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APOPTOSIS INDUCED BY PERSISTENT SINGLE-STRAND BREAKS IN THE MITOCHONDRIAL GENOME: CRITICAL ROLE OF EXOG (5' EXO/ENDONUCLEASE) IN THEIR REPAIR.

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Running title: Mitochondrial DNA Damage-Induced Apoptosis

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Reactive oxygen species (ROS), continuously generated as by-products of respiration, inflict more damage on the mitochondrial (mt) than on nuclear (nu) genome because of the former's non-chromatinized nature and proximity to ROS source. Such damage, particularly single-strand breaks (SSBs) with 5' blocking deoxyribose products generated directly or as repair intermediates for oxidized bases, is repaired via the base excision/SSB repair (BER)/SSBR pathway in both nu and mt genomes. Here we show that EXOG, a 5'exo/endonuclease and unique to the mitochondria unlike FEN1 or DNA2, which like EXOG have been implicated in the removal of the 5' blocking residue, is required for repairing endogenous SSBs in the mt genome. EXOG depletion induces persistent SSBs in the mt DNA, enhances ROS level, and causes apoptosis in normal cells but not in mt genome deficient rho0 cells. Thus, these data show for the first time that persistent SSBs in the mt genome alone could provide the initial trigger for apoptotic signaling in mammalian cells.

A mammalian cell contains up to several thousands copies of duplex, circular 16.5 kb mt genome within 80-700 mitochondria depending on the cell type (1,2). The mtDNA encodes essential subunits of the respiratory chain, tRNA and rRNAs, all of which are critical for maintaining oxidative phosphorylation (OXPHOS) (3). OXPHOS accounts for about 85% of oxygen consumed by the cell, and early reports estimated that under physiological conditions, ~5% of consumed oxygen is partially reduced to ROS (4). Although recent reports indicate a much lower level of ROS production in normal cells, even low persistent ROS level has long term detrimental effects (5,6). Thus, the mitochondria, the major cellular site for ROS generation, are under continuous oxidative stress that results in oxidative damage to DNA, as well as proteins and lipids (7).

ROS-induced DNA damage includes multitude of mutagenic oxidized bases and single-strand breaks (SSBs) containing 3' and 5' blocking groups in DNA, which are generated both directly or as intermediates during BER (8,9). Due to close proximity of the site of ROS generation and nonchromatinized state of the mt genome, the mutation rate in human mtDNA is 20- to 100-fold higher relative to the nu DNA (10). As summarized in recent reviews (11-13)

repair of oxidized base lesions or abnormal bases is initiated with their excision by a DNA glycosylase. A monofunctional glycosylase, such as uracil-DNA glycosylase (UDG), excises U from the DNA to generate an AP site which is then cleaved by AP-endonuclease (APE1) in mammalian cells, to generate 3' OH and nonligatable 5' deoxyribose phosphate (dRP) residues. In the nucleus, the 5' dRP could be removed by DNA polymerase β (pol β) via its intrinsic dRP lyase activity. In the mitochondria, the DNA polymerase γ (pol γ) with similar dRP lyase activity is also able to remove the dRP moiety (14). In the case of oxidized base repair by DNA glycosylases with intrinsic AP lyase activity, such as 8-oxoguanine-DNA glycosylase (OGG1), base excision is coupled to strand cleavage at the AP site with generation of 5' phosphate and 3' blocking phospho-α,β unsaturated aldehyde which is subsequently removed by the intrinsic 3' phosphodiesterase activity of APE1. This leaves a 3'OH that serves as the primer terminus for DNA repair synthesis. However, the absence of an aldehyde group in oxidized deoxyribose fragment at the 5' terminus after DNA strand break, as in the case of oxidized AP sites, precludes their removal by the dRP lyase activity of pol β and γ in the nucleus and mitochondria, respectively. In such a case, the 5' blocking group in nu DNA together with additional nucleotides are removed by flap endonuclease 1 (FEN1), a 5' exo/endonuclease. Thus, the resulting gap filling by a DNA polymerase and nick sealing by DNA ligase during BER could proceed via two subpathways: single-nucleotide (SN)-BER where only the damage base is replaced or long-patch (LP)-BER where 2-6 additional nucleotides at the 5' terminus are removed by a 5' exo/endonuclease followed by resynthesis. In the nucleus, DNA ligase3 (lig3) is involved in SN-BER after pol β fills in the single nucleotide gap. FEN1-mediated gap is likely to be filled in by replicative DNA polymerases δ/ε followed by nick sealing with DNA ligase1 (lig1) although pol β has also been implicated (15). In contrast to the situation in the nucleus with multiple DNA polymerases and ligases, their sole mt counterparts, pol γ and lig3, are responsible for both replication and repair of mtDNA (16).

Only SN-BER activity was known in the mitochondria until we and others discovered LP-BER activity in mt extract of mammalian cells; however, the identity of the mt 5' end-processing exo/endonuclease was not settled (17-20). Our group and Akbari et al reported that mt LP-BER is FEN1-independent. We detected the presence of an unknown 5' exo/endonuclease activity in mt extract of mammalian cells which generated short (3-4 nucleotides) fragments, distinct from those generated by FEN1 (18). In contrast, Liu et al have shown that repair of 2-deoxyribonolactone, a common AP site oxidation product, was dependent on FEN1 activity (19). Subsequently DNA2, a helicase/nuclease, which plays various roles in the processing of nu DNA intermediates during replication and repair in yeast, was shown to be present in the human mitochondria, and was proposed to process 5' flap intermediates in DNA, synergistically with FEN1 during mtDNA replication and LP-BER (21). In addition, another 5' exo/endonuclease, EXOG, which localized exclusively in mitochondria, was identified as a paralog of Endonuclease G (EndoG), with nuclease activity towards ssDNA; however, its role in the maintenance of mt genome was not determined (22).

In this report, we investigated the role of the three mt 5' exo/endonucleases and have shown that EXOG but not FEN1 nor DNA2 provides the critical 5'

exonuclease activity for mt BER/SSBR. EXOG depletion causing accumulation of persistent SSBs in the mt, but not in nu genome, increases oxidative stress and induces mt dysfunction, thereby activating the intrinsic apoptotic pathway. More importantly, we have shown for a first time that persistent SSBs in the mt genome alone trigger apoptosis in mammalian cells which is suppressed in cells deficient in mtDNA. Those results underscore the importance of mt genome integrity in cell survival.

Experimental procedures

Cell Culture. The human lines HeLa, MCF7 and HCT 116 (p53+/+) were grown in DMEM and McCoy's media, respectively. A549 cells were grown in F12 medium. Respiration-deficient (A549 rho0) cells were established by maintaining A549 cells in the culture medium containing 50 ng/mL ethidium bromide for >60 population doublings. When cells became respiration-deficient, the medium was supplemented with 50 μg/mL uridine, 120 μg/mL sodium pyruvate, and 50 ng/mL ethidium bromide (23). All cell lines were obtained from the American Type Culture Collection (ATCC). The culture media were supplemented with 50 units/mL penicillin, 50 μg/mL streptomycin and 10% heat-inactivated FBS.

