The p53 Pathway Promotes Efficient Mitochondrial DNA Base Excision Repair in Colorectal Cancer Cells

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Abstract
The tumor suppressor p53 plays a central role in the DNA damage response. p53 enhances base excision repair (BER), in part, through direct interaction with the repair complex. Mitochondrial DNA (mtDNA) is repaired by a mtBER pathway. Many colorectal cancers harbor mtDNA mutations that are associated with poor prognosis. In addition to modulating the apoptotic response, mitochondria-localized p53 also stimulates mtBER. However, the mechanisms by which p53 enhances colorectal cancer mtBER after stress remain unclear. To explore this, we used colorectal cancer cells isogenic for p53 (HCT116p53+/+ and HCT116p53−/−). p53+/+ cells more efficiently repaired H2O2-damaged DNA as measured by semiquantitative mtDNA displacement loop PCR. Mitochondrial extracts from p53+/+ cells more efficiently stimulated 32P-dCTP incorporation into a uracil-oligonucleotide. Recombinant p53 complemented p53−/− mitochondrial extract repair of uracil-32P-dCTP incorporation into a uracil-oligonucleotide. As a measure of DNA glycosylase activity, p53+/+ mitochondrial extracts more efficiently incised uracil-32P-dCTP incorporation into a uracil-oligonucleotide, although recombinant p53 could not stimulate oligonucleotide incision. p53 did not influence mitochondrial uracil or 8-oxo-G-containing oligonucleotides, although recombinant p53 could not stimulate uracil-oligonucleotide incision. p53 did not influence mitochondrial uracil or 8-oxo-G-containing oligonucleotides, although recombinant p53 could not stimulate uracil-oligonucleotide incision.

Introduction
The p53 tumor suppressor is a central mediator of the cellular damage response, with loss of p53 leading to defects in apoptosis, cell cycle arrest, and DNA repair (1). The p53 pathway plays a major role in maintaining genomic stability through transcriptional activation of DNA repair target genes (2, 3). The base excision repair (BER) pathway is important for the repair of mutagenic DNA base modifications caused by a variety of cellular stressors such as oxidation and alkylation (4). Moreover, the p53 pathway stimulates efficient BER at least in part through a transcription-independent process involving a direct interaction between p53 protein and the BER machinery (3, 5–9), although the precise mechanisms remain obscure.

In addition to the nuclear genome, cells harbor a mitochondrial genome. Mitochondrial DNA (mtDNA) mutations have been implicated in a variety of human diseases including cancer (10–13). Many human cancers have a several-fold higher rate of somatic mtDNA mutations than nuclear DNA; additionally, mutations in mtDNA repair genes have been found in tumors (10, 12–16). Moreover, mtDNA mutations can directly contribute to more aggressive behavior of tumor cells (17, 18). Some reports have estimated that nearly 70% of human colorectal cancers harbor mtDNA mutations (12, 13, 19, 20). Furthermore, mtDNA mutations have recently been described as a poor prognostic factor in colorectal cancer (21).

Disruption of the p53 axis plays an important role in colorectal tumor development and response to therapy (22–27). Despite these observations, the mechanisms that promote mtDNA repair in colorectal cancer, and the role of the p53 pathway in this process, remain to be clarified.

mtDNA is repaired by a short-patch BER process (16, 28). Existing as a 16.6 kb circular structure containing 13 oxidative phosphorylation genes, two rRNAs, and 22 tRNAs, mtDNA is prone to mutations because it is localized near the inner mitochondrial membrane in which reactive oxygen species are generated. Additionally, mtDNA lacks histone protection and, except for the displacement loop (D-loop), has minimal noncoding regions so that open reading frames are frequently mutated (10, 16, 29). mtBER is initiated by a DNA glycosylase to remove the damaged base and the apurinic/apyrimidinic site is processed by an apurinic/apyrimidinic endonuclease (or the apurinic/apyrimidinic-lyase function of some glycosylases). Correct nucleotide incorporation is catalyzed by DNA polymerase-γ and religation of the nicked strand by DNA ligase III (16, 29). p53 protein can stimulate nuclear BER by interacting with nuclear apurinic/apyrimidinic endonuclease and nuclear DNA polymerase-β, resulting in stabilization of the DNA polymerase-β and abasic DNA interaction (5, 6). Likewise, an intact p53 pathway stimulates mtBER in unstimmed mouse hepatocytes (30). However, the p53-mediated mechanisms that stimulate mtBER after stress in colorectal cancer remain unclear.

