Low-level p53 expression changes transactivation rules and reveals superactivating sequences

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Transcriptional activation by the tumor suppressor p53 is considered to depend on cellular level, although there are few systems where this dependence on cellular level of p53 has been directly addressed. Previously, we reported that transactivation from p53 targets was sensitive to both p53 amount and DNA sequence, with some sequences being responsive to much lower p53 levels than others when examined in yeast model systems or human cells. Because p53 is normally present at low levels and perturbations might lead to small increases, we examined transactivation under limiting p53. Unlike the positive relationship between transactivation and binding affinity found at target sequences at high cellular levels of human p53 in yeast, no such relationship was found at low levels. However, transactivation in the yeast system and the torsional flexibility of target sequences were highly correlated, revealing a unique structural relationship between transcriptional function and sequence. Surprisingly, a few sequences supported high transactivation at low p53 levels in yeast or when transfected into human cells. On the basis of kinetic and flexibility analyses the “supertransactivation” property was due to low binding off rates of flexible target sites. Interestingly, a supertransactivation response element can differentiate transcriptional capacities of many breast cancer-associated p53 mutants. Overall, these studies, which are relevant to other transcription factors, address the extent to which transcriptional properties of p53 target sequences are determined by their intrinsic physical properties and reveal unique rules of engagement of target sequences at low p53 levels.

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quence-specific DNA binding by transcription factors (TFs) to their target sites on DNA is a key step in many cellular functions such as transcriptional activation and cooperation (1). In response to cellular stress p53 acts as a TF by binding to DNA targets, leading to the expression of many genes that participate in a variety of biological processes including cell-cycle arrest, DNA repair, and apoptosis (1). After nearly 30 y, the mechanisms of p53 transactivation from different RE sequences in a constant chromatin environment (13). This system, which was created in diploid yeast (13), addresses both the ability of p53 to function in vivo isogenicomic yeast-based model system to address human p53 transactivation from different RE sequences in a constant chromatin environment (13). This system, which was created in diploid yeast (13), addresses both the ability of p53 to function from specific target sequences (i.e., on/off) and the extent of transactivation from these sites at variable levels of p53 protein. Here, we establish that low basal levels of p53 expression result in transactivation of a linked reporter. Further increases in p53 lead to increased transactivation in an RE-specific manner. Using in vitro binding affinities for various p53 constructs, we found a positive relationship between in vivo transactivation and 


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binding affinities, but only at high p53 levels. However, at low p53 levels transactivation was correlated with the DNA torsional flexibility of 29 natural and synthetic target sequences, revealing a unique structural relationship between transactivation function and p53 RE sequence.

In addition, we discovered two sequences with unusual properties for p53-driven transactivation. They are highly responsive to low basal levels of p53. At high levels, the maximum response was comparable to that for transactivation from other highly responsive REs such as p21-5’ and p53R2. The transcriptional capacity of many p53 mutants associated with breast cancers could be differentiated by transactivation from such an RE. We establish that the highly sensitive response to wild-type (WT) p53 is linked to an increased kinetic stability of p53 on such sequences. Thus, the DNA sequence itself can strongly affect p53 transactivation even under conditions where the number of p53 molecules is small, suggesting that DNA structural properties can be critical factors in p53-dependent gene regulation.

Results

Transactivation at High vs. Low Protein Expression Level. Recently, we developed a luciferase reporter system in diploid yeast that allows us to transactivation capacities to be addressed with target sequences at variable levels of expression under isogenic conditions (13). p53 expression is under a rheostatable GAL1 promoter that provides controlled and inducible expression of p53, depending on the carbon source. This assay provides the opportunity to address the relationship between p53 induction and transactivation capacity at high and low levels of p53 molecules. In Table S1 we estimate that p53 levels change from ~300 molecules per cell in the absence of galactose to ~33,500 molecules at high levels of expression (0.024% galactose). Analysis of the strength of transactivation (relative light units per microgram of protein) from 11 artificial consensus-related targets (Table S2 and Fig. L4) and 18 natural REs (Table S2 and Fig. 1B) under different levels of p53 expression revealed distinct patterns of transactivation. The pattern of change in transactivation level from the natural p53 REs at high protein levels (Fig. 1B) spans three orders of magnitude and is similar to in vitro protein binding patterns (7, 11, 12), with high-affinity binding sites such as p21-5’ and p53R2 providing high levels of transactivation and weak binding sites such as Rad51 remaining at low transactivation levels, regardless of p53 expression (Fig. 1B). Below the 0.004% level of galactose, all natural REs decrease dramatically in their transactivation level, most being below 10^{-4} light units/µg protein, whereas p21-5’ and p53R2 are slightly above (Fig. 1B). The consensus-like targets are less variable in transactivation at high protein levels and center around 10^{-4}-10^{-5} light units/µg protein (Fig. 1L4). However, at low and basal levels of protein the transactivation pattern of the consensus-like sequences changes dramatically from that observed for natural p53 REs with a wide range of transactivation levels (0.2-4.0 × 10^{-4} light units/µg protein) from the consensus-like targets.

Transactivation Capacity Correlates with Binding Affinity at High, but Not Low, Protein Expression Level. Our observation that at high p53 protein levels the transactivation correlates with in vitro binding (7, 11, 12) led to a statistical evaluation between these variables, using the Spearman rank correlation coefficient. Only 14 sequences were in common with the in vitro studies of Weinberg et al. (7). As shown in Fig. 2A, there is a strong (ρ = 0.87) and highly significant correlation (t test for the significance of the coefficient = -6.19, P value = 0.002) between transactivation level at 0.024% galactose (high induction of p53) and binding affinity measured for a p53 construct containing the DBD and the tetramerization domain (p53CT) (7). At low cellular levels of p53 (0.002% galactose) a weak (ρ = 0.41) and not significant correlation (t test = -1.58, P value = 0.134) is observed between these 14 sequences (Fig. S14A). A similar strong (ρ = 0.91) and significant (t test = -6.90, P value = 0.003) correlation at 0.024% galactose is observed when a p53 construct containing only the DBD was used in the binding affinity measurements (Fig. 2B), using 12 p53 REs for which we measured binding affinity (Table S3, and ref. 12) as well as transactivation levels. These correlations demonstrate that the relationship between transactivation and p53 binding is independent of whether p53 binding characteristics are measured with or without the tetramerization domain, as well as being independent of the method of measurement [electrophoretic mobility shift assay (EMSA), Fig. 2B, vs. fluorescence anisotropy, Fig. 2A].