Preparation of Whole Cell, Nuclear and Mitochondrial Extract, Western Blot and Immunoprecipitation. Whole cell, nuclear and mitochondrial extract were prepared as described earlier (24). Protein concentrations were determined using Bradford reagent (Bio-Rad) using bovine serum albumin (BSA) as the standard. Western analysis was performed as described in (25) using following antibodies: EXOG (Sigma), GAPDH (Cell Signaling), FEN1 (Bethyl Laboratories or GeneTex, Inc.), DNA Polγ (Abcam), DNA Lig3 (QED Bioscience Inc.), PARP (Santa Cruz Biotechnology), caspase-9 (Cell Signaling), caspase-8 (Cell Signaling), caspase-3 (Cell Signaling), 56KDa subunit of mt complex V and ND-37 37KDa subunit of mt complex I (Molecular Probe-Invitrogen). FLAG-peptide immunoprecipitation was performed as described earlier (18) using FLAG M2-agarose beads incubated with ~1 mg of mitochondrial extract isolated from HeLa cells transiently transfected with human EXOG containing FLAG on C-terminus. Human EXOG cDNA was cloned into p3x-FLAG-CMV-14 vector (SIGMA) as described in (22).

Protein Depletion with siRNA. HeLa, HCT 116 or A549 cells were transfected with 40 nM siRNA specific for FEN1 (Santa Cruz Biotechnology, cat# sc-37795), DNA2 (Santa Cruz Biotechnology, cat# sc-90458), EXOG (Invitrogen, cat# ENDOGL1HSS115057) or control using Lipofectamine 2000 (Invitrogen) per the manufacturer's protocol. To optimize the siRNA concentration for maximal target depletion, preliminary transfections were carried out with 10–100 nM for each siRNA. The level of depletion was calculated by densitometric analysis of Western blots relatively to loading control of three independent experiments using Gel Logic 2200 and Molecular Imaging Software (Kodak).

RT-PCR. The mRNA depletion of individual nucleases was evaluated by RT-PCR using OneStep RT-PCR Kit (QIAGEN) per the manufacturer's recommendations and following pairs of primers were used: EXOG 5'-TTT TCT GAG CGG CTT CG T-3' (sense) and 5'-TGA TCT TTT CCA GTC TGA GCA-3 (antisense); FEN1 5'-CCT GGC CAA ACT AAT TGC TGA-3' (sense) and 5'-TCC CCT TTT AAA CTT CCC TG-3 (antisense); DNA2 5'-TCC GCT CTG CTG

TTG ACA ATA-3' (sense) and 5'-TCA GTT TAT GTT TGG CTC TGG-3 (antisense). Preliminary assays were carried out to ensure the linearity of PCR amplification with respect to number of cycles and RNA concentration.

Assessment of Apoptosis, Superoxide Anion Level and Mitochondrial Membrane Depolarization. Early apoptotic, late apoptotic and necrotic populations were characterized using the Annexin V-PE Apoptosis Detection Kit (BD Pharmingen) per the manufacturer's protocol. MitoSOX Red (Molecular Probes) was used to assess the level of superoxide anion generated in the mitochondria, according to manufacturer's recommendations. Mt membrane depolarization was analyzed using the MitoProbe JC-1 Assay Kit (Molecular Probes). In all cases, the cells were analyzed by flow cytometry (Becton-Dickinson FACSCanto at UTMB Flow Cytometry and Cell Sorting Core).

Extracellular flux (XF) analysis. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA) as described (26). Briefly, on the day before XF analysis, siRNA-transfected MCF7 cells were trypsinized and sub-cultured in an XF24 cell culture plate at 4×10^4 cells/well and incubated in 5% CO_2 incubator at 37 °C for overnight. The cells were then washed and the growth medium was replaced with unbuffered DMEM. After incubating cells for another 60 min in a 37 °C incubator without CO_2, the OCR and ECAR measurements were performed simultaneously.

Quantification of DNA Damage. Gene-specific semi-quantitative PCR assays for measuring DNA damage were performed as described earlier (27) using LongAmp Taq DNA Polymerase (New England BioLabs). Preliminary assays were carried out to ensure the linearity of PCR amplification with respect to number of cycles and DNA concentration. Damage to mtDNA was normalized to mt genome copy number determined by amplification of a 211bp fragment.

DNA Repair Synthesis Assay. The DNA repair assay was carried out as described earlier (18,28). Briefly, the 20 μL assay mixture contained 20 μM each of 4 unlabeled dNTPs, 4 μCi [α-^{32}P]dATP, mitochondrial or nuclear protein extract (5–10 μg), and duplex oligo substrate in assay buffer. After incubation at 37°C for 30 min, the products were separated from the substrates by electrophoresis in a 20% acrylamide/7 M urea gel. The radioactivity in these bands was quantitated in a PhosphorImager (Molecular Dynamics) using ImageQuant software. Preliminary enzyme assays were carried out to ensure the linearity of the reaction with respect to both time and the amount of extract. To rule out strand displacement during LP-BER, we also performed repair assay using 3'-^{32}P labeled oligonucleotide (18). The specific activity of APE was calculated as previously reported (29). Recombinant EXOG was purified and its specific activity determined using gap- and flap-specific substrates as described earlier (18,30).

Statistical Analysis. At least three independent experiments were carried out in duplicates or triplicates for each assay. The results are presented as mean ± standard error (s.e.m.) and analyzed for statistical significance with one-way ANOVA.

RESULTS

EXOG Depletion Triggers Cell Death. To evaluate the role of mt 5' exo/endonucleases, we used appropriate siRNAs to individually knock down EXOG, FEN1 and DNA2 to 10-20% of their endogenous levels at 48 h post-transfection in HeLa and HCT 116 ($p53^{+/+}$) cells. The reduction in enzyme and mRNA levels were calculated by semi-quantitative RT-PCR and densitometric analysis of Western blots (Fig.1A,B). Massive cell death was observed only after depleting EXOG, but not FEN1 nor DNA2, as assessed by plasma membrane permeability and externalization of phosphatidylserine. Such analysis discriminates early apoptotic (Annexin V-positive, 7-Amino-actinomycin D (7-AAD)-negative), from late apoptotic (Annexin V-positive, 7-AAD-positive), or necrotic (Annexin V-negative, 7-AAD-positive) cell populations (31,32). We observed significant increase in the levels of early and late apoptotic but not necrotic cell populations at 48 h post-transfection in EXOG-depleted cells for both lines, but not after depleting FEN1 nor DNA2 (Fig.1C,D, and supplemental Fig.1A,B). Only prolonged (4-day) incubation with FEN1-siRNA caused apoptosis (data not shown), presumably due to FEN1's essential role in maintaining integrity of the nu genome and thus cell survival (19). On the other hand, we did not detect any type of cell death after DNA2 depletion, even after longer (4-day) siRNA treatment (data not shown). Although mtDNA replication is independent of the cell cycle (33), we tested the effect of depletion of each of the three 5' exo/endonucleases on the level of BrdU incorporation into DNA of HCT cells stained with 7-AAD at 48 h after siRNA transfection (34). Significant increase (~5-fold) in the sub-G_1 cell population was observed only after depletion of EXOG compared to the control (supplemental Fig.2A,B), which further supports our conclusion that EXOG depletion activates cell death.

