

A role for DNA-mediated charge transport in regulating p53: Oxidation of the DNA-bound protein from a distance

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Charge transport (CT) through the DNA base pairs provides a means to promote redox reactions at a remote site and potentially to effect signaling between molecules bound to DNA. Here we describe the oxidation of a cell-cycle regulatory protein, p53, from a distance through DNA-mediated CT. A consensus p53 binding site as well as three DNA promoters regulated by p53 were synthesized containing a tethered DNA photooxidant, anthraquinone. Photo-induced oxidation of the protein occurs from a distance; introduction of an intervening CA mismatch, which inhibits DNA-mediated CT, prevents oxidation of p53. DNA-mediated oxidation is shown to promote dissociation of p53 from only some promoters, and this sequence-selectivity in oxidative dissociation correlates with the biological regulation of p53. Under severe oxidative stress, effected here through oxidation at long range, p53 dissociates from a promoter that activates DNA repair as well as the promoter for the negative regulator of p53, Mdm2, but not from a promoter activating cell-cycle arrest. Mass spectrometry results are consistent with disulfide bond formation in p53 upon DNA-mediated oxidation. Furthermore, DNA-bound p53 oxidation is shown *in vivo* by up-regulation of p53 and subsequent irradiation in the presence of a rhodium photooxidant to give a new p53 adduct that can be reversed with thiol treatment. This DNA-mediated oxidation of p53 parallels that seen by treating cells with hydrogen peroxide. These results indicate a unique mechanism using DNA-mediated CT chemistry by which p53 activity on different promoters may be controlled globally under conditions of oxidative stress.

DNA electron transfer | oxidative stress | redox regulation

The protein p53 is a 393-aa tumor suppressor involved in controlling cellular pathways that include apoptosis, cell cycle arrest, and DNA repair (1–6). Functioning as a transcription factor controlling the expression of myriad downstream genes, p53 must distinguish between promoters to regulate pathways under different cellular pressures, such as hypoxia and oxidative stress (3). It is no surprise that a protein this crucial to cellular integrity also is tightly regulated; a critical negative regulator of p53 function is Mdm2, an E3 ubiquitin ligase that, when bound to p53, facilitates its degradation (4). How is the binding of p53 to its different promoters chemically controlled depending on different cellular stresses? Here we show that the binding of p53 to specific promoter sequences, including that encoding the Mdm2 gene, can be altered from a distance through the DNA-mediated oxidation of p53. Hence DNA-mediated charge transport (CT) is seen to provide a chemical basis to understand the global regulation of p53 under oxidative stress.

The sequence-specific activation of transcription by p53 depends on its ability to bind to a consensus sequence within the promoter region. The consensus sequences for p53 consist of two symmetric response elements PuPuPuC(A/T)(T/A)GPyrPyr spaced 0- to 13-bp apart (7). Many of the natural promoter sequences that bind p53 do not have a 100% consensus match. Conversely, some sequences that share 100% homology with the consensus sequence do not bind p53 (8). Most p53 mutations found in human tumors are located in the DNA-binding domain

and involve amino acid residues that contact DNA or are required for proper folding (9, 10). The strength of p53 binding to a specific DNA sequence also is influenced by post-translational modification (8) and the redox state of the protein (11–13). In particular, oxidative conditions decrease the specific affinity of p53 for certain promoters. In the DNA binding domain are 10 conserved cysteines, of which 3 are involved in zinc coordination (9). Interestingly, cysteine 277, seen hydrogen-bonded to the consensus sequence in the crystal structure of p53 bound to DNA, has been proposed to undergo disulfide bond formation with cysteine 275, which would alter p53 binding to DNA in a redox-sensitive manner (14). Cysteines 141 and 135, only 3.8-Å apart, also are in close proximity to the DNA bases and represent additional targets for disulfide bond formation that could alter DNA binding.

