Novel role of p53 in maintaining mitochondrial genetic stability through interaction with DNA Pol γ

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Mitochondrial DNA (mtDNA) mutations and deletions are frequently observed in cancer, and contribute to altered energy metabolism, increased reactive oxygen species (ROS), and attenuated apoptotic response to anticancer agents. The mechanisms by which cells maintain mitochondrial genomic integrity and the reason why cancer cells exhibit more frequent mtDNA mutations remain unclear. Here, we report that the tumor suppressor molecule p53 has a novel role in maintaining mitochondrial genetic stability through its ability to translocate to mitochondria and interact with mtDNA polymerase γ (pol γ) in response to mtDNA damage induced by exogenous and endogenous insults including ROS. The p53 protein physically interacts with mtDNA and pol γ , and enhances the DNA replication function of pol γ . Loss of p53 results in a significant increase in mtDNA vulnerability to damage, leading to increased frequency of *in vivo* mtDNA mutations, which are reversed by stable transfection of wild-type p53. This study provides a mechanistic explanation for the accelerating genetic instability and increased ROS stress in cancer cells associated with loss of p53.

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Introduction

Mitochondria play essential roles in energy metabolism, generation of reactive oxygen species (ROS), and regulation of apoptosis. Of the polypeptides that constitute the mitochondrial respiratory chain, 13 are encoded by mitochondrial DNA (mtDNA) (Anderson *et al*, 1981). The coordinated activity of

the respiratory chain components facilitates electron transfer for generation of ATP. mtDNA is vulnerable to mutagenic lesions induced by endogenous and exogenous DNA-damaging agents such as ROS and radiation, due in part to its close proximity to the respiratory chain, lack of histone protection, and limited DNA repair capacity (Marcelino and Thilly, 1999; Kang and Hamasaki, 2002). Defects in respiratory chain components arising from mtDNA mutations may result in decreased ATP production, affect the electron transport process, and cause electron leakage and increased formation of superoxide (O_2^-) (Staniek *et al*, 2002; McKenzie *et al*, 2004). O_2^- radicals, when produced in excess, interact with other radicals to form ROS that may cause damage to DNA, proteins, and lipids (Halliwell and Gutteridge, 1999). Defects in components of the respiratory chain that result in leakage of electrons could hence be potentially harmful to the cell. ROS have been suggested to play a major role in the pathogenesis of disorders associated with mtDNA mutations (McKenzie *et al*, 2004). It is evident that maintenance of integrity of the mitochondrial genome is of utmost importance to cells.

Under the influence of oncogenic signals, cancer cells are generally more active in metabolism than normal cells and generate more ROS, which impose a risk of damaging mtDNA and causing mitochondrial mutations. Malfunction of the respiratory chain subsequent to mtDNA mutations may in turn cause increased generation of ROS, leading to further DNA damage, genetic instability, and cancer progression (Pelicano *et al*, 2004; Singh, 2004). Indeed, increased mutations in mtDNA have been observed in cancer cells of various tissue origins (Wallace, 1999; Carew and Huang, 2002; Copeland *et al*, 2002; Nomoto *et al*, 2002; Carew *et al*, 2003). Since maintenance of mtDNA integrity is crucial to the normal functioning of the cell, and since mtDNA mutations are frequently observed in human cancers, a thorough understanding of the mechanisms by which cells maintain the stability of their mitochondrial genome is essential.

One of the key molecules involved in the maintenance of mitochondrial genomic stability is the mtDNA polymerase γ (pol γ), which is the sole DNA polymerase in mitochondria and plays an essential role in mtDNA replication and repair (Copeland *et al*, 2003; Kaguni, 2004). Human pol γ contains a 140 kDa catalytic subunit and a 55 kDa accessory subunit. Mutations in either the polymerase or the exonuclease domain of pol γ seem to be associated with mitochondrial malfunction and premature aging (Copeland *et al*, 2003; Trifunovic *et al*, 2004). Biochemical analyses have shown that such defects result in increased frequency of mtDNA mutations (Van Goethem *et al*, 2002; Copeland *et al*, 2003). However, the molecular details of the mechanisms by which pol γ ensures stability of the mitochondrial genome following mtDNA damage remain to be elucidated. Since multiple proteins participate in mtDNA replication and repair processes, it is likely that, in addition to the 3'–5' exonuclease activity of pol γ , other molecules may also play important roles in maintaining mtDNA integrity.

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The p53 tumor suppressor protein plays a central role in response to DNA damage, cell cycle regulation, and apoptosis. More than 50% of human cancers carry mutations in p53 (Vogelstein *et al*, 2000; Lane and Hupp, 2003). Recent studies demonstrated that, in addition to its role as a transcription factor, p53 protein can translocate to the mitochondria in response to certain stimuli, and induce transcription-independent apoptosis through direct interaction with Bcl-2 family proteins (Marchenko *et al*, 2000; Mihara *et al*, 2003; Chipuk *et al*, 2004; Erster *et al*, 2004). Additionally, it has also been reported that a small fraction of p53 is also present in the mitochondria of unstressed cells, although its function remains unclear (Mahyar-Roemer *et al*, 2004). The current study demonstrates that p53 exhibits physical and functional interactions with pol γ in response to mtDNA damage, and plays a novel role in maintaining mitochondrial genetic stability.