Contrary to the weak and lack of significant correlation of transactivation at low p53 levels and K_{th}, there is a strong (ρ = 0.66) and highly significant correlation (t test = 4.55, P value = 0.0006) between the torsional flexibility of p53 REs and transactivation (Fig. 2C). We recently showed that torsional (twist) flexibilities of p53 REs are important in p53 interactions with its RE domain. Moreover, we showed that the torsional flexibility of unmeasured sequences can be estimated using the dispersion of twist angle values from their average values, as determined from crystal structures of protein–DNA complexes (14). This approach is valid because the sequence-dependent torsional deformability described by Olson et al. (14) correlates with our experimental values (12). Using this methodology we assessed the relationship between transactivation at low cellular levels of p53 and calculated torsional flexibility (14, 15) of the entire sequence for each RE, using rank correlation calculations (Fig. 2C) for all binding sites studied here (29 sequences). Fig. 2C shows that the relationship between the calculated torsional flexibility (14, 15) of p53 sites and transactivation level is strong and highly significant, as detailed above. These torsional flexibility calculations were carried out along both decameric parts of all p53 sequences examined, regardless of

Fig. 1. Variation in p53 protein levels reveals distinct transactivation patterns for p53 toward different response elements (REs). The abilities of WT p53 to transactivate different consensus-like (A) and natural (B) REs with increased cellular levels of p53 were measured in 24-h growing cultures of cells incubated with increasing amounts of galactose. For basal levels (galactose 0%) cells were grown on media containing rafinose as a carbon source. Presented are the mean and SEM for 5–30 independent luciferase reporter assay repeats. Sequences are described in Table S2.
Con-A and GGG Are Supertransactivation Sequences. Among the sequences studied here, transactivation at the “Con-A” and “GGG” REs (Table S2) was nearly 10-fold higher than at p21-5’ at basal levels of p53 expression or when cells were incubated at low levels of galactose (up to 0.004% galactose, Fig. 1 A and B). We refer to them as “supertransactivation” REs. Transactivation of Con-A was high even under the repressed (glucose) levels of p53 (Fig. S3A). Changing the CWWG core from CATG (Con-A) to CTAG (Con-B) or CAAG (Con-C) abolished the high transactivation level at basal levels of p53 (Fig. L4). Furthermore, supertransactivation was dramatically reduced when one or more bases were inserted between half-site decamers (Fig. L4). There was limited transactivation at high levels of p53 protein from 3/4-site and 1/2-site derivatives of Con-A; however, none of the non-canonical versions of Con-A supported a significant level of transactivation at low levels of p53 (Fig. S3B). Thus, p53 capability for transactivation from the supertransactivation Con-A RE is dependent on the availability of a full-site RE, without spacers between half sites and with a CATG center. Neither of these supertransactivation REs was found among the >200,000 p53-like REs (16) in the human genome; however, the probability of any 20-base sequence in the genome is low.

To determine whether the differences at basal p53 levels between supertransactivation sequences and other RE sequences are due to increased binding of p53 to these specific REs, we investigated in vivo occupancy, using quantitative chromatin immunoprecipitation-PCR (ChIP-qPCR) in yeast (Fig. 3A). As shown in Fig. 3A, no significant difference in p53 binding to p21-5’ or Con-A REs was observed at high protein levels (0.024% galactose, solid bars). However, at basal p53 levels, when cells were grown in raffinose (shaded bars), there was an approximate fourfold difference in p53 binding to the Con-A sequence compared with the p21-5’ RE, consistent with the transactivation results. No p53 binding was found in cells not induced for p53 (grown in raffinose) and containing only the empty CORE cassette used to clone the p53 REs (Fig. 3A). Differential occupancy by p53 was also determined using standard ChIP-qPCR approaches (Fig. S4A).

p53 Requirements for DNA-Dependent Supertransactivation in Yeast and Human Cells. We analyzed transactivation at supertransactivation sequences by p53 structural mutants that are compromised for oligomerization and tetramerization. Although p53 tetramers are the functional unit essential for high-level transactivation, p53 can transactivate from half-site REs in yeast and human cells (13), and p53 dimers have been shown to bind consensus half-site sequences in vitro (17). The L344P mutation or deletion of the tetramerization domain (ΔTet, lacking residues 325–357) yields p53 monomers, whereas L344A results in a dimeric protein (summarized in ref. 13, see references in Fig. S3). As shown in Fig. S3C, these mutant proteins were not able to drive transactivation from the Con-A sequence at low or even high levels of p53. Thus, p53 capability for transactivation from the supertransactivation Con-A RE is also dependent on p53 being able to form tetramers.

We also determined the impact of p53 protein levels on transactivation from the supertransactivation sequences in human cells. Osteosarcoma SaOS2 cells, which are p53 null, were transfected with increasing amounts of p53 expression vector along with a constant amount of luciferase reporter constructs containing the p53 REs. The p53 protein levels were confirmed by Western blot analysis (Fig. 3B). We determined p53 transactivation from the supertransactivation sequences Con-A and GGG as well as from GGA and p21-5’ at low (5 ng) and high p53 levels (25 ng; Fig. 3C). These levels were chosen on the basis of transactivation from p21-5’ and Con-A REs with increasing amounts of transfected p53
targets transactivated as well as levels (20). Among these, 29
human target REs, where mutants could alter the spectrum of
activation from the Con-A RE, using a yeast ADE2 color assay
mutants associated with breast cancer for their ability to trans-
activate from the Con-A and GGG REs compared with that from the
expression resulted in an approximate threefold greater trans-
activation from Con-A and GGG REs compared with that from the
expression vector (Fig. S4B). As shown in Fig. 3C, low-level p53
expression resulted in an approximate threefold greater trans-
activation from Con-A and GGG REs compared with that from the
p21-5' RE and the GGA sequence. At high p53 levels there were no
significant differences between any of the REs (Fig. 3C).

Among the thousands of cancer-associated p53 mutations
identified (International Agency for Research on Cancer, version
R15, November 2010, ref. 18) many have very low transactivation
capabilities (19), which might be more responsive with the
supertransactivation Con-A RE. We examined 50 missense p53
mutants associated with breast cancer for their ability to trans-
activate from the Con-A RE, using a yeast ADE2 color assay
(Fig. S5 A and B and ref. 13) and the luciferase reporter assay
(Fig. S5C). The 50 mutants were previously analyzed for “func-
tional fingerprints” in terms of capability for transactivation from
11 human target REs, where mutants could alter the spectrum of
targets transactivated as well as levels (20). Among these, 29
mutants lacked transactivation capability and the functional
fingerprint of 21 mutants was altered. Using the ADE2 plate
assay and a high galactose level (0.128%), we found that the lack
of any transactivation capability by these mutants was best pre-
picted by Con-A, compared with any of the other 11 human p53-
RE targets used (Fig. S5F). Among the altered function mutants
but P151A and R174K exhibited the transactivation characteristic of WT p53 (Fig. S5B); however, they all retained
good induction from Con-A at higher p53 levels. The transactivation capability of several mutants was confirmed with the
luciferase reporter assay. Several mutants exhibited substantial
transactivation from the p21-5' and Con-A REs at elevated p53
levels; however, only Con-A supported high levels of trans-
avtivation at basal levels of some of the p53 mutants (Fig. S5C). Thus, transactivation from the Con-A sequence can be used
as a diagnostic tool to address transcriptional capabilities of
p53 mutants.

