cycle arrest, apoptosis, DNA repair, and other p53-dependent activities is well known. However, the mechanisms by which p53 induces a specific activity over another are unclear. Here, I have investigated mechanisms of differential target gene regulation by p53 family proteins and have found that stringent regulation of and by p53 family proteins, the characteristics of the target gene promoter, and histone deacetylases (HDACs) all play important roles in this process. Through the use of engineered mutants, I found that the p53 N-terminal activation domain 1 (AD1) and C-terminal basic domain (BD) inhibited the ability of p53 to induce Insulin-like Growth Factor Binding Protein 3 (IGFBP3), a pro-apoptotic p53 target gene. p53(ΔNΔBD), a natural occurring p53 form in which the inhibitory functional domains are deleted, was able to activate the IGFBP3 promoter. Similar to p53, the C termini of p63 and p73 were also inhibitory. The regulation of IGFBP3 by C-terminally truncated p53 family proteins was dependent upon the unique organization of the p53-responsive element half-sites within the IGFBP3 promoter. Furthermore, I found that HDAC activity, and not p53-DNA binding activity, governed the regulation of IGFBP3 by full-length p53 family proteins, since inhibition of HDACs restored the induction of IGFBP3 by exogenous full-length p53, p63, and p73 proteins. To determine if HDAC1 or HDAC2 was the HDAC involved, I generated stable cell lines which induc-
bly knockdown HDAC1 or HDAC2. Although knockdown of HDAC1 or HDAC2 did not affect the regulation of IGFBP3 by p53, I found that HDAC2 plays novel roles in the p53 pathway. Importantly, knockdown of HDAC2 inhibited MCF7 cell proliferation in a dose-dependent manner, induced cellular senescence, and inhibited p53 transcriptional activities through modulation of p53-DNA binding activity. In sum, I have found that HDACs facilitate the differential regulation of the pro-apoptotic target gene Insulin-like Growth Factor Binding Protein 3 (IGFBP3) by p53 family isoforms and that HDAC2 impacts the transcriptional activities of p53 by modulation of p53-DNA binding activity.
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INTRODUCTION

p53, a tumor suppressor gene, is the most commonly inactivated gene in human cancers (44). Germline mutation of p53 is causative of the familial cancer syndrome, Li Fraumeni Syndrome (3, 33). Patients with Li Fraumeni Syndrome develop multiple tumors at an early age (33). Furthermore, p53 knockout mice are tumor prone (14). Interestingly, mice engineered to express mutant p53 instead of wild-type p53 display a tumor spectra that is similar to that of patients with Li Fraumeni Syndrome (31, 43). p53 mediates tumor suppression by functioning as a sequence-specific transcription factor. While the ability of p53 to regulate target genes that mediate cell cycle arrest, apoptosis, DNA repair, and other p53-dependent activities is well known, the mechanisms by which p53 induces a specific activity over another remain unclear. Here, I have investigated mechanisms of differential target gene regulation by p53 family proteins.

The p53 Functional Domains

The p53 protein contains several functional domains: activation domain 1 (AD1), activation domain 2 (AD2), the proline-rich domain (PRD), the sequence-specific DNA-binding domain (DBD), the nuclear localization signal (NLS), the tetramerization domain (TD), and the C-terminal basic domain (BD) (Fig. 1) (reviewed in reference 23). Activation domain 1 is important for transactivation; this domain contains residues that contact the basal transcriptional machinery (34). Activation domain 2 is important for
Fig. 1. The functional domains in the p53 family proteins. (A) AD, activation domain; PRD, proline-rich domain; DBD, DNA-binding domain; NLS, nuclear localization signal; TD, tetramerization domain; BD, basic domain; SAM, sterile-α-motif domain. % denotes percent identity. (B) Genomic structure of p53, p63, and p73. Dotted lines denote alternative splicing. Black bars denote an alternative reading frame. p53 is transcribed from the upstream promoter, and ΔNp53 is generated by alternative splicing or the use of an internal translational start site. p53 contains AD1 and AD2, whereas ΔNp53 contains only AD2. TAp63/TAp73 isoforms are transcribed from the upstream promoter, and ΔNp63/ΔNp73 isoforms are transcribed from the cryptic promoter within intron 3. TAp63/TAp73 contain AD1. The unique residues and the PRD comprise AD2 in ΔNp63/ΔNp73. Numbers denote amino-acid position. C-terminal alternative splicing of p63 and p73 affects the composition of the SAM domain. Black bars denote an alternative reading frame.

p53-dependent apoptosis (9, 10, 56, 62). The proline-rich domain contains five PXXP motifs and is important for the induction of apoptosis and growth suppression (47, 55, 57, 61). The DNA-binding domain recognizes the canonical p53 responsive element which consists of two decamers (RRRC^A/\gamma_T^GGYYY) separated by a spacer of 0-13 bp, where R represents purine and Y pyrimidine (15). The majority of tumor-derived mutations in p53 are missense mutations within the DNA-binding domain, underscoring the importance of p53 DNA-binding for tumor suppression. All evidence suggest that the basic domain is an important regulatory domain. Previous studies have shown that deletion of the basic domain and peptides or antibodies targeted to the basic domain increase p53 sequence-specific DNA-binding activity \textit{in vitro} (21, 26, 27, 50, 51). In addition, the function of the basic domain is altered through post-translational modifications. Residues in the basic domain are phosphorylated (e.g. by Casein Kinase II and Protein Kinase C), acetylated (e.g. by p300/CBP and P/CAF), ubiquitinated (e.g. by Mdm2), and sumoylated (reviewed in reference 1, 30, 58).

While p53 predominantly exists as a single isoform, N- and C-terminally truncated p53 forms have been identified (Fig. 1) (reviewed in reference 4, 12). N-terminal truncation of p53 occurs through internal translational initiation at codon 40 and through the use of an alternate promoter within intron 4 to yield ΔNp53 and Δ133p53, respectively (6, 13). C-terminal truncation of p53 occurs through alternative splicing of intron 9 to yield p53β (also known as I9+p53) and p53γ (2, 6, 16). In addition, several studies suggest that N- and C-terminal cleavage of p53 may be induced by interaction with mismatched DNA or with the protease calpain (36, 40, 42). To date, the biological functions of these isoforms remain unclear; however, the realization that p53 functional domains
influence p53-dependent activities sheds new light on the functional importance of these isoforms.

The p53 Family

Recently, p63 and p73 proteins have been identified as p53 homologues (reviewed in reference 59). p53 family members share significant similarity at the amino acid level within the activation domain, the DNA binding domain, and the tetramerization domain (Fig. 1). Like p53, both p63 and p73 bind to the canonical p53 responsive element, transactivate p53 target gene expression, and induce apoptosis when overexpressed (38, 60). Unlike p53, the genes encoding p63 and p73 are rarely mutated in human cancer. Rather than displaying a propensity for tumor formation as the p53 knockout mouse (14), the p63 and p73 knockout mice demonstrate discrete developmental defects (38, 59, 60). p63 and p73 undergo alternative splicing of their C terminus, resulting in three p63 isoforms (α-γ) and seven p73 isoforms (α-η) (reviewed in reference 23). These isoforms are transcribed from an upstream promoter as well as from a cryptic promoter within intron 3, called the TA and ΔN isoforms, respectively. Differential expression of these isoforms is believed to facilitate differential target gene regulation by p63 and p73.

The p53 Signaling Pathway

Because p53 is a protein with the ability to induce cell death, the activity of p53 is tightly controlled. In the unstressed condition, the p53 protein is maintained at a low level. To date, the most critical negative regulator of p53 is Mdm2 (reviewed in reference 37). Mdm2, an E3-ubiquitin ligase, promotes the proteosomal degradation of p53 by
ubiquitinating C-terminal lysine residues within the basic domain of p53. Mdm2 also interferes with the transcriptional activity of p53 by directly binding and obscuring the N-terminal transcriptional activation domain 1 of p53. In addition to Mdm2, other ubiquitin ligases such as Pirh2 and COP have been found to influence the stability of p53 (reviewed in reference 24).

The p53 protein is modified and stabilized in response to cellular stress signals induced by DNA damage, hypoxia, ionizing and UV radiation, oncogene activation, and others (Fig. 2) (18, 30, 32, 35, 41). For example, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and rad3 related (ATR) phosphorylate p53 in response to ionizing and UV radiation, respectively. N-terminal phosphorylation of p53 abrogates the interaction between p53 and Mdm2, and thus induces the stabilization of p53 (52). The activated, stabilized p53 then mediates its effects through the activation of target genes that participate in a variety of pathways such as cell cycle arrest, apoptosis, DNA repair, inhibition of angiogenesis and metastasis, and others (Fig. 2) (reviewed in reference 22). For example, p53 induces the cyclin-dependent kinase inhibitor p21, which plays important roles in G1 and G2/M cell cycle arrest. The p53 target genes 14-3-3σ and Growth Arrest and DNA-Damage Inducible protein 45 (GADD45) also play roles in p53-mediated cell cycle arrest. p53 participates in DNA repair pathways by regulating the target genes p53R2, Xeroderma Pigmentosum group E (XPE), and XPC. p53 plays a role in both the intrinsic and extrinsic pathways of apoptosis by inducing pro-apoptotic factors such as Fas/FasL, KILLER/DR5, caspase 8, BAX, p53AIP1, NOXA, PUMA, Bid, and Apaf-1 (Fig. 2).
Fig. 2. The p53 pathway. See text for details.

Differential Target Gene Regulation by p53 Family Proteins

While p53 is known to regulate many target genes for tumor suppression, mechanisms by which p53 differentially regulates gene expression remain unclear. Knowledge of these mechanisms will shed light on the basic biology of p53 and will also yield insights into potential therapies that specifically activate the pro-apoptotic transcriptional program of p53. To study mechanisms by which p53 differentially regulates gene expression, I have taken two approaches. The first approach was to characterize the role of the p53 functional domains and the activities of p53 family isoforms in the transcriptional regulation of the p53 pro-apoptotic target gene Insulin-like Growth Factor Binding Pro-
tein 3 (IGFBP3). The second approach was to characterize the role of the co-repressor histone deacetylase 2 (HDAC2) in the regulation of p53 transcriptional activities.

IGFBP3, a member of the Insulin-like Growth Factor Binding Protein (IGFBP) family, is the major serum IGFBP. In addition, IGFBP3 is expressed by most cell types and has been found to inhibit growth in both IGF-dependent and -independent manners (8, 20, 25, 29, 46, 49, 53). The regulation of IGFBP3 by p53 was initially intriguing because although IGFBP3 was previously found to be regulated by p53 (5, 7), we found that full-length p53 only weakly induced IGFBP3 whereas p53 which lacked the N-terminal activation domain 1 and the C-terminal basic domain, called p53(ΔAD1ΔBD), strongly induced IGFBP3. This suggested that the p53 functional domains may play a role in target gene regulation. Indeed, I found that AD1 and the BD inhibited the ability of full-length p53 to induce IGFBP3. Furthermore, I found a common theme among the p53 family proteins: the C terminus is inhibitory, such that C-terminally truncated p53, p63, and p73 isoforms induced IGFBP3, whereas full-length isoforms did not. Importantly, I found that full-length endogenous and exogenous p53 family proteins were capable to induce IGFBP3 when histone deacetylases (HDACs) were inhibited with Trichostatin A (TSA). Thus, histone deacetylase activity inhibited the induction of IGFBP3 by full-length p53, potentially through AD1 and the BD.

The level of histone acetylation at a gene promoter is the result of the dynamic interplay between histone acetyl-transferases (HATs) and HDACs (reviewed in reference 11). HATs are transcriptional co-activators that are recruited to a gene promoter by a trans-activator (reviewed in reference 54). HATs acetylate lysine residues within N-terminal tails of core histones. Acetylation neutralizes the positive charge of the lysine
residue within the histone tail to weaken the electrostatic interaction between DNA and histone tails and promote the formation of transcriptionally active chromatin. Conversely, HDACs deacetylate lysine residues to promote the formation of transcriptionally inactive chromatin. In addition to histones, both HATs and HDACs have been identified to regulate the acetylation of many non-histone proteins (reviewed in reference 17).

HDACs, which are well recognized to function as co-repressors, are organized into four classes: class I (HDAC 1, 2, 3, 8), class II (HDAC 4, 5, 6, 7, 9, 10), class III (SIRT 1-7), and class IV (HDAC 11) (19, 39). HDACs in classes I, II, and IV contain a conserved catalytic domain and are commonly inhibited by HDAC inhibitors such as TSA and sodium butyrate (reviewed in reference 19). These inhibitors chelate the zinc cation within the enzyme active site (reviewed in reference 19). Class III HDACs, which contain an NAD-dependent catalytic domain, are insensitive to these agents (reviewed in reference 19). Since HDAC1 and HDAC2 are TSA-sensitive and have been found to interact with and inhibit the transcriptional activity of p53 (28), I chose to study the role of HDAC1 and HDAC2 in the p53 pathway. Through the use of stable MCF7 cell lines which inducibly knockdown HDAC1 or HDAC2, I found that HDAC2, but not HDAC1, plays important roles in the p53 pathway. I found that knockdown of HDAC2 inhibited cellular proliferation in a dose-dependent manner, induced cellular senescence, and augmented p53-DNA binding to enhance p53-dependent trans-activation and trans-repression of a subset of target genes.

In summary, I have explored mechanisms which facilitate differential target gene regulation by p53 family proteins. I have found that the organization of the p53-responsive elements within the target gene, the functional domains of p53 family pro-
teins, and the co-repressor HDAC2 all play important roles in differential target gene regulation by p53 family proteins.
THE C TERMINUS OF P53 FAMILY PROTEINS IS A CELL FATE DETERMINANT

by

KELLY LYNN HARMS AND XINBIN CHEN


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Abstract

The p53 tumor suppressor is the most commonly mutated gene in human cancers. The ability of p53 to induce cell cycle arrest, apoptosis, DNA repair, and other p53-dependent activities is well known; however, the mechanism by which p53 induces a specific activity over another is unclear. Here, we showed that stringent regulation of and by p53 family isoforms facilitates differential target gene expression and thus determines cell fate. Through the use of engineered deletion mutants, we found that activation domain 2 is required for induction of the proapoptotic target gene insulin-like growth factor binding protein 3 (IGFBP3) by p53 and that the basic domain inhibits induction of this gene by p53. Thus, for the first time we provide evidence that the basic domain of p53 is inhibitory in vivo as has been determined in vitro. We also showed that the in vivo inhibitory activity of the basic domain depends upon activation domain 1, such that combined deletion of activation domain 1 and the basic domain was required to alleviate the inhibition by the basic domain. Importantly, deletion of the inhibitory functional domains, namely N-terminal activation domain 1 and the C-terminal basic domain, is paralleled in nature. We found that the IGFBP3 promoter was activated by p53(ΔNΔBD), which mimics a naturally occurring N-and C-terminally truncated human p53 isoform, and by p53AS, a C-terminally truncated murine p53 isoform generated through alternative splicing, but not by full-length human or murine p53. In addition, we found that the C termini of p63 and p73 inhibit the induction of IGFBP3, such that C-terminally truncated p63 and p73 isoforms induce the expression of IGFBP3, whereas full-length ones cannot. We also demonstrated that IGFBP3 is an important effector of the apoptosis induced by N-and C-terminally truncated p53, such that knockdown of IGFBP3 by using an IGFBP3 neutral-
izing antibody or IGFBP3 small interfering RNA partially rescues the cell death induced by N-and C-terminally truncated p53. In addition, we identified that histone deacetylase activity, not p53 DNA binding ability, governs the regulation of IGFBP3 by full-length p53 family proteins, as inhibition of histone deacetylases restores the induction of IGFBP3 by exogenous full-length p53, p63, and p73 proteins. Furthermore, we found that activation of p53 or inhibition of histone deacetylases alone was not sufficient to induce IGFBP3; however, combined treatment endowed endogenous p53 with this activity. To better understand the significance of this regulation, we performed a microarray study and identified several target genes differentially regulated by full-length p53 and p53 lacking the N-terminal activation domain 1 and the C-terminal basic domain. Taken together, our data suggest a novel mechanism by which p53 family proteins differentially regulate gene expression and provide an insight for designing a combined therapy for cancer treatment.