Results and discussion

Loss of p53 leads to increased vulnerability to mtDNA depletion

During our studies of mtDNA damage and its mechanistic link to ROS generation and cellular responses to anticancer agents (Carew *et al*, 2003; Pelicano *et al*, 2003; Achanta and Haung, 2004), a surprising association between the status of cellular p53 and the vulnerability of mtDNA to exogenous damage came to our attention. In the process of deriving mitochondrial respiration-deficient cells (ρ^0) using a standard ethidium bromide (EtBr) method, which at low concentrations preferentially intercalates into mtDNA and causes its depletion (King and Attardi, 1996; Chandel *et al*, 1998; Pelicano *et al*, 2003), we consistently observed that cells lacking p53 function were significantly more vulnerable to ethidium-induced mtDNA depletion than cells with wild-type (wt) p53. The HCT116 p53 $^{-/-}$ cells, generated from the parental HCT116 cells by somatic disruption of p53 (Bunz *et al*, 1998), were susceptible to mtDNA depletion by chronic incubation with a low concentration of EtBr (100 ng/ml, 120 days) and developed a respiration-deficient phenotype (Figure 1A and C). In contrast, the same ethidium treatment did not cause mtDNA depletion or loss of mitochondrial respiration in HCT116 p53 $^{+/+}$ cells (Figure 1B and D), suggesting that p53 might somehow protect the integrity of the mitochondrial genome. Subsequent experiments showed that a higher concentration of ethidium (200 ng/ml) and longer exposure time (150 days) were needed to generate ρ^0 cells from the HCT116 p53 $^{+/+}$ cells (Table I). Similar results were observed in further experiments comparing the sensitivity of mtDNA to ethidium in several other cell lines harboring wt p53, mutant p53, or p53 deletion. HL-60 (p53-null) and Raji cells (mutant p53) were much more sensitive than ML-1 cells (wt p53) to ethidium-induced mtDNA damage (Table I).

We then used the isogenic HCT116 cells to evaluate the acute effect of p53 on mtDNA depletion by exposing the p53 $^{+/+}$ and p53 $^{-/-}$ cells to various concentrations of ethidium for a relatively short period (20 days instead of 120 days). The mtDNA content was measured by standard PCR analysis using a pair of primers specific for the mtDNA D-loop region as described previously (Carew *et al*, 2003). As shown in Figure 2A, there was a concentration-dependent

Figure 1 Comparison of ethidium-induced mtDNA depletion and loss of mitochondrial respiratory activity of p53 isogenic cells. (A, B) HCT116 p53 $^{+/+}$ cells and the isogenic p53 $^{-/-}$ cells were exposed to EtBr (100 ng/ml) for 120 days and several sub-clones of surviving cells were isolated and expanded as described in Materials and methods. The D-loop region of mtDNA from four individual clones (C1–C4), each derived from either parental (Par) p53 $^{+/+}$ or p53 $^{-/-}$ cells was analyzed by PCR as described previously (Carew *et al*, 2003). (C, D) Mitochondrial respiratory activity in parental cells and isolated clones was measured by an oxygen consumption assay (Pelicano *et al*, 2003). The slope of the oxygen consumption curve reflects the respiratory activity.

Table I Comparison of EtBr concentrations and exposure duration required for depletion of mtDNA and generation of respiration-deficient cells (ρ^0) from parental cells with different p53 status

Cell lines	p53 status	EtBr (ng/ml)	Time (day)
HL-60 (leukemia)	Null	50	21
Raji (leukemia)	Mut	50	21
ML-1 (leukemia)	wt	100	75
HCT116 p53 $^{-/-}$ (colon cancer)	Null	100	120
HCT116 p53 $^{+/+}$ (colon cancer)	wt	200	150

depletion of mtDNA in the p53 $^{-/-}$ cells, with about 80% of mtDNA diminished in cells treated with 1 μ g/ml of EtBr by day 20. In contrast, only a moderate decrease of mtDNA was seen in p53 $^{+/+}$ cells exposed to the same concentration of EtBr (Figure 2B). When the ethidium-treated cells were subcultured at day 20 in regular medium without EtBr, a large number of p53 $^{+/+}$ cells formed colonies, even in cells treated with relatively high concentrations (0.3–1 μ g/ml) of ethidium (Figure 2C). As the regular culture medium only supports the growth of respiration-competent cells (ρ^+), but not the respiration-defective (ρ^0) cells that require other nutrient supplements, these data suggest that the p53 $^{+/+}$ cells were able to sustain the acute exposure to ethidium and that their mitochondria remained competent in respiration. In contrast, few colonies from the p53 $^{-/-}$ cells treated with 0.3–1 μ g/ml ethidium survived in regular culture medium (Figure 2C). This suggests that the relatively high concentrations of ethidium caused severe damage to mtDNA in p53 $^{-/-}$ cells, leading to respiration deficiency and failure to survive in regular culture medium. When the ethidium-treated p53 $^{-/-}$ cells were cultured in special medium supplemented with high glucose, pyruvate, and uridine (ρ^0 medium) to enable

Figure 2 Absence of p53 leads to vulnerability of mtDNA to damage by EtBr. (A, B) HCT116 p53^{+/+} and p53^{-/-} cells were exposed to the indicated concentrations of EtBr for 20 days, DNA was isolated from the cells and the D-loop region of mtDNA was analyzed by PCR. (C) HCT116 p53^{+/+} and p53^{-/-} cells were exposed to the indicated concentrations of EtBr in McCoy's supplemented culture medium (see Materials and methods) for 20 days, with regular culture split every 3–4 days. After 20 days, the cells were cultured in SM medium (McCoy's + supplements) without EtBr for 6 days. The cells were then plated (3000/well) in duplicate sets. One set was incubated in regular culture medium (McCoy's + 10% FBS) to grow cells with competent mitochondrial respiratory function (ρ^+ cells). Another set of cells was cultured in medium supplemented with glucose, pyruvate, and uridine to allow the growth of respiration-competent cells as well as respiration-deficient (ρ^0) cells. The colonies growing in the supplemented medium were a mixture of ρ^+ and ρ^0 cells.

ATP generation through glycolysis, many small colonies formed, even in cells treated with 1 µg/ml EtBr (Figure 2C, indicated by the arrows). These data further indicate that ethidium caused severe mtDNA damage in p53^{-/-} cells, which did not survive in regular medium but were able to form colonies in special medium supplemented with nutrients for ρ^0 cells.

