p53 modulation of TFIIH-associated nucleotide excision repair activity

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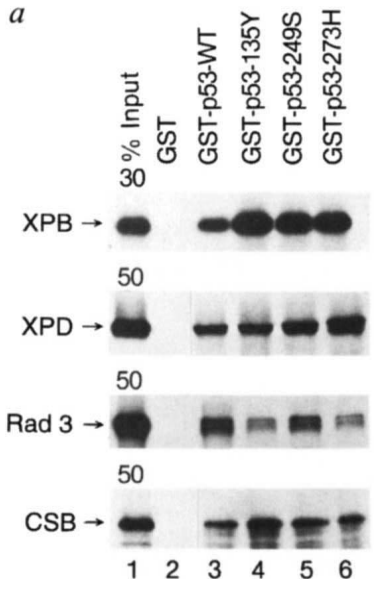
p53 has pleiotropic functions including control of genomic plasticity and integrity. Here we report that p53 can bind to several transcription factor IIH-associated factors, including transcription-repair factors, XPD (Rad3) and XPB, as well as CSB involved in strand-specific DNA repair, via its C-terminal domain. We also found that wild-type, but not Arg273His mutant p53 inhibits XPD (Rad3) and XPB DNA helicase activities. Moreover, repair of UV-induced dimers is slower in Li-Fraumeni syndrome cells (heterozygote p53 mutant) than in normal human cells. Our findings indicate that p53 may play a direct role in modulating nucleotide excision repair pathways.

Eukaryotes possess an intricate network of DNA repair systems which restore genomic integrity following DNA damage from endogenous and exogenous mutagens. Defects in this process can result in a mutator phenotype associated with tumorigenesis. The cancer-prone disorder, xeroderma pigmentosum (XP), results from a deficiency in repair of UV-induced DNA damage[1] and some oxidative lesions as well[2]. Three other autosomal recessive syndromes, Bloom's syndrome (BS), Fanconi's anaemia (FA) and ataxia telangiectasia (AT) are associated with DNA damage hypersensitivity and possible DNA repair deficiencies[3]. Germline mutations in the p53 tumour suppressor gene, as in individuals with Li-Fraumeni syndrome (LFS), are also associated with increased tumour risk[4]. Although p53 'knockout' mice appear normal at birth, they have a higher incidence of spontaneous tumours during their adult life[5], and their cells exhibit increased genomic instability when grown in culture[6,7]. Also, disruption of p53 function by either mutations or association with viral oncoproteins has been implicated in the development of many human cancers[8].

While the mechanism of p53 tumour suppression is still unclear, there is strong evidence that p53 plays an important part in cell-cycle regulation during times of genomic stress. For example, DNA damage, produced by UV, ionizing radiation or certain chemical mutagens that generate DNA strand breaks, can trigger p53 accumulation in mammalian cells [9,10]. p53 stimulates transcription of the genes for $p21^{WAF1}$, *GADD45* and cyclin G, whose products may induce G1 arrest, presumably allowing the cell to repair DNA damage before DNA replication[9,11–13]. $p21^{WAF1}$ directly inhibits DNA replication by binding to PCNA[14]. p53 can also induce apoptosis in some circumstances, presumably to remove cells that are incapable of repairing DNA damage[15]. Hence, p53 might act as a 'safeguard' in maintaining genomic integrity by either monitoring the G1 checkpoint[9] and/or directly interacting with repair proteins[16]. The search for an association between p53 and other cellular proteins may provide clues to the p53 tumour suppressor function.

We recently reported the *in vitro* interaction between p53 and XPB[16], a DNA helicase involved in nucleotide excision repair (NER) and recently found to be the largest subunit of human RNA polymerase II basal transcription factor, TFIIH[17]. TFIIH contains at least 5 subunits, including XPD, XPB, p62, p44, and p34. It possesses ATPase, kinase and helicase activities, and is required for initiation of gene transcription by RNA polymerase II[17–23]. Mutations in XPB and XPD are responsible for the UV sensitivity and predisposition to skin cancers associated with xeroderma pigmentosa XP-B and XP-D patients, respectively[24,25]. These patients also exhibit the clinical hallmarks of Cockayne syndrome (CS), a DNA repair disorder in which only transcription-coupled NER is defective when not associated with XP[24,26,27]. A majority of CS patients have mutations in CSB, another presumed helicase protein[28]. Clearly, the proteins encoded by these genes function as components of a multi-complex repair machinery.

To elucidate the functions of p53 and its possible role in NER, we have examined the hypothesis that p53 modulates the helicase activities of the TFIIH-based transcription/repair complex by binding to subunits that may have a role in NER — XPD and XPB. We have also examined p53 interaction with Rad3, the yeast homologue of XPD, and CSB, involved in transcription-coupled preferential-



