

COMMENTARY

p53 protein at the hub of cellular DNA damage response pathways through sequence-specific and non-sequence-specific DNA binding

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Our environment contains physical, chemical and pathological agents that challenge the integrity of our DNA. In addition to DNA repair, higher multicellular organisms have evolved multiple pathways of response to damage including programmed cell death—apoptosis. The p53 protein appears to sense multiple types of DNA damage and coordinate with multiple options for cellular response. The p53 protein activities depend upon its DNA binding. Specific p53 protein post-translational modifications are required for efficient sequence-specific binding and transcriptional activities. Non-sequence-specific DNA binding may involve a wide spectrum of p53 proteins and predominate as DNA damage is more severe or p53 protein is more highly induced. p53 protein is not strictly required for DNA damage sensing and repair. Rather, p53 protein may govern an apoptosis checkpoint through competition with DNA repair proteins for non-sequence-specific binding to exposed single-stranded regions in the DNA duplex. This model provides a framework for testing mechanisms of p53-mediated apoptosis dependent upon the p53 protein modification state, the level of p53 protein accumulation, the level of DNA damage and the capacity of the damaged cell to repair.

p53 protein is central to the cellular response of higher multicellular organisms to a variety of potentially damaging extracellular stimuli, including UV light, γ -irradiation, chemical carcinogens and chemotherapeutic agents. DNA repair machinery has evolved to maintain genomic integrity and stability after various kinds of DNA damage. Nucleotide excision repair (NER) eliminates pyrimidine dimers caused by UV light. Base excision repair (BER) targets base modifications caused by DNA hydrolysis and alkylation. Mismatch repair corrects errors of DNA replication. DNA strand breaks are remedied by DNA recombination using unbroken alleles as templates. The cell cycle checkpoint is a surveillance mechanism that coordinates with DNA repair by delaying progress through the cell cycle following DNA damage (1). This delay allows time for cells to repair DNA before replication or segregation of defective chromosomes, thus preventing propagation of heritable genetic errors. In prokaryotes, DNA damage blocks cell division by activating the 'SOS' feedback control system (2) that prevents cell division

Abbreviations: BER, base excision repair; CAK, cyclin-activated kinase; dsDNA, double-stranded DNA; NER, nucleotide excision repair; RPA, replication protein A; ssDNA, single-stranded DNA; sds-DNA, single-stranded/double-stranded DNA transition.

and increases the capacity to repair damaged DNA. In yeast, an increased genetic instability following γ -irradiation can be seen in mutants of *rad9* that have a defect in the G₂ checkpoint (3). In addition to cell cycle checkpoints, apoptosis evolved in multicellular organisms as a mechanism to ensure the integrity of the organism by eliminating cells with irreparable DNA damage. In this article, we will review the sequence-specific and non-sequence-specific DNA binding of p53 protein and comment on how p53 protein, as the guardian of the genome, might orchestrate the cellular response to DNA damage in concert with the DNA repair machinery in order to select among multiple effector pathways toward DNA repair, survival or apoptosis.

p53 as a guardian of the genome

The critical role of the p53 gene in maintaining the integrity of the genome is evident in that p53 is the most commonly altered gene in human cancer, with a mutation frequency exceeding 50% (4). Most mutations are missense mutations within the evolutionarily conserved DNA binding domain (5). Mutation of the p53 gene results not only in loss of p53 function but also in gain of oncogenic functions (6) and in adoption of a dominant-negative conformation able to inactivate the protein product of the normal allele through heterotetramerization (7). Germline mutations of p53 have been found in Li–Fraumeni syndrome, an inherited disorder with a high risk of developing a variety of cancers at an early age (8). The impact of p53 alterations on tumorigenesis is considerably more than the statistics for p53 gene mutation indicate, as wild-type p53 may be functionally inactivated by other mechanisms. The p53 protein can be inactivated by viral oncogenes and defects in the p53 activation pathway. Most DNA tumor viruses have evolved their own mechanisms in order to gain advantage to replicate their genome. E6 protein from human papillomavirus (HPV) in cervical carcinoma degrades p53 through ubiquitin-mediated proteolysis (9). Hepatitis B viruses (HBV) involved in the pathogenesis of 90% of human hepatocarcinoma encode HBXAg, which binds to the N-terminal domain of p53 and inactivates its transactivation activity (10). SV40 viral T antigen abolishes DNA binding activity of p53 by associating with the DNA binding domain of p53 (11), and adenoviral E1B protein inhibits transactivation activity of p53 protein (12). Although SV40 and adenovirus transform primarily rodent cells, they exemplify mechanisms by which cellular proteins yet to be recognized might inactivate p53 in human cancer.

Research over the last decade reveals that p53 protein is dispensable for normal development but is pivotal in cellular response to DNA damage. p53 activity is tightly controlled at negligible levels in normal cells. p53 protein is rapidly induced by DNA damage stimuli such as ionizing irradiation (13), UV light (14) and ribonucleotide depletion (15). The induction of p53 is achieved through a post-translational mechanism that reduces p53 turnover. Induced p53 functions as a transcription

factor for downstream genes that function in pathways of cell cycle regulation, apoptosis and DNA repair. Transactivation of the inhibitor of cyclin-dependent kinase (cdk) *p21* is one of the better understood mechanisms of p53 in response to DNA damage (16). In addition to cdk inhibition, p21 binds to proliferating-cell nuclear antigen (PCNA). The binding preferentially inhibits the processivity of DNA polymerase in DNA replication but not in DNA repair, which may contribute to coordinating growth arrest and DNA repair in the S phase (17).

