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Identification of a Functional *In Vivo* p53 Response Element in the Coding Sequence of the Xeroderma Pigmentosum Group C Gene

Kedar Hastak[1], Shanthi Adimoolam[1], Nathan D. Trinklein[2,3], Richard M. Myers[2,4], and James M. Ford[1,2]

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Abstract

The protein product of the xeroderma pigmentosum group C (XPC) gene is a DNA damage recognition factor that functions early in the process of global genomic nucleotide excision repair. Regulation of XPC expression is governed in part by p53 at the transcriptional level. To identify the regulatory elements involved in the p53-dependent control of XPC expression, we performed a quantitative PCR tiling experiment using multiple regularly spaced primer pairs over an 11-kb region centered around the XPC transcriptional start site. p53 chromatin immunoprecipitation was performed following ultraviolet irradiation, and DNA was analyzed for enrichment at each of 48 amplicons covering this region. A segment just upstream of the XPC translational initiation site was significantly enriched, whereas no enrichment of any other region was noted. *In vitro* promoter reporter assays and gel retardation assays were used to confirm the p53 responsiveness of this region and to define the minimal region with stimulating activity. We identified a p53 response element that has significant similarity to a consensus sequence, with 3 mismatches. This response element is unique in that part of the p53 binding site included the coding sequence for the first 2 amino acids in the XPC protein.

Keywords

p53, XPC, DNA damage, DNA repair

Introduction

The human nucleotide excision repair (NER) pathway is an evolutionarily conserved mechanism for DNA repair that removes a wide variety of bulky DNA adducts induced by environmental as well as endogenous sources.[1] The most relevant of the lesions repaired by NER include the ultraviolet (UV) irradiation–induced cyclobutane pyrimidine dimer (CPD) and 6-4 pyrimidine-pyrimidone photoproduct (6-4PP).[2] Two genetically distinct pathways for NER have been defined: the bulk removal of lesions over the entire genome, termed global genome repair (GGR), and the specific removal of transcription-blocking lesions from the transcribed strand of RNA polymerase II–transcribed genes, referred to as transcription-coupled repair (TCR).[1,3] Defects in NER have been attributed to the skin cancer–prone syndrome xeroderma pigmentosum (XP) as well as to other developmental disorders such as Cockayne syndrome and trichothiodystrophy.[1]

Multiple proteins in the NER pathway have been identified and well characterized in terms of their roles in recognition of the DNA adduct, excision of the lesion, gap filling, and ligation.[2,4,5] In addition to these NER factors, work from our laboratory has demonstrated that the tumor suppressor p53 is specifically required for GGR but dispensable for TCR of UV-C–induced CPDs.[6-8] Due to the differential modes of damage recognition in GGR versus TCR, we proposed that p53 may be involved in the damage recognition step in GGR and focused on the regulation of GGR-specific DNA lesion binding factors by p53 as a potential mechanism for p53-dependent NER. We previously demonstrated that p53 transcriptionally upregulates expression of the DDB2 gene,[9] found mutated in XP group E (XPE), and that overexpression of the DDB2 gene product enhances GGR in the context of a p53 deficiency.[10] XP group C (XPC) is

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[1]Department of Medicine, Stanford University School of Medicine, Stanford, CA, USA
[2]Department of Genetics, Stanford University School of Medicine, Stanford, CA, USA
[3]SwitchGear Genomics, Menlo Park, CA, USA
[4]HudsonAlpha Institute for Biotechnology, Huntsville, AL, USA

Corresponding Author:
James M. Ford, Stanford University Medical Center, Division of Oncology, 269 Campus Drive, 1115 CCSR Building, Stanford, CA 94305-5151, USA (Email: jmf@stanford.edu).

another GGR-specific gene whose protein product is involved in 6-4PP recognition[4] and, together with DDB2, is required for CPD removal.[11-14] We and others reported the transcriptional regulation of XPC by p53 in response to DNA damage and showed that the mRNA and protein products of XPC increased in a p53- and DNA damage–dependent manner.[15,16] Using consensus sequence searches and gel shift assays, we located a p53 response element in the upstream regulatory region of the XPC gene. In fact, promoter reporter studies using this DNA sequence demonstrated sequence-specific and p53-dependent activation of the firefly luciferase reporter gene. However, we observed through the use of chromatin immunoprecipitation (ChIP) assays that p53 lacked significant binding to this putative promoter element *in vivo*, thus questioning the validity of this proposed p53 binding element. More recently, Wei *et al.*[17] used a whole genome ChIP assay to identify p53 binding sites and also described a potential binding site within approximately 500 base pairs 5′ to the XPC gene coding region but did not resolve the actual binding sequence nor performed functional assays. We now report the use of genomic tiling ChIP assays to survey the entire XPC promoter and coding region and the identification of a functional p53 binding site with high resolution. We find that the p53 binding and regulatory site in XPC is uniquely located in its first exon at the translational start site and that p53 functionally regulates XPC-dependent NER activity through binding to this site.