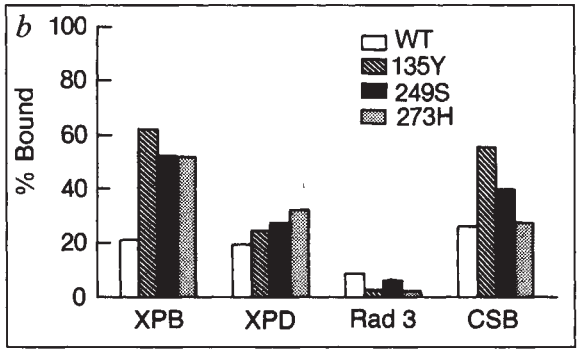


Fig. 1 Human wild-type and mutant p53 proteins are able to complex with XP proteins *in vitro*. *a*, GSH-beads loaded with either 4 µg of GST (lane 2) or 2 µg of GST-p53-WT (lane 3), GST-p53-135Y (lane 4), GST-p53-249S (lane 5), or GST-p53-273H (lane 6) were mixed with ^{35}S-labelled, *in vitro*-translated XPB, XPD, Rad3 (5 µl) or CSB (15 µl). Proteins which remained bound were analysed by SDS/PAGE. The authentic XP proteins immunoprecipitated by the specific antibodies were loaded in lane 1 as references. In lane 1, the indicated fraction of XPB, XPD, Rad3 or CSB were immunoprecipitated by anti-XPB polyclonal antibodies (Ab) as a 90 kD protein, MAb2F6 (anti-X mAb) as an 80 kD protein, anti-Rad3 polyclonal Abs as a 85 kD protein, or anti-CSB polyclonal Abs as a 170 kD protein, respectively. *b*, The percentage of total protein bound was quantitated by densitometry. The binding between experiments varies by less than 10%.

strand NER. We show here that wild-type p53 selectively associates *in vitro* with XPD (Rad3), XPB and CSB, and with XPB *in vivo*. Some p53 mutants found in human tumours also bind to these proteins *in vitro*. Wild-type p53 binds to XPB at a region containing helicase motif III, which is functionally indispensable for NER activity[29]. Finally, we show that LFS cells have a slower rate of gene-specific DNA repair than normal human cells. Our data suggest that p53 may modulate DNA repair by direct interaction with proteins involved in these pathways, and that p53 mutations may affect the cellular response to DNA damage resulting in less efficient DNA repair. This may be a possible mechanism by which p53 maintains genomic stability and prevents tumour formation.

Binding of p53 to NER factors

We initially determined whether p53 interacts with NER proteins *in vitro*. Human wild-type p53 was tagged at the N terminus with glutathione S-transferase (GST) and incubated with *in vitro* translated ^{35}S-labelled XPD, Rad3, XPB or CSB protein. ^{35}S-labelled proteins bound to p53 were isolated by glutathione-conjugated Sepharose beads (GSH-beads). XPD, XPB and CSB bind to GST-p53-WT with a relatively higher affinity (about 20% of input) than Rad3 (about 10% of input) (Fig. 1*a*, lanes 1 with 3). GST alone did not interact with any of the four proteins (lane 2). In parallel, GST tagged XPB also efficiently bound to *in vitro* translated p53 (data not shown). *In vitro*-translated REF1, a nuclear factor involved in DNA repair and redox pathways, as well as *in vitro*-translated luciferase and other non-relevant proteins present in the translation mix, did not bind to either GST-p53-WT or GST-XPB (data not shown). Binding is not mediated through single stranded (ss) DNA and RNA, as demonstrated by the treatment of *in vitro*-translated products with DNase and RNase prior to binding (data not shown).

Human p53 mutants found in cancers frequently exhibit different capacities to bind cellular and viral proteins as well as DNA[16,30]. Therefore, equal amounts of GST-tagged p53 mutants, (His135Tyr, Arg249Ser, Arg273His) were tested for binding to the *in vitro*-translated proteins used above. All the mutants tested had similar or increased binding to the human proteins but decreased binding to yeast Rad3, as compared to GST-p53-WT (Fig. 1*a*, compare lane 3 to lanes 4–6). This suggests that mutant p53 may exert a dominant-negative effect by binding to and sequestering the cellular targets of wild-type p53. It is interesting that the His135Tyr mutant, which has diminished binding to hepatitis B virus X protein (HBX)[16] and papillomavirus E6 protein[31], binds to XPD, XPB, Rad3 and CSB. HBX can also inhibit p53 binding to XPB presumably by binding to the same site of p53 that interacts with XPB, since HBX did not bind to XPB *in vitro*[16].

p53 binds to the XPB protein *in vivo*

We next determined whether the *in vitro* interaction between p53 and XPB occur *in vivo*. The low concentrations of transcription factor components including XPB and XPD require the use of highly sensitive methods to detect their binding to p53. Previous studies of p53 binding to XP protein[16,32] or TBP and TBP-associated proteins[33,34] reported only *in vitro* interactions. Therefore, we used the KT3 epitope-tagged XPB expression system[29] that allows one to measure a detectable level of the XPB protein by the KT3 antibody. A human hepatoblastoma line (HepG2) and a human adenovirus-transformed embryonal kidney cell line (293), that both contain high levels of wild-type p53, were transfected with the KT3-tagged XPB expression

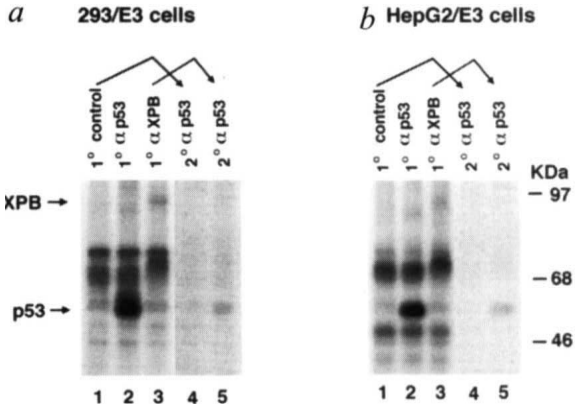


Fig. 2 Interaction between p53 and XPB proteins *in vivo* demonstrated by double immunoprecipitation (IP) analysis. 293/E3 cells (*a*) and HepG2/E3 cells (*b*) were immunoprecipitated at first with the normal mouse IgG antibody (lanes 1), the anti-p53 DO-1 mAb (lanes 2) or the KT3 mAb (lanes 3). The precipitates from lanes 1 and 3 were immunoprecipitated again with the DO-1 antibody and were loaded as lanes 4 and 5, respectively.