Depletion of EXOG Activates Intrinsic Apoptotic Pathway due to Mitochondrial Dysfunction. Apoptosis typically occurs via intrinsic or extrinsic pathway, which converge on activating the executioner caspases, caspase-3 and caspase-7 (35). Intrinsic apoptotic pathway usually involves loss of mt transmembrane potential and change in ROS generation (36,37). We tested how EXOG deficiency activated the apoptotic pathway at various times after siRNA transfection of HeLa cells. The EXOG level was reduced by 50% at 12 h after siRNA transfection, and by 80-90% after 36-48 h (Fig.2A). This was accompanied by significant decrease in the procaspase-9 level at 36 and 48 h post transfection, together with activation of caspase-3 but not of caspase-8 (Fig.2A). In addition, the 24KDa PARP cleavage product, an apoptosis hallmark (38,39), was detected at 36 and 48 h after EXOG depletion (Fig.2A). Increase in early apoptotic cells could be observed at 24 h after EXOG-siRNA transfection, and late apoptosis at 36 h (Fig.2B). The lack of off-target effect in activation of caspase-3 was tested by co-expression siRNA resistant EXOG which prevented caspase-3 activation (not shown). Depolarization of the mt membrane potential significantly increased at 36 and 48 h after transfection compared to control after depletion of EXOG but not of FEN1 nor DNA2 (supplemental Fig.3B,C,D). Moreover, superoxide generation, presumably due to the loss of mt membrane potential (40), was observed only in EXOG- but not FEN1- nor DNA2-depleted cells (Fig.2C and supplemental Fig.4A,B). These results indicate that EXOG depletion causes a loss of mt functions which in turn activates the intrinsic apoptotic pathway.

In order to test whether EXOG-depletion affects mt function, we knocked down EXOG in MCF7 cells and studied oxygen consumption using a Seahorse Flux analyzer. This instrument simultaneously measures the oxygen consumption rate (OCR), a measure of OXPHOS and extracellular acidification rate (ECAR) a measure of lactate production via glycolysis (26). MCF7 breast cancer cells were chosen for these experiments because of their caspase-3 deficiency (41,42) and therefore would be expected not to undergo apoptosis following loss of EXOG. Apoptosis could alter oxidative phosphorylation and might obscure any direct effects of EXOG-depletion. EXOG knockdown in MCF7 cells reduced basal OCR and ATP-linked OCR by over 2-fold (Fig.2D and supplemental Fig.5). Surprisingly, these cells did not show compensatory increase in glycolysis. These data indicate that loss of EXOG causes decline in OXPHOS capacity, and therefore overall bioenergetics in the cell.

EXOG Depletion Decreases Mt Genome Integrity. To evaluate the contribution of EXOG, FEN1 and DNA2 to the maintenance of mt genomic integrity, we measured the level of damage in mt and nu DNA by semi-quantitative PCR of long genomic fragments (27). This assay measures integrity of both mt and nu genome, by quantifying mostly SSBs since majority of oxidatively damaged base lesions in the genomes are by-passed by DNA polymerases (8,43). The mt-specific DNA fragment in EXOG-depleted HeLa cells had 25% and 50% less amplification relative to the control at 24 and 48 h after siRNA transfection, respectively (Fig.3A, and supplemental Fig.6A). In contrast, 20% less amplification was observed for a nu-specific DNA region in EXOG-depleted cells only at 48 h after siRNA transfection (Fig.3B, and supplemental Fig.6A). The level of nu DNA damage was further investigated by Comet analysis in alkaline condition of HeLa cell DNA (44), which showed no significant increase in the tail moment at 48 h after EXOG-siRNA transfection (supplemental Fig.S7). These results indicate that EXOG depletion causes accumulation of SSBs predominantly in the mt genome. On the other hand, FEN1 depletion did not affect the integrity of the mt genome under similar conditions, while accumulation of nu DNA damage was observed (Fig.3A,B, and supplemental Fig.6B). We conclude that FEN1's primary role is to maintain integrity of the nu genome, as was also suggested earlier (18,20,45). Interestingly, DNA2 depletion had no sustained effect on the integrity of the nu genome, although an early increase in mtDNA damage was detected (at 12 h post-transfection), which appears to have been subsequently repaired (Fig.3A,B, and supplemental Fig.6C). These results suggest that DNA2 is also involved in maintaining mt genome integrity, but its deficiency is eventually compensated by EXOG or other yet unknown 5' exonuclease(s) as suggested earlier (21).

EXOG Provides Critical 5' End-processing Activity for Mt LP-BER/SSBR. The repair of SSBs containing 5' blocking, oxidized deoxyribose fragments in mtDNA would require 5' end-processing by one of the 5' exo/endonucleases, which our results so far indicate to be EXOG. To confirm that EXOG contributes most if not all of the 5' end-processing activity, we examined repair activity of mt extract for the AP analog tetrahydrofuran (THF) incorporated in a duplex oligonucleotide (18). THF is an AP site mimic whose repair is initiated by APE1 to generate 3' OH and 5' THF-phosphate; the latter is resistant to 5'AP lyase activity of pol γ. Thus its removal prior to ligation

of strand break would require a 5' exonuclease. A 52nt band signifying complete repair was observed with mt extract from control, FEN1- or DNA2-depleted cells (Fig.4A). On the other hand, a 32-34nt band, corresponding to the repair intermediate product prior to repair synthesis, was generated with the mt extract from EXOG-depleted cells (Fig.4A). This indicates that EXOG provides the critical 5' end-cleaning activity which could not be substituted with FEN1 or DNA2. Thus, EXOG deficiency would generate SSBs as incomplete repair products in the mt genome as we have documented earlier. Furthermore, this observation indicates the lack of strand displacement in our assay which does not require 5' end-processing. We performed similar *in vitro* DNA repair synthesis assays with nu extract from control and EXOG-depleted cells, and did not observe any significant difference, which further confirms the role of EXOG as the 5' end-cleaning enzyme specifically for mt SSBR (Fig.4B). In addition, we observed similar levels of APE activity, the primary 3' end-cleaning enzyme in mammalian cells in mt extract from cells in which 5' exo/endonucleases were individually depleted (Fig.4C). Based on our previous observation that mt repair proteins form a complex for performing BER/SSBR (18), we isolated EXOG immunocomplexes from mt extract and demonstrated the presence of APE1, DNA lig3 and the catalytic subunit of DNA pol γ (Fig.4D). Since our previous report indicated the presence of an unknown 5' exonuclease generating short DNA products with gap or flap DNA substrates (18), we tested EXOG-specific activity using similar gap and flap DNA substrates. Regardless of the substrate type, we consistently observed 2nt reaction products (Fig.4E,F). In addition, we performed DNA repair synthesis assay with mt extract isolated from HeLa cell transfected with control or EXOG-specific siRNA and shown that the loss of full-length repair product with EXOG-depleted extract could be restored when recombinant EXOG protein was added to the reaction mixture (Fig.4G). These results clearly established EXOG as the major 5' exonuclease for mt LP-BER.

