Chemoptogenetic damage to mitochondria causes rapid telomere dysfunction

Wei Qian,a,b Namrata Kumara,c Vera Roginskayaa,b Elise Fouquerela,b,1 Patricia L. Opresko,b,d,1 Sruti Shivaa,e Simon C. Watkinsa,g, Dmitry Kolodziejnyib,i, Marcel P. Bruchezbi, and Bennett Van Houtena,b,c,2

aDepartment of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; bDepartment of Chemical Biology, University of Pittsburgh Medical Center (UPMC) Hillman Cancer Center, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; cMolecular Genetics and Developmental Biology Graduate Program, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; dDepartment of Environmental and Occupational Health, University of Pittsburgh Graduate School of Public Health, Pittsburgh, PA 15261; eVascular Medicine Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261; fDepartment of Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, PA 15261; gCenter for Biological Imaging, University of Pittsburgh, Pittsburgh, PA 15261; hDepartment of Biological Sciences, and Molecular Biosensors and Imaging Center, Carnegie Mellon University, Pittsburgh, PA 15213; iPresent address: Department of Biochemistry and Molecular Biology, Thomas Jefferson University and Sydney Kimmel Medical College, Philadelphia, PA 19107.

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Reactive oxygen species (ROS) play important roles in aging, inflammation, and cancer. Mitochondria are an important source of ROS; however, the spatiotemporal ROS events underlying oxidative cellular damage from dysfunctional mitochondria remain unresolved. To this end, we have developed and validated a chemoptogenetic approach that uses a mitochondrially targeted fluorogen-activating peptide (Mito-FAP) to deliver a photosensitizer MG-2I dye exclusively to this organelle. Light-mediated activation (660 nm) of the Mito-FAP–MG-2I complex led to a rapid loss of mitochondrial respiration, decreased electron transport chain complex activity, and mitochondrial fragmentation. Importantly, one round of singlet oxygen produced a persistent secondary wave of mitochondrial superoxide and hydrogen peroxide lasting for over 48 h after the initial insult. By following ROS intermediates, we were able to detect hydrogen peroxide in the nucleus through ratiometric analysis of the oxidation of nuclear cysteine residues. Despite mitochondrial DNA (mtDNA) damage and nuclear oxidative stress induced by dysfunctional mitochondria, there was a lack of gross nuclear DNA strand breaks and apoptosis. Targeted telomere analysis revealed fragile telomeres and telomere loss as well as 53BP1-positive telomere dysfunction-induced foci (TFIs), indicating that DNA double-strand breaks occurred exclusively in telomeres as a direct consequence of mitochondrial dysfunction. These telomere defects activated ataxia-telangiectasia mutated (ATM)-mediated DNA damage repair signaling. Furthermore, ATM inhibition exacerbated the Mito-FAP–induced mitochondrial dysfunction and sensitized cells to apoptotic cell death. This profound sensitivity of telomeres through hydrogen peroxide induced by dysregulated mitochondrial ROS reveals a crucial mechanism of telomere–mitochondria communication underlying the pathophysiological role of mitochondrial ROS in human diseases.

ATM signaling | DNA damage response | singlet oxygen | mitochondria | telomere

Significance

It is highly controversial whether secondary reactive oxygen species generated by dysfunctional mitochondria are able to diffuse across the cytoplasm to the nucleus and cause subsequent nuclear changes. We have developed a targeted chemoptogenetic technology to induce mitochondrial dysfunction by generating short-lived highly reactive singlet oxygen exclusively in the mitochondria, with precise spatiotemporal control by light stimulation. Through careful analysis of the events involving mitochondrial dysfunction and subsequent nuclear oxidative stress that resulted in specific telomere damage, we delineated the mechanism of mitochondrion–telomere axis of cellular damage. Our findings revealed a fundamental mechanism underlying the pathophysiological role of mitochondrial singlet oxygen, with important ramifications for understanding the role of mitochondrial oxidative stress in the aging and cancer among other human diseases.

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Conflict of interest statement: M.P.B. is a founder of Sharp Edge Labs, a company applying the FAP-fluorogen technology.

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fPresent address: Department of Biochemistry and Molecular Biology, Thomas Jefferson University and Sydney Kimmel Medical College, Philadelphia, PA 19107.

gTo whom correspondence may be addressed. Email: vanhoutenb@upmc.edu.

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cytoplasm and nuclear ROS accumulation (11). Among nuclear alterations, shortening of telomeres has been considered as a consequence of oxidative stress, although the role of mitochondria in oxidative telomere damage is not clear (12). Similarly, telomere damage as indicated by telomere dysfunction-induced foci (TIFs) has been observed in senescent cells with higher mitochondrial superoxide generation; however, the cause and effect relationship between mitochondrial ROS and telomere damage has not yet been established (13, 14).

