[Cancer Biology & Therapy 1:2, 145-149, March 2002]; ©2002 Landes Bioscience

Research Article

A Nucleotide Excision Repair Master-Switch: p53 Regulated Coordinate Induction of Global Genomic Repair Genes

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Received 10/10/01; Accepted 10/26/01

Previously published online as a CB&T "Paper in Press" at http://www.landesbioscience.com/journals/cancerbio/papersinpress/inpress12.html

KEY WORDS

DNA repair, Gene induction, Ionizing radiation, XP, GADD45a

ABSTRACT

The tumor suppressor gene p53 is mutated in many human cancers. One of its major roles is as a transcription factor, and its many effector genes control key cellular processes including cell cycle checkpoints and apoptosis. An important role in DNA repair is also emerging for both p53 itself and some of its effector genes. The products of two p53-regulated genes, GADD45a and DDB2, are now known to participate in the global genomic repair (GGR) sub-pathway of nucleotide excision repair (NER). We recently reported the induction of a third GGR gene, XPC, following exposure of normal human peripheral blood lymphocytes to γ-rays. We now show that XPC is induced in a variety of human cell lines in response to both ionizing and ultra-violet (UV) radiation and alkylating agents, and that this induction requires wild-type p53.

INTRODUCTION

Since the connection was first made between the tumor suppressor p53 and nucleotide excision repair,[1-3] it has become clear that certain p53-effector genes and perhaps p53 itself are involved in NER. However, unlike classical DNA repair-deficient cells, such as from xeroderma pigmentosum (XP) group A or Cockayne's syndrome, p53-deficient tumor lines do not show severe hypersensitivity to genotoxic agents whose damage is repaired by NER. This may reflect the competing defects in apoptosis that promote survival, which we have termed "the two faces of p53."[4,5] Another possibility is that p53 primarily affects only one subpathway of NER. Two NER subpathways have been described: "global genomic repair" (GGR), which operates genome wide and on the non-transcribed strand (NTS) of active genes, and "transcription-coupled repair" (TCR), which ensures rapid and efficient clearance of lesions from the transcribed strand of active genes.[6,7] After UV-type damage, a deficiency in TCR is associated with more cytotoxicity compared to a deficiency in GGR. For example, Cockayne syndrome cells, which are deficient in TCR only, and XP group A cells, which are deficient in both subpathways, show severe UV sensitivity, while typically milder sensitivity has been observed for XP group C[8,9] and E[10,11] cells, which are deficient only in GGR. Ford and Hanawalt have reported that p53-deficient cells show only a reduction in GGR[3] and more recently that the p48 XP group E (*DDB2*) gene is p53-regulated.[10,11] The p53-regulated[12] *GADD45a* gene has also been shown to play a role in the GGR pathway of NER.[1,13,14] We discuss here a further association between GGR and p53 involving *XPC* and *GADD45a*.

MATERIALS AND METHODS

Cell Culture and Treatment with DNA-Damaging Agents. The human myeloid leukaemia cell line ML-1, and other human cancer cell lines from the NCI cell screen panel were used in this study. The myeloid/lymphoid cell lines ML-1, SR, and Molt4, as well as RKO (colon carcinoma), A549 (lung carcinoma) and MCF7 (breast carcinoma) have all been shown to have wild-type p53 function.[15] The myeloid/lymphoid cell lines CCRF-CEM, HL60, and K562, as well as H1299 (lung carcinoma), RKO/E6 (colon carcinoma stabily expressing E6), MCF7/E6-1b (breast carcinoma stabily expressing E6) and T47D (breast carcinoma) have all been demonstrated to have absent or abnormal p53 function.[15,16] TK6 (ATCC CRL-8015) is a p53 wild-type B-lymphoblastoid cell line.[17] NH32 is a targeted p53 null cell line derived from TK6.[18]

All cell lines were grown in RPMI 1640 medium supplemented with 10% heat-inactivated (56°C for 45 minutes) fetal calf serum and 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified, 5% CO_2 atmosphere in a 37°C incubator. Cells were irradiated at approximately 2.96 Gy/min. to a total dose of 20 Gy using a Mark I-68 ^{137}Cs source (J.L. Shepherd and Associates, Inc.). Irradiated cells were incubated at 37°C for 2, 4, 8 or 24 hours, and RNA was extracted using a modified guanidine thiocyanate method.[19] For UV exposure, suspension cells were pelleted by centrifugation,

