Published in final edited form as:

Free Radic Biol Med. 2014 October ; 0: 30–39. doi:10.1016/j.freeradbiomed.2014.07.011.

DNA Double-Strand Breaks Activate ATM Independent of Mitochondrial Dysfunction in A549 Cells

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Abstract

Excessive nuclear or mitochondrial DNA damage can lead to mitochondrial dysfunction, decreased energy production, and increased generation of reactive oxygen species (ROS). Although numerous cell signaling pathways are activated when cells are injured, the ataxia telangiectasia mutant (ATM) protein has emerged as a major regulator of the response to both mitochondrial dysfunction and nuclear DNA double-strand breaks (DSBs). Since mitochondrial dysfunction is often a response to excessive DNA damage, it has been difficult to determine whether nuclear and/or mitochondrial DNA DSBs activate ATM independent of mitochondrial dysfunction. In this study, mitochondrial and nuclear DNA DSBs were generated in A549 human lung adenocarcinoma cell line by infecting with retroviruses expressing the restriction endonuclease *PstI* fused to a mitochondrial (MTS) or nuclear (NTS) targeting sequence and a hemagglutinin antigen epitope tag (HA). Expression of MTS-*PstI*-HA or NTS-*PstI*-HA activated the DNA damage response defined by phosphorylation of ATM, the tumor suppressor protein p53 (TP53), KRAB-associated protein (KAP)-1, and structural maintenance of chromosomes (SMC)-1. Phosphorylated ATM and SMC1 were detected in nuclear fractions whereas phosphorylated TP53 and KAP1 were detected in both mitochondrial and nuclear fractions. *PstI* also enhanced expression of the cyclin-dependent kinase inhibitor p21 and inhibited cell growth. This response to DNA damage occurred in the absence of detectable mitochondrial dysfunction and excess production of ROS. These findings reveal DNA DSBs are sufficient to activate ATM independent of mitochondrial dysfunction and suggest that the activated form of ATM and some of its substrates are restricted to the nuclear compartment, regardless of the site of DNA damage.

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Keywords

ATM; DNA damage; free radicals; mitochondrial dysfunction; oxidative stress

Introduction

During the process of ATP synthesis, mitochondria generate reactive oxygen species (ROS) by the escape of electrons predominantly from complexes I and III of the electron transport chain (ETC) [1]. Due to the close proximity of mitochondrial DNA (mitochondrial DNA) to the ETC, it has been hypothesized that mitochondrial DNA is more oxidatively damaged than nuclear DNA [2]. Because the mitochondrial genome encodes components of the ETC and ATP synthase, mitochondrial DNA damage may result in less efficient oxidative phosphorylation and a greater release of free radicals resulting in a “vicious cycle” of repetitive mitochondrial DNA damage and dysfunction [3]. Growing evidence implicate mitochondrial DNA mutations in cancer, aging, and other human diseases [4-11]. However, mitochondrial DNA mutational load (point mutations and deletions) is not greater in cells collected from patients with defects in oxidative phosphorylation when compared to healthy controls [12]. Therefore, although the “vicious cycle” hypothesis is an attractive concept, the relationship between mitochondrial DNA damage and dysfunction still remains unclear.

Mitochondrial dysfunction activates interorganellar or retrograde signaling to the nucleus that affects transcription of nuclear encoded proteins controlling mitochondrial growth and function [13]. For example, depletion of mitochondrial DNA, resulting in loss of mitochondrial respiration, alters cell signaling and nuclear transcription profiles in yeast [13, 14] and mammalian cells [15, 16]. Further, loss of mitochondrial membrane potential leads to increased cytosolic calcium levels and changes in transcription of genes involved in calcium storage and transport [15, 16]. Similarly, the recently described mitochondrial unfolded protein response (mtUPR) orchestrates mitochondrial to nuclear delivery of a transcription factor ATFS-1 in response to mitochondrial dysfunction [17]. Patients who suffer from diseases caused by mutations in mitochondrial DNA such as myoclonic epilepsy with ragged red fibers (MERRF) syndrome and myopathy, encephalopathy, lactic acidosis, and stroke like episodes (MELAS) provide further evidence for mitochondrial retrograde signaling. Both MERRF syndrome and MELAS stem from mutations in mitochondrial tRNA genes resulting in decreased translation of mitochondrial DNA-encoded proteins. To compensate for decreased mitochondrial function, mRNAs of nuclear encoded oxidative phosphorylation proteins are increased [18]. Other studies indicate that inhibition of complexes in the ETC (Complexes I [19], III [20], V[21]) alters nuclear transcription profiles. These as well as other studies (reviewed in [13, 22, 23]), suggest that mitochondrial dysfunction induced by either complete loss of the mitochondrial genome, mutations in mitochondrial genes encoding subunits of the ETC, or drugs that inhibit the ETC, alters cellular activity regulated by the nuclear genome.

The serine/threonine kinase ataxia telangiectasia mutated (ATM) plays a critical role in initiating the cellular signaling cascade in response to nuclear DSBs activating downstream signals to promote cell survival by regulating cell cycle checkpoints and DNA repair[24,

25]. Deficiency or mutation of ATM results in the rare autosomal recessive disorder Ataxiatelangiectasia (A-T), which is displayed as a spectrum of disease phenotypes including progressive neurological degeneration, predisposition to cancer, acute sensitivity to ionizing radiation, and a compromised immune response [26-29]. Recently, ATM has been localized to the mitochondrial compartment and has been implicated in also maintaining mitochondrial homeostasis [30, 31]. ATM deficient cell lines exhibit reduced mitochondrial respiratory capacity and diminished cytochrome c oxidase activity [31, 32]. In mouse thymocytes, loss of ATM altered mitochondrial morphology resulting in swollen, poorly organized cristae as well as increases in mitochondrial mass and mitochondrially-produced ROS [30]. Furthermore, cells and tissues from A-T patients display a reduction in mitochondrial DNA copy number suggesting a link between ATM and mitochondrial genome maintenance [33]. However, when considering the vicious cycle hypothesis, it remains unclear whether ATM is responding to mitochondrial dysfunction or DNA damage resulting from mitochondrial dysfunction.