To directly address the cellular consequences of mitochondrial dysfunction and subsequent ROS production, it is necessary to develop a highly targeted approach that can be tightly regulated to produce direct mitochondrial damage without spurious damage to other cellular components. One promising approach is to use chromophore-assisted light inactivation technology that combines light activation with a fluorescent moiety to produce ROS, particularly with newer genetically encoded photosensitizer proteins, such as KillerRed or MiniSOG (mini singlet oxygen generator) (15, 16). Genetic targeting of these fluorescent moieties to specific cellular compartments by fusion to organelle-specific targeting peptides has provided high spatial delivery. However, problems of spurious light activation of these intrinsic photosensitizer proteins or other intrinsic biological photosensitizers (e.g., flavins) persist (17). An ideal solution would combine light in the near-infrared (NIR) range, avoiding intrinsic chromophore activation, with an organelle-targeted fluorescent protein that is only activated by the addition of a high-affinity chemical ligand, avoiding issues related to continuous excitation of the photosensitizer by ambient light.

To this end, we have developed and validated a unique chemoptogenetic approach consisting of a binding-activated photosensitizer dye MG-2I (iodine-substituted malachite green analog) and a mitochondrial-targeted fluorogen-activating peptide (Mito-FAP) to produce on-demand, short-lived singlet oxygen in the mitochondrial matrix (18, 19). Unbound MG-2I has a low fluorescence and undetectable singlet oxygen generation; however, when bound to Mito-FAP and exposed to NIR light (660 nm), the resulting complex is highly fluorescent and produces singlet oxygen with a high quantum yield. This system provides precise spatiotemporal control of generation of singlet oxygen to only the mitochondria. Singlet oxygen is formed by the transfer of energy to ground-state triplet molecular oxygen through various enzymatic and nonenzymatic systems, such as processes mediated by photosensitizers, peroxidase enzymes, and radical termination reactions (20). Singlet oxygen is highly reactive and therefore, short-lived (2 to 4 μs), attacking a wide range of biological targets, including DNA, RNA, proteins, and lipids only in close proximity to the site of generation. Singlet oxygen directly oxidizes amino acid side chains of proteins, including His, Tyr, Met, Cys, and Trp, at physiological pH, causing loss of enzyme activities (20). Using the Mito-FAP system, we have directly addressed whether dysfunctional mitochondria are able to generate sufficient fluxes of ROS to attack other cellular compartments, including nuclear DNA. Our study revealed that damage to mitochondria inflicted by mitochondrial-targeted singlet oxygen produced a long-lived wave of secondary ROS, which caused mtDNA damage, ATM activation, cell cycle arrest, and rapid telomere dysfunction as characterized by S3BP1-positive TIFs, telomere fragility, and telomere loss in the apparent absence of general nuclear DNA strand breaks. Our finding provided direct evidence that mitochondrial dysfunction has profound effects on the nucleus, with important ramifications for the role of mitochondrial-generated ROS in aging and cancer.

Results
Mito-FAP System Induces Mitochondrial Dysfunction. We used HEK293 cells stably expressing Mito-FAP (18, 19) to investigate the cellular impact of singlet oxygen-induced mitochondrial dysfunction (Fig. L4). This chemoptogenetic approach consists of an inert fluorogen-activating peptide (FAP) targeted to the mitochondrial matrix (Fig. 1A, ii and iii) with mitochondrial leading sequences of COXIV and COXVIII (Fig. 1A, i) combined with an MG-2I dye that is activated by NIR light, producing singlet oxygen when bound by the targeted FAP (Fig. 1A, iv) (18, 19). As shown in Fig. 1B, 4 h after dye MG-2I (50 nM) and light exposure (660 nm, 5 min), we observed a reduction in basal oxygen consumption rate (OCR) of cells as well as a diminished response to mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) compared with MG-2I dye or light treatment alone. In comparison, we did not observe any effect of MG-2I and light on cells that do not express Mito-FAP (SI Appendix, Fig. S1), confirming the specificity of the Mito-FAP chemoptogenetic system. The decrease in OCR is also dependent on the duration of light exposure (SI Appendix, Fig. S2). Correspondingly, there was a compensatory increase of extra-cellular acidification rate (ECAR) in MG-2I- and light-treated cells (Fig. 1C). Sodium azide, a singlet oxygen quencher, when present during light activation was able to rescue basal oxygen consumption and partially restore the response to FCCP after MG-2I and light treatment (Fig. 1D), indicating that mitochondrial dysfunction induced by the Mito-FAP system is in part mediated by singlet oxygen. N-acetyl-cysteine (NAC), a scavenger of a wide range of free radicals, including both singlet oxygen and ROS (21, 22), also prevented MG-2I- and light-induced treatment provided a near complete protection of mitochondrial respiration (Fig. 1E) (both basal and response to FCCP), suggesting that ROS other than singlet oxygen alone are involved in the mitochondrial dysfunction observed 4 h after MG-2I and light treatment. Taken together, mitochondrial-targeted generation of singlet oxygen leads to a drastic decline of mitochondrial function, which can be mitigated by either singlet oxygen quenching or a broad-spectrum antioxidant.