In response to stress, p53 protein translocates to the mitochondria to promote apoptosis via transcription-independent mechanisms (31–37). p53 protein can also localize to the mitochondria independent of apoptosis—implying a nonapoptotic function for mitochondrial localized p53 protein (38, 39). Given that p53 enhances nuclear BER after stress (5–9), and that mtDNA mutations may play a role in colorectal cancer formation and response to therapy (12, 13, 19–21), the finding that an intact p53 pathway stimulates mtBER in unstimmed mouse liver mitochondria.

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(30) led us to investigate the role of p53 in enhancing mtBER in colorectal cancer cells after DNA damage.

In this report, we present new evidence that the p53 pathway stimulates colorectal cancer mtBER at the DNA glycosylase step and during nucleotide incorporation catalyzed by DNA polymerase-γ. p53 protein was detected in an inner mitochondrial membrane-containing particulate fraction and associated with components of the mtBER complex (28). Together, these results suggest that the p53 pathway is important for stimulating mtBER after stress, and provides new insight into the molecular pathways that influence mtDNA genomic stability in colorectal cancer.

Materials and Methods

Cell culture and reagents. Cells were grown in DMEM with 10% fetal bovine serum, 290 μg/mL L-glutamine, 100 units penicillin, and 100 μg streptomycin per mL at 37°C in 5% CO2. HCT116p53+/+ and HCT116p53−/− cells (40) were a gift from Dr. Bert Vogelstein (Johns Hopkins, Baltimore, MD). Recombinant p53, OGG1, APE1, or green fluorescent proteins (GFP), were generated by cloning the cDNAs into a fluorescent vector (Invitrogen, Carlsbad, CA) using standard techniques. After isopyrrolid-thio-d-N-galactopyranoside induction in bacteria, expressed proteins were purified by Ni-NTA agarose per manufacturer protocol (Qiagen, Valencia, CA) and stored at −80°C. Purified protein was quantitated using standard techniques by silver-stained SDS-polymerase-γ was from Novus Biologicals (Littleton, CO). Horseradish peroxidase–conjugated secondary antibodies were from Jackson ImmunoResearch Laboratory (West Grove, PA).

Mitochondrial extracts. Mitochondria were isolated using Percoll gradient centrifugation adapted from previous methods (41, 42). Briefly, cells were Dounce homogenized on ice in M-SHE buffer [0.21 mol/L mannitol, 0.07 mol/L sucrose, 10 mmol/L HEPES-KOH (pH 7.4), 1 mmol/L EDTA, 1 mmol/L EGTA, 0.15 mmol/L spermine, 0.75 mmol/L spermidine, 1 mmol/L DTT] with freshly added protease inhibitors (1 μg/mL of leupeptin, aprotinin, pepstatin A, 1 mmol/L phenylmethylsulfonyl fluoride). Nuclei were pelleted at 1,200 × g and the supernatant centrifuged at 10,000 × g for 15 minutes at 4°C to pellet mitochondria. Mitochondrial pellets were resuspended in 3% Ficoll 400/0.5× M SHE-buffer, layered over 6% Ficoll 400/1× M-SHE buffer and centrifuged at 10,400 × g for 25 minutes at 4°C. The pellets was resuspended in M-SHE-0.3 mg/mL digoxigenin buffer at 4°C for 15 minutes, centrifuged again at 10,500 × g for 15 minutes, and then washed once in M-SHE buffer. Extracts prepared by solubilizing mitochondria for 30 minutes at 4°C in lysis buffer [20 mmol/L HEPES (pH 7.4), 400 mmol/L KCl, 1 mmol/L EDTA, 5% glycerol, 0.5% Triton X-100, 2 mmol/L DTT, and fresh protease inhibitors]. Lysates were clarified by centrifugation at 16,000 × g at 4°C for 1 hour, concentrated to 5 to 10 mg/mL protein, and stored at −80°C. Mitochondrial subfractions were prepared as previously described with minor modifications (28). Briefly, purified mitochondria fractions (described above) were sonicated with 4–10-second bursts at 5 W on ice in 300 mmol/L NaCl, 10 mmol/L HEPES (pH 7.4), 1 mmol/L EDTA, 5% glycerol, and fresh protease inhibitors. Homogenates centrifuged at 100,000 × g for 1 hour and the particulate fraction (Mito-particulate) was resuspended at an equal volume to the supernatant (Mito-soluble).