Translocation of p53 to mitochondria and its interaction with pol γ

Several approaches were then used to test the possibility that p53 might translocate to mitochondria and facilitate mtDNA synthesis in response to mtDNA damage. We first analyzed p53 protein in the mitochondrial fractions isolated from cells before and after treatment with EtBr, which preferentially accumulates in the mitochondria due to the cationic charge of ethidium, and intercalates into mtDNA. As shown in Figure 3A and B, ethidium caused an increase of p53 protein in the mitochondrial fractions. This was observed in ML-1 cells (wt p53) and HCT116 p53^{+/+} cells. Blotting of mitochondrial protein Hsp60 demonstrated equal loading of the mitochondrial extracts. Analysis of the mitochondrial protein prepara-

tions for PCNA showed that there was little nuclear contamination (lanes 1–5), whereas the whole-cell extracts exhibited a strong PCNA signal (lane 6).

Confocal microscopic analysis was then used to confirm the mitochondrial localization of p53 and to evaluate its possible colocalization with the mtDNA polymerase γ . Using appropriate combination of antibodies (see Materials and methods), p53 and pol γ were detected as green and red fluorescent signals, respectively. The colocalization of these two proteins would therefore appear as a yellow signal. As shown in Figure 3C, the untreated HCT116 cells exhibited a background p53 signal (green) in the cytosol and nuclei, whereas pol γ (red signal) appeared as clusters of small dots exclusively in the cytosolic region (mitochondria). There was no significant colocalization of p53 and pol γ in the control cells. After treatment with EtBr, p53 accumulated in the mitochondria, and appeared colocalized with pol γ as yellow spots. However, a portion of the mitochondria still exhibited only strong pol γ signal without p53, suggesting that these mitochondria might not have significant DNA damage or that there was insufficient p53 transported into these mitochondria at the time of cell fixation.

The physical interaction of p53 and pol γ was further demonstrated by coimmunoprecipitation experiments, in which p53 was pulled down with pol γ (Figure 3D). Interestingly, this molecular interaction was substantially enhanced when cells were pretreated with EtBr, as evidenced by a stronger p53 band in the protein extracts prepared from ethidium-treated cells (Figure 3D, lanes 6 and 14).

Effect of ROS on mitochondrial translocation of p53 and its interaction with pol γ

Under physiological conditions, ROS are constantly generated in the mitochondria through the respiratory chain. This ROS production may significantly increase when cells are under metabolic stress and/or have mtDNA mutations (Pelicano *et al*, 2004). Owing to the physical proximity between the ROS generation site and mtDNA, an increase in ROS production would pose a risk of damaging mtDNA and lead to further malfunction of mitochondrial respiration. To evaluate the biological relevance of p53 in responding to endogenous ROS-mediated mtDNA damage, we used a biochemical approach to enhance ROS generation by interfering with the respiratory activity using rotenone, a complex I inhibitor known to increase mitochondrial ROS (Degli Esposti, 1998; Pelicano *et al*, 2003). As shown in Figure 4A, treatment of HCT116 cells with rotenone resulted in a concentration-dependent increase of O_2^- radical. Incubation with 100 and 500 nM rotenone caused increases of O_2^- to 150% ($P < 0.001$) and 210% ($P < 0.001$) of the control, respectively. This increased ROS generation was accompanied by an increase of p53 protein levels in the mitochondrial fraction, as demonstrated by Western blot analysis (Figure 4B). Immunofluorescent staining and confocal microscopic analysis showed that, following exposure to 100 nM rotenone for 24 h, there was a substantial accumulation of p53 signal (green fluorescence) in the mitochondria, which predominantly colocalized with pol γ (red stain) and appeared as yellow spots (Figure 4C). To test if the mitochondrial translocation of p53 and its interaction with pol γ following rotenone treatment were mediated by ROS, cells were incubated with rotenone in the presence of the ROS scavenger *N*-acetylcysteine

Figure 3 Ethidium induces mitochondrial translocation of p53 and its interaction with pol γ . **(A)** ML-1 cells (wt p53) and **(B)** HCT116 p53 +/+ cells were incubated with EtBr as indicated below. Protein extracts from the mitochondrial fractions were assayed for p53 by Western blotting. The mitochondrial protein Hsp60 was used as a loading control. The nuclear protein PCNA was also blotted to show that nuclear protein contamination in the mitochondrial fractions was negligible. Lane 1, mitochondrial fraction from control cells; lanes 2–3, mitochondrial fractions from cells treated with 0.3 μ g/ml ethidium for 12 and 24 h, respectively; lanes 4–5, mitochondrial fractions from cells treated with 1 μ g/ml ethidium for 12 and 24 h, respectively; lane 6, total protein extracts from control cells. **(C)** Confocal microscopic analysis of intracellular localization of p53 and pol γ . HCT116 p53 +/+ cells were incubated with or without EtBr (1 μ g/ml, 24 h), fixed, and labeled for p53 (green) and pol γ (red) as described in Materials and methods. The majority of p53 outside the nuclear region was colocalized with pol γ in the ethidium-treated cells, as evidenced by the yellow signal. **(D)** Coimmunoprecipitation of p53 with pol γ . Protein extracts (1 mg) from cells treated with or without ethidium (1 μ g/ml, 12 h) were incubated with 1.5 μ g of rabbit polyclonal anti-DNA pol γ antibody or control rabbit IgG at 4°C for 5 h, and the protein complexes were separated using magnetic beads coated with protein A as described under Materials and methods. The supernatant (S), last wash (W), and pellet (P) were assayed for p53 by Western blotting.

