P53 Dependence of Topoisomerase I Recruitment *in vivo*

Yinghui Mao,[1] Shoshiro Okada,[1] Long-Sheng Chang,[2] and Mark T. Muller[1*]

[1]Department of Molecular Genetics, The Ohio State University,

[2]Department of Pediatrics, Children's Hospital, Columbus, Ohio 43210, USA

* To whom correspondence should be addressed at: Department of Molecular Genetics, the Ohio State University, 484 West 12th Avenue, Columbus, OH 43210, USA.

Phone: (614) 292-1914

Fax: (614) 292-4702

E-mail: muller.2@osu.edu

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Abstract

DNA damage is attended by rapid recruitment of endogenous type I topoisomerase (topo I) into covalent cleavage complexes with genomic DNA *in vivo*. In contrast, endogenous topoisomerase II alpha and beta are not stimulated by DNA damage. We show that topo I and p53 physically interact at arrested topo I-genomic DNA covalent complexes *in vivo,* suggesting that p53 directly stimulates topo I activity and damage to the genome of the afflicted cell. Moreover, cells that express wild type p53 are most proficient at recruiting topo I following DNA damage; however, the p53 dependence is conditional since topo I recruitment following DNA damage can be restored if p53 mutant cells (containing a single mutant allele) are artificially held in G_1. In contrast, p53 null mutants do not recruit topo I after DNA damage under any conditions (although camptothecin dependent topo I/DNA complexes readily form in the nulls). These results show that topo I activation following DNA damage is p53 dependent. It also depends upon the cell cycle in a way that is very different from that observed with DNA replication dependent, camptothecin mediated DNA breaks. The data suggest a model where p53 activates topo I which inflicts additional genomic damage after the initial UV damage events. Topoisomerases therefore contribute to the p53 commitment to apoptosis and topo I might assist in elimination of DNA damaged cells as part of the cellular proofreading function inherent in the p53 pathway.

Key words: Topoisomerase I/p53/topoisomerases/DNA damage/DNA repair

Introduction

Cellular responses to DNA damage include activation of cell cycle arrest, DNA repair, and in some cases cell death by apoptosis (1-3). The *p53* tumor suppressor gene has been proposed as a genomic 'guardian' by exerting cell cycle checkpoint control in response to DNA damage (4). Following exposure to a wide variety of DNA-damaging agents, p53 expression is 'activated' by one or more post-translational mechanisms that result in a rapid increase in the cellular level of this protein. Functional p53 is required for activation of a G_1 checkpoint and the resulting growth arrest is thought to allow cells time to repair DNA prior to replication (5-7) or in some cells eradicate DNA damage laden cells which may be precancerous (8). In addition, p53 may influence DNA repair through GADD45, which stimulates the DNA synthesis associated with the excision repair process by forming complexes with PCNA (9). Moreover, p53 may play a more direct role in DNA repair by binding to ERCC3 excision repair factor and several TFIIH-associated factors *in vitro* (10). The positive effects of p53 (i.e., events that enhance the excision repair pathway) compared to negative effects (i.e., events that lead to eradication of DNA damaged cells) may both operate to varying extents depending upon the degree of DNA damage and tissue environments. Recently, it was reported that wild-type p53 can bind and increase the catalytic activities of topoisomerase I (topo I) *in vitro* and *in vivo* (11,12). Given that topo I is also a DNA damaging agent, we examined p53 effects on topo I-genomic DNA interaction following DNA damage.

Eukaryotic topo I makes single strand breaks in DNA, followed by one or more cycles of controlled rotation followed by resealing (13). Topo I participates in a variety of DNA templating activities, such as transcription (14-17) and DNA replication (18,19),

presumably to reduce torsional stress in the template. Topo I is also thought to influence genomic instability through illegitimate recombination (20-23). Although topo I is not an essential gene in yeast (24), it is required for embryonic development in *Drosophila melanogaster* (25) and mice (26); therefore, topo I is essential in the context of a multicellular organism.

There have been hints that topo I participates in post-DNA damage response. Distortions in the helix, such as abasic sites and UV photoproducts inhibit the catalytic activity of topo I and/or trap topo I on DNA *in vitro* (27,28). Previously, our group reported that DNA damage by UV irradiation specifically stimulates the formation of covalent complexes between topo I and DNA *in vivo* (29). We refer to this phenomenon as the topo I /DNA damage response and in the present work, evidence is presented that this response is strongly dependent upon cell cycle checkpoints activated by p53 following UV damaged DNA. These findings suggest a model whereby topoisomerase I and p53 might cooperate to eliminate damaged genomes from the organism.