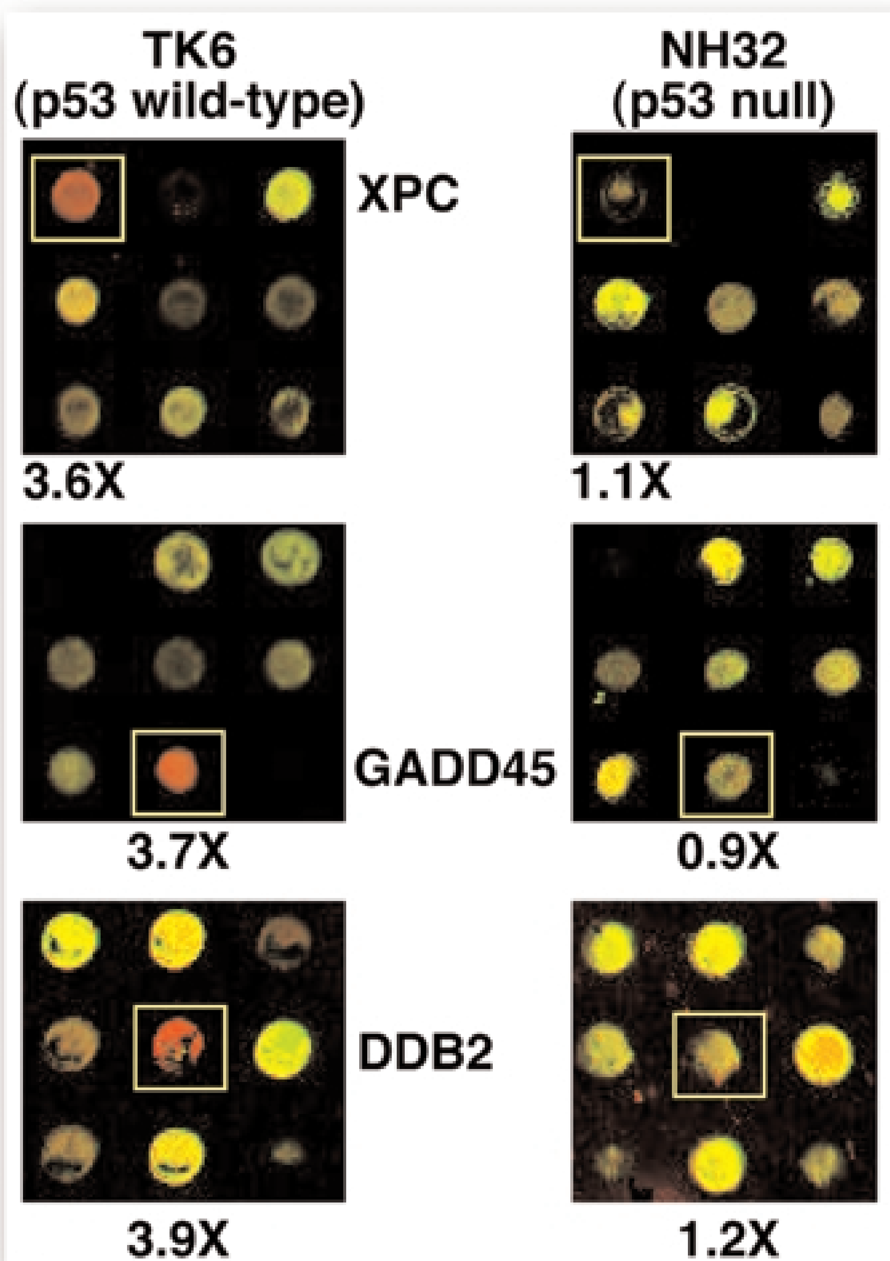

Figure 1. Sections of microarrays hybridized to RNA isolated from unirradiated (shown in green) and 8 Gy gamma-irradiated (red) TK6 or NH32 cells 4 hours after irradiation. Red spots result from greater hybridization of a specific transcript from the irradiated sample as compared to the control, while transcripts that remain unchanged following irradiation produce yellow spots. Yellow boxes indicate the position of the indicated gene in each array section shown. The red:green ratio calculated from the hybridization intensity of the irradiated:control samples for each of the genes of interest is indicated beneath the respective panel. This number represents the fold-induction above basal expression resulting from irradiation in this experiment.