(NAC). As shown in Figure 4C, addition of NAC prevented the translocation of p53 to mitochondria in rotenone-treated cells; the cellular localization of p53 and pol γ in cells coincubated with rotenone and NAC was similar to that in the control cells. NAC alone did not cause detectable change in cellular p53 distribution (data not shown). These data suggest that the rotenone-induced p53 localization to mitochondria was mediated by ROS.

For comparison, exogenous hydrogen peroxide (H_2O_2), which has a longer half-life than O_2^- and is able to enter the cells and reach both mitochondria and the nucleus, was added to the cell culture and its effect on p53 localization

was analyzed. As shown in Figure 4C, H_2O_2 caused an increase of p53 in both mitochondria and the nucleus. This is consistent with the ability of H_2O_2 to damage mtDNA and nuclear DNA (nDNA), and the previously established role of p53 in response to nDNA damage, and was in contrast to rotenone, which increased endogenous O_2^- generation in the mitochondria and caused translocation of p53 almost exclusively to mitochondria.

To test if the increase of mitochondrial ROS generation induced by rotenone might preferentially cause oxidative mtDNA damage, triggering p53 translocation to mitochondria, we used dot-blot analysis of mtDNA for the presence of

Figure 4 Increased mitochondrial generation of ROS and mtDNA damage causes p53 localization to mitochondria and interaction with pol γ . **(A)** HCT116 p53^{+/+} cells were incubated with various concentrations of rotenone, and increased O₂⁻ generation was measured by flow cytometry analysis as described previously (Huang *et al*, 2000). Green curve, background fluorescence without labeling with dihydroethidium; black curve, control cells labeled with dihydroethidium for 60 min; red curve, cells treated with 100 nM rotenone for 8 h and labeled with dihydroethidium for 60 min; orange curve, cells treated with 500 nM rotenone for 8 h and labeled with dihydroethidium for 60 min. The numbers indicate the mean O₂⁻ contents for the respective curves (displayed in log scale). **(B)** Protein extracts of mitochondria isolated from the control or rotenone-treated cells were assayed for p53 by Western blotting. Mitochondrial protein Hsp60 was also measured as a loading control. Lane 1, mitochondria from control cells; lanes 2–3, mitochondria from cells treated with 100 nM rotenone for 12 and 24 h, respectively; lane 4, mitochondria from cells treated with 300 nM rotenone for 24 h. **(C)** HCT116 p53^{+/+} cells were untreated or treated with 100 nM rotenone (Rot) alone for 24 h or in combination with 3 mM NAC, or with 50 μ M H₂O₂ for 24 h as indicated. NAC was preincubated 1 h before addition of rotenone. The cells were fixed and immunostained for p53 (green) and pol γ (red), and visualized by confocal microscopic analysis as described in Materials and methods.

8-oxo-dG as an indicator of ROS-mediated damage in comparison with nDNA. MtDNA and nDNA from control and rotenone-treated cells were immobilized on a membrane, and immunoblotted with an antibody specific for 8-oxo-dG as described in Materials and methods. As illustrated in

Figure 5, rotenone treatment resulted in a significant increase in 8-oxo-dG signal in mtDNA in a time-dependent manner, whereas nDNA isolated from the same cells showed no increase in 8-oxo-dG signal. These results suggest that rotenone was able to selectively cause mtDNA damage, and that

Figure 5 Preferential oxidative mtDNA damage induced by rotenone. (A) HCT116 cells were treated with 300 nM rotenone for 12–24 h as indicated. nDNA and mtDNA were isolated, dot-blotted onto a nitrocellulose membrane, and assayed for 8-oxo-dG residues in DNA using a specific antibody against 8-oxo-dG as described in Materials and methods. (B) Quantitation of oxidative mtDNA damage induced by rotenone. The intensity of the dots of the mtDNA samples was measured by densitometry. The bars show the relative dot intensity compared to the control sample. Data represent mean $\pm$ s.d. from three separate experiments.

the mitochondrial localization of p53 was a specific response to oxidative damage in mtDNA. Taken together, the data shown in Figures 3–5 suggest that the primary signal that caused localization of p53 to mitochondria was likely triggered by mtDNA damage. Although a preferential degradation of p53 in the nucleus might also explain the non-nuclear distribution of p53 seen in Figures 3 and 4, this is unlikely, since the overall p53 signal was increased after treatment with ethidium or rotenone.

Physical association of p53 with mtDNA

We next used mtDNA immunoprecipitation assay (mtDIP assay) and PCR analysis to test if p53 localizes to the mitochondrial matrix and physically associates with mtDNA. As illustrated in the upper panel of Figure 6, our mtDIP-PCR analysis is based on the principle of chromatin immunoprecipitation (ChIP) assay. After HCT116 p53 +/+ cells were treated with EtBr (1 μ g/ml) or rotenone (300 nM), mitochondria were isolated and proteins that interact with mtDNA were chemically crosslinked with formaldehyde as described in Materials and methods. The samples were then subjected to immunoprecipitation with anti-p53. The precipitated DNA was isolated and analyzed by PCR for mtDNA, using a pair of primers specific for the mitochondrial D-loop region. Nonspecific IgG antibody was used as a negative control, and mtDNA (before immunoprecipitation) from each sample was used as positive control (input) for the PCR reaction. As shown in Figure 6, specific mtDNA PCR signals were observed in p53-precipitated samples (lanes 2, 5, and 8), but not in the control IgG samples (lanes 3, 6, and 9), indicating that the immunoprecipitation was p53-specific.