RESULTS

UV-irradiation stimulates endogenous topo I-DNA covalent complex. The ICT bioassay is an antibody-based method that detects endogenous topo I-DNA covalent complex formation *in vivo* (29-31). The method has been successfully used to directly evaluate the action of endogenous topo I on genomic DNA in response to DNA repair-related activity in the absence of topoisomerase poisons that drive topo I into cleavable complexes. For example, we demonstrated that DNA damage (UV irradiation) stimulates topo I-DNA covalent complex formation in the absence of the topo I poison, camptothecin (CPT). We refer to this as the topo I/DNA damage response (29). The kinetics of response were analyzed in MCF-7 cells after exposure to 10 J/m^2 of UV irradiation. Topo I-DNA covalent complexes were detected rapidly after UV treatment (29). As shown in **Figure 1**, within 30 minutes, the level of topo I-DNA complex increased 1.5-2 fold over that of untreated controls, and the complexes continued to increase through the time when nucleotide excision repair was maximal. Within 5 hours, topo I-DNA complexes reached peak level (about 3-4 fold over no incubation), then gradually receded within one cell cycle. The kinetics of complex formation closely paralleled the p53 profile of response after UV irradiation (5,6). To confirm that p53 accumulated in the nucleus after UV irradiation, MCF-7 cells were stained with an anti-p53 antibody and examined by indirect immunofluorescence microscopy. UV treated cells displayed strong nuclear fluorescence; however, untreated controls presented minimal background nuclear staining (data not shown).

The topo I/DNA damage response does not require new protein synthesis. The elevation in endogenous topo I covalent complexes could be a result of new protein synthesis consequently giving higher levels of total topo I. Alternatively, pre-existing topo I could be activated by p53 or chromatin remodeling factors (32). To examine dependence on new protein synthesis, we determined the effects of cycloheximide on the topo I/DNA damage response. MCF-7 cells were treated (cycloheximide, CPT, UV) as described in **Figure 2** and the DNA isolated from the ICT analysis and probed with antibody to topo I. The data show that blocking protein synthesis had no effect on topo I-DNA complexes that were elevated post-UV damage. Camptothecin induced complexes were slightly reduced (<20%) by blocking translation; however, this may be explained by concomitant arrest of S-phase since it is known that camptothecin mediated toxicity is greater in S-phase (33,34).

Topo I/DNA damage response occurs in cell lines with wild-type and mutant p53. Data in **Figure 1** demonstrated that the topo I/DNA damage response parallels the nuclear accumulation of p53, suggesting a role for p53 in the response. To test whether the topo I/DNA damage response is related to p53, we evaluated the response in cell lines with different p53 status. MCF-7 cells express wild type p53, whereas SK-BR-3 cells carry a deletion in one *p53* allele and a point mutation (amino acid 175) in the remaining allele, encoding a non-functional p53 protein which is defective in sequence specific DNA binding (35,36). The HL60 cell line is p53-null. As expected, MCF-7 cells (wild type p53) showed an elevation in topo I-DNA complex formation following DNA damage (**Figure 3A**). In contrast, SK-BR-3 cells and HL60 cells that lack functional p53,

did not show UV-induced topo I-DNA complex formation (**Figure 3B, 3C**). Western blotting data reveal that these results can not be due to differences in topo I protein levels between these cell lines, since all cell lines contain similar amounts of cellular topo I before and after UV treatment (data not shown). Moreover, treating SK-BR-3 cells and HL60 cells with CPT results in similar covalent complex formation relative to the wild type MCF-7 cells (**Figure 3**). Finally, we tested topo I recruitment in transgenic mouse cell lines from animals that were heterozygous for p53 (one allele deleted) compared to homozygous null mutants and obtained consistent results (data not shown). From these collective results, we conclude that deployment of topoisomerase I onto the genome following UV exposure is related to the status of p53.

Restoration of the topo I/DNA damage response by p53. To explore a specific link between p53 and topo I deployment after DNA damage, we corrected the p53 defect in SK-BR-3 cells. An expression plasmid containing wild-type p53 under control of the CMV promoter was transfected into SK-BR-3 cells and p53 expressing clones were selected and expanded as stable cell lines (**Figure 4**). Three different clones, all expressing p53 (based upon Western blotting) were examined for topo I recruitment following DNA damage. These cell lines gave rise to high levels of topo I-DNA complex formation after UV irradiation (**Figure 4B**), compared to the parental SK-BR-3 cells (**Figure 4A**). These data clearly demonstrate a direct link between p53 and the topo I/DNA damage response since the p53 mutant (parental) line and restored clone should be otherwise isogenic. In addition, CPT-induced topo I-DNA covalent complexes were enhanced in the SK-BR-3/hp53 cells. This result suggests that p53 may be directly

stimulating topo I activity *in vivo*, and/or possibly recruiting topo I activity to sites of DNA damage, which is consistent to previous reports demonstrating that p53 increases the catalytic activity of topo I *in vitro* (11,12).