resuspended in "RPMI saline" (100 mM NaCl; 5.4 mM KCl; 11 mM d-glucose; 5.6 mM Na_2HPO_4; 0.4 mM $MgSO_4$; 0.4 mM $CaCl_2$) and irradiated as previously described[20] with germicidal lamps at a dose rate of 2.41 J/m^2/sec. to a dose of 8 J/m^2. The cells were resuspended in fresh medium and incubated at 37ºC for 4 to 8 hours before harvesting the RNA. For methyl methanesulfonate (MMS) treatment, MMS (Aldrich) was added to the medium to a concentration of 25, 50 or 100 mg/ml and incubated for 4 hours, when either the RNA was harvested, or the MMS medium was removed and replaced with fresh medium for longer incubations.

Microarray Hybridization and Analysis. 100-200 µg of whole-cell RNA was labeled and hybridized to 6728 element microarrays as described previously.[21] In brief, probes were prepared by PCR amplification of IMAGE consortium clones and arrayed on poly-L-lysine coated glass slides. Fluorescently labeled cDNA was prepared from control and γ-irradiated TK6 or NH32 whole-cell RNA by a single round of reverse transcription (BRL Superscript II) in the presence of fluorescent dNTP (Cy3 dUTP or Cy5 dUTP, Amersham). Probes and targets were hybridized together for 16 hours in 3x SSC at 65°C in the presence of the blockers human CoT1 DNA, yeast tRNA and polydeoxyadenine. Hybridized slides were washed at room temperature once in 0.5X SSC, 0.01% SDS for 5 minutes and again in 0.06X SSC for 5 minutes. Cy3 and Cy5 fluorescences were scanned using a pre-release laser confocal scanner (Agilent Technologies), and images were analyzed using the DeArray program to calibrate relative ratios and develop confidence intervals for their significance.[22] The ratios were normalized to those of a set of 88 internal controls[23] with a theoretical ratio of 1.0. The variance in the housekeeping set was used to determine the significance of expression changes following irradiation.

Quantitative RNA Hybridization Analysis. Serial dilutions of the RNAs were dotted onto nylon membranes, hybridized with cDNA probes at 55°C in a buffer containing 50% formamide (Hybrisol I, Oncor), and washed under standard conditions as previously described.[24] For hybridization probes, cDNA inserts were excised or PCR amplified from pHul45 (*GADD45a*), Image Clone 701112 (*XPC*) and Image Clone 753447 (*DDB2*) and labeled with ^{32}P using random primers (Stratagene). Hybridization was quantitated on a phosphorimager (Molecular Dynamics), and relative signal levels, normalized to the polyA content of each sample, were determined using the RNA-Think program.[24,26] With this approach, the values for relative RNA are directly proportional to RNA abundance and differences of 1.5-fold or more can be reliably measured.[24,27] Results obtained with this sensitive approach also agreed well with those obtained by RNAse protection determinations.[28]

RESULTS

Microarray hybridization of RNA from control and 8 Gy γ-ray treated TK6 (p53 wild-type) cells shows clear induction of genes in the GGR pathway, including XPC, GADD45a, and DDB2 (Fig. 1). In contrast, identically treated NH32 cells (a p53-null derivative of TK6), while exhibiting similar patterns of hybridization to surrounding targets on the arrays, show no induction of these genes by microarray hybridization. Genes for XP complementation groups involved in transcription-coupled repair, including XPA,

