Mitochondrial Dysfunction Impairs Tumor Suppressor p53 Expression/Function*‡

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Recently, mitochondria have been suggested to act in tumor suppression. However, the underlying mechanisms by which mitochondria suppress tumorigenesis are far from being clear. In this study, we have investigated the link between mitochondrial dysfunction and the tumor suppressor protein p53 using a set of respiration-deficient (Res−) mammalian cell mutants with impaired assembly of the oxidative phosphorylation machinery. Our data suggest that normal mitochondrial function is required for γ-irradiation (γIR)-induced cell death, which is mainly a p53-dependent process. The Res− cells are protected against γIR-induced cell death due to impaired p53 expression/function. We find that the loss of complex I biogenesis in the absence of the MWFE subunit reduces the steady-state level of the p53 protein, although there is no effect on the p53 protein level in the absence of the ESSS subunit that is also essential for complex I assembly. The p53 protein level was also reduced in undetectable levels in Res− cells with severely impaired mitochondrial protein synthesis. This suggests that p53 protein expression is differentially regulated depending upon the type of electron transport chain/respiratory chain deficiency. Moreover, irrespective of the differences in the p53 protein expression profile, γIR-induced p53 activity is compromised in all Res− cells. Using two different conditional systems for complex I assembly, we also show that the effect of mitochondrial dysfunction on p53 expression/function is a reversible phenomenon. We believe that these findings will have major implications in the understanding of cancer development and therapy.

Mitochondrial dysfunction is associated with aging, degenerative diseases, and cancer (1–3). One of the key functions of mitochondria is to make ATP by the process of oxidative phosphorylation (OxPhos), which is carried out by four electron transport chain (ETC)/respiratory chain (RC) complexes (I–IV) and the ATP synthase (complex V). The OxPhos machinery consists of over 100 nuclear and mitochondrial DNA-encoded proteins (4). Thirteen proteins encoded by mitochondrial (mt) DNA are core proteins of complexes I and III–V that are synthesized inside mitochondria. Complex II is an exception as all of its subunits are encoded by nuclear genes. Mutations in both mtDNA and nuclear genes encoding OxPhos complexes are associated with almost all types of cancers (1, 3). Somatic mutations in complex I subunit-encoding genes are frequently associated with oncocytesomas (5, 6). A recent study with head and neck cancers suggests that there are no mutational hot spots in the mtDNA (7). However, other studies suggest that mtDNA polymorphisms may predispose certain populations to cancer (8–12). The association of germ line mutations in complex II subunits with hereditary paragangliomas and pheochromocytomas constitute the strongest evidence for implicating mitochondrial metabolism in tumorigenesis (13, 14). Furthermore, the association of reduced expression of several subunits of OxPhos complexes with various cancers also suggests a possible role of mitochondrial metabolism in tumor progression (15, 16).

Mitochondria have been recently proposed to act as tumor suppressors (17, 18). However, the exact molecular mechanisms by which mitochondria suppress tumorigenesis are not clear. In tumors associated with mutations in complex II subunits, accumulation of succinate is suggested to be the underlying cause of tumorigenesis because it promotes hypoxic adaptations via regulating prolyl hydroxylases (19, 20). The association of mutations in fumarate hydratase, another TCA cycle enzyme capable of modulating succinate metabolism, in leiomyomas, renal cell carcinomas, and Leydig cell tumors also supports the role of TCA cycle intermediates in tumorigenesis (21–23). It has been observed that succinate and fumarate can impair the activities of prolyl hydroxylases, which regulate the hypoxia-inducible factor 1 alpha (HIF1α) (22). Furthermore, the inactivation of tumor suppressors (e.g. PTEN, phosphatase

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2 The abbreviations used are: OxPhos, oxidative phosphorylation; ETC, electron transport chain; RC, respiratory chain; Gy, gray; MEF, mouse embryonic fibroblast; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; BHT, butylhydroxytoluene; NAC, N-acetylcysteine; MnTE-2-PyP, Mn(III) meso-tetrakis(N-ethyl-2-pyridyl) porphyrin; Dox, doxycycline; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)aminojethanesulfonic acid.
and tensin homolog) and activation of genes implicated in survival signaling (e.g. AKT1, HIFα) by ETC/RC deficiencies can contribute to the process of tumorigenesis (24). PTEN gets inactivated by redox changes because of mitochondrial dysfunction (24). Whether mitochondrial dysfunction can regulate tumor suppressor p53 in a way that favors tumorigenesis is not known. Considering that the tumor suppressor PTEN protects p53 from its inhibitor Mdm2 (25), it is possible that p53 function could be down-regulated by ETC/RC deficiencies.

Impaired p53 signaling is the most common genetic alteration found in cancer and it plays a critical role in apoptosis (26). p53 controls cell fate by transcription-dependent and transcription-independent mechanisms following stress signals such as DNA damage, oncogene activation, and hypoxia (27–29). p53 has also been implicated in regulating cellular metabolism (30). It regulates glycolysis and oxidative metabolism in opposing directions (30–32), and therefore it is considered as one of the molecular mediators of the Warburg effect, the switch from oxidative metabolism toward glycolysis in cancer cells (33). Although most studies have focused on the downstream effects of p53 on cellular metabolism (32), very little is known about how p53 activity is affected by mitochondrial dysfunction (34).

In this study, we present evidence that mitochondrial dysfunction suppresses p53 expression and function reversibly. The suppression of p53 by impaired mitochondrial function renders cells resistant to γ-irradiation (γIR)-induced death. These data demonstrating that the p53-mediated cell death requires normal mitochondrial function suggest that impaired mitochondrial function can directly promote tumorigenic cascades as well as impair responses to chemotherapies.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Rotenone was from Calbiochem. All other reagents were from Sigma unless otherwise stated.

**Cells and Culture Conditions**—The respiration-deficient (Res−) cells used in this study have been described elsewhere (see Table 1 for details) (35–38). As these Res− cells completely rely on glycolysis for their bioenergetic needs, they must be grown in the presence of plenty of glucose with frequent change of the culture medium (39–42). The generation of B2-MWFF1 cells using the pTre-On vector has been described recently (35, 38). The G18-ESSS cells were also developed by the same strategy. Briefly, cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1% minimal essential medium nonessential amino acids (Mediatech, Inc., Manassas, VA), and 1% antibiotic mix (penicillin/streptomycin, Invitrogen) at 37 °C in a humidified atmosphere of 5% CO2, 95% air. In light of this study, caution must be taken to avoid the chronic presence of antibiotics such as tetracycline (and its derivatives) that can impair mitochondrial protein synthesis in cell culture medium. In inducible cells (B2-MWFF1 and G18-ESSS), complex I assembly was induced using 1 μg/ml doxycycline (Dox) as described previously (35, 38). Cells were harvested after washing once with Ca2+, Mg2+-free phosphate-buffered saline (PBS), pH 7.4, using trypsin/EDTA (Invitrogen).

