The role of human DNA glycosylase NEIL2 and the single-strand break repair protein polynucleotide kinase 3'-phosphatase in maintenance of the mitochondrial genome

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Running title: Role of NEIL2 and PNKP in mitochondrial genome repair

ABSTRACT

The repair of reactive oxygen species (ROS)-induced base lesions and single-strand breaks (SSBs) in the nuclear genome via the base excision (BER) and SSB repair (SSBR) pathways, respectively, is well characterized, and important for maintaining genomic integrity. However, the role of mitochondrial (mt) BER and SSBR proteins in mt genome maintenance is not completely clear. Here we show the presence of the oxidized base-specific DNA glycosylase NEIL2 and the DNA end-processing enzyme polynucleotide kinase 3'-phosphatase (PNKP) in purified human mitochondrial extracts (ME). Confocal microscopy revealed co-localization of PNKP and NEIL2 with the mitochondria-specific protein cytochrome c oxidase subunit 2 (MT-CO2). Further, chromatin immunoprecipitation analysis showed association of NEIL2 and PNKP with the mitochondrial genes MT-CO2 and MT-CO3 (cytochrome c oxidase subunit 3); importantly, both enzymes also associated with the mitochondria-specific DNA polymerase γ (Poly). In-cell association of NEIL2 and PNKP with Poly was further confirmed by Proximity Ligation Assays. PNKP-depleted ME showed a significant decrease in both BER and SSBR activities, and PNKP was found to be the major 3'-phosphatase in human ME. Furthermore, individual depletion of NEIL2 and PNKP in the human HEK293 cells caused increased levels of oxidized bases and SSBs in the mt genome, respectively. Taken together, these studies demonstrate the critical role of NEIL2 and PNKP in maintenance of the mammalian mitochondrial genome.

INTRODUCTION

Reactive oxygen species (ROS)-induced genomic damage includes a plethora of oxidized bases, abasic (AP) sites and DNA single-strand breaks (SSBs) that are often mutagenic and are etiologically linked to various pathophysiologies, including sporadic cancer, and a multitude of age-related degenerative diseases (1,2). All of these DNA base lesions are primarily repaired by the evolutionarily conserved base excision repair (BER) pathway in both the nucleus and mitochondria (3). BER for oxidized bases is initiated by the recognition and cleavage of the base lesion from DNA by a DNA glycosylase; the resulting AP site is then cleaved by the intrinsic lyase activity of the glycosylase. In mammalian cells, five oxidized base-specific DNA glycosylases have been identified, belonging to two families based on their reaction mechanisms and structural conservation of their catalytic motifs. OGG1 and NTH1, of the Nth family, remove base lesions...
only from duplex DNA and have β-elimination activity, generating 3’-deoxyribose phosphate (3’-dRP) and 5’-phosphate (5’-P) groups at the resulting SSB (4). In contrast, the recently discovered NEILs 1-3 of the Nei family are active with duplex as well as single-stranded DNA (occurring transiently during DNA replication and transcription). Our recent studies now show that NEIL2 preferentially repairs oxidative damage from the transcribed genes, and NEIL1 primarily associates with the replication-associated proteins, thus indicating its involvement in repair during DNA replication (5,6). NEIL1 and 2 have β,δ-elimination activity, generating 3’-phosphate (3’-P) and 5’-P groups at the SSB site. In the ensuing step, the 3’-dRP and 3’-P blocking groups are removed by AP endonuclease 1 (APE1) or Polynucleotide kinase 3’-phosphatase (PNKP), respectively, in the two distinct subpathways (7-9). In the mammalian cell nucleus DNA polymerase β (Polβ) or DNA polymerase δ/ε (Polδ/ε) then fills in the gap before nick sealing by DNA ligases (LigaseIIIα/LigaseI) (10).

Besides being generated as intermediates of oxidized base repair, SSBs are also generated in the genome by oxidative stress, ionizing radiation and various chemotherapeutic agents, generating strand breaks with a variety of nonligatable “dirty” ends (11-13). Such DNA ends are among the most toxic and mutagenic lesions in mammalian genomes, because they are refractory to DNA polymerases and DNA ligases; the conventional 3’-OH and 5’-P ends must be restored for gap filling and DNA ligation to occur. Most SSBs are repaired via a SSBR pathway consisting of four basic steps: SSB detection, DNA end processing, DNA gap filling, and DNA ligation (3,14,15). A number of enzymes are available to process the SSBs, depending on the nature of the blocking groups at both the ends (14,15). Those with 3’-phosphate termini are noteworthy because they are one of the major SSBs induced by oxidative stress, and are also generated as intermediates of NEIL-mediated BER (7,8). PNKP, with dual 3’-phosphatase and 5’-kinase activities, is required for processing 3’-P and 5’-OH at strand breaks (16,17). 5’-OH is generated by some nucleases, as intermediates of topoisomerase cleavage, and also during DNA replication (16,18,19). Persistent SSBs caused by the deficiency of 5’ and/or 3’ end-cleaning enzymes may result in a severe phenotype, including cell death (15). PNKP is thus a key enzyme for processing both 3’ and 5’ termini in SSBR.