Introduction

The tumor suppressor p53 is the most commonly mutated gene in human cancers (43). After activation by cellular stresses, p53, a sequence-specific transcription factor, functions to transactivate genes that mediate cell cycle arrest, apoptosis, DNA repair, inhibition of angiogenesis and metastasis, and other p53-dependent activities (24, 30). The p53 protein contains several functional domains: activation domain 1 (AD1) within residues 1 to 42, activation domain 2 (AD2) within residues 43 to 63, the proline-rich domain (PRD) within residues 64 to 91, the sequence-specific DNA binding domain (DBD) within residues 100 to 300, the nuclear localization signal within residues 316 to 325, the
tetramerization domain (TD) within residues 334 to 356, and the C-terminal basic domain (BD) within residues 364 to 393.

AD1 is important for transactivation; this domain contains residues that contact the basal transcriptional machinery (34). Previously, along with others we identified AD2 and characterized the requirement of AD2 for p53-dependent apoptosis (7, 8, 61, 70). In addition, along with others we have shown that the PRD is necessary for the induction of apoptosis and contributes to growth suppression (50, 60, 62, 67). The C-terminal BD has been subjected to extensive analysis. All evidence suggests that the BD is an important regulatory domain. Previous studies have shown that deletion of the BD and peptides or antibodies targeted to the BD increase p53-specific DNA binding activity in vitro (23, 25, 26, 55, 56). In addition, the function of the BD is altered through posttranslational modifications such as phosphorylation by casein kinase II or protein kinase C and acetylation by p300/CBP as well as through interactions with other proteins such as the calcium binding protein S100b and the DNA repair proteins XPB and XPD (reviewed in references 4 and 30).

Recently, the p63 and p73 proteins have been identified as p53 homologues (2, 28, 44, 59, 63). p53 family members share significant similarity at the amino acid level within the AD, the DBD, and the TD. Like p53, both p63 and p73 bind to the canonical p53-responsive element, transactivate p53 target gene expression, and induce apoptosis when overexpressed (reviewed in reference 64). Unlike p53, the genes encoding p63 and p73 are rarely mutated in human cancer (39, 65). Rather than displaying a propensity for tumor formation as in the p53 knockout mouse model (14), the p63 and p73 knockout animals demonstrate discrete developmental defects (39, 65). In addition, p63 and p73
undergo alternative splicing of their C termini, resulting in three p63 isoforms (α to γ) and seven p73 isoforms (α to η). These isoforms are transcribed from an upstream promoter as well as from a cryptic promoter within intron 3, called the TA and ΔN isoforms, respectively (reviewed in reference 64). Differential expression of these isoforms is believed to facilitate differential target gene regulation by p63 and p73.

With the realization that each p63 and p73 isoform functions in similar yet different manners, new light is shed on the functional importance of previously identified p53 isoforms. Although different mechanisms generate p53 isoforms, mouse and human p53 exist as both N- and C-terminally truncated forms (reviewed in reference 11). N-terminal truncation of human and mouse p53 yields ΔNp53 and Δ40p53, respectively. Human ΔNp53 is generated through alternative splicing of intron 2 with the subsequent use of the translational start site at codon 40 (21). Mouse Δ40p53 lacks the first 40 amino acids and is generated through the use of an internal translational start site at codon 41. C-terminal truncation of human and mouse p53 yields I9+p53 and p53AS and occurs through alternative splicing of introns 9 and 10, respectively (1, 18, 32). In addition, several studies strongly suggest that calpain and interaction with mismatched DNA induce both N- and C-terminal cleavage of p53 (31, 37, 40, 42, 45). To date, the biological functions of these p53 isoforms remain unclear.

Insulin-like growth factor binding protein 3 (IGFBP3) belongs to the IGFBP family. There are six known members (reviewed in reference 17). Previously, IGFBP3 was shown to be regulated by p53 (5). IGFBP3 contains eleven decamers of the p53-responsive element within the promoter (3) and two canonical p53-responsive elements, called box A and box B, within introns 1 and 2, respectively (5). Since IGFBP3 is the ma-
jor serum IGFBP, many IGF-dependent functions have been described; however, recent
evidence supports the IGF-independent functions of IGFBP3. Interestingly, IGF-
independent functions oppose the IGF-dependent functions, such that IGFBP3 directly
inhibits growth, induces apoptosis, and sensitizes cells to apoptosis (6, 22, 29, 53).

Here, we showed that p53 isoforms differentially regulate target gene expression.
Through the use of engineered deletion mutants, we first found that p53 functional do-
 mains dictate target gene specificity. We found that AD2 is required for, and the basic
domain inhibits, induction of IGFBP3 by p53. Subsequently, we found a common theme
among the p53 family of transcription factors: the C terminus of p53 family isoforms in-
hibits transactivation of IGFBP3, such that C-terminally truncated p53, p63, and p73 iso-
forms induce the expression of IGFBP3, whereas full-length ones cannot. We showed
that IGFBP3 is an important mediator of p53(ΔAD1ΔBD)-dependent apoptosis. We also
determined that histone deacetylase (HDAC) activity governs the induction of IGFBP3 by
full-length p53 family proteins, as the inhibition of HDACs restores the ability of exoge-
nous full-length p53, p63, and p73 isoforms to induce IGFBP3. Interestingly, we found
that the simultaneous stabilization of p53 through the DNA damage pathway and the in-
hibition of HDACs restore the ability of endogenous full-length p53 to induce IGFBP3.
Our data suggest a mechanism by which p53 family isoforms differentially interact with
regulatory proteins to facilitate a highly regulated gene expression profile.
Materials and Methods

*Plasmids and Mutagenesis*

Human p53 proteins encoding full-length p53, p53(R249S), p53(ΔAD1ΔBD), p53(ΔAD1), p53(ΔBD), p53(AD1−ΔBD), p53(ΔAD2ΔBD), p53(AD2−ΔBD), and p53(ΔD1AD2−ΔBD) were as previously described (69). All cDNAs, except p53(R249S) and p53(AD1−ΔBD), were N-terminally hemagglutinin (HA)-tagged. To generate p53(ΔC20), a human cDNA fragment that encodes residues 1 to 373 was amplified by the 5′ end primer 5HA (GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TGA GGA GCC GCA GTC AGA TCC) and the 3′ end primer C373 (AGA ATT CTC ACT TTT TGG ACT TCA G). To generate p53(ΔC10), a human cDNA fragment that encodes residues 1 to 383 was amplified by the 5′ end primer 5HA and the 3′ end primer C383 (AGA ATT CTC AGA GTT TTT TAT GGC G). To generate ΔNp53, a human cDNA fragment that encodes residues 40 to 393 was amplified by the 5′ end primer ΔNp53 (AGA ATT ACC ATG GAT GAT TTG ATG CTG TCC CCG G) and the 3′ end primer C393 (AGA ATT CTC AGT CTG AGT CAG GCC CTT CTG TC). To generate p53(ΔNΔBD), the 3′ end cDNA fragment starting from the StuI site in ΔNp53 was replaced with the corresponding cDNA fragment in p53(ΔBD). To generate I9+p53, a human cDNA fragment that encodes an alternative splice form of p53 was generated by using the 5′ end primer 5hp53 (AGA ATT CAC CAT GGA GGA GCC GCA GTC AGA TCC TA) and the consecutive 3′ end primers I9-up (TTG AAA GCT GGT CTG GTC CTG AAG GGT GAA ATA TTC) and I9-dn (AGA ATT CTT AAC AAT TTT CTT TTT GAA AGC TGG TCT GGT C). Full-length murine p53 was generated by using 5′ end primer 5mp53 (AGA ATT CAC CAT GAC TGC CAT GGA GGA GTC) and the 3′ end
primer mC387 (TGA ATT CTC AGT CTG AGT CAG GCC CCA C). p53(A135V), a murine cDNA that encodes a naturally occurring p53 DNA binding mutant was as previously described (38). To generate Δ40p53, a murine cDNA fragment that encodes residues 41 to 387 was amplified by using the 5' end primer Δ40p53 (AGA ATT CAC CAT GGA CGA TCT GTT GCT GCC) and the 3' end primer mC387. To generate p53AS, a murine cDNA fragment that encodes an alternative splice form of p53 was generated by using the 5' end primer 5mp53 and the consecutive 3' end primers AS-up (TTG ATC AAG GCT TGG AAG GCT CTA GGC TGG AGG CTG GAG TGA GCC CT) and AS-dn (TGA ATT CCT AGC AGT TTG GGC TTT CCT CCT TGA TCA AGG GGA AG).

cDNAs encoding murine p63 isoforms, p63α, ΔNp63α, p63γ, and ΔNp63γ were kindly given by C. Di Como (20). All p63 proteins were N-terminally Myc-tagged as previously described (20). All p63-expressing constructs were confirmed by DNA sequencing. cDNAs encoding human p73 isoforms, p73α, ΔNp73α, p73β, and ΔNp73β were as previously described (35).

The luciferase reporter constructs under the control of the p21 promoter, IGFBP3 box A, and IGFBP3 box B were as previously described (5, 41). Luciferase reporter constructs under the control of the IGFBP3 promoter, pGL2/–1165 IGFBP3 and pGL2/–705 IGFBP3, were kindly given by Xiao-Fan Wang. Deletion constructs of the IGFBP3 promoter in the luciferase reporter vector pGL2 were generated by PCR and confirmed by sequencing. To generate pGL2/–256 IGFBP3, a genomic fragment of the IGFBP3 promoter spanning nucleotides (nt) –256 to +72, with +1 being the transcriptional start site, was amplified by using the 5' end primer –256 BP3 (AGG TAC CTG GCC GGG CAC
ACC TTG) and the 3' end primer GLprimer2 (Promega). pGL2/–183 IGFBP3, pGL2/–116 IGFBP3, and pGL2/–91 IGFBP3 were amplified by using the 5' end primers –183 BP3 (AGG TAC CCG GCC GAG TCT CGA GCT G), –116 BP3 (AGG TAC CCA GCC GTG CCT GCG CCG A), or –91 BP3 (AGG TAC CCC TCC CAA CCC CCA CTC C) with the 3' end primer GLprimer2.

To generate constructs expressing small interfering RNAs (siRNAs) targeting the IGFBP3 coding region, pSuper-si-IGFBP3-CR1 and pSuper-si-IGFBP3-CR2 64-nt oligonucleotides were annealed and cloned into pSuper. With the siRNA targeting region shown in bold, the oligonucleotides for pSuper-si-IGFBP3-CR1 were the forward oligonucleotide 5'─GAT CCC CGA GCA CAG ATA CCC AGA ACT TCA AGA GAG TTC TGG GTA TCT GTG CTC TTT TTG GAA A-3' and the reverse oligonucleotide 5'─AGC TTT TCC AAA AAG AGC ACA GAT ACC CAG AAC TCT CTT GAA GTT CTG GGT ATC TGT GCT CCG G-3'. The oligonucleotides for pSuper-si-IGFBP3-CR2 were the forward oligonucleotide 5'─GAT CCC CGA GCA TTG CCC GGA GCT GC TTT TTG GAA A-3' and the reverse oligonucleotide 5'─AGC TTT TCC AAA AAG GGG AAG GAG GAC GTG CAC TCT CTT GAA GTG CAC GTC CTC CTT CCC CGG G-3'. The oligonucleotides for the control siRNA targeting rat p53 were the forward oligonucleotide 5'─GAT CCC CGA TTG CCC GGA GCT GCT TCA AGA GAG CAG CTC CGG GCA ATG CTC CTT TTT GGA AA-3' and the reverse oligonucleotide 5'─AGC TTT TCC AAA AAG AGC ATT GCC CGG AGC TGC TCT CTT GAA GCA GCT CCG GGC AAT GCT CCG GG-3'.
Cell Lines

The culture, transfection, and generation of MCF7 cell lines were performed as previously described (70). Individual clones were screened for the inducible expression of HA-tagged p53(ΔBD) by Western blot analysis with monoclonal antibodies against p53. Individual clones were screened for the inducible expression of Myc-tagged p63γ and Myc-tagged ΔNp63γ by Western blot analysis with monoclonal antibodies against Myc. The MCF7 cell lines p53-24, p53(ΔAD1ΔBD)-15, p63α-11, ΔNp63α-9, p73α-2, and p73β-31 were as previously described (13, 68, 69). EB and H1299 cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum.

Luciferase Assay

A dual luciferase reporter assay was used to determine the transcriptional activity of p53, engineered p53 mutants, and p53 family isoforms. The luciferase reporter constructs used were pGL2/p21-A (41), which is regulated by the p21 promoter with two p53-responsive elements; pGL2/IGFBP3-Box A (5), which contains the p53-responsive element within intron 1 of the IGFBP3 gene; pGL2/IGFBP3-Box B (5), which contains the p53-responsive element within intron 2 of the IGFBP3 gene; and pGL2/–1165 IGFBP3, pGL2/–705 IGFBP3, pGL2/–256 IGFBP3, pGL2/–183 IGFBP3, pGL2/–116 IGFBP3, and pGL2/–91 IGFBP3, which are regulated by the IGFBP3 promoter. In all, 1 μg of a luciferase reporter, 1 μg of pcDNA3 control vector or pcDNA3 vector that expresses p53 family isoforms, and 25 ng of Renilla luciferase assay vector pRL-CMV (Promega, Madison, Wis.) were cotransfected by using the calcium phosphate method into MCF7, H1299, or EB cells. A dual luciferase assay was performed in triplicate ac-
cording to the manufacturer's instructions (Promega). The fold increase in relative luciferase activity is a product of the luciferase activity induced by p53 family isoforms divided by that induced by an empty pcDNA3 vector.

**Western Blot Analysis**

Western blot analysis was performed as previously described (70) with anti-p53 monoclonal antibodies DO-1, PAb1801, PAb240, and PAb421; antiactin polyclonal antibody (Sigma); anti-p21 polyclonal antibody (C-19) (Santa Cruz); anti-HA polyclonal antibody (Y11) (Santa Cruz); anti-HA monoclonal antibody (HA.11) (Covance); and anti-Myc monoclonal antibody (9B11) (Cell Signaling). To analyze IGFBP3 protein, conditioned medium was collected from MCF7 cells induced or uninduced to express p53 and mutants for 72 h. The conditioned medium was concentrated by using a VivaSpin 6-ml concentrator (Vivascience), resuspended with 2x sodium dodecyl sulfate sample buffer, and boiled for 5 min. Western blot analysis was then performed as described by using anti-IGFBP3 polyclonal antibody (Upstate).

**RNA Isolation, Northern Blot Analysis, and Reverse Transcription-PCR (RT-PCR)**

Total RNA was isolated by using Trizol reagents (Invitrogen). Northern blot analyses were performed as described (70). The p21, glyceraldehyde-3-phosphate dehydrogenase (GADPH), and aquaporin 3 probes were prepared as previously described (66, 70); the IGFBP3 cDNA probe was made from a 700-bp EcoRI-XhoI fragment. First-strand cDNA was synthesized by using iScript (Bio-Rad) according to the manufacturer's instructions. The level of the transcripts for IGFBP3 and GADPH were determined by
PCR. The primers used to amplify GAPDH were as previously described (9). The primers used to amplify a 942-bp IGFBP3 cDNA fragment were the 5' end primer 5-BP3-CR (AGA ATT CTG TAC TGT CGC CCC ATC C) and 3' end primer 3-BP3-CR (AGA ATT CCG TCT ACT TGC TCT GCA TGC).