**Plasmids and Cell Transfection**—The p53-GFP fusion protein described by Norris and Haas (43) was a generous gift from Dr. D. Joseph Jerry. The GFP-expressing plasmid (pEFGP-N3) and p53 reporter (p53-Luc) were from Clontech and Stratagene, respectively. The p53-Luc reporter plasmid expresses firefly luciferase from a synthetic promoter that contains 14 repeats of the p53-enhancer element (TGCGTGGACTTGCCTGG). Unless otherwise stated, the cells were transfected at ~50% confluency with the indicated plasmids using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. The GFP-expressing (pEFGP-N3) and p53 reporter (p53-Luc) plasmids were used at 4 and 8 μg/ml concentrations, respectively, with 10 μl of Lipofectamine 2000 in 500 μl of transfection mixture.

**Cell Death Assays**—Propidium iodide and an Alexa Fluor 488-conjugated anti-annexin V antibody were used to monitor the cell death by flow cytometry using the FACSCalibur FACSscan (BD Biosciences). Cells were γIR-treated with the indicated dose(s) using the Gammator B (Radiation Machinery Corp., Parsippany, NJ). About 1 h prior to γIR, the culture medium was replaced with fresh medium with or without indicated drug(s), and cell death was monitored 24 h post-γIR. All cells (floating and attached) were collected with minimal handling and stained using propidium iodide and annexin V for 15 min at room temperature following the manufacturer’s instructions (Vybrant apoptosis assay kit 2, Molecular Probes). Each experiment was performed in triplicate and repeated at least three times.

**Protein Analyses**—Cells were lysed in RIPA lysis buffer (10 mm Tris, pH 7.2, 150 mm NaCl, 1% Triton X-100, and 0.1% SDS) containing protease inhibitors (P8430, Sigma) and phosphatase inhibitors mixtures (P5726 and P2850, Sigma). A microplate-based BCA protein assay kit (Thermo Scientific) was used to determine protein concentrations. Western blot analyses were performed after separating the proteins on 10–20% SDS-polyacrylamide gels (Bio-Rad) and transferring them onto PVDF membrane (0.2 μm) using the iBLOT apparatus (Invitrogen). The following primary antibodies were used at the indicated dilutions: anti-total p53 (DO-1) (1:1000, Santa Cruz Biotechnology), anti-actin (1:5000, Abcam), and anti-HA epitope (1:1000, Covance Babco). Proteins were visualized using secondary antibody conjugated with HRP (1:4000, Santa Cruz Biotechnology), and the ECLPlus chemiluminescence reagent (Bio-Rad).

**p53 Activity Assay**—p53 activity was assayed using a luciferase reporter assay as described with a few modifications (44). Cells were seeded in triplicate in 35-mm plates at a density of 60,000 cells/ml (G7 and G18) or 50,000 cells/ml (all other cell lines) a day before transfection with the pEFGP and p53-Luc plasmids. Nontransfected cells were used as negative controls. 24 h post-transfection, the cells were exposed to 10 Gy, and 6 h later, the cells were harvested for analyses. Unless otherwise stated, all activity assays were performed with 10 Gy γIR to minimize the potential risk associated with DNA damage at a higher dose (e.g. 40 Gy), which may block the transcription. After aspirating the supernatant, the cell pellet was resuspended in 300 μl of annexin V binding buffer (Vybrant apoptosis assay kit 2, Molecular Probes) and then divided into three
equal aliquots. The first aliquot was used to determine transfection efficiency via FACS analysis. The second and third aliquots were pelleted, and the supernatants were removed. One pellet was resuspended in 100 μl of RIPA buffer to determine protein concentration and used for Western blotting. The other was resuspended in the passive lysis buffer to assess the luciferase activity according to the manufacturer’s instructions (Promega) using a TD-20/20 luminometer (Turner Designs). The relative p53 activity was determined as shown in Equation 1,

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luciferase \text{ activity (arbitrary units)} = \frac{\text{(protein concentration)} \times \text{(transfection efficiency)}}{\text{(Eq. 1)}}
\]

The results are reported as the average of averages ± S.D. for each condition from at least three independent experiments. Each experiment had assays performed in quadruplicate for each condition.

**Splenocytes and Thymocytes Cultures**—Splenocytes and thymocytes were harvested from adult p53+/+ and p53−/− C57BL/6 mice according to previously established protocols (45, 46). Whole spleen and thymus were collected from euthanized mice and placed into DMEM. Tissues were minced and gently rubbed between two acid-etched slides to release individual cells. Cells were transferred to T25 flasks for 2 h in a standard tissue culture CO2 incubator at 37 °C to remove adherent cells. After 2 h, nonadherent cells were collected and plated at a density of 100,000 cells per 60-mm plate. Cell death following 1 Gy γIR was monitored by flow cytometry as described above. All animal procedures were in compliance with the IACUC.

**In Situ Respirometry**—The degree of respiratory inhibition by rotenone was determined using a 24-well extracellular flux (XF24) analyzer from Seahorse Biosciences as described previously (47). HEK293T cells were seeded at 15,000–30,000 cells/well 24–48 h prior to measurements and then gently washed with and incubated in the respiration buffer (all in mM): 120 NaCl, 3.5 KCl, 1.3 CaCl2, 0.4 KH2PO4, 20 Na-TES, 5 NaHCO3, 1.3 Na2SO4, 2 MgCl2, and 15 d-glucose, containing 0.4% fatty acid-free BSA. The determination of the rates of oxygen consumption by the XF24 analyzer is based on oxygen-dependent fluorescence quenching of a fluorophore and the corrections for back diffusion of oxygen in measuring wells (see Ref. 47 for details). The XF24 respirometer allows up to four sequential additions of desired drugs using injection ports A–D. Respiration assays were performed with repeated cycles of 1 min of mixing, 1 min of waiting, and 3 min of measurement before and after the additions of different drugs. Full correction algorithm (Akos) was applied to determine the respiration rates (47). The maximal respiratory capacity was determined in the presence of 3 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), which was determined after careful titrations in the presence and absence of 1 μg/ml oligomycin, a complex V inhibitor. In our experience although the presence of oligomycin does not change the required FCCP concentration for maximal respiratory stimulation (data not shown), it can impair respiratory capacity up to ~45% (see supplemental Fig. S1D). Similar effect is observed in almost all other cells we have tested, including primary neurons (data not shown). Therefore, the oligomycin was excluded from the experiments toward determining the maximal respiratory capacity of cells. This effect is observed irrespective of the substrate used such as glucose versus pyruvate.