**Binding Kinetics and the Molecular Basis of Supertransactivation.**

What is the molecular origin of the supertransactivation phe-
nomenon? The two supertransactivation sequences do not reach
the high-affinity levels of well-established targets such as p21-5' and GGA (Table S3, Fig. S2, and refs. 7, 8, and 11), as noted
above. The Con-A and GGG sequences exhibit a high degree of
torsional flexibility (12); more than other p53 REs; however, the
difference in torsional flexibility between these sequences and
the GGA target may not account fully for the supertransac-
tivation phenomenon. We, therefore, determined the binding
stability (i.e., the kinetic off rate) of WT p53DBD toward four
DNA-binding sites: the supertransactivation targets, Con-A and
GGG, and high-affinity targets p21-5' and GGA (Fig. 4 and Fig.
S6A). Analysis of the tetramer band in Fig. S6A (Table S4) shows
that there is a biphasic dissociation kinetic behavior of p53 tet-
ramers from all four sequences. The half-life of fraction “A” was
always fast (a few seconds) and variable between experiments
(with a curve-fitting error of the same magnitude or larger),
whereas the half-life of fraction “B” was slower and reproducible
between experiments and, hence, had a low curve-fitting error.
This biphasic behavior means that there are two linked macro-
scopic dissociation processes and that the kinetics of dissociation
are determined by a mechanism involving binding intermediates.
On the basis of the half-lives of complexes undergoing process B
dissociation (Fig. 4 and Table S4), the supertransactivation se-
quences form more kinetically stable complexes with p53DBD,
with off rates that are twice as large as those with the other
sequences.

These observations point to kinetic stability of p53DBD tet-
ramers on Con-A and GGG being responsible for the super-
transactivation behavior. We then asked whether the same trend
between kinetic stability and supertransactivation is observed for
p53DBD dimers, using DNA targets with just one specific dec-
amerized half site (HS), flanked by nonspecific sequences (Table S4
and Fig. S6B). Table S4 shows that p53DBD forms stable dimers
on the supertransactivation sequence Con-A HS and the non-
supertransactivation sequence GGA HS, but not on the super-
transactivation sequence GGH HS.

**Discussion.** The level of a transcription activator is expected to in-
fluence the extent of target gene expression. Despite that expectation, there are few examples in which it has been possible to directly ex-
amine this issue. Here, we used a rheostate p53 expression
system along with various physical analyses to address specifically
three aspects of the relationship between transactivation and p53
target sequence: high vs. low p53 level, impact of cancer-associated
mutations, and unique supertransactivation sequences.

**Transactivation at Low p53 Levels.** At high p53 levels, the positive relationship between transactivation and DNA-binding affinity
that we observed suggests that under conditions of constant chromatin environment, binding affinity is a major determinant
of transactivation. However, this relationship breaks down at low
p53 levels and suggests that under conditions of limited stress the
factors responsible for p53-dependent transactivation are different
from those at highly induced p53 levels. Under conditions of low

![Fig. 3. Validation of supertransactivation response at low levels of p53 in yeast and mammalian cells.](image-url)
p53 levels we found that transactivation levels are largely determined by the torsional flexibility of the DNA sequences. We attribute this difference to the functional form of p53 being a tetramer. p53 dimerizes during cotranslation in polysomes (21) and under 50 nM protein is normally present as a dimer in solution (22, 23). Thus, the functional tetramer form is induced by DNA binding (12, 23, 24). Our estimate (Table S1) is that 0.024% galactose corresponds to 33,500 p53 molecules. Assuming a yeast cell radius of 2–4 μm, the estimated number of molecules corresponds to ~40 nM of p53 within the cell at high protein expression levels (levels in human cells appear to be sometimes higher; Table S1). When the protein level is low, the chance that two dimeric p53 molecules will arrive together at the RE to bind as a tetramer is low. Therefore, at low protein levels the binding of the tetrameric form can be accomplished only when the first dimer species is bound stably enough on the DNA, such that it is able to stay bound until the next dimer molecules arrives at the RE. Torsional flexibility of the RE can contribute to the kinetic stability of p53 dimers on the RE, because we have previously shown (12) that torsional flexibility of p53 REs facilitates the reorientation of two p53 monomers within each dimer to stabilize intradimer interactions with concomitant decrease in binding cooperativity. Hence, at low levels of expression of p53 and on torsionally flexible DNA targets, the complex of p53 dimers on DNA can transiently accumulate as intermediates. Therefore, the transactivation level at low protein concentrations is correlated with the torsional flexibility of the target site and not with the binding affinity of the p53/DNA complex. Consistent with our previous study (12), all target sites on the right side of Fig. 2B (above “ranked” position 17) have the very torsionally flexible CATG center, whereas those on the left (ranked ≤17) have other DNA motifs at the center.

**Supertransactivation.** Many studies have addressed the function of p53 toward a variety of REs that have different capabilities in supporting transactivation. Using our system that examines p53 function over a broad range of protein expression, we discovered that two sequences were highly effective at supporting high levels of transactivation even under very low expression conditions, which has the potential for greatly increasing the functionality of p53.

The supertransactivation character of Con-A and GGG REs at low protein expression requires a full-site consensus binding element and depends on the ability of p53 to form a tetramer, the same structural requirements necessary for high response at high protein levels. Transactivation with few molecules suggests unique properties of these supertransactivation REs. Other REs often require at least 10 times more p53 to achieve even low levels of transactivation, and several weak REs required ~100 times more p53 protein over basal levels to reach transactivation levels comparable to those obtained from Con-A or GGG REs at basal p53 expression. As reported for other REs with high transactivation responses, a CATG core motif and absence of spacers between half sites are required for a strong response by the supertransactivation REs. However, these requirements are not enough for the supertransactivation phenomenon, as discussed below.

We found that torsional flexibility is a prerequisite for high transactivation at low protein levels. However, this amount of torsional flexibility is not sufficient, because the GGA and AGG targets also have above average torsional flexibilities (12). The supertransactivation phenomenon additionally requires kinetic stability of the tetrameric complex, i.e., low off rate of p53 from full-site RE, which is observed only for the GGG and Con-A targets. A low off rate might be due to DNA breathing (base pair opening), which would be lower in GGG and Con-A relative to other REs, because they contain either no A-T base pairs (GGG) or only one (Con-A), outside the central CATG region. If so, it may be possible to identify additional supertransactivation sequences using the yeast approaches described here.

The Con-A RE was originally derived from a mutagenesis approach of three tandem repeats of the upstream p53-binding element of the human RGC sequence (CCAGGCAAGT)3 done to determine the DNA-binding sequence specificity of wild-type p53 (25). The GGG RE is a self-complementary sequence derived from the Con-A RE (11). The observation that neither of the supertransactivation REs is found in the genome (the closest sequence found has 17 of the 20 nt without a mismatch) may indicate that such sequences would lack the p53 stress response associated with the other targets. Previously, we observed with a bead-binding assay that p53 binding is ~2-fold greater for Con-A RE than p21-5′ RE (26), using basal levels of p53 from untreated nuclear U2OS cell extracts. Consistent with this, we show here that at low levels, transactivation from p53 is 10-fold better in vivo to the Con-A RE than to p21-5′ RE (Fig. 1), which is generally considered one of the most responsive natural p53 REs at basal p53 expression levels (27).