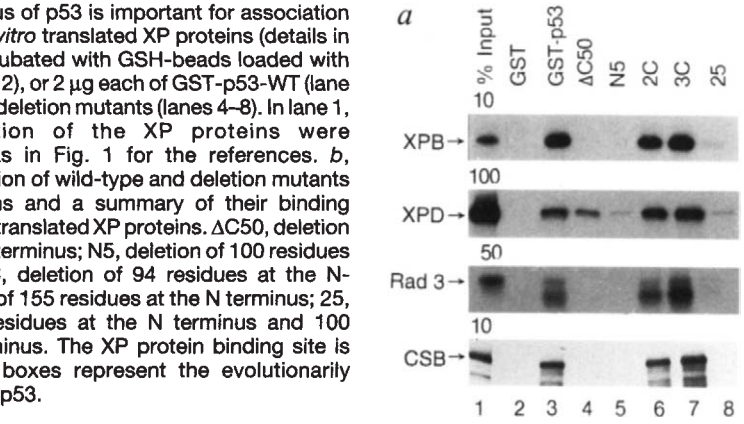


Fig. 3 Carboxyl terminus of p53 is important for association with XP proteins. A. *In vitro* translated XP proteins (details in Fig. 1 legend) were incubated with GSH-beads loaded with either 4 μg of GST (lane 2), or 2 μg each of GST-p53-WT (lane 3), or various GST-p53 deletion mutants (lanes 4–8). In lane 1, the indicated fraction of the XP proteins were immunoprecipitated as in Fig. 1 for the references. *b*, Schematic representation of wild-type and deletion mutants of human p53 proteins and a summary of their binding properties with *in vitro*-translated XP proteins. ΔC50, deletion of 50 residues at the C terminus; N5, deletion of 100 residues at the C terminus; 2C, deletion of 94 residues at the N-terminus; 3C, deletion of 155 residues at the N terminus; 25, deletion of both 94 residues at the N terminus and 100 residues at the C terminus. The XP protein binding site is indicated. The black boxes represent the evolutionarily conserved domains of p53.

vector. Resulting cells were subjected to a double immunoprecipitation (IP) by a KT3 antibody followed by a p53 antibody (DO-1). In the first IP, the KT3 antibody specifically precipitated the XPB protein (Fig. 2, lane 3) and the low p53 signal could not be distinguished due to background (compare lane 3 to 1). However, when these samples were subjected to a second IP by DO-1 antibody, p53 was detected only in the KT3-precipitated sample (lane 5), and not in the normal mouse IgG-precipitated sample (lane 4) from both cell types. These data indicate that p53 is able to bind to the XPB protein *in vivo*.

C terminus of p53 is responsible for binding

Defining the protein domains required for various interactions can provide clues to the significance of the interaction, especially if the domains are functionally important. We used deletion mutants of p53 tagged with GST for this purpose. Deletion of the 50 C-terminal amino acids abolished binding of XPB, Rad3 or CSB, and reduced binding of XPD, while deletion of up to 155 N-terminal amino acids had no detectable effect (Fig. 3). XPD binding diminished with deletion of the 100 C-terminal amino acids (Fig 3*a*, compare lane 5 to lane 3).

Initial mapping studies suggested that a region of XPB containing the helicase motif III was responsible for interacting with p53. (XPB contains seven putative helicase motifs which are conserved among the helicase superfamily and are indispensable for NER activity[29].) We used the Chou-Fasman and the Robson-Garnier methods[35,36] to predict possible secondary structures for the two interacting regions of p53 and XPB. The conserved helicase motif III consists of a 3–6 residue turn containing 1–3 acidic (negatively charged) residues, which are likely to be exposed on the protein's surface, and which are separated by two α helices or β sheets, depending on the particular member of the superfamily (Fig 4*c*). Sequence analysis of the C-terminal p53 domain reveals that it contains a stretch of basic (positively charged) amino acids (residues 367–387) which are evolutionarily conserved from *Xenopus* to human. This region is likely to form an α-helix with all the positively charged residues facing one side (Fig. 4*b*). We hypothesize that this positively charged α-helical domain of p53 may be in direct contact with the negatively charged turn of helicase motif III in XPB.