**ChIP Assay**

A chromatin immunoprecipitation (ChIP) assay was performed as previously described (36). After 24 h of induction (+) or no induction (−) of full-length p53 or p53(ΔAD1ΔBD) in MCF7 cells, chromatin was cross-linked in 1% formaldehyde in phosphate-buffered saline (PBS), and nuclei were extracted. Chromatin was sonicated to yield 500- to 1,000-bp DNA fragments and immunoprecipitated with a mixture of anti-HA polyclonal antibody (Y11) (Santa Cruz) and anti-p53 PAb1801 monoclonal antibody. After reverse cross-linking and phenol-chloroform extraction, DNA fragments bound by p53 were purified over a QIAGEN column. PCR was performed to visualize the enriched DNA fragments. Primers designed to amplify the 11 decamers of the p53-responsive element within the IGFBP3 promoter were the 5' end primer –282BP3 (TGC TGA GGT GGC CTG GAG T) and the 3' end primer +51BP3 (TCC AGG CAG GAA GCG GCT GAT C). Primers that amplify the 5' p53-responsive element within the p21 promoter were as previously described (36).

**Trypan Blue Dye Exclusion Assay**

MCF7-p53-24 or MCF7-p53(ΔAD1ΔBD)-15 cells were seeded at a density of 2 x 10^4 cells/well in 24-well plates in the presence or absence of tetracycline. At 12 h after
plating, anti-rabbit polyclonal antibody (Sigma) or anti-IGFBP3 polyclonal antibody (Upstate) was added to the cell culture supernatant at a dilution of 1:250. At 24 h after antibody addition, both floating cells in the medium and live cells on the plate were collected and concentrated by centrifugation. After staining with trypan blue (Sigma) for 10 min, both live (unstained) and dead (stained) cells were counted in a hemocytometer. The percentage of dead cells was calculated as the number of dead cells divided by the total number of cells counted.

**Proliferation Assays**

A CellTiter96 nonradioactive cell proliferation assay (Promega) was used to perform an MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide] assay. MCF7-p53(ΔAD1ΔBD)-15 cells were seeded at a density of 5 x 10^3 cells/well in 96-well plates in the presence and absence of tetracycline. To use IGFBP3 siRNA to characterize the contribution of IGFBP3 during MCF7-p53(ΔAD1ΔBD)-15-mediated cell death, after adherence to the plate, cells were transfected with 50 ng (per 96-well plate) of either a control siRNA expression vector targeting rat p53 or a mix of siRNA expression vectors targeting two locations within the coding region of IGFBP3 by using FuGENE 6 transfection reagent (Roche). At 44 h after plating, cells were labeled with the dye solution for 4 h and permeabilized for at least 1 h, and the absorbance at 570 nm was read by a microplate reader. To use IGFBP3 neutralizing antibody, at 18 h after plating, anti-rabbit polyclonal antibody (Sigma) or anti-IGFBP3 polyclonal antibody (Upstate) was added to the cell culture supernatant at a dilution of 1:250. At 72 h after plating, samples were processed as
above, and absorbance at 570 nm was determined. \( P \) values were determined by a Student's \( t \) test.

A CellTiter-Glo luminescent cell viability assay (Promega) was used to determine the cellular ATP content. MCF7-p53(ΔAD1ΔBD)-15 cells were transfected with pSuper empty vector or a mix of siRNA expression vectors targeting two locations within the coding region of IGFBP3 by using FuGENE 6 transfection reagent (Roche). At 24 h after transfection, cells were seeded at a density of \( 5 \times 10^3 \)/well in 96-well plates in the presence and absence of tetracycline. At 36 h after plating, the CellTiter-Glo luminescent cell viability assay (Promega) was performed according to the manufacturer's instructions. The relative luciferase unit is expressed as a percentage of the product of luciferase activity induced by ATP content in the presence of p53 divided by that induced in the absence of p53. \( P \) values were determined by a Student's \( t \) test.

**Drug Treatments**

To verify that cisplatin stabilizes endogenous p53 and that trichostatin A (TSA) increases acetylation of histones, MCF7 cells were treated with 50 \( \mu \)M cisplatin (Sigma) dissolved in \( \text{H}_2\text{O} \) and 100 ng of TSA (Upstate) per ml dissolved in 75% ethanol. To determine the effect upon IGFBP3 expression, MCF7 cells were treated with 50 \( \mu \)M cisplatin and 100 ng of TSA per ml for 12 h. To determine whether exogenous p53, p63\( \alpha \), or p73\( \alpha \) induces IGFBP3 in the presence of TSA, MCF7 cells were induced or uninduced to express p53, p63\( \alpha \), or p73\( \alpha \) for 12 h prior to treatment with 100 ng of TSA per ml for another 12 h.
**Affymetrix Gene Chip Analysis**

Total RNA from MCF7 cells induced and uninduced to express full-length p53 and p53(ΔAD1ΔBD) was isolated, labeled, and hybridized to an Affymetrix gene chip (U133 plus 2.0).

**Results**

**IGFBP3 Is Induced by p53(ΔAD1ΔBD) but Not by Full-Length p53**

p53 contains several functional domains (Fig. 1A). During the characterization of these domains by using stable inducible cell lines, we have previously reported that p53(ΔAD1ΔBD), which lacks the N-terminal AD1 and the C-terminal BD, is more potent than full-length p53 to induce apoptosis in MCF7 breast adenocarcinoma cells (69). We chose MCF7-HA-p53-24 and MCF7-HA-p53(ΔAD1ΔBD)-15 cell lines because the proteins expressed by these lines are equivalent, although they are expressed at levels two-fold higher than endogenous p53 levels induced by cisplatin (Fig. 1B and data not shown). While the BD has been characterized as a negative regulatory domain in vitro (23, 25, 56), to date, no one has been able to demonstrate the negative activity of this domain in vivo. We reasoned that the study of p53(ΔAD1ΔBD) might shed insight into how the BD functions as a negative regulator. Interestingly, we found that IGFBP3, a proapoptotic target gene, was induced by p53(ΔAD1ΔBD) but not by full-length p53, as analyzed by Northern blotting (Fig. 1C). To demonstrate that both full-length p53 and p53(ΔAD1ΔBD) are transcriptionally active, we analyzed the expression of p21. Both full-length p53 and p53(ΔAD1ΔBD) were able to induce p21. GAPDH was used as a
FIG. 1. IGFBP3 expression is induced by p53(ΔAD1ΔBD), but not by full-length p53. (A) Schematic representation of p53 functional domains: AD1, AD2, PRD, sequence-specific DBD, the nuclear localization signal (NLS), TD, and the C-terminal BD. Numbers indicate amino acid residues. (B) The level of p53 and actin was assayed by Western blot analysis by using anti-HA monoclonal and antiactin polyclonal antibodies, respectively, in MCF7-HA-p53-24 and MCF7-HA-p53(ΔAD1ΔBD)-15 cells in the absence (–) or presence (+) of p53 or p53(ΔAD1ΔBD) for 24 h. (C) A Northern blot was prepared by using total RNAs isolated from MCF7-HA-p53-24 and MCF7-HA-p53(ΔAD1ΔBD)-15 cells in the absence (–) or presence (+) of p53 or p53(ΔAD1ΔBD) for 24 h. The blot was sequentially probed with cDNAs derived from IGFBP3, p21, and GAPDH genes.

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loading control. These data suggest that AD1 and the BD inhibit the ability of full-length p53 to induce IGFBP3.

IGFBP3 was shown to be regulated by p53 in EB-1 cells, a colorectal carcinoma cell line that expresses exogenous p53 under control of the metallothionein promoter (5). Because we identified a unique situation where p53(ΔAD1ΔBD), but not full-length p53, induced IGFBP3, we wanted to characterize the mechanism regulating the ability of full-length p53 to induce IGFBP3 in MCF7 cells. Interestingly, O-glycosylation masks the PAb421 epitope of p53 in EB-1 cells and the resulting O-glycosylated p53 is activated for DNA binding in vitro (57).

The IGFBP3 Promoter Is Activated by p53(ΔAD1ΔBD) But Not by Full-Length p53

The canonical p53-responsive element contains two decamers [RRRC(A/T)(A/T)GYYY] separated by a spacer of 0 to 13 bp, where R represents purine and Y represents pyrimidine (15). IGFBP3 is a unique p53 target gene in that it contains 11 tandem decamers of the p53-responsive element within the proximal promoter, with each decamer separated by ≤9 bp (3). Thus, the IGFBP3 promoter potentially contains 10 tandem canonical p53-responsive elements spanning the region from nt –246 to –92, with +1 as the transcriptional start site. In addition, IGFBP3 contains two canonical p53-responsive elements, called box A and box B, within introns 1 and 2, respectively (5) (Fig. 2A).

To determine which regions within the IGFBP3 gene are responsive to p53(ΔAD1ΔBD), we analyzed the ability of transiently overexpressed HA-p53(ΔAD1ΔBD) and full-length HA-p53 to activate the IGFBP3 promoter as well as box
FIG 2. Activation of the IGFBP3 promoter by p53(ΔAD1ΔBD) requires all 11 decamers of the p53-responsive element. (A) Schematic representation of the IGFBP3 locus and luciferase reporter constructs. CMV, cytomegalovirus. (B) Schematic representation of the p21 luciferase reporter construct. (C) The IGFBP3 promoter is activated by p53(ΔAD1ΔBD) but not by full-length p53 in MCF7 cells. MCF7 cells were cotransfected with 1 µg of the luciferase reporter under control of the IGFBP3 promoter, box A, or box B and 1 µg of empty pcDNA3 or pcDNA3 vector expressing HA-p53 or HA-p53(ΔAD1ΔBD). (D) The IGFBP3 promoter is activated by p53(ΔAD1ΔBD) but not by full-length p53 in EB cells. The experiment was performed as described above except that EB cells were used.

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FIG. 2 con’t. Activation of the IGFBP3 promoter by p53(ΔAD1ΔBD) requires all 11 decamers of the p53-responsive element. (E) The 11 decamers of the p53-responsive element function as a unit. MCF7 cells were cotransfected with 1 µg of a luciferase reporter under control of various lengths of the IGFBP3 promoter and 1 µg of empty pcDNA3 or pcDNA3 vector expressing HA-p53 or HA-p53(ΔAD1ΔBD). The number between each decamer indicates the number of base pairs of separation. (F) HA-p53 and HA-p53(ΔAD1ΔBD) are expressed at equal levels. MCF7 cells were transfected with 5 µg of empty pcDNA3 or pcDNA3 vector expressing HA-p53 or HA-p53(ΔAD1ΔBD). HA-p53 and HA-p53(ΔAD1ΔBD) were detected with anti-HA monoclonal antibody. Actin was detected with antiactin polyclonal antibody.


A and box B in MCF7 cells by using a dual luciferase reporter assay. We found that the –1165 and –705 IGFBP3 reporter constructs (Fig. 2A), which contain the IGFBP3 promoter from nt –1165 to +73 and from nt –705 to +73, respectively, were activated by p53(ΔAD1ΔBD) but not by full-length p53 (Fig. 2C and E). Surprisingly, reporters under the control of box A or box B linked to a minimal cytomegalovirus promoter (Fig. 2A) were only weakly activated by p53(ΔAD1ΔBD) and full-length p53 (Fig. 2C).
luciferase reporter under the control of the p21 promoter with two canonical p53-responsive elements (Fig. 2B) was activated by both full-length p53 and p53(ΔAD1ΔBD), albeit to a lesser extent by the latter (Fig. 2C). Equal levels of p53 protein are expressed by HA-p53 and HA-p53(ΔAD1ΔBD) as detected by Western blot analysis (Fig. 2F). These data suggest that the p53-responsive elements within the IGFBP3 promoter are primarily responsible for the induction of IGFBP3 by p53(ΔAD1ΔBD).

To determine whether our observation was cell type specific, we tested the ability of full-length p53 and p53(ΔAD1ΔBD) to activate the IGFBP3 promoter in EB colon and H1299 non-small cell lung carcinoma cells. We found the same pattern of activation in EB and H1299 cells: the IGFBP3 promoter was activated by p53(ΔAD1ΔBD) but not by full-length p53 (Fig. 2D and data not shown). Again, the p21 promoter was activated by both p53(ΔAD1ΔBD) and full-length p53 (Fig. 2D and data not shown).

Because IGFBP3 is unique in that the promoter contains 11 tandem decamers of the p53-responsive element, we wanted to determine which decamers were necessary or sufficient for activation by p53(ΔAD1ΔBD). Thus, we generated four new reporter constructs: –256 IGFBP3, –183 IGFBP3, –116 IGFBP3, and –91 IGFBP3 (Fig. 2A). We found that the –256 IGFBP3 reporter, which contains all 11 decamers, was strongly activated by p53(ΔAD1ΔBD) (Fig. 2E). Surprisingly, we found that the 11 decamers function as a unit. Deletion of four upstream decamers inhibited activation of the –183 IGFBP3 reporter by p53(ΔAD1ΔBD) (Fig. 2E). In addition, the presence of only two decamers, recapitulating the canonical p53-responsive element, was not sufficient for activation of the –116 IGFBP3 reporter by p53(ΔAD1ΔBD) (Fig. 2E). Similarly, no activity was detected when all 11 decamers were deleted as in the –91 IGFBP3 reporter (Fig. 2E). Con-
sistent with the above data, full-length p53 did not strongly activate any of the reporter constructs (Fig. 2E). Taken together, these data and results obtained by Northern blot analysis (Fig. 1C) indicate that p53(ΔAD1ΔBD) but not full-length p53 is capable of transactivating the IGFBP3 gene.

*Activation of the IGFBP3 Promoter by p53 Requires the Presence of AD2 and Deletion of the BD*

The mechanism by which p53 induces a specific activity over another remains unclear in p53 research. We reasoned that p53 functional domains may play a role in target gene selection. To this end, we used deletion and point mutation of p53 to analyze the requirements of p53 functional domains for activation of the IGFBP3 promoter. Because p53(ΔAD1ΔBD) lacks two functional domains, we first wanted to determine which domain is inhibitory. We found that deletion of only AD1 resulted in activity similar to full-length p53 (Fig. 3A); however, deletion of only the BD enabled strong activation of the IGFBP3 promoter (Fig. 3A). We then wanted to identify the inhibitory residues within the BD. We found that p53(ΔC20), which lacks the C-terminal 20 amino acids, strongly activated the IGFBP3 promoter, albeit less than p53(ΔBD) (Fig. 3A).

p53(ΔC10), which lacks the C-terminal 10 amino acids, had activity similar to that of full-length p53 (Fig. 3A). p53(R249S), a tumor-derived DNA binding mutant, served as a negative control and did not activate the IGFBP3 promoter. p53 protein was expressed by all p53 constructs used in these studies (data not shown).

Next, we wanted to characterize the N-terminal requirement. We reasoned that AD1 is not required because p53(ΔAD1ΔBD) strongly activates the IGFBP3 promoter.
FIG. 3. Activation of the IGFBP3 promoter by p53 requires the presence of AD2 and deletion of the BD. Schematic representations of p53 and mutants are shown at left. Graphs (right) show increases (n-fold) in activation of the –256 IGFBP3 reporter by p53 and mutants. MCF7 cells were cotransfected with 1 µg of luciferase reporter under control of the IGFBP3 promoter and 1 µg of empty pcDNA3 or pcDNA3 vector expressing p53 and various mutants. (A) The BD inhibits p53 activation of the IGFBP3 promoter. (B) AD1 is dispensable, but an inactive AD1 is inhibitory for p53 activation of the IGFBP3 promoter. (C) AD2 is required for activation of the IGFBP3 promoter.

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To test this, we used a double point mutation in AD1 (Gln^{22}-Ser^{23}), resulting in a well-defined AD1-deficient mutant (AD1−) that is incapable of interacting with the transcriptional machinery (34). We found that p53(AD1−ΔBD), which contains the nonfunctional AD1 and lacks the BD, was not able to activate the IGFBP3 promoter, whereas p53(ΔBD), which lacks only the BD, was active (Fig. 3B). Thus, AD1 is dispensable, but an inactive AD1 is inhibitory. This finding suggests that the conformation of p53 might be important for the induction of IGFBP3.