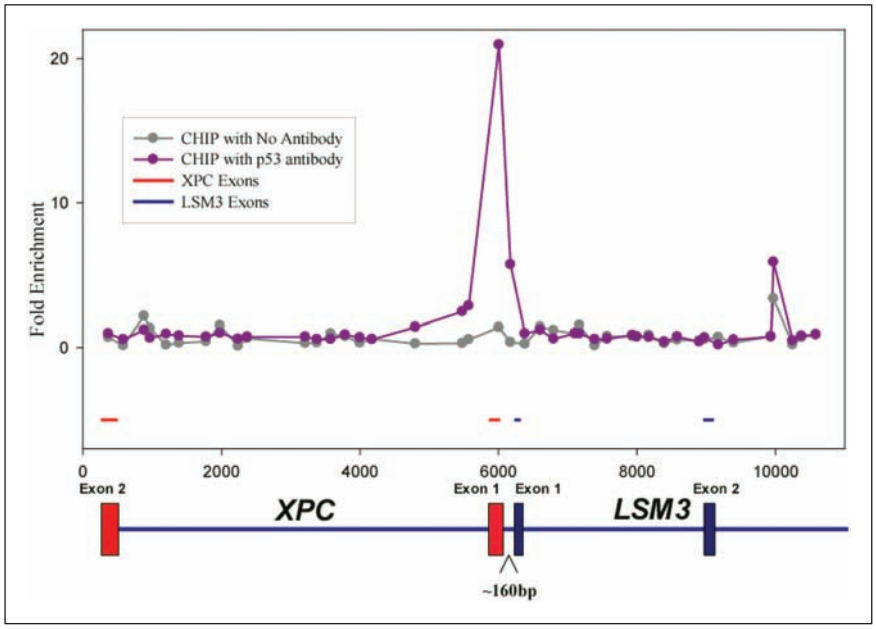

Figure 1. p53 chromatin immunoprecipitation (ChIP) with quantitative PCR tiling across the XPC gene locus. The graph represents the fold enrichment of a given amplicon and is plotted relative to the location of the amplicon in the XPC or LSM3 genes. The XPC exons are represented in red and the LSM3 exons in blue. Forty-eight primer pairs were constructed to generate evenly spaced amplicons over an 11-kb region of the genome centered around the shared promoter region of XPC and LSM3. HCT116 p53+/+ cells were irradiated with 15 J/m^2 of ultraviolet irradiation and harvested 16 to 24 hours later, following DNA protein cross-linking as detailed in Materials and Methods. Each data point represents the fold enrichment from ChIP DNA either in the presence or absence of an anti-p53 antibody.

Results

p53 ChIP assays reveal a p53 binding site upstream of the XPC coding sequence. In previous work, we identified a putative p53 response element 1,700 bp upstream from the XPC transcription start site that exhibited p53 binding activity and promoter activity in *in vitro* studies.[15] However, ChIP assays failed to demonstrate significant *in vivo* occupancy by p53 at this region (data not shown). Therefore, we designed multiple primer pairs across an 11-kb region to determine the enrichment of amplicons due to *in vivo* binding of p53 to potential regulatory sequences (Suppl. Table S1). Chromatin cross-linked DNA was isolated from HCT116 cells 16 to 24 hours after 15 J/m^2 of UV irradiation. Fold enrichment of p53-bound amplicons was determined by dividing the value obtained from each real-time PCR reaction standard curve to the average of all values below the 95th percentile, which is representative of the population that was not enriched. Figure 1 indicates the location of exons 1 and 2 of XPC and exons 1 and 2 of a neighboring divergent gene, LSM3. A distinct peak was noted at the start of exon 1 of XPC.

p53 interaction with the XPC promoter is through direct p53-DNA binding. Since initial sequence analyses did not reveal any potential p53 binding sites in the regulatory or intronic regions of XPC, we determined if the p53 ChIP enrichment was due to the direct binding of p53 to DNA or indirectly through a protein-protein interaction. A ChIP assay was performed using 087 p53 mutant human fibroblast cells (087 mut) that harbor a mutation in codon 248 of p53, resulting in a deficiency in the sequence-specific DNA binding activity of the expressed p53 protein. Figure 2A indicates that the p53 protein was expressed and detectable using conventional immunoprecipitation (IP) from both the HCT116 p53 wild-type (wt) cell line and the 087 mut cell