To test this hypothesis, we used peptides corresponding either to helicase motif III or to the p53 C-terminal domain for competition. Peptide #464 (residues 464–478 of XPB) efficiently competed *in vitro*-translated XPB from GST-p53 (Fig. 4*a*, compare lane 4 to lane 1), while a non relevant peptide #99 (from HBV) failed to do so

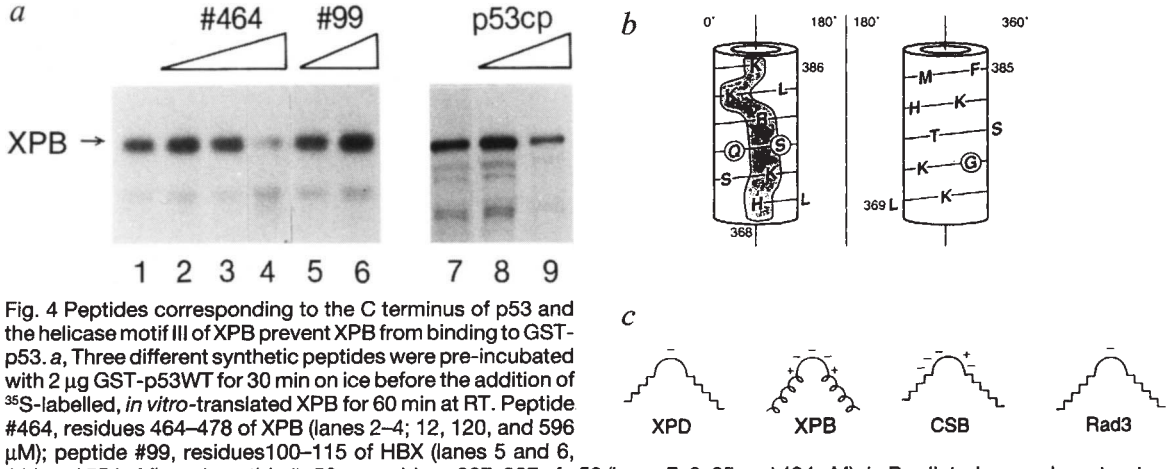


Fig. 4 Peptides corresponding to the C terminus of p53 and the helicase motif III of XPB prevent XPB from binding to GST-p53. *a*, Three different synthetic peptides were pre-incubated with 2 μg GST-p53WT for 30 min on ice before the addition of ^{35}S-labelled, *in vitro*-translated XPB for 60 min at RT. Peptide #464, residues 464–478 of XPB (lanes 2–4; 12, 120, and 596 μM); peptide #99, residues100–115 of HBX (lanes 5 and 6, 111 and 554 μM), and peptide #p53cp, residues 367–387 of p53 (lanes 7–9; 85 and 424 μM). *b*, Predicted secondary structure of amino acids 368–385 of p53. Open circle, amino acids which destabilize the α-helix. Shaded area, basic amino acids. *c*, Predicted secondary structure of helicase motif III of XP proteins. –, Negatively charged residues; +, positively charged residues; zig-zag lines, β-sheet; coiled lines, α-helix; curved lines, turn.