**Induction of γIR Resistance in Respiration-competent (Res⁺) Cells Using Drugs Affecting Mitochondrial Function**—Res⁺ cells were treated with either the complex I inhibitor rotenone or mitochondrial protein synthesis inhibitor chloramphenicol prior to γIR to recapitulate the effects of complex I deficiencies (as in B2 and G18 cells) or impaired mitochondrial protein synthesis (as in G7 cells), respectively, on γIR sensitivity. The rotenone was added at the indicated concentration 1 h prior to or after γIR (1–20 h), whereas the chloramphenicol (50 μg/ml) was added 24–48 h prior to γIR, and then relative cell death was monitored 24 h later as described above.

**Measurements of Mitochondrial Superoxide**—Mitochondrial superoxide production was monitored by using a superoxide-sensitive probe, the MitoSox (48, 49). Cells were treated with 0.2 μM MitoSox that showed detectable linear increase in fluorescence following rotenone treatment (see supplemental Fig. S3F). Live cells were imaged at 37 °C using Nikon Eclipse TE2000-U microscope equipped with a temperature enclosure and YFP/HQ (excitation), 590LP (emission), and 565LP (dichroic) filters. The relative levels of superoxide production in different cells were determined by flow cytometry (FACSCalibur FACSscan, BD Biosciences). γIR-treated cells in the presence and absence of superoxide dismutase mimetic, the Mn(III) neso-tetrakis(N-ethyl-2-pyridyl) porphyrin (MnTET-2-PyP) (48) were compared. Vehicle (DMSO) or 25 μM MnTET-2-PyP (1 h)-treated cells were incubated with 0.2 μM MitoSox for at least 30 min prior to irradiation and then analyzed after ~45 min. All experiments were performed in the respiration buffer.

**Real Time PCR**—RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s description, and the PCR was performed as described previously (44, 50). Isolated RNAs were reverse-transcribed using SuperScript III (Invitrogen) and analyzed for relative gene expression levels by quantitative PCR with Brilliant II SYBR Green quantitative PCR master mix (Stratagene). As the sequence information for hamster p53 targets is unavailable, we designed primers for hamster β-actin and p21 cDNA amplification and confirmed the specificity by sequencing. The primers used were as follows: 5′-AGGTATTCTGACCCCTGAGTACC-3′ (forward, hamster β-actin) and 5′-CAGCTCATAGCTTCTTCCAG-3′ (reverse, hamster β-actin); 5′-GAACCGTGGAACTTTGACTCG-3′ (forward, hamster p21) and 5′-GCGTTGGAGTGAAGATAAATCTG-3′ (reverse, hamster p21). The primers for mouse genes were based on primer design data base (Roche Applied Science). These were as follows: 5′-GATAGCGCGGCTGCTTGTCT-3′ (forward, Bax) and 5′-GGCTCGAAGTGTGGAGAGGA-3′ (reverse, Bax); 5′-TCTCCGAGGACTGATGTCGCAAA-3′ (forward, p21) and 5′-GGCAGCTTTGGTCCCGTTCATC-3′ (reverse, p21). The primers for mouse genes were based on primer design data base (Roche Applied Science). These were as follows: 5′-TCAAGCGGGAGGGATCAG-3′ (reverse, Puma); 5′-CAGATCCACGCGATATCCA-3′ (forward, p21) and 5′-GGCACACTTTGGCTCTTG-3′ (reverse, Puma). The real time PCRs were performed using Mx3005P (Stratagene),
Reversible Regulation of p53 by Mitochondrial Dysfunction

| Cell line            | Description                                      | Respiratory competence | Ref. |
|----------------------|--------------------------------------------------|------------------------|------|
| B2                   | Complex I-deficient CCL16-B2 Chinese hamster cells. Null for the MWFE subunit (Ndufa1 gene encoded) | Res           | 63   |
| B2-MWFE              | B2 cells complemented with HA epitope-tagged wild type hamster MWFE protein (MWFE-HA)                      | Res′           | 42   |
| B2-S55A              | B2 cells complemented with HA epitope-tagged MWFE bearing point mutation S55A                           | Res′ ~50%      | 35   |
| B2-MWFE             | B2 cells with Dox-inducible expression of MWFE HA                                                      | Conditionally Res′ | 35, 38 |
| G7                   | V79-G7 Chinese hamster cells with impaired mitochondrial protein synthesis, gene unknown             | Res′           | 54   |
| G18                  | Complex I-deficient V79-G18 Chinese hamster cells. Null for the ESSS subunit (Ndufb11 gene encoded)    | Res′           | 37   |
| G18-ESSS             | G18 cells with Dox-inducible expression of ESSS-HA                                                    | Conditionally Res′ | P. Potluri, unpublished data |
| G3                   | The parental V79 cells from which the G7 and G18 mutants were derived                                  | Res′           | 101  |

and the results were analyzed relative to actin expression as described previously (51).

**ATP Assays**—ATP concentrations in different samples were determined using the luciferin-luciferase assay (52). ATP was extracted with either boiling water or the extraction reagent provided with the ATP bioluminescent somatic cell assay kit (FLASC) from Sigma. Cells were plated at 35,000 in 30-mm plates. 24 h later they were treated with 40 Gy γIR and then harvested at the indicated times for ATP assays. Harvested cells were diluted to a final concentration of 2.5 × 10⁶/ml, and ATP was assayed following the manufacturer’s instructions for FLASC kit using a TD-20/20 luminometer (Turner Designs). An ATP standard curve was used to determine relative ATP levels in the samples.

**RESULTS**

**Mitochondrial Dysfunction Protects against γIR-induced Cell Death**—To determine whether mitochondrial dysfunction protects cells from p53-mediated cell death, we used several Res− mutants that have been previously characterized (see Table 1) (35–37, 42). Because γIR-induced death is primarily mediated by the p53 pathway (45), the γIR was used as genotoxic stress to measure the level of protection offered by mitochondrial dysfunction. All experiments were done in the presence of plenty of glucose (4.5 mg/ml) as Res− cells rely on glycolysis for their bioenergetic needs. Dose-response curves for γIR-induced death in Res+ (B2-MWFE, G3) and Res− (B2, B2-S55A, G7, and G18) cells are shown in Fig. 1. Clearly, the Res− (B2, B2-S55A, G7, and G18) cells were protected from γIR-induced cell death at all doses tested. B2-S55A cells, which have partial (~50%) complex I deficiency (35), were partially protected (Fig. 1A) compared with B2 cells in which complex I assembly is completely lacking due to the absence of the MWFE subunit (42). Serine 55 in the MWFE protein is an evolutionarily conserved potential complex I regulatory site for protein kinase A-mediated phosphorylation (35, 53). However, in the cells used here, we have not found any evidence of acute regulation of cellular respiration by this residue in the presence of cAMP agonists.3 Like B2 cells, the G18 cells, with complete complex I deficiency because of the absence of a different subunit, the ESSS (37), were also protected from γIR-induced death (Fig. 1B). Likewise, the G7 cells with severe OxPhos defects resulting from the complete absence of mitochondrial protein synthesis were also protected (Fig. 1, B, E, and F) (54).