**Binding Kinetics and Implications for the Mechanism of RE Binding of p53.** Kinetic analysis of four p53 targets revealed that p53 tetramers dissociated with a biphasic behavior, indicating that dissociation proceeds through a mechanism involving binding intermediates. Petty et al. (28) suggested an induced-fit binding mechanism of p53 to its REs. This mechanism entails two binding (or dissociation) steps: a rigid body interaction followed by a conformational change. Our Binding curves show a considerably greater biphasic character to p53 off rate than for the WT p53 complex studied by Petty et al. (28). This may be due to the p53 construct used here as well as to the conditions of the binding interactions. The biphasic behavior may be due to quaternary changes in p53 conformation (11, 29) and/or changes in the L1-loop conformation observed in several p53 structures upon DNA binding (11, 28, 30, 31). However, they may also be due in part to other kinds of binding intermediates that do not involve an induced-fit mechanism, for example a dimer intermediate on the way to forming or abolishing the functional tetrameric form of p53. From Table S4 it is clear that different intermediates occur when tetramers dissociate from p53 full sites, compared with dissociation of dimeric complexes from p53 half sites, on the basis of differences in the biphasic characteristics of the dissociation process; however, the nature of the various species is unknown at present.

p53 Cancer-Associated Mutants and Implications of Supertransactivation. Many p53 mutants retain transcriptional capability and can even result in a “change of spectrum” of the transactivated REs (32, 33). Importantly, transactivation from the Con-A sequence appears to distinguish transcriptional capability between many p53 mutations associated with breast cancers and WT p53 (20). The supertransactivation Con-A sequence appears diagnostic of...
residual transactivation function of mutant p53 proteins. Among the 50 cancer-associated p53 mutants examined, transactivation by mutant p53 from Con-A predicts transactivation from at least one additional RE (Fig. S5A). However, in the absence of transactivation from Con-A, even at high p53 expression, there is also no transactivation from any of an additional 11 natural REs studied (Fig. S5A). The supertransactivation property of Con-A might be useful for determining which p53 mutants could be remedied.

The present results at low p53 levels lead us to raise a general question of whether such sequences exist for other transcription factors. Our findings establish the possibility of screening for supertransactivation simply by presenting potential sequences with an associated reporter at low levels of the relevant transcription factor, such as NKX2.5, in yeast (34) or mammalian cells. Supertransactivation response sequences may have general diagnostic and therapeutic utilities for p53 as well as for other transcription factors.

Materials and Methods
A detailed description of materials and methods is available in SI Materials and Methods.

Protein and DNA. Human p53 core domain (residues 94–293, referred to as p53BDDB) was a kind gift from Zippora Sheinkman (Weizmann Institute of Science, Rehovot, Israel). All DNA sequences for EMSA were synthesized by Sigma Genosys and purified by a reverse-phase cartridge.

Binding Experiments. Binding affinity and dissociation kinetics measurements were conducted as previously described (12, 35, 36). In kinetics experiments radiolabeled hairpin duplexes (8 μM) were incubated with 270 nM or 160 nM p53BDDB (for half-site and full-site sequences, respectively) in the same binding buffer used in X0 measurements before adding unlabeled competitor DNA of the same sequence (1.58 μM).

Twist Flexibility Calculations. The dispersion of averaged structural parameters from their values in protein–DNA complexes (14), measured by the SD, was taken as a measure of the flexibilities of individual DNA base pair steps. The flexibility calculation was carried out on the entire group of p53 REs (with or without spacers), using a sliding window of dinucleotides.

Statistical Analysis. In testing the strength of relationships between variables, we calculated Spearman’s rank correlation coefficient (denoted by ρ) as a nonparametric measure of correlation (37). We assumed that the relationship between the variables being compared is monotonic. Reported P values are two-tailed.

Transactivation Reporter and ChIP Assays in Yeast and Human Cells. The reporter assays are described in ref. 13. Details of the qualitative ADE2 reporter assay and the quantitative luciferase reporter assay are presented in Fig. S6. For the chromatin immunoprecipitation assays p53 DO-1 (Santa Cruz Bio-technology) and H3 ab1791 (Abcam) antibodies were used. Human SaOS2 osteosarcoma cells (HTB-85; American Type Culture Collection) were routinely maintained following standard conditions and procedures for culturing mammalian cells.

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Supporting Information

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SI Materials and Methods

Protein and DNA. Human p53 core domain [residues 94–293, referred to as p53 DNA-binding domain (p53DBD)] was a kind gift from Zippora Shakked (Weizmann Institute of Science, Rehovot, Israel). The cloning, transformation, overexpression, and purification are as described by Kitayner et al. (1). Protein concentration was determined spectroscopically, as previously described (2). Before the binding studies, the fraction of p53DBD molecules active for DNA binding was determined as described previously (2, 3). All DNA sequences for the electrophoretic mobility shift assay (EMSA) were synthesized by Sigma Genosys and purified by a reverse-phase cartridge.

Binding Affinity Measurements. Binding affinity measurements were conducted as previously described (2). In short, radio-labeled and gel-purified hairpin duplexes (<0.1 nM) and increasing amounts of p53DBD were incubated on ice for 2 h in a buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM ATP, 25 units/mL BSA, 10% (vol/vol) glycerol, 10 mM DTT, and 200 mM KCl. Complexes were resolved from free DNA by electrophoresis on native gels (6%, 37.5:1 acrylamide:bisacrylamide ratio). The gels were run at 550 V and 4 °C in a running buffer containing 1× TG [25 mM Tris-HCl (pH 8.3), 190 mM glycine], until the bromophenol blue dye migrated 7 cm. Dried gels were quantified using a Fuji FLA 5000 phosphoimage, as described previously (2). We analyzed the system separately for each bound band, using TotalLab v2 (Nonlinear Dynamics), using a regular two-binding-site model (2). Association binding constants were calculated using nonlinear least-squares methods of parameter estimation (SigmaPlot; Jandel Scientific). For the p53AIP1 target that showed only the tetramer binding species, we assumed zero values to account for the unobserved dimer band. Thus, the following equations were used (4):

\[ \Theta_0 = 1 / \left(1 + K_{a1} \cdot [P] + K_{a2} \cdot [P]^2 \right) \]  
\[ \Theta_1 = K_{a1} \cdot [P] / \left(1 + K_{a1} \cdot [P] + K_{a2} \cdot [P]^2 \right) \]  
\[ \Theta_2 = K_{a2} \cdot [P]^2 / \left(1 + K_{a1} \cdot [P] + K_{a2} \cdot [P]^2 \right) \]