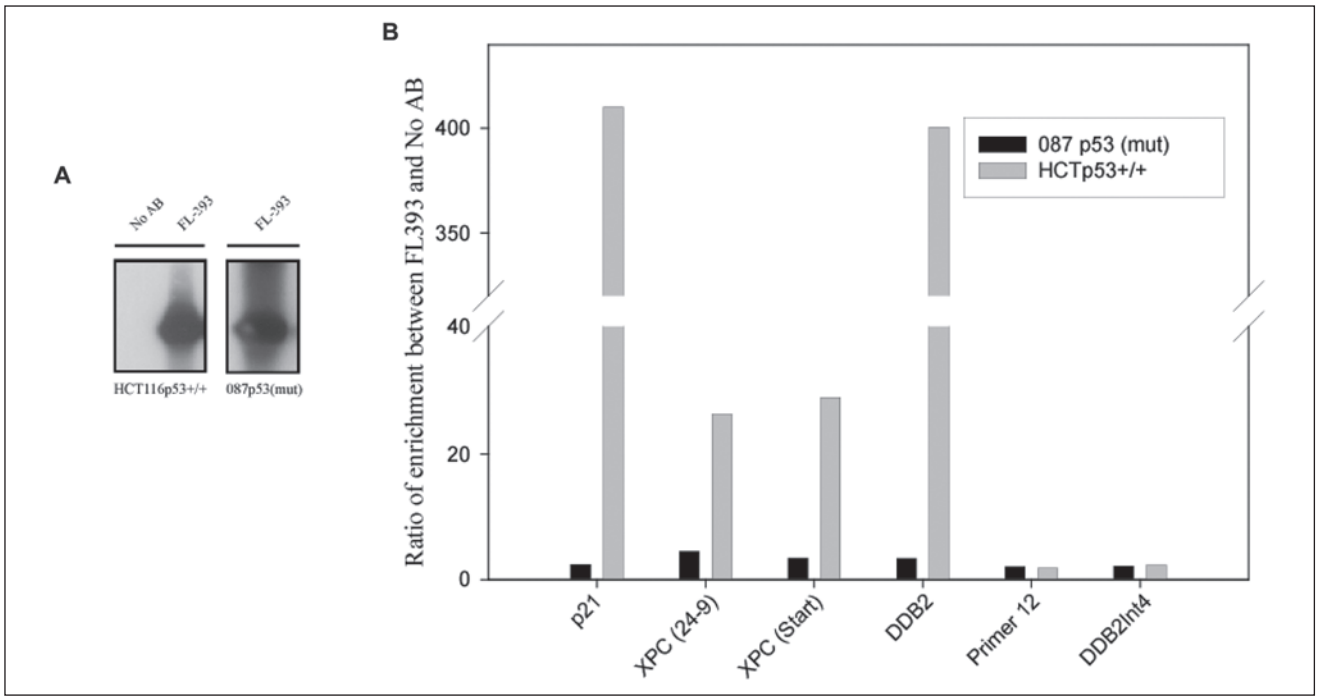

Figure 2. Relative enrichment of p53 binding sites in HCT116 p53+/+ and 087 mut cells. (**A**) Immunoprecipitable p53 is observed at similar levels in both HCT116 p53+/+ and 087 mut cells. Anti-p53 (FL-393) antibody was used for the chromatin immunoprecipitation (ChIP), and anti-p53 (DO-1) antibody was used to probe the Western blot. (**B**) The experimental Ct value for a given amplicon was fit to the standard curves generated using that primer pair, and the quantity of template containing that specific amplicon was calculated. Fold enrichment of a given amplicon by ChIP was represented as the ratio of the experimental promoter quantity to a no-antibody control. p21 and DDB2 primer pairs were used to test the identified p53 response elements in the respective promoters. XPC1 and XPC2 are 2 primer pairs that generate amplicons around the XPC translational start site. Primer 12 and DDB2Int4 are negative controls that result in amplicons not enriched by p53 (Suppl. Table S1).

line. In addition to the conventional IP, a ChIP assay was also performed using the 087 mut cells. The ChIP DNA was analyzed using primer pairs to p21 and DDB2 p53 response elements as controls and to the XPC1 and XPC2 primers around the XPC start site and 2 previously tested negative control primers, primer 12 and DDB2 intron 4 (Suppl. Table S2). Figure 2B indicates the comparative enrichment of known or putative p53 binding sites in p21, DDB2, and XPC. Although significant binding of p53 to the p21, DDB2, and XPC promoter sites was noted in p53 wt HCT116 cells, such binding was completely absent in the 087 mut cells.

p53 binds specifically in vitro *to a region in exon 1 of XPC.* Electrophoretic mobility shift assays were used to study the binding properties of p53 to various segments centered around the translational start site of XPC. The locations of the probes studied relative to the initiator codon of XPC are shown in Figure 3A. Binding of transiently overexpressed p53 in H1299 nuclear extracts to the various probes is indicated in Figure 3B. p21 and GADD45 probes were used as positive controls. A strong band shift was seen in the extracts with p53, which is absent in extracts lacking p53. This band was supershifted in the presence of the pAb421 anti-p53 monoclonal antibody. A similar binding of p53 to XPCGS1 was seen, whereas no such binding is observed when XPCGS2 or XPCGS3 probes were used, thus implying that the 8 bp in XPCGS1 that are missing in XPCGS2 are important for p53 binding. Figure 3C shows the specificity in binding of p53 to the XPCGS1 probe. Excess cold probe outcompeted the shifted band, whereas a nonspecific probe of similar length failed to compete. The band was also further supershifted by a second p53 antibody, DO-1.

The XPC p53 binding sequence directs p53-dependent luciferase reporter gene expression in vitro. Luciferase reporter assays were used to determine whether the identified p53 binding region in the XPC promoter displayed a p53-dependent transcription-enhancing activity. The reporter constructs were created by cloning in varying lengths of the XPC promoter region as represented in Figure 4A. Five constructs (pGL3XPCREV del1-5) contained inserts of progressively shorter length approaching the XPC translational start site, and 5 additional constructs (pGL3X-PCFOR del1-5) contained increasingly shorter sequences localizing away from the start site. The results of the luciferase activity assay for the wt reporter (pGL3XPC) and the deletion mutants are summarized in the graph in Figure 4A.