To determine whether the protection from γIR-induced cell death in Res− cells is a consequence of the loss of ETC/RC assembly or activity, we measured γIR-induced death in the presence of rotenone, which specifically inhibits complex I activity when used in the low nanomolar (<100 nM) range (55). Higher rotenone concentrations affect the microtubule assembly. Our studies with primary neurons have shown that significant respiratory inhibition can be detected with rotenone concentration as little as 10 nM (48). Therefore, we decided to test the effect of 0–50 nM rotenone on cellular respiration using a microplate-based respirometry assay (47). To determine the relevance of our findings in relation to human pathophysiology, we wanted to test the effect of mitochondrial impairments in HEK293T cells first. As expected, there was a clear dose-dependent decline of basal respiration (Fig. 2A) as we have observed with primary rat neurons (48). Because the FCCP-stimulated respiration is not very stable despite careful FCCP titrations, we believe that accurate determination of the extent by which the respiratory capacity (FCCP-stimulated maximal respiration) is restricted is only possible by treating cells with rotenone before FCCP stimulation as shown in Fig. 2A. As expected, a dose-dependent decline of the respiratory capacity of HEK293T cells was seen. About 42.4 ± 4.8% decline in the respiratory capacity could be observed even with as little as 5 nM rotenone (Fig. 2A). 50 nM rotenone could inhibit 68.0 ± 5.3% of the basal and 72 ± 6.9% of the maximal HEK293T cell respiration. The B2-MWFE and G3 cells also showed similar rotenone sensitivity (data not shown). A side-by-side comparison of rotenone effects on FCCP-stimulated respiration showed relatively more inhibition when the rotenone was added before FCCP stimulation (supplemental Fig. S1, A–C). Our estimates suggest that 50 nM rotenone could inhibit 79.4 ± 4.3% of the complex I-dependent respiration in B2-MWFE, G3, and HEK293T cells.

To determine the effect of rotenone on γIR-induced death, the HEK293T cells were treated with 0–50 nM rotenone 1 h prior to γIR, and the extent of death was quantified 24 h later via flow cytometry as described under “Experimental Procedures.” The relationship between the rotenone concentration and γIR doses on HEK293T cell sensitivity is shown in Fig. 2B. Rotenone protected cells from γIR-induced death in a dose-dependent manner compared with controls. The dose-dependent protection was clearer at lower radiation doses. 50 nM rotenone gave

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3 N. Yadava, unpublished data.
maximal protection. Similar dose-dependent rotenone-mediated protection was also observed in G3 (Fig. 2D) and B2-MWFE (data not shown) cells. These data support the observations made from complex I-deficient Res−/H11002 (B2, B2-S55A, and G18) cells in which the OxPhos machinery is impaired by genetic mutations (Fig. 1) (35–37, 42). Furthermore, to determine the temporal effects of mitochondrial dysfunction on γIR-induced death, we treated HEK293T cells with rotenone at different time points after γIR, and we then determined the relative level of protection. In cells treated at or after 1 h post-γIR, the protective effect of rotenone declined drastically (Fig. 2C). A similar protection profile was also observed with G3 (Fig. 2E) and B2-MWFE (data not shown) cells. These data clearly suggest that only the pre-existing respiratory inhibition is protective. The degree of protection with 50 nM rotenone was comparable with that offered by genetic impairments in Res− (B2, G7, and G18) cells with severe ETC/RC deficiencies (Fig. 2D).

The p53 is a tumor suppressor, which directs radiation-induced cell death by both transcription-dependent and -independent mechanisms (28, 29, 45). To investigate whether γIR-induced death is indeed p53-dependent, the mouse embryonic fibroblasts (MEFs) derived from p53 wild type (p53+/+) and null (p53−/−) C57BL/6 mice were irradiated and assayed for cell death (46). As expected, the p53−/− MEFs were completely protected from the γIR-induced death, although the p53+/+ MEFs were highly susceptible (Fig. 3A). Furthermore, the level of protection by 50 nM rotenone was comparable with that offered by the complete loss of p53 protein in p53−/− MEFs (Fig. 3A).

Recent studies have suggested that the p53 can quickly translocate to mitochondria following irradiation (56). Thus, it is conceivable that in our fibroblast lines the high dose γIR (40 Gy) is inducing a direct mitochondrial interaction with p53, which is responsible for efficient cell death in control cells, whereas the loss of mitochondrial function by pre-existing...
ETC/RC defects in Res− (B2, G18, and G7) and rotenone-treated cells may prevent this direct action.

To test whether mitochondrial dysfunction indeed impairs the direct actions of p53 on mitochondria, we determined the effect of ETC/RC inhibition in murine thymocytes and splenocytes in which the direct effects of p53 on mitochondria have been established (56). The thymocytes and splenocytes were isolated from age-matched p53−/−/− and p53+/+/+ C57BL/6 mice, and the cell death was monitored 24 h post-γIR (46). The data from thymocytes (Fig. 3B) and splenocytes (Fig. 3C) were also in agreement with the data from MEFs showing comparable protection by the complete loss of p53 and rotenone pretreatment (Fig. 3A). These data clearly suggest that complex I inhibition suppresses p53 function that results in protection against γIR-induced death. Furthermore, this effect was observed across different cell types (fibroblasts, splenocytes, and thymocytes) and species (mouse: MEFs, splenocytes, and thymocytes; hamster: lung fibroblasts-B and G series cells; and human: HEK293T cells).

**Mitochondrial Dysfunction Alters p53 Expression and Function**—Our experimental results demonstrated that the role of p53 was critical in determining the γIR sensitivity of cells (Fig. 3) as expected (45). Thus, it remained to determine whether mitochondrial dysfunction affected p53 expression...
(protein levels) or function in Res− cells. This distinction was explored by monitoring the expression of p53 protein in Res− cells by Western blot analyses. Toward this goal, cells with and without γIR (10 Gy, 6 h) were analyzed for the relative levels of the p53 protein. Although p53 protein could be detected in B2-MWFE (Res+) and B2-S55A (Res−, ~50% complex I-deficient) cells, it was undetectable in B2 (Res−) cells with complete complex I deficiency (Fig. 4A, top panel) under identical conditions. As in B2 cells (Fig. 4A, top panel), the p53 protein was also not detected in G7 cells that have impaired mitochondrial proteins synthesis (Fig. 4B, top panel). In contrast to B2 cells, the p53 was detectable at near normal level in G18 cells due to a null mutation in a different subunit, the ESSS (Fig. 4B, top panel) (37). At this stage, we do not know the underlying basis for why the complex I deficiency in B2 versus G18 cells results in different fates of p53. When Res+ (MEFs and HEK293T) cells were treated with 50 nM rotenone, the induction of p53 protein following γIR was also blocked (Figs. 3A and 4D). Because the G18 Res− cells expressing normal levels of p53 were also protected from γIR-induced death (Fig. 1B) and rotenone treatment compromised γIR sensitivity in Res− cells (G3, B2-MWFE, HEK293T, and p53+/− MEFs, thymocytes, and splenocytes) (Figs. 2 and 3), we concluded that p53 activity may be suppressed in the presence of mitochondrial dysfunction.