Mitochondrial Singlet Oxygen Induces Delayed Decrease of ETC Activities. Singlet oxygen damage to mitochondria after photodynamic therapy has been suggested to cause lipid damage, rapid loss of inner mitochondrial membrane potential, subsequent cytochrome c release, and cell death (23). Using a monovalent broad-spectrum antioxidant.

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Mitochondrial Singlet Oxygen Triggers a Secondary Wave Generation of Superoxide and Hydrogen Peroxide. The duration of singlet oxygen generation in mitochondria by the mitochondrial-targeted Mito-FAP system can be precisely controlled by the time of exposure to light, which in our study, is 5 min. The lifetime of singlet oxygen in most solvents is in the microsecond range (25). Since we did not detect immediate damaging effects of singlet oxygen on mitochondrial function (Fig. 2) and because NAC had a higher protective effect against MG-2I and light-induced mitochondrial dysfunction than sodium azide, we, therefore, hypothesized that oxidative damage by singlet oxygen to mitochondria initiates a secondary wave generation of ROS to amplify the damaging effects. Four hours after MG-2I and light exposure, we observed a significant increase in MitoSox signal (79.3% of cells exhibited increased superoxide generation) compared with MG-2I or light exposure alone (0.3%) (Fig. 3A), indicating stimulation of superoxide after singlet oxygen insult. Importantly, 5-min exposure to singlet oxygen was able to initiate a chronic flux of superoxide generation that lasted for at least 48 h (74.8% of cells at 24 h and 70.7% of cells at 48 h showed elevated superoxide), suggesting the occurrence of a continuous cycle of ROS generation in mitochondria. MitoTEMPO, a mitochondrial-targeted superoxide scavenger (26), was able to suppress MitoSox signal (36% reduction), further confirming the generation of superoxide in mitochondria (Fig. 3B).

To assess the potential sites of superoxide generation within the ETC, we used several inhibitors against specific ETC components. While both rotenone (Complex I inhibitor) and antimycin A (Complex III inhibitor) further enhanced superoxide generation by MG-2I and light treatment (SI Appendix, Fig. S3), 2-thienyltrifluoroacetone (TTFA; Complex II inhibitor) had no effect. Since singlet oxygen did not immediately suppress the activity of these ETC complexes and because these inhibitor treatments increased rather than decreased superoxide production compared with previous reports (27, 28), our data suggested that a secondary wave of superoxide and hydrogen peroxide makes the ETC more prone to inhibitor-mediated ROS production.

Singlet oxygen, due to its high reactivity, does not diffuse far (≈20 nm) from its site of generation before it reacts with other moieties (29, 30). Superoxide anion generated in mitochondrial matrix, due to its negative charge, is not able to cross the mitochondrial inner membrane. However, superoxide is rapidly converted to hydrogen peroxide by MnSOD in mitochondria. Hydrogen peroxide is freely diffusible, causing oxidation in other cellular compartments beyond mitochondria. We monitored the cellular redox status of cysteine that can be readily oxidized by hydrogen peroxide using a ratiometric single-cell redox imaging method, in which free sulfhydryl groups are first labeled with thiol-reactive dye Alexa Fluor 647-maleimide followed by reduction of oxidized thiols (present and unreactive as disulfides during the initial labeling) by Tris(2-carboxyethyl)phosphine (TCEP) and subsequent labeling of these cysteine that can be readily oxidized by hydrogen peroxide using a broad-spectrum ROS scavenger NAC (10 mM; E) added 15 min before exposure to MG-2I dye (50 nM) and light (5 min). Four hours after treatment, OCR was determined by a Seahorse Extracellular Flux Analyzer. Data show the representative results of at least 3 experiments with similar results. Data are represented as mean ± SD of at least 6 wells. MLS, mitochondrial leading sequence.
Mitochondrial Dysfunction Induces Nuclear DNA Replication Stress without Cell Death. We next sought to determine the biological consequences of this secondary wave of ROS produced by the singlet oxygen-injured mitochondria. We observed a significantly reduced cell growth after treatment with MG-2I dye and light (50 nM MG-2I with light exposure for 5 min). Seventy-two hours after treatment, the cell number in the group treated with the combination of MG-2I and light was only doubled, which is around 7-fold lower than in control cells or cells treated with dye or light alone (Fig. 4C). To analyze if the reduced cell growth is due to enhanced cell death, we performed the Annexin V apoptosis assay. We did not detect a significant apoptosis at 24 or 48 h in cells treated with MG-2I and light (Fig. 4B), indicating a cell cycle delay due to dysfunctional mitochondria. We thus investigated cell cycle changes by monitoring 5-bromo-2′ deoxyuridine (BrdU) incorporation and mitotic phosphorylation of histone H3 (Fig. 4C). Starting from 4 h after MG-2I and light treatment, we observed a decrease in the cellular intensity of BrdU signal (the amount of BrdU incorporated per cell) followed by a reduced number of cells incorporating BrdU over the next 24- to 72-h period (45.6% of cells are BrdU positive in control vs. 20.4% in cells 72 h posttreatment). There was no significant change in the number of mitotic cells as revealed by the unaltered number of cells positive for phosphorylated histone H3. We also observed a time-dependent increase in the phosphorylation of RPA32 at Ser4/Ser8, a marker of replication stress, and a decrease of Cyclin E, an S-phase cyclin, demonstrating nuclear DNA replication stress as a result of mitochondrial dysfunction (Fig. 4D).