Mt DNA-depleted Cells are Resistant to Cell Death due to EXOG Deficiency. Our studies so far indicate that the accumulation of unrepaired SSBs generated in mtDNA induces the intrinsic apoptotic pathway. To test this further, we utilized A549 cells in which the mtDNA was depleted to an undetectable level (rho0) prior to EXOG knockdown (Fig.5A). SiRNA transfection resulted in >80% reduction of EXOG polypeptide in both, parental and rho0 cell lines (Fig.5B). While EXOG depletion caused 3- and 4-fold increase in early and late apoptotic cell population in mtDNA containing normal A549 cells, there was no significant increase in the number of apoptotic cells in A549 rho0 cells compared to control-siRNA treated cells (Fig.5C,D). To confirm that apoptosis could be induced in A549 rho0 cells, we incubated these cells with 50 μM etoposide, a nuclear DNA topoisomerase II inhibitor (46) and observed significant fraction of apoptotic cells (supplemental Fig.8). In addition, depolarization of the mt membrane potential in A549 rho0 cells was not affected after EXOG-depletion in contrast to that in A549 cells (Fig.5E). These results together with those in Fig. 2, indicate that mt dysfunction due to EXOG-depletion is directly linked to mt genome integrity and thus provide the first direct evidence that persistent SSBs specifically in the mt genome activate intrinsic apoptosis.

DISCUSSION

Our results show that EXOG is an essential component of the BER/SSBR

in the mitochondria whose depletion causes accumulation of unrepaired SSBs specifically in the mt genome, triggering intrinsic apoptotic pathway. We propose that compromising the integrity of the mt genome causes mt dysfunction reflected by depolarization of mt membrane potential, OXPHOS decline and spiraling increase in ROS level that further damages mt genome as the immediate target, and subsequently the nu genome (Fig.6). Here we have also shown that EXOG forms a complex with mt repair proteins, APE1, DNA pol γ and DNA lig3, to carry out SSBR and its activity could not be compensated by other mt 5' exonucleases, in support of our previous results (18). We should point out that the timeline of cellular events due to EXOG-depletion warrants detailed analysis because some of these events could occur simultaneously.

EXOG was identified as a dimeric mt-specific enzyme, with a bona fide mitochondrial leader sequence, which in contrast to EndoG possesses 5' exonuclease activity (22,30). Moreover, while mammalian EndoG acts on single- or double-stranded DNA and RNA at similar rates, EXOG was found to prefer single-stranded DNA substrate (22). Such difference in substrate specificity suggests distinct cellular functions of EXOG and EndoG. EndoG plays a role in cell proliferation and DNA recombination (47) in addition to its well established function in cell death (32). Our results suggest a similar, critical role of EXOG in maintaining mt genome integrity as a component of the mt BER/SSBR machinery. The roles of two other two 5' exo/endonucleases, DNA2 and FEN1, mt genome repair appears to be more complex. Yeast DNA2 (yDNA2), with 5'-3' DNA-dependent helicase and ATPase activity on forked DNA, together with exonuclease activity with protruding ssDNA, was identified as an essential nuclear protein (48,49). Both DNA2 and FEN1 were proposed to be involved in maturation of Okazaki fragments during DNA replication in yeast (50). The recombinant human DNA2 (hDNA2) shows similar enzymatic activity as yDNA2 (51) and interacts with FEN1 suggesting their complementary role during nu DNA replication (52). Interestingly, in contrast to the situation in yeast, hDNA2 was initially proposed to be localized exclusively in the mitochondria (21) although its nuclear localization was subsequently established (53). Depletion of hDNA2 reduced the level of mtDNA replication intermediate (53). Interaction of hDNA2 with DNA pol γ, the only DNA polymerase in mitochondria involved in both replication and repair, further supports its important role in biogenesis of mt genome (21). It was suggested that hDNA2 together with FEN1 processes flap intermediates during mt DNA replication and repair (19).

Mouse and human cells depleted of individual DNA glycosylases do not show strong cellular effect/phenotype despite of significant accumulation of multiple oxidatively damaged bases (54-57). It thus appears that extensive accumulation of oxidative DNA base lesions, in nu or mt genomes, does not limit life span of mouse nor cause severe cellular effect (58,59). On the other hand, cells depleted from mt DNA pol γ, DNA lig3, FEN1 or APE1 are embryonic lethal in mouse or causes apoptosis in cultured cells (60-63). Thus maintaining integrity of mt and nu genomes via faithful SSBR is a prerequisite for cell survival. Here we have shown that EXOG is critical for integrity of mt genome and its depletion triggers intrinsic apoptotic pathway. More importantly, this trigger is absent in cells deficient in mtDNA. While the rho0 cells do not exist naturally, they provide a unique opportunity for studying

the role of mtDNA in cellular processes (64-66). However, how mt damage triggers cellular signaling activate the apoptotic warrants further investigation. In response to nu DNA damage, eukaryotic cells activate a kinase-based checkpoint signaling network to arrest cell cycle progression and recruit repair machinery or trigger programmed cell death or senescence if the damage is extensive (67-69). The DNA damage response network can be divided into two major protein kinase signaling branches which function through the upstream kinases, ATM and ATR which are critical initiators of the G_1/S, intra-S and G_2/M cell-cycle checkpoints through activation of their downstream effector kinases Chk2 and Chk1, respectively (69-72). Phosphorylation of Chk2 was recently shown to be significantly enhanced in cells with mtDNA damage caused by menadione treatment leading to G_2-M cell cycle arrest (73).

In conclusion, we have provided the first evidence that mt genome damage alone activates programmed cell death. Furthermore, depletion of EXOG could unravel the signaling pathways activated by mtDNA damage. EXOG could be explored as a therapeutic target to initiate tumor cell death.