To determine the effect of mitochondrial dysfunction on p53 transactivation activity, we used a luciferase-based p53 reporter assay as described under “Experimental Procedures.” In this assay, the luciferase expression was driven by multiple copies of a p53 response element isolated from the human p21 promoter. The p21 gene is a well-established transcriptional target of p53. The normal and Res− cells were transfected with the reporter plasmid, and 24 h post-transfection they were 10 Gy γIR-treated. The relative luciferase activity was measured 6 h post-γIR. At 6 h after γIR, maximal p53 activity was found (data not shown). Although in Res+ cells (B2-MWFE, G3) the p53 activity was up-regulated over 6-fold following γIR, the induction was not observed in Res− cells (B2, G7, and G18) with severe ETC/RC deficiencies (Fig. 4, A and B, bottom panels; supplemental Table S1). The induction of p53 activity in B2-S55A cells with partial complex I deficiency was significantly lower (~2-fold) compared with B2-MWFE cells (6-fold). This is in agreement with the partial γIR sensitivity of B2-S55A cells (Fig. 1A). Because Ser-15 phosphorylation status did not correlate with p53 transactivation activity across different cells in our hands, it is not shown. To determine whether rotenone also impairs p53 activity in Res+ cells (B2-MWFE, G3, HEK293T), we measured the p53 activity in cells pretreated with rotenone. As expected, rotenone pretreatment abolished the γIR-dependent induction of p53 activity in Res− cells (Fig. 4C; supplemental Table S1). Also, the presence of rotenone lowered the γIR-induced increase in p53 protein level in HEK293T cells (Fig. 4D) and MEFs (Fig. 3A).

To determine whether complex I deficiencies can influence endogenous expression of p53 target genes, we monitored p21 expression in Res+ versus Res− cells by real-time PCR as described under “Experimental Procedures.” Our data in Fig. 4 (E and F) suggest that complex I deficiency can influence the expression of endogenous p53 targets. As expected, based on

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**FIGURE 3.** p53 dependence of γIR sensitivity and the relative protection by rotenone compared with complete p53 loss. Cell death was assayed 24 h post-γIR in the wild type (p53+/+) and null (p53−/−) cells derived from C57BL/6J mice. A, MEFs. Top panel. Western blot analysis of the total p53 protein. Actin was monitored as loading control. Bottom panel, relative levels of death in p53+/+ and p53−/− MEFs induced by 40 Gy γIR. B, p53-dependent 1 Gy γIR-induced death in thymocytes. C, p53-dependent 1 Gy γIR-induced death in splenocytes. Because of the differences in survival between p53+/+ and p53−/− thymocytes and lymphocytes following isolation, their control values were set to 100%. Note that thymocytes and splenocytes are very sensitive to radiation. Thus, they were treated with 1 Gy γIR. Control, untreated cells; IR, γ-irradiated; Rot, 50 nM rotenone treated, Rot+IR, rotenone (50 nM) plus IR-treated.
basal p53 activities in B2 and G18 Res<sup>-</sup> cells, p21 expression was significantly lower in B2 cells compared with their controls (B2-MWFE), although there was no significant difference in G18 (Res<sup>-</sup>) versus G3 (Res<sup>+</sup>) cells. Furthermore, γIR did not induce p21 expression in Res<sup>-</sup> (B2 and G18) cells, although there was a low level induction in normal cells (B2-MWFE and G3). Because of the lack of sequence information for hamster p53 targets, we could not monitor expression of other genes in these cells. Alternatively, we monitored the effect of rotenone on γIR-induced expression of selected p53 genes in mouse splenocytes and thymocytes, which are also protected in the presence of rotenone (Fig. 2, B and C). Splenocytes and thymocytes are highly radiation-sensitive cells. The γIR-induced p53 response is well characterized in these cells (45, 57). The inhibition of p21 induction by γIR in B2-MWFE cells in the presence of rotenone suggested that rotenone could affect the expression of p53 targets. To test this, we used splenocytes and thymocytes from the p53 wild type (p53<sup>+/+</sup>) and null (p53<sup>-/-</sup>) C57BL/6 mice and monitored the expression of p21, Bax, and Puma, which are well known p53 targets induced by γIR (58). A time course analysis suggested that the maximal induction of all three genes was achieved 6 h post-γIR (1 Gy) in both cell types (data not shown). Therefore, 6 h was selected for comparative analyses in the presence and absence of 50 nM rotenone. The p53<sup>+/+</sup> C57BL/6
spleenocytes and thymocytes served as negative controls. Data in Fig. 5 show a significant inhibition of all three genes following IR in cells treated with 50 nM rotenone. However, in contrast to the inhibition of IR-induced response, the basal expression of p21, Bax, and Puma in spleenocytes was significantly increased (Fig. 5, A–C). This level of increase by rotenone alone did not result in increased cell death in spleenocytes, although the impaired IR response was sufficient to protect the cells (Fig. 3C). There was no such increase in basal expressions of p21, Bax, and Puma in thymocytes (Fig. 5, D–F). As expected in p53<sup>−/−</sup> cells, no significant inductions in these genes were found (Fig. 5). Taken together, our data clearly suggest that the respiratory inhibition either because of genetic mutations or pharmacological impairments can suppress p53 expression/function.

Effect of Complex I Deficiency on p53 Expression/Function Is Reversible—To eliminate the possibility that the p53 gene in Res<sup>−</sup> cells may be mutated, we used a conditional complex I assembly system (B2-MWFE<sup>i</sup>) recently described by us to determine whether restoring complex I assembly would result in gain-of-p53 function and IR sensitivity (35, 38). In B2-MWFE<sup>i</sup> cells, complex I assembly was induced with 1 μg/ml Dox in the medium for 48 h. Then cells were allowed to recover without Dox for 24 h and treated with 40 Gy IR. Data in Fig. 6A show the induction of the MWFE protein as indicator of complex I assembly, which led to the recovery of p53 protein expression, IR sensitivity (Fig. 6B), and activity (Fig. 6C). The assembly of complex I under these conditions has been established in previous studies and confirmed in this study (data not shown). Under this paradigm, the cellular respiration of stably trans-
fected cells (B2-MWFE) and Dox-induced cells (B2-MWFEi) were comparable (data not shown). A similar observation was made with another conditional system, the G18-ESSSi (Fig. 6, D–F), which expresses the ESSS protein using the same inducible strategy in Res−/H11002 G18 cells (35). It should be noted that in G18 cells, the p53 protein level was not reduced (Fig. 4B, top panel), although the /H9253 IR-induced p53 activity was missing (Fig. 4B, bottom panel), which correlated with /H9253 IR resistance (Fig. 1B). In G18-ESSSi cells as well the restoration of complex I assembly, which is indicated by the ESSS protein expression (Fig. 6D) by Dox treatment, restored /Hr sensitivity (Fig. 6E), and p53 activity (Fig. 6F). As expected, the restored p53 activity following the induction of MWFE and ESSS proteins in B2-MWFEi and G18-ESSSi cells is rotenone sensitive (supplemental Fig. S2). The gain-of-p53 activity (/H11011 6-fold post-/H9253 IR) in both inducible systems (B2-MWFEi and G18-ESSSi) confirms that normal ETC/RC function is required for p53-expression/function (Fig. 6, C and F).