G_1 arrest restores the topo I/DNA damage response in some p53 mutant cell lines. DNA damage is known to stimulate a p53 dependent cell cycle checkpoint response; therefore, p53 mutant cells would not be expected to display growth arrest following UV irradiation. We next examined whether the topo I/DNA damage response in p53 mutants might be restored by artificially imposing a cell cycle blockade following UV exposure. Different p53 mutants were examined. First, we tested HL-60 cells which are null for p53. The topo I/DNA damage response was clearly missing from these cells, both in G1 arrested and growing cells (Fig. 5). Controls show that topo I/DNA complexes readily form following CPT treatment as expected; thus, topo I is clearly active in the null cells.

We also examined the topo I response in SK-BR-3 cells which as noted above carry a deletion in one p53 allele and a point mutation (amino acid 175) in the other. Consequently, this cell possesses a p53 mutant protein that retains its ability to interact with topo I (REF); however, since the mutation destroys DNA binding, the mutant p53 cannot stimulate expression of genes downstream of the p53 activation events. The topo I/DNA damage response was compared in G1 arrested and exponentially growing SK-BR-3 cells. The topo I damage response was essentially undetectable in exponentially growing SK-BR-3 cells (Figure 6A). In contrast, cells that were serum arrested in G1, clearly demonstrated a topo I/DNA damage response (Fig. 6B) and upon release from

arrest, the damage response was again lost (Fig. 6C). Cells containing this particular p53 mutation actively recruit topo I following UV damage when arrested in G1. Furthermore, these data suggest that the topo I/DNA damage response involves at least two distinct aspects of p53: 1) a cell cycle checkpoint that facilitates the topo I/DNA damage response (and/or topo I recruitment following recruitment), and 2) a direct interaction between topo I and p53 (see also Fig. 7 below).

Topo II isoforms respond differentially to UV damage. Topo II complex formation was not stimulated by UV damage (29); however, given that cell cycle checkpoints are activated after UV damage, we modified the experiment to examine the effects of UV damage on topo II isoforms (p170, p180), which are differentially regulated in the cell cycle (37,38). The ICT technique will detect topo II-DNA covalent complexes only in the presence of a topo II poison like VP16 and both endogenous forms of p170 and p180 are trapped on the genome (**Table 1**). In p53 wild type cells, UV treatment reproducibly reduced p170 complexes, whereas p180 complexes were unaffected. Thus, although topo II is not directly stimulated by DNA damage (like topo I), VP16 mediated activity was clearly altered in the case of p170.

P53 Binds topo I-DNA Covalent Complexes *in vivo*. It has been reported that the p53 protein and topo I physically interact (11,12). To determine whether p53 is physically associated with topo I when the latter is arrested in a cleavable complex *in vivo*, we modified the ICT bioassay to examine protein/protein binding in vivo. When topo I-DNA complexes were maximal (4 h post-UV treatment), MCF-7 cells were exposed to dimethylsuberimidate (DMS), a bifunctional protein/protein cross linking

reagent, to test for the presence of p53 in the DNA peak of the CsCl gradient (**Figure 7**). If p53 binds topo I, which itself is covalently trapped on the genome, then we should detect p53 associated with DNA in the ICT-CsCl gradient (**Figure 7A**). The presence of topo I and p53 was examined by Western blotting of the DNA peak fractions (pooled). The p53 signal, detected in the absence of DMS, reflects the background signal for p53 (since the signal did not increase with increasing DNA concentration). In contrast, exposure to DMS resulted in a significant increase in p53 signal when 2 μg of genomic DNA was used, and a commensurate increase in p53 signal observed using 6 μg of DNA (**Figure 7B**). Note however, that these data do not unambiguously prove that p53 is physically dragged into the DNA peak by topo I since p53 could be trapped by linkage to another (as yet unknown) DNA binding protein. While this possibility seems remote given the known association between p53 and topo I *in vitro* (11, 12) an additional control was carried out to examine this possibility. DNA peak fractions off the CsCl gradient were pooled and digested extensively with DNase I to release bound proteins. We then performed an immunoprecipitation with an anti-p53 anitbody, recovered the precipitate and probed with topo I antibody. The data clearly show complex formation between topo I and p53 (**Figure 7C).** To the best of our knowledge, this is the first demonstration that endogenous p53 and topo I are together at the site of the topo-DNA covalent (cleavable) complex *in vivo*.