Oxidative damage to DNA arising at long range through DNA-mediated CT now has been demonstrated by using a variety of pendant, distally bound photooxidants (15–19). We have shown that oxidative damage to DNA can occur at least 200-Å away from the tethered oxidant (16). Although this reaction displays a shallow distance dependence, it is exquisitely sensitive to perturbations in the intervening base-pair stack; DNA binding proteins, intervening mismatches, and base lesions can serve to attenuate charge migration (20). This sensitivity of DNA CT to stacking perturbations has led to its application in developing novel electrochemical sensors (21, 22). DNA CT also has been proposed to play a biological role in the detection of base lesions by DNA repair proteins (23). Moreover, long-range oxidative DNA damage through CT has been demonstrated to occur within nucleosomes and within the cell nucleus (24, 25). DNA CT furthermore can be harnessed to promote a variety of redox reactions on DNA triggered from a distance. We have shown that thymine dimers in DNA can be repaired at long range through DNA-mediated CT (26). Most recently, we have determined that DNA CT can be used to promote the formation of disulfide bonds from thiols incorporated into the DNA backbone (27). It is this chemistry, triggered from a distance, that we considered also might be useful in promoting reactions of proteins bound to DNA. Because p53 contains cysteine residues in close proximity within the DNA binding domain, we wondered whether we could selectively oxidize the DNA-bound protein and, in so doing, alter DNA binding through long-range CT. This chemistry from a distance, mediated by DNA, then would provide a completely novel mechanism to globally regulate p53 binding. Fig. 1 schematically illustrates this general chemistry.

Results and Discussion

Photooxidation of p53 Bound to Different DNA Sequences. To test whether DNA-bound p53 could be oxidized from a distance, a

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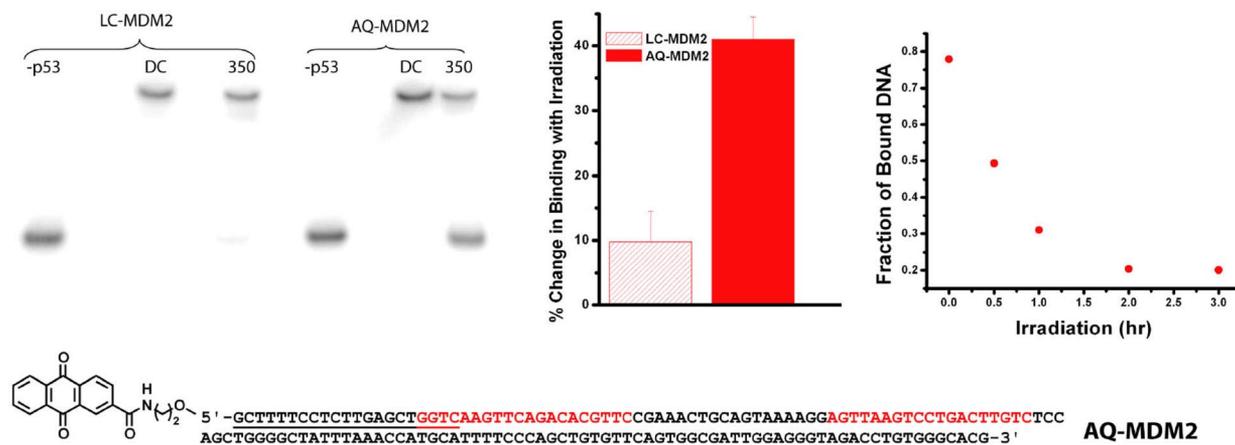


Fig. 4. Oxidation of p53 bound to the *Mdm2* promoter. The gel-shift assay (Upper Left) shows an autoradiogram after electrophoresis under non-denaturing conditions of LC-MDM2, the sequence without appended oxidant and AQ-MDM2, the sequence containing a tethered AQ both in the absence of irradiation (DC) and with irradiation for 45 min at 350 nm (350) in the presence of p53. Lanes marked –p53 contain only DNA. Quantification of the percentage change in binding also is shown in a bar graph (Upper Center), where the error bars reflect standard deviations from multiple trials. A time course for p53 dissociation from AQ-MDM2 also is shown (Upper Right) as a function of irradiation. The *Mdm2* sequence tethered with AQ is indicated (Lower) (the complementary ^{32}P -labeled strand is not shown), where the p53 binding sites are highlighted in red and the primers used are underlined. Samples contained 0.125 μM duplex, 0.5 μM p53, 5 μM dAdT, 0.1% np-40, 0.1 mg/ml BSA in 20 mM Tris-Cl (pH 8), 20% glycerol, 100 mM KCl, and 0.2 mM EDTA.