Figure 6 Physical interaction of p53 with mtDNA in whole cells. HCT116 cells (p53 +/+) were incubated with or without EtBr (1 μ g/ml) or rotenone (300 nM) for 24 h. Mitochondria were isolated and subjected to mtDIP-PCR assay using p53 antibody for immunoprecipitation (nonspecific IgG antibody as control) and mitochondrial D-loop-specific primers for PCR detection of mtDNA as described in Materials and methods. The PCR products were analyzed by agarose gel electrophoresis. Lanes 1–3, PCR products from mitochondria isolated from control cells without treatment with ethidium or rotenone; lanes 4–6, PCR products from mitochondria of cells treated with ethidium; lanes 7–9, PCR products from mitochondria of cells treated with rotenone. Note: In, input control (mtDNA before immunoprecipitation); p53, immunoprecipitation with p53 antibody; IgG, immunoprecipitation with control IgG antibody; N, nucleus; M, mitochondria.

There was a basal level of p53 associated with mtDNA in the absence of exogenous stress. This is consistent with the data in Figure 3B and C, where p53 is detected in the mitochondria of control cells, likely reflecting a response to the endogenous ROS-mediated mtDNA damage. Incubation of cells with ethidium led to a detectable increase of the PCR signals (lane 5 compared to lane 2). Interestingly, there was no significant increase of the mtDNA PCR signal in the cells treated with rotenone (lane 8), likely due to the ROS-mediated damage to mtDNA template that is known to retard the PCR reaction (Mambo *et al*, 2003; Santos *et al*, 2003), thus masking the increased association between p53 and mtDNA.

p53 enhances the DNA replication function of pol γ

An *in vitro* DNA primer extension assay was employed to further test the functional effect of p53 on DNA polymerization activity of pol γ , using double-stranded deoxyoligonucleotides containing sequences identical to the first 40 nucleotides of the mtDNA replication origin as the primer/template. As illustrated in Figure 7A, pol γ in the mitochondrial extracts from p53 $^{-/-}$ cells exhibited DNA replication activity, as evidenced by the incorporation of [α - 32 P]dATP into the 40-base polymerization product (lane 3). p53 itself exhibited

Figure 7 p53 enhances the DNA replication function of pol γ *in vitro*. (A) *In vitro* DNA primer extension by pol γ in the presence and absence of p53 and EtBr. Mitochondrial protein extracts containing pol γ were isolated from HCT116 p53^{-/-} cells as described in Materials and methods. The primer/template DNA sequence is shown on top of the figure. Reaction mixtures containing the indicated components were incubated at 37°C for 20 min, and analyzed on a denaturing polyacrylamide gel. (B) The radioactivity of each 40-base product band from (A) was quantified using a phosphorimager, and expressed as % intensity of the respective control samples. Each bar represents mean $\pm$ s.e. of three separate determinations. *Indicates statistically significant increase in band intensity. (C) Effect of p53 on *in vitro* DNA primer extension by pol γ using DNA primer/template containing mismatched nucleotides. The reaction conditions were similar to those described in (A), except that ethidium was excluded, and the last three nucleotides at the 3'-end of the primer were altered to create a 3-nucleotide mismatched region as indicated above (slanted letters). The reaction mixtures containing the indicated amount of p53 protein were incubated at 37°C for 20 min, and analyzed on a 15% denaturing polyacrylamide gel. (D) The radioactivity of the 40-base product band in (C) was quantified using a phosphorimager. The intensity of each band was expressed as % of the control band (without p53). Each bar represents means $\pm$ s.e. from three separate gel analyses. *Indicates statistically significant increase in band intensity.

no DNA replication activity (lane 2). However, addition of p53 protein to the mitochondrial extracts from p53^{-/-} cells resulted in an approximately 25% increase of the DNA replication activity (lane 4). Addition of ethidium to the *in vitro* reaction substantially suppressed the activity of pol

γ (lane 5), suggesting that intercalation of EtBr into the DNA primer/template may alter the DNA conformation and retard replication. Under these conditions, p53 significantly enhanced the polymerization activity of pol γ in a concentration-dependent manner (lanes 6–8). The quantitative data from multiple gels are summarized in Figure 7B. This 25–50% increase of pol γ activity in the presence of p53 is statistically significant ($P < 0.05$), and may contribute to the p53-dependent protection of mtDNA observed in Figures 1 and 2. However, the exact role of this relatively small increase of enzymatic activity *in vivo* is still unclear.

As the DNA polymerization assay described above used mitochondrial extracts as the source of pol γ , it is important to rule out the possibility that the observed DNA polymerization activity might be due to a contamination of the mitochondrial extracts with nuclear DNA polymerases. In a control experiment, addition of 0.3–3.0 μ M aphidicolin, which is known to effectively inhibit nDNA synthesis at these concentrations, did not inhibit DNA polymerization catalyzed by the mitochondrial extracts (data not shown). This result suggests that the major enzyme activity was from pol γ (insensitive to aphidicolin) rather than from nuclear DNA polymerases.

The above observations led us to examine the effect of p53 on the polymerization activity of pol γ using DNA primer/template that contained other conformational changes such as mismatched nucleotides. Mismatches arise in DNA either due to mispairing during replication or incorporation of an unpaired nucleotide opposite to a modified base such as an oxidized guanine. We altered the nucleotide sequence of the primer to create a mismatch of three nucleotides between the template and the primer at the 3'-end (Figure 7C). This DNA construct required the excision of the unpaired nucleotides by the 3'–5' exonuclease activity of pol γ for normal polymerization (primer extension). In the absence of p53, only a moderate polymerization was detected as a light band at 40-base position (Figure 7C, lane 3). Addition of p53 protein significantly increased the polymerization product (Figure 7C–D), suggesting that p53 was able to enhance the overall excision and polymerization activities of pol γ . Interestingly, a recent study showed that p53 is also able to enhance the base excision repair activity of mouse liver mitochondrial extracts on uracil-containing DNA (de Souza-Pinto *et al*, 2004). Since mitochondrial extracts contain multiple proteins, it would be interesting to use the recently described mtDNA replisome containing recombinant pol γ , TWINKLE, and mtSSB proteins (Korhonen *et al*, 2004) to test the effect of p53 on mtDNA replication in this defined system, which may further reveal the minimum requirements for p53 to stimulate mtDNA replication activity.