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References

1. Robin, E. D., and Wong, R. (1988) *Journal of cellular physiology* **136**, 507-513
2. Miller, F. J., Rosenfeldt, F. L., Zhang, C., Linnane, A. W., and Nagley, P. (2003) *Nucleic acids research* **31**, e61
3. Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J., Staden, R., and Young, I. G. (1981) *Nature* **290**, 457-465
4. Turrens, J. F. (2003) *J Physiol* **552**, 335-344
5. Staniek, K., and Nohl, H. (2000) *Biochimica et biophysica acta* **1460**, 268-275
6. St-Pierre, J., Buckingham, J. A., Roebuck, S. J., and Brand, M. D. (2002) *The Journal of biological chemistry* **277**, 44784-44790
7. Cadenas, E., and Davies, K. J. (2000) *Free radical biology & medicine* **29**, 222-230
8. Wallace, S. S. (2002) *Free radical biology & medicine* **33**, 1-14
9. Caldecott, K. W. (2008) *Nat Rev Genet* **9**, 619-631
10. Pesole, G., Gissi, C., De Chirico, A., and Saccone, C. (1999) *Journal of molecular evolution* **48**, 427-434
11. Almeida, K. H., and Sobol, R. W. (2007) *DNA repair* **6**, 695-711
12. Fortini, P., and Dogliotti, E. (2007) *DNA repair* **6**, 398-409
13. Hegde, M. L., Hazra, T. K., and Mitra, S. (2008) *Cell Res* **18**, 27-47
14. Longley, M. J., Prasad, R., Srivastava, D. K., Wilson, S. H., and Copeland, W. C. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95**, 12244-12248
15. Liu, Y., Kao, H. I., and Bambara, R. A. (2004) *Annual review of biochemistry* **73**, 589-615
16. Pinz, K. G., and Bogenhagen, D. F. (1998) *Molecular and cellular biology* **18**, 1257-1265
17. Stierum, R. H., Dianov, G. L., and Bohr, V. A. (1999) *Nucleic acids research* **27**, 3712-3719

18. Szczesny, B., Tann, A. W., Longley, M. J., Copeland, W. C., and Mitra, S. (2008) *The Journal of biological chemistry* **283**, 26349-26356
19. Liu, P., Qian, L., Sung, J. S., de Souza-Pinto, N. C., Zheng, L., Bogenhagen, D. F., Bohr, V. A., Wilson, D. M., 3rd, Shen, B., and Demple, B. (2008) *Molecular and cellular biology* **28**, 4975-4987
20. Akbari, M., Visnes, T., Krokan, H. E., and Otterlei, M. (2008) *DNA repair* **7**, 605-616
21. Zheng, L., Zhou, M., Guo, Z., Lu, H., Qian, L., Dai, H., Qiu, J., Yakubovskaya, E., Bogenhagen, D. F., Demple, B., and Shen, B. (2008) *Molecular cell* **32**, 325-336
22. Cymerman, I. A., Chung, I., Beckmann, B. M., Bujnicki, J. M., and Meiss, G. (2008) *Nucleic acids research* **36**, 1369-1379
23. Marchetti, P., Susin, S. A., Decaudin, D., Gamen, S., Castedo, M., Hirsch, T., Zamzami, N., Naval, J., Senik, A., and Kroemer, G. (1996) *Cancer research* **56**, 2033-2038
24. Szczesny, B., Tann, A. W., and Mitra, S. (2010) *Mechanisms of ageing and development* **131**, 330-337
25. Ramana, C. V., Boldogh, I., Izumi, T., and Mitra, S. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95**, 5061-5066
26. Qian, W., and Van Houten, B. (2011) *Methods (San Diego, Calif)* **51**, 452-457
27. Santos, J. H., Meyer, J. N., Mandavilli, B. S., and Van Houten, B. (2006) *Methods in molecular biology (Clifton, N.J)* **314**, 183-199
28. Das, A., Wiederhold, L., Leppard, J. B., Kedar, P., Prasad, R., Wang, H., Boldogh, I., Karimi-Busheri, F., Weinfeld, M., Tomkinson, A. E., Wilson, S. H., Mitra, S., and Hazra, T. K. (2006) *DNA repair* **5**, 1439-1448
29. Izumi, T., and Mitra, S. (1998) *Carcinogenesis* **19**, 525-527
30. Kieper, J., Lauber, C., Gimadutdinow, O., Urbanska, A., Cymerman, I., Ghosh, M., Szczesny, B., and Meiss, G. (2010) *Protein expression and purification* **73**, 99-106
31. Patel, V. A., Longacre, A., Hsiao, K., Fan, H., Meng, F., Mitchell, J. E., Rauch, J., Ucker, D. S., and Levine, J. S. (2006) *The Journal of biological chemistry* **281**, 4663-4670
32. Buttner, S., Eisenberg, T., Carmona-Gutierrez, D., Ruli, D., Knauer, H., Ruckenstuhl, C., Sigrist, C., Wissing, S., Kollroser, M., Frohlich, K. U., Sigrist, S., and Madeo, F. (2007) *Molecular cell* **25**, 233-246
33. Falkenberg, M., Larsson, N. G., and Gustafsson, C. M. (2007) *Annual review of biochemistry* **76**, 679-699
34. Lizard, G., Miguet, C., Gueldry, S., Monier, S., and Gambert, P. (1997) *Annales de pathologie* **17**, 61-66
35. Taylor, R. C., Cullen, S. P., and Martin, S. J. (2008) *Nat Rev Mol Cell Biol* **9**, 231-241
36. Tait, S. W., and Green, D. R. (2010) *Nat Rev Mol Cell Biol* **11**, 621-632
37. Bratton, S. B., Walker, G., Srinivasula, S. M., Sun, X. M., Butterworth, M., Alnemri, E. S., and Cohen, G. M. (2001) *The EMBO journal* **20**, 998-1009
38. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., and et al. (1995) *Nature* **376**, 37-43
39. Boulares, A. H., Yakovlev, A. G., Ivanova, V., Stoica, B. A., Wang, G., Iyer, S., and Smulson, M. (1999) *The Journal of biological chemistry* **274**, 22932-22940
40. Kroemer, G., Zamzami, N., and Susin, S. A. (1997) *Immunology today* **18**, 44-51
41. Yang, X. H., Sladek, T. L., Liu, X., Butler, B. R., Froelich, C. J., and Thor, A. D. (2001) *Cancer research* **61**, 348-354