\[ \Theta_0 = 1 / \left(1 + K_{a1} \cdot [P] + K_{a2} \cdot [P]^2 \right) \]  
\[ \Theta_1 = K_{a1} \cdot [P] / \left(1 + K_{a1} \cdot [P] + K_{a2} \cdot [P]^2 \right) \]  
\[ \Theta_2 = K_{a2} \cdot [P]^2 / \left(1 + K_{a1} \cdot [P] + K_{a2} \cdot [P]^2 \right) \]

where \( K_{a1} \) and \( K_{a2} \) are the intrinsic microscopic binding constants for each half site and \( K_{a12} \) is the cooperativity constant (4). \( K_{a1} \) increases the measure (or decrease) in the binding affinity of the second dimer relative to that of the first dimer due to the cooperativity of the binding interaction. For targets with identical half sites, and assuming equipartition of binding free energies, an apparent dimer-equivalent association constant can be calculated by taking the square root of the \( K_{a2} \) value. The reported values (Table S3) are the dissociation binding constants \( (K_{D}\text{p53}) \), which are the reciprocal of the association binding constants. Even though \( k_1 \) is not equal to \( k_2 \) when the two half sites are not identical [for natural response elements (REs)], this measure gives an estimation for an averaged dimer equivalent tetramer \( K_{D} \) also for these sites.

Dissociation Kinetics Experiments. Dissociation kinetics experiments were carried out as previously determined (5, 6). In short, individual sequences were embedded in hairpin constructs. Radio-labeled hairpin duplexes (0.4 nM) were incubated with 270 nM or 160 nM p53DBD (for half-site and full-site DNA sequences, respectively) for 2 h at 4 °C. In the same binding buffer used for \( K_{D} \) measurements, before adding unlabeled linear DNA duplex competitor DNA of the same sequence (1.58 μM). Ten or 11 time points were then taken for each sequence that was adjusted according to the half-life derived from initial experiments. The gels were run using the same conditions used for \( K_{D} \) experiments. Dried gels were quantified using a Fuji FLA 5000 phosphoimage, as previously described (5, 6). In experiments using full-site p53 REs, we quantified only the upper bound band, corresponding to a bound tetramer, because the lower bound band (a bound dimer) cannot be quantified accurately, because it has contributions from gel instabilities in the bound tetramer bands. \( F(t) \), the fraction of bound tetrameric complex at the different time points, was calculated from the equation \( F(t) = (P_{\text{bound}} \text{complex} (t))/(P_{\text{bound}} \text{complex} (t) + (P_{\text{bound}} \text{free} (t))) \), where \( P_{\text{bound}} \) is the photostimulated luminescence and \( bg \) is the background. In \( F(t)/F(0) \) was plotted as a function of time \( (t) \) after the addition of the unlabeled competitor. The data were fitted to a two-phase first-order kinetic equation \( F(t)/F(0) = A e^{-k_1 \cdot t} + B e^{-k_2 \cdot t} \), where \( A \) and \( B \) are fractions of molecules dissociating with rate constants \( k_1 \) and \( k_2 \), respectively. The half-life of complexes dissociating by the \( B \) process was calculated from \( t_{1/2} = \ln(2)/k_2 \). We obtained a better fit to a biphasic kinetic equation for experiments using full-site as well as half-site p53 REs.

Chromatin Immunoprecipitation (ChIP) Assay in Yeast. Yeast strains were grown in the presence of raffinose (no p53 induction) or with 0.024% galactose (p53 induction, as previously described in ref. 7). The chromatin immunoprecipitation assays using p53 DO-1 (Santa Cruz Biotechnology) and H3 ab1791 (Abcam) were performed as described in ref. 8 with minor modifications. Anti-mouse IgG (Santa Cruz) was used as negative control. Chromatin was sonicated to yield an average size of 300- to 500-bp DNA fragments, using a Misonix horn device following manufacturer’s recommendations (Osinica). After cross-link reversal, ChIPpied DNA fragments were detected by quantitative real-time PCR, using SYBR Green I and a 7900HT Sequence Detection System (Applied Biosystems). For ChIP-PCR, the immunoprecipitated DNA fragments were amplified in the presence of described primers, using REDTaq ReadyMix PCR Mix (Sigma) following the manufacturer’s recommendations. Products were analyzed on a 1.5% agarose gel. The primers used in this study were (5′ – 3′) ChIP 1-F: TTTGTATGGCGGAATT-GACTTT, ChIP 1-R: ACTAAAGTGCTGCGCCATC, and ChIP 3-R: TGAGAGTGTGGCCTG.

Jordan et al. www.pnas.org/cgi/content/short/1205971109
Transfections and Luciferase Assays in Human Cell Lines. pC53-SN3 plasmid coding for human p53 cDNA under the control of CMV promoter and the control vector pCMV-Neo-Bam were kindly provided by Bert Vogelstein (Johns Hopkins University, Baltimore, MD). Luciferase reporter constructs containing the desired p53 RE from selected sequences were constructed in pGL3-Promoter backbone (Promega) as previously described (9) and the identity of the inserts was confirmed by DNA sequencing. For luciferase assays p53 null SaOS2 cells were seeded in 24 wells and transfected using Fugene-6 reagent (Roche) according to manufacturer’s instructions with 200 ng of reporter constructs along with the indicated amount of p53 expression vector. pRL-SV40, a reporter plasmid coding for Renilla reniformis luciferase (Promega), was used as a control of transfection efficiency in the luciferase reporter assay. Total plasmid DNA per well was adjusted to an equal level by adding the empty vector pCMV-Neo-Bam. Forty-eight hours posttransfection extracts were prepared using the Dual Luciferase Assay System (Promega) following the manufacturer’s protocol and luciferase activity was measured on a Victor Wallac multilabel plate reader (PerkinElmer). Relative luciferases activities for each construct were defined as the mean value of the firefly luciferase/Renilla luciferase ratios obtained from three independent experiments performed in triplicate.

Immunoblot Analysis. Whole-cell extracts from SaOS2-transfected cells were quantified using the Bradford protein assay (BioRad). Equal amounts of whole-cell extracts were separated on 4–12% BisTris NuPAGE and transferred to polyvinylidene difluoride membranes (Invitrogen). The blots were probed with primary antibodies (Santa Cruz Biotechnology) for p53 (DO-1) and Actin (C-11). Bands were detected using horseradish peroxide-conjugated secondary antibodies (Santa Cruz Biotechnology) and the enhanced chemiluminescence (ECL) detection system (Amersham).

Fig. S1. Relationship of transactivation from p53 REs to different DNA-related characteristics at high vs. low p53 expression levels. (A) Transactivation at low p53 expression levels is not correlated to binding affinity of p53CT to p53 REs. (B) Transactivation at high p53 expression levels is not correlated to DNA torsional flexibility of p53 REs. Torsional flexibility calculations were carried out on both decameric parts of all p53 sequences examined, excluding spacer sequences; hence, the ranking of identical sites differing only in the spacer sequence is identical. For other details see Fig. 2 in the main text.