Therefore, this oxidative dissociation, despite being triggered from distance, is sequence-selective.

The fact that only certain sequences are affected by long-range oxidation provides a mechanism through which oxidized p53 can discriminate among promoters depending on cellular conditions. Oxidative stress can lead to the generation of guanine radicals throughout the genome through DNA-mediated CT. The observation that binding to the *p21* promoter is not strongly affected by DNA-mediated CT suggests that, with oxidative stress, this cell-cycle arrest protein still is transcribed. In contrast, based on these results, the transcription of *Gadd45* would be expected to be down-regulated; with substantial generation of guanine radicals, the DNA would be damaged beyond the capabilities of repair. In fact, it has been shown that, depending on cellular stress, these two genes are differentially activated by p53; with ionizing radiation, binding of p53 to the *p21* promoter is maintained, but dissociation occurs from the *Gadd45* promoter (14, 29).

The E3 ubiquitin ligase Mdm2 is a crucial protein involved in the regulation of p53 activity (4). Binding of Mdm2 to p53 promotes its ubiquitination and signals for its transport to the cytoplasm where it is degraded. In turn, p53 transcribes the *Mdm2* gene, resulting in a negative feedback loop for p53 transcriptional activity. Understandably, conditions of severe oxidative stress are known to lead to decreased transcription of *Mdm2*, ensuring higher levels of p53 and therefore increased transcription of proapoptotic genes (30).

A 150-bp fragment containing the human *Mdm2* promoter (LC-MDM2) was constructed as was an analogous fragment with tethered AQ (AQ-MDM2). LC-MDM2 and AQ-MDM2 sequences were incubated with p53 and either irradiated at 350 nm for 45 min or left in the dark. The fraction of bound DNA is found to decrease as a function of irradiation for AQ-MDM2, where long-range CT occurs, but remains relatively unaltered for LC-MDM2, the fragment lacking the photooxidant (Fig. 4). Increasing the extent of irradiation results in increased dissociation of p53 from the *Mdm2* promoter, as can be seen in an irradiation time course monitored by gel-shift assay. Thus, it appears that the dissociation of p53 can be triggered by photo-induced DNA CT with the photooxidant bound ~ 60 Å away. Importantly, this result supports the idea that, under conditions of oxidative stress, where p53 is essential, its level may be

regulated through DNA-mediated oxidation, which, in turn, leads to the inhibition of the transcription of its negative regulator. It should be emphasized that, in response to oxidative stress, the down regulation of the p53–Mdm2 interaction already has been specifically demonstrated (31); these results provide a chemical mechanism for that regulation.

Mass Spectrometry Supports the Oxidation of DNA-Bound p53. To confirm that the dissociation of p53 from certain DNA sequences is caused by chemical oxidation of the protein, we analyzed peptides resulting from a tryptic digest of p53 after irradiation in the presence of AQ-Con-1. Digestion with trypsin results in multiple peptide fragments, one of which contains cysteine 141, a residue located near the DNA binding region of the protein. This fragment, with a mass of 1,854 Da, is detected in the absence of irradiation and corresponds to a fragment with cysteine 141 in the reduced state (Fig. 5). After 3 h of irradiation at 350 nm of AQ-Con-1 in the presence of p53, however, this peak is lost. A likely explanation is that DNA-mediated oxidation promotes formation of a disulfide bond between cysteine 141 and the nearby cysteine 135 (associated with another tryptic peptide) that yields a much larger M_r fragment after trypsin digestion; cysteine, in fact, has the lowest oxidation potential of all of the residues and is significantly lower than guanine. That disulfide bond formation occurs specifically with DNA-mediated oxidation is supported by reappearance of the 1,854- M_r fragment with the addition of DTT (data not shown); this reversal is not quantitative. It is noteworthy that the 1,854- M_r fragment additionally contains a tryptophan, a residue that also may be easily oxidized in an electron transfer reaction (32). However, tryptophan oxidation would be expected to yield a fragment of $\sim 1,870$, which we do not observe. Oxidation of cysteines 275 and 277 had earlier been proposed as a possible redox regulatory switch associated with p53 binding (14). Trypsin digestion of p53 also leads to two fragments with M_r s of 1,708 and 1,709, one of which contains both cysteine 275 and 277. We could not reliably resolve these peaks from one another and from the cysteine 275–277 disulfide product. It therefore is difficult to determine whether cysteines 275 and 277 also undergo oxidation; certainly other oxidation products that we cannot resolve are possible. Nonetheless, the specific changes in fragmentation pattern we observe with irradiation, particularly the reversible loss of the 1,854- M_r