Mammalian mitochondria contain their own 16.5kb circular DNA molecule, which is subjected to continuous attack by endogenous ROS because of its proximity to the site of ROS generation via mt electron transport system complexes. Furthermore, unlike nuclear DNA mtDNA lacks protective histones, and hence is more susceptible to oxidative damage (20,21,22). Oxidative damage and SSBs in the mt genome have been implicated in various human degenerative diseases and in aging (23-25). Hence, mtDNA repair is critical for normal cellular functioning. Mitochondria have their own DNA repair systems, and repair of oxidized bases via BER has already been demonstrated for a number of cell types (26). Several mt BER proteins have recently been identified; they are either identical to those found in the nucleus or are nuclear BER protein isoforms that arise from variant RNA splicing. Among the DNA glycosylases for oxidized bases, OGG1 and NTH1 have been shown to localize in mitochondria. Bohr’s laboratory recently identified NEIL1 in mitochondria from mouse liver (27). While OGG1/NTH1-initiated BER involving APE1 has been fairly well characterized, NEIL-initiated BER in mitochondria and the protein components involved in this pathway have not been established (28). More importantly, little is known about the SSBR in mitochondria. DNA polymerase γ is the only DNA polymerase in mammalian mitochondria, and is thus essential for both mt genome replication and repair (29).

Here we provide evidence for the presence of NEIL2 and PNKP in mitochondria isolated from human cells. We also demonstrate a role of PNKP in NEIL-mediated BER of oxidized bases, as well as in mt SSBR. The accumulation of oxidized bases and SSBs in the mt genome of NEIL2- and PNKP-depleted cells, respectively, indicated these proteins’ critical roles in maintaining mt genomic integrity.

**EXPERIMENTAL PROCEDURES**
Cell culture and generation of a stable NEIL2-FLAG expressing HEK293 cells - Human embryonic kidney (HEK293) cells were grown at 37°C and 5% CO₂ in DMEM media containing 10% fetal bovine serum and 100 units/mL each of penicillin and streptomycin. HEK293 cells stably expressing NEIL2-FLAG at low levels comparable to the endogenous NEIL2 protein levels were generated as described before (5). Human neuroblastoma SH-SY5Y cells were grown in DMEM:F12 (1:1) media (30).

Depletion of PNKP and NEIL2 in HEK 293 cells- We have developed micro RNA-adapted shRNAmir constructs for targeting endogenous PNKP. Five different single-stranded 97-mer template oligonucleotides containing the human miR30 loop sequences and 5' and 3' flanking and antisense sequences targeting human PNKP transcripts were designed using the “Oligo Retriever” program as described previously (31-33). Each of the single-stranded oligonucleotides was PCR-amplified with common 5' and 3' PCR primers: (5’- CAGAAGGCTCGAGAAGGTATATTGTGACAGTGAGCG-3’ and 5’- CTAAAGTAGCCCCTTAATTCCGAGGCTAGGCA-3’). The 128bp PCR products were purified from an agarose gel, digested with XhoI and EcoRI, and then ligated with the retroviral vector LMP (Open Biosystem). Each individual shRNAmir construct for PNKP was transiently transfected, and the targeting efficiency of each clone was verified by Western blotting and real-time (RT)-PCR analyses. Targeted inactivation resulted in extensive cell death, so shRNAmir constructs that downregulated PNKP by ~60% were used to target PNKP in the experiments described here.

NEIL2 was depleted in HEK293 cells using siRNA (SIGMA; GAAUGAACCUAGAGCGGUG). Cells were harvested 48h after transfection and depletion of NEIL2 was confirmed by Western blotting.

Isolation and purification of mitochondria- Mitochondria were isolated using the Mitochondria Isolation Kit (Pierce Protein Research Product, Thermo Scientific, cat. no. 89874) per the manufacturer’s protocol with an optimized Dounce homogenization procedure. Isolated mitochondria were washed with PBS, treated with trypsin (1 mg/mL in PBS) for 15 minutes at room temperature to remove contaminating proteins adhered to the outer surface of mitochondria, then extensively washed with PBS. The washed mitochondria were lysed in 50 mM Tris, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM DTT and 1% Triton X-100. Different fractions (cytosolic, nuclear and mitochondrial) were analyzed on 10% SDS-PAGE and tested for the presence of NEIL2 (with rabbit polyclonal antibody [Ab], see ref 5) and PNKP (rabbit polyclonal Ab, gift from Dr. Michael Weinfeld), and also tested for the purity of the fractions by successive Western blotting using nucleus- (RNA Pol II, Santa Cruz Biotechnology), cytoplasm- (lactate dehydrogenase, Santa Cruz Biotechnology), and mitochondria- (70kDa subunit of complex II, C II-70kDa, Molecular Probes) specific antibodies.

Immunofluorescence- Human neuroblastoma SH-SY5Y cells were grown on microscopic coverslip and were fixed in 4% paraformaldehyde/PBS, blocked with 10% goat serum for 60 min, and incubated with primary Abs for NEIL2, PNKP (rabbit polyclonal) or mt-specific MT-CO2 (mouse monoclonal, Santa Cruz Biotechnology) at 4°C overnight. After incubation with Alexa fluor 488 (Green) or 568 (Red)-conjugated secondary Ab, the coverslips were mounted in prolong gold antifade with DAPI (Invitrogen). Images were taken using a Nikon Plan Fluor60x0.5-1.25 oil objective mounted on a Nikon Eclipse TE2000 confocal microscope equipped with a Himamatsu EM-CCD camera. The image processing was limited to contrast enhancement.