Fig. S2. Binding affinity measurements of p53DBD/REs complexes. DNA targets were imbedded in DNA hairpin constructs (concentration <0.1 nM). Upper bands show protein-bound DNA and lower bands show unbound DNA. The number below each gel is the concentration of p53DBD monomers active for DNA binding. The gels are representative examples of three to six independent experiments conducted with each site.
Characterization of Con-A RE supertransactivation response at low levels of p53 protein. (A) Low-level transactivation is p53 dependent. To ascertain whether the low-level transactivation from the Con-A sequence was dependent on p53, transactivation from Con-A RE was assessed for WT p53 (+) and a strain lacking p53 (−). Individual diploid colonies were grown overnight in YPDA medium, washed, diluted, and inoculated into synthetic medium containing 2% glucose or 2% raffinose, which corresponds to repressed and noninduced conditions, respectively. Overnight-growing cultures were harvested and transactivation was assessed with a quantitative luciferase assay. (B) Supertransactivation at basal levels of p53 requires a full-site RE. To assess whether WT p53 could maintain a low-level response with noncanonical REs, the ability of WT p53 to transactivate from a Con-A 1/2 site or derivation of the 3/4 site was compared with its ability to transactivate from the full-site RE. Arrows indicate a 1/4 site. (C) p53 tetramerization is required for the supertransactivation. To determine whether the low-level p53 response was due to a p53 dimer binding and transactivating from the Con-A sequence, several mutations that affected the ability of the protein to oligomerize were assessed for transactivation. 344A is a dimer protein, 344P is a monomer, and ΔTet eliminates the tetramerization domain (1–6). As determined with a luciferase assay, the three mutants were not able to function from the Con-A RE at any level of p53 analyzed. Presented in A–C are the mean and SEM of eight biological replicates.

Fig. S4. (A) p53 occupancies at Con-A and p21-5′ at low and high p53 levels. Occupancies of p53 at p21-5′ and Con-A REs were evaluated in yeast cells by ChIP PCR assay at low (raffinose) and high (0.024% galactose) levels of p53 protein after 24 h of growth. Yeast cells with either RE but lacking p53 (denoted “CORE”) were used as a control. Presented is a representative gel of two independent biological repeats with the PCR products of the Input DNA (input) and ChIP DNA (p53, IgG antibodies) obtained from two different sets of designed primers surrounding the p53 RE region (ChIP 1 and ChIP 3 primers; see SI Materials and Methods for sequences). ChIP with a histone-3 antibody served as an internal negative control. (B) Supertransactivation driven by Con-A RE in human cells. A luciferase reporter assay was assessed in human p53 SaOS2 cells. Luciferase reporter constructs containing p21-5′ and Con-A REs were cotransfected along with increasing amounts of the CMV-p53 WT expression vector. The AIP RE luciferase construct provided an internal control. At 48 h posttransfection, induction of the luciferase reporter was assessed. Relative luciferase activity was compared with the pGL3-P plasmid lacking the p53 RE (mock). Presented is the average and SD for three independent experiments.
Fig. S5. (Continued)
Fig. S5. Subtle defects of p53 missense mutations revealed by transactivation from Con-A. (A) Transactivation capacities of WT and mutant p53 were assessed from the Con-A RE, using the ADE2 plate color assay at decreasing levels of p53 induction (described below). (B) Transactivation from Con-A of "altered-function" breast cancer mutants (1) at varying levels of p53, using the ADE2 plate reporter assay (described below). (C) Transactivation capacities of several p53 missense mutations from the Con-A sequence were assessed with the luciferase assay as described below. Presented are the mean and SEM of four biological repeats.

Method: p53 transactivation assessment with a qualitative ADE2 color reporter system (2). Single-colony isolates of the p53-inducible RE-ADE2 reporter strains were streaked onto an YPDA control plate containing glucose and high levels of adenine and grown to equivalent amounts at 30 °C. The plates were then replica-plated onto a series of nine experimental plates containing selective media with low levels of adenine (5 mg/L), 2% raffinose, and various amounts of galactose (0, 0.001, 0.002, 0.004, 0.008, 0.016, 0.032, 0.064, and 0.128%). Transactivation capacities for the p53 mutants were determined after 3 d at 30 °C by the ability of the mutant to produce a change in colony pigmentation. Transactivation of the ADE2 gene, which is a direct readout of p53 interaction with the specific RE, results in large white colonies where decreased or loss of transactivation of the ADE2 results in pink and red colonies, respectively. Colony pigmentation was scored on a scale of 1–5, where 1 is no apparent transactivation (red colonies) and 5 is strong transactivation (white colonies).

Method: p53 transactivation assessment with the quantitative luciferase reporter system (construction described in ref. 2). Individual colonies of cells with the p53-inducible RE reporter (2) were inoculated into 5 mL rich media, YPDA plus adenine (200 mg/L), and grown overnight at 30 °C with shaking. The overnight culture was diluted 1:50 in H2O. For each measurement, 1 mL of the diluted culture was spun down, washed of residual glucose with H2O, and resuspended in 2 mL synthetic complete-LYS media (plus 2% raffinose or raffinose supplemented with increasing amounts of galactose (0, 0.002, 0.004, 0.008, 0.010, 0.012, 0.016, 0.020, 0.024, 0.028, or 0.032%). These cultures were grown overnight (~18 h) at 30 °C to ~2–4 x 10^7/mL (late log to early stationary). The 2-mL cultures were spun down and the supernatant was aspirated. The remaining pellet was resuspended in 100 μL reporter lysis buffer (Promega) and an equivalent amount of 425–600 μm acid-washed glass beads was added (Sigma). Samples were homogenized for 30 s in the Biospec Products minibead beater, briefly incubated on ice, and spun for 20 min at 16,000 × g relative centrifugal force in an Eppendorf 5415R centrifuge to separate the soluble protein fraction. The standard protocol recommended by the manufacturer (Promega) was performed for the luciferase assay system, starting with 10 μL of protein extract. Luciferase activity was measured from 96-well, white optiplates (Perkin-Elmer) in a Wallac Victor2 multilabel counter (Perkin-Elmer). Light units were standardized per microgram protein as determined by a Bio-Rad protein assay.