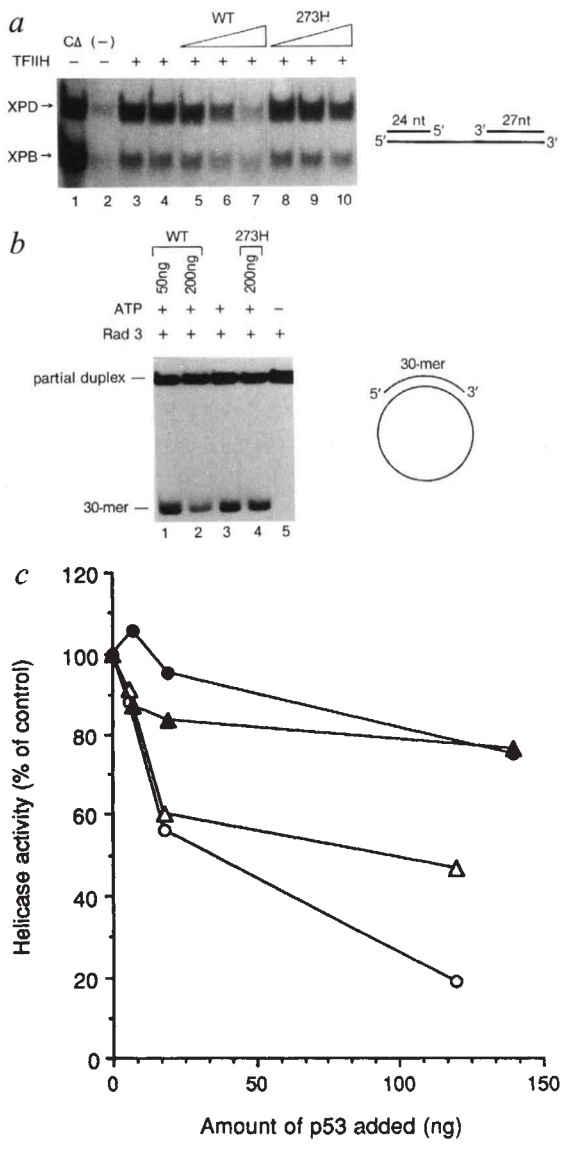


Fig. 5 Wild-type but not mutant p53 inhibits helicase activity of XPD and XPB, the major components of TFIIH, as well as the activity of Rad3. *a*, Substrate is indicated on the right; the 27 nt (top band) is displaced by XPD (5'→3' helicase) and the 24 nt (bottom band) is displaced by XPB (3'→5' helicase). The ^{32}P-labelled substrate was incubated with highly purified TFIIH (HAP fraction) in the absence (lanes 3 and 4) and in the presence of 6, 18 and 120 ng of baculovirus-produced wild-type p53 (lanes 5–7), or 7, 21, and 140 ng of mutant Arg273His (lanes 8–10). In lane 1, the substrate was heated for 2 min at 100 °C (CΔ) and in lane 2, the substrate was loaded directly (–). *b*, The Rad3 DNA helicase activity was measured as described[57] using 90 ng Rad3 protein in the absence (lane 3) or presence of 50 ng or 200 ng of p53WT (lanes 1 and 2), or 200 ng of mutant Arg273His (lane 4). Helicase activity of 90 of Rad3 protein was assayed without 1 mM ATP (lane 5). *c*, The quantitative results obtained from densitometry analysis of the autoradiography shown in Fig. 5*a* with the amounts of oligomers displayed by XP proteins and expressed in the helicase activity as a function of p53 doses. ○, XPD+WT; △, XPB+WT; ●, XPD+Arg273His; ▲, XPB+Arg273His.

(compare lanes 5 and 6 to lane 1). Similar results were obtained with peptide corresponding to residues of 479 to 493 of XPB (data not shown). As expected, peptide #p53cp (residues 367 to 387 of p53) also competed *in vitro*-translated XPB from GST-p53 (Fig. 4*a*, compare lane 9 to lane 7). We conclude that motif III of XPB directly interacts with the p53 C-terminal domain.

Effect of p53 on helicase activity of TFIIH, Rad3

As p53 inhibits many DNA and RNA helicases[37–39], we investigated whether p53 alters the helicase activity of XPD and XPB within the functional TFIIH complex and of recombinant Rad3 protein[21]. Native TFIIH, purified from HeLa cell nuclear extract as described[17], was shown to be transcriptionally active and contained both ATP-dependent 5'-3' (contributed by XPD) and 3'-5' (contributed by XPB) helicase activities[17,18,21]. This system allows us to assay simultaneously and distinguish between XPD and XPB helicase activity within the native TFIIH complex (see Fig. 5 legend). A highly purified human wild-type recombinant p53 protein produced from baculovirus effectively inhibits intrinsic TFIIH helicase activity in a dose-dependent manner (Fig. 5*a*, both orientation, compare lanes 5–7 to lanes 3 and 4; Fig. 5*c*), although XPD was affected to a greater degree than XPB (Fig. 5*c*). An identical result was observed by the recombinant p53 produced from *Pichia Pastoris* (data not shown). No inhibition was observed with addition of the mutant Arg273His (Fig. 5*a*, compare lanes 9 and 10 to lanes 3 and 4; Fig. 5*c*), even though it binds to XPD and XPB proteins (Fig. 1).

p53 contains DNA single-strand (ss) annealing activity[39,40], due to the preferential binding of p53 to ssDNA ends (90% of total binding)[40]. The binding of two DNA ends to a p53 oligomer may align DNA fragments and promote renaturation[40]. To distinguish between this annealing activity and direct anti-helicase activity following binding, we used a single-strand circular DNA hybridized to a short oligonucleotide as a substrate. Wild-type p53, but not mutant Arg273His, inhibited Rad3 helicase activity to a similar degree (Fig. 5*b*, compare lane 2 to lanes 1, 3 and 4), similar to the observation with TFIIH. This is consistent with the hypothesis that p53 inhibits TFIIH helicase activity directly by binding to helicase proteins.

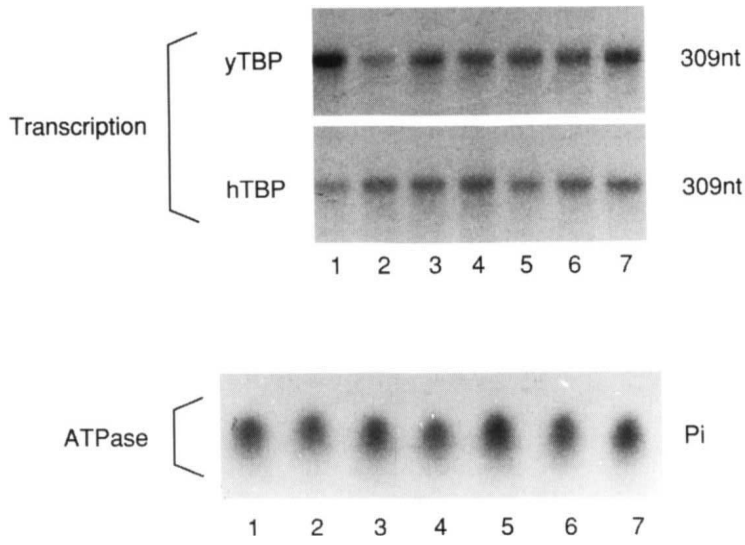


Fig. 6 The TFIIH-associated *in vitro*-transcription of RNA poly II and ATPase activities are not inhibited by p53. The purified TFIIH was preincubated for 20 min at 4 °C without (lane 1) or with 6, 18 or 120 ng of baculovirus-produced wild-type p53 (lanes 2–4), or 7, 21, and 140 ng of mutant Arg273His (lanes 5–7). The transcription reaction was them completed by addition of RNA II, DNA template, nucleotides and the other basal transcription factors including either yeast TBP (yTBP) or human TFIID (hTBP). The specific transcript of a 309 nt either by yTBP or hTBP is indicated. The ATPase activity of the TFIIH with or without p53 was measured as described in Methods. Pi, inorganic phosphate that is liberated from ATP.