Chromatin Immunoprecipitation (ChIP) and Re-ChIP assay- Cells were cultured in DMEM medium containing 10% FBS. ChIP analysis was performed using a chromatin immunoprecipitation assay kit (Upstate Cell Signaling Solution, Millipore Temecula, CA) per the manufacturer's protocol, and re-ChIP assays were performed as described (5,34). Briefly, the cells (~10⁶ cells) were treated with formaldehyde (1% final concentration) for 10 min for cross-linking, then washed twice with...
PBS and lysed in 200 µL of 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS with a protease inhibitor mixture and sonicated to generate ~400-bp-long DNA fragments, and the supernatants diluted with 20 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.01% SDS, and protease inhibitors. The pre-cleared supernatant was then incubated overnight at 4°C with Ab to FLAG or PNKP or RNAP II (Santa Cruz Biotechnology), as indicated in Fig 3. The immunocomplexes were precipitated with salmon sperm DNA/protein G-agarose and the agarose beads washed sequentially in a low-salt wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.01% SDS), a high-salt wash buffer (same as low-salt wash buffer except containing 500 mM NaCl), LiCl wash buffer, and 20 mM Tris-HCl, pH 8.0, 1 mM EDTA buffer. The immunocomplexes were extracted from the beads with elution buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1.0% SDS, and 100 mM NaHCO3).

The re-ChIP assays were performed as described (5,34). Briefly, the eluant of the primary immunocomplex obtained with the first Ab was diluted 10-fold with dilution buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, and protease inhibitors), then subjected to further immunoprecipitation with the 2nd Ab. Immunocomplexes were again extracted from the beads with elution buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1.0% SDS, and 100 mM NaHCO3).

In Situ Proximity Ligation Assay (PLA; Duolink)- HEK293 cells were grown overnight in 16-well chamber slides, fixed with 4% paraformaldehyde, permeabilized with 0.2% tween-20, and incubated with primary Abs for NEIL2 (mouse monoclonal, Abnova) or PNKP (mouse monoclonal, gift from Dr. Michael Weinfeld) and Poly (anti-POLG1, rabbit polyclonal; Agrisera AB, Sweden) and subjected to PLA assays using the Duolink PLA kit from OLink Bioscience, performed according to the manufacturer’s instructions. The nuclei were counterstained with DAPI and the PLA signals were visualized in a fluorescence microscope at 20x magnification (Olympus).

Expression and purification of recombinant proteins- Recombinant NEIL2, PNKP, LigIIIα and Poly were purified as described previously (7,35-39) and the purified proteins were stored in 50% glycerol-containing PBS at -20°C.

Analysis of the DNA glycosylase activity of NEIL2 and the 3'-phosphatase activity of PNKP- Base excision and strand cleavage activities of DNA glycosylases in ME or using purified NEIL2 were measured using a 5'-32P-labeled 51-mer oligo with 5-OHU in an 11-nt long bubble oligo (5-OHU·B11, Table 1) in 10 µl of BER buffer containing 25 mM Hepes-KOH (pH 7.6), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 100 µg/ml bovine serum albumin (BSA) and 5% glycerol at 37°C for 15 min, followed by electrophoresis in a 20% Urea-PAGE, and the radioactive bands were analyzed in a PhosphorImager. The 3'-phosphatase activity of PNKP was assayed as we previously described (7).

DNA-trapping assay- DNA-trapping reactions were performed for 30 min at 37°C by incubating 10 µg of ME or the purified proteins with 32P-labeled bubble substrate oligo (5-OHU·B11) in a reaction mixture containing 25 mM HEPES, pH7.5; 50 mM KCl, 1 mM EDTA and 50 mM NaCNBH3. (40). The trapped complexes were separated by 12% SDS-PAGE, and the gels were dried on DE81 paper for PhosphorImager analysis of radioactivity.

Repair of an oxidized base or SSB using ME- Repair of the oxidized base lesion 5-OHU was measured in ME (from control or PNKP-depleted cells) using 2 pmol of lesion-containing duplex oligo (5-OHU·G, Table 1). The 20 µL reaction mixture also contained 1 mM ATP, 25 µM unlabelled dNTPs and 10 µM [α-32P]-dNTPs (the concentration of the corresponding cold dNTP was lowered to 15 µM, unless otherwise specified) in BER buffer (7), and the
reaction mixture was then incubated for 30 min at 37°C. For measuring repair of an SSB, we generated a circular plasmid substrate containing a single SSB with 3'-P and 5'-P at the strand break (Fig 6B). Briefly, pUC19CPD plasmid, which contains two recognition sequences for the restriction enzyme N.BstNB1 32 nt apart (41), was completely digested with N.BstNB1 (New England BioLabs). The plasmid was partially denatured by heating at 65°C for 10 min to remove the 32-nt oligo (5'-GCG GAT ATT AAT GTG ACG GTA GCG AGT CGC TC-3') and mixed with a biotinylated complementary oligo. The annealed, biotinylated 32 nt duplex was removed from the plasmid using streptavidin-agarose Dyna beads (Sigma). The resulting gapped plasmid was extracted with phenol/chloroform and ethanol-precipitated to remove the N.BstNB1. The plasmid was redissolved in TE buffer containing 50 mM NaCl, annealed with U-containing 5'-phosphorylated 32 nt oligo (5'-pGCG GAT ATT AAT GTG ACG G U A GCG AGT CGC TC-3'), then ligated using T4 DNA ligase. The covalently closed Form-I plasmid containing U was then purified by CsCl-centrifugation and converted to a 1-nt gapped plasmid with 3'-P/5'P ends (pUC19CPD-SSB) by treatment with Udg and Fpg. The SSB repair was measured using 200 ng of plasmid substrate as described for 5-OHU repair.