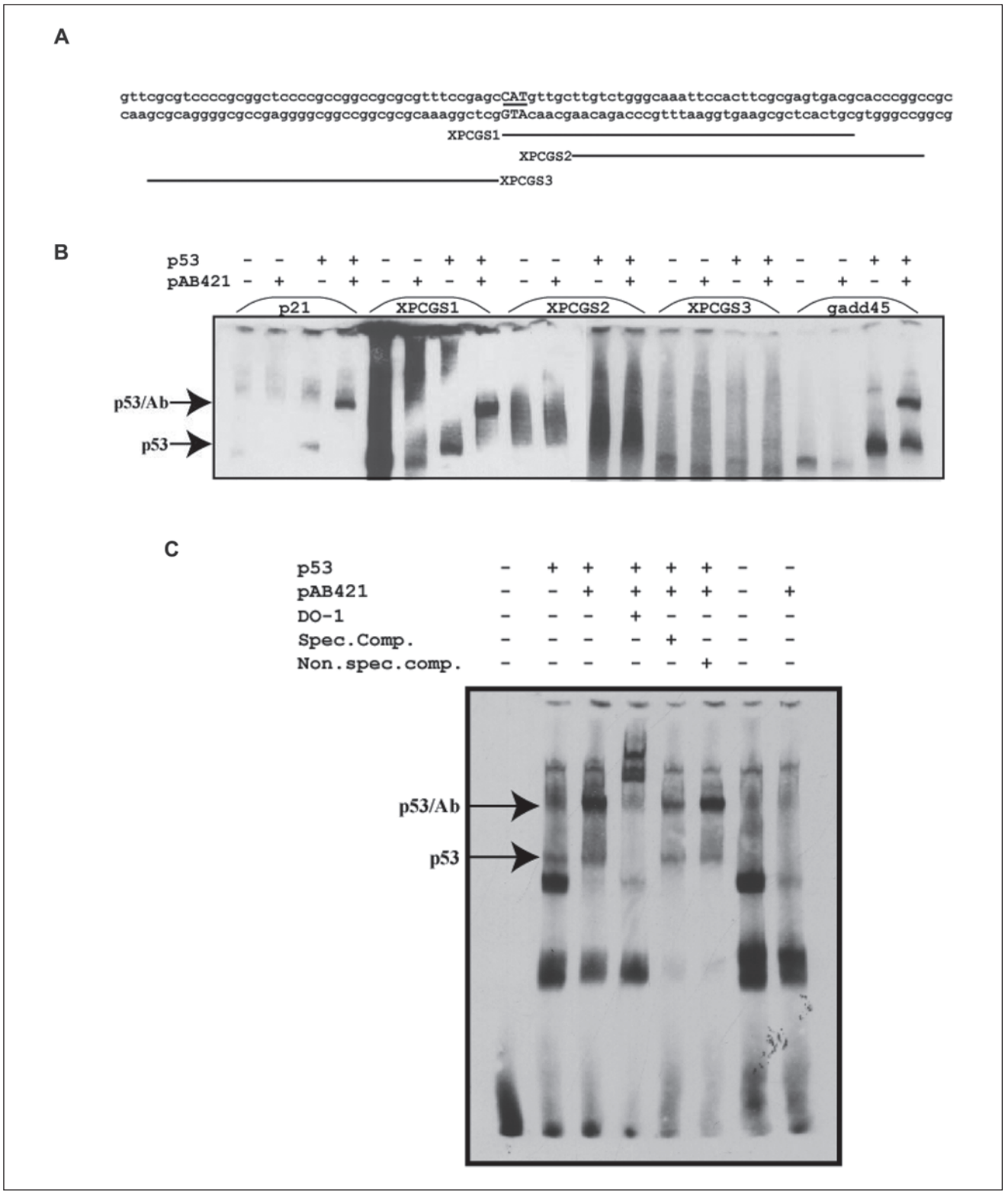

Figure 3. *In vitro* binding of p53 to a sequence in exon 1 of XPC. (**A**) Shown are the locations of the 3 probes, XPCGS1, XPCGS2, and XPCGS3, used for the gel retardation assay in relation to the XPC translational start site. (**B**) There were 10 ug of nuclear extracts from H1299 cells with or without transiently expressed p53 that were used in EMSA binding reactions both in the absence and presence of an activating p53 antibody, pAb421. The binding conditions are as described in experimental procedures. For each probe, the first 2 lanes are ±p53 antibody and without p53, whereas the next 2 lanes are in the presence of transiently expressed p53. The arrows indicate the p53 and p53/AB bands shift. (**C**) The specificity of binding to XPCGS1 is indicated in this image. The p53 and p53/AB shifted bands are indicated by the arrows. Lane 4 indicates a further supershift by the addition of a second p53 antibody, DO-1. The p53/AB band is outcompeted by a specific cold probe (lane 5), but a nonspecific competitor has no effect on binding (lane 6). The last 2 lanes are negative controls with H1299 nuclear extracts in the absence of p53.