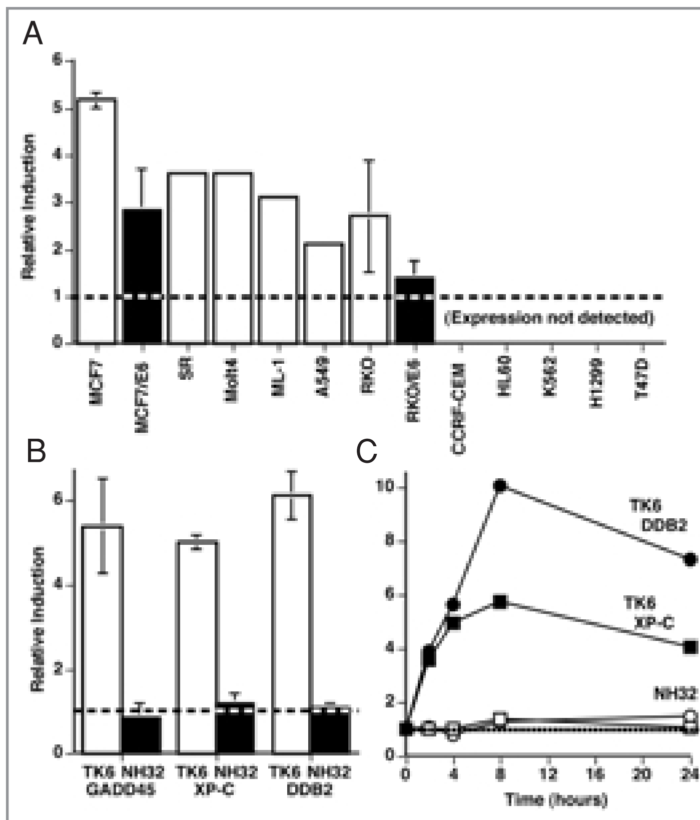

Figure 2. Induction of XPC RNA by 20 Gy g-rays in a panel of human tumor cell lines, assayed four hours after treatment. The white bars represent cell lines with wild-type p53 function, while the black bars represent cell lines with p53 function abrogated by expression of HPV E6. Expression of XPC was not detected in the 5 p53-mutant cell lines tested. Where error bars are shown they are the standard error of the mean for 4 independent experiments. The dashed line indicates the relative level of XPC in the unirradiated controls.
Induction of GADD45a, XPC and DDB2 four hours after irradiation with 10 Gy g-rays in the human lymphoblastoid cell line TK6 (wild-type p53; white bars) and in NH32 (p53-/- null derivative of TK6; black bars). The bars are the mean of three independent experiments, and error bars are standard errors. The dashed line indicates the relative levels in unirradiated controls. Induction of XPC and DDB2 over time following irradiation of TK6 and NH32 with 10 Gy g rays. The dashed line shows levels in unirradiated controls.

XPB and XPG, were not induced in response to γ-rays regardless of p53 status in these two cell lines, or in any of 36 additional human tumor cell lines screened.

While *GADD45a* and *DDB2* have been previously reported to be dependent on wild-type p53 function for ionizing radiation induction, the p53 dependence of *XPC* induction has not been reported. We further surveyed the radiation responsiveness of the *XPC* gene using single probe quantitative hybridization in a panel of human tumor cell lines. Six p53-wild type lines showed significant induction of *XPC* by ionizing radiation, while no measurable expression was detected in five p53 mutant cell lines (Fig. 2A). Additionally, when p53 function was abrogated by expression of HPV E6 expression in two p53-wt lines, radiation induction of *XPC* was ablated. Repeated experiments confirmed both the induction of *XPC, DDB2* and *GADD45a* in the p53 wild-type TK6 cell line, and the complete loss of this induction in the p53-null derivative, NH32 (Fig. 2B). These genes also showed similar kinetics of expression over time after exposure of TK6 cells to10 Gy γ-rays (Fig. 2C). In contrast, transcript levels in NH32 cells remained unchanged up to 24 hours after exposure, indicating that loss of p53 results in loss of responsiveness of these genes rather than simply delayed response.

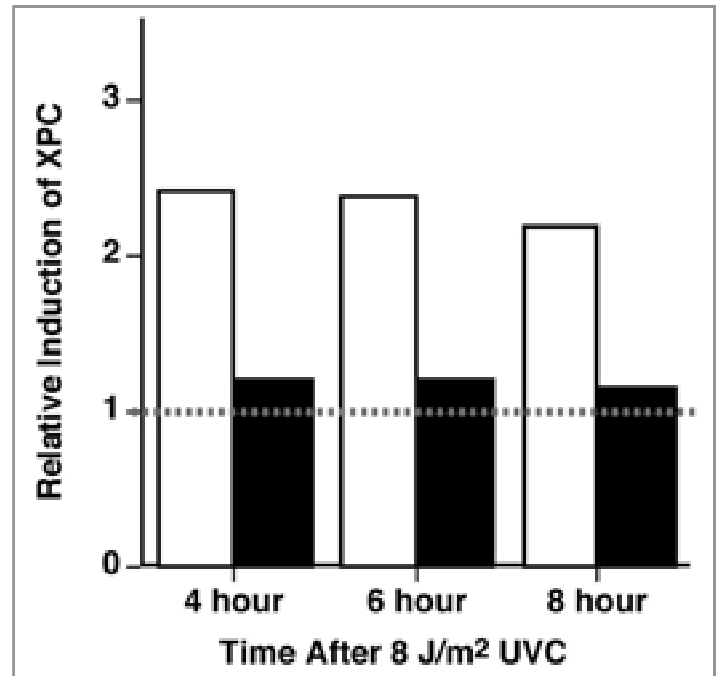

Figure 3. Induction of XPC over time by UV irradiation of TK6 (white bars) and NH32 (black bars). The dotted line indicates the level in un-exposed controls.