Since there is evidence that mitochondrial DNA mutations precede impairment of mitochondrial function, the response to damaged mitochondrial DNA may be a potential early sensor of pending mitochondrial dysfunction and increasing oxidative stress. However, to our knowledge, there are no studies that attempt to define retrograde signaling in the absence of a change in mitochondrial function. Persistent expression of DNA endonucleases in the mitochondria of cell lines and transgenic mice causes mitochondrial deletions and dysfunction such as often seen in aged individuals [34-39]. To test the early response to mitochondrial DNA DSBs, we fused a mitochondrial targeting sequence (MTS) amino-terminal to the restriction endonuclease *PstI* followed by a hemagglutinin antigen (HA) tag (MTS-*PstI*-HA), and expressed it in A549 human lung adenocarcinoma cells. The human mitochondrial genome encodes two *PstI* restriction endonuclease recognition sites at positions 6910 and 9020 within complex IV subunit I and complex V subunit F6 coding regions, respectively. As a control, we also constructed a nuclear-targeted *PstI* construct with the SV40 nuclear localization sequence (NLS-*PstI*-HA). Although leaky intracellular trafficking of *PstI* limited our ability to cleanly distinguish the response to mitochondrial versus nuclear DNA damage, we discovered DNA strand breaks are sufficient to activate ATM and the DNA damage response (DDR) independent of mitochondrial dysfunction and excess production of ROS.

Materials and methods

Development of retroviruses expressing *PstI*

PstI cDNA was optimized for translation by the mammalian ribosome using the following codons: Ala (GCC), Arg (CGC), Asn (AAC), Asp (GAC), Cys (TGC), Gln (CAG), Glu (GAG), Gly (GGC), His (CAC), Ile (ATC), Leu (CTG), Lys (AAG), Met (ATG), Phe (TTC), Pro (CCC), Ser (TCC), Thr (ACC), Trp (TGG), Tyr (TAC) and Val (GTG). The cDNA was produced by Integrated DNA Technologies (Coralville, IA) and used as a template to create the targeted *PstI* expressing constructs. The mitochondrial *PstI* construct consists of the mitochondrial targeting sequence (MTS) from human cytochrome c oxidase subunit VIII fused to *PstI* followed by hemagglutinin antigen (HA) tag sequence (MTS-*PstI*-

HA). MTS-*PstI*-HA was generated using a primer containing the *EcoRI* recognition site, cytochrome oxidase subunit VIII MTS, followed by 18 base pairs (bp) of *PstI* (5'-GATCGAATTCATGTCCGTCCTGACGCCGCTGCTGCTGCGGGGCTTGACAGGCTCGCCCCGGCGGCTCCCAGTGCCGCGCGCCAAGATCCATTCGTTGATGAAGGAGCTGAAGCTG-3'), in combination with a reverse primer containing the *SalI* recognition sequence, HA tag sequence followed by the last 18-bp of *PstI* (excluding the stop codon) (5'-GATCGTCGACAGCGTAATCTGGAACATCGTATGGGTAGGACAGCTCGGGCTTCTTG-3'). The nuclear targeted *PstI* construct consists of the SV40 nuclear localization sequence (NLS) in-frame with *PstI* fused to a HA tag sequence (NLS-*PstI*-HA). Similarly, NLS-*PstI*-HA was created with a primer containing the *EcoRI* recognition site, SV40 NLS, and 18-bp of the *PstI* (5'-GATCGAATTCATGCCCAAGAAGAAGCGAAAGGTCATGAAGGAGCTGAAGCTG-3') and reverse primer described above. The PCR products were cloned into pIRES2-EGFP (Clontech, Mountain View CA). The MTS-*PstI*-HA and NLS-*PstI*-HA sequences were subcloned from the pIRES-GFP plasmid into the pBabe-IRES-EGFP-puromycin constructs (Addgene, Cambridge, MA).

Cell culture, transfections, and infections

A549 human lung adenocarcinoma cell line (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco's modified Eagle medium (DMEM, high glucose) with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 µg/ml streptomycin (Invitrogen, Grand Island, NY) in 5% CO₂ at 37°C. pBabe-IRES-EGFP-puromycin (empty virus control) and pBabe-IRES-EGFP-puromycin containing either the MTS- or NLS-*PstI*-HA cDNAs were cotransfected with the pVSV-G coat producing plasmid into human epithelial kidney Phoenix amphotropic cells (compliments of Dr. Garry Nolan, Stanford University) using the CaPO₄ method (Clontech). Media was harvested one day later and used to infect target cells for 18 hours. Infected cells were washed two times with 1× Hanks Balanced Salt solution (HBSS) and cultured in fresh medium. This was designated time zero for all experiments. For experiments where end points were analyzed at four days post infection, transduced cells were selected using 1 µg/ml puromycin (Invitrogen), except when flow cytometry was used to gate on infected cells expressing GFP.