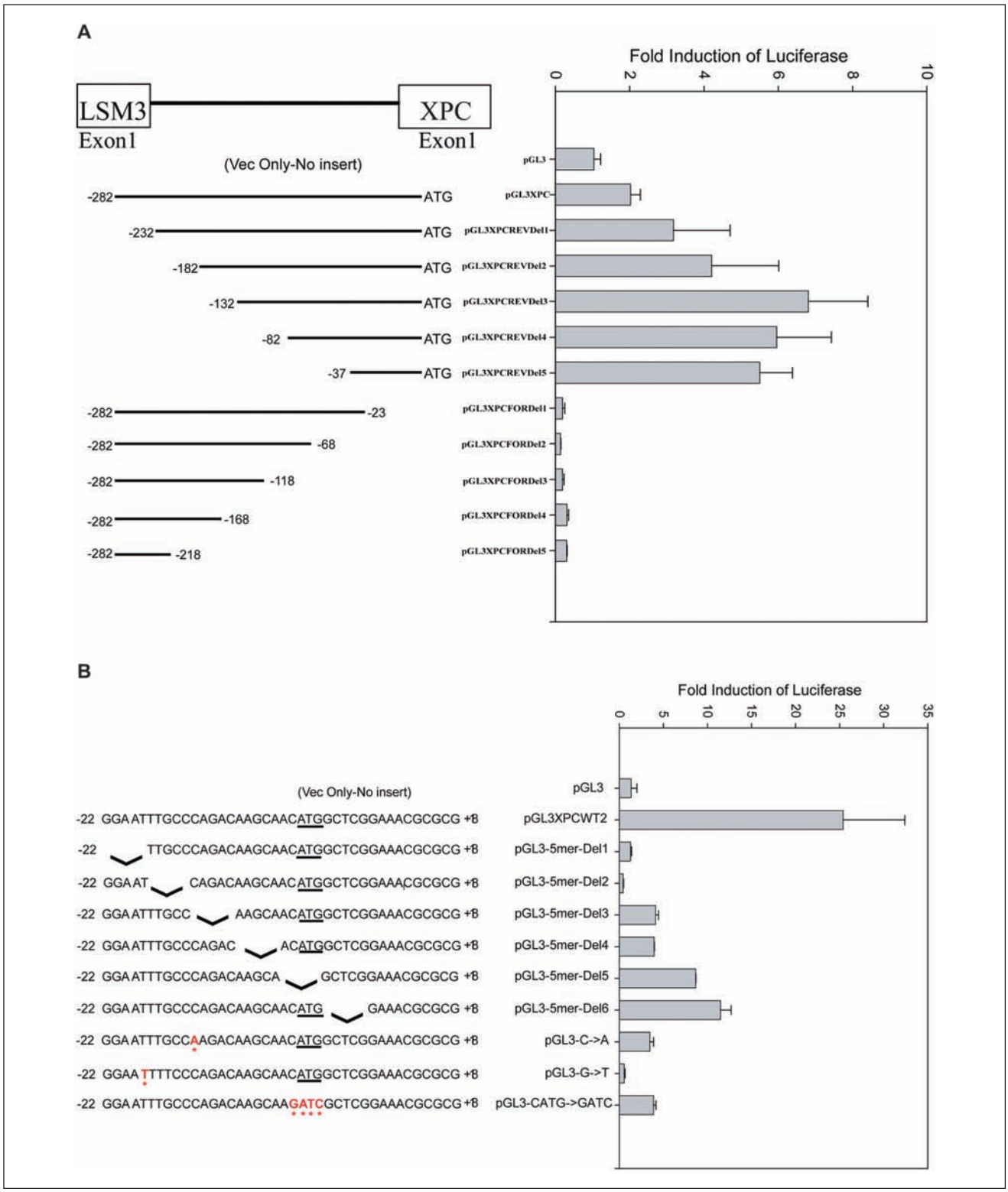

Figure 4. Promoter activity and p53-dependent reporter gene expression by the enhancer element in exon 1 of XPC. (**A**) The p53-dependent induction of luciferase expression using various deletions of the promoter region of XPC is represented in this image. Rev del1-5 retains the XPC translational start site and contains varying lengths of the promoter region. For del1-5 lacks the XPC translational start site and varying lengths of the promoter from the XPC start site. The locations of the deletions are represented on the left-hand side and are shown relative to the start site of XPC. The fold induction is represented as the ratio of the firefly luciferase value (after normalization with the corresponding Renilla luciferase value) in the presence of p53 to that in the absence of p53. (**B**) A 40mer sequence around the XPC translational start site (pGL3XPCWT2) was used to construct 5-bp deletions as well as point mutations to determine the essential elements of the p53 enhancer element in the XPC gene. The sequences are depicted on the left, with the XPC start site underlined, and the deletions are shown by the gaps. The point mutants are represented in red, with an asterisk indicating the substituted base pair. The fold inductions are calculated as described in Figure 4A. Experiments were done 3 independent times in triplicate. Data are representative of one such experiment, and error bars represent standard deviation.

The fold induction of luciferase by a given construct is represented as the fold increase in the ratio between the firefly and Renilla luciferase signals in the presence versus the absence of p53 expression following transient transfections in p53–/– HCT116 cells. The pGL3XPCREV del1-5 constructs displayed a gradual increase in the p53-dependent expression of luciferase with the narrowing of the insert to the specific p53 enhancer region. pGL3XPCFOR del1-5 showed no dependence of p53 activity on luciferase expression in all 5 constructs, implying that the p53 response element is localized to the region within the first 37 nucleotides upstream of the XPC initiator codon. An additional set of 40mers were created omitting various sequences around the XPC translational start site, and the minimal sequence required for maximal p53 responsiveness was determined (data not shown), part of which included the start of the XPC coding sequence. This sequence is represented as pGL3XPCWT2 in Figure 4B. Six 5mer deletions within this sequence were constructed, and their relative contributions to p53 activity are shown in the graph. In addition, 3 point mutants were created as shown in the figure, and all 3 sites appeared to be required for full p53 function, although to slightly varying degrees. Overall, these smaller deletions serve to further confirm the importance of this region in the p53-dependent regulation of the XPC gene.

The XPC p53 binding sequence is crucial for NER. After identifying the p53 binding region, we wanted to investigate the effect of this p53 response element on functional NER activity. Therefore, we transfected each of the six 5mer deletion vectors along with wt XPC and control pGL3 vector into HCT116 cells and studied the repair of CPDs and 6-4PPs following UV-C irradiation. As shown in Figure 5, all 5mer del1-5 constructs exhibited lesser enhancement of CPD and 6-4PP repair 24 hours after UV irradiation compared to the wt construct, whereas del6 had similar repair efficiency as wt XPC. Each cell line was compared to a 0-hour control at which time no repair had occurred, and the percentage of repair at 24 hours was calculated. Thus, transient overexpression of wt XPC is enough to increase DNA repair efficiency in HCT116 cells. Moreover, deletions closer to the transcription start site exhibited lesser enhancement of CPD and 6-4PP repair. This shows that p53-XPC binding is critical for repair of UV-induced lesions.

A p53 response element is located in the coding sequence of XPC. Following identification of the minimal element required for maximal p53-dependent *XPC* gene activity, sequence analysis was performed using the p53 consensus binding element,[18] and a region of significant similarity was identified as shown in Figure 6. This sequence had 2 p53 half-sites with a 7–base pair spacer and contained 3 mismatches from the consensus. The protein translation for part of the sequence is also indicated, as the identified response element includes the start of the XPC protein coding sequence.