As NER is not a major pathway for the repair of DNA damage caused by ionizing radiation, it was of interest to see if *XPC* also showed similar p53-dependent regulation in response to UV radiation. UV exposure of TK6 cells resulted in induction of the *XPC* gene, but there was no response in the p53-null line, even several hours after the peak response occurred in TK6 (Fig. 3). The response to the base-damaging agent methylmethane sulfonate (MMS) was also investigated. Exposure to 50 μg/ml MMS, a dose previously found to give maximal induction of many genes, resulted in induction of all three genes in the p53 wild-type cell line (Fig. 4A). There was no response of *DDB2* or *XPC* in the p53 null line, NH32, although a marginal response was seen for *GADD45a,* as has been previously reported in p53-deficient cells.[29] When NH32 cells were treated with increasing doses of MMS, a marginal response was seen for *XPC* but not *DDB2*, at 100 mg/ml, the highest dose used. At this extremely toxic dose, the response of most genes begins to fall off, as was seen for both of these genes in TK6 (Fig. 4B).

DISCUSSION

We recently reported ionizing radiation induction of the *XPC* gene in normal human lymphocytes.[30] As most of the other genes robustly induced in that study were known to be p53 regulated, we looked to see if the ionizing radiation induction of *XPC* was also dependent on p53. We show here that *XPC* is induced by ionizing and UV radiation and MMS in a p53-dependent manner. The similarity of DNA-damage responsiveness of the three major GGR genes *XPC*, *DDB2*, and *GADD4*a, and their strict dependence on wild-type p53 function indicates a central role for p53 in the regulation of GGR.

Although the role of Gadd45a in DNA repair has not been as well defined as that of the XP proteins, many lines of evidence suggest a role for Gadd45a in NER. Reduction of Gadd45a protein expression has been found to reduce NER,[1,13] and more recently GGR and NTS repair were found to be significantly reduced in cells from *Gadd45a*-null mice.[14,31] While GGR and NTS repair were