Immunoblotting, cell fractionation, and antibodies

For whole cell extracts, cells were harvested using lysis buffer containing 0.05% SDS [40]. Lysates were sonicated at 20% maximal power with a stepped microtip (Sonics Vibra Cell, Newtown CT). Cellular fractions were obtained by harvesting the cells in lysis buffer from the Cell Fractionation Kit-Standard (Abcam, Cambridge, MA). Protein concentrations were determined using BCA assay (Thermo Scientific, Rockford, IL) and extracts were diluted with 3× Laemmli Buffer (Laemmli at 1× contains 50 mM Tris (pH 6.8), 1% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). Samples were separated using SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membrane (Pall Life Sciences, Pensacola, FL). All transfers were done using Tris/glycine buffer. All primary antibodies were diluted in 5% (w/v) milk in Tris-buffered saline with 0.1% (v/v) tween (TBS-T) at the following concentrations: β-actin at 1:10,000 (Sigma-

Aldrich, St. Louis, MO); ATM, ATM (phosphoserine 1981), TP53 (phosphoserine 15), histone H3 and histone H2AX at 1:1000 (BD Cell Signaling, San Jose, CA); γ -H2AX (phosphoserine 139) at 1:2500 (EMD Millipore, Billerica MA); TP53 (DO1) at 1:1000 (Novacastra Laboratories, UK), complex IV subunit I at 1:3000 and complex V subunit F6 at 1:1,000 (Mitosciences, Eugene, OR); p21 at 1:500 (BD Pharminogen, Franklin Lakes, NJ); KAP1 at 1:10,000, KAP1 (phosphoserine 824) at 1:2000, SMC1 at 1:4000, and SMC1 (phosphoserine 975) at 1:1000 (Bethyl Laboratories, Inc., Montgomery TX). Secondary horseradish peroxidase (HRP) conjugated antibodies (Southern Biotech, Birmingham, AL) were incubated in 5% (w/v) milk in TBS-T (1:7000). HRP was detected using the ECL Plus Western Blotting Detection kit (GE Healthcare, UK) and developed using Blue Sensitive film (Laboratory Products Sales, Rochester, NY).

Southern blotting

Cells were washed, trypsinized, re-suspended in DNA lysis buffer (200 mM NaCl, 20 mM EDTA, 40 mM Tris pH8, 0.5% SDS, 0.5% β -mercapthoethanol, 20 mg/mL proteinase K), and incubated overnight at 55°C. DNA was extracted twice with phenol:chloroform:isoamyl alcohol and precipitated with isopropanol overnight at -20°C. DNA was collected via centrifugation, washed once with 70% ethanol, and re-suspended in sterile water. DNA (10 μ g) was digested overnight at 37°C with *EcoRV* and *SacII* $-/+$ *PstI* (New England Biolabs, Inc., Ipswich, MA) before separating by gel electrophoresis on a 0.8% 1 $\times$ Tris-acetate (TAE) agarose gel containing ethidium bromide. DNA was transferred to a Magna Graph 0.45-micron nylon membrane (Osmonics, Inc., Minnetonka, MN). The 1499-bp probe for total mitochondrial DNA was generated by PCR using primers (5'-GGTCACACGATTAACCCAAG-3') and (5'-GTTGGTTGATTGTAGATATTGG-3'). To detect *PstI*-induced mitochondrial double-strand breaks, the 744-bp probe was generated by PCR using primers (5'-GCCCCACTTCTTACCACAAGG-3') and (5'-GCTCAGGTGATTGATACTCC-3'). DNA probes were isolated in low-melt agarose and labeled using the Prime-A-Gene labeling system (Promega, Madison, WI) with α -³²P-dCTP. Membranes were hybridized with probes overnight at 65°C, subsequently washed with 2 $\times$ SSC, 0.1% SDS, and visualized via Blue Sensitive film (Laboratory Products Sales, Rochester, NY).

Measuring mitochondrial function by oxygen consumption

Asynchronously dividing cells were infected with control, mito*PstI*, or nuc*PstI* viruses. Cells were extensively washed free of virus 18 hours later and cultured for 24 hours in virus-free media. Cells were then trypsinized and re-plated at 40,000 cells per well the night before analysis in 24 well polystyrene plates (Seahorse Biosciences, North Billerica, MA). One hour prior to analysis cell culture medium was changed to unbuffered DMEM (Invitrogen) supplemented with 25 mM glucose, 1 mM sodium pyruvate, and 4 mM L-glutamine. Oxygen consumption was measured every 12 seconds over two, five minute intervals using a Seahorse XF24 Analyzer (Seahorse Biosciences) under the following conditions: resting or baseline, after FCCP (Sigma-Aldrich) (500 nM) treatment (maximal OCR), and after Antimycin A (Sigma-Aldrich) (5 μ M) treatment (non-mitochondrial OCR). Oxygen consumption rates (OCRs) were calculated using the Seahorse XF24 software (adapted from [41]). After measurements, cells in each well were lysed and BCA analysis

was used to determine protein concentration. OCR readings were normalized to total protein in each well. The non-mitochondrial OCR was then subtracted from the baseline and maximal OCRs to obtain the mitochondrial specific OCRs.

Mitochondrial Membrane Potential and Redox Staining

Cells were harvested via trypsinization and washed with phosphate-buffered saline (PBS) (Cellgro, Herndon, VA). Cells were incubated in 5 μ M DHE (Invitrogen), 5 μ M mitoSOX (Life Technologies), or 50nM tetramethylrhodamine, ethyl ester (TMRE) for 15-30 minutes at 37°C. Cells were pelleted, washed, and resuspended in PBS. Fluorescence intensity was detected using a LSR II flow cytometer (BD Biosciences, San Jose, CA).