In the absence of the DMS cross linker, we detected topo I recruitment on the genome as expected (Fig. 7C); however, treatment with the protein/protein cross linker further stimulated the topo I signal in the DNA peak anywhere from 2-5 fold (in different

experiments) suggesting that topo I may, be interacting with itself or in some kind of self protein/protein clustering mode as a complex.

Discussion

Previously, we reported that UV-induced DNA damage stimulates topo I-DNA covalent complex formation *in vivo* (29). A direct role for topoisomerase I in the nucleotide excision repair process was proposed because repair-deficient XPD cells are additionally compromised in their ability to recruit topoisomerase onto the genome after UV irradiation. We have now extended our initial observations and relevant findings can be summarized as follows.

(i) The kinetics of topo I covalent complex formation following UV irradiation of intact cells closely parallels the nuclear accumulation of p53. In response to DNA damage, cells normally undergo a p53-dependent cell cycle arrest, DNA repair, or apoptosis. Stimulation of topo I activity by p53 (11,12) suggests a functional relationship between these two proteins involving in DNA damage response.

(ii) MCF-7 cells, expressing wild-type p53, are proficient in the topo I/DNA damage response. In contrast, exponentially growing SK-BR-3 cells expressing mutant p53 show very little topo I response. These data suggest that p53 may stimulate topo I-DNA interactions leading to arrested covalent complexes on the genome. It is important to note that cells expressing mutant p53 sustain just as much DNA damage (UV adducts) as do wild-type p53 expressing cells per given UV dose, however p53 wild type cells are much more proficient with regard to topo I deployment as measured by ICT following damage. Lanza et al., (39) and work from this lab (29) confirmed that topo I cleavage/religation equilibrium is sensitive to helical distortions associated with UV lesion formation. Since UV damaged DNA can stabilize topo I cleavable complexes, and since p53 activates topo

I activity on DNA, topo I might assist in promoting the cell down a path of apoptosis by inflicting genome damage (see points that follow).

(iii). Topo I/DNA damage can be effectively restored in SK-BR-3 (mutant) cells when providing a wild-type *p53* gene. Since a single gene replacement is sufficient to restore topo I-DNA complex formation after DNA damage, we conclude that p53 is a key component in the response. All of the p53 wild type revertants we tested were proficient in the topo I/DNA damage response, thereby arguing against clonal variation independent of p53.

(iv). The topo I/DNA damage response is conditionally dependent upon the p53 status. In one particular p53 mutation (point mutation in the DNA binding domain) cells that are exponentially growing do not recruit topo I following DNA damage; however, by blocking cell cycle traverse in G1, topo I-DNA complex formation post-UV treatment could be restored. This is consistent with the previous report indicating that post-UV survival of p53 mutant cells is enhanced by imposing a cell cycle checkpoint (7). In addition, it has been reported that this particular p53 mutant can still physically interact with topo I. Interpretation of these collective results is difficult owing to the complexity of the pathways and additional data will be required to clarify the situation. Basically there are two models. One model suggests that p53 may promote cell cycle arrest to facilitate DNA repair, as proposed by Linke et al. (7). Topo I being a repair factor and also responsive to helical distortion or abasic sites (28) could explain why cleavable complexes are elevated during the periods of excision repair. Another model suggests that p53-driven checkpoints stably withdraw DNA damaged cells from the cycle to ensure that DNA replication does not proceed into or through damaged template. In this case,

topo I, perhaps under the stimulatory influence of p53 (11,12), might contribute to the DNA damage by forming covalent complexes which we detect in our ICT bioassay. In this way, topo I and p53 cooperate to inflict irreparable damage and permanent withdrawal of the cell from the replicative pool of cells.