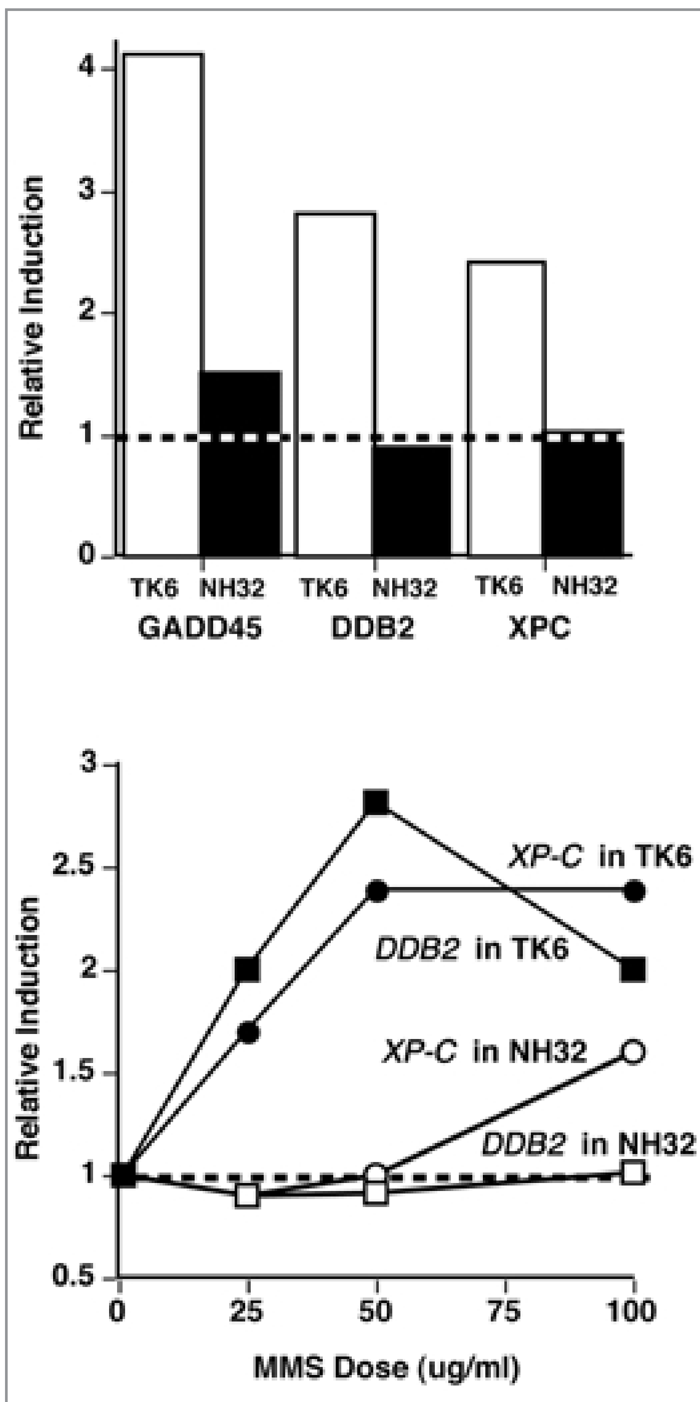

Figure 4. Induction of GADD45a, XPC and DDB2 by a 4 hour exposure to 50 mg/ml MMS (maximal induction, which occurred 12 hours after the start of treatment, is shown.) Dose-dependent induction of XPC and DDB2 in TK6 and NH32 cells. RNA was harvested 12 hours after the start of a 4 hour treatment with each dose of MMS to obtain maximal levels of induction.

reduced in *GADD45a*-null cells to levels even lower than those for *Tp53*-null cells, TCR was equivalent to wt.[14] Both XPC and XPE probably function as damage recognition factors[32] and this may also be the case for Gadd45. Like the p48 protein encoded by *DDB2*, Gadd45 protein is not required for in vitro NER using purified DNA template.[33,34] Addition of p48 stimulated in vitro NER,[35] and addition of Gadd45 or the related protein MyD118 was associated with a small increase in the same type of assay.[36] However, Gadd45 produced a much larger increase in repair using nuclear extracts containing chromatin-related proteins.[1,14] Interestingly, Gadd45 can bind to nucleosomes, particularly UV-irradiated nucleosomes, and may function as a chromatin accessibility factor.[37] XPC has also been implicated in nucleosome unfolding during NER,[38] and thus both proteins may have a role in damage recognition in chromatin.

The regulation of GGR genes by p53 further defines another role for p53 as "guardian of the genome." Loss of normal p53 function would affect the expression of the NER genes *Ddb2, GADD45a, Xpc,* and also *p53R2*.[39] In the case of the latter gene, loss of damage-induced ribonucleotide reductase action would probably affect GGR more than TCR, since TCR proceeds more rapidly, using the available deoxynucleotide pool. Defective GGR is associated with increased carcinogenesis; e.g., *Xpc*-null mice show increased UV carcinogenesis (reviewed in ref. 40). In addition, exposure to DMBA, whose damage is repaired by NER, has recently been found to be associated with increased carcinogenesis and an increased mutation frequency in *GADD45a*-null mice.[31] Since the cytotoxicity of DNA-damaging agents repaired by NER depends largely on the proficiency of TCR in the cells, this presents a challenge for cancer therapy to exploit the GGR deficiency in p53-deficient tumors, which represent the majority of human tumors. One possibility would be the use of DNA interstrand crosslinking agents, a class of highly cytotoxic agents that can interfere with DNA synthesis (with resultant cytotoxicity) by producing lesions both within and outside of active genes. Unlike typical single-strand lesions caused by most other classes of cytotoxic agents, which can be tolerated outside of actively transcribed genes, interstrand crosslinks, as is the case for double-strand breaks, can be extremely toxic anywhere in the DNA. As TCR does not efficiently repair this sort of lesion in bulk DNA, the cell must depend on GGR for its repair. In this case, the GGR deficits present in p53-deficient tumors may specifically enhance the efficacy of this class of drugs as cancer therapeutic agents.

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