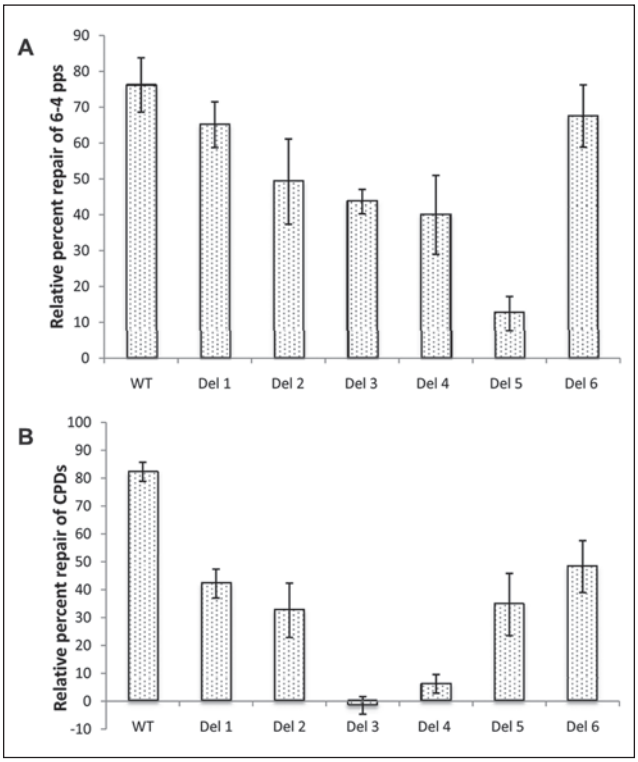

Figure 5. The XPC p53 binding sequence is crucial for nucleotide excision repair. Cells were transfected with either wild-type XPC or XPC del vectors as indicated, followed by irradiation with 20 J/m^2 of ultraviolet C (UV-C). Repair of cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) was measured by ELISA and the percentage lesions 24 hours after UV irradiation compared to that at 0 hours, termed relative repair. (**A**) Relative repair of 6-4PPs in HCT116 cells. (**B**) Relative repair of CPDs in HCT116 cells. Experiments were done 3 independent times in triplicate. Data are representative of one such experiment, and error bars represent standard deviation.

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p53 consensus :   RRRCWWGYYYNNNNNNNRRRCWWGYYY
Mismatches    :      *           *        *
XPC p53RE     :   GAATTTGCCCAGACAAGCAACATGGCT
Translation   :                        M  A
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Figure 6. Sequence analysis of the XPC start site. Shown is an alignment of the XPC start site with the consensus binding sequence of p53. A p53 response element is present with 3 mismatches; however, of note is that part of the response element codes for the first 2 amino acids in XPC.

Discussion

The XPC NER gene product is important in human carcinogenesis and aging.[5,19,20] The role of XPC as an early DNA damage recognition factor in GGR of UV photoproducts has been well established,[4,21,22] and XPC appears to function in the removal of oxidative DNA damage as well.[20] The mode of XPC regulation following DNA damage is not clearly understood. Although XPC appears to preferentially bind 6-4PPs but not CPDs *in vitro*, it is essential for repair of CPDs both *in vitro* as well as *in vivo*.[4,11,14,23] We and others demonstrated that XPC is a UV-inducible factor and that induction of XPC mRNA and protein levels requires functional p53.[15,16] In that report, we identified a putative p53 response element 1,700 bp proximal to the XPC gene with an intriguing sequence structure. Using *in vitro* gel shift assays, we showed specific binding of p53 to this response element. We subsequently investigated this element further using *in vitro* promoter reporter assays, and the identified site indeed appeared functional. However, when *in vivo* binding of p53 to this response element was tested using ChIP, none could be detected, thus questioning the functional activity of this XPC promoter region. Wei *et al*.[17] used a whole genome ChIP assay together with paired-end ditag sequencing to map potential p53 binding sites across the genome and described a site within approximately 500 base pairs of the XPC start site but did not provide resolution at the sequence level.

To identify functional p53 response elements in the XPC regulatory domains, we simultaneously analyzed potential p53 binding *in vivo* across an 11-kb genomic region centered around the XPC transcriptional start site. Multiple evenly spaced primer pairs allowed us to scan this entire region and aided in the identification of a single p53 binding site that would likely have been overlooked by sequence analysis, especially given the large size of the XPC gene, the location of the element, and the number of deviations from the consensus sequence. This binding was due to a direct interaction of p53 with the promoter because mutant p53 lacking sequence-specific DNA binding did not associate with the XPC promoter *in vivo*. This observation was further confirmed by *in vitro* gel retardation assays that showed that p53 selectively bound to this element but not to any of the adjoining sequences. Reporter assays demonstrated that the identified region is capable of functioning as an enhancer to mediate p53-dependent transcription and facilitated in the identification of critical residues in the response element that arbitrate p53 binding.

The approach we took was powerful for several reasons. It combined the enhanced sensitivity of real-time PCR along with the ChIP assay and enabled the identification of strong and relatively weaker p53 transcription factor binding sites. In comparison to the p53 response element in p21, the binding of p53 to the XPC genomic element is weaker, which is not surprising given that XPC is not induced to the same level as p21 following DNA damage.[15] Nevertheless, the regulation of XPC expression is clearly relevant to functional NER activity, as shown in Figure 5. The transient overexpression of wt XPC is enough to enhance DNA repair of CPDs and 6-4PPs. Moreover, as transient transfections result in gene overexpression, the expression of deleted XPC vectors was enough to mask the effect of native XPC proteins in HCT116 cells. Thus, we were able to show that XPC gene binding by p53 is critical for efficient DNA repair activity. Secondly, large regions of the genome were simultaneously analyzed from a single ChIP assay, with much higher resolution than reported using whole genome approaches.[17,24] The finer details of the critical residues involved in binding can then be elucidated using *in vitro* approaches as demonstrated in this study. The final point to be noted is that transcription factor binding sites can also be located in uncommon regions, such as the coding sequence of genes as seen with the case of XPC, and genome-wide scanning of p53 binding sites typically does not include exonic regions of the genome.[24] This report, to our knowledge, is the first observation of a p53 binding site in the protein coding sequence of its target gene. Whether the location of the p53 response element as part of the coding sequence of XPC or other genes has any bearing on the extent or mode of gene regulation awaits further investigation.

It is also of interest that the regulation of XPC gene expression is governed by a small promoter region of approximately 160 nucleotides that is shared by the divergent *LSM3* gene, a member of the LSM superfamily of genes involved in RNA processing.[25] Whether LSM3 is also transcriptionally regulated by p53 is currently being investigated; however, our preliminary results suggest that the shared promoter element can function in a bidirectional manner. Bidirectional promoters often co-regulate the expression of their respective genes.[26] Thus, it is possible that the identified p53 response element, although proximal to the XPC gene start site, may regulate expression of the LSM3 gene.