Fig. S6. Dissociation kinetics measurements of p53DBD/REs complexes. (A) Representative EMSA gels showing dissociation of p53DBD from p53 full-site binding sequences. (B) Representative EMSA gels showing dissociation of p53DBD from p53 half-site (HS) binding sequences. DNA targets were embedded in hairpin constructs (0.4 nM) and incubated with 270 nM or 160 nM p53DBD (for half-site and full-site DNA sequences, respectively). The number below each gel denotes the time after adding linear double-stranded competitor DNA (1.58 μM).
Table S1. Number of p53 molecules per cell at different levels of galactose in a diploid yeast cell reporter system

<table>
<thead>
<tr>
<th>% galactose</th>
<th>p53 molecules per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>~300</td>
</tr>
<tr>
<td>0.004</td>
<td>460</td>
</tr>
<tr>
<td>0.008</td>
<td>5,500</td>
</tr>
<tr>
<td>0.012</td>
<td>37,800</td>
</tr>
<tr>
<td>0.016</td>
<td>24,000</td>
</tr>
<tr>
<td>0.024</td>
<td>33,500</td>
</tr>
</tbody>
</table>

A diploid strain containing GAL1::WT p53 and the p21-5′RE-luciferase reporter was grown overnight in synthetic complete medium, diluted, washed, and inoculated into 2 mL selective medium containing either raffinose (basal expression) or raffinose plus 0.002–0.024% galactose to induce p53 for 24 h. The titer was measured and protein concentrations in lysates were determined with a standard Bradford assay (BioRad), to estimate total protein per cell. The level of p53 was estimated by Western immunoblot analysis (DO-1 and pAb1801 antibodies; Santa Cruz Biotechnology) by comparing to known amounts of p53 (10, 20, and 40 ng) (BD Biosciences Phar-mingen). To estimate the amount of p53 per microgram of total cell lysate, pixel values were measured from autoradiographs of increasing exposure lengths. The measured pixel values were calculated for samples and compared with pixel values obtained from known amounts of purified p53 (10, 20, and 40 ng). The total pixel value was the sum of pixels for the predominant p53 band that runs at 53 kDa and possible degradation products detected by the antibodies. The pixel sum enabled a direct assessment of p53 molecules per cell. Assuming that the radius of an average yeast cell is between 2 and 4 μm, the amount of p53 molecules in our rheostable system (from 300 to 33,500) corresponds to 0.4–40 nM within the cell. Specific data including gels and autoradiogram measurements are available upon request. Note that for human cells, measurements of basal levels of p53 extend from 17,000 to 200,000 molecules (~60–500 nM), depending on the cell line (1–3). Even for low p53 levels, a significant fraction of p53 protein can be found bound to selected sites (for example, p21/CDKN1A promoter) under basal conditions (4). The basal p53 levels are similar to those of other transcription factors such as estrogen receptor, Myc, and NF-κB that also range from 10,000 to 300,000 molecules per nucleus (5). Depending on cell type and treatment, p53 levels can increase up to 20-fold after activation by stress signals (2, 6).

Table S2. p53 response element sequences examined in this study

<table>
<thead>
<tr>
<th>p53 RE</th>
<th>RRCCWWGGYYY*</th>
<th>Spacer</th>
<th>RRCCWWGGYYY*</th>
<th>Source</th>
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<td>Con-A</td>
<td>GGGCATGTCC</td>
<td></td>
<td>GGGCATGTCC</td>
<td>(1)</td>
</tr>
<tr>
<td>Con-A + 1 nt</td>
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<td>c</td>
<td>GGGCATGTCC</td>
<td>This study</td>
</tr>
<tr>
<td>Con-A + 2 nt</td>
<td>GGGCATGTCC</td>
<td>cc</td>
<td>GGGCATGTCC</td>
<td>This study</td>
</tr>
<tr>
<td>Con-A + 5 nt</td>
<td>GGGCATGTCC</td>
<td>cccctg</td>
<td>GGGCATGTCC</td>
<td>This study</td>
</tr>
<tr>
<td>Con-B</td>
<td>GGGCATGTCC</td>
<td></td>
<td>GGGCATGTCC</td>
<td>(1)</td>
</tr>
<tr>
<td>Con-C</td>
<td>GGGCATGTCC</td>
<td></td>
<td>GGGCATGTCC</td>
<td>(1)</td>
</tr>
<tr>
<td>GAGA</td>
<td>GGGCATGTCC</td>
<td></td>
<td>GGGCATGTCC</td>
<td>(2)</td>
</tr>
<tr>
<td>GGG + 2 nt</td>
<td>GGGCATGTCC</td>
<td>gc</td>
<td>GGGCATGTCC</td>
<td>(2)</td>
</tr>
<tr>
<td>AGG</td>
<td>AGGGAGTCTCT</td>
<td></td>
<td>AGGGAGTCTCT</td>
<td>(2)</td>
</tr>
<tr>
<td>GGA</td>
<td>GGGCATGTCC</td>
<td></td>
<td>GGGCATGTCC</td>
<td>(2)</td>
</tr>
<tr>
<td>GGA + 2 nt</td>
<td>GGGCATGTCC</td>
<td>gc</td>
<td>GGGCATGTCC</td>
<td>(2)</td>
</tr>
<tr>
<td>Con-J</td>
<td>GGGCATGTCC</td>
<td></td>
<td>GGGCATGTCC</td>
<td>(3)</td>
</tr>
<tr>
<td>Con-K</td>
<td>GGGCATGTCC</td>
<td></td>
<td>GGGCATGTCC</td>
<td>(3)</td>
</tr>
<tr>
<td>14-3-3 B51</td>
<td>AGGCATGTCC</td>
<td>cAnCAGTCCC</td>
<td>(4)</td>
<td></td>
</tr>
<tr>
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<td>tAGCAttAGCCC (not a decamer)</td>
<td>AGGCATGTCC</td>
<td>(4)</td>
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<tr>
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<td>tctCTTGGCC</td>
<td></td>
<td>GGGCTTGGTCg</td>
<td>(5, 6)</td>
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<tr>
<td>BaxA</td>
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<td>g</td>
<td>AGGCACAGCTt</td>
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<tr>
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<td>GGGACCAGCCt</td>
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<td>GADD45</td>
<td>GAAATGCTCC</td>
<td></td>
<td>AACATGCTGt</td>
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</tr>
<tr>
<td>MDM2 R1</td>
<td>GCTCAAGTCCg</td>
<td>c</td>
<td>GGGACATGCTt</td>
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</tr>
<tr>
<td>MDM2 R2</td>
<td>GAGCTAAGTCt</td>
<td>c</td>
<td>tGGCATGCTT</td>
<td>(7)</td>
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<td>MMP2</td>
<td>AGCAAGCCTC</td>
<td></td>
<td>GACTGTTCt</td>
<td>(5, 6)</td>
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<td>Noxa</td>
<td>AGGGCTGGCC</td>
<td></td>
<td>cGGGAGTCTg</td>
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</tr>
<tr>
<td>p21-3'</td>
<td>GAAGAGAaCT</td>
<td></td>
<td>GGGCATGTCT</td>
<td>(5, 6)</td>
</tr>
<tr>
<td>p21-5'</td>
<td>GAAATGCTCC</td>
<td></td>
<td>cAGCATGTt</td>
<td>(5, 6)</td>
</tr>
<tr>
<td>p53R2</td>
<td>tGACATGCTC</td>
<td></td>
<td>AGGCATGTCT</td>
<td>(5, 6)</td>
</tr>
<tr>
<td>PA26</td>
<td>GGAACATGCT</td>
<td></td>
<td>AGGCATGTCT</td>
<td>(5, 6)</td>
</tr>
<tr>
<td>PCNA</td>
<td>GGAACATGCT</td>
<td></td>
<td>GGGCTTGGTC</td>
<td>(5, 6)</td>
</tr>
<tr>
<td>PIDD</td>
<td>AGGCAATGCTc</td>
<td>gctgctg</td>
<td>GGGCATGTCT</td>
<td>(5, 6)</td>
</tr>
<tr>
<td>PUMA B52</td>
<td>tGCAATGCTC</td>
<td></td>
<td>tGGCATGCTC</td>
<td>(5, 6)</td>
</tr>
<tr>
<td>Rad51</td>
<td>AAACATGCG</td>
<td></td>
<td>AATCAACGCT</td>
<td>(5, 6)</td>
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*Bases in uppercase letters agree with the published consensus sequence.