So far, more than 20 p53 downstream genes have been identified (18). They are involved in diverse cellular activities, such as *p21* in G₁ growth arrest, 14-3-3 σ in a G₂/M checkpoint, BAX and p53-induced genes (PIGs) in apoptosis, and GADD45 and XPE in DNA repair. The common feature of these downstream genes is that they contain one or more p53 consensus binding sites in their regulatory regions. The p53 consensus binding site contains two or more copies of a 10 bp half-site 5'-PuPuPuC(A/T)(T/A)GpyPyPy-3' (19). p53 protein binds to the complete consensus site as a tetramer. Crystallographic analysis of p53 protein binding to its consensus DNA revealed that four evolutionarily conserved regions within the DNA binding domain directly contact the major and minor grooves of the p53 consensus DNA (20). The residues that are most frequently mutated in human cancers make critical contributions to DNA binding by directly contacting DNA or fostering conformation to support DNA binding. That >90% of p53 mutations are located within the sequence-specific DNA binding domain (5) indicates the importance of p53 DNA binding in response to DNA damage. Therefore, it is important to understand how p53 binding to DNA is regulated.

Regulation of p53 sequence-specific binding to DNA

Covalent modification

p53 protein induction through post-transcriptional modification in response to DNA damage is a mechanism that permits rapid activation and avoids relying on transcription from a potentially damaged DNA template. Both the N- and C-terminal domains of the p53 protein are subjected to extensive covalent modifications such as phosphorylation, acetylation and sumoylation, as shown for human p53 in Table I. These modifications contribute to p53 regulation by affecting its DNA binding, degradation, localization, oligomerization and association with cellular factors. Since there are many excellent reviews about p53 modifications (21), we will focus mainly on modifications that regulate p53 DNA binding. Phosphorylation of S378 by PKC enhances p53 binding to DNA similarly to the C-terminal p53 activating antibody PAb421 (22). Phosphorylation of S392 is correlated with increased DNA binding, probably by facilitating p53 tetramerization. While phosphorylations of C-terminal amino acids generally enhance p53 sequence-specific binding to DNA, there are conflicting results. Phosphorylation of S315 by cdk enhances p53 binding to specific DNA and confers binding site preference (23). However, dephosphorylation of this site reportedly can facilitate tetramerization and nuclear localization, which are critical for p53 function (24). It remains to be tested whether phosphorylation of S376 is correlated with DNA binding regulation. Enhanced p53 binding to specific DNA has been associated with phosphorylation of S376 by cyclin-activated kinase (CAK) in one report (25), and with 14-3-3 σ binding to p53, which is dependent upon de-

phosphorylation of S376, in another report (26). These studies suggest that p53 modification is a complex process involving multiple enzymes acting in sequence. Although not a direct effect, p53 DNA binding can be increased by phosphorylation of N-terminal amino acids (27). Phosphorylation of S15 and S37 recruits p300/CBP protein that acetylates lysines 373 and 382 at the C-terminus, resulting in activation of p53 binding to specific DNA (28). Phosphorylation of S15 and S37 by Chk1 and Chk2 is heavily dependent upon tetramerization of p53 (29). Tetramerization of p53 protein is facilitated by phosphorylation of p53 at S392 and dephosphorylation at S315 (24). Regulation of p53 at multiple levels provides a means for p53 protein to select among downstream events in response to diverse genotoxic stress signals. Testing this cascade of events in cells responding to DNA damage is a major ongoing challenge. An even greater challenge is to determine how p53 modifications might confer specificity in transactivation of 20 or more p53 downstream genes. The DNA sequence variation in the p53 binding sites in these genes and the complexity of possible p53 modifications provide a mechanism for specificity in relaying signals from different genotoxic and oncogenic pathways to different downstream genes.

p53-associated factors

Although the DNA binding domain of p53 binds well to specific DNA (30), the binding is highly subject to regulation by other regions of p53 protein. It has been hypothesized that p53 DNA binding is subject to allosteric regulation by C-terminal domains that lock p53 tetramers in a latent state for DNA binding (31). Such inhibition can be overcome by C-terminal modifications other than phosphorylation, such as proteolytic truncation, alternative splicing and association with other cellular factors (32). The potential for p53 regulation by p53-associated factors has been long implicated from *in vitro* DNA binding activation by PAb421, which recognizes C-terminal amino acids 372–381. A number of cellular and viral proteins have been identified as p53-associated factors. While the biological relevance of these associations remains to be verified, several cellular factors have been shown to bind to the C-terminal region of p53 and activate DNA binding activity. 14-3-3 σ is a p53 downstream gene that mediates G₂ arrest by sequestering phosphorylated cdc25C (33). Upon dephosphorylation of S376, 14-3-3 σ binds to p53 and enhances its DNA binding activity, thus forming a positive feedback loop for p53 (26). c-Abl is a non-receptor tyrosine kinase that is activated by DNA damage and mediates phosphorylation of JNK and p38 MAPK (34,35). Both JNK and p38 MAPK are kinases that phosphorylate p53 in a DNA damage-dependent manner. Recently, Nie *et al.* reported that c-Abl is capable of binding to p53 and stabilizing its DNA binding (36). While activation of p53 DNA binding by c-Abl is independent of its kinase activity, the direct activation of p53 DNA binding may synergize with the c-Abl kinase cascade-mediated p53 activation and amplify the response to DNA damage. Although the N-terminal domain of p53 is frequently targeted for transcriptional repression by cellular and viral oncogenes (12,37,38), there is evidence that the N-terminal association may participate in regulation of p53 DNA binding in other ways. Replication protein A (RPA) is a single-stranded DNA (ssDNA) binding protein that binds to the N-terminus of p53. An *in vitro* study by Miller *et al.* showed that association with RPA blocks p53 binding to specific DNA (39). The association between RPA and p53 can be disrupted with