Immunoprecipitation of p53 and DNA polymerase-γ. Cells infected with p53 expressing adenovirus, or control adenovirus, were collected into PBS and centrifuged at 1,000 × g. Cell pellets were resuspended on ice for 20 minutes in 20 mL M-SHE buffer plus fresh protease inhibitors, vortexed, and sonicated with four 10-second 5-W bursts. Nuclei were cleared by centrifugation at 2,000 × g and mitochondria were pelleted by centrifuging the supernatant at 10,000 × g for 20 minutes. Mitochondria lysed in 1% NP40, 0.1% deoxycholate, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl 1 mmol/L EDTA, 1 mmol/L Na3VO4, 1 mmol/L NaF, and freshly added protease inhibitors. Immunoprecipitations done with 2 μg of nonimmune rabbit IgG or anti-p53 antisera overnight at 4°C. Twenty microliters of a protein A/G slurry were added and rotated at 4°C for 2 hours, beads were washed five times with lysis buffer, and immunoblotted as previously described (43).

p53 immunoprecipitation and D-loop PCR assay. Cells were treated with 0 or 50 μmol/L of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for 24 hours, cross-linked with 1% formaldehyde/PBS for 15 minutes, and the reaction was stopped with 0.125 mol/L of glycine for 5 minutes. Cells lysed in 5 mL lysis buffer [150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mmol/L Tris (pH 8.0), 5 mmol/L EDTA] with fresh protease inhibitors. Lysates were sonicated on ice and clarified by centrifuging for 10 minutes at 13,000 × g. Extracts were precleared with 50 μL of protein A-Sepharose/salmon sperm DNA slurry (1:1) for 30 minutes and incubated with 2 μg of anti-p53 overnight at 4°C. Fifty microliters of an A/G-agarose slurry was then incubated for 2 hours at 4°C and beads were washed twice with cold radioimmunoprecipitation assay buffer, four times with cold immunoprecipitation wash buffer [100 mmol/L Tris (pH 8.0), 500 mmol/L LiCl, 1% NP40, 0.1% deoxycholic acid], and twice with cold radioimmuno-precipitation assay buffer. One hundred and fifty microliters of immunoprecipitation elution buffer (50 mL NaHCO3 with freshly made 1% SDS) were added and rocked for 15 minutes at 25°C, after which beads were spun-out, and supernant transferred to a fresh tube. Another 150 μL immunoprecipitation elution buffer was added to the beads as done previously and the supernatants were combined. Cross-links reversed with 1 mL of 10 mg/mL RNase A and 12 μL of 5 mol/L NaCl with incubation at 65°C for 6 hours. DNA was purified by phenol–chloroform extraction, ethanol precipitation, and dissolved in 100 μL H2O. D-loop PCR amplification was done with 25 cycles (at 94°C for 15 seconds and 65°C for 45 seconds) and analyzed on a 2% TAE agarose gel. Forward primer, 5′-ctcgactgaggctccggac-3′; reverse primer, 5′-tgttgctgactaattagcaggt-3′.

D-loop gel mobility shift assay. A 0.4 kb D-loop region was PCR amplified and labeled with 32P-dCTP and terminal deoxynucleotidyl transferase (TdT). 8-oxo-dG lesions induced with photo-activated methylene blue as previously described (44). DNA binding assays done in 1× electromobility gel shift assay (EMSA) buffer [12 mmol/L HEPES (pH 7.9), 60 mmol/L NaCl, 1 mmol/L EDTA, 0.2 mmol/L EDT, 8% glycerol, 0.6 μg/mL poly dI-dC] with 0.1 μg labeled D-loop DNA and 50 ng recombinant mitochondrial transcription factor A (mtTFA; a gift from Dr. Kimitoshi Kohno, University of Occupational and Environmental Health, Kitakyushu, Japan) or p53 protein in a 20 μL volume. Reactions incubated at 25°C for 20 minutes, DNA/protein complexes were resolved on a 5% polyacrylamide-0.5× Tris-borate EDTA/2.5% glycerol gel, and images were captured by autoradiography.

In vivo repair of hydrogen peroxide–damaged mtDNA. Semiquanti-tive PCR using mitochondrial D-loop primers were carried out as described previously with minor modifications (44). Briefly, PCR was done with 15 ng of total DNA, 1 μCi of 3,000 Ci/mmol/L 32P-dCTP, 200 μmol/L deoxynucleotide triphosphate (dNTP), 20 pmol primer, and 2.5 units of Taq enzyme in a 50 μL volume for 25 cycles (94°C for 10 seconds and 72°C for 15 minutes). Amplification products were resolved on 6% PAGE, imaged with autoradiography, and quantitated by scanning densitometry as previously described (43).