Because AD1 is dispensable, we wanted to characterize the role of AD2. We have previously found that AD2 is important for the induction of apoptosis (70). Interestingly, we found that deletion of AD2 (ΔAD2) or mutation of AD2 (AD2−) abolished the activity of p53(ΔBD) at the IGFBP3 promoter (Fig. 3C). Further strengthening the importance of AD2 was the finding that mutation of AD2 (AD2−) abolished the activity of p53(ΔAD1ΔBD) at the IGFBP3 promoter (Fig. 3C). Taken together, these data suggest that a functional AD2 is required for activation of the IGFBP3 promoter.

*The Activity of Naturally Occurring p53 Isoforms Correlates with the Activity of Our Engineered p53 Mutants*  
p53 has been reported to exist as both individual and combined N- and C-terminally truncated isoforms (Fig. 4A). To determine whether the behavior of our engineered p53 mutants represents the behavior of naturally occurring p53 isoforms, we generated untagged constructs expressing human and mouse p53 isoforms that have been reported to exist in the literature, namely human ΔNp53, mouse Δ40p53, human I9+p53, human p53(ΔNΔBD), and mouse p53AS (Fig. 4A). Similar to p53(ΔAD1) which lacks
the first 42 amino acids, human ΔNp53 lacks the first 39 amino acids. ΔNp53 is generated through alternative splicing of intron 2. The resulting exon contains three stop codons; thus, the translational start site of ΔNp53 is at codon 40 of full-length p53 (21). Mouse Δ40p53 lacks the first 40 amino acids and is generated through the use of an internal translational start site at codon 41 (reviewed in reference 11). As expected, the activity of ΔNp53, Δ40p53, and p53(ΔAD1) was analogous, with all constructs having similar levels of activity at the IGFBP3 promoter compared to full-length human and mouse p53 (Fig. 4B and C). I9+p53 is generated through alternative splicing of intron 9 and results in the substitution of 62 C-terminal amino acids by 10 unique amino acids (18). Because alternative splicing abolishes the TD, I9+p53 should be transcriptionally inactive. As expected, I9+p53 did not activate the IGFBP3 promoter (Fig. 4B). C-terminal cleavage of p53, detectable by the lack of anti-p53 PAb421 epitope located within amino acids 371 to 380, occurs through interaction with single-stranded DNA (ssDNA) or cleavage by calpain (42, 45). We reasoned that p53(ΔBD), which lacks amino acids 364 to 393, mimics a C-terminally cleaved p53. It is also likely that a p53 protein exists that is alternatively spliced at the N terminus and is subsequently truncated at the C terminus through interaction with ssDNA or calpain. Thus, we generated p53(ΔNΔBD). As expected, the activities of p53(ΔNΔBD) and p53(ΔAD1ΔBD) were analogous, with both constructs strongly activating the IGFBP3 promoter (Fig. 4B). In addition, we found that mouse p53AS activated the IGFBP3 promoter, whereas full-length mouse p53 did not (Fig. 4C). p53AS is generated through alternative splicing of intron 10 and results in the substitution of 26 C-terminal amino acids by 17 unique amino acids. Mouse p53(A135V), a naturally occurring DNA binding mutant, was inactive and served as a negative control (Fig. 4C).
FIG. 4. The activity of naturally occurring p53 isoforms correlates with the activity of engineered mutants. (A) Schematic representation of human (top) and murine (bottom) p53 isoforms. Numbers indicate exons. Dotted lines indicate alternative splicing. Human ΔNp53 is generated through alternative splicing of intron 2. The alternative exon (EII) contains three stop codons; thus, translation begins at codon 40. Additionally, human ΔNp53 is generated through the use of an internal translational start site at codon 40. Mouse Δ40p53 is generated through the use of an internal translational start site at codon 41. Human I9+p53 and mouse p53AS are generated through alternative splicing of introns 9 and 10, respectively. (B) Naturally occurring human p53 isoforms activate the IGFBP3 promoter. Shown is a schematic representation of human p53 isoforms (left). MCF7 cells were cotransfected with 1 μg of luciferase reporter under control of the IGFBP3 promoter and 1 μg of empty pcDNA3 or pcDNA3 vector expressing p53 and various mutants (right). (C) Naturally occurring mouse p53 isoforms activate the IGFBP3 promoter. The schematic representation and graphs are analogous to those described for panel B.

FIG. 4 con’t. The activity of naturally occurring p53 isoforms correlates with the activity of engineered mutants. (D and E) p53 isoforms are expressed from various p53 constructs. MCF7 cells were transfected with empty pcDNA3 or pcDNA3 vector expressing various human p53 isoforms. H1299 cells were transfected with empty pcDNA3 or pcDNA3 vector expressing various mouse p53 isoforms. p53 was detected with anti-p53 monoclonal antibodies. Actin was detected with antiactin polyclonal antibody.


p53 protein was expressed by these constructs as detected by Western blot analysis (Fig. 4D and E). Taken together, these data suggest that naturally occurring p53 isoforms differentially regulate IGFBP3. Unfortunately, we were not able to detect endogenous levels of naturally occurring isoforms by Western blot analysis. However, we cannot rule out the possibility that undetectable levels exist and may possess significant function.

*IGFBP3 Expression Is Induced by C-Terminally Truncated p63 and p73 isoforms, p63γ and p73β, But Not by Full-Length Ones*

Since the C terminus of p53 plays a critical role in regulating IGFBP3, we reasoned that the C termini of p63 and p73 might also affect the regulation of IGFBP3. Both
p63 and p73 undergo alternative splicing of their C termini, resulting in three p63 isoforms (α to γ) and seven p73 isoforms (α to η) (Fig. 5A). These isoforms, called the TA and ΔN isoforms, are transcribed from the upstream promoter as well as from a cryptic promoter within intron 3, respectively. Thus, many naturally occurring p63 and p73 proteins are generated through alternative splicing and the use of two transcriptional start sites.

To determine whether the N and/or the C termini of p63 and p73 modulate the regulation of IGFBP3, previously characterized stable MCF7 cell lines were analyzed for their ability to regulate IGFBP3 as follows: for cell lines inducibly expressing the Myc-tagged p63 isoforms, p63α (clone 11), ΔNp63α (clone 9), p63γ (clone 19), or ΔNp63γ (clone 18) was used; for cell lines expressing the HA-tagged p73 isoforms, p73α (clone 2) or p73β (clone 31) was used. Equal levels of p63 are expressed by the representative clones as detected by Western blot analysis (Fig. 5B). Interestingly, we found that IGFBP3 was strongly induced by the C-terminally truncated isoforms p63γ and p73β but not by the full-length or the ΔN isoforms, as detected by Northern blotting (Fig. 5C). We also analyzed the ability of the p63 and p73 isoforms to regulate the well-characterized p53 target gene, p21. Similar to the regulation of IGFBP3 by p63 and p73 isoforms, the C-terminally truncated isoforms p63γ and p73β strongly induce p21. In addition, p21 is induced by full-length p73α, albeit weakly. Thus, a pattern emerges for p53 family regulation of IGFBP3: the C terminus of the p53 family is inhibitory for the induction of IGFBP3.
FIG. 5. The C termini of p63 and p73 facilitate differential regulation of IGFBP3. (A) Schematic representation of p53 family functional domains and isoforms. SAM, sterile-α-motif domain; TA, isoforms transcribed from the upstream promoter; and ∆N, isoforms transcribed from the cryptic promoter within intron 3. Dotted lines indicate alternative splicing. Percent identity is indicated. (B) The levels of p63 and actin were assayed by Western blot analysis by using anti-Myc monoclonal and antiactin polyclonal antibodies, respectively, in MCF7-Myc-p63α-11, MCF7-Myc-∆Np63α-9, MCF7-Myc-p63γ-19, and MCF7-Myc-∆Np63γ-18 cells in the absence (−) or presence (+) of p63 for 24 h. (C) IGFBP3 is induced by p63γ and p73β but not by p63α, ∆Np63α, ∆Np63γ, or p73α. A Northern blot was prepared by using total RNAs isolated from cells uninduced (−) or induced (+) to express various p63 or p73 isoforms for 24 h. The blot was sequentially probed with cDNAs derived from IGFBP3, p21, and GAPDH genes.

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FIG. 5 con’t. The C termini of p63 and p73 facilitate differential regulation of IGFBP3. (D and E) p63γ and p73β activate the IGFBP3 promoter. MCF7 cells were cotransfected with 1 µg of luciferase reporter under control of the IGFBP3 promoter, box A, or box B and 1 µg of empty pcDNA3 or pcDNA3 vector expressing various p63 (D) or p73 (E) isoforms. (F) The 11 decamers function as a unit in response to p63γ and p73β. (G and H) Proteins are expressed from various p63 and p73 constructs. MCF7 cells were transfected with 5 µg of empty pcDNA3 or pcDNA3 vector expressing Myc-tagged p63 isoforms or HA-tagged p73 isoforms. Levels of p63, p73, and actin were determined by Western blotting with anti-Myc monoclonal antibody, anti-HA monoclonal antibody, and antiactin polyclonal antibody, respectively.

**p63γ and p73β Activate the IGFBP3 Promoter**

To determine which regions within the IGFBP3 gene are responsive to p63γ and p73β, we analyzed the ability of various p63 and p73 isoforms to activate the IGFBP3 promoter as well as box A and box B in MCF7 cells by using a dual luciferase reporter assay. We found that the –705 IGFBP3 reporter was activated by p63γ but not by p63α, ΔNp63α, or ΔNp63γ (Fig. 5D). None of the p63 isoforms analyzed strongly activated box A or box B (Fig. 5D). Similarly, the –705 IGFBP3 reporter was activated by p73β but not by p73α, ΔNp73α, or ΔNp73β. None of the p73 isoforms analyzed strongly activated box A or box B (Fig. 5E). Next, we wanted to determine which decamers within the IGFBP3 promoter were necessary or sufficient for activation by p63γ and p73β. We found that all 11 decamers were required for activation of the IGFBP3 promoter. Deletion of four upstream decamers, as with the –183 IGFBP3 construct, severely inhibited p63γ and p73β activity at the IGFBP3 promoter (Fig. 5F). No activity was detected when 9 or all 11 decamers were deleted, as with the –116 IGFBP3 or –91 IGFBP3 construct, respectively (Fig. 5F). Thus, we found that both p63γ and p73β behave similarly to p53(ΔAD1ΔBD). Protein was expressed by these p63 and p73 constructs as detected by Western blotting (Fig. 5G and 5H). Taken together, these data and results obtained by Northern blot analysis (Fig. 5C) indicate that p63γ and p73β, but not the α or ΔN isoforms, utilize the IGFBP3 promoter to induce IGFBP3.

**IGFBP3 Is an Important Effector of p53(ΔAD1ΔBD)-Dependent Cell Death**

Because p53(ΔAD1ΔBD) is more potent than full-length p53 to induce apoptosis in MCF7 cells (69) and IGFBP3 is induced by p53(ΔAD1ΔBD) but not by full-length p53 (Fig. 1C), we wanted to characterize the contribution of IGFBP3 in p53- and
p53(ΔAD1ΔBD)-dependent apoptosis. To do this, we performed a trypan blue dye exclusion assay and found that cell death induced by p53(ΔAD1ΔBD) was reduced by 36% with the addition of the IGFBP3 neutralizing antibody to the cell culture supernatant compared to the cells incubated with the rabbit control antibody (Fig. 6A). Cell death induced by full-length p53 was not reduced by incubation with the IGFBP3 antibody (Fig. 6A). In addition, we performed an MTT assay and found that incubation with the IGFBP3 neutralizing antibody increases the survival of cells expressing p53(ΔAD1ΔBD) in comparison to cells incubated with the rabbit control antibody (Fig. 6B). To confirm these data, we measured cell viability in the presence of p53(ΔAD1ΔBD) with and without knockdown of IGFBP3 by siRNA. We used transient transfection of vector-expressed siRNA hairpins that target two locations within the IGFBP3 coding region. Using the MTT assay, we found that transient knockdown of IGFBP3 with siRNA increases the viability of cells expressing p53(ΔAD1ΔBD) in comparison to cells transfected with control siRNA targeting rat p53 (Fig. 6C). In addition, we measured cellular ATP as an indication of cell viability and found that siRNA targeting IGFBP3 increases the viability of cells expressing p53(ΔAD1ΔBD) compared to cells transfected with empty pSuper vector (Fig. 6D). To demonstrate the extent of the transient knockdown of induced IGFBP3 levels, RT-PCR was performed on p53(ΔAD1ΔBD)-15 cells transiently transfected with a mix of the IGFBP3-siRNA expression vectors pSuper-si-IGFBP3-CR-1 and pSuper-si-IGFBP3-CR-2 or a control siRNA expression vector targeting rat p53 and were subsequently induced or uninduced to express p53(ΔAD1ΔBD). A 39% reduction of induced IGFBP3 levels was detected by RT-PCR (Fig. 6E). Although we cannot completely abolish induced IGFBP3 levels by using transient transfection of pSuper-si-IGFBP3-CR-1 and
FIG. 6. IGFBP3 is an effector of p53(∆AD1∆BD)-mediated apoptosis. (A and B) Neutralizing IGFBP3 antibody reduces cell death induced by p53(∆AD1∆BD) but not by full-length p53. Results are shown of a trypan blue dye exclusion assay of MCF7-p53-24 and MCF7-p53(∆AD1∆BD)-15 cells and MTT assay of MCF7-p53(∆AD1∆BD)-15 cells induced or uninduced to express the p53 protein in the presence of anti-IGFBP3 neutralizing antibody or anti-rabbit control antibody. Experiments were performed as described in Materials and Methods. (C and D) Knockdown of IGFBP3 by siRNA enhances viability of cells induced to express MCF7-p53(∆AD1∆BD)-15. Shown are the results of an MTT assay and an ATP luminescence assay of MCF7-p53(∆AD1∆BD)-15 cells induced or uninduced to express the p53 protein transiently transfected with vector-expressed siRNA against IGFBP3 (mix of two siRNAs targeting the coding region of IGFBP3) or with control siRNA against rat p53 or empty pSuper vector. Experiments were performed as described in Materials and Methods. (E) A 39% reduction of the p53(∆AD1∆BD)-induced IGFBP3 mRNA level by IGFBP3 siRNA. Levels of the transcripts for IGFBP3 and GAPDH with transient transfection of vectors expressing siRNA against IGFBP3 (mix of two siRNAs targeting the coding region of IGFBP3) or control siRNA against rat p53 were determined by RT-PCR with 28 cycles.

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pSuper-si-IGFBP3-CR-2, we do detect a modest but reproducible reduction of cell death induced by p53(ΔAD1ΔBD) in the presence of IGFBP3 siRNA (Fig. 6C and D). We suspect that the reduction of cell death would be greater given more efficient IGFBP3 knockdown. Taken together, these data suggest that IGFBP3 is an important effector of p53-dependent apoptosis.

*Full-Length p53 and p53(ΔAD1ΔBD) Bind to the IGFBP3 Promoter In Vivo*

To gain insight into how the BD regulates p53 target gene selection, we wanted to characterize the mechanism that enables p53(ΔAD1ΔBD), but not full-length p53, to induce IGFBP3. First, we wanted to characterize the induction of IGFBP3 by p53(ΔBD), because deletion of the BD renders p53 capable of activating the IGFBP3 promoter (Fig. 3A). Thus, we generated several stable MCF7 cell lines inducibly expressing HA-tagged p53(ΔBD) in the tetracycline-repressible system. A representative cell line, clone 3, was selected for presentation. Western blot analysis showed that the level of p53(ΔBD) protein expressed in this line was comparable to that of full-length p53 and p53(ΔAD1ΔBD) in M7-p53-24 and M7- p53(ΔAD1ΔBD)-15 cell lines, respectively (Fig. 7A). To demonstrate that p53(ΔBD) is transcriptionally active, we analyzed the expression of p21. Like full-length p53 and p53(ΔAD1ΔBD), p53(ΔBD) was able to induce p21 (Fig. 7B). Surprisingly, we found that p53(ΔBD) did not significantly induce IGFBP3, as determined by Northern blot analysis (Fig. 7B). Although unexpected, our result is not unprecedented as deletion of the BD has been shown to activate DNA binding in vitro (25); however, to date deletion of the BD has not been shown to enhance the expression of a target gene. Western blot analysis further confirmed that p53(ΔBD) was not capable of inducing
FIG. 7. IGFBP3 is induced by p53(ΔAD1ΔBD) but not by full-length p53 or p53(ΔBD).