Chloramphenicol Treatment Recapitulates the Protective Effect of Impaired Mitochondrial Protein Synthesis Observed in G7 Cells—To determine whether G7 cells are protected because of the impaired mitochondrial protein synthesis or because of an unknown mutation in the p53 gene, we treated the parental Res+, the G3 cells, with a mitochondrial protein synthesis inhibitor, the chloramphenicol. Cells were pretreated with 50 μg/ml chloramphenicol for 48–72 h prior to /H9253 IR because we hypothesized that the impaired mitochondrial protein synthesis would affect the p53 expression/function via blocking the assembly of OXPhos machinery. This should show an effect on the p53 protein expression and /H9253 IR sensitivity similar to the effects observed in G7 cells (Figs. 1B and 4B). Cell death was monitored 24 h post-/H9253 IR as described under “Experimental Procedures.” As expected, there was a progressive increase in cellular protection by chloramphenicol treatment for 24–48 h (Fig. 7A). This could be due to slow turnover/disappearance of ETC/RC complexes as shown by us and others (38, 59). The resistance to /Hr-induced death in 48-h chloramphenicol-treated cells could be correlated with the corresponding decline of the p53 protein levels (Fig. 7B) similar to that observed in G7 cells (Fig. 4B, top panel). The human HEK293T cells also show a similar decline in p53 levels following chloramphenicol treatment, which result in their protection from /H9253 IR-induced death and the suppression of p53 activity (data not shown).

Ectopic Expression of p53 in Res− Cells Does Not Restore /Hr Sensitivity—One of the therapeutic strategies currently proposed for treating some cancers involves the restoration of nor-
mal p53 protein function by ectopic expression or using re-activating drugs (60). Such a strategy may face problems, if the tumors have pre-existing mitochondrial dysfunction, which often is the case as suggested by the prevalence of mtDNA mutations in almost all types of cancers (1, 3). Thus, it was essential to test whether ectopic expression of p53 could restore γIR sensitivity in Res− cells. Therefore, the Res− B2 and G18 cells were transfected with wild type human p53-GFP, and the sensitivity to γIR-induced death was examined. The p53−/− MEFs were used as positive controls for the rescue experiments. Although p53-GFP rescued the γIR sensitivity in p53−/− MEFs, no rescue was evident in p53-GFP transfected complex I-deficient B2 and G18 cells (Fig. 8A). As expected, the γIR sensitivity in MEFs correlated with the restoration of p53 activity, although no activity was detected in the p53-transfected Res− (B2 and G18) cells (Fig. 8B). These data along with those in Fig. 6 confirm that without restoring the ETC/RC function, it is not possible to rescue p53 function in the presence of mitochondrial dysfunction. These results also support our hypothesis that mitochondrial ETC/RC activity is an important upstream regulator of the tumor suppressor protein p53.

Effect of Antioxidants on p53 Activity and Radiation Sensitivity of Res− Cells—The protection by rotenone, which increases mitochondrial superoxide production in cells (supplemental Fig. S3F) (48) suggested that pre-existing oxidative stress may play a role in the protection against γIR-induced death. To determine the role of oxidative/redox stress in p53 suppression and γIR sensitivity, we monitored the ability of N-acetylcysteine (NAC) to alleviate glutathione redox stress, and butylhydroxytoluene (BHT) and MnTE-2-PyP could have restored the p53 activity and γIR-sensitivity of cells (supplemental Fig. S3F) (48) suggested that pre-existing oxidative stress may play a role in the protection against γIR-induced death. To determine the role of oxidative/redox stress in p53 suppression and γIR sensitivity, we monitored the ability of N-acetylcysteine (NAC) to alleviate glutathione redox stress, and butylhydroxytoluene (BHT) and MnTE-2-PyP could have restored the p53 activity and γIR-sensitivity of cells. Therefore, the Res− B2 and G18 cells were transfected with wild type human p53-GFP, and the sensitivity to γIR-induced death was examined. The p53−/− MEFs were used as positive controls for the rescue experiments. Although p53-GFP rescued the γIR sensitivity in p53−/− MEFs, no rescue was evident in p53-GFP transfected complex I-deficient B2 and G18 cells (Fig. 8A). As expected, the γIR sensitivity in MEFs correlated with the restoration of p53 activity, although no activity was detected in the p53-transfected Res− (B2 and G18) cells (Fig. 8B). These data along with those in Fig. 6 confirm that without restoring the ETC/RC function, it is not possible to rescue p53 function in the presence of mitochondrial dysfunction. These results also support our hypothesis that mitochondrial ETC/RC activity is an important upstream regulator of the tumor suppressor protein p53.

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FIGURE 7. Effect of chloramphenicol on γIR sensitivity of cells. A, protection of the γIR-sensitive G3 (Res−) cells by the chloramphenicol, a mitochondrial protein synthesis inhibitor. Cells were pretreated with 50 μg/ml chloramphenicol for 24 and 48 h and then γIR-treated (40 Gy) with the chloramphenicol present in the medium. Cell death was assayed 24 h later. B, chloramphenicol addition (48 h prior to γIR) reduced the p53 protein level in G3 cells comparable with that seen in G7 cells. The total p53 level was monitored using the DO1 antibody, while using actin as the loading control. Western blot for G3 cells corresponding to the 48 h data set in A are shown. Ctr, untreated controls; γIR, γIR-treated; Chl, chloramphenicol treated; Chl+IR, chloramphenicol plus γIR-treated.