42. Yang, X. H., Edgerton, S., and Thor, A. D. (2005) *International journal of oncology* **26**, 1675-1680
43. Meyer, J. N. (2010) *Ecotoxicology (London, England)* **19**, 804-811
44. Singh, N. P., McCoy, M. T., Tice, R. R., and Schneider, E. L. (1988) *Experimental cell research* **175**, 184-191
45. Shen, B., Singh, P., Liu, R., Qiu, J., Zheng, L., Finger, L. D., and Alas, S. (2005) *Bioessays* **27**, 717-729
46. Panduri, V., Weitzman, S. A., Chandel, N. S., and Kamp, D. W. (2004) *Am J Physiol Lung Cell Mol Physiol* **286**, L1220-1227
47. Huang, K. J., Ku, C. C., and Lehman, I. R. (2006) *Proceedings of the National Academy of Sciences of the United States of America* **103**, 8995-9000
48. Budd, M. E., and Campbell, J. L. (1995) *Proceedings of the National Academy of Sciences of the United States of America* **92**, 7642-7646
49. Bae, S. H., and Seo, Y. S. (2000) *The Journal of biological chemistry* **275**, 38022-38031
50. Budd, M. E., and Campbell, J. L. (1997) *Molecular and cellular biology* **17**, 2136-2142
51. Masuda-Sasa, T., Imamura, O., and Campbell, J. L. (2006) *Nucleic acids research* **34**, 1865-1875
52. Kim, J. H., Kim, H. D., Ryu, G. H., Kim, D. H., Hurwitz, J., and Seo, Y. S. (2006) *Nucleic acids research* **34**, 1854-1864
53. Duxin, J. P., Dao, B., Martinsson, P., Rajala, N., Guittat, L., Campbell, J. L., Spelbrink, J. N., and Stewart, S. A. (2009) *Molecular and cellular biology* **29**, 4274-4282
54. Arai, T., Kelly, V. P., Minowa, O., Noda, T., and Nishimura, S. (2006) *Toxicology* **221**, 179-186
55. Nilsen, H., Rosewell, I., Robins, P., Skjelbred, C. F., Andersen, S., Slupphaug, G., Daly, G., Krokan, H. E., Lindahl, T., and Barnes, D. E. (2000) *Molecular cell* **5**, 1059-1065
56. Takao, M., Kanno, S., Shiromoto, T., Hasegawa, R., Ide, H., Ikeda, S., Sarker, A. H., Seki, S., Xing, J. Z., Le, X. C., Weinfeld, M., Kobayashi, K., Miyazaki, J., Muijtjens, M., Hoeijmakers, J. H., van der Horst, G., and Yasui, A. (2002) *The EMBO journal* **21**, 3486-3493
57. Vartanian, V., Lowell, B., Minko, I. G., Wood, T. G., Ceci, J. D., George, S., Ballinger, S. W., Corless, C. L., McCullough, A. K., and Lloyd, R. S. (2006) *Proceedings of the National Academy of Sciences of the United States of America* **103**, 1864-1869
58. Jang, Y. C., and Remmen, V. H. (2009) *Experimental gerontology* **44**, 256-260
59. Perez, V. I., Bokov, A., Van Remmen, H., Mele, J., Ran, Q., Ikeno, Y., and Richardson, A. (2009) *Biochimica et biophysica acta* **1790**, 1005-1014
60. Hance, N., Ekstrand, M. I., and Trifunovic, A. (2005) *Human molecular genetics* **14**, 1775-1783
61. Puebla-Osorio, N., Lacey, D. B., Alt, F. W., and Zhu, C. (2006) *Molecular and cellular biology* **26**, 3935-3941
62. Kucherlapati, M., Yang, K., Kuraguchi, M., Zhao, J., Lia, M., Heyer, J., Kane, M. F., Fan, K., Russell, R., Brown, A. M., Kneitz, B., Edelmann, W., Kolodner, R. D., Lipkin, M., and Kucherlapati, R. (2002) *Proceedings of the National Academy of Sciences of the United States of America* **99**, 9924-9929
63. Izumi, T., Brown, D. B., Naidu, C. V., Bhakat, K. K., Macinnes, M. A., Saito, H., Chen, D. J., and Mitra, S. (2005) *Proceedings of the National Academy of Sciences of the United States of America* **102**, 5739-5743

64. Appleby, R. D., Porteous, W. K., Hughes, G., James, A. M., Shannon, D., Wei, Y. H., and Murphy, M. P. (1999) *European journal of biochemistry / FEBS* **262**, 108-116
65. Buchet, K., and Godinot, C. (1998) *The Journal of biological chemistry* **273**, 22983-22989
66. Stuart, J. A., Hashiguchi, K., Wilson, D. M., 3rd, Copeland, W. C., Souza-Pinto, N. C., and Bohr, V. A. (2004) *Nucleic acids research* **32**, 2181-2192
67. Abraham, R. T. (2001) *Genes & development* **15**, 2177-2196
68. Harper, J. W., and Elledge, S. J. (2007) *Molecular cell* **28**, 739-745
69. Jackson, S. P., and Bartek, J. (2009) *Nature* **461**, 1071-1078
70. Bartek, J., and Lukas, J. (2003) *Cancer cell* **3**, 421-429
71. Kastan, M. B., and Bartek, J. (2004) *Nature* **432**, 316-323
72. Shiloh, Y. (2003) *Nature reviews* **3**, 155-168
73. Koczor, C. A., Shokolenko, I. N., Boyd, A. K., Balk, S. P., Wilson, G. L., and Ledoux, S. P. (2009) *The Journal of biological chemistry* **284**, 36191-36201

FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Effects of Mt 5' Exo/Endonuclease Depletion on Cell Viability. (A) The mRNA levels of EXOG, FEN1 and DNA2 as measured by semi-quantitative RT-PCR and (B) Western analysis for EXOG and FEN1 in HeLa cells at 48h after transfection. The DNA2 polypeptide was not analyzed due to lack of high quality, commercial antibodies. (C) Viability of HeLa cells after transfection with individual siRNAs was measured by staining with Annexin V and 7-AAD, followed by flow cytometry. (D) Quantification of early apoptotic, late apoptotic and necrotic populations in EXOG-, FEN1- and DNA2-depleted HeLa cells. The mean result of three independent experiments is shown.

Fig. 2. EXOG depletion Induces Mitochondrial Dysfunction and Programmed Cell Death. (A) Western analysis of EXOG depletion and caspase activation at various times after transfection of HeLa cells with EXOG-siRNA compared to control-siRNA. GAPDH was used as a loading control. Proteins separated on 12.5% SDS-PAGE were probed with antibodies as indicated. (B) Quantitation of early apoptotic, late apoptotic and necrotic cell populations in HeLa cells at 12, 24, 36 and 48 h post-transfection with EXOG siRNA. The mean result of three independent experiments is shown. (C) The level of superoxide anion was measured by MitoSOX Red staining in EXOG siRNA-transfected HeLa cells compared to control. The mean results ± s.e.m. of three independent experiments is shown. * indicates $p<0.05$ compared to control. (D)

Oxygen consumption (OCR) and extracellular acidification rate (ECAR) were measured 48 hours after EXOG-siRNA transfection of MCF7 cells as described in Experimental Procedures. The inhibitors were injected sequentially at indicated time points, A, oligomycin (1μg/ml), B, FCCP (0.3 μM), C, 2DG (100 mM) and D, rotenone (1 μM). The data are representative from two independent experiments performed in replicates of five. Basal OCR is the mean of first four time point prior to injection A. ATP-linked OCR is the basal OCR (mean of points 1-4) minus the oligomycin level of OCR (mean of points 5-7). Student's T-test was used to compare mean values, $p< 0.001$.