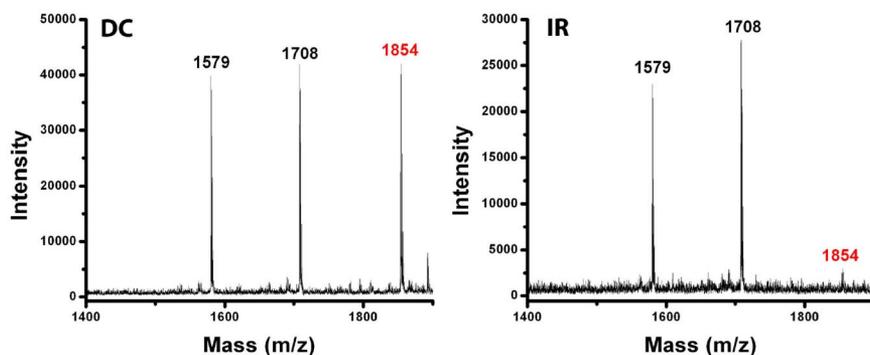


Fig. 5. MALDI-TOF mass spectrometry of a p53 tryptic digest after incubation with AQ-Con-1 without (DC) or with (IR) 3 h of irradiation at 350 nm. Samples contained 0.3 μM duplex and 1.2 μM p53 in 20 mM Tris-Cl (pH 8), 20% glycerol, 100 mM KCl, and 0.2 mM EDTA. The fragment with a mass of 1,854 corresponds to the fragment TCPVQWVDSTPPGTR containing cysteine 141.

fragment, establish clearly that DNA-bound p53 is chemically changed consistent with disulfide bond formation. Photoactivation of the tethered AQ therefore leads to oxidation of the DNA-bound protein from a distance.

Photooxidation of p53 *in Vivo* with a Rhodium Intercalator. To examine directly whether p53 is oxidized *in vivo* as a result of DNA-mediated oxidation, we carried out photooxidation studies on HCT116 cells by using $[\text{Rh}(\text{phi})_2\text{bpy}]^{3+}$ ($\text{phi} = 9,10$ -phenanthrenequinone diimine). This rhodium complex, which binds DNA by intercalation, was the first photooxidant used in establishing long-range oxidative DNA damage by DNA-mediated CT (15). We also have found that, as with AQ, photoactivation of the DNA-tethered rhodium complex can promote oxidation of DNA-bound p53 (data not shown). Importantly, this Rh complex also has been used in demonstrating oxidative DNA damage by CT in HeLa cell nuclei (25). Therefore, to test for DNA-mediated oxidation of p53 *in vivo*, HCT116 cells were treated with cisplatin to up-regulate p53 (33), incubated with the rhodium complex for different lengths of time, and photolyzed. After protein denaturation and SDS/PAGE analysis of total cellular protein, changes to p53 were analyzed

by Western blotting analysis. As can be seen in Fig. 6, the banding pattern of p53 remains relatively unaltered when the cells are irradiated without rhodium. However, when cells are incubated with rhodium and light to initiate the photooxidation reaction, a modified p53 band is clearly apparent above the parent band at long rhodium incubation times. Furthermore, when the oxidized protein samples are treated with 2-mercaptoethanol, a reagent that disrupts disulfide bonds, the modified p53 adduct is reversed. The ability to reversibly form the p53 adduct supports the hypothesis of DNA-mediated disulfide bond formation in the regulation of p53.