However, at levels of p53 which inhibit helicase activity (Fig. 5), no alteration of the ATPase activity associated with TFIIH and Rad3 (data not shown) or basal transcriptional activity associated with TFIIH (Fig. 6) was detected.

DNA repair is less efficient in Li-Fraumeni cells

Our data indicate that both wild-type and mutant p53 bind to XPD and XPB proteins, although only wild-type p53 inhibits their helicase activity. This suggests a role for p53 in initiating the NER pathway, and predicts that p53 defects may result in less efficient DNA repair. To test this hypothesis, we examined gene-specific repair of UV-induced PDs from normal fibroblasts and fibroblast from a LFS family (heterozygous for a mutation at codon 245 of *p53*). These cells exhibit comparable population doubling times and growth potentials when compared to normal cells (data not shown).

The initial dimer frequency (IDF) per 10 kb in the dihydrofolate reductase (DHFR) gene of normal cells following UV irradiation (10 J m^{-2}) was 0.69±0.03, similar to our previous work[41]. The IDF in the LFS cell strains 2673 and 2675 was 0.71±0.26 and 0.67±0.09, respectively. The repair of PDs was measured at 4, 8 and 24 h following irradiation. Both normal human fibroblast cell strains (GM00038A and GM02987) remove PDs very efficiently (Fig. 7). In GM00038A strain, repair was 31%±1 at 4 h, 60%±1 at 8 h and 82%±7 at 24 h, respectively, consistent with previous reports[41]. In contrast, repair in cell strain from the kindred proband (2675) was only 2%±7 at 4 h, 24%±9 at 8 h, and 66%±1 at 24 h (Fig. 7). Repair in cell strain from the father (2673) was -7.3%±29 at 4 h, 20%±17 at 8 h, and 52%±14 at 24 h (Fig. 7). Hence, repair of *DHFR* in both LFS cell strains was significantly less at all time points than that observed in the two normal controls, and especially after 4 and 8 h time points. However, no difference in RNA synthesis recovery was visible in these cells following UV exposure, suggesting that these cells may have a similar sensitivity to UV light (data not shown).

Discussion

p53 as an NER factor-binding protein. p53 binds to the XPB and XPD helicases, that form part of the transcription-repair complex, TFIIH. This conclusion is based on the observations that: (i) GST-p53-WT binds specifically to *in vitro*-translated XPD (Rad3), XPB, or CSB proteins,

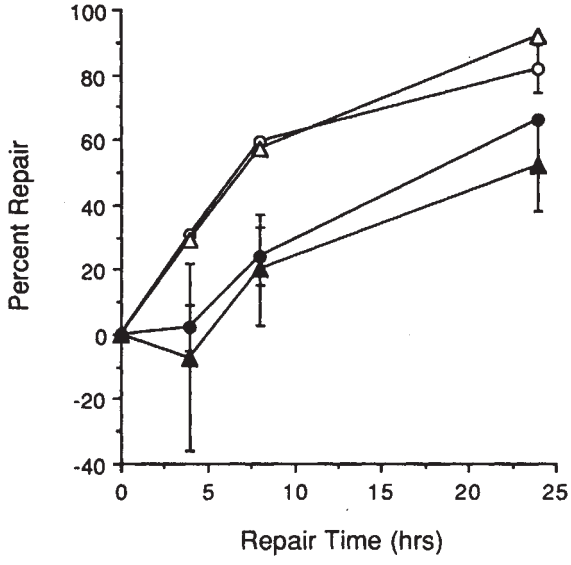


Fig. 7 Average repair in *DHFR* studied in two normal fibroblast strains and two LFS-derived strains after treatment with 10 J m^{-2} UV. Gene-specific repair is calculated using the actual IDF obtained for each experiment since it was previously shown that this yields reproducible results[41]. Each repair time point is the average of values obtained for that time point in duplicate from three separate experiments (normal cells) or from two experiments (LFS cells). △, GM02987 (normal cells); ○, GM00038A (normal cells); ▲, father (LFS cells); ●, proband (LFS cells).

but not to truncated XPB protein lacking helicase motifs Ia, II, III, and not to other non-relevant peptides; (ii) GST-XPB also binds to *in vitro*-translated p53; (iii) GST-p53-135Y binds to the tested XP proteins although it has diminished binding to *in vitro*-translated HB viral X protein[16] and human papillomavirus E6 protein[31]; (iv) GST-p53 deletion mutants have diverse binding activity to XPD, XPB and CSB; and (v) p53 is able to bind to XPB *in vivo*. The fact that p53 binds to these helicases which are involved in NER suggests that p53 has an important role in the maintenance of genomic integrity not only by controlling the cell cycle but also by direct participation in the NER pathway. Xiao and colleagues recently reported that p53 can bind to TFIIH *in vitro* via its activation domain and the p62 subunits of TFIIH[32].