Suppression of ethidium- and ROS-induced mitochondrial mutations *in vivo* by p53

To test the ability of p53 to suppress ethidium- or ROS-induced mitochondrial mutations in whole cells, we employed a previously described assay system using chloramphenicol (CAP) to select cells with mutations in the mitochondrial 16S ribosomal RNA (16S rRNA) gene, which produces an altered 16S rRNA and renders the cells resistant to CAP (Blanc *et al*, 1981; Kearsey and Craig, 1981). As illustrated in Figure 8, exposure to ethidium (30–100 ng/ml) did not cause the appearance of CAP-resistant colonies in

Figure 8 p53 suppresses ethidium- and ROS-induced mitochondrial mutations *in vivo*. HCT116 p53 +/+ cells, p53-/- cells, or p53-/- cells transfected with wt p53 (rescue, see Materials and methods) were incubated with 0, 30, or 100 ng/ml of EtBr for 3 days, and then in fresh McCoy's medium without ethidium for an additional 3 days. The cells were plated in six-well plates at the indicated cell density and incubated with 1 mM CAP for 14 days to select surviving colonies with mtDNA mutations in the 16S rRNA gene as described previously (Kearsey and Craig, 1981). After a 14-day incubation period with a replacement of culture medium containing 1 mM CAP on day 7, the surviving colonies were fixed, stained with 5% Giemsa staining solution and photographed. For induction of mitochondrial mutations by rotenone (Rot), cells were first incubated with 100 nM rotenone for 8 h, washed, and then cultured in fresh medium without rotenone for 40 h. This chemical exposure was repeated for three cycles. CAP-resistant mutants were selected as described above.

HCT116 p53 +/+ cells, whereas the same concentrations of ethidium induced the formation of multiple colonies resistant to CAP in the p53-/- cells. Interestingly, treatment with 100 ng/ml of EtBr did not result in more CAP-resistant colonies than incubation with 30 ng/ml of ethidium, possibly due to the toxic effect of EtBr at this concentration. Similarly, an increase in mitochondrial mutations was observed when the p53-/- cells were exposed to ROS stress by rotenone. Repeated exposure of p53-/- cells (5000 cells/well) to 100 nM rotenone led to the formation of a significant number of mutant colonies (Figure 8). In contrast, the same treatment of rotenone did not cause detectable mutants in p53 +/+ cells.

We then performed 'rescue' experiments to further test the role of p53 in suppressing mtDNA mutations in whole cells induced by ethidium and ROS stress. HCT116 p53-/- cells were transfected with a wt p53-expressing vector (pcDNA3.1/Zeo), stable transfectants were selected, and p53 expression was verified as described in Materials and methods. As shown in Figure 8, when the 'rescued' HCT116p53-/- cells expressing p53 were exposed to EtBr or rotenone followed by selection with CAP, no mutant colonies emerged. The data are similar to that of HCT116 p53 +/+ cells, and further support the conclusion that p53 is able to suppress mtDNA mutations in whole cells.

Based on our observations, we propose a model in Figure 9 that illustrates the novel role of p53 in maintaining mitochondrial genetic integrity, and provides a mechanistic link

between the loss of p53 function, genetic instability, and cancer progression with respect to ROS generation and ROS-mediated DNA damage. The mitochondrial respiratory chain is a major source of ROS due to electron bifurcation (Staniek *et al*, 2002). These endogenous ROS may damage mtDNA, leading to increased mtDNA mutations, which in turn cause malfunction of the respiratory chain components encoded by mtDNA, and thus further increase electron leakage and ROS generation. This would result in an amplification of ROS stress and cause further damage to mtDNA and nDNA, leading to genetic instability in cancer cells (Pelicano *et al*, 2004).

The role of p53 as a tumor suppressor protein that responds to DNA damage and transcriptionally activates molecules involved in cell cycle arrest, DNA repair, or apoptosis is well established (Vogelstein *et al*, 2000; Lane and Hupp, 2003). The central function of p53 in cells has fueled interests in defining its mechanism of action and regulation, and in determining how its inactivation facilitates cancer progression (Hupp *et al*, 2000). Here we report a novel function for p53 in maintaining mitochondrial genetic stability, which likely contributes to its role as a major tumor suppressor molecule. Translocation of p53 protein to mitochondria has been observed previously in other experimental systems, and was demonstrated to play a role in apoptosis under certain conditions (Marchenko *et al*, 2000; Mihara *et al*, 2003; Chipuk *et al*, 2004; Erster *et al*, 2004). Our study suggests that mitochondrial p53 may have a funda-

Figure 9 Schematic illustration of the novel role of p53 in maintaining genetic stability. (1) mtDNA is vulnerable to exogenous and endogenous DNA-damaging agents such as chemicals and ROS. (2) Mutations of mtDNA lead to mitochondrial respiratory malfunction and increased free radical generation. (3) The increase in ROS serves as a constant source of mutagens that cause further damage to mtDNA, leading to further amplification of ROS stress. (4) ROS also damages nDNA and promotes genetic instability and cancer progression. The ability of p53 to interact with mitochondrial pol γ and enhance mtDNA integrity provides a mechanism to suppress the ROS amplification circuit. This novel function, together with the previously demonstrated activity in regulation of the cell cycle and apoptosis in response to DNA damage, represents the important role of p53 as a major tumor suppressor molecule.

mental role in maintaining mtDNA integrity by interacting with pol γ and mtDNA, and enhancing the DNA replication function of pol γ . It is of interest to note that, in a recent report, Heyne *et al* (2004) identified a sequence within the human mitochondrial 16S rDNA region that can function as a p53-binding motif, and suggested a role for p53 in binding to, and regulating mtDNA.