To ensure that the visualized p53 oxidation product is general and not specific to Rh treatment, hydrogen peroxide also was used as an oxidant in HeLa cells in parallel with Rh. As observed with HCT116 cells, irradiation without rhodium or the presence of rhodium but without irradiation causes no change in the p53 banding pattern of HeLa cells. However, addition of the rhodium photooxidant and irradiation leads to a lower-mobility oxidation product (Fig. 7). Importantly, analogous changes in p53 are evident on addition of 10 mM hydrogen peroxide to the HeLa cells and to a comparable level as to cells treated with rhodium and irradiated. Because the rhodium photooxidant is known to promote both long-range oxidative DNA damage in cell nuclei at comparable high concentrations and the DNA-mediated oxidation of p53 *in vitro*, these chemical changes to p53 seen *in vivo* with rhodium photoactivation likely reflect p53 oxidation at long range through DNA-mediated CT. Moreover, this DNA-mediated oxidation appears to effect the same changes in p53 as

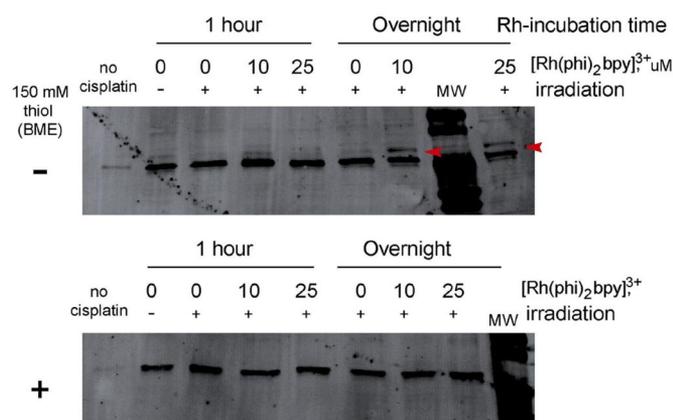


Fig. 6. Reversible oxidation of p53 from HCT116 cells visualized by Western blotting analysis. After treatment of HCT116 cells with cisplatin to up-regulate p53, cells were incubated with different concentrations of the rhodium photooxidant for either 1 h or overnight and irradiated with a solar simulator. Cellular protein was analyzed by SDS/PAGE followed by Western blotting analysis. Cells irradiated without rhodium show no p53 adduct bands. Overnight incubation of irradiated cells with either 10 or 25 μM $[\text{Rh}(\text{phi})_2\text{bpy}]^{3+}$ lead to a new, lower-mobility p53 adduct (red arrowhead, upper gel). Addition of 2-mercaptoethanol removes the oxidation-dependent p53 adduct (lower gel).

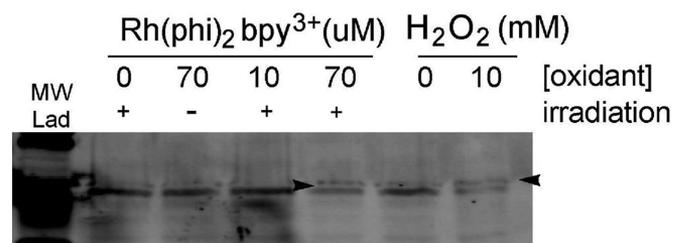


Fig. 7. Western blotting analysis of oxidized p53 derived from HeLa cells. Cells were incubated with hydrogen peroxide for 30 min at 37°C (no irradiation) or with $[\text{Rh}(\text{phi})_2(\text{bpy})]^{3+}$ for 30 min at 37°C and then irradiated with a solar simulator (UV cut-off filter <345 nm). Total protein was analyzed by SDS/PAGE followed by Western blotting with anti-p53 antibody and a fluorescent secondary antibody. Cells irradiated without rhodium, or in the presence of rhodium but without irradiation, show little change in the p53 banding pattern. However, incubation with 70 μM rhodium with irradiation or hydrogen peroxide yields a change in the p53 profile with a new, lower-mobility band as indicated by an arrowhead.

does the oxidative stress associated with treatment of cells with peroxide. Significantly, then, these experiments illustrate that oxidative stress mediated through DNA can impart a cellular damage response that leads to oxidation of p53.