(A) Secreted IGFBP3 is induced by p53(ΔAD1ΔBD). Cell extracts and concentrated conditioned medium were prepared from cells uninduced (−) or induced (+) to express p53, p53(ΔAD1ΔBD), or p53(ΔBD). Levels of p53, actin, and secreted IGFBP3 in p53-24, p53(ΔAD1ΔBD)-15, and p53(ΔBD)-3 cell lines were determined by Western blotting with anti-p53 monoclonal antibodies, antiactin polyclonal antibody, and anti-IGFBP3 polyclonal antibody, respectively. (B) p53(ΔAD1ΔBD) transactivates the gene encoding IGFBP3. A Northern blot was prepared by using total RNAs isolated from cells uninduced (−) or induced (+) to express p53, p53(ΔAD1ΔBD), or p53(ΔBD) for 24 h. The blot was sequentially probed with cDNAs derived from IGFBP3, p21, and GAPDH genes. (C and D) Schematic representation of the IGFBP3 and p21 promoters with the location of the transcriptional start site, the TATA box, p53-responsive elements, and primers used for ChIP assays. (E) p53 and p53(ΔAD1ΔBD) bind the IGFBP3 promoter in vivo. A ChIP assay was performed as described in Materials and Methods. (F) p53 and p53(ΔAD1ΔBD) bind the p53-responsive element within the p21 promoter in vivo.

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IGFBP3, since secreted IGFBP3 was substantially increased by p53(∆AD1∆BD) but not by full-length p53 or p53(∆BD) (Fig. 7A). Although we do detect a slight increase in the secreted IGFBP3 protein level when full-length p53 or p53(∆BD) is expressed, a corresponding increase in IGFBP3 mRNA level is not detected. Thus, we attribute the modest induction of IGFBP3 protein by full-length p53 and p53(∆BD) to posttranslational stabilization of the IGFBP3 protein that may be effected by another p53 target gene. As hinted by the study of AD1, where AD1 is dispensable but an inactive AD1 is inhibitory (Fig. 3B), these data indicate that the conformation of the p53 protein is important for the regulation of IGFBP3.

To determine whether p53 DNA binding is the mechanism governing the regulation of IGFBP3 by p53, we performed ChIP assays. After a 24-h induction of full-length p53 or p53(∆AD1∆BD), chromatin was cross-linked, sonicated, and immunoprecipitated with a mixture of anti-HA polyclonal and anti-p53 PAb1801 monoclonal antibodies. To visualize the enriched DNA fragments, PCR was performed to amplify the region of the IGFBP3 promoter spanning the 11 decamers as well as the upstream p53-responsive element within the p21 promoter (Fig. 7C and D). Chromatin prepared without induction of full-length p53 or p53(∆AD1∆BD) was used as a negative control. We found that both full-length p53 and p53(∆AD1∆BD) bound to the IGFBP3 promoter (Fig. 7E); however, only p53(∆AD1∆BD) induces IGFBP3 (Fig. 1C). No enrichment of the DNA fragment containing the IGFBP3 promoter was detected in the samples not induced to express full-length p53 or p53(∆AD1∆BD). As expected, both full-length p53 and p53(∆AD1∆BD) bound to the p21 promoter (Fig. 7F). Thus, p53 DNA binding is not the mechanism by which IGFBP3 is induced by p53(∆AD1∆BD) but not by full-length p53.
HDAC Activity Inhibits the Ability of Full-Length p53 Family Isoforms to Induce IGFBP3

Because full-length p53 binds the IGFBP3 promoter in vivo, we reasoned that under certain circumstances, full-length p53 would become competent to induce IGFBP3. Such circumstances might include the addition of an activating posttranslational modification, removal of an inhibitory posttranslational modification, dissociation from an inhibitory p53-associated protein, or a combination of these effects. To this end, we used MCF7 cells to characterize the regulation of IGFBP3 by endogenous p53 activated by the DNA damaging agent cisplatin in the presence and absence of the HDAC inhibitor TSA.

Western blotting showed that endogenous p53 protein was stabilized within 4 h upon DNA damage with 50 µM cisplatin, and the stabilization continued throughout 24 h (Fig. 8A). p21 protein was detected as early as 8 h after cisplatin treatment and increased throughout 24 h (Fig. 8A). HDAC inhibition, evidenced by increased levels of acetylated histone H3, was detected within 2 h upon treatment with 100 ng of TSA per ml, and the inhibition continued throughout 24 h (Fig. 8B). Because MCF7 cells did not tolerate a combined treatment of cisplatin and TSA for more than 12 h, we chose this time point for future analyses. We found that activation of p53 by cisplatin or inhibition of HDAC activity by TSA was not sufficient to induce IGFBP3, as detected by Northern blotting (Fig. 8C). However, the combined treatment with cisplatin and TSA enabled endogenous full-length p53 to adopt the conformation necessary to induce IGFBP3 (Fig. 8C). In contrast, p21 was induced upon DNA damage, but its expression was inhibited by the combined treatment (Fig. 8B and C).

To verify our observation that inhibition of HDAC activity restores the ability of endogenous p53 to induce IGFBP3, we tested the ability of exogenous full-length p53
FIG. 8. HDAC activity inhibits the induction of IGFBP3 by full-length p53 family isoforms. (A) Time course of p53 stabilization by the DNA damage agent cisplatin. MCF7 cells were treated with 50 µM cisplatin, and cell extracts were collected at the time points indicated. p53, p21, and actin levels were determined by Western blotting by using anti-p53 monoclonal antibodies, anti-p21 polyclonal antibody, and antiactin polyclonal antibody, respectively. (B) Time course of p53 stabilization with cisplatin and/or HDAC inhibition by TSA. MCF7 cells were treated with 50 µM cisplatin and/or 100 ng of TSA per ml, and cell extracts were collected at the time points indicated. p53, acetylated histone H3, p21, and actin levels were determined by Western blotting by using anti-p53 monoclonal antibodies, anti-acetylated histone H3 polyclonal antibody, anti-p21 polyclonal antibody, and antiactin polyclonal antibody, respectively. (C) Inhibition of HDACs enables induction of IGFBP3 by endogenous p53. A Northern blot was prepared by using total RNAs isolated from MCF7 cells treated as indicated for 12 h. The blot was sequentially probed with cDNAs derived from IGFBP3, p21, and GAPDH genes. (D) Inhibition of HDACs restores the ability of exogenous p53 to induce IGFBP3. A Northern blot was prepared by using total RNAs isolated from cells uninduced (-) or induced (+) to express p53 or p73β in the presence or absence of 100 ng of TSA per ml. The blot was sequentially probed with cDNAs derived from IGFBP3 and GAPDH genes.

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and p73α to induce IGFBP3 in the presence of TSA. As noted previously, IGFBP3 was not induced by full-length p53 or p73α (Fig. 1C, 5C, and 8D). However, TSA treatment restored the ability of exogenous full-length p53 and p73α to induce IGFBP3 in the M7-p53-24 and M7-p73α-2 cell lines, respectively (Fig. 8D). Similarly, TSA treatment rendered full-length p63α competent to induce IGFBP3 (data not shown). We note that a slight increase in IGFBP3 mRNA was detected in the presence of TSA alone in M7-p53-24 and M7-p73α-2 cell lines (Fig. 8D). It is possible that a small amount of p53 or p73 was expressed in the uninduced condition, leading to the modest induction of IGFBP3 in the presence of TSA. Taken together, these data demonstrate that HDACs inhibit the ability of full-length p53 family proteins to induce IGFBP3 and that this inhibition can be overcome by combination treatments that simultaneously activate p53 and inhibit HDACs.

**Differential Target Gene Regulation by Full-Length p53 and p53(ΔAD1ΔBD)**

p53 family isoforms differentially regulate IGFBP3. Specifically, AD1 and the C-terminal BD inhibit induction of IGFBP3 by p53. Importantly, this inhibition can be overcome with inhibition of HDACs. To better understand the significance of this regulation, we wanted to determine whether other target genes are also under similar control by p53 isoforms. Thus, we performed Affymetrix gene chip analysis by using MCF7 cells induced and uninduced to express full-length p53 or p53(ΔAD1ΔBD). We identified several common target genes such as p21, FDXR, and others that were induced by full-length p53 and p53(ΔAD1ΔBD) (Table 1). We also identified several differentially regulated target genes that were induced by p53(ΔAD1ΔBD) but not by full-length p53 and vice
versa (Table 1). Importantly, Northern blot analysis confirmed that the water and glycerol transporter aquaporin 3 (AQP3) is regulated similarly to IGFBP3, such that AQP3 is induced by p53(∆AD1∆BD) but not by full-length p53 (Fig. 9). Although AQP3 has not been linked to apoptosis to date, we cannot rule out the possibility that the increased expression of AQP3 may be proapoptotic by altering the water balance of the MCF7 cells.

Discussion

How p53 differentially regulates target gene expression and thus chooses cell fate is complex. Previous studies have shown that the level of p53 (8, 48), the sequence of p53-responsive elements (47, 58), the proapoptotic AD2 (61, 70), the PRD (50, 69), and

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Common target gene

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Differentially regulated gene

p53-associated proteins, such as ASPP1 and ASPP2 (33, 52), play an important role in this process. In this study, we have identified a common theme among the p53 family proteins: the C terminus is inhibitory, such that C-terminally truncated p53, p63, and p73 isoforms induce the expression of IGFBP3, an important effector of apoptosis, whereas full-length isoforms cannot. Thus, for the first time, we provide evidence that the BD of p53 is inhibitory in vivo as has been described in vitro. We have also shown that the in vivo inhibitory activity of the BD depends upon AD1, such that alleviation of inhibition by the BD requires deletion of AD1. We attribute the inhibition to HDAC activity. Inhibition of HDAC activity restores the ability of endogenous and exogenous full-length p53, p63, and p73 isoforms to induce IGFBP3. Moreover, inhibition of HDAC activity further enhances induction of IGFBP3 by C-terminally truncated p53 family isoforms (data not

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FIG. 9. Differential target gene regulation by full-length p53 and p53(ΔAD1ΔBD). A Northern blot was prepared by using total RNAs isolated from cells uninduced (−) or induced (+) to express p53 or p53(ΔAD1ΔBD) for 24 h. The blot was sequentially probed with cDNAs derived from AQP3, IGFBP3, p21, and GAPDH genes.

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shown). We also identified several target genes differentially regulated by
p53(ΔAD1ΔBD) and full-length p53, emphasizing the importance of p53 isoforms and
functional domains for target gene selection. In summary, we have found that differential
target gene selection requires the coordination of p53 family isoforms with the promoter
environment.

*p53 Family Isoforms Differentially Regulate Target Gene Expression*

Our studies indicate that coordinated and integrated signals are required for the
induction of the proapoptotic target gene IGFBP3 by the p53 family. We showed that p53
functional domains regulate the ability of p53 to activate the IGFBP3 promoter, such that
AD2 is required and AD1 and the BD are inhibitory (Fig. 3). Interestingly, deletion of the
inhibitory functional domains, namely the N-terminal AD1 and the C-terminal BD, is par-
alleled in nature. N-terminal truncation of p53 has been reported to occur (i) through al-
ternative splicing of human p53 to yield ΔNp53/p47 (21); (ii) through the use of the inter-
nal translational start site at codon 40 and 41 in human and mouse p53, yielding ΔNp53
and Δ40p53, respectively (11, 12); (iii) through cleavage by calpain to yield a 42-kDa
human and 41-kDa mouse p53 (31, 45); (iv) through interaction with mismatched double-
stranded DNA to yield mouse p40(ΔN) (40); and (v) through interaction with an N- and
C-terminally truncated p53 protein to yield mouse p50(ΔN23) (42). C-terminal truncation
of p53 has been reported to occur (i) through alternative splicing to yield human I9+p53
and murine p53AS (1, 18); (ii) through cleavage by calpain (45); and (iii) through interac-
tion with the 3’ end of ssDNA to yield murine p50(ΔC) and p40(ΔC) (42). Combined N-
and C-terminal truncation has been reported to occur through interaction with mis-
matched DNA to yield p35, which is reported to have intrinsic protease ability (42), and through cleavage by calpain to yield a 33-kDa murine p53 (45). We postulate that combined N- and C-terminal truncation could occur sequentially. Alternative splicing or internal translational initiation at codon 40 generates ΔNp53, which is subsequently truncated at the C terminus through interaction with ssDNA or calpain. In the case of mouse p53, internal translational initiation of the p53AS transcript at codon 41 generates Δ40p53AS, which is analogous to p53(ΔAD1ΔBD). We found that both ΔNp53 and Δ40p53 behave similarly to p53(ΔAD1) (Fig. 4B and C). I9+p53 lacks the TD and, as expected, is transcriptionally inactive (Fig. 4B). Interestingly, full-length mouse p53 and p53AS behave similarly to full-length p53 and human p53(ΔBD): mouse p53AS activates the IGFBP3 promoter, whereas full-length mouse p53 cannot (Fig. 4C). In addition, p53(ΔNΔBD), which mimics a sequentially truncated p53 isoform, is extremely active at the IGFBP3 promoter (Fig. 4B). Thus, for the first time, we showed that p53 isoforms do have different functions. In addition, using Affymetrix gene chip analysis, we identified several target genes differentially regulated by full-length p53 and p53(ΔAD1ΔBD) (Table 1).

Based on these data, we suggest a model according to which specific environmental stimuli trigger the generation of p53 isoforms that will differentially regulate target gene expression (Fig. 10A). Genotoxic stress induces DNA damage. The DNA damage pathway activates and stabilizes full-length p53, which in turn induces p53-dependent cell cycle arrest through induction of p21. The arrest allows a cell time to repair its damaged DNA. However, if the damage is extensive, free 3' ends of the damaged DNA could induce N- and/or C-terminal cleavage of ΔNp53 and full-length p53 to yield
p53(ΔNΔBD). Since p53(ΔNΔBD) is resistant to negative regulation at the IGFBP3 promoter, IGFBP3 is induced. IGFBP3, along with other proapoptotic target genes, shifts the balance from p53-dependent cell cycle arrest to p53-dependent apoptosis. Thus, we propose that certain stimuli can alter the expression pattern of specific p53 isoforms and, subsequently, the expression pattern of p53 target genes (Fig. 10A). In support of this model, DNA damage has been shown to heighten the apoptotic response without affecting p53 levels (8). In addition, a recent study demonstrated that DNA damage-induced p53-dependent neuronal cell death was prevented upon calpain inhibition (54) and thus highlights the potential functional importance of calpain-dependent p53 isoforms.

Further strengthening the validity of our model is the evidence we found that p63 and p73 isoforms function in an analogous manner to p53 isoforms: the p63 and p73 C-terminally truncated isoforms, p63γ and p73β, but not full-length isoforms, induce the expression of IGFBP3 (Fig. 5C). Thus, the question arises, How are these isoforms differentially regulated? Since the regulation of alternative splicing has not been thoroughly studied, we propose a model where certain signals would activate alternative splicing to preferentially upregulate p63γ or p73β, which would carry out a specific transcriptional program, namely the induction of IGFBP3 and other proapoptotic target genes, and subsequently apoptosis (Fig. 10A). Therefore, future work needs to be done to characterize the regulation of alternative splicing.