Cell proliferation analysis

Cells were pulse labeled with 5-bromo-2'-deoxyuridine (BrdU) labeling reagent (Invitrogen, 1:100 in pre-warmed DMEM) for 1.5 hours and then fixed in 1% paraformaldehyde overnight at 4°C. Cells were permeabilized with 0.1% tritonX and subsequently treated with 0.3 mg/ml DNase (Sigma-Aldrich) in PBS for 30 minutes at 37°C. The cells were then incubated with anti-BrdU antibody (Invitrogen, 1:50 in PBS + 2% FBS), washed in PBS, and resuspended in PBS containing 2% FBS and 5 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Fluorescence intensity of BrdU and DAPI positive cells expressing GFP expression was evaluated on a LSR II flow cytometer.

Cell death and senescence

Cell death was measured using the Annexin V-APC Apoptosis Detection Kit I (BD PharMingen, San Diego, CA). Briefly, cells were harvested and stained with Annexin V-APC and 7AAD, and analyzed on LSR II (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). GFP positive, transduced cells were gated and 7AAD versus Annexin V fluorescence was plotted. Senescence was determined using a Senescence β -gal staining kit from Mirus Bio LCC (Madison, WI).

Quantitative real-time PCR

RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA) and converted into cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real time PCR was performed using SYBR green incorporation with a CFX96 Real-Time C1000 Thermal Cycler (Bio-Rad, Hercules, CA). A dilution series was performed to ensure primer efficiency was between 90 and 110%. A dissociation melt curve was performed with every run to ensure single band contribution to the fluorescent signal. Primers used were as follows: p21: 5'-CTGGAGACTCTCAGGGTCGAA-3' and 5'-GGATTAGGGCTTCCTCTTGGA-3' PUMA: 5'-ATGCCTGCCTCACCTTCATCA-3' and 5'-AGCACAGGATTCACAGTCTGGG-3' Bax: 5'-TGCTTCAGGGTTTCATCCAG-3' and 5'-GGCGGCAATCATCCTCTG-3' GAPDH: 5'-TGGTATCGTGGAAGGACTCATGAC-3' and 5'-ATGCCAGTGAGCTTCCCGTTCAGC-3'.

Fold change was determined using the following equation: fold change = $2^{-[(Ct^{target})_{treated} - (Ct^{target} - Ct^{reference})_{untreated}]}$ with GAPDH used as the reference gene.

Statistical analysis

Values are represented as means $\pm$ standard deviations unless otherwise stated. Significance was determined using unpaired two-tailed T-tests or three way analyses using ANOVA on GraphPad Instat 3 for MacIntosh. *P*-value of less than 0.05 was considered significant.

Results

Expression of the *PstI* endonuclease in A549 cells

Retroviruses were created that expressed mitochondrial-targeted *PstI* (MTS-*PstI*-HA) or nuclear-targeted *PstI* (NLS-*PstI*-HA) in-frame with a carboxy-terminal hemagglutinin antigen (HA) tag that was placed upstream of an IRES-EGFP sequence (Fig. 1A). A549 cells were infected for 18 hours, washed, and replated for an additional 48 hours. MTS-*PstI*-HA and NLS-*PstI*-HA were faintly detected using an antibody against the HA tag when cells were washed free of virus (0 hours) and readily detected by 24 and 48 hours post-infection (Fig. 1B). Increased expression of *PstI* correlated with increased expression of EGFP, which was also used to confirm infection with the control virus lacking *PstI*. Consistent with *PstI* damaging DNA, increased phosphorylation of H2AX (γ H2AX) was observed in cells expressing MTS-*PstI*-HA and NLS-*PstI*-HA, but not the empty vector (Fig. 1B). To confirm *PstI* was properly targeted within the cell, cytoplasmic, mitochondrial and nuclear fractions were prepared 48 hours after cells were washed free of virus. The presence of mitochondrial complex IV subunit I (also called cytochrome oxidase I) and nuclear histone H3 were used to assess purity of the fractions (Fig. 1C). Although mitochondrial and nuclear fractions were relatively pure, MTS-*PstI*-HA was detected in both mitochondrial and nuclear fractions. NLS-*PstI*-HA was detected in all three fractions. Immunofluorescence staining with anti-HA antibody confirmed leaky targeting of MTS-*PstI*-HA and NLS-*PstI*-HA (data not shown). This implies that neither the MTS-*PstI*-HA nor NLS-*PstI*-HA was exclusively localized to their expected intracellular compartments.

Mitochondrial-targeted *PstI* generates mitochondrial DNA double-strand breaks without disrupting mitochondrial respiration

While the sub-cellular localization of MTS- and NLS-*PstI*-HA was not restricted to their designated compartments, their ability to selectively cause mitochondrial DNA DSBs was evaluated by Southern blot analyses (Fig. 2A and 2B). Total cellular DNA was collected when virus was removed from the cells (time 0), digested with *SacII* and *EcoRV*, and hybridized with a probe spanning the *PstI* recognition site at position 9020 of the mitochondrial genome. The 6.1 kb band detected represents mitochondrial DNA that has not been cleaved by *PstI*, while cleavage of *PstI* at position 9020 in the mitochondrial genome produces a 3.9 kb band. A 4.0 kb band is generated by cleavage at both *PstI* recognition sites followed by ligation eliminating the *PstI*-*PstI* intervening sequence. The linear *PstI*-*PstI* fragment produces a 2.1 kb band. Using this approach, a faint but reproducible 3.9/4.0 kb band was consistently detected in DNA isolated from cells infected with MTS-*PstI*-HA, but not with the control or NLS-*PstI*-HA. To confirm that mitochondrial DNA post-infection was sensitive to *PstI*-induced cleavage, these samples were also incubated with *PstI* *in vitro*. Loss of the 6.1 kb band with appearance of the 3.9/4.0 kb band indicate that the mitochondrial *PstI* recognition sites were not resistant to cleavage. Furthermore, to

investigate whether there was a significant change in mitochondrial DNA copy number, the DNA was hybridized with a probe within the *SacII* recognition sites. A 1.7 kb band was consistently detected in all samples confirming that mitochondrial DNA was not being lost.