FIGURE 8. Ectopic expression of p53 does not restore the γIR sensitivity in Res− cells. A, relative rescues of the γIR sensitivity in Res− (B2 and G18) cells and p53−/− MEFs. GFP positive % live cells are reported with nonirradiated controls set to 100%. B, relative level of p53 activity rescue in Res− (B2 and G18) cells and p53−/− MEFs. Transiently (24 h) co-transfected cells with the p53-GFP and p53 activity reporter were γIR-treated and analyzed for relative levels of death as described under “Experimental Procedures.” Cells were treated in triplicate from which the two sets were used for the determination of cell death (24 h post-γIR (40 Gy), A), and the remaining set was used for the p53 activity assay (6 h post-γIR (10 Gy), B). The p53−/− MEFs from C57BL/6 mice were used as positive controls to demonstrate the rescue of the γIR sensitivity and p53 activity. Ctr, non-γIR-treated transfected cells; γIR, γIR-treated transfected cells.
γIR sensitivity correlated with the lack p53 activity (Fig. 9, C and F) and protein recovery (supplemental Fig. S3G) by NAC, BHT, and MnTE-2-PyP treatments. At lower doses (10 Gy), although the B2-MWFE cells were not protected by antioxidants, the G3 cells were partially protected (Fig. 9D). The reason for this difference between the B2-MWFE and G3 cells is not clear. Both cells show increased superoxide production following γIR treatment that can be quenched by 25 μM MnTE-2-PyP (supplemental Fig. S3, A and D). The superoxide production was monitored using flow cytometry with MitoSox that reports mitochondrial superoxide (48, 49). There were expected differences between the Res− cells. Although in G7 cells no increase in superoxide production following γIR was noticed, there was a significant increase in B2 and G18 cells (supplemental Fig. S3, B, C, and E). However, this increase was relatively modest compared with control cells. Because G7 cells have combined defects of complexes I and III–V, they were not expected to produce superoxide. However, in B2 and G18 cells with isolated complex I deficiencies, superoxide generation may be possible by the ETC/RC downstream to complex II or by the partial assembly of a complex I peripheral arm (63). At this stage, it is unclear whether the increased superoxide production in B2 and G18 cells following γIR is coming from partial complex I assemblies or the ETC/RC downstream to complex II. The failure of MnTE-2-PyP to protect the control cells while successfully quenching the superoxide production suggests that lower mitochon-

**FIGURE 9.** Effect of antioxidants on γIR sensitivity and p53 activity. A–C, antioxidant effects on γIR sensitivity (A and B) and p53 activity (C) of B2-MWFE versus B2 cells. D–F, antioxidant effects on γIR sensitivity (D and E) and p53 activity (F) of G3 versus G18 cells. Cells were treated with 30 mM NAC, 200 μM BHT, or 25 μM MnTE-2-PyP 2.5 h before γIR. Cell death was monitored 24 h post-IR at 10 Gy (A and D) or 40 Gy (B and E). p53 activity was monitored 6 h post-IR as described under “Experimental Procedures.”
Mitochondrial Dysfunction Impairs p53-mediated Cell Death—Mitochondrial dysfunction confers opposing cell fates in cancer versus degenerative diseases. Although mitochondrial dysfunction predisposes cells to death in degenerative diseases, it provides survival advantages to cancer cells. This suggests that mitochondrial dysfunction elicits different responses under different pathophysiological conditions. We have recently shown that even minimal impairments of complex I can lead to neuronal death under excitotoxic stress with high bioenergetic demand by causing bioenergetic deficit (48). In this study we have tested how mitochondrial dysfunction can affect cell fate under a genotoxic stress, such as γIR in mitotic cells. Our data suggest that pre-existing mitochondrial dysfunction protects cells from γIR-induced death by suppressing p53 expression/function (Figs. 1 and 2). The cellular protection in the presence of suppressed p53 is not surprising as p53 is a key player in radiation-induced cell death (Fig. 3) (45) and cells can meet their bioenergetic demands solely via glycolysis (39, 40). The level of cellular protection is increased with the degree of ETC/RC impairment. It is mediated either by the down-regulation of p53 protein expression (B2 and G7 cells) or function (G18 and rotenone-treated Res− cells) (Fig. 4). To our knowledge this is the first study describing reversible suppression of p53 expression/function by complex I deficiencies (Fig. 6). This study provides compelling evidence that mitochondrial metabolism can indeed regulate p53, and it completes a regulatory feedback loop in association with positive regulation of OxPhos by p53 via complex IV assembly factor SCO2 (30). This may be the reason why p53 has to make sure that mitochondria function normally by its recently described mtDNA repair activities (64–66).

Our data showing resistance to γIR-induced death is in agreement with the decreased susceptibility to staurosporine-induced death in cells without mtDNA as shown by Dey and Moraes (67). Impaired ETC, particularly at complexes I and II, is also found to protect against the tumor necrosis factor-induced cell death (61, 68). However, the protection offered by rotenone is in disagreement with a few studies showing increased cell death (autophagy or apoptosis) (55, 69, 70). These differences could be due to the experimental conditions used. Although in our study we have used rotenone in low nanomolar concentrations with a secondary genotoxic stress, others have treated cells chronically with rotenone without a secondary stress. Some of these studies have used higher doses of rotenone that affect cell death (non-neuronal) by impairing microtubule assembly (55, 70) and others have used neuronal cells that may differ in cellular physiology (69). This is supported by the observation that chronic rotenone exposure still potentiates death in dopaminergic neurons in the absence of a functional complex I even in low nanomolar range (71). It is noteworthy that none of these studies examined p53 expression/function following rotenone treatments. Thus, our studies described here provide a novel insight into the mechanisms by which mitochondrial dysfunction can induce pathophysiology.

Our knowledge of how ETC/RC impairments regulate p53 expression/function is limited (34). As the p53 is considered an important player in regulating cellular oxidative/redox stress and itself being a redox-regulated protein (72), it is expected that ETC/RC impairments may affect the p53 expression/function. A few studies have shown the opposite effect of ETC/RC inhibition on p53 expression/function (73, 74). It is found that complex I inhibition up-regulates p53 in dopaminergic neurons (73), although in MCF-7 cancer cells, it blocks p53 up-regulation in response to hypoxia (74). However, a recent study found that only complex III inhibitors could induce p53 via impairing pyrimidine biosynthesis (in the absence of secondary stress), which does not depend on the functional status of the ETC/RC (75). In our study, the complex I deficiencies induced either by rotenone treatment or by genetic mutations clearly suppress the p53 induction following a genotoxic stress (Figs. 4 and 5). These studies clearly establish that ETC/RC function is an important regulator of the p53 expression/function that may be relevant to tumorigenesis. This is also supported by the down-regulation of p53 protein in mammary epithelial cells lacking mtDNA (MCF12Ap′) with mitochondrial dysfunction (76).

The comparable level of protection by mitochondrial dysfunction and p53 loss suggests that all apoptotic functions of p53 (transcription-dependent and -independent) could be compromised by mitochondrial ETC/RC defects (Figs. 3 and 5) (27, 29, 77). Although at this stage we do not know the exact mechanisms by which p53 is inactivated by mitochondrial dysfunction, a multitude of possibilities exists (see below). At least in B2 cells, the reduced level of p53 protein is at the post-transcriptional level as we did not find any reduction in p53 mRNA expression (data not shown). However, in G7 (Res−) or chloramphenicol-treated (Res−) cells this possibility is not excluded. How the loss of the MWFE and ESSS subunits differentially regulates p53 protein expression is an interesting observation that will be followed up in future studies using molecular genetic approaches.