Implications. The activation and functions of p53 are exquisitely sensitive to the cellular environment. Indeed, increasing levels of oxidative stress can cause p53 to transcribe either proapoptotic or antiapoptotic genes, depending on cellular pressures, thus allowing the cell either to survive and undergo repair under conditions of low stress, or undergo apoptosis under conditions of high stress (34). Our results provide a chemical basis to understand these opposing biological responses. The oxidation of DNA-bound p53 from a distance, generated by photooxidation in our experiments and presumably through charge migration among guanine radicals within the cell, is sequence-selective, requiring the intimate association of p53 with DNA to promote charge transfer from the DNA and protein oxidation. Without specific protein/DNA interaction, p53 oxidation and subsequent dissociation from the DNA does not occur. Hence DNA-mediated CT, reflecting the extent of oxidative stress in the cell, can provide a general route at long range to regulate p53 binding to different promoters.

Methods

DNA Synthesis. The consensus sequence with and without a mismatch, oligonucleotides containing the p21 and GADD45 promoter region, as well as the primers for the Mdm2 sequence were synthesized on an ABI 394 DNA synthesizer using standard phosphoramidite chemistry. Sequences not containing AQ were synthesized with the trityl group intact and were deprotected and cleaved from the resin in NH_4OH overnight at 60°C . The oligonucleotides were subsequently purified by HPLC before and after trityl group removal in 80% acetic acid and characterized by MALDI-TOF mass spectrometry. In synthesizing strands containing the AQ photooxidant, including the primer for Mdm2, an AQ derivative [AQ-2-carboxylic acid (2-hydroxyethyl) amide], AQ-1 was used, which was converted into its respective phosphoramidite and incorporated onto the 5' end of the sequence with a 15-min coupling time (35). Strands were cleaved from the resin and deprotected in NH_4OH at 60°C overnight. AQ-DNA was purified once by HPLC and characterized by MALDI-TOF mass spectrometry. The oligonucleotides were quantified by using UV-visible spectroscopy and annealed to their respective duplexes by heating equal moles of complementary strands to 90°C for 5 min and slow-cooling to ambient temperature in a buffer containing 5 mM sodium phosphate and 50 mM NaCl, pH 7. The resulting duplexes were purified on a 10% native acrylamide gel at 4°C to remove excess single-strand. The desired duplex band was excised from the gel by UV shadowing and eluted into $0.1\times$ Qiagen buffer over night at 4°C . The DNA then was ethanol-precipitated and resuspended in a buffer containing 5 mM sodium phosphate and 50 mM NaCl, pH 7.

The 150-bp duplex containing the human Mdm2 promoter was constructed from a HeLa DNA template, and primers were synthesized as described above for standard PCR amplification. The primers used were 5'-GCTTTTCCTCTTGAGCTG-GTC-3' (forward) or the AQ-tethered analogue and 5'-CGTGCCACAGGTCTACC-3' (reverse). After incubation at 95°C for 4.5 min, 40 cycles were performed as follows: 94°C for 30 s, 55°C for 1 min, and 72°C for 30 s. The PCR product was purified by using a QIAquick PCR purification kit (Qiagen), desalted with a microbiospin-6 chromatography column (Bio-Rad), and quantified by agarose gel electrophoresis running a 1-kb ladder standard for comparison. DNA strands were labeled at the 5' end with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and polynucleotide kinase, and excess ATP was removed by using a microbiospin-6 chromatog-

raphy column (Bio-Rad). A small amount of the labeled duplex was mixed with a known concentration of cold duplex.