Interestingly, all the p53 mutants from human cancers tested also bind to XPD, XPB and CSB proteins to a similar or greater extent than WT, in contrast to the p53 mutants that have generally lost the sequence-specific DNA binding activity needed for transcriptional activation. This is most easily understood by the gain-of-function concept. Mutant p53 could bind to important cellular targets that are normally occupied by WT p53 protein, resulting in a dominant-negative phenotype in the presence of WT p53 or a dominant-oncogenic phenotype in the absence of WT[42]. In agreement with this concept, cells homozygous for mutant p53 are genetically more unstable than heterozygous and wild-type cells[6,7]. However, it is not clear how the binding of WT p53 or mutant p53 to XPD, XPB and CSB proteins and hence the potential modulation of NER activity, relates to the phenotype of genomic stability. One possibility could be through modulation of the XP helicase activity by interaction with helicase motifs that are important in their NER activity[29,43]. p53 binds to helicase motif III of XPB, which may be essential for the unwinding activity, and is a common feature for many other helicases[43]. p53 can bind to many cellular DNA helicases[44], although the identities of these proteins are not known.

The positively charged C-terminal p53 domain is evolutionarily conserved and is thought to be important for the negative regulation of p53 function[45]. Several proteins, including casein kinase II and protein kinase C, that can activate the latent function of the protein, bind to this region[45,46]. Regulation of WT p53 through modification of the C-terminal domain may also occur through alternative splicing of murine p53 protein *in vivo*[47], or by the *in vitro* truncation of the C-terminal 30 amino acids[45]. The common feature that the p53 C-terminal domain interacts with these XP proteins suggests that they are functionally related. It will be of interest to determine if binding of XPD, XPB and CSB proteins to the C-terminal domain activates p53 and if such activation is important in the modulation of NER activity.

p53 inhibits the helicase activity of XPD and XPB proteins. Although p53 can inhibit TFIH helicase activity, it did not inhibit its ATPase or transcriptional activities at the same dose in our *in vitro*-reconstituted system. No inhibition of the Rad3 ATPase activity by p53 was observed until the concentration was 10-fold higher than that used in the helicase assay (data not shown). One possibility is that motif III is an important catalytic domain of the helicase, and p53 physically blocks its action on the substrate while the helicase continues to hydrolyze ATP in an attempt to melt DNA. It has been reported that the helicase and ATPase activities can be uncoupled[49]. Although ATPase activity is required for helicase activity, alteration of the helicase function may not affect the ATPase activity.

p53 modulation of NER activity. The repair of UV-induced PDs from LFS cells is less efficient than in normal cells, strongly suggesting an important role for p53 in the NER pathway. The difference in repair between LFS cells and normal cells could not be explained by arrest of the cells at the G1-checkpoint as a significant difference was observed within 4 h after UV exposure while the RNA synthesis recovery was similar to normal cell strains, and heterozygous LFS cells still retain their G1-checkpoint[6,7] (M. Tainsky, personal communication). This differential repair was also observed at several other loci (Evans *et al.*, manuscript in preparation)[50], suggesting that p53 could modulate both transcription-coupled and overall genome repair pathway. It is possible that p53 inhibition of the helicase activity associated with XP proteins might provide a mechanism for efficient damage recognition, and might serve as a failsafe or alternative sensor to the multiprotein complex involving XPA, ERCC1 and other proteins[51]. DNA damage is known to trigger the accumulation of p53 protein which has been shown to bind preferentially to single-stranded DNA ends[39,40]. The notion of p53 as a monitor of DNA damage is supported by the phenotype of mice deficient for the DNA repair ERCC1 protein that exhibits a high level of the p53 protein in liver cells[52]. In addition, p53 may block the translocation of helicases from the TFIIH complex as they scan through DNA, resulting in the formation of a stable helicase-damaged DNA complex which would stimulate the formation and activity of the repair complex. The inhibition of helicases by p53 may facilitate the initiation of NER, leading to more rapid and effective repair. Moreover, we can further speculate that the inhibition of the TFIIH-associated helicase activity by p53 may lead to apoptosis (program cell death), since TFIIH is essential for cell viability.

The potential role of p53 in DNA repair is further strengthened by observations that two cancer-prone syndromes—ataxia telangiectasia and Bloom syndrome—are defective in their p53 response to UV and IR[9,10]. These patients are deficient in various damage-response genes which act upstream of p53. As in LFS, AT and BS patients develop cancers at a very young age. Thus, a new hypothetical model emerges: DNA damage increases p53 levels in cells. p53 may also act as a sensor that binds to damaged regions and recruiting the NER machinery by trapping a major component of the repair complex, TFIIH, at sites where it is needed. p53 could also interact with proteins involved in post-incision processes, such as PCNA and RPA. In fact, colocalizes p53 with several proteins, including DNA polymerase α, PCNA, DNA ligase 1 and RPA[53]. p53 also binds RPA *in vivo*[54], suggesting that p53 may be intimately involved in the assembly of the multi-complex repair machinery in many different circumstances requiring DNA repair. Inactivation of p53 could lead to an increase in mutation frequency[55–57], resulting from inefficient NER and genomic instability, manifested by gene amplification, aneuploidy and chromosomal aberrations that are associated with malignant progression. Consistent with this hypothesis is the finding that expression of mutant and wild-type p53 is more mutagenic

than expression of a mutant p53 alone (Tainsky, M.A., personal communication). Wild-type conformation of the temperature-sensitive murine p53 (Val^{135}) reduces the frequency of UV-induced point mutations in a lambda phage shuttle vector chromosomally integrated in murine L cell line (Glazer, P.M., persersonal communication). The mutation frequency of *hprt* locus is at least 100-fold higher in cells from p53-deficient mice than cells from normal mice (Wyllie, A.H., personal communication). Cells lacking wild-type p53 show increased rates of gene amplification[6,7]. p53 binds to CSB, another potential helicase that is considered to be primarily involved in transcription-coupled NER[28], suggesting that p53 may also have a role in this pathway.