Table I. p53 protein post-translational modifications and effects

Modification	DNA damage	Enzyme	Function	Reference
Ser15-P	γ and UV	ATM, ATR	p300 binding	(111,112)
The18-P	γ and UV	CK-1	Mdm2 association	(113)
Ser20-P	γ and UV	Chk2	Mdm2 association	(114)
Ser33-P	γ and UV	p38	Apoptosis	(115)
Ser33-P	γ and UV	CAK	Acetylation	(28,110)
Ser37-P	γ and UV	ATR	Acetylation	(28,111)
Ser46-P	UV	p38	Apoptosis	(115)
Thr81-P	ROS	JNK	Apoptosis	^a
Ser315-P	NA	cdk	DNA binding	(23)
Ser315_P	NA	Cdc14 phosphatase	Nuclear localization	(116)
K-320-Ac	γ and UV	PCAF	DNA binding	(28)
K373-Ac	γ and UV	CBP/p300	DNA binding	(117)
Ser376_P	IR	dephosphorylation	Binding to 14-3-3 σ	(26)
Ser376-P	NA	CAK	DNA binding	(25)
Ser378-P	NA	PKC	PAb421 like activation	(22)
K382-Ac	γ and UV	CBP/p300	DNA binding	(28)
K386-Sumo	UV	E1 and hUbc9	Stabilization	(118,119)
Ser392	UV	CKII, p38	Tetramerization	(24,120)

The DNA damaging agents used are listed but do not imply specificity which may vary with cell type. Positions of amino acids in human p53 are shown. ' _P', dephosphorylation.

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ssDNA or DNA repair-dependent phosphorylation of RPA (40). While C-terminal associations are generally linked with enhanced DNA binding, the outcomes of the N-terminal interactions are less consistent. Zauberman *et al.* found that Mdm2 protein association abolishes p53 binding to specific DNA in cell lysates as determined by means of the McKay assay (41). However, the complex of p53–DNA–Mdm2 was detected with purified recombinant proteins by EMSA (42). Nevertheless, the authenticity and biological relevance of these associations need to be evaluated in the context of the general transcription machinery. p53 is associated with several general transcription factors including TFIID, TFIIF and p300/CBP (43–45). These transcription factors form multi-protein complexes that span the N- and C-terminal domains of the p53 protein. For example, the p62 polypeptide component of TFIIF binds to the p53 N-terminus, whereas XPD and XPB of TFIIF bind the C-terminus. It has been found that p53 DNA binding is stabilized by the association with TFIID and p300/CBP. The association between p53 and these general transcription factors may more closely reflect the nature of p53 regulation in the chromatin context.

Chromatin architecture

Unfolding chromatin structure is required for transcription. Histone acetyltransferase activity is associated with a number of transcriptional activators, such as p300/CBP (CREB binding protein), PCAF (p300/CBP associated factor) and TAFII250 (TATA binding protein associated factor) (46). In addition to acetylating p53 protein at lysine 373 and 382 (28), binding to N-terminally phosphorylated p53 could position p300/CBP to open up nucleosomes for recruitment of other transcription factors to promoters of p53 downstream genes. p53 binding sites in p53 downstream genes vary in position relative to the promoter. Two p53 binding sites in the WAF-1 gene are located between 2 and 3 kb upstream of the transcription start site (16). Two p53 binding sites are separated by 17 bp and located 1.5 kb downstream of the start site within the first intron of the Mdm2 gene (47). p53 binding sites in the cyclin G gene are separated by a half kilobase interval in the first exon (48). DNA loop formation was described as a mechanism of

transactivation mediated by the stacking of tetramers through the central domain of p53 protein (49). Recently, we have observed active DNA binding by p53 protein in the absence of PAb421 for the endogenous Mdm2 p53 binding sequence (two p53 binding sites separated by 17 bp) and for that sequence in which the two Mdm2 p53 binding sites were replaced with Waf-1 sites (121 and unpublished data). Moreover, p53 binding to the double sites could not be fully competed by either single site. p53 binding to DNA, like that of other DNA binding proteins, represents a dynamic on/off state (50). The chance of dissociating two p53–DNA complexes simultaneously is much lower than that for single binding. Therefore, the formation of a multi-p53–DNA complex may provide a stable scaffold for transcription machinery as well as enhance the complexity for p53 regulation.