Finally, our work provides strong evidence that XPC is a *bona fide* p53 target gene involved in p53-dependent NER. The regulation of XPC is complex, and not only is it UV inducible at the transcriptional level,[15] but XPC protein activity is also affected by multiple posttranslational factors. XPC localization to sites of UV photoproducts, and in particular CPDs, requires adequate levels of the p53-regulated DDB2 gene product.[14,27,28] In addition, XPC is ubiquitylated rapidly following DNA damage, and this may regulate its ability to form DNA damage recognition and repair complexes at the sites of UV photoproducts.[29-31] Therefore, p53 regulates NER through multiple interactions, both transcriptionally and posttranslationally. The role of p53 in regulating DNA repair is critical to DNA damage response pathways and in human tumorigenesis.

Materials and Methods

Cell culture and transfections. HCT116 human colon carcinoma cells, wt or with a homozygous knockout of the p53 gene (p53–/–), were cultured in McCoy's 5A modified medium supplemented with 10% fetal bovine serum and antibiotics at 37°C and 5% CO_2. H1299 p53-null human lung carcinoma cells and 087 mut cells were grown in DMEM (high glucose) containing 10% serum and antibiotics at 37°C and 5% CO_2.

For the reporter studies, HCT116 p53–/– cells were seeded at a density of 0.05×10^6 per well in a 24-well dish 2 days prior to transfection. Transfections were performed using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA), as per the manufacturer's instructions, using 0.4 μg of each construct per well. The appropriate reporter and co-reporter vectors were transfected at a ratio of 50:1, and the reporter and p53 expression plasmid or the corresponding empty expression plasmid were transfected at a ratio of 1:1. Transfections were carried out in triplicate.

ChIP assay. The p53 ChIP assay was performed using the ChIP assay kit (EMD Millipore, Billerica, MA) with minor modifications. Briefly, HCT116 wt cells or 087 mut cells were grown in 15-cm dishes to 80% confluency. Cells were UV irradiated or not with a dose of 15 J/m^2 and harvested immediately or incubated for varying lengths of time after UV irradiation in growth medium. At the appropriate time points, cells were trypsinized and cross-linked with 1% formaldehyde (in PBS) for 5 minutes at room temperature. Cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M for 10 minutes. Cells were then washed twice with PBS and lysed in 1 mL of lysis buffer containing a protease inhibitor cocktail (Sigma, St. Louis, MO). The lysates were sonicated to shear the chromatin to fragments of approximately 500 to 1,000 bp in length. Lysates were diluted 1:10 in ChIP dilution buffer and precleared with Protein A agarose (PAA) beads (Upstate Biotechnology) for 1 hour with rocking at 4°C. Chromatin-bound p53 was then incubated with 3 μg of FL-393 anti-p53 polyclonal antibody at 4°C overnight with gentle rocking, and the antibody-antigen immune complexes were precipitated by incubation with PAA beads for 1 hour. Immunocomplexes were washed sequentially with low and high salt buffers, with a LiCl wash followed by 2 washes with TE buffer. Samples were rocked on ice with the wash buffers for 5 minutes each. The protein-antibody complexes were eluted (1% SDS, 0.1 M $NaHCO_3$) and the cross-links reversed by the addition of NaCl and incubation at 65°C for 4 hours. ChIP DNA was purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA).

Real-time quantitative PCR and p53 binding analysis. Quantitative PCR was carried out to measure the amounts of template containing a particular amplicon as defined by a specific primer pair. Primers were designed using Primer3 (Broad Institute, Cambridge, MA) to generate evenly spaced amplicons over an 11-kb region centered around the XPC transcriptional start site. The primer sequences are included in the supplementary material (Suppl. Table S1). For each primer pair, 4 genomic DNA standards ranging from 60 ng to 60 pg in a 10-fold dilution series were included in the reaction to determine primer efficiency, along with the no-antibody control DNA and the p53 ChIP DNA. Real-time PCR reactions were carried out in buffer containing 3.5 mM $MgCl_2$, 0.125 mM dNTPs, 0.5 μM each of forward and reverse primers, 0.5X SyBr Green (Molecular Probes, Eugene, OR), and 1 U Stoffel fragment (Applied Biosystems, Foster City, CA), or alternatively, a commercial SyBr green master mix (Stratagene, La Jolla, CA) was used. Template DNA was diluted 1:10 and added to a final volume of 20 μL. Product accumulation was measured over 40 cycles using the ABIPrism 7900HT sequence detection system (Applied Biosystems), and the threshold cycle (Ct) for each reaction was used to determine the enrichment of a given amplicon over a control amplicon. The threshold cycle is the cycle at which the fluorescent signal reaches an arbitrarily set threshold near the middle of the log-linear phase of amplification. The experimental Ct value for a given amplicon was fit to the standard curves generated using that primer pair, and the quantity of template containing that specific amplicon was calculated. Fold enrichment of a given amplicon by ChIP was represented as the ratio of the experimental promoter quantity to a negative control promoter quantity.

Gel retardation assays. The p53 response elements in the p21, GADD45, and XPC promoters were used as probes to detect sequence-specific p53 binding *in vitro*. The probes used were as follows: p21: 5′-TGG CCA TCA G GAACA TGT CCC AAC ATG TTG AGC TCT GGC A-3′; Gadd45: 5′-TGG TAC AGA ACA TGT CTA AGC ATG CTG GGG ACT G-3′; XPCGS1: 5′-CAT GTT GCT TGT CTG GGC AAA TTC CAC TTC GCG AGT GAC G-3′; XPCGS2: 5′-TTG TCT GGG CAA ATT CCA CTT CGC GAG TGA CGC ACC CGG C-3′; XPCGS3: 5′-CGC GTC CCC GCG GCT CCC CGC CGG CCG CGC GTT TCC GAG C-3′. The underlined sequences represent established p53 response elements. Nuclear extracts from H1299 cells alone and H1299 cells transiently overexpressing p53 were prepared using the CelLytic Nuclear extraction kit (Sigma). Briefly, cells were washed and lysed at 4°C in hypotonic lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) containing 10 mM DTT and protease inhibitors. To the swollen cells in lysis buffer, Ipegal CA-630 (Sigma) was added to a final concentration of 0.6%. The lysates were vortexed and centrifuged for 30 seconds at 10,000 rpm. The supernatants (cytoplasmic fraction) were collected, and the nuclei pellet was resuspended in extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 10 mM DTT,