Table S3. Binding affinity of p53DBD/DNA complexes examined in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence*</th>
<th>Dimer</th>
<th>Averaged dimer equivalent tetramer</th>
<th>Cooperativity constant(^{1,2})</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>Con-A (Con1)</td>
<td>cGGGCAATGCTCCGGGCAATGCTCtg</td>
<td>314 (14)</td>
<td>64 (4)</td>
<td>25 (2)</td>
<td></td>
</tr>
<tr>
<td>GGG (Con4)</td>
<td>cGGGCAATGCTCCGGGCAATGCTCtg</td>
<td>378 (22)</td>
<td>72 (6)</td>
<td>29 (3)</td>
<td></td>
</tr>
<tr>
<td>GGG + 2 nt</td>
<td>cGGGCAATGCTCGGGCAATGCTCtg</td>
<td>249 (15)</td>
<td>134 (13)</td>
<td>3.6 (0.5)</td>
<td></td>
</tr>
<tr>
<td>GGA (Con5)</td>
<td>cGGGCAATGCTCCGGGCAATGCTCtg</td>
<td>175 (18)</td>
<td>36 (4)</td>
<td>23 (1)</td>
<td></td>
</tr>
<tr>
<td>GGA + 2 nt</td>
<td>cGGGCAATGCTCGGGCAATGCTCtg</td>
<td>188 (17)</td>
<td>101 (9)</td>
<td>3.6 (0.5)</td>
<td></td>
</tr>
<tr>
<td>AGG (Con6)</td>
<td>cGGGCAATGCTCGGGCAATGCTCtg</td>
<td>647 (94)</td>
<td>88 (11)</td>
<td>57 (11)</td>
<td></td>
</tr>
<tr>
<td>p21-3'</td>
<td>GGGCAATACGCTCCAGAATGCTGAG</td>
<td>310 (25)</td>
<td>34 (1)</td>
<td>83 (12)</td>
<td>This work</td>
</tr>
<tr>
<td>PUMA B52</td>
<td>CTGGCAATGCTCCAGAATGCTCtg</td>
<td>695 (96)</td>
<td>109 (11)</td>
<td>50 (3)</td>
<td>This work</td>
</tr>
<tr>
<td>p3SAIP1</td>
<td>cCTGGCTCCAGGCGGCAATGCTCtg</td>
<td>30 (6) μM(^1,5)</td>
<td>480 (70)</td>
<td>~3,800 (450)</td>
<td>This work</td>
</tr>
</tbody>
</table>

*Alternate half sites are in boldface type and underlined. Nonspecific sequences outside the 20-bp REs of consensus-related sites and in spacer sequences are in italics.
\(^{1}\)The values in parentheses are the SEM. For details see Beno et al. (2) and SI Materials and Methods.
\(^{2}\)Defined in SI Material and Methods.
\(^{3}\)The values are an average of three to seven independent experiments conducted with each sequence.
\(^{4}\)K\(_D\) estimated by assuming zero values for the unobserved dimer bands. See SI Materials and Methods for details.
\(^{5}\)From Beno et al. (2).
\(^{6}\)The sequences are from Kitayner et al. (1) and are analyzed separately here for dimers and tetramers.

http://www.pnas.org/cgi/content/short/1205971109
Table S4. Binding stability of p53DBD/DNA complexes examined in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence*</th>
<th>“A” fraction†</th>
<th>“B” fraction†</th>
<th>t_{1/2} of fraction B (min)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGG (Con4)</td>
<td>cGGGCAATGCCGGCCATGCCCTg</td>
<td>0.36 (0.05)</td>
<td>0.64 (0.05)</td>
<td>12 (2)</td>
</tr>
<tr>
<td>Con-A (Con1)</td>
<td>cGGGCAATGCCGGCCATGCCCTg</td>
<td>0.52 (0.03)</td>
<td>0.48 (0.03)</td>
<td>12 (2)</td>
</tr>
<tr>
<td>GGA (Con5)</td>
<td>cGGACAATGGCCGGCCATGCCCTg</td>
<td>0.60 (0.02)</td>
<td>0.40 (0.02)</td>
<td>5.1 (1)</td>
</tr>
<tr>
<td>p21-5</td>
<td>cGGACAATGGCCGGCCATGCCCTg</td>
<td>0.45 (0.03)</td>
<td>0.55 (0.03)</td>
<td>5.2 (0.8)</td>
</tr>
<tr>
<td>GGA HS‡</td>
<td>tggttgGAGCCATGCCTgggtg</td>
<td>0.79 (0.03)</td>
<td>0.21 (0.03)</td>
<td>6 (1)</td>
</tr>
<tr>
<td>Con-A HS‡</td>
<td>tggttgGAGCCATGCCTgggtg</td>
<td>0.71 (0.02)</td>
<td>0.29 (0.02)</td>
<td>24 (6)</td>
</tr>
<tr>
<td>GGA HS‡</td>
<td>tggttgGAGCCATGCCTgggtg</td>
<td>0.75 (0.01)</td>
<td>0.25 (0.01)</td>
<td>25 (2)</td>
</tr>
</tbody>
</table>

*Alternate half sites are in boldface type and underlined. Nonspecific sequences outside the specific consensus sites are in italics.

†The data were fitted to a two-phase first-order kinetic equation: \( F(t)/F(0) = Ae^{-kt_1} + Be^{-kt_2} \). \( F(t) \) is the fraction of bound tetrameric complex at the different time points and \( F(0) \) is the fraction of bound tetrameric complex at time 0. \( A \) and \( B \) are the fractions of molecules dissociating with rate constants \( k_1 \) and \( k_2 \), respectively. The half-life of complexes dissociating by the \( B \) process was calculated from \( t_{1/2B} = \ln 2/k_2 \). The values are an average of four to nine independent experiments conducted with each sequence; the values in parentheses are the SEM. We obtained a better fit to a biphasic kinetic equation for experiments, using full-site as well as half-site p53 REs.

‡HS is half site, i.e., only one specific dimeric binding site.