p53 binding to DNA is affected not only by DNA structure but also by chromatin and nuclear proteins. High mobility group protein-1 (HMG-1) is a non-histone chromosomal protein that has been shown to activate p53 DNA binding (51), perhaps mediated by DNA bending that stabilizes p53–DNA complex formation. Unlike other p53 activators, HMG-1 is not present in the p53–DNA complex. HMG-1 may allow p53 to overcome an energy barrier to bend DNA in an intermediate step of p53 binding to DNA. DNA bending is a common mechanism for transactivation. It helps not only the formation of stable DNA–protein complexes but also the assembly of transcription complexes in the chromatin context. However, our understanding of p53 regulation at the chromatin level is largely limited to speculations from *in vitro* studies and will depend upon new development in technology for *in vivo* evidence. Recently, Rubbi and Milner provided evidence that p53 is preferentially localized at sites of active RNA synthesis (52). p53 protein also associates with the centrosome, first shown by Brown *et al.* (53). Deppert found that mutant p53 protein anchors to matrix associated regions (MAR elements) (54), but so far no one has demonstrated wild-type p53 associations with nuclear matrix or chromatin *in situ*. With more powerful analytical microscopy and image analysis, p53 localization and associations in cells will be further defined and contribute to a better understanding of p53 regulation and activity.

Redox modification

Hainaut and Milner have shown that specific DNA binding of p53 is highly dependent upon the reduction state of p53 protein (55). p53 protein has a unique zinc finger that is composed of C176, H179, C238 and C242. The zinc finger forms a DNA binding interface by bridging two loop-helix structures in the DNA binding domain. There are an additional seven cysteine residues involved in direct DNA binding and conformation in the DNA binding domain. Mutations of these cysteine residues result in the loss of p53 DNA binding activity (56). The presence of cysteine residues in these critical positions of p53 protein renders it susceptible to regulation by cellular redox status. p53-specific binding to DNA requires a thiol reducing agent like dithiothreitol (DTT). Oxidation of p53 by thiol oxidants like diamide abolishes p53 binding to specific DNA. A similar effect can be achieved by metal chelation since zinc binding is correlated with thiol reduction. Considering oxidative stress caused by DNA damage and other genotoxic stimuli, it is essential for the cell to maintain a reductive state for p53 function. The cellular redox state is maintained in a highly efficient manner by antioxidant molecules and enzymes like glutathione, thioredoxin and superoxide dismutase. In addition to scavenging oxidants, they participate in transcription regulation in response to oxidative stress. For example, thioredoxin as a thiol reducing molecule is capable of stimulating DNA binding activity of various transcription factors like NF κ B (57) and AP-1 (58). More recently, thioredoxin has been shown to enhance p53 DNA binding and transactivation (59). Further, p53 transcriptional activity is suppressed in yeast strains with thioredoxin reductase mutation or deletion (60,61). More interestingly, p53 binding to specific DNA is activated by Ref-1, a dual functional protein that serves as an A/P endonuclease in BER and modulates DNA binding of various transcription factors in response to oxidative stress (62). Since DNA damage is associated with the production of oxygen free radical species, the regulation of p53 by Ref-1 may provide a mechanism to coordinate DNA repair and redox states. In addition to being modulated by redox proteins, p53 participates in redox metabolism by transactivation of reducing molecules like glutathione peroxidase and repression of oxidating molecules like nitric oxide synthase (63,64). Accumulating evidence suggests that the p53 pathway integrates signals not only from DNA strand breaks but also from oxidative stress pathways.

Single-stranded DNA

In addition to activation by the above mechanisms, ssDNA stimulates p53 binding to specific DNA. Jayaraman and Prives found that p53 binding to the GADD45 p53 binding site is enhanced by ssDNA detected by a DNase I protection assay (65). It is even more interesting that only short oligonucleotides, up to 40 bases in length, are capable of activating p53 sequence-specific DNA binding. Such activation can be completely reversed by increasing the length of the oligomer to 66 bases. p53 protein has been found to bind ssDNA and facilitate its annealing and strand transfer (66). Deletion analysis revealed that the C-terminal domain of p53 protein binds to the end of the DNA strand, whereas the central domain contributes to binding the internal segment of ssDNA (67). The inhibitory effect of the long oligonucleotides may be attributed to an ability to bind the central domain, therefore competing with specific DNA binding (68). The binding site for ssDNA has been mapped to residues 361–382 of p53 protein, including the region of p53 activated by Pab421

binding and S378 phosphorylation. These data imply that removal of negative control imposed by the C-terminal domain is the mechanism by which ssDNA binding activates p53.

ssDNA segments are the by-products or intermediates of DNA repair and DNA replication. Production of ssDNA by 5' to 3' exonuclease at the end of a DNA strand break is the initial step to repair double-stranded DNA (dsDNA) breaks by homologous recombination (69). ssDNA gaps are also formed after excision of damaged DNA during NER and BER. This ssDNA is vulnerable to nuclease attack and active for DNA recombination. Therefore, it is well protected by ssDNA binding proteins in both DNA repair and replication. Accumulation of ssDNA is evident in cells subjected to DNA damage. Considerable evidence suggests that ssDNA mediates the SOS response in bacteria and cell cycle checkpoints in yeast (70). Activation of p53 by ssDNA seems to be an attractive model for p53 to coordinate cellular response to DNA damage. However, it is almost impossible for p53 activated at the damage site to transactivate distant downstream genes, and the short ssDNA generated by excision repair is rapidly degraded in the cell. A more plausible model for ssDNA binding effects on p53 protein may be activation of p53 protein for enhanced non-sequence-dependent binding to adjacent dsDNA.