In vitro repair incorporation assay. Incorporation assay as previously described with minor modifications (44). Briefly, mitochondrial extracts from cells treated with MNNG were incubated with either 8-oxo-dG- or uracil–containing double-stranded oligonucleotides (43). Alternatively, a uracil-containing 1.3 kb BER template was generated by PCR amplification of pGEM-ZZI (Promega, Madison, WI) using a uracil-containing forward primer. Forward primer, 5′-tcaggtttcaagggccttc tagaagcgactcagcgc-3′; reverse primer, 5′-tcatacagaggagggagtagcagcgc-3′. Assays done at 32°C in 50 μL buffer containing 45 mL/mmol/L HEPES-KOH (pH 7.8), 70 mmol/L KCl, 7.4 mmol/L MgCl2, 0.9 mmol/L DTT, 0.4 mmol/L
p53 Stimulates mtBER in Damaged Colorectal Cancer Cells

Results

p53 enhances in vivo repair of H2O2 damaged mtDNA in colorectal cancer cells. mtDNA mutations are frequent in colorectal cancers (12, 13, 19) and correlate with poor prognosis (21). p53 inactivation also plays an important role in colorectal cancer (22, 27, 46). Because p53 enhances mtBER in undamaged mouse cells (30), we wondered if p53 played a role in stimulating mtBER in damaged colorectal cancer cells. Using colorectal cancer cell lines made isogenic for p53 by homologous recombination (HCT116p53+/+ and HCT116p53−/−) cells growing exponentially (−), after 200 μmol/L H2O2 for 1 hour (0), or after 24 hours recovery in normal media (24 hr, top). Quantitation of changes in 32P-dCTP incorporation into PCR products at the indicated time points after H2O2 damage relative to baseline untreated cells (bottom). Columns, means of triplicate experiments; bars, SD. *: P = 0.015; #: P = 0.875 (unpaired two-tailed f test).

Figure 1. p53 pathway enhances in vivo repair of H2O2-damaged mtDNA. A, semiquantitative PCR amplification with and without damage (top). Titration of 32P-dCTP radiolabeled PCR amplification products using mtDNA D-loop–specific primers with increasing amounts of indicated input DNA (bottom). B, semiquantitative PCR using 15 ng input of DNA prepared from HCT116p53+/+ or HCT116p53−/− cells growing exponentially (−), after 200 μmol/L H2O2 for 1 hour (0), or after 24 hours recovery in normal media (24 hr, top). Quantitation of changes in 32P-dCTP incorporation into PCR products at the indicated time points after H2O2 damage relative to baseline untreated cells (bottom). Columns, means of triplicate experiments; bars, SD. *: P = 0.015; #: P = 0.875 (unpaired two-tailed t test).
below. These results suggest that an intact p53 pathway could enhance in vivo repair of H2O2-damaged mtDNA.

The p53 pathway enhances total in vitro mtBER of colorectal cancer cells. Because HCT116p53+/+ colorectal cancer cells more efficiently repair H2O2-damaged mtDNA in vivo (Fig. 1), we wished to determine if p53 stimulated mtBER in these cells—given that p53 could localize to the mitochondria (31–36, 38) and directly stimulate mtBER in undamaged cells (30). To measure differences in the BER capacity of mitochondrial extracts derived from p53+/+ or p53−/− colorectal cancer cells, we first isolated mitochondria free of nuclei (Fig. 2A). Lack of detectable PCNA or lamin B nuclear proteins shows no detectable nuclear contamination. DNA polymerase-γ protein in the mitochondrial, but not nuclear, fraction shows mitochondrial enrichment (47). Similar to previous reports (32–36, 38, 39), we detected p53 in mitochondrial extracts from p53+/+ cells (Fig. 2A, top). To measure mtBER capacity between HCT116p53+/+ and HCT116p53−/− cells, mitochondrial extracts were prepared from both cell lines after treatment with sublethal doses of the DNA-damaging agent (MNNG). The administration of 50 μmol/L of MNNG for 24 hours did not induce apoptosis as determined by the percentage of sub-G₀ cells using flow cytometry and caspase-3 cleavage (Fig. 2A, bottom). Extracts were then added to a uracil-containing BER template in the presence of 32P-dCTP (Fig. 2B, left), and incorporation of radiolabel into the full-length DNA template was used to measure differences in total mtBER capacity. Mitochondrial extracts prepared from MNNG-damaged p53+/+ cells more efficiently stimulated radiolabel incorporation than p53−/− extracts (Fig. 2B, right). The addition of recombinant p53 protein to p53−/− mitochondrial extracts stimulated the incorporation of 32P-dCTP into a uracil-oligonucleotide (Fig. 3A), and 32P-dGTP incorporation into an 8-oxo-G oligonucleotide (Fig. 3B). Control recombinant GFP did not stimulate radiolabel incorporation into either oligonucleotide. These results suggest that the p53 pathway, at least in part, directly stimulates in vitro total mtBER in human colorectal cancer cells.