Analysis of oxidized bases and SSBs in the mt genome by PCR amplification assay-Mitochondrial genome-specific semi-quantitative PCR assays of long DNA fragments for measuring DNA damage were performed as described earlier (42) using LongAmp Taq DNA Polymerase (New England BioLabs) and amplifying an 8.9kb region of mt DNA. Preliminary assays were carried out to ensure the linearity of PCR amplification with respect to the number of cycles and DNA concentration. Damage to mt DNA was normalized to mt genome copy number determined by amplification of 211bp fragment using the specific primers (Table 1). Unrepaired oxidized bases in DNA from NEIL2-depleted cells were measured by digestion with Fpg/EndoIII to generate strand breaks before the PCR analysis (43).

RESULTS
The presence of NEIL2 and PNKP in mammalian mitochondria- We previously reported the unusual activity of NEIL1 and NEIL2 in excising lesions from DNA bubble structures [unlike OGG/NTH1, which are active only with duplex DNA (44)]. Interestingly, we also found a similar DNA glycosylase activity in the purified ME from HEK293 cells (Fig 1A, lane 2). To test the presence of NEILs in mitochondria, we first analyzed trapped complex formation using ME from HEK293 cells with a 5-OHU-containing bubble substrate (5-OHU.B11). These glycosylases form a transient Schiff’s base at the AP site (after excision of the base lesion) that can be trapped with NaCNBH3 or NaBH4. Fig 1B shows the formation of two distinct trapped complexes with the ME (lane 4). The presence of similarly sized trapped products in parallel trapping assays with recombinant NEIL1 (lane 2) and NEIL2 (lane 3) suggested that both NEILs are present in mitochondria. The presence of NEIL1 in mitochondria has already been reported without detailed characterization (27). To provide evidence that NEIL2 also contributes to the repair of oxidative damage in the mitochondrial genome, we compared DNA glycosylase/AP lyase activity (on 5-OHU.B11) with the ME prepared from control vs. NEIL2-depleted cells (siRNA-mediated; Fig. 1C). Fig 1D shows an ~50% decrease in activity with NEIL2-depleted compared to control ME (lane 2 vs. 3). The mitochondrial presence of NEIL2 was further confirmed by Western analysis of the ME from HEK293 cells using anti-NEIL2 Ab [Fig 1E; (5)]. We have shown previously that NEIL-initiated repair in the nucleus utilizes PNKP, not APE1, for processing the β,δ-elimination product 3'-P at the strand break (7,8). We thus postulated that PNKP should be present in the mitochondria; indeed, it was found to be present in the ME (Fig 1E, lane 3). Lane 4 contained recombinant NEIL2 and PNKP (10 ng). Quantitating the band intensities on the blots indicated that 30 µg of ME contained ~20ng of PNKP and ~4ng of NEIL2. Our data thus suggest that PNKP is a relatively abundant DNA repair protein in mitochondria. PNKP is known to be involved in multiple repair pathways.
(BER, SSBR and double-strand break repair), so its abundance may be a requirement for the cells.

One inherent challenge in studying mitochondrial proteins is the difficulty of removing nuclear and cytosolic contaminants from purified mitochondria. Some proteins originally shown to be present in the mitochondria could in fact be adventitiously associated with the mitochondrial outer membrane. However, such extraneous proteins are susceptible to trypsin, which does not degrade the matrix proteins (45,46). In this study we treated the mitochondrial pellet with trypsin before extraction in mt lysis buffer. To confirm the purity of the mitochondrial fraction, we subjected the mitochondrial, cytosolic and nuclear fractions to Western analysis using Abs specific for each (Fig 1E). Lactate dehydrogenase (LDH), specific for the cytosolic fraction, was absent in the mitochondrial and nuclear fraction. An RNA Polymerase II (RNAP II) Ab was used to check for contamination by the nuclear fraction; we observed that isolated mitochondria were free from contamination by the cytosolic or nuclear protein, and both NEIL2 and PNKP were indeed present in the ME. Finally, the identity of the mitochondrial fraction was confirmed by the presence of 70kDa subunit of complex II (C II-70kDa).

To further confirm that NEIL2 and PNKP localize to the mitochondria in human cells, we co-stained SH-SY5Y cells with the mitochondria-specific MT-CO2 Ab and Abs for NEIL2 or PNKP. Upon immunofluorescence microscopy we observed significant co-localization of NEIL2 (Fig 2A) or PNKP (Fig 2B) with MT-CO2, indicating the presence of a substantial fraction of NEIL2 and PNKP in mitochondria. Taken together, these results provide the first evidence for the presence of both NEIL2 and PNKP in mammalian mitochondria.