Gel Mobility-Shift Assay. Human p53 used in binding experiments was purchased from Protein One. Protein was allowed to bind to the DNA by incubating $0.5\ \mu\text{M}$ p53 with $0.25\ \mu\text{M}$ labeled duplex in the presence of $5\ \mu\text{M}$ poly dAdT (Amersham Pharmaceuticals), 0.1% np-40, 0.1 mg/ml BSA (New England Biolabs) in 20 mM Tris-Cl (pH 8), 20% glycerol, 100 mM KCl, and 0.2 mM EDTA for 1 h at ambient temperature. The samples subsequently were either irradiated at 350 nm for 30 or 45 min by using a 1,000-W Hg/Xe lamp with a 320-nm long pass filter and monochromator or stored in the dark before gel loading. Samples containing the Mdm2 promoter complexed with p53 were loaded onto a 4–20% gradient acrylamide gel (Bio-Rad) in 0.3% TBE with 0.1% Triton-X, whereas those containing the consensus sequence, p21, or GADD45 promoters were run on an 8% acrylamide gel. The gradient gels were electrophoresed for 3 h at 50 V at 4°C , vacuum-dried, and imaged by using a Storm 820 phosphorimager (Molecular Dynamics/GE Healthcare), whereas the 8% gels were electrophoresed for 1.5 h at 50 V at 4°C and directly imaged as described above. The fraction of bound DNA was quantified by using ImageQuant 5.2. Each experiment was performed at least two times, and the resulting quantifications were averaged.

Mass Spectrometry of Oxidized p53. Samples containing p53 ($1.2\ \mu\text{M}$) were incubated with $0.3\ \mu\text{M}$ AQ-Con-1 for 1 h in 20 mM Tris-Cl (pH 8), 20% glycerol, 100 mM KCl, and 0.2 mM EDTA. The samples then were either irradiated at 350 nm on the 1,000-W Hg/Xe lamp for 3 h or stored in the dark. The samples were digested with $0.4\ \mu\text{g}$ of trypsin (Promega) at 37°C overnight. Digestion was quenched with $4\ \mu\text{l}$ of acetic acid. The resulting peptides were concentrated and desalted with a C-18 zip tip (Millipore) and mixed with an α -Cyano matrix. Samples were analyzed by using a Voyager De-Pro MALDI (PerSeptive Biosystems) mass spectrometer and calibrated against Sequazyme standards.

p53 Oxidation in Vivo. Oxidation of HCT cells was carried out by incubating 1 million cells with cisplatin containing complete RPMI 1640 medium (10-cm² culture flask, 5 ml of medium, 1 h at 37°C , 0.75 mg/ml cisplatin). After two washes with PBS, complete RPMI 1640 medium, with $[\text{Rh}(\text{phi})_2\text{bpy}]^{3+}$ where appropriate, was added. After incubation with $[\text{Rh}(\text{phi})_2\text{bpy}]^{3+}$, cells were washed and resuspended in 2 ml of PBS. Rh-containing samples then were irradiated by using a 1,000-W Orion Solar Simulator for 30 min (the cell culture flask eliminates wavelengths below 350 nm). PBS was removed, and 350 μl of mammalian protein extraction reagent with protease inhibitors (Pierce) was added. Protein samples were centrifuged, and an equal volume of Laemmli sample buffer was added and heated to 95°C for 5 min. Samples with 2-mercaptoethanol contained 150 mM.

Experiments with HeLa cells (5 million) were treated with trypsin and washed twice with PBS. Cells were resuspended in 50 μl of PBS, and an appropriate concentration of oxidant was added (see Fig. 7); the cells were incubated at 37°C for 30 min. Rh-containing samples then were irradiated by using the solar simulator outfitted with a UVB/UVC-blocking filter and a glass filter that eliminates wavelengths $<345\ \text{nm}$ for 30 min and were prepared similarly to the HCT116 cells.

Total cellular protein samples were electrophoresed on a 10% Tris-HCl gel. The gel was transferred via electroblotting to a poly(vinylidene difluoride) membrane. The membrane was blocked for 1 h by incubation in a 5% milk/Tris-buffered saline

with Tween solution (TBS-T). The membrane was washed with TBS-T followed by an anti-p53 antibody (sc-126; Santa Cruz Biotechnologies) and incubated overnight at 4°C. After several washes with TBS-T, the membrane was incubated with Alexa Fluor 680 goat anti-mouse IgG (Molecular Probes) for 1 h in TBS-T with 0.01% SDS at room temperature. After several

washes, the membrane was imaged with a 700-nm filter Li-Cor imaging system.

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