The p53 pathway enhances DNA glycosylase, but not apurinic/apyrimidinic endonuclease, activity of colorectal cancer mitochondria. Because p53 stimulated total mtBER in colon cancer cells (Figs. 1–3), we wondered if p53 stimulated specific mtBER steps. The first step is catalyzed by a DNA glycosylase to remove the damaged base (reviewed in ref. 16). To determine if p53 could stimulate oxoguanine glycosylase (mtOGG1) activity, we compared the ability of HCT116p53+/+ and HCT116p53−/− mitochondrial extracts to incise a radiolabeled 8-oxo-G-oligonucleotide (Fig. 4A). Without dNTPs, the glycosylase incision generates a 32P-labeled 24-mer (from a 48-mer). p53+/+ mitochondrial extract incision activity did not significantly increase if cells were pretreated with MNNG (27-36% incised; Fig. 4A, lanes 1 and 2). MNNG damage to cells only

Figure 2. Mitochondrial extracts from p53+/+ cells enhance total in vitro BER. A, indicated immunoblots on crude nuclear fractions (lanes 1-4) and purified mitochondrial fractions (lanes 5-8) from HCT116p53+/+ cells treated with the indicated μmol/L amounts of MNNG for 24 hours (top). Caspase 3 Western blot and percentages of sub-G₀ cells measured by flow cytometry on HCT116p53+/+ cells after treatment with indicated amounts of MNNG for 24 hours (bottom). B, left, total BER measured by incorporation of 32P-dCTP into a PCR product. Right, autoradiogram of 32P-dCTP incorporation into the uracil-containing 1.3 kb PCR product stimulated by mitochondrial extracts prepared from HCT116p53+/+ or HCT116p53−/− cells pretreated with the indicated μmol/L amounts of MNNG for 24 hours (top). Quantification of changes in 32P-dCTP incorporation stimulated by mitochondrial extracts into the BER template after pretreatment of cells with MNNG relative to baseline untreated cells (bottom). Points, means of triplicate experiments; bars, SD. *, P = 0.040 (unpaired two-tailed t-test).
modestly stimulated incision activity of p53+/+ extracts (67-82% incised; Fig. 4A, lanes 3 and 4). Positive and negative incision controls, in the absence of mitochondrial extracts, are shown in the right-hand panel. To measure mtUDG activity (48), we measured the abilities of p53+/+ and p53−/− mitochondrial extracts to incise a radiolabeled uracil-oligonucleotide (Fig. 4B). p53+/+ mitochondrial extracts had higher basal incision activities compared with p53−/− extracts (72% versus 47%; Fig. 4B, lane 3 versus 1). Pretreatment of cells with MNNG did not significantly stimulate template incision by either extract (Fig. 4B, lane 1 versus 2; lane 3 versus 4). Positive and negative incision controls, in the absence of mitochondrial extracts, are shown in the right-hand panel. Addition of recombinant p53 protein could not reproducibly complement the p53−/− mitochondrial extract incision of either 8-oxo-G or uracil-oligonucleotides (data not shown).

DNA glycosylase generates an apurinic/apyrimidinic site recognized by apurinic/apyrimidinic endonuclease, which cleaves the phosphate backbone for the nucleotide incorporation step (16). To determine if p53 stimulates mitochondrial apurinic/apyrimidinic endonuclease activity, we measured the ability of p53+/+ and p53−/− mitochondrial extracts to incise a radiolabeled tetrahydrofuran-oligonucleotide (Fig. 4C). Tetrahydrofuran oligonucleotides are an apurinic/apyrimidinic site model that is resistant to 3′-β-lyase activity of some DNA glycosylases; thus, incision predominantly reflects apurinic/apyrimidinic endonuclease activity (49). Without dNTPs, apurinic/apyrimidinic endonuclease incision generates a 32P-labeled 26-mer (from a 50-mer). p53−/− extracts had similar basal incision activities as p53+/+ extracts (Fig. 4C, lanes 1 and 3) which did not increase with MNNG pretreatment of cells (Fig. 4C, lanes 2 and 4). Positive and negative incision controls, in the absence of mitochondrial extracts, are shown in the right-hand panel. Recombinant p53 protein did not stimulate tetrahydrofuran-oligonucleotide incision by p53−/− extracts (data not shown). Together, these results suggest that an intact p53 pathway can enhance the DNA glycosylase step, but not the apurinic/apyrimidinic endonuclease step, possibly through an indirect mechanism.