**NEIL2 and PNKP associate with the mitochondrial genes-** To further confirm the association of NEIL2 and PNKP with the mtDNA, we carried out chromatin immunoprecipitation (ChIP, Fig 3A) followed by a second ChIP (re-ChIP, Fig 3B) in NEIL2-FLAG expressing HEK293 cells to resolve whether the proteins were enriched on the same region of the mtDNA. We first confirmed the presence of NEIL2-FLAG in mt extract using anti-FLAG ab (SIGMA) (Supplemental Fig S1), and then immunoprecipitated the cross-linked protein-DNA complexes separately with anti-FLAG Ab and PNKP Ab, washed the IPs, eluted the bound immune-DNA complexes, and amplified the precipitated DNA by PCR using mt gene-specific (MT-CO2 and MT-CO3) primers. Amplification of these mt-specific genes was observed for both NEIL2 and PNKP (Fig 3A. i, ii). Lack of any amplification of mt genes in IP using anti-RNAP II Ab or IgG served as controls (Fig 3A. iii, upper panel). As expected, amplification of nuclear β-actin gene fragment was observed in the RNAP II IP (Fig 3A. iii, lower panel). In the re-ChIP assay, we subjected the cross-linked chromatin fraction to first IP with anti-FLAG and PNKP Ab separately and prepared them for a 2nd immunopulldown (re-ChIP) with anti-Polγ Ab or IgG. Amplification was observed for NEIL2-Polγ sequential immunoprecipitation (lane 3), but not in the case of IgG (lane 2), indicating the specific association of NEIL2 and Polγ on the mt genome (Fig 3B). Similarly, we demonstrated an association between PNKP and Polγ on the mt genome (Fig 3B, lane 5).

To further confirm the association of NEIL2 or PNKP with Polγ, we used an in situ proximity ligation assay (PLA), in which the close physical association of two proteins is visualized by a fluorescent signal (Olink Bioscience, Uppsala, Sweden). This is a relatively new technique to study interaction of endogenous proteins. In this assay, two proteins were immunostained with two primary Abs that were raised in two different host species, such as one in mouse (in this case NEIL2 and PNKP) and the other in rabbit Ab (Polγ). A species-specific 2nd Ab, each containing a short oligo (PLA probe), was then allowed to bind to the primary Ab. When the two Abs are in close proximity (<40 nm), the oligos in the PLA probes can be amplified and visualized with a fluorescent probe as distinct foci. The assay has been shown to be highly specific for physically interacting endogenous proteins in a complex (47-49). We detected fluorescent signals for both NEIL2-Polγ and PNKP-Polγ (Fig 4).
interactions between NEIL2-Polγ and PNKP-Polγ were observed in the perinuclear compartments, as expected. No signals were detected when control IgGs were used in place of specific primary Abs. Taken together, these data clearly demonstrated the co-association of NEIL2 and PNKP with Polγ on the mitochondrial genome.

**PNKP is the major 3'-phosphatase in the mitochondria-** To provide direct evidence for PNKP’s role in repairing mtDNA, we have depleted PNKP using microRNA in the HEK293 cell line, which showed a ~60% reduction in its PNKP level, as determined by Western analysis as well as by qPCR (Fig 5A, lane 2; right panel). We then examined the 3'-phosphatase activity in MEs from control and PNKP-depleted cells using an oligo substrate containing an SSB with 3'-P, as described in Experimental Procedures (Fig 5B). PNKP’s 3'-phosphatase activity would release the 3'-P, which was analyzed by separation on denaturing PAGE. Robust 3'-phosphatase activity was observed in ME from cells expressing control miRNA (Fig 5B, lanes 6-7); however, ME from PNKP-depleted cells had only a residual 20-30% 3'-phosphatase activity (lanes 4-5) compared to that from control miRNA-treated cells, clearly indicating that PNKP was the major 3'-phosphatase activity in the ME.

**PNKP is required for both BER and SSBR in mitochondria-** We next examined the role of PNKP in the repair of oxidized bases and SSBs in MEs from control and PNKP-depleted cells. Repair of 5-OHU was measured as described before by analyzing the incorporation of [α-32P]-dCMP after processing of the 3'-P by PNKP. We first reconstituted SSB repair using the recombinant mt BER components, PNKP, Polγ and LigIII (Fig 6B, lane 2). As for oxidized BER, ME from control cells showed robust SSB repair (Fig 6B, lanes 3-4), and a marked decrease in repair was observed with ME from PNKP-depleted cells (lanes 5-6). Addition of recombinant PNKP (50 and 100 fmol) restored the SSB repair in PNKP-depleted ME (lanes 7-8). Together, these data show that PNKP plays a critical role in both BER and SSBR in mitochondria.

**Evidence for the role of NEIL2 and PNKP in mt genome maintenance-** To provide direct evidence for NEIL2’s and PNKP’s role in repairing mitochondrial genomes, the relative levels of base damage in the mitochondrial genes were measured in NEIL2- and PNKP-depleted and control cells using a semi-quantitative long amplification PCR assay (42). To measure unrepaired oxidized bases in NEIL2-depleted cells (Fig 7), the isolated DNA was digested with Fpg and Nth (*E. coli*) to generate SSBs at the sites of base lesions. However, genomic DNA from PNKP-depleted cells did not need to be treated with Fpg/Nth, because these cells accumulate SSBs even without the enzymatic treatment. Fig 7 shows that there was a 37.5±6.5% decrease in PCR amplification, due to unrepaired oxidized base damage in the mitochondrial DNA of NEIL2-depleted cells. Similarly, a 40±4.2% decrease in amplified product was observed with PNKP-depleted cells, indicating a substantial increase in unrepaired SSBs in mtDNA of PNKP-depleted cells (Fig 7) compared to their respective controls. Before analysis, the DNA was normalized for mt copy number by PCR of a 211bp region of the mt genome (43). Taken together, these results strongly suggest an important role of NEIL2 and PNKP in the maintenance of mt genomic integrity.