As further evidence that mitochondrial DNA DSBs were taking place, we evaluated expression of the two mitochondrial encoded genes that contain *PstI* sites. Relative to control virus, MTS-*PstI*-HA reduced expression of complex IV subunit I by 48 hours and complex V subunit F6 after 24 hours (Fig. 2C). Although mRNA levels of these genes also declined in cells expressing *PstI* targeted to the nucleus, it was less than that observed in cells expressing mitochondrial targeted *PstI*. Despite a reduction in mRNA expression, the expression of the respective proteins did not change (Fig. 2C and data not shown), which is consistent with other studies showing that the half-life of respiratory chain subunits is very slow [42]. To determine whether mitochondrial DNA DSBs and reduced expression of targeted genes affected mitochondrial function, the oxygen consumption rate (OCR) was measured in cells infected with control, MTS-*PstI*-HA, and NLS-*PstI*-HA retroviruses. Despite the continued expression of *PstI*, the mitochondrial OCR was not affected between cells expressing control, MTS-, or NLS-*PstI*-HA after 24 (data not shown) or 48 hours (Fig. 3A). Similarly, expression of MTS-*PstI*-HA or NLS-*PstI*-HA did not significantly cell morphology (data not shown) or alter mitochondrial membrane potential as defined by staining cells with tetramethylrhodamine, ethyl ester (TMRE), a positively charged dye that rapidly accumulates in active mitochondria (Fig. 3B). Expression of MTS-*PstI*-HA or NLS-*PstI*-HA also did not affect superoxide production measured by mitoSOX or dihydroethidium (DHE) staining (Fig. 3C and 3D). Thus, targeted expression of *PstI* to the mitochondria was sufficient to cause mitochondrial DNA DSBs without compromising mitochondrial function and ROS production.

DNA damage inhibits cellular proliferation

DNA damage activates a variety of molecular signals that inhibit cell proliferation and promote apoptosis. To further evaluate the cellular response *PstI* expression, A549 cells were infected with control, MTS-*PstI*-HA or NLS-*PstI*-HA. Infection with MTS-*PstI*-HA and NLS-*PstI*-HA retroviruses reduced cellular expansion by 48 hours with significant differences observed at 72 and 96 hours post infection compared to the control infected cells ($n = 3$; $P < 0.001$, in both cases) (Fig. 4A). Reduced number of cells at 48 and 72 was not attributed to senescence, defined by β -galactosidase staining, or apoptosis, as defined by Annexin V and 7AAD staining (data not shown). However, by 96 hours post-infection, the percent of dead cells increased from 3.3 ± 1.4 in the control to 9 ± 4.6 and 8.3 ± 4.9 in the MTS-*PstI*-HA and NLS-*PstI*-HA expressing populations, respectively ($n = 3$, $P < 0.03$). To assess whether growth arrest was occurring at earlier times, cells infected with control, MTS-*PstI*-HA or NLS-*PstI*-HA retroviruses were pulse labeled with bromodeoxyuridine (BrdU) at 48 hours. Expression of MTS-*PstI*-HA and NLS-*PstI*-HA significantly inhibited BrdU labeling ($n = 4$; $P < 0.001$, in both cases) (Fig. 4B). Consistent with growth inhibition being mediated in part through a p53-checkpoint, siRNA depletion of TP53 prior to infection with MTS-*PstI*-HA and NLS-*PstI*-HA retroviruses impaired expression of the cyclin-dependent kinase inhibitor p21 and partially enhanced BrdU incorporation ($n = 6$; $P = 0.009$ and 0.002 , respectively) (Fig. 4C).

DNA damage stimulates phosphorylation of ATM in the nucleus

In response to DNA DSBs, ATM undergoes autophosphorylation at serine 1981 (ATM-pSer1981) and becomes an active kinase that phosphorylates TP53 and other substrates. Expression of MTS-*PstI*-HA or NLS-*PstI*-HA increased ATM-pS1981 at 24 and 48 hours post-infection (Fig. 5A). Increased ATM phosphorylation correlated with increased phosphorylation of TP53-pS15, KAP1-pS824, an interacting partner of the KRAB (Kruppel-associated box) domain-containing zinc finger transcription factor family, and SMC1-pS957, a protein involved in maintaining chromosome structure was also observed. To determine the localization of these proteins, sub-cellular fractions were prepared from cells harvested 48 hours post-infection. Purity of cytoplasmic, mitochondrial, and nuclear enriched fractions were confirmed by blotting with Cytochrome c oxidase (or Complex IV) subunit 1 (Cox1) and nuclear histone H3 antibodies (Fig. 5B). ATM was predominantly detected in both mitochondrial and nuclear enriched fractions, with a small amount present in cytoplasmic fractions. However, ATM-pS1981 was detected in nuclear, but not mitochondrial, fractions of cells expressing MTS-*PstI*-HA or NLS-*PstI*-HA. SMC1 was detected primarily in the nuclear fraction of cells infected with control (C), mito*PstI* (M), or nuc*PstI* (N) viruses. In contrast, the SMC1-pS957 predominated in nuclear fractions of cells expressing mito*PstI* or nuc*PstI* but not the control virus (Fig. 5C). TP53 and KAP1 and their phosphorylated forms were predominantly localized to both nuclear and mitochondrial fractions. The phosphorylated form was only detected in mitochondria and nuclear compartments of cells expressing mito*PstI* (M) or nuc*PstI* (N) but not in controls.