**p53 stimulates colorectal cancer mitochondrial nucleotide incorporation.** To investigate the role of p53 in the nucleotide
incorporation step catalyzed by mitochondria localized DNA polymerase-\( \gamma \), we measured the ability of HCT116p53+/+ and HCT116p53−/− mitochondrial extracts to stimulate \(^{32}\)P-dCTP incorporation into a 50-mer oligonucleotide containing a one-base gap opposite a guanine (Fig. 5). p53+/+ extracts stimulated higher incorporation into a 50-mer oligonucleotide containing a one-base glycosylase incision assay. In the absence of mitochondrial extracts, we measured the ability of HCT116p53+/+ or HCTp53−/− mitochondrial extracts to stimulate \(^{32}\)P-dCTP incorporation compared with p53−/− extracts (Fig. 5A, lanes 1 and 3), whereas MNNG pretreatment of cells only slightly increased incorporation (Fig. 5A, lanes 2 and 4). However, the addition of recombinant p53 protein to p53−/− extracts stimulated incorporation (Fig. 5B, lanes 1-4). Incubation of extracts with N-methylmaelamide, an inhibitor of the 140 kDa catalytic subunit of mtDNA polymerase-\( \gamma \), attenuated incorporation (Fig. 5B, lanes 5 and 6). Addition of control recombinant GFP did not stimulate incorporation (Fig. 5B, lanes 7-9). Because nucleotide incorporation into the gap template also requires DNA ligase activity, we examined if the ligation step was influenced by p53 (Fig. 5C). p53+/+ or p53−/− mitochondrial extracts were incubated with a molar excess of a \(^{32}\)P-poly-T single-strand 18-mer relative to a poly(A) single-strand 150-mer oligo, and DNA ligase activity was measured by the generation of radiolabeled 18 bp multi-mers. There was no difference in the extracts DNA ligase activity, with or without MNNG pretreatment (Fig. 5C, left, lanes 3-6). The addition of recombinant p53 protein to p53−/− extracts did not stimulate ligation (Fig. 5C, right, lanes 1-3). T4 DNA ligase served as a positive control and no extract as a negative control. Together, these results suggest that p53 directly stimulates nucleotide incorporation catalyzed by DNA polymerase-\( \gamma \), independent of DNA ligase, in human colorectal cancer cells, and is consistent with previous reports using mouse liver mitochondrial extracts.

p53 protein present in the compartment in which mtBER occurs. Because mtBER is localized to an inner mitochondrial subfraction (28), we wondered if p53 protein could be detected in this fraction (Fig. 6A). We first purified p53+/+ mitochondria using methods described in Fig. 2A. No detectable nuclear contamination was shown by the absence of PCNA (Fig. 6A, top). Purified mitochondria were sonicated and subfractionated by centrifugation into particulate and soluble fractions (28). As a quality control, enrichment of OGG1 was detected in the inner mitochondrial membrane–containing particulate fraction (Fig. 6A, bottom) as previously described (28). We also detected DNA polymerase-\( \gamma \) and p53 protein in the particulate fraction (Fig. 6A). These results are consistent with the observations that p53 plays a direct role in mtBER.

To determine if p53 associates with mtDNA, we cross-linked HCT116p53+/+ cells with formaldehyde, followed by sonication and immunoprecipitation with anti-p53 antisera. PCR amplification of immunoprecipitates with D-loop primers showed an increase in PCR product if cells were pretreated with MNNG (Fig. 6B, lanes 5 and 6). As a specificity control for p53 immunoprecipitation, HCT116p53−/− cells could not generate a PCR product using D-loop primers (Fig. 6B, lanes 2 and 3). We did EMSA, using the D-loop region and recombinant p53 protein, to investigate if p53 directly bound this region (Fig. 6C). As a positive control, mtTFA bound and retarded migration of the labeled mtDNA D-loop template as previously described (lane 3, Fig. 6C; ref. 37). However, recombinant p53 protein did not retard D-loop migration under these in vitro conditions (lanes 1 and 2, Fig. 6C). Together, these results suggest that p53 protein associates within an inner mitochondrial membrane enriched fraction although whether p53 binds the D-loop directly, or indirectly as part of a complex, remains unknown.