**DISCUSSION**

PNKP was cloned and characterized many years ago (16,17); however, the functional significance of its presence in mitochondria, and more specifically its role in mt SSBR and NEIL-initiated BER have not been examined. To our
knowledge, our studies here showed for the first time the presence of NEIL2 and PNKP in mitochondria, and provided evidence for their critical roles in the repair of mt genomic damage. The mt forms of NEIL2 and PNKP identified in this study are identical to their nuclear forms. Analysis of the protein sequences of NEIL2 and PNKP showed no canonical mitochondrial-targeting sequences. However, this should not be troubling, as more than 50% of mitochondrial proteins do not use the classical import pathway that requires the recognition of a specific sequence (50). In addition, many nuclear proteins (for example, NF-kB, p53, BRCA1 and AP-1 etc.) have been detected in mitochondria despite lacking canonical mitochondrial-targeting sequences, indicating the existence of still unknown mechanisms of intracellular trafficking (51-55).

It is important to note that there is a general skepticism about unequivocally establishing the presence of a protein in mitochondria, because it is hard to remove protein contaminants associated with the mt outer membrane from the preparation, even after repeated banding by equilibrium centrifugation (56). Minimal extra-mitochondrial contamination was achieved in this study by treating the fractionated intact mitochondria with trypsin, followed by its inactivation with trypsin inhibitor (28,46). The resulting mitochondrial preparations were meticulously tested for nuclear and cytoplasmic contamination using Abs specific for proteins exclusively present in these compartments (Fig 1E).

DNA damage repair is a highly regulated and multistep process, and the proteins involved in the pathway act in concert (57). To understand this co-ordinated series of events, it is thus important to identify the interacting proteins and their functional association to form a repair complex. We have previously identified such a BER complex in the nucleus, involving NEIL2, PNKP, Polβ, and LigIIIα (8). In this study we have shown that NEIL2 and PNKP are in close proximity with the mitochondrial Polγ. Furthermore, PNKP, Polγ and LigIII are the minimal protein components required for mitochondrial SSBR, as determined by in vitro reconstitution of complete SSBR with purified proteins (Fig 6B). Notably, depletion of NEIL2 or PNKP caused an ~40% increase in endogenous DNA damage accumulation, even though the level of the proteins (NEIL or PNKP) was depleted by only 60-70%. These data suggest that inactivation or complete depletion of either NEIL2 or PNKP would cause severe damage to the mitochondrial genome, and hence strongly implicate these proteins in mt genomic maintenance.

To examine PNKP’s role in repair of the mt genome, we used PNKP-depleted ME (by ~60%) to demonstrate that PNKP is the major 3'-phosphatase in mitochondria (Fig 5B). This is an important finding, because 3'-P is one of the major blocked ends, as we have discussed previously. The PNKP-depleted ME also showed significantly less efficient total BER and SSBR (Fig 6), thus highlighting the role of PNKP in both NEIL-mediated BER and SSBR in mitochondria. The importance of SSBR is demonstrated by the observation that two of the proteins involved in this pathway, aprataxin and tyrosyl-DNA phosphodiesterase 1 (TDP1), are mutated in hereditary neurodegenerative diseases (58-60). Recent reports of the presence of TDP1 and aprataxin in mitochondria further underscore the importance of end-processing activity in mt genome maintenance (53,61). It has recently been shown that mutation in or lower levels of PNKP cause an autosomal recessive disease [MCSZ, (62)] characterized by microcephaly, intractable seizures, and developmental delay. Whether mt genomic damage contributes to the pathogenesis of MCSZ should be an important area for future studies.

Thus, understanding how mt proteins repair oxidized bases and SSBs is critically important, because any decrease of repair capacity leading to the accumulation of mt genomic damage could contribute to the onset of various diseases and/or pathologies.
FOOTNOTES

The article contains one supplemental figure and figure legend.

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Key words: Base excision repair, Mitochondria, NEIL2, Oxidative DNA damage, PNKP, Single-strand break repair.