p53 non-sequence-specific binding to DNA and biological relevance

In addition to binding specifically to DNA at p53 consensus sites, p53 binds non-specifically to DNA. Besides ssDNA noted above, p53 is capable of binding to a DNA duplex with free ends, nicked DNA generated by DNase I, DNA damaged by γ -irradiation (71), a DNA duplex with insertion-deletion lesion (IDL) mismatches (72), DNA with Holliday junctions (73), triple-stranded DNA (74) and DNA with single-stranded gaps (C.Prives, 10th p53 Workshop). These DNA structures represent the intermediates of DNA damage and DNA repair. Binding to non-specific DNA was primarily mapped to the C-terminal domain of p53 protein, and non-sequence-specific DNA binding by the C-terminal polypeptide cannot be competed by specific DNA (30). The C-terminal domain of p53 protein is responsible for binding to ssDNA, as mentioned above, but binding to ssDNA activates p53 to bind both sequence-specifically and non-sequence-specifically to DNA. A 2 nt overhang of a DNA segment is sufficient to activate its binding to p53 protein (67). Although the C-terminal domain of p53 protein is sufficient for non-sequence-specific DNA binding, the binding affinity is always lower than that of the full-length p53 protein, implying participation of the central domain. Crystallographic analysis of the p53 central domain reveals a flexible structure ideal for binding to dsDNA. Indeed, the central domain is capable of binding non-sequence-specifically to DNA. p53 protein with the last 30 amino acids truncated can bind non-sequence-specifically to dsDNA (68). While it remains to be determined for ssDNA binding, p53 binding to non-specific dsDNA is largely dependent upon wild-type conformation (75). p53 protein binding to a DNA duplex with IDL mismatches has been reported to be wild-type specific (76). Such binding can be competed by DNA with a p53 consensus site (unpublished data). Thus, p53 binding non-sequence-specifically to DNA is likely to be synergistic between the C-terminal binding to ssDNA and the central domain binding to dsDNA. A survey of DNA templates

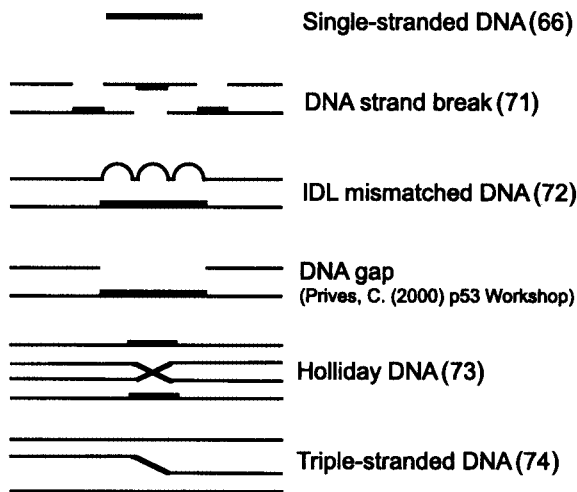


Fig. 1. Structures in DNA targeted for non-sequence-specific binding by p53 protein. ssDNA regions are indicated in bold. The structures shown, with the exception of ssDNA itself, have the common feature of ssDNA adjacent to dsDNA and are referred to as sds-DNA. IDL indicates insertion-deletion lesion.

targeted by p53 non-sequence-specific binding reveals a general structure composed of ssDNA in a DNA duplex context, as summarized in Figure 1. We will refer to this region of ssDNA and adjacent dsDNA as a single-stranded/double-stranded DNA transition (sds-DNA). This common structure may explain the ability of p53 protein to bind DNA damage induced by so many different agents.

The sds-DNA transitions are generated by DNA repair and DNA replication. Such DNA structures are recognized by DNA repair proteins and checkpoint proteins to mediate the cellular response to DNA damage. Unwinding the damaged DNA duplex to form a 'bubble' with ss- and dsDNA junctures is essential for NER. Both the Y-shaped DNA and the DNA bubbles are recognized and incised by the UV light response-induced DNA repair protein UvrBC in the absence of UvrA (77). Analysis of DNA-dependent protein kinase (DNA-PK) by electron crystallography suggests that DNA-PK binds to sds-DNA with separate binding sites, one for dsDNA and one for ssDNA (78). Binding to sds-DNA activates its kinase activities. There is evidence supporting the concept that sds-DNA is the signal to activate p53 protein. Induction of p53 has been found to be dependent upon the presence of unwound DNA intermediates from NER (79). Microinjection of dsDNA with 4 nt 5' overhangs but not of DNA duplexes with blunt ends induces p53-dependent growth arrest in fibroblasts (80). While it is clear that p53 activation by sds-DNA is mediated by checkpoint proteins with phosphatidylinositol-3 kinase activity like ATM, ATR and DNA-PK, the biological relevance of p53 binding to sds-DNA is still a matter of speculation. Albrechtsen *et al.* have recently reviewed activation status of p53 protein with particular attention to the role of p53 protein in DNA repair (81). The following sections will discuss possible biological functions of p53 protein non-sequence-specific binding to DNA.

Is p53 a damage sensor?