Previously, we have shown that the ΔN isoforms of p63 and p73 contain a unique AD (13, 35) that differs from the AD in the TA isoforms. The AD in TAp63 and TAp73 shares 22 and 29% identity, respectively, to AD1 in p53 (Fig. 5A). Interestingly, we have shown that the p53 AD1 is inhibitory and AD2 is required for activation of the IGFBP3
FIG. 10. A model of apoptosis for p53 family isoforms. Ac, acetyl.

Note: Adapted from “The C Terminus of p53 Family Proteins Is a Cell Fate Determinant” by K.L Harms and X. Chen, 2005, Molecular and Cellular Biology, 25, p. 2028. Copyright 2005 by the American Society for Microbiology. Adapted with permission.
promoter (Fig. 3B and C). Similarly, we found that the AD in the TA but not the ΔN iso-
forms is active in inducing IGFBP3 (Fig. 5C). Although the AD present in the TA iso-
forms is highly similar to AD1 in p53, perhaps the AD of TA possesses a wider range of functions, such that the critical residues or tertiary structure important for AD2 function are also contained within the TA AD. The fact that the ΔN isoforms cannot induce IGFBP3 suggests a functional difference between the ADs within the ΔN and TA iso-
forms.

The Promoter Environment Contributes to Differential Target Gene Selection

Our studies indicate that the promoter sequence in terms of the structure and num-
ber of the p53-responsive elements and the promoter topology in terms of the associated proteins are important for target gene selection. The structure of the p53-responsive ele-
ment within the IGFBP3 promoter is unique in that it contains 11 tandem decamers of the p53-responsive element. We found that the 11 decamers function as a unit, such that all are required for full activation of IGFBP3 by p53(ΔAD1ΔBD), p63γ, and p73β (Fig. 2E and 5F). Since only two decamers constitute the canonical p53-responsive element, these data suggest that the unique arrangement is important for the differential regulation of IGFBP3 by p53 family isoforms. Similar to IGFBP3, p53-induced gene 3 (PIG3) is a proapoptotic target gene and contains a unique p53-responsive element (46). p53 acti-
vates the PIG3 promoter through a pentanucleotide microsatellite sequence (10). Interest-
ingly, the pentanucleotide sequence is polymorphic, and increasing numbers of repeats confer greater responsiveness to p53 (10). To further support the importance of the re-
sponsive element, studies show that p53-responsive elements located within cell cycle
Arrest genes are more highly activated by p53 than the ones located in proapoptotic genes (47). It has been postulated that the spacing between the decamers is critical. Responsive elements found within cell cycle arrest genes do not contain interspersed sequences between the decamers, but the ones within proapoptotic genes do (47, 58).

Our studies also demonstrate that the coordination of p53 family isoforms with the promoter environment is important for transcriptional activation of IGFBP3. We found that full-length p53 and p53(ΔAD1ΔBD) bind to the IGFBP3 promoter in vivo, as determined by the ChIP assay (Fig. 7E); however, only p53(ΔAD1ΔBD) is capable of inducing IGFBP3 (Fig. 1C). We found that inhibition of HDAC activity restores the ability of exogenous full-length p53 to induce IGFBP3 (Fig. 8D). In addition, we found that p63 and p73 isoforms behave in a similar fashion: HDAC activity inhibits the induction of IGFBP3 by full-length p63 and p73 isoforms, p63α and p73α, but not by C-terminally truncated isoforms, p63γ and p73β (Fig. 8D and data not shown). Our data suggest a model according to which full-length p53 and p53(ΔAD1ΔBD) bind to the IGFBP3 promoter, but full-length p53 cannot recruit the basal transcriptional machinery due to its association with HDAC activity (Fig. 10B). Although the acetylation status of p53 has been shown to modulate its own activity (27), our model focuses on the ability of p53 to interact either directly or indirectly with HDACs that target the chromatin but not full-length p53, as p53(ΔAD1ΔBD), which is extremely active in inducing IGFBP3, lacks acetylation sites. In support of our model, recent studies have demonstrated that p53 target gene expression requires more than just p53 binding: p53 first binds to the p21 promoter and recruits p300, which then acetylates the p21 proximal promoter and cooperates with p53 to induce p21 (16, 36).
In our model, p53 family isoforms differentially interact with regulatory proteins to facilitate target gene expression. Differential protein interactions may depend upon the conformation of the p53 protein or specific epitopes, either gained or lost. In support of the importance of conformation, many studies have identified p53 mutants that can induce apoptosis but not cell cycle arrest and vice versa (19, 49, 51). For example, mutant p53(121F) is a more potent inducer of transcription-dependent apoptosis than full-length p53 (51). Although the structure of p53(121F) was not determined by crystallography, because full-length p53 is a fluid molecule and will not crystallize, we speculate that the conformation of p53(121F) is different from that of the full-length p53. Thus, p53(121F) may escape regulation by certain proteins, potentially HDACs or protein complexes with HDAC activity, to enable the induction of proapoptotic target genes. Interestingly, p53 has been shown to interact with mSin3A, a component of the transcriptional repression complex, as well as HDAC-1, HDAC-2, and HDAC-3 (27, 71). While mSin3A interacts with the PRD of p53 (71), the physical interaction between p53 and HDAC-1, HDAC-2, and HDAC-3 has not been mapped. Thus, it is possible that the HDACs interact with the N and C termini of p53, and thus the IGFBP3 promoter bound by p53(ΔAD1ΔBD) escapes this negative regulation. While many questions still remain and many mechanisms are yet to be discovered, one point becomes clear: the ability of the p53 family to induce the expression of proapoptotic target genes is under stringent regulation.

*Therapeutic Implications*

Restoration of p53 functions, especially p53-dependent apoptosis, is an attractive cancer therapeutic strategy. To exploit p53-dependent apoptosis for the destruction of
cancer cells, many studies have focused on strategies that preferentially activate p53 to induce proapoptotic target genes. Here, we found that treatment of MCF7 cells with the DNA damage agent cisplatin was sufficient to stabilize p53 levels with the subsequent induction of p21; however, cisplatin alone was not sufficient to enable p53 induction of IGFBP3, an important effector of apoptosis. However, combined treatment with cisplatin and the HDAC inhibitor TSA enabled IGFBP3 expression (Fig. 8C). Thus, our data presented here shed new insight into how we can modulate p53 activity to induce apoptosis in tumor cells, which can be explored for the design of combined therapies for cancer treatment.

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HISTONE DEACETYLASE 2 MODULATES P53 TRANSCRIPTIONAL ACTIVITIES
THROUGH REGULATION OF P53-DNA BINDING ACTIVITY

by

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Abstract

Histone deacetylase (HDAC) inhibitors are emerging as promising cancer therapeutics. HDAC inhibitors have been found to induce cellular activities that are strikingly similar to p53-mediated responses to genotoxic stress. For example, HDAC inhibitors induce cell cycle arrest, apoptosis, and cellular senescence. Since at least eleven HDACs are affected by the current HDAC inhibitors, which HDAC is critical for tumor cell survival and proliferation remains unknown. Thus, we sought to characterize the distinct roles of HDAC1 and HDAC2 in the p53 pathway. Through the use of stable MCF7 cell lines which inducibly express shRNA targeting HDAC1 or HDAC2, we found that HDAC2, but not HDAC1, plays important roles in the p53 pathway. Specifically, we found that knockdown of HDAC2, but not HDAC1, inhibited cellular proliferation in a dose-dependent manner and induced cellular senescence. Importantly, we found that knockdown of HDAC2, but not HDAC1, enhanced p53-dependent trans-repression and trans-activation of the c-Myc proto-oncogene and a subset of target genes, respectively. Furthermore, we found that the enhancement was due to increased p53-DNA binding activity but not alterations in p53 stability or post-translational modification(s). Thus, for the first time, our data suggest that HDAC inhibitors function through the p53 pathway, at least in part, by activating p53-DNA binding.

Introduction

The p53 tumor suppressor, a transcription factor, is well known to regulate target genes that mediate cell cycle arrest, apoptosis, senescence, DNA repair, and other responses to genotoxic stress (1, 2). The ability of p53 to regulate gene expression is under
stringent control. The p53 protein is maintained at a low abundance in non-stressed cells by the E3 ubiquitin ligases Mdm2, Pirh2, and COP (3, 4). DNA damage-induced phosphorylation and acetylation of p53 promote the stabilization and transcriptional activities of p53 (5-7). Because p53 has the potential to induce many responses, we and others have investigated mechanisms of differential target gene regulation by p53. Through the use of histone deacetylase (HDAC) inhibitors, we have previously shown that HDACs play a role in differential target gene selection by p53 family proteins (8).

HDACs play important roles in many diverse processes such as transcriptional regulation, protein-protein interaction, protein sub-cellular localization, and organismal aging (9, 10). HDACs, which deacetylate histones and non-histone proteins, are organized into four classes: class I (HDAC 1, 2, 3, 8), class II (HDAC 4, 5, 6, 7, 9, 10), class III (SIRT 1-7), and class IV (HDAC 11) (9, 11). HDACs in classes I, II, and IV contain a conserved catalytic domain and are commonly inhibited by HDAC inhibitors such as Trichostatin A (TSA) and sodium butyrate (10). These inhibitors chelate the zinc cation within the enzyme active site (10). Class III HDACs, which contain an NAD-dependent catalytic domain, are insensitive to these agents (10). Recent evidence demonstrates that HDAC inhibitors induce apoptosis and inhibit proliferation in tumor cells (12-16). Although HDAC inhibitors are in phase I/II clinical trials and are emerging as promising cancer chemotherapeutics (9), which HDAC(s) is critical for tumor cell survival and proliferation remains unknown.

p53 has recently been found to play an important role in mediating the effects of HDAC inhibitors. Upon HDAC inhibition, p53 has been found to be stabilized and acetylated at lysines 320, 373, and 382 (15-17). Although several lines of evidence suggest
that HDAC inhibitors activate the p53 pathway, the role of p53 during HDAC inhibition and mechanisms by which individual HDACs regulate p53 activity remain unclear.

In this study, we sought to characterize the function of individual HDACs on p53 activity. Through the use of stable MCF7 cell lines which inducibly express shRNA targeting HDAC1 or HDAC2, we found that knockdown of HDAC2, but not HDAC1, induced G1 arrest and inhibited cellular proliferation. Furthermore, knockdown of HDAC2 induced cellular senescence. Importantly, we found that knockdown of HDAC2, but not HDAC1, enhanced trans-repression and trans-activation of a subset of target genes by endogenous p53. Investigation into the mechanism revealed that knockdown of HDAC2 enhanced the ability of p53 to bind DNA \textit{in vivo}. Thus, for the first time, we found that HDAC2 negatively regulates p53 activity by inhibiting p53-DNA binding.

\textbf{Results}

\textit{Knockdown of HDAC2, but Not HDAC1, Inhibits Cellular Proliferation and Induces Cellular Senescence}

To characterize the role of individual HDACs on p53 activity, we generated stable MCF7 cell lines which inducibly express shRNA targeting HDAC1 or HDAC2 in the tetracycline-inducible system. Western blot analysis demonstrated that HDAC1 and HDAC2 protein levels were significantly decreased upon expression of shRNA targeting HDAC1 and HDAC2, respectively (Fig. 1A). Interestingly, we found that knockdown of HDAC2, but not HDAC1, resulted in a G1 arrest (Fig. 1B). Consistent with the arrest in G1, cellular proliferation (Fig. 1C) and colony formation (Fig. 1D) were inhibited upon knockdown of HDAC2. We also found that HDAC2 affected colony formation in a
Fig. 1. Knockdown of HDAC2, but not HDAC1, induces G1 cell cycle arrest, inhibits cellular proliferation, and induces cellular senescence. (A) The abundance of HDAC1 and HDAC2 is reduced in MCF7 stable cell lines upon tetracycline-inducible expression of shRNA targeting HDAC1 and HDAC2, respectively. The levels of HDAC1, HDAC2, and actin were assayed by Western blot analysis with antibodies against HDAC1, HDAC2, and actin, respectively, in MCF7-si-HDAC1-7 and MCF7-si-HDAC2-10 cells grown +/− tetracycline for 3d. (B) Knockdown of HDAC2, but not HDAC1, induces G1 arrest. The percentages of MCF7-si-HDAC1-7 and MCF7-si-HDAC2-10 cells grown +/− tetracycline for 4d were determined by propidium iodide staining and FACS analysis. (C) Knockdown of HDAC2 inhibits cell proliferation. Growth rates for parental MCF7, MCF7-si-HDAC1-7, and MCF7-si-HDAC2-10 cells grown +/− tetracycline were measured by Coulter cell counter.
Fig. 1 con’t. Knockdown of HDAC2, but not HDAC1, induces G1 cell cycle arrest, inhibits cellular proliferation, and induces cellular senescence. (D) Colony formation assay for parental MCF7, MCF7-si-HDAC1-7, and MCF7-si-HDAC2 clones B18, B38, and 10. Cells were grown +/- tetracycline for 12d, fixed, and stained with crystal violet. The levels of HDAC1, HDAC2, and actin were assayed by Western blot analysis in MCF7-si-HDAC2 clones B18, B38, and 10 grown +/- tetracycline for 3d. (E and F) Knockdown of HDAC2 induces cellular senescence. MCF7-si-HDAC2-10 cells were grown +/- tetracycline, fixed, and stained for senescence-associated β-gal activity at the indicated time. Phase contrast microscopy of representative colonies (E) and quantitation of SA-β-gal positive colonies (F) are shown. Error bars denote standard error.
dose-dependent manner, since more effective knockdown of HDAC2 resulted in more marked growth inhibition (Fig. 1D). In contrast, knockdown of HDAC1 or tetracycline had no effect on cellular proliferation or colony formation (Fig. 1C and D). Furthermore, we found that knockdown of HDAC2 resulted in cellular senescence (Fig. 1E and F). In sum, the inhibition of proliferation and the induction of cellular senescence upon knockdown of HDAC2 suggest that HDAC2 plays an important role in the promotion of tumorigenesis and that HDAC2 is a critical target of HDAC inhibitors for cancer therapy.