Discussion

Many human diseases, cancers, and aging are being attributed to a “vicious cycle” of progressive mitochondrial dysfunction, DNA damage, and ROS production that ultimately leads to altered cell function and even death [6, 11, 43, 44]. A recent study suggests that mitochondrial DNA damage precedes mitochondrial dysfunction, which further contributes to additional DNA damage [12]. Hence, there is need to understand how cells respond to DNA damage so as to prevent mitochondrial dysfunction and ROS production. The ATM protein has emerged as a central mediator of the cellular response to nuclear DNA DSBs and mitochondrial dysfunction. However, its role in signaling in response to mitochondrial DNA damage is not known, in part, because studying mitochondrial DNA damage separate from mitochondrial dysfunction has been challenging. Using *PstI* endonuclease to damage DNA, we provide evidence that DNA double strand breaks stimulate phosphorylation of ATM and the DDR response in the absence of mitochondrial dysfunction and ROS production. Although leaky trafficking of *PstI* endonuclease limited our ability to make definitive conclusions about ATM activation in response to nuclear versus mitochondrial DNA damage, phosphorylated ATM was not detected in mitochondria despite damage to mitochondrial DNA. These findings provide new evidence that DNA damage can activate ATM in the absence of mitochondrial dysfunction and suggest that damage to mitochondrial DNA activates ATM in the nucleus through retrograde signaling.

The generation of experimentally induced site-specific DSBs has been used to determine chromosomal aberrations and mutational spectra, as well as cellular consequences due to

DSB formation [34-39, 45]. To determine whether mitochondrial DNA DSBs activate a DDR and the resulting cellular consequences, we generated constructs to target the restriction endonuclease *PstI* to the mitochondrial and nuclear compartments. Unfortunately, we were not able to selectively target *PstI* to mitochondria and nuclear compartments using well-characterized targeting sequences specific for these organelles. This implies *PstI*, a protein that is not endogenously expressed in eukaryotes, may interact with other proteins that contain strong nuclear or mitochondrial targeting sequences. Despite our inability to restrict the localization of *PstI* to the designated compartments, we were able to generate mitochondrial DNA DSBs in the absence of detectable mitochondrial dysfunction. Southern blot analyses indicate that MTS-*PstI*-HA, while not the control or NLS-*PstI*-HA, results in detectable mitochondrial DNA DSBs. However, only a small amount of breaks were detected relative to total mitochondrial DNA. This observation could be due to the following scenarios: 1) MTS-*PstI*-HA is not efficiently cleaving mitochondrial DNA; 2) mitochondrial DNA DSB repair is efficiently repairing the cleaved DNA; 3) some fraction of the induced *PstI* break is not sufficiently detectable due to processing at the break site. Moreover, the amount of *PstI*-induced mitochondrial DNA DSBs are likely to be an underestimate of the actual amount of breaks because we are only monitoring the *PstI* site at position 9020. Efficient DNA repair seems to be the most plausible reason for why few breaks were observed because they were not observed at 24 and 48 hours, even after the virus was removed from the cells (data not shown). In other words, mitochondrial DNA DSBs detected at time 0 when virus was washed from the plates but not at 24 and 48 hours even though expression of *PstI* was maximally expressed at these times. Targeting *PstI* to mitochondria may therefore provide a useful model to understand how mitochondrial DNA DSBs are repaired.

Previous reports suggest that in diseases associated with mitochondrial mutations or deletions, a threshold of over half of the mitochondrial genome must be depleted prior to loss of mitochondrial function [46-48]. As described above, a limited amount of mitochondrial DNA DSBs was detected by Southern blot analysis in cells expressing MTS-*PstI*-HA but not NLS-*PstI*-HA. This limited amount of damage was sufficient to reduce mRNA levels of complex IV subunit I and complex V subunit F6 over time. Greater loss was observed in cells expressing mitochondrial-targeted *PstI* than nuclear *PstI*. Such differences may reflect less damage to mitochondrial DNA by leaky trafficking of NLS-*PstI*-HA or damage to nuclear encoded genes that affect mitochondrial gene expression. The modest reduction in complex IV subunit I and complex V subunit F6 mRNA expression may also not have been sufficient to reduce expression of their proteins below a threshold that results in mitochondrial dysfunction. In fact, MTS-*PstI*-HA and NLS-*PstI*-HA did not affect abundance of complex IV subunit I present in mitochondrial fractions when compared to that of cells infected with control virus (Fig. 1C). Regardless, reduced expression of complex IV subunit I and complex V subunit F6 mRNAs was not sufficient to cause overt mitochondrial dysfunction defined by change in the rate of oxygen consumption or superoxide levels. The limited number of breaks present at any time, plus the observation that DSBs were not detected after the virus is removed from the cells suggests that there were too few breaks at any given time to adversely affect mitochondrial homeostasis. Alternatively, changes in mitochondrial oxygen consumption rate were not detected at 48

hours because cancer cell lines are typically less reliant on mitochondria to produce energy. While A549 cells may be harder than primary cells, prolonged expression of mito*PstI* for 4 days reduced oxygen-consumption rates in A549 cells (data not shown). This is consistent with findings by other investigators who observed mitochondrial dysfunction when mitochondrial-targeted DNases were over-expressed for weeks and months [34-39]. Furthermore, mitochondrial DNA damage has been shown to decrease the capacity of mitochondrial respiration [49, 50]. Together, these findings suggest acute mitochondrial DNA damage can activate the DNA damage response prior to and independent of overt mitochondrial dysfunction.