Mitochondrial Dysfunction Leads to Activation of the ATM Pathway in the Absence of Overall Nuclear DNA Strand Breaks. Since a significant flux of hydrogen peroxide generated by mitochondria reached the nucleus, we sought to investigate whether DNA damage and repair signaling that may be involved in the cell cycle changes. After MG-2I and light exposure, we observed phosphorylation of ATM and Checkpoint kinase 2 (Chk2) (Fig. 5A). Activation of ATM has also been known to be directly induced by hydrogen peroxide (32, 33). As shown in Fig. 5B, the antioxidant NAC suppressed the phosphorylation of both ATM and Chk2 after MG-2I and light treatment. However, the lack of ATM dimer formation after MG-2I and light treatment (Fig. S4A), which is activated by DNA double-strand breaks but not oxidant stress as a result of mitochondrial dysfunction (Fig. 4E).

Alexa Fluor 555/647 (oxidized thiols/reduced thiols) in the nuclear region of the cells after MG-2I and light treatment, indicating the oxidation of nuclear protein after mitochondrial damage. A 24% increase in the fluorescence of a nuclear H2O2 sensor, Hyper-nuc, after treatment with MG-2I and light, compared with control, also supports the presence of H2O2 in the nucleus as a result of prolonged mitochondrial dysfunction (Fig. 3E).
detected after MG-2I and light exposure (SI Appendix, Fig. S4B), confirming the occurrence of nuclear DNA double-strand breaks, although to a limited extent. However, as revealed by an alkaline Comet assay, surprisingly, no significant overall nuclear DNA strand breaks (including both single- and double-strand breaks) were observed after MG-2I and light treatment compared with 20-min H$_2$O$_2$ (100 μM) treatment (Fig. 5C). The lack of detectable nuclear DNA strand breaks by the Comet assay is also consistent with our DNA damage assay by qPCR, which showed no reduction in the amplification of nuclear polymerase β gene (Fig. 2F). These results suggested the presence of a specific type of DNA strand break that activates an ATM response but is undetectable to the Comet assay.

To understand the role of ATM in cellular dysfunction induced by mitochondrial damage, we studied the effect of ATM inhibitor (ATMi) KU55933 on cell death and cell cycle changes after photosensitization. KU55933 further enhanced mitochondrial generation of superoxide (Fig. S5D) and synergistically induced apoptosis in cells treated with MG-2I and light (Fig. 5E) (e.g., 24.5% of Annexin V-positive cells were observed in cells treated with MG-2I + light+ 10 μM ATMi vs. 3.7% of Annexin V-positive cells were observed in cells treated with ATMi alone and 7.3% of Annexin V-positive cells were observed in cells treated with MG-2I and light). A protective ATM role on injured mitochondria after MG-2I and light treatment is consistent with the previous observation of mitochondrial dysfunction in ATM knockout cells (34). Furthermore, Fig. 5F revealed that 22% of cells undergo mitosis after treatment with MG-2I + light + ATMi. In contrast, the majority of cells treated with MG-2I + light showed S-phase delay (Fig. 4C), and only 5.3% of those cells undergo mitosis (Fig. 5F). Thus, the result in Fig. 5F indicated that the inhibition of ATM overrides replication stress-mediated S-phase delay after MG-2I and light treatment, forcing cells to progress into mitosis under replicative stress. The combination of enhanced mitochondrial superoxide generation and forced mitotic entry may underlie the mechanism of synergistic cell killing by the combination of ATM inhibition and FAP-bound MG-2I activation.

**Discussion**

In this study, we have provided direct evidence that mitochondrial dysfunction induced by mitochondrial-targeted singlet oxygen is
able to initiate a persistent secondary wave of superoxide and hydrogen peroxide generation. Importantly, hydrogen peroxide generated by mitochondria can diffuse to the nucleus and is sufficient to cause preferential telomere dysfunction but not overall nuclear DNA damage (Fig. 7).

Many environmental factors, such as heavy metals, sunlight, and pesticides, are known to cause mitochondrial dysfunction, ROS generation, and/or telomere damage, leading to pathological conditions (37–41). However, the relationship between mitochondria and telomere injury remained elusive, partly due to the inability of experimentally restricting damage exclusively to either compartment within a living cell. We have previously established a light-activated photosensitizer system that targets FAP to various cellular compartments combined with irradiation with light to precisely control the generation of singlet oxygen causing damage to those distinct compartments (18). In this study, by using Mito-FAP, we were able to initiate mitochondrial damage by generating singlet oxygen exclusively in mitochondria. The mitochondrial damage resulting from singlet oxygen generated in the mitochondria is probably due to initial direct oxidation of mitochondrial ETC proteins by singlet oxygen followed by the secondary generation of superoxide and hydrogen peroxide from dysfunctional mitochondrial respiration. Significantly, by confining the initial insult to the mitochondria to a short duration during which light was applied, we were able to follow the subsequent flux of distinct ROS and provide direct evidence that mitochondrial-generated hydrogen peroxide is able to travel to the nucleus and damage macromolecules inside the nucleus.