**HDAC2 Does Not Affect p53 Stability**

Since HDAC inhibitors have been found to induce the stabilization of p53 (15-17), we wanted to determine if knockdown of HDAC1 or HDAC2 affected p53 stability. We found that knockdown of HDAC1 or HDAC2 did not affect the stabilization of p53 in cells treated with the chemotherapeutic agent cisplatin or the Mdm2-inhibitor nutlin (Fig. 2A; also see 4C and 4D). Although a small increase in the abundance of p53 was detected upon knockdown of HDAC2 together with cisplatin treatment, this increase was not consistently observed and was not detected upon knockdown of HDAC2 together with nutlin treatment (Fig. 2A, compare lanes 9 and 10 with 11 and 12). Furthermore, knockdown of HDAC2 did not enhance the acetylation of lysines 373 and 382 upon cisplatin treatment (Fig. 2A). This is consistent with evidence that p53 can be deacetylated by HDAC1 and HDAC3, but not by HDAC2 (18-20). In line with this, knockdown of HDAC2 did not prolong the half-life of p53 (Fig. 2B). Taken together, these data suggest that HDAC1 or HDAC2 alone does not greatly affect p53 stabilization in response to cisplatin or nutlin.
Fig. 2. Knockdown of HDAC1 or HDAC2 does not affect p53 stability. (A) MCF7-si-HDAC1-7 and MCF7-si-HDAC2-10 cells were grown +/- tetracycline for 3d and treated with 50 µM cisplatin or 5 µM nutlin for 13h. The level of HDAC1, HDAC2, p53, p53-AcK373/382, and actin was assayed by Western blot analysis. (B) Knockdown of HDAC2 does not lengthen the half-life of p53. MCF7-si-HDAC2-10 cells were grown +/- tetracycline for 3d and treated with cyclohexamide (CHX) for the indicated time. The level of p53 and actin was assayed by Western blot analysis.
HDAC2 Inhibits p53-Dependent Trans-Repression of c-Myc

Because knockdown of HDAC2 resulted in growth arrest (Fig. 1 B-D), we sought to identify HDAC2 target genes. We found that c-Myc mRNA was reduced upon knockdown of HDAC2, but not HDAC1 (Fig. 3A). The abundance of c-Myc protein was also reduced upon inducible as well as transient knockdown of HDAC2 (Fig. 3B and C). This is consistent with reports that c-Myc is repressed by HDAC inhibitors (21-23). Therefore, for the first time, we have identified that knockdown of HDAC2, but not HDAC1, results in the repression of c-Myc (Fig. 3A and B). Since p53 is known to directly repress c-Myc (24), we sought to determine the role of p53 in the repression of c-Myc upon knockdown of HDAC2. To do this, we transiently knocked-down HDAC2 in MCF7 cells and in MCF7 cells in which p53 was stably knocked-down. We found that the repression of c-Myc upon knockdown of HDAC2 was partly dependent on p53 (Fig. 3C). Furthermore, we characterized the binding of p53 to the c-Myc promoter by chromatin immunoprecipitation (ChIP) analysis and found that in the unstressed condition, the extent of p53 bound to the p53-responsive element within the c-Myc promoter was markedly increased upon knockdown of HDAC2 (Fig. 3D and E). mSin3A, a component of the p53-repression complex, was also found to interact with the c-Myc promoter, but the interaction was not affected by HDAC2 knockdown (Fig. 3E). Consistent with the repressed state of the c-Myc promoter, the level of histone H3 acetylation was diminished upon knockdown of HDAC2 (Fig. 3F). Immunoprecipitation with rabbit-IgG served as a negative control in the ChIP assay (Fig. 3E & F).
Figure 3. Knockdown of HDAC2 represses c-Myc in a p53-dependent manner. (A) Knockdown of HDAC2, but not HDAC1, represses c-Myc mRNA. A Northern blot was prepared using total RNAs isolated from MCF7-si-HDAC1-7 and MCF7-si-HDAC2-10 cells grown +/- tetracycline for 3d. The blot was sequentially probed with cDNAs derived from c-Myc and GAPDH genes. (B) Knockdown of HDAC2 represses c-Myc protein. The level of HDAC1, HDAC2, c-Myc, and actin was assayed by Western blot analysis in MCF7 parental and MCF7-si-HDAC2-10 cells grown +/- tetracycline for 3d. (C) Repression of c-Myc upon knockdown of HDAC2 is partially p53-dependent. MCF7 parental and MCF7 cells in which p53 was stably knocked-down (MCF7-p53KD-3) were transiently transfected with scrambled or HDAC2 siRNA oligos for 3d. The level of HDAC2, c-Myc, p53, and actin was assayed by Western blot analysis. s.e. and l.e. denote short and long exposure, respectively. (D) Schematic representation of the human c-Myc promoter and location of transcriptional start sites, p53-responsive element, and primers used for ChIP assay. Lower case italicized nucleotides identify mismatches from p53 consensus binding site. (E) p53 binds the c-Myc promoter in vivo upon knockdown of HDAC2. ChIP assay was performed using MCF7-si-HDAC2-10 cells grown +/- tetracycline for 3d. Chromatin was immunoprecipitated with p53, mSin3A, and control rabbit IgG antibodies. The fragment containing the p53-responsive element in the c-Myc promoter was amplified by PCR. (F) Knockdown of HDAC2 resulted in decreased acetylation of histone H3 at c-Myc promoter. ChIP assay was performed using Ac-histone H3 and control rabbit IgG antibodies.
HDAC2 Inhibits p53-Dependent Trans-Activation of a Subset of Target Genes

Because HDAC2 inhibited p53-dependent trans-repression of c-Myc, we investigated whether HDAC1 or HDAC2 could also affect p53-dependent trans-activation. First, we sought to determine if HDAC1 or HDAC2 inhibited IGFBP3 or AQP3 induction by p53, since HDAC inhibitors restore the ability of endogenous p53 to induce IGFBP3 and possibly AQP3 in MCF7 cells (8). To test this, HDAC1 or HDAC2 was inducibly knocked-down in the absence or presence of cisplatin or nutlin. Western blot analysis demonstrated that p53 was stabilized upon treatment with cisplatin or nutlin and that HDAC1 and HDAC2 protein levels were decreased upon knockdown of HDAC1 and HDAC2, respectively (Fig. 2A). As a positive control, IGFBP3 and AQP3 were strongly induced by an N- and C-terminally truncated p53 and by p73, respectively (Fig. 4A). However, knockdown of HDAC1 or HDAC2 was not sufficient to activate endogenous p53 to induce IGFBP3 or AQP3 in MCF7 cells (Fig. 4A). Thus, a combination of HDAC1, HDAC2 and/or another HDAC is involved in repressing endogenous p53 to induce IGFBP3 and AQP3.

To identify other genes regulated by HDAC1 or HDAC2, we performed a microarray study. Although 2% of genes can be affected upon HDAC inhibition (25), only a small number of genes was found to be affected \( \geq 2 \)-fold upon knockdown of HDAC1 or HDAC2 (Table 1). As an internal control, the expression of HDAC1 and HDAC2 was found to be decreased \( > 2 \)-fold upon knockdown of HDAC1 and HDAC2, respectively (Table 1). Since we have previously found Dickkopf-1 (DKK1) to be a p53 target gene (26), we chose DKK1 for further analysis. Indeed, knockdown of HDAC2, but not HDAC1, led to a modest DKK1 induction (Fig. 4A). Strikingly, we found that knock
Fig. 4. Knockdown of HDAC2, but not HDAC1, enhances p53 transcriptional activity for a subset of target genes. (A) Knockdown of HDAC2 enhances p53 activity for DKK1 and p21, but not for IGFBP3 or AQP3 genes. A Northern blot was prepared using total RNAs isolated from MCF7-si-HDAC1-7 and MCF7-si-HDAC2-10 cells grown +/- tetracycline for 3d and treated with 50 µM cisplatin or 5 µM Nutlin as indicated. The blots were probed with cDNAs derived from IGFBP3, AQP3, DKK1, p21, and GAPDH genes. Total RNA isolated from MCF7 cells expressing p53( AD1 BD) and p73 was used as a positive control for induction of IGFBP3 and AQP3, respectively. (B) Knockdown of HDAC2 enhances p53 activity for Mdm2 and FDXR genes. A Northern blot was prepared using total RNAs isolated from MCF7-si-HDAC2-10 cells treated as indicated. The blot was sequentially probed with cDNAs derived from Mdm2, FDXR, and GAPDH genes. (C and D) Knockdown of HDAC2 enhances the induction of p21 protein by p53 in MCF7 and RKO cells. The level of HDAC1, HDAC2, p53, p21, and actin was assayed by Western blot analysis in MCF7-si-HDAC2-10 cells (C) treated as indicated and in RKO cells (D) transiently transfected with siRNA oligos and treated with nutlin as indicated.
Table 1. Genes affected by knockdown of HDAC1 or HDAC2

<table>
<thead>
<tr>
<th>Target gene</th>
<th>GenBank accession no.</th>
<th>Fold change in expression</th>
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<tbody>
<tr>
<td>HDAC1 knockdown</td>
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<tr>
<td>HDAC1</td>
<td>NM_004964.2</td>
<td>-2.8</td>
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<tr>
<td>S100A8</td>
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<td>AF101051.1</td>
<td>+5.4</td>
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<tr>
<td>DKK1</td>
<td>NM_012242.1</td>
<td>+2.6</td>
</tr>
<tr>
<td>HDAC2</td>
<td>NM_001527.1</td>
<td>-5.3</td>
</tr>
<tr>
<td>DUSP5</td>
<td>NM_004419.2</td>
<td>-2.7</td>
</tr>
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</table>

+/- indicates fold increase or decrease in gene expression, respectively.

down of HDAC2 together with cisplatin or nutlin treatment led to a robust DKK1 induction whereas treatment with cisplatin or nutlin alone led to a modest DKK1 induction (Fig. 4A). The induction of the p21, Mdm2, and FDXR genes by p53 was also further enhanced upon knockdown of HDAC2 (Fig. 4A and B). In contrast, knockdown of HDAC1 did not enhance DKK1 induction by p53 (Fig. 4A). Furthermore, the induction of p21 was diminished upon knockdown of HDAC1 together with nutlin treatment compared to nutlin treatment alone (Fig. 4A). This is consistent with our early report demonstrating a lower induction of p21 mRNA upon co-treatment of MCF7 cells with cisplatin and TSA compared to treatment with cisplatin alone (8). Thus, HDAC2 regulates p53 transcriptional activity, at least for a subset of p53 target genes.

To further characterize the role of HDAC2 in the p53 pathway, we examined the effect of HDAC2 on p21 induction by p53. Consistent with the level of p21 mRNA, p21 protein was induced to a greater extent upon knockdown of HDAC2, either inducibly in MCF7 cells or transiently in RKO colon carcinoma cells, together with nutlin treatment compared to nutlin treatment alone (Fig. 4C & D). We also found that knockdown of HDAC2 led to a modest activation of the p21 promoter and that knockdown of HDAC2
further enhanced the activation of the p21 promoter by p53 (Fig. 5B). Conversely, over-expression of HDAC2 repressed the ability of p53 to activate the p21 promoter in Hct116-p53−/− and MCF7 cells (Fig. 5C and 5D). It is important to note that in addition to somatic knockout of p53, Hct116-p53−/− cells lack HDAC2. Interestingly, the basal p21 promoter activity was repressed by overexpression of HDAC2 in a manner that was dependent on endogenous p53 (Fig. 5C and 5D). Taken together, HDAC2 inhibits the induction of p21 by p53.

**HDAC2 Inhibits p53-DNA Binding Activity**

Since knockdown of HDAC2 did not alter the stability or acetylation of p53 (Fig. 2A & B), enhanced p53 transcriptional activities upon knockdown of HDAC2 are not likely due to p53 abundance. Because knockdown of HDAC2 enhanced p53-DNA binding at the c-Myc promoter (Fig. 3E), we investigated whether HDAC2 could also affect p53-DNA binding activity at p53-induced genes by ChIP analysis. Indeed, we found that knockdown of HDAC2 together with nutlin or cisplatin treatment resulted in enhanced p53-DNA binding to the p53-responsive element within the p21 promoter when compared to p53-DNA binding upon nutlin or cisplatin treatment alone (Fig. 6A, 6B, & Supplemental Fig. 1A). The increase in p53-DNA binding was associated with a concomitant increase in binding of the co-activator p300 to the p21 promoter (Supplemental Fig. 1B). Furthermore, knockdown of HDAC2 together with nutlin treatment resulted in enhanced p53-DNA binding to the p53-responsive element within the Mdm2 promoter and to a putative p53-responsive element near the transcriptional start site of the DKK1 gene (Fig. 6C). Anti-mouse and mouse IgG2a antibodies served as negative controls in the ChIP
Fig. 5. The level of HDAC2 modulates p53 activity at the p21 promoter. (A) Schematic representation of the p21 luciferase reporter with p53-responsive elements and TATA box shown. (B) Knockdown of HDAC2 activates the p21 promoter and further enhances activation of the p21 promoter by p53. MCF7-si-HDAC2-10 cells were grown +/- tetracycline for 4d transfected with the p21 luciferase reporter, the internal control pRL-CMV, and either empty pcDNA3 or p53/pcDNA3 as indicated for 24h. Fold increase in luciferase activity is shown. (C and D) HDAC2 diminishes the activation of the p21 promoter by p53. Hct116 p53-/- (C) or MCF7 (D) cells were transfected with the p21 luciferase reporter, the internal control pRL-CMV, and pcDNA3, HDAC2, or p53 as indicated for 24h. DNA amounts were normalized with pcDNA3. Fold increase in luciferase activity is shown. Error bars denote standard error.
Fig. 6. HDAC2 inhibits p53-DNA binding. (A and B) Knockdown of HDAC2 enhances p53-DNA binding at the endogenous p21 promoter upon treatment with nutlin (A) and cisplatin (B). ChIP assay was performed using MCF7-si-HDAC2-10 cells grown +/- tetracycline for 3d and treated with 5 µM Nutlin (A) or 50 µM cisplatin (B) as indicated. Chromatin was immunoprecipitated with anti-p53 (either a mixture of DO1, PAb-1801, PAb-240, and PAb-421 or DO1 alone), anti-Ac-histone H3, control anti-mouse, or control mouse IgG2a antibodies as indicated. p53-RE1 within the p21 gene was amplified by PCR. (C) Knockdown of HDAC2 enhances p53-DNA binding at DKK1 and Mdm2 genes. ChIP assay was performed as in (A). A potential p53 responsive element near the transcriptional start site of the DKK1 gene and p53-RE1 within the Mdm2 gene were amplified by PCR. The GAPDH promoter was amplified and served as an additional negative control for p53-DNA binding.
assay (Fig. 6). As an additional control, p53 was not found to bind the GAPDH promoter (Fig. 6C).

While it is well accepted that the binding of an activator facilitates the hyper-acetylation of core histones at some promoters (27), surprisingly, we found that the level of histone H3 acetylation did not parallel the increase in binding of p53 and p300 to the p21 promoter. When compared to the unstressed condition, we found that the level of histone H3 acetylation increased upon treatment with nutlin or cisplatin regardless of HDAC2 status (Fig. 6A & Supplemental Fig. 1A). However, the level of histone H3 acetylation was diminished upon knockdown of HDAC2 together with nutlin or cisplatin treatment compared to nutlin or cisplatin treatment alone (Fig. 6A & Supplemental Fig. 1A). Thus, knockdown of HDAC2 together with cisplatin or nutlin treatment correlated with increased p53- and p300-binding and decreased histone H3 acetylation at the p21 promoter. This suggests that a corepressor, such as HDAC2, exerts a unique impact on the promoter for gene regulation.

Discussion

HDAC inhibitors, which have been shown to inhibit proliferation and to induce apoptosis and cellular senescence (13-15, 28, 29), are emerging as promising cancer therapies (9). Here we have identified that HDAC2, but not HDAC1, is a critical target for HDAC inhibition. We showed that inducible knockdown of HDAC2, but not HDAC1, induced G1 arrest, inhibited cellular proliferation, and induced cellular senescence. Our data is consistent with recent studies which have shown that transient knockdown of HDAC2 inhibits proliferation and induces apoptosis (30, 31). Importantly, we
have found that c-Myc is repressed upon knockdown of HDAC2. Since c-Myc is required for cellular proliferation in some cell types (32-34), it is likely that the repression of c-Myc plays an important role in the proliferation defect upon knockdown of HDAC2. Furthermore, through our microarray study, we identified and confirmed that claudin-1 (CLDN1), a component of the tight junction, was induced upon knockdown of HDAC2 (Table 1, Supplemental Fig. 2A & B). Since CLDN1 expression has been found to be increased in senescent compared to proliferating mammary epithelial cells (35), perhaps CLDN1 plays a role in the senescence induced upon knockdown of HDAC2. Taken together, these data suggest that HDAC2 promotes cellular proliferation and prevents cellular senescence and is thus a critical target of HDAC inhibitors.

Supplemental Fig. 1. Knockdown of HDAC2 augments p53- and p300-binding and diminishes acetylation of histone H3 at the p21 promoter. (A) ChIP assay was performed using MCF7-si-HDAC2-10 cells grown +/- tetracycline and treated with 50 M Cisplatin as indicated. Chromatin was immunoprecipitated with anti-p53 (mix of DO-1, PAb-1801, PAb-240, and PAb-421), anti-acetylated histone H3, or control anti-mouse antibodies. p53-RE1 within the p21 gene was amplified by PCR. (B) ChIP assay was performed using MCF7-si-HDAC2-10 cells treated as indicated. Chromatin was immunoprecipitated with anti-p300 antibodies. p53-RE1 within the p21 gene was amplified by PCR.
Supplemental Fig. 2. Knockdown of HDAC2 induces Claudin-1, but not Claudin-3 or Claudin-4. (A) A Northern blot was prepared using total RNAs isolated from MCF7-si-HDAC2-10 cells grown +/- tetracycline for 3d. The blot was sequentially probed with cDNAs derived from Claudin-1 and GAPDH genes. (B) The level of claudin-1, claudin-3, claudin-4, and actin was assayed by Western blot analysis with antibodies against claudin-1 (Zymed Laboratories Inc.), claudin-3 (Zymed Laboratories Inc.), claudin-4 (Zymed Laboratories Inc.), and actin in MCF7-si-HDAC2-10 cells grown +/- tetracycline for 3d.