protease inhibitor). Following agitation on a vortexer for 15 to 30 minutes, the samples were centrifuged at 20,000*g* for 5 minutes, and the supernatant (nuclear fraction) was removed and stored at –80°C. The binding reactions were set up with 10 μg of nuclear extract with or without 1 μg of pAb421 anti-p53 antibody for 20 minutes at room temperature in a buffer containing 10 mM Tris.HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM $MgCl_2$, and 0.05% NP-40, followed by another 20 minutes of incubation with the addition of 20 fmol of biotin end-labeled DNA probe. Biotin labeling of probes was carried out using the Biotin 3′ end DNA labeling kit (Pierce, Rockford, IL) as per the manufacturer's instructions. Single-stranded complementary oligos were individually labeled and annealed for 1 hour at room temperature to produce double-stranded probes. For specific or nonspecific competition or antibody supershifts, the respective unlabeled DNA probe (50-fold excess) or antibody (2 μg) (DO-1, Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction mixture prior to the addition of the labeled probes. The free probe and protein-DNA complexes were separated on a 4% neutral polyacrylamide gel in 0.5 × TBE at 120 V and 4°C. Transfer to nylon N+ membranes was carried out in 0.5 × TBE at 380 mA at 4°C for 1 hour, cross-linked, and detected by the Lightshift EMSA kit (Pierce) using streptavidin-HRP binding and chemiluminescent detection.

Reporter plasmid constructs. The 290-bp region between the 2 translational start sites of the XPC and LSM3 genes was PCR amplified and subcloned into the KpnI/BamHI site of the pGL3 basic vector (Promega, Fitchburg, WI) upstream of the firefly luciferase coding sequence to determine both the basal and p53-dependent promoter activity of this fragment. This construct was labeled as pGL3XPC. A similar length fragment from intron 1 of XPC not including the translational start site was cloned into the vector and labeled pGL3XPCnull. Sequential deletions of the insert in pGL3XPCREV from both ends were created, namely pGL3XPCfor.del 1-5 and pGL3XPCrev.del 1-5, with pGL3XPCrev.del5 being a 40mer right at the XPC translational start site. The minimal construct required for maximal reporter expression was labeled pGL3XPCWT2 using this as template, smaller 5mer deletions within this sequence (pGL3-5merDel 1-6) as well as point mutants (pGL3-C->A, pGL3-G->T and pGL3-CATG->GATC) were constructed to pinpoint the specific nucleotides critical for its function as a p53 response element. The point mutants were generated using the QuikChange XL site-directed mutagenesis kit (Stratagene, Santa Clara, CA). Supplementary Table S2 provides a summary of all the primer sequences used to generate the pGL3 constructs. To control for transfection efficiencies, the phRG-TK vector (Promega) containing the coding sequence for the Renilla luciferase enzyme was co-transfected along with the various pGL3 constructs. For p53 expression, the p53 coding sequence of wt p53 was cloned into the BamHI site of the pIRES2-EGFP (Clontech, Mountain View, CA) mammalian expression vector.

Luciferase reporter assays. The dual luciferase assay system (Promega) was used to assay for reporter gene expression driven by the inserted response element. Briefly, cells were lysed in the passive lysis buffer provided 24 hours following transfection. There was 2 μL of the lysate added to 50 μL of luciferase assay reagent II, and the firefly activity was recorded manually using a luminometer. The signal was quenched using 50 μL of the Stop & Glo Reagent (Promega), and the Renilla activity was recorded. The relative ratios of the 2 luciferase signals were used to calculate the fold induction of reporter expression in the presence of p53.

Western blotting. Immunoblot analysis was performed to determine p53 expression in 087 mut cells. Antigen-antibody-PAA complexes following IP were washed as described earlier, boiled in 25 μL of 1X SDS loading dye, and electrophoresed on a 10% SDS-PAGE gel. Following transfer, the membranes were probed with anti-p53 antibody (DO-1, 1:2,000) (Santa Cruz Biotechnology) and a horseradish peroxidase–conjugated anti-mouse secondary antibody (1:5,000) (Pierce). Protein bands were detected using the supersignal chemiluminescent substrate (Pierce) and autoradiography (Eastman Kodak Co., Rochester, NY). To confirm the overexpression of XPC, HCT116 cells were transiently transfected with empty vector or vector containing wt XPC. Forty-eight hours after transfection, cells were lysed, and immunoblot assay was performed to determine the expression of XPC using anti-XPC (Abcam, Cambridge, UK) antibody (Suppl. Fig. S1).

NER assay. HCT116 wt cells were grown overnight in 6-well plates (in triplicate). Cells were then transiently transfected with either empty pGL3 vector or pGL3XP-CWT2 or pGL3-5merDel 1-6 (as described above) using Fugene 6 (Roche Diagnostics, Indianapolis, IN), as per the standard protocol. Forty-eight hours after transfection, cells were rinsed with PBS, followed by exposure to 20 J/m^2 of UV-C irradiation. Genomic DNA was extracted (QIAamp DNA mini kit, Qiagen) at 0 to 24 hours. Repair of CPDs and 6-4PPs was measured using ELISA. Briefly, genomic DNA was distributed in triplicate onto microtiter plates precoated with 0.003% protamine sulfate. DNA lesions were detected with either 1:5,000 TDM-2 (for CPDs) or 1:5,000 64M-2 (for 6-4PPs), a gift from Dr. Toshio Mori.[32] The signals were amplified and subsequently developed with 3,5,3′,5′-tetramethylbenzidine (TMB) (Sigma). Absorbance was measured at 450 nm. Each experiment was repeated 3 independent times, and representative data are shown.

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