One of the advantages of the Mito-FAP system is the ability to fine tune the oxidative damage by adjusting the light exposure (SI Appendix, Fig. S2). With a condition that only suppresses cell growth without inducing significant cell death (Fig. 4), the Mito-FAP system allowed us to dissect the signaling events in the nucleus after mitochondrial dysfunction in a more physiologically relevant manner in contrast to many oxidative agents that often lead to gross nuclear DNA cleavage and cell death. While it has been suggested that oxidative stress causes telomere damage, such as telomere shortening and uncapping, it was not clear whether ROS generated by mitochondria under pathophysiological stress conditions is sufficient to directly cause telomere alterations (12). In our study, after an increase of mitochondrial ROS triggered by MG-2I and light treatment, we were able to detect gross nuclear DNA damage by either qPCR-based DNA damage assay or alkaline Comet assay for DNA single-strand breaks (Fig. 5E), which is consistent with previous reports (8, 9). However, targeted analysis of telomere lesions revealed telomeres as a preferential damage site for mitochondrial-generated ROS within the nuclear DNA. It is worth noting that DNA strand breaks at telomeres are not detectable by Comet. The absence of telomere damage in p3 cells after MG-2I and light treatment (Fig. 6C) and the protective effect of NAC (Fig. 6D) further demonstrate that ROS is essential in mitochondrial dysfunction-induced telomere damage. These results provide clear evidence that mitochondrial dysfunction directly leads to oxidative stress-mediated preferential telomere damage in the absence of general nuclear DNA damage.

The mechanism of the high susceptibility of telomere DNA to mitochondrial-generated oxidative stress has several potential causes. First, Linn and coworkers (42, 43) have shown that telomeric sequences are highly susceptible to hydrogen peroxide damage due to increased affinity for Fe2+ atoms that are bound to DNA to mediate Fenton chemistry. They reported that the preferential strand cleavage of DNA treated with hydrogen peroxide occurs at the nucleoside 5′dG moieties in the sequence RGGG, a sequence found in telomere repeats (35). In fact, plasmids containing 81 telomeric repeats are 7-fold more sensitive to damage by hydrogen peroxide plus iron (35). Second, mitochondria are the major site for the biosynthesis of iron-sulfur (Fe/S) clusters, which are critical prosthetic groups for several key proteins involved in nuclear maintenance, such as DNA polymerases and helicases (44, 45). RTERR, regulator of telomere elongation helicase 1, an Fe/S cluster-containing helicase, is an essential helicase for the control of telomere length and DNA repair on telomeres (46). When under oxidative stress, mitochondrial dysfunction can directly impair the biosynthesis of Fe/S clusters. In addition, the secondary ROS originated from mitochondria can cause potential Fe/S cluster conversion or complete cluster loss in proteins required for telomere maintenance.

The observation of 53BP1 recruitment to the TIFs 48 h after mitochondrial injury indicated direct DNA damage or uncapping at telomeres that are caused by mitochondrial-generated ROS. It has been reported that double-strand breaks at telomeres induced by a targeting Fok1-TRF1 system activate ATM kinase, which then leads to the accumulation of DNA damage and repair factors, such as 53BP1, to the break sites (36). Recruitment of 53BP1 in telomere DNA damage foci is known to increase chromatin mobility, promoting nonhomologous end joining DNA repair (36). Since we were not able to detect overall DNA strand breaks in
nuclear DNA by the Comet assay and because phosphorylation of KAP1 is mediated by double-strand breaks and not oxidation (33), the weak but detectable phosphorylation of KAP1 observed in Fig. 5D is likely the direct consequence of double-strand breaks at telomeres. Furthermore, the cell cycle arrest that we observed after Mito-FAP/MG-2I and light exposure (Fig. 4) may be partly due to telomere damage, as activation of ATM kinase after telomere damage has been shown to lead to cell cycle arrest (47). Our results demonstrating that ATM kinase inhibitor overrode cell cycle arrest caused by Mito-FAP/MG-2I and light exposure, leading to cell death (Fig. 5F), are also consistent with the role of ATM in telomere damage and cell cycle alterations.