A prevalent hypothesis about the biological relevance of p53 binding to altered DNA structures is that p53 is a damage sensor. Accordingly, p53 recognizes damaged DNA and recruits DNA repair machinery to the damaged site. The identification of DNA repair proteins XPB and XPD as p53-associated

factors as well as the phosphorylation of p53 protein by CAK (which in addition can activate p53 sequence-specific binding) strongly favor this idea. Although p53 binding to damaged and mismatched DNA resembles mismatch repair sensor MSH2, there is no evidence to show that p53 protein participates in mismatch repair. Each DNA repair machinery complex has its own factor to sense damaged DNA. The binding to damaged DNA by the sensor is avid and highly specific so as to target the appropriate DNA repair machinery to the particular type of DNA damage. For example, XPC-hHR23B is a damage sensor for NER that binds specifically to pyrimidine dimers (with a K_d value of 5×10^{-9}) and specifically recruits XPA and TFIIH to the damage site (82). p53 protein offers no obvious specificity to a particular repair machinery as it binds to different types of DNA structures and to proteins involved in different types of repair, like XPB and XPD for NER, and Rad51 for DNA recombination. Since DNA repair proteins already have components to sense DNA damage, the cell does not require p53 protein strictly as a damage sensor.

Is p53 a DNA repair effector?

If p53 protein is not primarily a damage sensor, is it directly involved in repair? Several biochemical properties associated with p53 non-sequence-specific binding to DNA have suggested a direct involvement in DNA repair. p53 protein is able to mediate ssDNA reannealing and DNA strand transfer (66,71,83). These activities resemble DNA strand invasion and single-strand annealing in homologous recombination. Homologous recombination is a well conserved cellular mechanism to repair damaged DNA and to mediate chromatin exchange in crossover during normal cellular processes like antibody generation and meiosis. Recombination is also a common cause of chromosomal abnormality in cancer development. Results from *in vivo* studies show that both inter- and intra-chromosomal recombination are significantly inhibited by wild-type p53 (84,85). This indicates that p53 may function as a regulator of DNA recombination by recognizing DNA ends and reannealing ssDNA to block abnormal recombination. However, it is possible that DNA recombination can be enhanced by high levels of p53 protein through binding at the C-terminus. This domain of mutant p53 remains intact in most cases, and stabilization of the mutant makes it highly available for this genetically destabilizing activity. We have suggested that increased non-homologous recombination may contribute to the 'gain of function' phenotype associated with p53 mutants as implicated by p53 C-terminal activation of topoisomerase I catalytic activity (86). Both *in vitro* and *in vivo* studies have shown that p53 protein is capable of rejoining DNA with double strand breaks, indicating its role in non-homologous end-joining (NHEJ) (80,87,88). Although NHEJ is required in preventing chromosomal translocation (89,90), the lack of specificity of DNA end rejoining by p53 protein may contribute to its 'gain of function' phenotype, as mutant p53 is equally active in rejoining DNA ends (87,88).

Although p53 binds to ssDNA and mismatched DNA, there is no direct evidence to support the involvement of p53 in either NER or mismatch repair in cell free systems, and there are no obvious DNA repair defects in p53 knock out mice. These observations suggest that p53 as guardian of the genome is not primarily involved in DNA repair. However, Offer *et al.* recently provided evidence that p53 participates in BER (91). Incorporation of radiolabeled dNTP into depurinated plasmids

in nuclear extracts (indicative of BER activity) was enhanced in cells expressing wild-type p53 and reduced in cells expressing mutant p53 proteins. While there is no experimental evidence to show how p53 participates in BER, Mummenbrauer *et al.* have found that wild-type p53 protein possesses intrinsic 3' to 5' exonuclease activity in the presence of magnesium ions (92). This activity is localized to the central domain of p53 protein based on deletion analysis and structural similarity to the catalytic domain of exonuclease III in *Escherichia coli*. The 3' to 5' exonuclease activity is a common mechanism to ensure sequence fidelity during DNA replication and DNA repair. This activity provides a molecular basis for p53 involvement not only in BER but also in other DNA repair machinery complexes where proofreading is necessary. The exonuclease activity of p53 seems to be mutually exclusive to its specific DNA binding activity. While the C-terminal domain negatively controls both specific DNA binding and exonuclease activity of p53, the exonuclease activity of p53 is inhibited by the activation events for specific DNA binding, such as phosphorylation or PAb421 association. Inhibition of 3' to 5' exonuclease activity by PAb421 and phosphorylation events that activate p53 suggest its association with latent p53 proteins (81). However, binding to non-specific DNA is also affected by these activation events. As noted above, both latent and activated p53 proteins are capable of such binding, indicating a more general function of p53 proteins binding non-sequence-specifically to DNA.

Does p53 protein mediate an apoptosis checkpoint?