While NLS-*PstI*-HA co-fractionated with mitochondria, we did not detect mitochondrial DNA DSBs suggesting that either this construct was unable to penetrate the mitochondrial matrix or was insufficient in generating mitochondrial DNA DSBs above the limit of detection. On the other hand, both NLS-*PstI*-HA and MTS-*PstI*-HA proteins were detected in nuclear fractions. Although 732,000 potential *PstI* recognition sites are predicted in the nuclear genome, DNA double strand breaks were not detected in several nuclear genes that contain *PstI* sites (data not shown). Our inability to detect damage at a given *PstI* site is not surprising given the difficulty in identifying DSBs using another restriction enzyme I-*PpoI*, which results in cleavage of only 10% of the less than 300 I-*PpoI* recognition sites in human nuclear DNA [45, 51]. Even though nuclear DSBs were not detected via Southern blot, cellular responses to nuclear DSBs, including increased levels of phosphorylated γ H2AX suggest *PstI*-induced nuclear DNA DSBs. We found expression of *PstI* produced cell cycle arrest as defined by elevated expression of p21, reduced BrdU incorporation, and reduced number of cells over time. Apoptosis was not observed until 96 hours post-infection and senescence, which has been linked to unrepaired DSBs in the nuclear compartment [52], was never observed. Taken together, this model results predominately in cell-cycle arrest, presumably to protect and repair the *PstI*-induced DSBs. Some cell death was observed by 96 hours. Since DNA DSBs were not readily detected despite the continued expression of *PstI*, death likely reflects a response to damage or a failure to effectively repair damage rather than an accumulation of DNA DSBs.

Despite the fact that DNA DSBs were not detected at later time points, activation of ATM, defined by phosphorylation on serine 1981 persisted with time. ATM activation correlated with increased phosphorylation of TP53, KAP1, SMC1, and γ H2AX. The activation of these proteins, especially TP53, is consistent with the expected cellular response to DNA DSBs [45]. Surprisingly, phosphorylated ATM and SMC1 were detected in nuclear but not mitochondrial fractions, whereas the unphosphorylated forms were detected in both compartments. Conversely, phosphorylated TP53 and KAP and their unphosphorylated forms were detected in both nuclear and mitochondrial fractions. Given that mitochondrial DNA DSBs were detected, this suggests that mitochondrial DNA damage drives retrograde signaling of ATM and some of its substrates in the nucleus. Interestingly, low doses of hydrogen peroxide can stimulate ATM phosphorylation in mitochondria without activating the DNA damage response defined by phosphorylation of H2AX [53]. Similarly, uncoupling mitochondria using CCCP stimulates ATM phosphorylation in mitochondria without stimulating phosphorylation of the ATM substrates TP53, KAP1 or SMC1 [30]. In that

study, the authors hypothesized ATM has an independent function in the mitochondrial compartment separate from nuclear or mitochondrial DNA damage. Our finding that DNA DSBs stimulate phosphorylation of TP53, KAP1, and SMC1 support this conclusion and suggest phosphorylation of these DDR proteins reflects a response to DNA damage and not just mitochondrial dysfunction. The cellular response to DNA damage appears to be very sensitive because transfection of purified DNase or plasmids containing a single nick is sufficient to activate p53 [54, 55]. Since p53 levels are responsive to small amounts of DNA damage and small amounts of mitochondrial DNA damage were detected in the current study, it is unlikely that the mitochondrial genome was not sufficiently damaged to activate the ATM pathway. However, it remains to be determined if more damage to the mitochondrial genome is required to stimulate phosphorylation of ATM in the mitochondria.

In summary, the current findings reveal mitochondrial and nuclear DNA damage can activate ATM and the DDR response in the absence of mitochondrial dysfunction. Although leaky trafficking of *PstI* limits our ability to distinguish the cellular response to nuclear or mitochondrial DNA DSBs, phosphorylated ATM was predominantly localized to the nucleus even in cells where mitochondrial DNA damage was detected. This supports the concept that mitochondrial DNA damage activates retrograde signaling to the nucleus that may influence nuclear dependent responses designed to promote mitochondrial DNA repair, and hence limit mitochondrial dysfunction and ROS production.

Acknowledgments

We thank Dr. Craig Jordan and Dr. John Ashton for assistance with creating retrovirus, Dr. Miguel Martins for contributing the pBabe-IRES-GFP-puro plasmid to Addgene for public use, and Dr. Garry Nolan for creating and sharing the Phoenix retrovirus producing cells. We are grateful to Emily Resseguie for critically reviewing this manuscript and the data. This work was supported by National Institutes of Health grants R01HL067392 and R01HL091968 (MAOR), and R01HL071158 (PSB). Training Grants T32HL66988 supported J. Gewandter and T32ES07026 supported L. Kalifa. NIH Center grant P30ES01247 supported equipment used for some of the experiments.

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Abbreviations

ATM	ataxia telangiectasia mutant
DSBs	double strand breaks
HA	hemagglutinin antigen epitope tag
KAP1	KRAB-associated protein-1
MTS	mitochondrial targeting sequence
NTS	nuclear targeting sequence
ROS	reactive oxygen species
SMC1	structural maintenance of chromosomes-1
TP53	tumor suppressor protein p53

Highlights

- Expression of PstI endonuclease created DNA double strand breaks in mitochondrial and nuclear DNA of A549 cells.
- PstI stimulated the DNA damage response defined by phosphorylation of ATM, TP53, and cell cycle arrest.
- DNA double strand breaks did not affect mitochondrial oxygen consumption or production of ROS.
- DNA damage can activate ATM signaling independent of mitochondrial dysfunction.