(v) In support of the second model described above, our *in vivo* crosslinking data show that p53 and topo I form a complex at the cleavable site. While others have reported p53/topo I binding (11,12), we show that p53 and topo I are in complex at the actual site of DNA cleavage/religation in chromatin. Our crosslinking experiments will only detect p53 bound to topo I that has been covalently coupled (trapped) on the genome through its catalytic cycle of action on DNA. This type of experiment reveals that topo I and p53 are in a complex immediately prior to (or during) the arrest of the covalent 'cleavable' complex. We speculate that topo I and p53 are part of a multi-protein complex and that p53 directly stimulates topo I cleavages (12). Our DMS crosslinking data also suggest that topo I itself is in a self-cluster modality since the amount of topo I detected in the DNA peak can be increased by protein-protein crosslinking. Since topo I is a monomeric protein, it is surprising that it can be detected in a cluster of activity unless its function is to pepper the genome with single strand breaks. It is also possible that topo I interacts with other p53 interactive proteins in chromatin. For example, it has been proposed that p53 plays a direct role in DNA repair by stimulating other DNA binding proteins; p53 binds GADD45, which forms complexes with PCNA, to directly stimulate the DNA synthesis associated with the nucleotide excision repair process (9,40). The reduction of nucleotide excision repair ability in gadd45$^{-/-}$ mouse embryo fibroblasts fits nicely with the idea of a role for Gadd45 in cellular DNA repair (32). Recently, it was also shown

that GADD45 can facilitate topo I in the presence of core histones *in vitro* (32). These data suggest p53 /GADD45 might enhance the ability of topo I to recognize or be recruited to altered chromatin structures formed as a result of DNA damage.

(vi) The topo I/DNA damage response does not require new protein synthesis (**Figure 2**) indicating that pre-existing topo I is affected. Elevated topo I-DNA complexes may be explained by the formation of a higher affinity DNA substrate (viz., helical distortions) (29,39), alterations that promote topo I access to underlying DNA in chromatin (chromatin remodeling, for example) (32), or direct stimulation of topo I by p53 (11,12). It is not unreasonable to propose that all these events cooperate to give the topo I/DNA damage response.

(vii) Topo II isoforms are clearly not stimulated to form covalent complexes following DNA damage like topo I (29). To examine topo II with the ICT bioassay, it was necessary to treat the cells with a topo II poison to trap complexes. UV irradiation clearly had a strong impact on p170 since covalent complexes were reduced on average four fold. There was no detectable effect on p180. The most likely explanation is that DNA damage imposed a cell cycle checkpoint that affected the p170 isoform which is strongly cell cycle regulated, while the p180, which is not a periodic enzyme, was not altered.

In summary, there are two models that explain p53 and cell cycle dependent topo I activity following DNA damage. First, topo I may be an active participant in excision repair. We previously showed that the topo I/DNA damage response is aborted in the repair-deficient XPD cells (29). Nucleotide excision repair is a complex process that involves damage recognition, incision of the damaged strand, excision of the lesion containing oligonucleotides, synthesis of new DNA, and ligation. Topo I could be

involved at any one (or all) of these steps; however, it is also likely that some chromatin activation or restaging is necessary prior to topo I activity at repair patches, since topo I cannot compete with bulk chromatin for DNA access. In this regard, Gadd45, a p53 responsive factor, might drive local chromatin modifications to facilitate topo I accessibility (32). A second model is that topo I contributes to the general demise of the cell by contributing to genomic damage and subsequent p53 dependent elimination through apoptosis. In this model, topo I assists in forcing cell cycle checkpoints by inflicting DNA cleavage complexes and elimination of cells destined to be precancerous. The clustering of topo I on the genome following DNA damage (Fig. 7) and the p53 stimulation of topo I cleavage activity support this model. These two models are not mutually exclusive and it is possible that DNA damaged cells exist in a balance between repair (or resurrection) and apoptosis. The outcome of the process is stochastic and depends on a number of unknown factors in the p53 pathway in addition to the cell cycle phase and degree of damage. Additional experiments are ongoing to elucidate the models.

Materials and Methods

Reagents. The topo I antibody is a human antibody against topo I isolated from serum of scleroderma patients. The topo IIα antibody is a rabbit polyclonal antibody directed against the 170 kDa form of human topo II. The topo IIβ antibody is a mouse monoclonal antibody to the 180 kDa form of human topo II. The topo IIα andibody was donated by TopoGEN, Inc. (Columbus, OH) and the topo IIβ antibody was a kind gift from Dr. A. Kikuchi. The anti-p53 antibody (Pab 421) was obtained from Oncogene Science (Uniondale, NY). Camptothecin (CPT) and etoposide (VP16) were also donated by TopoGEN, Inc. G418 was purchased from Life Technologies, Inc., Rockville, MD. Dimethylsuberimidate (DMS) and Cyclohemimide were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture. The MCF-7 and SK-BR-3 cell lines in this study are derived from human mammary adenocarcinoma. MCF-7 cells express wild type p53 (36,40), whereas SK-BR-3 cells carry a deletion in one of the *p53* alleles and a point mutation at amino acid 175 in the remaining allele, encoding a non-functional p53 protein which is defective in sequence specific DNA binding (35,36). Both cell lines are cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (CellGro, Inc., Herndon, VA). SK-BR-3/hp53 cells are a clonal isolate of SK-BR-3 cells containing the human p53 gene under control by the cytomegalovirus (CMV) immediate early promoter. HL60 is a p53-null human leukemia cell line and cultured in RPMI medium 1640 supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY).