Both mitochondrial and telomere functions are critical for cellular senescence, apoptosis, aging, and cancer (48). Using mitochondrial-targeted chemoptogenetic tools, we established that disrupted mitochondrial function is sufficient to damage telomeres through ROS intermediates in the absence of gross nuclear DNA damage. Such mitochondria–telomere axes of damage could serve as an originating event (for example, in malignant transformation) by producing conditions that favor survival of cells that possess high levels of telomere repair and maintenance capacity, a common feature of oncogenic cells. Since progeroid transgenic mice with critically short telomeres have mitochondrial dysfunction and increased production of ROS (49), our observation also suggests a cross-talk between dysfunctional mitochondria and dysfunctional telomeres that may set up a continuous cycle of cellular events, further amplifying this reciprocal damage in the cell. The prolonged mitochondrial generation of ROS after Mito-FAP/MG-2I and light treatment in the absence of significant apoptosis may be the manifestation of the occurrence of such a chronic cycle of damage. Hydrogen peroxide may, therefore, serve as a direct mediator of mitochondrial retrograde signaling pathway, regulating telomere-related cellular function in response to mitochondrial functional alterations under oxidative stress. The mitochondrial oxidative damage, persistent generation of ROS, and the resulting telomere dysfunction after initial transient singlet oxygen exposure may underlie the molecular processes of cancer, photoaging, and other human pathologies.

Materials and Methods

Cell Culture. HEK293 cells expressing Mito-FAP dLS** (Mito-FAP, FAP cloned downstream of mitochondrial-targeting sequence of COXIV/COXVII) fused with a fluorescent protein mCerulean3 for visualization (18) were cultured in Dulbecco’s modified Eagle medium (DMEM) media supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin-streptomycin, 1 mM sodium pyruvate, 50 μg/mL uridine, and 50 ng/mL endothelium-derived factor (EDF) at least 4 wk (50). pHyPer-nuc plasmids were obtained from Evrogen. Transfection was performed using FuGENE 6 (Roche Diagnostics) according to the manufacturer’s instructions.

FAP Activation of MG-2I Dye. HEK293 Mito-FAP cells were incubated at 37 °C for 15 min in phenol red free DMEM media containing MG-2I at a final concentration of 50 nM. Cells were then exposed to 660-nm (0.1-W/cm²) light for 5 min to stimulate the production of singlet oxygen. Phenol red medium was then removed, and fresh growth medium was applied for all subsequent procedures.

Stimulated Emission Depletion Microscopy (STED) Imaging. HEK293 cells stably expressing a mitochondrial-directed FAP were plated in an uncoated Mattek glass-bottomed 35-mm culture dish (Mattek Corporation) and infected with adenovirus coding for an mNEON-TOM20 construct. This protein is specifically localized to the mitochondrial outer membrane. Mito-FAP was imaged using MG-ester. Images were collected using a Leica 3× STED microscope using a white light laser tuned at 488 nm (mNEON) and 640 nm (FAP). Depletion was with the 775-nm line.

Western Blot Analysis. Western blot was performed as we previously described (51). Primary antibodies against ATM and p-ATM were obtained from Sigma-Aldrich. Drp1 was from BD Biosciences. Phospho-Drp1 (Ser616), phospho-Chk2 (Thr68), and Chk2 were from Cell Signaling Technology. Phospho-ATM (S1981) was from Epitomics. Complex I subunit NDUF53, Complex I subunit NDUF86, and Complex IV subunit II were from abcam.

Cell Proliferation and Cytotoxicity Assay. Cell proliferation was determined using a CyQUANT Direct Cell Proliferation Assay kit (Invitrogen) according to the manufacturer’s instructions. FITC-Annexin V Apoptosis Detection Kit (BD PharMingen) was used to quantify apoptotic cells according to the manufacturer’s instructions.

Cell Cycle Analysis. Cell cycle analysis was performed as we previously described (52). At various time points after treatment with MG-2I alone, light alone, or the combination of MG-2I and light, S-phase cells were pulse labeled with 10 μM BrdU for 30 min at 37 °C. Cells were then fixed in 70% cold ethanol overnight at 4 °C. DNA was denatured in 2 N HCl containing 0.5% Triton X-100 and neutralized with 0.1 M Na$_2$B$_4$O$_7$. Cells were then stained with FITC-labeled anti-BrdU antibody (BD Biosciences) followed by Alexa Fluor 647-conjugated phosphohistone H3 (Ser-10) antibody (Cell Signaling
is the Damage to and 5 (200 mM NaOH, 1 mM EDTA) with the CometAssay ES.

- Extracellular Flux Analysis. OCR and ECAR were measured using a Seahorse XF96 Extracellular Flux Analyzer (Seahorse Bioscience) essentially as previously described (53). After treatment, cells were seeded in XF96 cell culture plates at 8 × 10^4 cells per well in the presence of Cell-Tak cell and tissue attachment solution. For monitoring mitochondrial membrane potential, cells were incubated with 5 μM MitoSox (Invitrogen) or 2 μM JC-1 (Invitrogen), respectively, for 30 min at 37 °C. After washing with PBS, cells were collected by trypsinization and centrifugation. Cells were then labeled with Alexa Fluor 555 by incubating cells for 30 min in 1 mM Alexa Fluor 647-maleimide, and 0.02% Triton X-100 in PBS, resulting in the labeling of the mitochondrial membrane. Slides were then labeled with Alexa Fluor 555 by incubating cells for 30 min in 5 mM TCEP in PBS to remove excess dye, and then, incubated for 30 min in 5 mM TCEP in PBS to reduce disulfide bonds in proteins. Reduced disulfide bonds were then labeled with Alexa Fluor 555 for 30 min in 1 mM Alexa Fluor 555-maleimide. After 3 additional washes in PBS for 5 min, cells were examined and photographed by a laser-scanning confocal microscope, Olympus FLUOVIEW FV-1000, with a PlanApo N 60× objective and numerical aperture (NA) = 1.42 (Olympus). Ratiometric analysis on the fluorescence intensity of Alexa Fluor 555/647 SS/SH (disulfide/thiol) was performed using NIS-Elements software (Nikon).