Fig. 3. Accumulation of DNA Damage in the Mt Genome after Depletion of Mt 5' Exonucleases in HeLa cells. Quantitation of amplified mt and nu DNA fragments for all three nucleases is shown in (A) and (B), respectively. Damage to mt genome was normalized according to mt genome copy number. The mean results ± s.e.m. of three independent experiments is shown. *indicates $p<0.05$ compared to control.

Fig. 4. EXOG Provides Critical 5' Exonuclease Activity for Mt LP-BER. DNA repair synthesis assays with mt (A) and nu (B) extract of HeLa cells after depletion of EXOG, FEN1 or DNA2 and 52nt THF-containing oligo duplex. (C) APE1 endonuclease activity was measured using ^{32}P-5' label 43nt long THF oligo. APE1's specific activity in mt extract of EXOG-, FEN1- or DNA2-depleted cells of three independent experiments is shown. Nc, represents control reaction without extract. APE1^{R}, reaction with recombinant APE1 protein. (D) The EXOG-FLAG immunocomplex isolated from HeLa cells contained mt DNA Polγ, DNA Lig3 and APE1. The 37KDa subunit of NADH dehydrogenase (ND-37), input control. Specific EXOG activity with gap (E) and flap (F) DNA substrates in comparison to FEN1. Increasing amount of recombinant proteins were used (100, 200 and 400 fmol). (G) DNA repair activity in mt extract of HeLa cells transfected with control or EXOG-specific siRNA using 52nt THF oligo duplex.substrate; 100 or 200 fmol recombinant EXOG was added to assay mixture as indicated The substrate and product were separated with 20% acrylamide/8M urea gels (for A, B, C, E, F and G). M, ^{32}P-5' labeled marker oligos.

Fig. 5. Mt Genome Deficient Cells are Resistant to Cell Death due to EXOG Deficiency. (A) The level of mtDNA in A549 rho0 cells was assayed by PCR amplification of long nu and mt-specific DNA fragments indicating undetectable level of mtDNA in A549 rho0 cells. (B) Western blot analysis showing the level of EXOG depletion in A549 and A549 rho0 cells. (C) Viability of A549 and A549 rho0 cells after EXOG-depletion was measured by staining with Annexin V and 7-AAD at 48 h after siRNA transfection. (D) Quantitation of early apoptotic and late apoptotic cells in EXOG-depleted A549 and A549 rho0 cells at 48 h after siRNA transfection (shown as fold differences). The mean results ± s.e.m. of three independent experiments is shown. * indicates $p<0.05$ compared to control. (E) Analysis of the mt membrane potential in EXOG-depleted A549 (upper panel) and A549 rho0 (lower panel) compared to control cells 48 h after siRNA transfection measured by flow-cytometry.

Fig. 6. Model for Cell Death Triggered by Accumulation of SSBs in the Mt Genome.