Transfection. Freshly plated MCF-7 and SK-BR-3 cells at 50 to 80% confluence were transfected with 2 μg of DNAs per 35-mm dish, using SuperFect transfection reagent (QIAGEN, Valencia, CA), according to the manufacturer's instructions. For stable transfection of the wild-type *p53* gene into SK-BR-3 cells, cells were split into fresh medium containing 400 μg/ml G418 (CellGro, Inc., Herndon, VA) until resistant colonies formed. Colonies were recovered using Scienceware cloning cylinders (Fisher Scientific) and then checked for p53 expression by Western blotting.

UV irradiation of cells. For UV treatment of cells, culture medium was aspirated and cell monolayers were washed several times with room temperature PBS (140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4 and 1.75 mM KH_2PO_4). The cells were then exposed to a germicidal lamp emitting at 254 nm light at a fluence rate of 2 $J/m^2/s$. Immediately following irradiation, medium was replaced and cells were incubated at 37°C. At indicated times after UV treatment, cells were processed according to the ICT bioassay protocol.

***In vivo* complex of topo (ICT) bioassay.** The method for detecting topo-DNA covalent complexes *in vivo* is described in detail elsewhere (29-31). Following DNA purification on CsCl gradients, DNA concentrations were measured by fluorometry and fixed concentrations of DNA were blotted onto a slot blot device which was subsequently probed with a topo I antibody. Signals were illuminated using [^{125}I]-protein A (ICN, Costa Mesa, CA) and quantified using phosphoimage analysis.

For analysis of p53-topo I complexes, the ICT bioassay was modified as follows: at 4 h post-UV, MCF-7 cells were exposed to 10 mM dimethylsuberimidate (DMS) at room temperature for 1h to induce protein/protein cross linking. The ICT bioassay was then carried out and the presence of p53 in the DNA peak of CsCl gradients was determined by Western slot blotting.

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Legends to Figures

Figure 1. **Time course of the topo I/DNA damage response.** MCF-7 cells were UV irradiated (10 J/m^2), and returned to normal growth conditions at 37°C. At indicated times post-irradiation, cells were lysed according to the ICT bioassay as described in **Materials and Methods**. DNA was recovered from the CsCl gradient, pooled, and concentrations were determined by fluorometry. Three concentrations of DNA (6, 2, and 0.6 µg of DNA) were placed on an immuno slot blot. Topo I levels associated with the DNA were measured using an anti-topo I antibody probe as describe in **Materials and Methods**. Markers on the left correspond to 6, 2, and 0.6 µg DNA per slot and times of harvest after UV exposure (hours) are marked along the top.

Figure 2. **Topo I/DNA damage response in the presence of cycloheximide.** MCF-7 cells were pretreated with 50 µM cycloheximide (C-hex) for 30 min, then treated with CPT (10 µM for 30 min), UV irradiation (10 J/m^2, then incubated for 4 h), or untreated (control), followed by the ICT bioassay using topo I antibody as described in **Materials and Methods**. Three DNA concentrations (10, 3.3, and 1.0 µg) spotted on the blot are indicated on the left.

Figure 3. **Topo I deployment in p53 wild type and mutant cells after DNA damage.** MCF-7 cells (p53 wild-type), SK-BR-3 cells (p53 mutant), and HL60 cells (p53 null) were either treated with CPT (10 µM for 30 min), UV irradiation (10 J/m^2, then incubated for 4 h), or untreated (control), followed by the ICT bioassay using an anti-topo I antibody

as probe (see **Materials and Methods**). **Panel A:** topo I-DNA complexes in MCF-7 cells; **Panel B:** topo I-DNA complexes in SK-BR-3 cells; **Panel C**: topo I-DNA complexes in HL60 cells. The DNA concentrations (6, 3, 1.5, and 0.75 μg) spotted onto the blot are shown on the left.

Figure 4. **Restoration of the topo I/DNA damage response in SK-BR-3/hp53 cells.** SK-BR-3 cells were stably transfected with wild type p53 gene and assayed for the topo I/DNA damage response as described in "Materials and Methods". These p53 revertant cells (SK-BR-3/hp53-2, 5, 12, **panel B**), or the parental p53 mutant cell line (**panel A**) were treated with CPT (10 μM for 30 min), UV irradiation (10 J/m^2, then incubated for 4 h) or untreated (control), followed by the ICT bioassay with the anti-topo I antibody probe (see **Materials and Methods**). Three DNA concentrations (6, 2, and 0.6μg) were tested.