- Mitochondrial Membrane Potential and ROS Generation. To measure mitochondrial-generated ROS or mitochondrial membrane potential, cells were incubated with 5 μM MitoSox (Invitrogen) or 2 μM JC-1 (Invitrogen), respectively, for 30 min at 37 °C. After washing with PBS, cells were collected and suspended in PBS containing 1% bovine serum albumin (BSA). The fluorescence intensity of Mitosox and JC-1 was measured using an Accuri C6 flow cytometer (BD Accuri Cytometers).

- qPCR-Based Mitochondrial and Nuclear DNA Damage Analysis. Damage to mtDNA and nuclear DNA was assessed 24 h after Mito-FAP/MG-2I dye and light treatment as we described previously (54). Briefly, total cellular DNA was isolated with a DNeasy Blood & Tissue Kit (Qiagen), and the concentration of isolated DNA was determined by PicoGreen (Invitrogen). qPCR reaction mixtures contained 15 ng of template DNA. Tricarbonate qPCR reactions for each treatment condition were performed with an LA PCR kit (Takara Bio Company) in a Biometra Professional standard thermocycler 96 (Biometra). The primer nucleotide sequences were as follows: for the 221-bp fragment of mitochondrial genome: 5′-CCCACAAAAACCATTTAACACCCCA-3′ and 5′-TTTTATCTGCGAGATGTTGGGATG-3′; for the 8.9-kb fragment of mitochondrial genome: 5′-TCTAACGGCCCTTTGAGGGAAGC-3′ and 5′-TCTTCTATTCCAGTGGATTCTTGAATG-3′, and for the 12.2-kb human 13S rDNA fragment: 5′-CCATGGAGCTTCCTTCCCTC-3′ and 5′-GAGTCAGTTCGCTGCTG-3′. The amounts of the PCR products were quantified using PicoGreen to indicate real-time amplification efficiency, which is dependent on the damage frequency of DNA template. Lesion frequency per DNA strand of treated samples was calculated based on the formula: λ = -(lnF₀/Fₚ), where F₀ is the fluorescence values of treated samples and Fₚ is the fluorescence values of control samples.

- Ratiometric Redox Immunocytochemistry. The cellular oxidative status was measured by a ratiometric approach previously reported (31). After treatment, HEK293 Mito-FAP cells were fixed for 30 min in the dark in 4% parformaldehyde, 1 mM N-ethylmaleimide (NEM), 2 μM Alexa Fluor 647-maleimide, and 0.02% Triton X-100 in PBS, resulting in the labeling of the sulfhydryl group with Alexa Fluor 647. Cells were washed 3 times for 5 min in PBS to remove excess dye and then, incubated for 30 min in 5 mM TCEP in PBS to reduce disulfide bonds in proteins. Reduced disulfide bonds were then labeled with Alexa Fluor 555 by incubating cells for 30 min in 1 mM Alexa Fluor 555-maleimide. After 3 additional washes in PBS for 5 min, cells were examined and photographed by a laser-scanning confocal microscope, Olympus FLUOVIEW FV-1000, with a PlanApo N 60× oil immersion objective and numerical aperture (NA) = 1.42 (Olympus). Ratiometric analysis on the fluorescence intensity of Alexa Fluor 555/647 SS/SH (disulfide/thiol) was performed using NIS-Elements software (Nikon).

- Alkaline Comet Assay. The single-cell gel electrophoresis or Comet assay was performed with a CometAssay kit (Trevigen). Twenty-four hours after treatment with MG-2I alone, light alone, or the combination of MG-2I and light, cells were collected by trypsinization and centrifugation. Cells were then washed once in ice-cold PBS and suspended at 100,000 cells per 1 mL in ice-cold PBS. Cells were combined with molten low melting agarose (1% LMP; at 37 °C) at a ratio of 1:10 (vol/vol) and immediately spread onto a 20-well CometSlide. Slides were placed flat at 4 °C in the dark for 10 min to allow gelling. Slides were immersed in 4 °C lysis solution overnight. After draining excess lysis buffer, slides were gently immersed in freshly prepared alkaline unwinding solution, pH > 13 (200 mM NaOH, 1 mM ethylenediaminetetraacetic acid [EDTA]), for 20 min at room temperature. Electrophoresis was performed at 21 V for 30 min in alkaline electrophoresis solution pH > 13 (200 mM NaOH, 1 mM EDTA) with the CometAssay ES system. After electrophoresis, slides were immersed twice in distilled water (dH₂O) for 5 min each followed by 70% ethanol for 5 min at room temperature. After drying overnight at room temperature, slides were stained with SYBR Gold for 30 min at room temperature. Images were taken with an
Olympus BX51 microscope and analyzed by Trevisgen’s Comet Analysis Software. At least 200 cells from each treatment condition were scored.