p53 interacts with nuclear DNA polymerase-\( \beta \) to stimulate nuclear BER (6). We therefore wondered if p53 interacted with mtDNA polymerase-\( \gamma \) given that p53 also localizes to an inner mitochondrial subfraction (Fig. 6A; ref. 28). In contrast to a recent

**Figure 4.** Intact p53 pathway enhances an in vitro mitochondrial DNA glycosylase incision assay. A, autoradiogram of a \(^{32}\)P-labeled 8-oxo-G–containing oligonucleotide after incubation with mitochondrial extracts prepared from HCT116p53+/+ or HCTp53−/− cells pretreated with or without MNNG. Oligo incision by recombinant GFP (negative control) and recombinant OGG1 protein (positive control), in the absence of mitochondrial extracts (right). B, autoradiogram of a \(^{32}\)P-labeled uracil-containing oligonucleotide after incubation with mitochondrial extracts prepared from HCT116p53+/+ or HCTp53−/− cells pretreated with or without MNNG. Oligo incision by recombinant GFP (negative control) and recombinant UDG and endo IV protein (positive control), in the absence of mitochondrial extracts (right). C, autoradiogram of a \(^{32}\)P-labeled tetrahydrofuran-containing oligonucleotide after incubation with mitochondrial extracts prepared from HCT116p53+/+ or HCT116p53−/− cells pretreated with or without MNNG. Oligo incision by recombinant GFP (negative control) and recombinant APE1 (positive control), in the absence of mitochondrial extracts (right). Percentage incised: incised oligo / (incised oligo + full-length oligo).
we were unable to coimmunoprecipitate endogenous p53 and endogenous DNA polymerase-γ in HCT116p53+/+ cells (data not shown). Therefore, we infected HCT116p53–/– cells with a p53 expressing adenovirus, isolated mitochondrial fractions, and immunoprecipitated with anti-p53 antisera (lane 3, Fig. 6D). In addition to the expected detection of p53 protein (bottom), a 140 kDa band of the predicted size of the DNA polymerase-γ catalytic subunit protein was detectable (top). To control for specificity, coimmunoprecipitations from control adenovirus–infected HCTp53–/– cells (lane 2, Fig. 6D) did not detect DNA polymerase-γ over the background (top) or p53 (bottom). As a further control, immunoprecipitation of Ad-p53-infected cells with nonimmune IgG did not detect either p53 or DNA polymerase-γ (lane 1, Fig. 6D). These results suggest that, at least when artificially overexpressed in HCT116p53–/– cells, p53 protein can associate with DNA polymerase-γ. However, whether this interaction occurs under physiologic conditions remains to be shown under our experimental conditions.

**Discussion**

We have shown that the p53 pathway promotes efficient mtBER in colorectal cancer cells in response to damage using both in vivo and in vitro repair assays. Given that mtDNA mutations (a) are frequent in colorectal cancer (12, 13, 19, 20), (b) predict poor prognosis (21) and, (c) contribute to aggressive tumor behavior (17, 18), our findings provide novel insight and opens new avenues for investigation into an important mechanism.
that could influence colorectal cancer development and response to therapy.

Our data expands the role of p53 beyond promoting nuclear BER (3, 5–9) and provides a mechanism by which p53 could promote mitochondrial genomic stability in colorectal cancer cells. This is consistent with a recent report that p53 enhances mtBER in undamaged mouse hepatocytes (30). It also seems that specific steps in colorectal cancer mtBER are enhanced by p53. Using in vitro incision assays as a read-out for mtDNA glycosylase activity, p53+/+ mitochondrial extracts had enhanced glycosylase activity compared with p53−/− extracts (Fig. 4A and B). Additionally, nucleotide incorporation was more efficiently stimulated by p53+/+ mitochondrial extracts compared with p53−/− extracts (Fig. 5A and B). In contrast, p53 status did not influence the apurinic/apyrimidinic endonuclease or DNA ligase steps (Figs. 4C and 5C). Interestingly, although DNA damage stimulated p53-mediated total mtBER in vivo (measured by semiquantitative D-loop PCR; Fig. 1), or in vitro (measured by an incorporation assay; Fig. 2), DNA damage only minimally stimulated the DNA glycosylase or DNA polymerase-γ steps—at least as measured with in vitro BER assays (Figs. 4 and 5). Thus, it remains unclear if p53 stimulates specific mtBER steps in response to damage. One possibility is that the in vitro assays do not completely reconstitute mtBER, and suggests that more complex mechanisms influence the p53-mtBER axis. However, this might also reflect the possibility that p53 has both genomic maintenance, as well as a DNA damage response, functions for the mitochondrial genome.