Abbreviations: Ab, antibody; APE1, apurinic/apyrimidinic (AP) endonuclease1; BER, base excision repair; ChIP, Chromatin immunoprecipitation; 3'-dRP, 3'-deoxyribose phosphate; ME, mitochondrial extract; mt, mitochondrial; MT-CO2, mitochondrial cytochrome c oxidase subunit 2; MT-CO3, mitochondrial cytochrome c oxidase subunit 3; NTH1, endonuclease three homolog 1; OGG1, 8-oxoguanine DNA glycosylase 1, NEIL, Nei-like; PLA, proximity ligation assay; 3'-P, 3'-phosphate; 5'-P, 5'-phosphate; PNKP: polynucleotide kinase 3'-phosphatase; Pol, DNA polymerase; ROS, reactive oxygen species; SSB, single-strand-break; SSBR, single-strand-break repair.
FIGURE LEGENDS

Fig 1. Identification of NEIL2 and PNKP in mitochondria. A. A 5' 32P-labeled 51-mer oligo (5-OHU-B11, Table 1A) was used for DNA glycosylase/AP lyase assay with purified ME (10 µg, lane 2). Lane 1, no protein; lane 3, purified NEIL2 (20 fmol) as a positive control. B. Trapping assay of purified NEIL1 (20 fmol, lane 2), NEIL2 (lane 3) and ME (10 µg, lane 4) with 5' 32P-labeled 5-OHU.B11 oligo. Trapped complexes of the NEILs and free DNA are indicated. C. Western analysis of NEIL2 depletion in HEK293 cell extracts (10 µg) by NEIL2-specific siRNA; tubulin as loading control. D. DNA glycosylase/AP lyase activity (with 5' 32P-labeled 5-OHU.B11) in the ME (5 µg) prepared from control (lane 2) vs. NEIL2-depleted (lane 3) cells. E. Western blot analysis of cytosolic, nuclear and mt fractions from HEK293 cells. Abs specific for RNAP II, LDH and the 70kDa subunit of the electron transport chain complex II (C II-70kDa) were used as nuclear, cytosolic and mt markers, respectively, to show the purity of the mt preparation. Cytosolic, nuclear and mitochondrial fractions were loaded in equal amounts (30 µg). Purified NEIL2 and PNKP (10 ng) were used as reference.

Fig 2. Colocalization of NEIL2 and PNKP in the mitochondria. Mitochondrial presence of NEIL2 (A) and PNKP (B) was investigated in human neuroblastoma SH-SY5Y cells using Abs for NEIL2 (rabbit polyclonal, see ref 5), PNKP (rabbit polyclonal) and mitochondria-specific MT-CO2 (mouse monoclonal, Santa Cruz Biotechnology), the nuclei are counterstained with DAPI (Blue). Red: NEIL2 and PNKP; Green: Mitochondria; and Yellow: overlap of Red and Green showing significant co-localization of NEIL2 and PNKP with MT-CO2. Other details are in the Experimental Procedure section.

Fig 3. A. ChIP assay. After sonication and immunoprecipitation of cross-linked chromatin separately with Ab for FLAG (i) or PNKP (ii) or RNAP II (iii), the IPs were washed, the bound protein-DNA complexes eluted, and the precipitated DNA amplified by PCR using mitochondrial (MT-CO2 and MT-CO3) or nuclear (β-actin) gene-specific primers. B. Re-chIP assay. The bound fractions from the 1st IP were eluted, divided into two aliquots, and subjected to a 2nd IP with IgG (as control) or with a specific Ab. PCR amplifications were performed using the specific primers as shown in Table 1B.

Fig 4. Detection of NEIL2 and PNKP (mouse Ab) interaction with Polγ (rabbit Ab) in HEK293 cells by Proximity Ligation Assays. Upper panel, PNKP (mouse monoclonal, gift from Dr. Michael Weinfeld) with Polγ (anti-POLG1; Agrisera AB, Sweden) or IgG (rabbit Ab); lower panel, NEIL2 (mouse monoclonal, Abnova) with Polγ (rabbit Ab) or IgG (rabbit Ab).

Fig 5. Representative gel showing the 3'-phosphatase activity of PNKP in mitochondria. The substrate for PNKP’s 3'-phosphatase assays was generated as we have described previously (7) A. Western analysis of PNKP in control (lane 1) and PNKP-specific miRNA-expressing cells (lane 2). Upper panel, RNAP II as control. PNKP transcript levels in control vs. miRNA treated cells were quantitated by qPCR (right panel). B. A 32P-labeled 3′ phosphate-containing oligo (0.5 pmol) was used to measure the 3′-phosphatase activity of PNKP in MEs (5, 10 µg) prepared from control miRNA-expressing (lanes 6, 7) and PNKP-depleted cells (lanes 4, 5). Lane 1, substrate alone; lanes 2, 3; purified PNKP (50 and 100 fmol) as positive control. S, 32P-labeled 3′-P-containing oligo substrate; P, released phosphate. The histogram show quantitation of percent (%) product (32Pi) released.

Values for these histograms and those in all other figures represent the mean from at least three independent experiments. The radioactive bands were quantitated using Quantity One Software from Bio-Rad. Standard error of the mean were calculated using Microsoft Excel 7.0.

Fig 6. Representative gel showing BER and SSBR using mt extract. A. Efficient repair of 5-OHU lesion in a duplex oligo (top), measured by incorporation of [α-32P]-dCMP after excision of 5-OHU. Lane 1, no protein; lanes 2-3, ME from control cells (5 and 10µg); lanes 4-5, PNKP-depleted ME (5 and 10µg). Addition of purified PNKP (50 and 100 fmol) restored efficient repair (Lanes 6-7). Lane 8, size markers.
Histogram (bottom) represents quantitation of the repair products, with lane 2 arbitrarily set as 1. **B.** A plasmid DNA containing a single U was treated with Udg/Fpg to generate 3'-P and 5'P with a one nucleotide gap to assess PNKP’s 3'-phosphatase activity. Repair was then monitored by incorporation of $[\alpha-^{32}P]$-dCMP in the presence of Polγ and LigIIIα. The repaired product was digested with N.BsrNI, and analyzed by denaturing gel electrophoresis. Other details are provided in Experimental Procedures. Lane 1, purified (50 fmol) Polγ and Lig IIIα; lane 2, reconstitution of SSBR using purified PNKP, Polγ and LigIII (50 fmol each). Lanes 3-4, ME (5 and 10 µg); lanes 5-6, PNKP-depleted ME (5 and 10µg); lanes 7-8, PNKP-depleted ME plus purified PNKP (50 and 100 fmol). Lane 9, size marker. Quantitation of the radioactive bands (lanes 3-8) is represented in a histogram (bottom) with lane 3 arbitrarily set as 1. A schematic representation of generating a 3'-P-containing plasmid DNA substrate is shown (top).