**Electron Transport Chain Complex Activity Assay.** The activity of citrate synthase was accessed by measuring the conversion of oxaloacetate with acetyl coenzyme A (CoA) to citrate + CoA as previously described (55). The production of CoA was coupled to its reaction with dihydroxyacetone phosphate (DHAP), and the product of this reaction (DHAP-CoA) formed a colored compound with formation that was kinetically monitored spectrophotometrically at 412 nm. Lysed cells (10 to 30 μg) were equilibrated at 37 °C in a reaction mix containing 400 μM DTNB, 200 μM acetyl-CoA, 100 mM Tris, pH 8.0, and 0.1% Triton X-100. Reactions were initiated by the addition of 200 μM oxaloacetate. The increase in absorbance at 412 nm (10 min) and the rate were expressed as picomoles per minute per milligram of protein based on the extinction coefficient of 13,600 (mole per minute per liter) for DTNB-CoA.

The activities of the 4 ETC complexes were performed essentially as described previously (55). Complex I converts NADH to ubiquinone using succinate as a substrate. Complex I activity was measured by adding exogenous NADH and ubiquione to the cell as substrates and monitoring the rotenone (10 μM)-sensitive decrease in the absorbance of NADH at 340 nm. Lysed cells were equilibrated at 37 °C in a reaction mixture containing 25 mM KPO4, pH 7.2, 10 mM MgCl2, 2.5 mM/ml BSA, 1 mM KCN, and 0.1 mM NADH. Reactions were initiated by the addition of decylubiquinone (50 μM), and the decrease in absorbance at 340 nm was monitored (10 min), after which rotenone (10 μM) and the reaction were monitored for an additional 10 min. The rotenone-sensitive rate was expressed as picomoles per minute per milligram of protein based on the extinction coefficient of 6,180 (mole per minute per liter) for NADH.

Complex II activity was measured using succinate and ubiquinone as substrates, coupling the (a) Complex II-catalyzed transfer of an electron from succinate to ubiquinone and the (b) ubiquinol reduction of the dye 2,6-dichlorophenolindophenol (DCPIP), which is monitored at 600 nm. Lysed cells were equilibrated at 37 °C in a reaction mixture containing 50 mM KPO4, pH 7.4, 20 mM succinate, 0.1 mM EDTA, 1 mM KCN, 10 μM rotenone, and 0.12 mM DCPIP. Reactions were initiated by 50 μM decylubiquinone, and the decrease in absorbance at 600 nm was monitored for 10 min, after which TTFA was added to a final concentration of 0.1 mM and the reaction was monitored for an additional 10 min. The TTFA-sensitive rate was expressed as picomoles per minute per milligram of protein based on the extinction coefficient of 1.6,180 (mole per minute per liter) for NADH.

Complex III activity was measured using oxidized cytochrome c and ubiquinone or ubiquinol as substrates and monitoring the reduction of cytochrome c at 550 nm. Lysed cells were added to a reaction containing 50 mM KPO4, pH 7.4, 1 mM EDTA, 5 mM MgCl2, 2 mM KCN, 10 μM rotenone, and 15 μM oxidized cytochrome c. Reactions were initiated by the addition of 15 μM ubiquinol. The increase in absorbance at 550 nm was monitored for 3 min. The first order rate constant (k) for the oxidation of cytochrome c was calculated and activity was expressed as k/min/mg. All complex activities are shown as a percentage of the control condition.

Complex IV activity was measured using reduced cytochrome c as the substrate and monitoring the oxidation of cytochrome c at 550 nm. Lysed cells were equilibrated to 30 °C in 10 mM KPO4, pH 7.0, and the reaction was initiated by the addition of 50 μM reduced cytochrome c. The decrease in absorbance at 550 nm was monitored for 3 min. The first order rate constant (k) for the oxidation of cytochrome c was calculated and activity was expressed as k/min/mg. All complex activities are shown as a percentage of the control condition.
hybridization wash buffer (70% formamide, 10 mM Tris HCl, pH 7.5) for 15 min each. After 3 washes in PBS, cells were incubated with DAPI (1:10,000) for 10 min at room temperature. Cells were rinsed once with PBS and dH2O before being mounted on slides with Prolong Diamond Anti-Fade (Molecular Probes).

Images were acquired on a Nikon Ti inverted fluorescence microscope with a 2.0 μm. Images were deconvoluted and analyzed using NIS Elements Advance research software. The nuclei were labeled using the region of interest tool. Using the measurement tab, a separate binary layer was created for the 53BP1 foci and the telomere foci. The intersection tool was then used to create a third binary layer that identified the 53BP1 foci overlapping the telomere foci. The intensity threshold for each channel was kept the same for all the same samples. The foci counts were exported to Excel for analysis.