Although our work and others (30, 51) show that the p53 pathway enhances mtBER, the precise mechanisms remain to be clarified. p53 protein directly stimulates nuclear BER after genotoxic damage by interaction with the nuclear BER complex at abasic sites (3, 5–9). Thus, it is tempting to speculate that p53 may similarly play a direct role in mtBER especially given the recent findings describing p53 translocation to mitochondria in stressed (31–37) as well as nonstressed cells (38, 39). We found that the addition of recombinant p53 protein to colorectal cancer...
mitochondrial extracts stimulated in vitro repair incorporation (requiring completion of all BER steps; Fig. 3). However, it remains possible that in response to sublethal stress, p53 transcriptionally activates target genes that directly, or indirectly, enhance mtBER. Indeed, our inability to stimulate mtDNA glycosylase activity with added recombinant p53 protein (data not shown) suggests that p53 has an indirect role at this step—even though p53+/+ mitochondrial extracts had higher basal glycosylase activity than p53−/− extracts (Fig. 4A and B). This is consistent with a prior report demonstrating that recombinant p53 protein could not stimulate mtDNA glycosylase function of mouse hepatocytes (30). Interestingly, in that report mitochondria from p53+/+ and p53−/− mouse livers had no differences in DNA glycosylase basal activity (30). This is in contrast to human colorectal cancer cells (Fig. 4) and suggests that the cell context could influence the role of p53 at the glycosylase step. Whether such differences are specific to colorectal cancer remains to be determined.

We found that p53 protein stimulates repair of a one-nucleotide gap-oligonucleotide (Fig. 5A and B). This most likely occurs through stimulation of nucleotide incorporation by DNA polymerase-γ because the final DNA ligase step was not affected by p53 (Fig. 5C). These findings are consistent with data using undamaged mouse liver mitochondria (30), although the precise mechanisms remain elusive. We detected p53 protein within the inner mitochondrial compartment (Fig. 6) where mtDNA and mtBER components localize (28). However, we were unable to show that p53 protein could directly bind an 8-oxo-G D-loop by EMSA (Fig. 6C) even though p53 protein was detected in association with the D-loop region in intact cells (Fig. 6D). This implies that the p53 protein could indirectly associate with mtDNA as part of a repair complex rather than by direct mtDNA binding. This is supported by the finding that p53 cannot bind a uracil-containing oligonucleotide (30). However, it is possible that the ability of p53 to bind damaged DNA is subject to complex regulation not reconstituted using an in vitro EMSA. Indeed, previous reports show that p53 protein from damaged cell extracts could be pulled down with an 8-oxo-G oligonucleotide (5). We also investigated whether p53 could bind mtDNA polymerase-γ because p53 can bind nuclear DNA polymerase-β (6). Despite multiple attempts, we could not detect binding between the endogenous p53 and DNA polymerase-γ proteins in HCT116p53+/+ cells (data not shown), although we were able to communoprecipitate overexpressed p53 and endogenous DNA polymerase-γ (Fig. 6D). Perhaps under certain conditions, p53 may interact with DNA polymerase-γ to stimulate mtBER. However, this result must be interpreted cautiously as it requires overexpression of p53. Interestingly, a very recent report described binding between endogenous DNA polymerase-γ and endogenous p53 in HCT116p53+/+ cells, and that this association was enhanced by pretreatment of cells with ethidium bromide (51). These studies imply that the potential interactions between p53, DNA polymerase-γ, and mtDNA involves complex mechanisms that are highly dependent on system and cell context. Ultimately, understanding the molecular mechanisms of how p53 modulates mtBER will require the demonstration that recombinant, transcriptionally translated p53 can translocate into translocation-competent mitochondria using rigorously defined cell-free mitochondrial protein import assays.

Our data which shows that an intact p53 pathway stimulates specific steps during mtBER in colorectal cancer cells provides significant mechanistic insight into the clinical observations that colorectal cancers have a high frequency of mtDNA mutations which negatively affect survival (12, 13, 19–21). Given that loss of p53 function plays an important role in colorectal cancer development and response to therapy (22–26), our findings suggest that attenuation of specific mtBER steps may be important in colorectal cancer biology and is consistent with recent reports demonstrating that p53 promotes mitochondrial genomic stability (51) and that mtDNA mutations promote aggressive tumor behavior and apoptosis resistance (17, 18). The ultimate role for p53 stimulation of colorectal cancer mtBER remains to be determined. Nevertheless, our observations open new avenues for investigation that may eventually provide new diagnostic/prognostic tools and novel therapeutic strategies for colorectal cancer patients.

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