The failure of G18 cells to activate p53 following γIR suggests that one or more steps involved in p53 activation are impaired due to ETC/RC deficiency. This seems plausible as p53 activation is regulated by a number of interdependent post-translational modifications (78, 79). The analysis of differential and reversible post-translation modifications of p53 in response to ETC/RC deficiencies is an interesting topic, which we believe should be followed in detail in a separate investigation. These modifications are expected to be different from the Ser-15 phosphorylation, which did not correlate well with p53 activity in conjunction with mitochondrial impairments (data not shown).

As ETC/RC deficiencies are often found associated with increased oxidative/redox stresses, in Res− cells they could have been the underlying cause for p53 suppression and γIR resistance. However, our data suggest that the oxidative/redox stress is not associated with the radioresistance of Res− cells and p53 suppression (Fig. 9). This leaves open two other major possibilities that may influence the p53 expression/function. First, after γIR an acute bioenergetic deficit may affect the p53
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function. A role for the ATP/ADP switch in regulating the p53 function has been suggested by an in vitro study (80). Second, mitochondrial membrane potential (Δψm) may play a role in regulating the intracellular ionic (e.g., Ca2+) homeostasis that in turn could affect p53 expression/function (81–83). Because p53 is a Zn2+-binding protein (84), it would be interesting to investigate whether ETC/RC deficiencies can affect p53 function by deregulating Zn2+ homeostasis. Intracellular regulation of another ion, Cu2+, has been shown to affect the p53 function (85). Furthermore, it has been recently observed that Δψm can affect the p53 function at the mitochondria (86). Irrespective of how p53 function is regulated, it is important to note that transcriptional activity of p53 is an important read-out for overall apoptotic functions of p53, because the DNA-binding domain is implicated in its direct action on mitochondria (28). This is probably via controlling p53 conformation (84, 85). Because these possibilities (ATP deficit and ionic homeostasis) are interlinked with Δψm (87), it requires a separate investigation with real time measurements. We plan to address these issues by testing cell-permeable substrates to bypass complex I for generating Δψm, and then determining the recovery of p53 expression/function. Toward this end, we have promising results with monomethyl succinate that rescues the γIR sensitivity in complex I-deficient B2 and G18 cells but not in G7 cells, which have defects in complex I and III–V (data not shown). Monomethyl succinate feeds electrons to complex II in intact cells by elevating intracellular succinate level.

Contrary to what one would simply expect, a transient decline in ATP level is observed only in Res− cells (supplemental Fig. S4). This decline could be either a consequence of the activation of the apoptotic program or perturbed ionic homeostasis in the presence of functional ETC/RC (81, 88). Although in Res− cells the ATP level declines by 60–70%, no decline is observed in Res− cells suggesting that glycolysis alone can compensate for ATP demand. Moreover, in Res− cells the ATP demand may be lower as they are protected because of suppression of the p53 (this study) and dysfunctional ETC/RC, which may prevent major changes in ionic homeostasis following γIR and thus cost less bioenergetically (81). The transient nucleotide (ATP) depletion may be a key requirement in the progression of apoptosis as suggested previously (88). Our data point toward the possibility that functional ETC/RC may play a significant role in this phenomenon apart from regulating p53 expression/function via a dynamic interplay between cellular bioenergetics and Ca2+ or other ions homeostasis as suggested above.

Cancer, Aging, and Mitochondrial Dysfunction—The data presented in this study show how mitochondrial dysfunction can affect p53 expression/function and its effect on γIR-induced cell death. These findings may have serious implications as the p53 pathway is most frequently impaired in cancers either due to mutations in the p53 gene itself or indirectly through proteins that modulate its activity (27). In relation to cancer development, it is expected that mitochondrial dysfunction would down-regulate p53 expression/function to relieve the suppression of glycolysis by its target genes, e.g., TIGAR (31, 32). Although we have not measured the expression of TIGAR in Res− cells, its up-regulation is plausible. Without negative feedback regulation of p53, the cells with severe mitochondrial dysfunction would experience bioenergetic deficits because of the repression of glycolysis by p53. The metabolic switch from OxPhos to glycolysis in the presence of reduced p53 expression supports the assumption that the p53 suppression is a viable option to meet the bioenergetic needs of cells (89). Thus, de-repression of glycolysis may be essential in mitotic cells, which may have limited OxPhos capacities (the ability to make ATP, as measured by ADP-stimulated respiration). Our studies suggest that in many fibroblasts, there is a very little spare OxPhos capacity compared with ETC/RC capacity (measured by protophor FCCP stimulation).4 This observation in association with the acute decline of ATP in normal cells (supplemental Fig. S4) suggests that ETC/RC activity may transiently increase the ATP demand following γIR by altering the ionic homeostasis as suggested above.

As tumorigenesis is a multistep process involving four to seven rate-limiting, stochastic events that determine age-dependent incidence (90), it is possible that functional mitochondria can play an active role in suppressing tumorigenesis by keeping p53 functional (this study). This view is supported by studies that demonstrate that mitochondrial dysfunction can initiate molecular and biochemical changes leading to gain of several phenotypic hallmarks of cancer as follows: specifically by (i) inhibiting tumor suppressors PTEN (24) and p53 (this study); (ii) activating oncogenic pathways such as AKT1 and HIF1α (24, 91–93); (iii) inducing extracellular matrix remodeling to allow invasiveness (94); (iv) conferring resistance to cell death (67); and (v) forcing metabolic shift toward glycolysis (95). Thus, in light of perturbations in cellular physiology/signaling by ETC/RC defects, it is anticipated that mitochondrial dysfunction could be tumorigenic, whereas normal mitochondrial function would maintain the regulatory surveillance by p53 (this study) in response to oncogenic stress and prevent adaptive changes that could favor cancer development. Thus, this study supports the current view that functional mitochondria could act as tumor suppressors via regulating p53 expression/function. Conversely, the defective ETC/RC can promote tumorigenesis as suggested by a few recent studies (76, 96). The impairment of p53 (this study) along with the activation of AKT1 by mitochondrial dysfunction may also be relevant to chemo resistance that is very often observed during cancer therapy (97, 98).

Considering that age is a major risk factor for tumorigenesis (99) and mitochondrial function declines with age (100), it is possible that mitochondrial dysfunction plays a key role in tumor initiation as originally suggested by Otto Warburg in 1956 (33). Warburg proposed that an “irreversible injury” to the ETC/RC was an initiating event in cancer development. It is noteworthy that like mitochondrial function, p53 function also declines with age by an unknown mechanism (58). From this perspective it would be interesting to explore the role of mitochondrial dysfunction preceding p53 functional decline as the underlying cause to explain the increased incidence of tumorigenesis with age.

4 C. Kim, unpublished results.
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