Methods

Plasmids. GST-p53-WT encodes GST fused to human wild type p53. GST-p53-135Y,–249S, and –273H encode GST fused to mutant p53 His135Tyr, Arg249Ser and Arg273His, respectively[16]. ΔC50 was made by PCR amplification of the first 1028 nucleotides (nt) of p53 from pC53-SN (a gift of B. Vogelstein, Johns Hopkins University), and insertion into the *Bam*HI site of pGEX-2T (Pharmacia LKB) and encodes GST fused to residues 1–353 of p53. N5, 2C, 3C, and 25, kindly provided by B. Stillman (Cold Spring Harbor Laboratory), encode GST fused to residues 1–293, 94–393, 155–393 and 94–293 of p53, respectively[58]. pZAP10 encodes a XPB cDNA and was used for *in vitro* synthesis of XPB protein. pGEM3zf(+)T7XPD was used for *in vitro* synthesis of XPD protein. pGEM4z-SP6ccaccRAD3 was used for *in vitro* synthesis of Rad3 protein. pcBLsSE6 was used for *in vitro* synthesis of CSB.

Purification of recombinant proteins. GST fusion proteins were produced in *E. coli* and purified on glutathione-Sepharose 4B beads (GSH-beads) according to the manufacturer (Pharmacia LKB). The purified fusion proteins immobilized on the surface of GSH-beads were stored at 4 °C in phosphate-buffered saline, pH 7.4, containing 1% Triton X-100 for up to two months. Protein concentrations were determined by Coomassie blue staining of SDS/PAGE and comparison to molecular weight standards (Bio-Rad) run on the same gel. Highly purified baculovirus-produced p53-WT and p53-273H proteins were kindly provided by C. Prives (Columbia University), and were proved to be the biologically active forms (data not shown). *Pichia Pastoris*-produced wild-type p53 was a gift of P. Abarzua (Hoffman La Roche). To label the *in vitro* translated proteins, the corresponding plasmids were used in a one-step *in vitro* transcription and translation system (Promega) at r.t. (RT) for 90 min in the presence of [^{35}S]cysteine (Dupont).

***In vitro* protein binding and analysis of protein complexes.** Binding assays were as described[16]. For peptide competition study, binding assays were done by preincubation of GST-p53-WT containing beads with various concentrations of peptides for 30 min on ice followed by addition of *in vitro*-translated proteins. Representative data are shown in Figs 1, 3 & 4, all the binding experiments were repeated three or more times. The binding between experiments varies by less than 10%.

KT3-tagged XPB expression and immunoprecipitation. HepG2 and 293 cells were maintained as described[16]. Cells were cotransfected with pKT3-WT[29] and a pCMVneo plasmid by lipofection[16]. Stable transfectants were selected into 400 μg ml^{-1} of G418 containing medium. Two weeks after transfection, mass populations of G418-resistant colonies were harvested, expanded in culture as the HepG2/E3 cells and 293/E3 cells, and used for immunoprecipitation prior to third passage. The expression of the XPB protein (90 kD) in these cells was verified by immunoprecipitation of cell lysates with KT3 antibody followed by western blot with polyclonal anti-XPB antibodies (data not shown). For detection of the p53-XPB complex, cells were labelled with ^{35}S-methionine (150 μCi ml^{-1}) for 2 h and cell lysates prepared as described[16] were subjected to a double immunoprecipitation approach for detection of the p53-XPB complex *in vivo*. In first IP, cell lysates were incubated with the KT3 antibody (1 μg ml^{-1}) and the protein G plus A agarose beads for 1 h at 4 °C. Following extensive washes with IP buffer, immunoprecipitates were eluted from beads by heating with 1X gel loading buffer at 90 °C for 10 min. The IP buffer (25 volume) was added, and eluted proteins were incubated at 4 °C for 1 h. Samples were then immunoprecipitated by addition of the DO-1 antibody as described above. The immunoprecipitates were analyzed on the SDS-PAGE.

Purification of TFIIH and Rad3 proteins, and functional assay. Purification of TFIIH and all other basic transcription factors from Hela cells required for the transcription run-off assay were described[18]. The helicase and ATPase assays of TFIIH are essential as described[18,21]. Purification of Rad3 as well as its ATPase and helicase assays were described elsewhere[59].

Gene-specific damage and repair of PDs. The normal cell strains, GM00038A and GM02987, were obtained from the Coriell Institute for Medical Research (Camden, NJ). The LFS cell strains derived from the proband, designated 2675 and the strain derived from his father designated 2673, were obtained from the Family Studies Branch of the National Cancer Institute. These members of a larger LFS kindred have been shown by Srivastava and colleagues to be heterozygous for a mutation at codon 245 of the p53 gene[60]. The methodology for the examination of gene-specific repair has been described in detail[61]. Gene-specific repair was studied in fragments of *DHFR*. The 1.8 kb *DHFR* probe, obtained from G. Attardi (California Institute of Technology) detects a 21.5 kb fragment.

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