Figure 5. **Topo I/DNA damage response in G_1 arrested p53 mutant cells.** SK-BR-3 cells were arrested in G_1 phase using double thymidine block. Both exponentially growing cells (**panel A**) and G_1 arrested cells (**panel B**) were treated with CPT (10 μM for 30 min), UV irradiation (10 J/m^2, then incubated for 4 h) or untreated (control). Cells either arrested in G_1 phase using serum starvation or released were treated with UV irradiation 10 J/m^2, then incubated for 4 h. After release, cells traversed the replicative cycle was determined by pulse label with [^{3}H]-thymidine (**panel C**). ICT bioassays were

performed using an anti-topo I antibody as described in **Materials and Methods**. Three DNA concentrations (6, 2, and 0.6 μg) spotted on the blot are indicated on the left.

Figure 6. **Topo I/DNA damage response in G_1 arrested p53 null cells.** HL60 cells were arrested in G_1 phase using double thymidine block. Both exponentially growing cells (**panel A**) and G_1 arrested cells (**panel B**) were treated with CPT (10 μM for 30 min), UV irradiation (10 J/m^2, then incubated for 4 h) or untreated (control).

Figure 7. **Topo 1-DNA-p53 covalent complex formation *in vivo*.** MCF-7 cells were treated with UV (10 J/m^2), then incubated for 3 h at 37°C. The cells were scraped up and placed in 1.7 ml microcentrifuge tubes, washed with PBS twice, and resuspended in Hepes buffer (pH 8.5-9.0). DMS was added to 10 μM and the cells were incubated for 1 h at room temperature. The cells were lysed with detergent and DNA was purified from CsCl according to the ICT bioassay. Two DNA concentrations (6 and 2 μg) were spotted onto the blot which was probed with either anti-topo I or anti-p53 antibodies. A diagram of the expected results is illustrated in **panel A**: In the absence of DMS, only topo I will be covalently trapped on genomic DNA; in the presence of the cross-linker, p53 should be detected in the DNA peak if p53/topo I make physical contact that can be crosslinked with DMS. **Panel B** shows the Western slot blotting results using either p53 antibody or topo I antibody. Low DNA concentration is on the right and high DNA concentration is on the left side of each blot as indicated on the top. **Panel C** shows the western slot

blotting results with the topo I antibody after immunoprecipitating the pooled DNA fractions with the p53 antibody.

References

1. Canman, C. E., Gilmer, T. M., Coutts, S. B., and Kastan, M. B. (1995) *Genes Dev.* **9,** 600-611
2. Smith, M. L., and Fornace Jr., A. J. (1996) *Am. J. Pathol.* **148,** 1019-1022
3. Kaufmann, W. K., and Paules, R. S. (1996) *FASEB J.* **10,** 238-247
4. Cox, L. C., and Lane, D. P. (1995) *BioEssays* **17,** 501-508
5. Lane, D. (1994) *Int. J. Cancer* **57,** 623-627
6. Levine, A. J., Perry, M. E., Chang, A., Silver, A., Dittmer, D., Wu, M., and Welsh, D. (1994) *Br. J. Cancer* **69,** 409-416
7. Linke, S. P., Klarkin, K. C., and Wahl, G. M. (1997) *Cancer Res.* **57,** 1171-1179
8. Hill, L. L., Ouhtit, A., Loughlin, S. M., Kripke, M. L., Ananthaswamy, H. N., and Owen_Schaub, L. B. (1999) *Science* **285,** 898-900
9. Smith, M. L., Chen, I.-T., Zhan, Q., Bae, I., Chen, C.-Y., Gilmer, T. M., Kastan, M. S., O'Connor, P. M., and Fornace, A. J. (1994) *Science* **266,** 1376-1380
10. Wang, X. W., Yeh, H., Schaeffer, L., Roy, R., Moncollin, V., Egly, J. M., Wang, Z., Friedberg, E. C., Evans, M. K., Taffe, B. G., Bohr, V. A., Weeda, G., Hoeijmakers, J. H. J., Forrester, K., and Harris, C. C. (1995) *Nat. Genet.* **10,** 188-195
11. Albor, A., Kaku, S., and Kulesz-Martin, M. (1998) *Cancer Res.* **58,** 2091-2094
12. Gobert, C., Bracco, L., Rossi, F., Olivier, M., Tazi, J., Lavelle, F., Larsen, A. K., and Riou, J. F. (1996) *Biochemistry* **35,** 5778-5786