Fig. 1.

Characterizing expression of MTS-*PstI*-HA and NLS-*PstI*-HA in A549 cells. (A) Cartoon model showing the MluV promoter driving expression of an empty cassette as control, the MTS-*PstI*-HA, or NLS-*PstI*-HA cDNA followed by an internal ribosome entry site (IRES) and the enhanced green fluorescence protein (EGFP). (B) A549 cells were infected with control, MTS-*PstI*-HA, or NLS-*PstI*-HA retroviruses. Virus was removed 18 hours later when medium was replenished. Cells were immediately harvested (0 hours), 24, and 48 hours later. The expression of the HA tag, EGFP, H2AX, γ-H2AX, and β-actin was detected in lysates prepared from cells infected with control (lane C), MTS-*PstI*-HA (lane M), or NLS-*PstI*-HA (lane N) retrovirus. (C) Cells were infected with control (C), MTS-*PstI*-HA (M), or NLS-*PstI*-HA (N) retroviruses for 18 hours, washed, and harvested 48 hours later. Cytoplasmic (lane 1), mitochondrial (lane 2), and nuclear (lane 3) enriched fractions were prepared and immunoblotted with antibodies against HA tag, complex IV subunit 1 (CIV SI), or histone H3.

Fig. 2.

Expression of MTS-*PstI*-HA causes mitochondrial DNA double strand breaks. (A) Schematic of the human mitochondrial genome with designated restriction endonuclease recognition sites. Dotted lines indicate location where probes anneal. (B) Total DNA was purified from A549 cells infected with control (lane C), MTS-*PstI*-HA (lane M), or NLS-*PstI*-HA (lane N) retrovirus for 18 hours. The DNA was digested with *SacII* and *EcoRV* without (-) or with (+) *PstI* *in vitro* as controls. Bold and skinny arrows indicate *PstI*-induced DSB or deletion product of 3.9 and 2.1 kb respectively that were detected in lane M of the -*PstI* lanes. Both products were detected in all samples when *PstI* was added (+) to the digests. (C) A549 cells were infected with control, MTS-*PstI*-HA or NLS-*PstI*-HA retroviruses for 18 hours, harvested as time 0, or plated in fresh media for an additional 24 and 48 hours. The mRNA levels of cytochrome IV subunit I and cytochrome V subunit F6 were evaluated by qRT-PCR and graphed relative to the control virus. ($n = 3$, * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$).

Fig. 3.

Expression of MTS-*PstI*-HA does not significantly disrupt mitochondrial respiration. A549 cells were infected with control, MTS-*PstI*-HA or NLS-*PstI*-HA retroviruses for 18 hours. Virus was removed and the cells were cultured in fresh medium for an additional 48 hours. (A) The oxygen consumption rate (OCR) was determined on a Seahorse Bioanalyzer before and after sequentially injecting FCCP and antimycin A. The OCR for each condition was normalized to total protein. Data represent mean OCR ± SEM from 2 independent experiments with at least 6 samples per experiment. (B) Cells were stained with TMRE and the mitochondrial membrane potential was graphed as fold change over control virus. (C) Cells were stained with mitoSOX and mitochondrial superoxide production was graphed as fold change over control virus. (D) Cells were stained with DHE and cytoplasmic superoxide production was graphed as fold change over control values.

Fig. 4.

Expression of MTS-*PstI*-HA or NLS-*PstI*-HA inhibits growth of A549 cells. (A) Cells were infected with control, MTS-*PstI*-HA, or NLS-*PstI*-HA retroviruses for 18 hours. Cells were cultured in fresh medium and counted 24, 48, 72, and 96 hours later. Values represent mean $\pm$ SD ($n = 3$, * $P < 0.001$). (B) Cells were pulse labeled with BrdU 48 hours post-infection, fixed, and stained with antibody against BrdU and DAPI dye. After gating on the GFP+ population using flow cytometry, BrdU fluorescence intensity was graphed versus DNA content defined by DAPI fluorescence. Inset values in the representative histograms represent the percentage of BrdU+ cells in 10,000 GFP+ cells. The average number of BrdU+ cells relative to the control population was graphed ($n = 4$, $P < 0.001$, in both cases). (C) A549 cells were transfected with siRNAs targeted a luciferase control sequence or TP53 24 hours prior to viral infections. Cells were infected with virus, washed 18 hours later, replated in fresh media for 48 hours, and then pulse labeled with BrdU for 2 hours. SiRNA targeting reduced p53 expression of p53 and the p53-target gene p21. The average number of BrdU+ cells relative to control cells transfected with luciferase siRNA was graphed ($n = 6$, *** $P < 0.0001$, ** $P = 0.002$, * $P = 0.009$).

Fig. 5.

DNA DSBs induce phosphorylation of ATM in the nucleus. (A) Cells were infected with control (lane C), MTS-*PstI*-HA (lane M), or NLS-*PstI*-HA (lane N) retroviruses for 18 hours. Lysates were harvested immediately (0), 24, and 48 hours post-infection and blotted with antibody against ATM, ATM-pS1981, TP53, TP53-pS15, KAP1, KAP1-pS824, SMC1, SMC1-pS957 and β -actin used as a loading control. (B and C) Cytoplasmic (lane 1), mitochondrial (lane 2), and nuclear (lane 3) enriched fractions were prepared from cells harvested 48 hours post-infection. Fractions were immunoblotted with the same antibodies plus antibodies against complex IV subunit I (CIV SI) and histone H3 used to assess purity of mitochondrial and nuclear fractions, respectively.