If p53-mediated DNA repair is critical for maintaining genomic integrity, p53 and its related DNA repair function should be well conserved from bacteria to humans. However, p53 and newly discovered members of the p53 gene family are present only in multicellular organisms. While p73 and p63 family members bind to the p53 consensus (93,94), to date there are no reports of their capacity for non-sequence-specific binding, for example, to mismatched DNA. The p53 homolog in *Drosophila* is the most primitive relative of p53 identified so far. *Drosophila* p53 mediates DNA damage-induced apoptosis but not growth arrest, suggesting that the primitive function of p53 is to mediate apoptosis in genome maintenance (95–97). Eliminating cells with damaged DNA by apoptosis is vital for multicellular organisms to prevent genetic transformation (98). It is generally accepted that p53-mediated apoptosis and p53-induced cell cycle checkpoints are the two major mechanisms of p53 protein activity as a tumor suppressor. The p53-mediated cell cycle checkpoint is activated by checkpoint proteins well conserved in yeast such as ATM, ATR and Chk2. However, the mechanism of p53-mediated apoptosis is largely unknown, despite the identification of apoptosis-related genes as downstream transcriptional targets of p53. So far it is not clear why p53 mediates growth arrest in certain cells but apoptosis in others responding to the same DNA damage, or how a given cell type determines its response to different DNA damaging agents or even to different doses of the same agent. Clearly, there are certain signals sensed by p53 protein that trigger the apoptosis pathway. Given the role of p53 as a guardian of the genome, unrepaired DNA should be a legitimate signal for p53 protein to direct the cell towards the apoptosis pathway. There is evidence that when DNA damage is not repaired, activation of p53 can lead to apoptosis (90). Binding to sds-DNA as defined above suggests a biochemical basis whereby p53 protein might

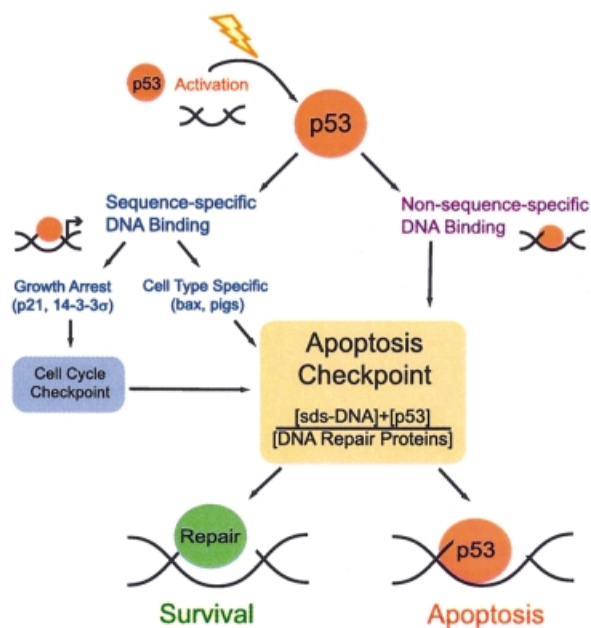


Fig. 2. Model: p53 protein mediates an apoptosis checkpoint in response to DNA damage. p53 protein is activated by DNA damaging stimuli through multiple post-translational modifications. Cell type and/or DNA damage-specific responses are dependent upon transcriptional and non-transcriptional effects of DNA binding by p53 proteins. An apoptosis checkpoint is determined by thresholds of p53 proteins and damaged DNA (sds-DNA) relative to a constant level of ubiquitous DNA repair proteins (see text).

monitor an apoptosis checkpoint in response to a variety of types of DNA damage. Rather than sensing the damage primarily to recruit repair proteins, p53 protein may sense the equilibrium between the amounts of damaged DNA and available DNA repair proteins. This equilibrium would be dependent upon p53 protein modification state, the level of p53 protein accumulation, the level of DNA damage and the capacity of the damaged cell to repair. Since sds-DNA is also targeted by DNA repair proteins like RPA and Rad51, it is tempting to propose that p53 proteins and DNA repair proteins are competing for sds-DNA dynamically, as reflected in the equation: $[sds-DNA] + [p53]/[DNA\ repair\ proteins] \Rightarrow Apoptosis$. According to the scheme represented in Figure 2, DNA damage leads to accumulation of activated p53 proteins with increased affinity for DNA. Distinct p53 modifications may target different genes, such that certain cells with a higher capacity to repair or less capacity for rapid renewal might arrest, repair and survive. Because the levels of repair proteins generally far exceed those of p53 protein, sites of damaged DNA would be occupied predominantly by DNA repair proteins if the damage is mild. However, even limited levels of p53 protein may bind damaged DNA above the threshold of DNA repair protein availability. Alternatively, high levels of p53 induced by DNA damage or oncogenic events could compete with DNA repair proteins for a limited amount of DNA lesions, triggering apoptosis. There is abundant evidence that apoptosis is highly correlated with the level of p53 protein and the extent of DNA damage (99). Furthermore, increased apoptosis has been linked to p53 when DNA repair is inhibited by inactivation of poly(ADP-ribose) polymerase (PARP), a ubiquitous DNA binding protein involved in repair and possibly in apoptosis (100). It is possible to test the proposed apoptotic checkpoint equation by microinjection of fluorescein-labeled

sds-DNA into cells. Huang *et al.* have reported that micro-injection of a plasmid with a long gap or DNA with a 5' overhang into fibroblasts induced p53-dependent growth arrest (80). Cells with apoptotic potential could be used to test the hypothesis that sds-DNA dosage and extent of association of p53 and sds-DNA are directly correlated with p53-dependent apoptosis. Comparison of fibroblasts refractory to apoptosis with epithelial cells competent for apoptosis might provide clues as to how cell type-specific transcriptional events lead to survival or apoptosis. While the apoptosis checkpoint controlled by p53 binding non-sequence-specifically to DNA is hypothetical, it provides a framework for explaining how p53-mediated apoptosis is dependent upon cell type or extent of DNA damage and why apoptosis is more likely to be induced in cells less robust for DNA repair proteins.