Interestingly, there were no significant differences in background mitochondrial mutations between the p53 +/+ and p53 -/- cells under normal culture conditions without exogenous stress (Figure 8). This is consistent with the observations by Zhou *et al* (2003) in their study of COXII regulation by p53. However, when cells are exposed to DNA-damaging agents such as increased endogenous ROS generation or chemicals, it is essential that the damaged mtDNA be promptly repaired. Thus, the ability of p53 to localize to the mitochondria and interact with pol γ to protect mtDNA integrity is likely to reduce the risk of mitochondrial malfunctions in the presence of endogenous ROS or exogenous DNA-damaging agents, preventing further amplification of ROS-mediated DNA damage. This also provides a mechanistic explanation for the accelerating genetic instability associated with the loss of p53 in the late stage of cancer development. Mutations in p53 have been reported to occur in over 50% human tumors (Vogelstein *et al*, 2000). The increase in endogenous ROS generation due to mtDNA mutations, and the subsequent mitochondrial malfunction in cancer cells with p53 mutations, may contribute significantly to the various genetic alterations observed during cancer progression.

Since p53 mutations are frequent in human cancer, it would be interesting to determine whether mutant p53 might retain its ability to localize to mitochondria and guard the integrity of mtDNA. The observation that Raji cells with p53 mutation at codon 213 (Arg $\rightarrow$ His) were

sensitive to ethidium-induced mtDNA depletion similar to the p53-null HL-60 cells (Table I) suggests that this particular mutant p53 might not offer significant protection. It would be of great interest in further studies to examine various types of mutant p53 molecules for their ability to protect mtDNA integrity.

Materials and methods

Damage of mtDNA and isolation of respiration-deficient cell clones

A standard EtBr method was used to induce mtDNA damage and depletion as described previously (King and Attardi, 1996; Chandel *et al*, 1998; Pelicano *et al*, 2003). HCT116 p53 +/+ cells and the isogenic p53 -/- cells were exposed to 100 ng/ml of EtBr in McCoy's medium supplemented with 10% FBS, 0.47% glucose, 50 μ g/ml uridine, and 1 mM pyruvate for 120 days, with regular culture split every 3–4 days. At the end of 120 days, cells were plated at low density and individual colonies were isolated for analysis of mtDNA and mitochondrial respiration. The cell clones were expanded and maintained in medium supplemented with nutrients (without EtBr) to support the growth of p⁰ cells. The methods to analyze mtDNA depletion and respiration are described below.

Analysis of mtDNA depletion and mitochondrial respiratory activity

DNA was isolated using the standard phenol:chloroform:isoamyl extraction method, and the mtDNA D-loop region was amplified by PCR using the following primers:

sense: 5'-CACCTATTAACCACTCACG-3';
antisense: 5'-TGAGATTAGTAGTATGGGAG-3'.

The nuclear gene GAPDH was analyzed as a control

(sense: 5'-CGGAGTCAACGGATTGTGGCC-3';
antisense: 5'-GTGGCAGAGATGGCATGGAC-3').

The PCR reactions were carried out and the reaction products were analyzed as described previously (Carew *et al*, 2003). Mitochondrial respiration in whole cells was measured by an oxygen consumption assay as described previously (Pelicano *et al*, 2003).

Isolation of mitochondria, immunoprecipitation and immunoblotting

ML-1 cells and HCT116 cells were treated with EtBr or rotenone as indicated. Mitochondria were isolated from control and treated cells as described previously (Doda, 1998; Xu *et al*, 2005). Briefly, cells were harvested, washed once with cold PBS, and re-suspended in 3 volumes of isolation buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 70 mM sucrose, 210 mM mannitol, and protease inhibitors). After incubating in an ice-bath for 10 min, the cell suspension was homogenized with 15 strokes in a 2 ml glass homogenizer. The samples were centrifuged twice at 1500g at 4°C for 5 min to remove nuclei and cell debris. The supernatants were centrifuged at 15 000g for 15 min to separate the mitochondrial and cytosolic fractions. Mitochondria were washed once with the isolation buffer before use. p53 protein in the mitochondrial fractions was assayed by immunoblot analysis using a p53 antibody (Ab-6, Oncogene Research Products). The mitochondrial Hsp60 protein (antibody N-20, Santa Cruz Biotechnology) and nuclear PCNA (antibody Clone-24, BD Transduction Labs) were also blotted to ensure equal loading of mitochondrial protein and the absence of nuclear protein contamination.

For immunoprecipitation, cell pellets or isolated mitochondria were re-suspended in PBS containing protease inhibitors and sonicated at 10 W for 5 sec three times. The samples were incubated with 0.3 M NaCl in an ice-bath for 10 min, followed by centrifugation at 5000g. The supernatants were adjusted to 0.2 M NaCl. Immunoprecipitation was performed by incubating 1 mg of protein extracts with 1.5 μ g of rabbit polyclonal anti-pol γ antibody (Ab-3, NeoMarkers) or rabbit IgG (Calbiochem) as control for 5 h at 4°C, followed by pull-down with protein-A-coated magnetic beads, and analysis of p53 by immunoblotting.

Immunocytochemistry and confocal microscopic analysis

HCT116 p53+/+ cells were grown on glass coverslips in the presence or absence of EtBr, rotenone, NAC (Sigma Aldrich), H₂O₂, or their combination as indicated. The samples were fixed with cold methanol at -20°C for 5 min, washed with PBS, and then incubated with 5% BSA for 30 min at room temperature before adding 1° antibodies: mouse anti-p53 (Ab-6) or rabbit anti-DNA pol γ (Ab-3). The samples were incubated at room temperature for 3 h, washed, and then incubated with 2° antibodies (FITC-labeled anti-mouse or rhodamine-labeled anti-rabbit, Calbiochem) for 1 h at room temperature. The coverslips were washed five times with PBS, mounted on glass slides using 50% Vectashield mounting medium (Vector Laboratories), and analyzed using an Olympus FluoView 500 confocal laser-scanning microscope.