**Fig 7.** Quantitation of mitochondrial DNA damage in NEIL2- and PNKP-depleted cells. Upper panel: Representative gel showing PCR-amplified fragments of the long amplicon (8.9kb region) and 211 bp region. The relative levels of endogenous DNA damage were calculated by quantitating the long amplicon (8.9kb region) PCR product after normalizing for mt copy number by PCR of a 211bp region of mt genome. Bottom panel: Quantitation of the amplified products is represented in histograms with the amplicon from control siRNA- or miRNA-treated cells arbitrarily set as 100. The DNA from NEIL2-depleted cells was digested with Fpg/EndoIII before analysis. Other details are provided in the Experimental Procedures. *, p<0.01.
Table 1. DNA substrate and primer sequences used in the study

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<tr>
<th>A. DNA substrate sequence</th>
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<td><strong>5-OHU-containing bubble (X:5-OHU)</strong></td>
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<tr>
<td>![Image of DNA sequence]</td>
</tr>
<tr>
<td><strong>5-OHU-containing duplex (X:5-OHU)</strong></td>
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<td><strong>Primer-template substrate for DNA polymerase activity</strong></td>
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<td>5'-GCTAGCTTGGAATCGTATCATGTACAC TCGTG TGCCGTG TAGACCGTGCC-3'</td>
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<td>R: 5'-GAATGATTCAACAAAATGGCGGTAAT-3'</td>
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<th>C. Primer sequences used for PCR amplification assay</th>
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<td><strong>MT-8.9kb</strong></td>
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<td>F: 5'-TCTAAGCCTCCTTTATCGAGCCGA-3'</td>
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<td>F: 5'-CCCGACAAAAACCCATTACTAAACCCCA-3'</td>
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<td>R: 5'-CCCGACAAAAACCCATTATTAACCCCA-3'</td>
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REFERENCES


Fig 1

A. 5'-[32P]-OHU

B. No Enzyme

C. Control siRNA

D. ME

E. Nuclear, Cytoplasmic, Mitochondrial, NEIL2 & PNKP (pure)
Fig 2

A.  NEIL2  MT-CO2  Merge  Merge+DAPI

B.  PNKP  MT-CO2  Merge  Merge+DAPI
Fig 3

A. ChIP assay

(i) Input IgG NEIL2

(ii) Input IgG PNKP

(iii) Input IgG RNAPII

B. Re-ChIP assay

1stIP NEIL2 NEIL2 PNKP PNKP

2ndIP Input IgG Pol IgG Pol

MT-CO2 MT-CO3

MT-CO2 β-actin
Fig 4

NEIL2 (Mouse)  PNKP (Mouse)

Poly (Rabbit)  IgG (Rabbit)
Fig 6

A.

5'-GCGGATATTAA TG TGA(U)GGT AGCGAGTCGCTC-3'  
32 nt

17 nt

Udg+Fpg

PNK

3'P

OH

PNKP

B.

32P-dCMP incorporation

5'-GCGGATATTAA TG TGA(U)GGT AGCGAGTCGCTC-3'

17 nt

32 nt

Udg+Fpg

PNK

3'P

OH

PNKP

B.A.

32P-dCMP incorporation

ME (Con)

ME (PNKP-depleted)

PNKP (pure)

1 2 3 4 5 6 7 8

- + ++ - - - -

- - + ++ ++ ++

- - - - - + ++

Marker

Repaired product

Unligated products

-51nt

-25nt

Fold change

(repaired product)

1 2 3 4 5 6 7

A.

B.

Repaired product

Unligated products

-32nt

-17nt

Fold change

(repaired product)

1 2 3 4 5 6 7 8

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Fig 7

<table>
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<th>Control miRNA</th>
<th>PNKP miRNA</th>
<th>Control siRNA</th>
<th>NEIL2 siRNA</th>
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<td>3</td>
<td>4</td>
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8.9 kb

211 bp

PCR amplification of mt genome (8.9kb)

Control miRNA  PNKP miRNA

Control siRNA  NEIL2 siRNA

*
The role of human DNA glycosylase NEIL2 and the single-strand break repair protein polynucleotide kinase 3' phosphatase in maintenance of the mitochondrial genome
Santi M. Mandal, Muralidhar L. Hegde, Arpita Chatterjee, Pavana M. Hegde, Bartosz Szczesny, Dibyendu Banerjee, Istvan Boldogh, Rui Gao, Maria Falkenberg, Claes Gustafsson, Partha S. Sarkar and Tapas K. Hazra

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