The function of HDAC2 as a co-repressor is well established. HDAC2 plays an important role in transcriptional repression by the mSin3a and NURD complexes as well as by transcription factors such as Mad, YY1, p53, and others (19, 36-39). In this study, we have identified a novel function for HDAC2 whereby HDAC2 modulates p53-DNA binding activity and thus affects the ability of p53 to trans-activate and trans-repress a subset of target genes. We showed that knockdown of HDAC2 enhanced the induction of p21, DKK1, Mdm2, and FDXR, but not IGFBP3 or AQP3, by p53. Furthermore, knockdown of HDAC2 repressed c-Myc in a manner that was partly p53-dependent. Investigation into the mechanism revealed that enhanced trans-activation and trans-repression by p53 was due to augmented p53-DNA binding activity but not alterations in p53 stability or acetylation.
The precise mechanism by which HDAC2 inhibits p53-DNA binding remains unclear. Several hypotheses exist: (i) HDAC2 directly inhibits p53-DNA binding activity, (ii) HDAC2 deacetylates p53 at unknown lysine residue(s) to inhibit p53-DNA binding activity, or (iii) HDAC2 modulates chromatin structure which affects p53-DNA binding activity. Since HDAC2 has been found to directly interact with p53 (19), the first hypothesis is formally possible. Evidence suggests that acetylation of C-terminal lysine residues in p53 enhances p53-DNA binding activity in vitro and in vivo (40-42). Although cisplatin-induced acetylation of lysines 373 and 382 was not altered upon knockdown of HDAC2, HDAC2 may act on the eighteen other lysine residues within p53. Thus, the second hypothesis is formally possible. However, several lines of evidence suggest that p53 acetylation and stabilization are linked (6, 20). Since p53 stability was not affected upon knockdown of HDAC2, p53 acetylation may not be responsible for the augmented p53-DNA binding activity. Unexpectedly, we found that the level of histone H3 acetylation was diminished upon knockdown of HDAC2. The decrease in histone H3 acetylation correlated with the increase in p53-DNA binding activity. Thus, our data may support the third hypothesis where HDAC2, although a deacetylase, impacts p53-DNA binding through the modulation of histone acetylation and chromatin composition. In line with this, HDAC2 has been shown to interact with topoisomerase II which plays an important role in chromatin structure (38). Furthermore, Myc has recently been identified to influence global chromatin structure whereby lack of Myc led to a striking decrease in overall histone acetylation (43). Perhaps knockdown of HDAC2 induces an initial p53-independent repression of c-Myc which then triggers a feedforward loop of decreased histone H3 acetylation and increased p53-DNA binding activity. Regardless, the reduced
acetylation of histone H3 may enhance the electrostatic interactions between p53 and chromatin, thereby stabilizing the p53-DNA interaction.

In summary, we showed that knockdown of HDAC2 inhibited cellular proliferation, induced cellular senescence, and augmented p53-DNA binding to enhance p53-dependent trans-activation and trans-repression of a subset of target genes. This is the first report, to our knowledge, to demonstrate that HDAC2 negatively regulates p53-DNA binding activity. Future studies need to address the precise mechanism by which HDAC2 inhibits p53-DNA binding as well as whether HDAC2 affects the DNA binding activity of mutant p53 or other p53 family proteins. Since the expression of HDAC2 has been found to be increased in cancers (30, 31, 44), our study suggests that HDAC2 is a critical target of HDAC inhibitors in cancer therapies.

Materials and Methods

Plasmids and Reagents

HAp53/pcDNA3 and p21A/pGL2 were as described (8). To generate tetracycline-inducible Pol III driven expression of shRNA targeting HDAC1 or HDAC2, oligos were designed, annealed, and cloned into pTER vector. For HDAC1 shRNA, oligos were

HDAC1-si-CR-F: 5’GAT CCC C GC AGA TGC AGA GAT TCA ACT TCA AGA
GAG TTG AAT CTC TGC ATC TGC TTT TTG GAA A-3’

and HDAC1-si-CR-R: 5’-AGC TTT TCC AAA AA G CAG ATG CAG AGA TTC AAC TCT CTT GAA GTT
GAA TCT CTG CAT CTG CGG G-3’ with HDAC1 targeting sequence in **BOLD**. For HDAC2 shRNA, oligos were HDAC2-CR-644-F: 5’GAT CCC C AGA TCA GGA TTC TGT TA T TCA AGA GA AAC AGA ATC CTG ATG CTT TTT TTG GAA

**TTC TGT TAT TCA AGA GAT AAC AGA ATC CTG ATG CTT TTT TTG GAA**
A-3’ and HDAC2-CR-644-R: 5’-AGC TTT TCC AAA AAA AGC ATC AGG ATT
CTG TTA TCT CTT GAA TAA CAG AAT CCT GAT GCT TGG G-3’ with HDAC2 targeting sequence in **BOLD**. Murine HDAC2 was subcloned from pME18s into pcDNA3 using EcoRI/XhoI. Reagents were tetracycline in EtOH at 2 µg/ml, doxycycline (Sigma) in H2O at 1 µg/ml, nutlin-3 (Cayman Chemical Company, Ann Arbor, MI) in EtOH, *cis*-Diammineplatinum(II) dichloride (Sigma) in H2O, and cyclohexamide (Sigma) in EtOH at 10 µg/ml. 21bp annealed RNA oligos (HDAC2 siRNA: AAG CAU CAG GAU UCU GUU A and scrambled HDAC2 siRNA: GGC CGA UUG UCA AAU AAU U) were purchased from Dharmacon RNA Technologies.

**Cell Culture and Transfection**

MCF7, Hct116 p53−/−, and MCF7-GPX2-p53KD-3 cell lines were maintained as described (8, 45-47). Stable cell lines were generated as described (47). Individual clones were screened to identify MCF7 clone with stable integration of pcDNA6/TR (Invitrogen), named MCF7-TR7. Individual clones were screened to identify tetracycline-inducible knockdown of HDAC1 or HDAC2 by Western blot analysis, named MCF7-si-HDAC1-7, MCF7-si-HDAC2-10, MCF7-si-HDAC2-B18, and MCF7-si-HDAC2-B38. For transient knockdown of HDAC2, MCF7 or MCF7-p53KD-GPX2-3 (with stable p53-knockdown and tet-inducible GPX2 expression) cells were transfected with 50 nM oligos using siLentFect (BioRad) in the absence of tetracycline for 3d.
**Western Blot Analysis**

Western blot analysis was performed as described (47) using anti-p21 (C19; Santa Cruz), anti-Ac-p53(AcK373/382) (Upstate), anti-actin (Sigma), anti-HDAC1 (E210; Upstate), anti-HDAC2 (3F3; Upstate), anti-c-Myc (9E10.2), and anti-p53 (DO-1, PAb1801, PAb240, and PAb421) antibodies.

**Growth Rate, Colony Formation Assay, and DNA Histogram Analysis**

Growth rate was assayed as described (47). Briefly, 3x10⁴ cells were seeded +/- tetracycline in triplicate. Attached cells were counted at the indicated time. For colony formation assay, 500 cells were seeded +/- tetracycline in triplicate. Cells were fixed in methanol: glacial acetic acid (7:1), washed in H₂O, and stained with crystal violet (0.2 g/L). DNA histogram analysis was performed as described. Briefly, 1x10⁵ cells were seeded +/- tetracycline in triplicate, harvested, fixed in 100% ethanol, stained with propidium iodide, and analyzed by FACS.

**Senescence-Associated β-Galactosidase Assay**

1x10³ MCF7-si-HDAC2-10 cells were seeded +/- tetracycline in triplicate. Cells were fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS, washed in PBS, and stained O/N with 40 mM citric acid/sodium phosphate (pH 6.0), 150 mM NaCl, 2 mM MgCl₂, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mg/ml X-gal. % of SA-β-gal positive colonies were scored.
RNA Isolation and Northern Blot Analysis

Total RNA was isolated using Trizol reagents (Invitrogen). Northern blot analysis was performed as described (8). The IGFBP3, AQP3, DKK1, GAPDH, Mdm2, p21, and FDXR probes were prepared as described (8, 26, 47, 48); the 338bp e-Myc cDNA probe was amplified using primers as described (49) and cloned into pGEM-Teasy.

Luciferase Assay

To determine the effect of HDAC2 knockdown on p53 activity, MCF7-si-HDAC2 cells were grown +/- tetracycline for 4d and co-transfected in triplicate with p21A/pGL2, internal control Renilla luciferase vector pRL/CMV, and either empty pcDNA3 or HAp53/pcDNA3 for 24h. To determine the effect of HDAC2 on p53 activity, Hct116 p53-/- and MCF7 cells were co-transfected in triplicate with p21A/pGL2, pRL/CMV, and an equal concentration of pcDNA3, p53, and HDAC2 expression vectors as indicated for 24h. The dual luciferase reporter assay (Promega) was performed. Fold increase in luciferase activity is shown. Standard error was determined.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was performed as described (8). Briefly, MCF7-si-HDAC2-10 cells were grown +/- tetracycline for 3d and treated with 5 µM nutlin or 50 µM cisplatin as indicated. Chromatin was crosslinked and sonicated. DNA was quantitated and equal amounts of chromatin were immunoprecipitated with anti-mSin3A (K20; Santa Cruz), anti-AcH3 (Upstate), anti-p53 DO-1, or a mix of anti-p53 (DO-1, PAb1801, PAb240, and PAb421) antibodies. Control antibodies were non-immune rabbit IgG (Sigma), mouse
IgG2a (UPC-10; Sigma), and anti-mouse (Sigma). Immunoprecipitated DNA fragments were purified over a QIAGEN column and analyzed by PCR. Primers that amplify the GAPDH promoter and the 5’ p53-responsive elements within the p21 and Mdm2 promoters were as described (8, 50, 51). Primers that amplify a 208bp fragment spanning the p53-responsive element within the human c-Myc gene were the 5’end primer Myc-1765-5’ (TGA GGG ACC AAG GAT GAG AAG AAT G) and 3’ end primer Myc-1104-3’ (TGA AAG TGC ACT GTA TGT AAC CCG C). Primers that amplify a 189bp fragment spanning the +1 site of the DKK1 gene were the 5’ end primer DK1-2996F (CAG TCA GGA CTC TGG GAC CGC AGG G) and the 3’ end primer DK1-3185R (GCC GCT ACC ATC GCG ACA AAG ACC C).

Affymetrix Gene Chip Analysis

Total RNAs from MCF7-si-HDAC1-7 and MCF7-si-HDAC2-10 cells grown +/- tetracycline for 4d were isolated, labeled, and hybridized to an Affymetrix gene chip (U133 plus 2.0).

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References


SUMMARY

p53 is a tumor suppressor gene. Depending on context, p53 regulates transcription of distinct but overlapping sets of genes which mediate different p53-dependent activities such as apoptosis or cell cycle arrest. Mechanisms that facilitate differential target gene regulation by p53 family proteins are complex. Previous studies have shown that the level of p53 (10), the p53 functional domains (62), p53-associated proteins such as ASPP1 and ASPP2 (48), as well as characteristics of the target gene promoter (45) all impact this process. In these studies, I have found that HDACs play important roles in the regulation of p53 transcriptional activities. Specifically, HDACs facilitate differential target gene regulation by p53 family isoforms, and HDAC2 modulates the DNA-binding activity of p53.

Through the study of the differential transcriptional regulation of IGFBP3 by p53 family proteins, I found that the C terminus of p53 family proteins was inhibitory, such that C-terminally truncated p53, p63, and p73 isoforms induced IGFBP3 whereas full-length isoforms did not. For p53, the N-terminal activation domain 1 was also inhibitory. The unique organization of eleven half-sites of the p53-responsive element within the IGFBP3 promoter was critical for the regulation of IGFBP3 by C-terminally truncated p53 family proteins. Although the C terminus did not affect the ability of p53 to bind the IGFBP3 promoter, the inhibitory function of the C terminus in p53 family proteins was linked to the activity of HDACs. Inhibition of HDAC activity with TSA restored the abil-
ity of endogenous and exogenous full-length p53, p63, and p73 isoforms to induce IGFBP3. Since we showed that IGFBP3 is an important pro-apoptotic target gene, the data suggest that the induction of p53-mediated apoptosis is under stringent regulation by HDAC activity as well as by the expression of p53 isoforms.

This is the first study to show that HDAC activity inhibits the ability of full-length p53 family proteins to regulate the pro-apoptotic target gene IGFBP3, thus many questions are raised. For example, which HDAC(s) governs the regulation of IGFBP3 by p53. While I have found that HDAC2 modulates p53-DNA binding activity to impact the transcriptional activity of p53 for a subset of target genes, neither HDAC1 nor HDAC2 was the HDAC that inhibited IGFBP3 induction by p53. Since p53-DNA binding is not the critical factor that governs the regulation of IGFBP3 by p53, HDACs must function in additional manners to regulate p53 activity. Also, if HDACs function to limit p53-mediated apoptosis by inhibiting the induction of IGFBP3 by p53, what regulates HDAC activity to promote p53-mediated apoptosis? Since p63 and p73 play important roles in development, the physiological consequence of differential IGFBP3 regulation by p63 and p73 isoforms remains unknown. In the study, I also identified several genes that were differentially regulated by full-length p53 and by p53 which lacks the N-terminal activation domain 1 and the C-terminal basic domain, called p53(∆AD1∆BD). It will be interesting to learn if these genes are regulated by p53 family isoforms in a manner similar to IGFBP3 as well as if these genes function in a pro-apoptotic manner.

In the second study, I found that HDAC2 modulates p53 transcriptional activities through the regulation of p53-DNA binding activity. First, I found that knockdown of HDAC2 induced cellular senescence and inhibited MCF7 cell proliferation in a dose-
dependent manner. Importantly, I found that knockdown of HDAC2 induced the repression of c-Myc in both p53-dependent and -independent manners. Furthermore, knockdown of HDAC2 enhanced the induction of p21, Mdm2, ferrodoxin reductase (FDXR), and dickkopf-1 (DKK1) by p53. The enhancement of p53 trans-repression and trans-activation was due to an enhancement in p53-DNA binding activity and not alterations in p53 stability or post-translational modification(s). Thus, HDAC2 negatively regulates p53 transcriptional activities by affecting p53-DNA binding activity.

This is the first study to show that HDAC2 modulates p53-DNA binding activity to impact p53 trans-activation and trans-repression of a subset of target genes. My data suggest that HDAC2 affects chromatin structure to modulate the p53-DNA interaction. However, the precise mechanism by which HDAC2 inhibits p53-DNA binding remains unclear and needs further investigation. Future studies should also address whether HDAC2 or another HDAC affects DNA-binding by mutant p53 or by p63 and p73. Since I have found that HDAC2 regulates p53 activities for a subset of genes, it will be important to characterize the role of HDAC2 in the regulation of other p53 target genes. Also, what regulates the activity of HDAC2 in the p53 pathway? Since HDAC2 has been found to be overexpressed in cancers and I have found that HDAC2 affects MCF7 cell proliferation in a dose-dependent manner, it will be interesting to learn if aberrant overexpression of HDAC2 in tumor cells serves as a mechanism to promote tumorigenesis by inhibiting p53 activity. HDAC inhibitors are emerging as promising cancer chemotherapeutic agents, but the molecular basis remains unclear. For the first time, our data suggest that HDAC inhibitors activate the p53 pathway, at least in part, through activation of p53-DNA binding activity.
In sum, I have found that HDACs play important roles in regulating the transcriptional activity of full-length p53 family isoforms and that HDAC2 plays an important role in the regulation of p53-DNA binding activity.