The transcriptional and non-transcriptional p53-dependent arms of the p53 response to DNA damage could, theoretically, be exercised independently or cooperatively. Mainly transcriptional events might occur in cases where p53 is induced at very low levels of sds-DNA. Non-transcriptional events might predominate in severely damaged cells because transcriptional activities of p53 protein are compromised by conformational changes due to oxidative stress (101). Cell type-specific responses may require a cooperation of events in which accumulation of proteins encoded by p53 downstream target genes tip the balance toward survival (either due to reversible or prolonged cell cycle arrest) or apoptosis. While apoptosis can be regulated at the transcriptional level by p53, p53-dependent apoptosis can be achieved in a transcription-independent manner. Examples of this are p53-mediated apoptosis in the presence of inhibitors of transcription and translation (102,103) and apoptosis mediated by p53 mutants lacking transcriptional activities (104). Although the N-terminal proline-rich domain and the C-terminus of p53 protein are required for p53-dependent apoptosis (99,105), the central domain must participate in p53-mediated apoptosis, as tumor cells with p53 mutations are resistant to apoptosis in general. Furthermore, certain p53 mutants (Ala143, His273) with substantial transactivation activities are defective in apoptosis (106,107). Using a p53 DNA binding assay applicable to cells and tissues, we found that p53 mutant (His273) in human colon carcinoma cell SW480 has lost its ability to bind non-specific DNA while retaining the ability to bind specific DNA (unpublished data). This associates the defect in p53 binding non-sequence-specifically to DNA with the defect in p53-mediated transcription-independent apoptosis and is worthy of exploration in other p53 mutants.

Due to the lack of sequence-dependence, apoptosis mediated by sds-DNA binding should be through protein-protein interactions instead of transactivation. Several lines of evidence coming from the laboratory of Harris and others indicate that DNA repair machinery is involved in p53-mediated apoptosis. p53-mediated apoptosis is abrogated in cells with either XPB or XPD mutations and can be restored by introduction of wild-type XP genes (108). Attenuation of p53-mediated apoptosis was also observed in cells with a defect in WNR, a DNA helicase mutated in Werner syndrome (109). At first glance, these observations seem contrary to the hypothesis above, since repair defects might be expected to result in a higher frequency of sds-DNA and increased p53 binding and apoptosis. However, the increased p53 binding to sds-DNA may occur, but apoptosis as its consequence may be blocked by the repair protein defect. These repair protein defects may

provide clues as to how the signal of p53 binding to sds-DNA is transduced and linked to the apoptosis pathway. XPB and XPD mutants are associated with attenuated apoptosis whereas CSB mutants result in enhanced apoptosis. p53 protein has been found to be associated with XPB, XPD and CSB. Inhibition of the helicase activities of these proteins by association with p53 suggests that enhancement of DNA repair is not the mechanism by which p53 maintains genomic integrity, at least not through the association with these repair proteins. Since DNA repair is not necessary in cells committed to apoptosis, these associations may be primarily involved in apoptosis. The association between p53 and DNA repair proteins suggests transcription-independent apoptosis. Another possibility is that p53 binding non-sequence-specifically at sites of DNA damage mediates p53 modifications by the DNA repair machinery. Such modifications may confer specificity of p53 DNA binding to transactivate pro-apoptotic genes. Evidence supporting this hypothesis comes from p53 modification by CAK, a component of TFIIH (25,110). This would be a transcriptional means to convey the apoptotic signal if p53 protein dissociates from the complex upon modification. The availability of functionally distinct p53 mutants and model systems such as the simpler organism *Drosophila* will provide tools to dissect the mechanism of p53-mediated apoptosis.

Clearly, p53 is a multifunctional protein with multiple potential modifications and biochemical properties. A major challenge in the field is how to correlate its biochemical properties with its biological behaviors in cells and, more significantly, *in vivo*. Remaining questions include: how are the transcriptional and non-transcriptional arms of p53 response to DNA damage coordinated in specific cell types; what is the nature of the interactions between p53 proteins and DNA repair proteins or other constitutive or induced proteins required for p53-dependent apoptosis; how do p53 and other proteins at the postulated apoptosis checkpoint trigger the next steps in the apoptosis cascade. To answer these questions, DNA binding studies must be extended from the test tube to the cells, requiring increasing sensitivity. Molecular profiling of p53 downstream genes by means of DNA chip technology, affinity assays of cellular p53 protein binding to a range of specific and non-specific DNA templates and identification of discrete p53 proteins modified through phosphorylation and acetylation by means of specific antibodies will make a powerful combination for defining the p53 pathway in response to different genotoxic stresses in cells and tissues. Application of these approaches will permit defining the p53 response to DNA damage through sequence-specific and non-sequence-specific binding to DNA. It may reveal how p53 selectively turns on the expression of certain sets of genes through sequence-specific DNA binding or, in the face of overwhelming DNA damage, uses a variety of DNA repair machinery components to trigger apoptosis through non-sequence-specific DNA binding. Finally, these approaches may uncover how specific post-translationally modified p53 proteins integrate the response to different upstream signals. To fully understand how p53 proteins orchestrate the cellular response to DNA damage, it may be essential to view the location and extent of p53 binding to specific DNA and to damaged DNA and the association of p53 protein with other proteins in the chromatin context in cells responding to DNA damage.

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