Figure 1

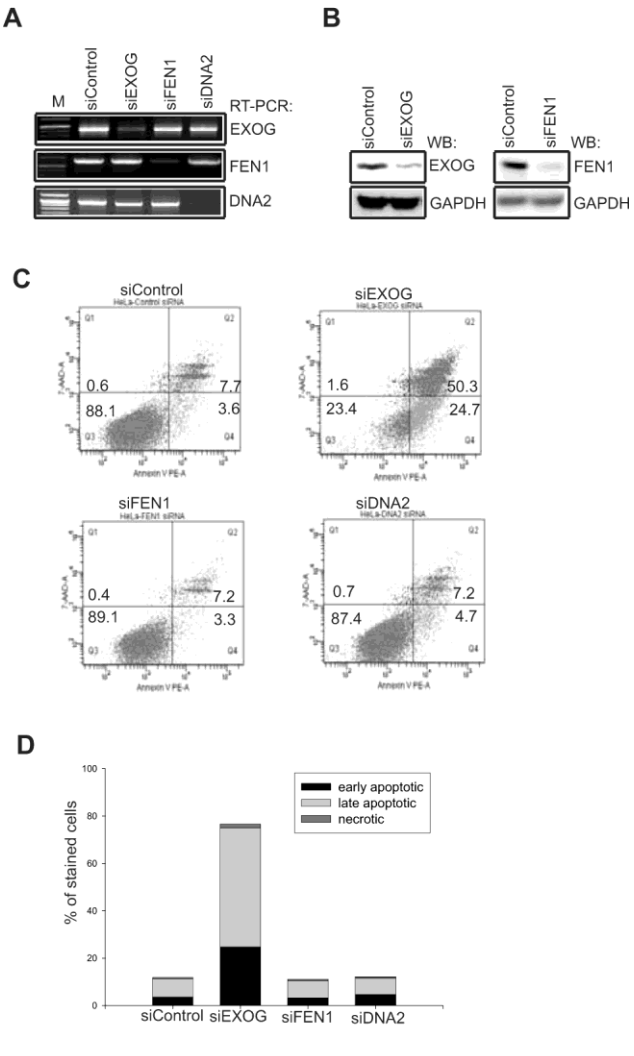


Figure 2

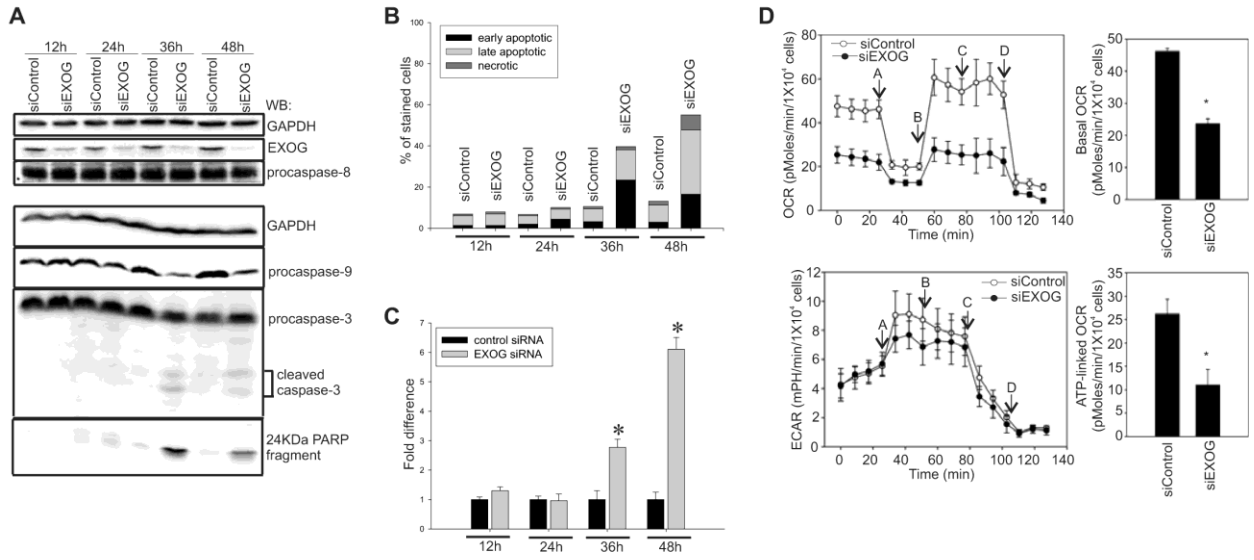


Figure 3

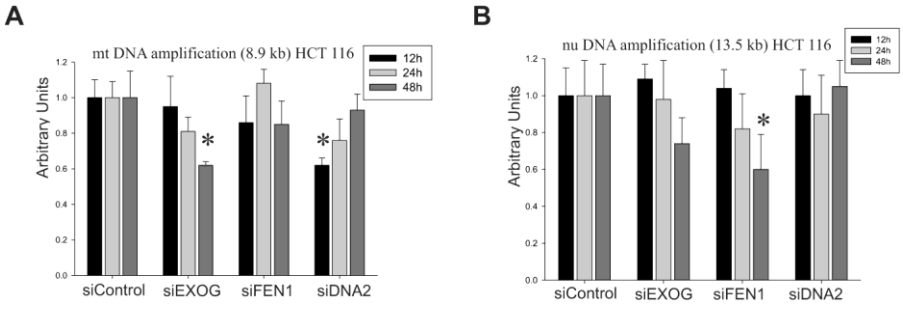


Figure 4

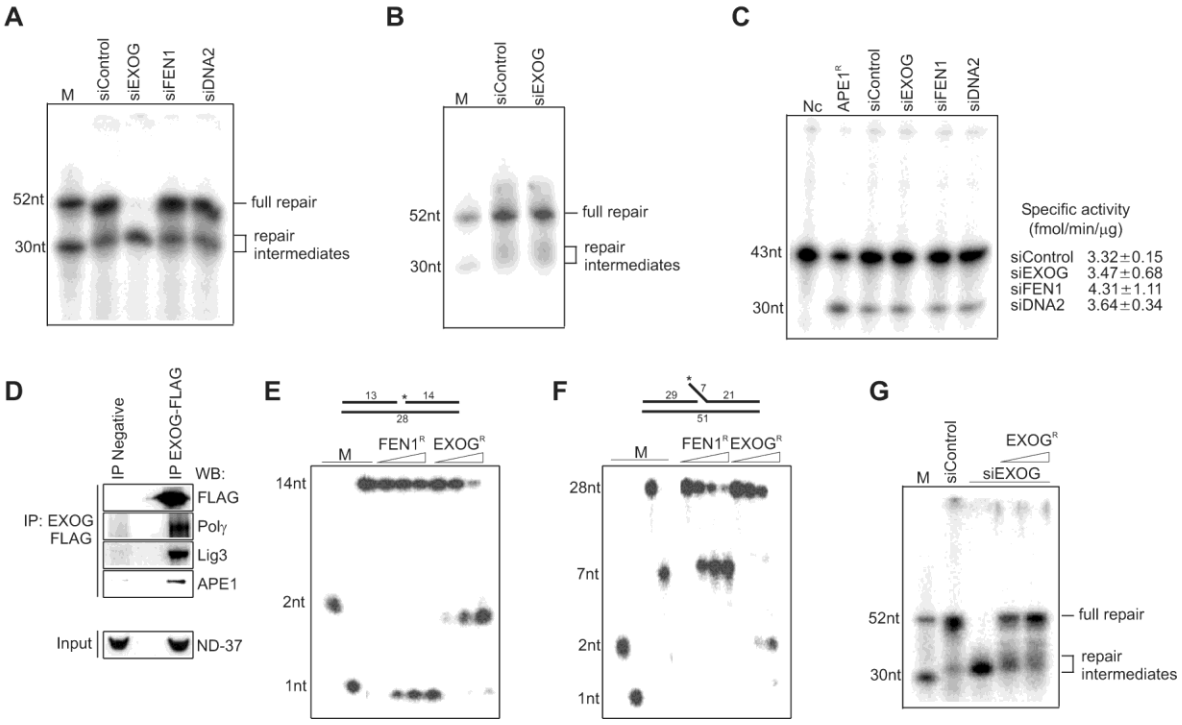


Figure 5

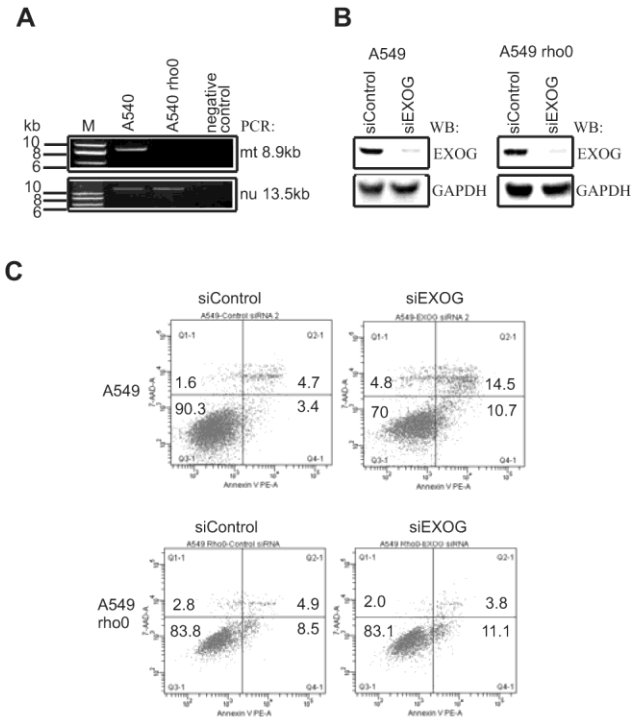
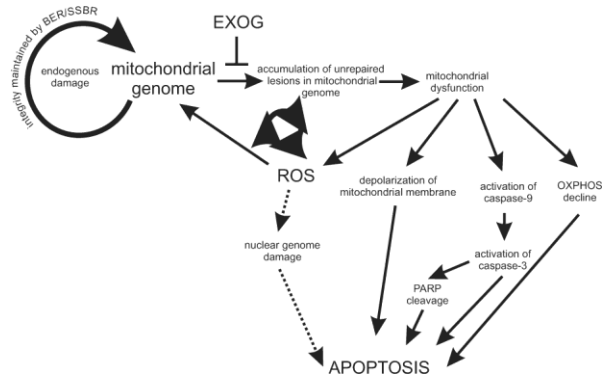


Figure 6



Apoptosis induced by persistent single-strand breaks in the mitochondrial genome: Critical role of EXOG (5′ EXO/Endonuclease) in their repair

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