13. Stewart, L., Redinbo, M. R., Qiu, X., Hol, W. G. J., and Champoux, J. J. (1998) *Science* **279**(5356)**,** 1534-1541

14. Muller, M. T., Pfund, W. P., Mehta, V. B., and Trask, D. K. (1985) *EMBO J.* **4,** 1237-1243

15. Merino, A., Madden, K. R., Lane, W. S., Champoux, J. J., and Reinberg, D. (1993) *Nature* **365,** 227-232

16. Wu, H.-Y., and Liu, L. F. (1991) *J. Mol. Biol.* **219,** 615-622

17. Kertzschmarr, M., Meisterernst, M., and Roeder, R. G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 11508-11512

18. Holm, C., Covey, J. M., Kerrigan, D., and Pommier, Y. (1989.) *Cancer Res.* **49,** 6365-6368

19. D'Arpa, P., Beardmore, C., and Liu, L. F. (1990) *Cancer Res.* **50**(6919-6924)

20. Wang, J. C., Caron, P. R., and Kim, R. A. (1990) *Cell* **62,** 403-406

21. Wang, J. C. (1991) *J. Biol. Chem.* **266,** 6659-6662

22. Zhu, J., and Schiestl, R. H. (1996) *Mol. Cell. Biol.* **16,** 1805-1812

23. Wang, J. C. (1996) *Annu. Rev. Biochem.* **65,** 635-692

24. Yanagida, M., and Sternglanz, R. (1990) in *DNA topology and its biological effects* (Cozzarelli, N. R., and Wang, J. C., eds), pp. 299-320, Cold Spring Harbor Laboratory Press, N.Y.

25. Lee, M. P., Brown, S. D., Chen, A., and Hsieh, T.-S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 6656-6660

26. Morham, S. G., Kluckman, K. D., Voulomanos, N., and Smithies, O. (1996) *Mol. Cell. Biol.* **16**(12)**,** 6804-6809

27. Pedrini, A. M., and Ciarrocchi, G. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1787-1791

28. Pourquier, P., Ueng, L.-M., Kohlhagen, G., Mazumder, A., Gupta, M., Kohn, K. W., and Pommier, Y. (1997) *J. Biol. Chem.* **272**(12)**,** 7792-7796

29. Subramanian, D., Rosenstein, B. S., and Muller, M. T. (1998) *Cancer Res.* **58**(5)**,** 976-84

30. Subramanian, D., Kraut, E., Staubus, A., Young, D. C., and Muller, M. T. (1995) *Cancer Res.* **55,** 2097-2103

31. Subramanian, D., Furbee, C. S., and Muller, M. T. (1999) in *Protocols for DNA Topoisomerases* (Bjornsti, M., and Osheroff, N., eds) Vol. in press

32. Carrier, F., Georgel, P. T., Pourquier, P., Blake, M., Kontny, H. U., Antinore, M. J., Gariboldi, M., Myers, T. G., Weinstein, J. N., Pommier, Y., and Fornace, A. J., Jr. (1999) *Mol. Cell. Biol.* **19**(3)**,** 1673-85

33. Li, L. H., Fraser, T. J., Olin, E. J., and Bhuyan, B. K. (1972) *Cancer Res.* **32**(12)**,** 2643-2650

34. Liu, L. F. (1989) *Annu. Rev. Biochem.* **58,** 351-375

35. Kovach, J. S., McGovern, R. M., Cassady, I. D., Swason, S. K., Wald, L. E., Vogelsteim, B., and Sommer, S. S. (1991) *J. Natl. Cancer Inst.* **83,** 1004-1009

36. Blagosklonny, M. V., Toretsky, J., and Weckers, L. (1995) *Oncogene* **11**(5)**,** 933-939

37. Woessner, R. D., Mattern, M. R., Mirabelli, C. K., Johnson, R. K., and Drake, F. H. (1991) *Cell Growth Differ.* **2,** 209-214

38. Capranico, G., Tinelli, S., Austin, C. A., Fisher, M. L., and Zunino, F. (1992) *Biochim. Biophys. Acta. Gene Struct. Express.* **1132,** 43-48

39. Lanza, A., Tornaletti, S., Rodolfo, C., Scanavini, M. C., and Pedrini. (1996) *J. Biol. Chem.* **271,** 6978-6986

40. Kastan, M. B., Zhao, Q., El-Deiry, W. S., Carrier, F., Jacks, T., Welsh, W. V., Plunkett, B., Vogelstein, B., and Fornace, A. J. J. (1992) *Cell* **71,** 587-597