DNA primer extension assays

The effect of p53 on the DNA polymerization activity of DNA pol γ was tested using an *in vitro* DNA primer extension assay. Mitochondria were isolated from HCT116 p53-/- cells as described above, and lysed in hypotonic buffer with a brief sonication. The samples were centrifuged at 12,000 r.p.m. for 10 min to remove unbroken mitochondria and debris, and the mitochondrial protein extracts in the supernatants were used as the source of DNA pol γ. The DNA primer-template pair used in this assay contains the first 40 nucleotides of the mtDNA replication origin sequence:

5'-CAGATACTGCGACATAGGGT-3';
3'-GTCTATGACGCTGTATCCACGAGGCCGAGGTCGACAGC-5'.

The reaction mixtures (20 μl) contained 10 mM Tris-HCl, pH 7.4, 8 mM MgCl₂, 0.1 mM DTT, 100 μg BSA/ml, 50 μM each of dCTP, dGTP, and dTTP, 1 μM dATP, and 4 μl of [α-³²P]dATP (10 μCi/μl), the indicated amount of recombinant human wt p53 protein (BD Pharmingen), mitochondrial extract, and EtBr. After incubation at 37°C for 20 min, the reactions were stopped by adding equal volume of loading buffer (95% formamide, 20 mM EDTA, and 0.05% bromophenol blue) and heating at 90°C for 5 min. The samples were separated on a 15% denaturing polyacrylamide gel. The [³²P]radioactivity associated with each 40-base product band was quantified using a phosphorimager.

Dot blot assay for oxidative DNA damage

HCT116 p53+/+ cells were treated with 300 nM rotenone for 12–24 h, and mitochondria were separated from nuclei by differential centrifugation as described above. The nuclear and mitochondrial pellets were incubated in a buffer (25 mM EDTA, 10 mM Tris, pH 6.8, and 10 μl of 2 mg/ml proteinase K) at 45°C overnight, followed by ethanol precipitation of DNA. The mtDNA and nDNA were dissolved in TE buffer and dot-blotted onto nitrocellulose membranes using the Bio-Dot apparatus (Bio-Rad Laboratories), followed by immobilization at 65°C for 30 min. The membrane was washed with PBS-T buffer (1 × PBS containing 0.1% Tween), blocked with 5% milk for 1 h, and then incubated with anti-8-oxo-dG antibody (Clone IF7, Trevigen) overnight, followed by washing with PBS-T three times and incubation with HRP-anti-mouse antibody for 1 h. 8-Oxo-dG signal was visualized by chemiluminescence. The intensity of the dots was quantified using a densitometer.

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MtDIP-PCR assay of p53-mtDNA interaction

We adapted the principle of ChIP assay to detect the possible interaction between p53 and mtDNA. Mitochondria were isolated from HCT116 p53+/+ cells treated with or without EtBr or rotenone as indicated. The isolated mitochondria were incubated with 1% formaldehyde for 30 min and then washed with PBS. The crosslinking reaction was stopped by addition of 125 mM glycine, followed by sonication and incubation in an ice-bath for 10 min. The samples were centrifuged at 5000g for 10 min. One-tenth the supernatant was used as input control for the PCR reaction. The remaining supernatant was diluted with 4 volumes of IP buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1% NP-40, 0.5% Triton X-100, and protease inhibitors), and incubated at 45°C for 30 min, and centrifuged at 2000g for 5 min. The supernatant was incubated with p53 antibody (2 μg) or nonspecific IgG at 4°C overnight, followed by mixing with protein G-conjugated agarose beads (preblocked with 200 μg/ml of BSA) at 4°C for 1 h. The beads were pulled down, and washed sequentially with the following buffers: buffer I (20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), buffer II (buffer I containing 500 mM NaCl), buffer III (10 mM Tris, pH 8.0, 0.25 M LiCl, 1 mM EDTA, 1% NP-40, 1% deoxycholate), and buffer IV (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA). Both the input control samples and the immunoprecipitated samples were incubated with elution buffer (1% SDS, 0.1 M sodium bicarbonate) at 65°C for 15 min, centrifuged at 2000g for 5 min. The supernatant was incubated with 20 μl of 5 M NaCl at 65°C overnight to reverse the crosslinking. The samples were then incubated with 25 mM EDTA, 10 mM Tris (pH 6.8), and 10 μl of 2 mg/ml proteinase K at 45°C for 1 h, and extracted with phenol-chloroform-isoamyl alcohol, followed by ethanol precipitation. The purified DNA was dissolved in water and used for PCR to detect the presence of mtDNA, using a pair of primers specific for the D-loop of mtDNA: 5'-CACCTATTAACTCACTCAGC-3' (forward) and 5'-TGAGATTAGTAGTATGGGAG-3' (backward). The PCR reactions were performed as described previously (Carew *et al*, 2003).

Transfection of HCT116 p53-/- cells with p53

A full-length p53 DNA construct was synthesized by PCR amplification of the wt p53 plasmid (Invitrogen Life Technologies), using the following primers: sense 5'-TCGAATTCGCCACCATGGAGGAGCCG CAGTCAGAT-3' and antisense 5'-GCGAATTCTCAGTCTGAGTCAGGC CCTTCTGT-3'. The amplified full-length p53 DNA construct was inserted into a pcDNA3.1/Zeoцин plasmid vector (Invitrogen Life Technologies). After verifying DNA sequence and orientation, the p53 construct was transfected into HCT116 p53-/- cells using the Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies). The transfectants were selected in the presence of 250 μg/ml of zeocin for 15 days, and the surviving clones were pooled. The expression of p53 protein and its upregulation by radiation in the stable transfectants was confirmed by Western blot analysis.

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