The repair of reactive oxygen species-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 Nei-like 2 (NEIL2) and the DNA end-processing enzyme polynucleotide kinase 3'-phosphatase (PNKP) in purified human mitochondrial extracts (MEs). Confocal microscopy revealed co-localization of PNKP and NEIL2 with the mitochondrial-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 mitochondrion-specific DNA polymerase γ. In cell association of NEIL2 and PNKP with polymerase γ 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 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.

Reactive oxygen species-induced genomic damage includes a plethora of oxidized bases, apurinic/apyrimidinic (AP) sites, and DNA single strand breaks (SSBs) that are often mutagenic and are etiologically linked to various pathophysiologicals, 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; they belong 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 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). NEILs 1 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’-deoxyribose phosphate and 3’-P blocking groups are removed by AP endonuclease 1 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 (ligase IIIα/ligase I) (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, which generate 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 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 inter- mini are noteworthy because they are one of the major SSBs.

Although OGG1/NTH1-initiated BER involving AP endonuclease 1 has been fairly well characterized, NEIL-initiated 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 nuclease 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 16.5-kb circular DNA molecule, which is subjected to continuous attack by endogenous reactive oxygen species because of its proximity to the site of reactive oxygen species generation via mt electron transport system complexes. Furthermore, unlike nuclear DNA, mtDNA lacks protective histones and hence is more susceptible to oxidative damage (20–22). 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 and co-workers (27) recently identified NEIL1 in mitochondria from mouse liver. Although OGG1/NTH1-initiated BER involving AP endonuclease 1 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 for 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 the critical roles of these proteins in maintaining mt genomic integrity.

### EXPERIMENTAL PROCEDURES

#### Cell Culture and Generation of Stable NEIL2-FLAG-expressing Human Embryonic Kidney (HEK293) Cells

HEK293 cells were grown at 37 °C and 5% CO2 in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. HEK293 cells stably expressing NEIL2-FLAG at low levels comparable with the endogenous NEIL2 protein levels were generated as described before (5). Human neuroblastoma SH-SY5Y cells were grown in DMEM:F-12 (1:1) (30).

#### Depletion of PNKP and NEIL2 in HEK293 Cells

We have developed microRNA-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’-CAGAAG-GCTCGAGAAGGTATATTGCTGTTGACAGTAGGCGG-3’ and 5’-CTAAAGTGACCCTTGAATTCCGAGTAGTGAGCG-3’. The 128-bp 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 down-regulated PNKP by ~60% were used to target PNKP in the experiments described here.

NEIL2 was depleted in HEK293 cells using siRNA (Sigma; GAUUGAACCUCAGAGCGGUG). Cells were harvested 48 h 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, catalog number 89874) according to 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 min at room temperature to remove contaminating proteins adhered to the outer surface of mitochondria, and 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 by 10% SDS-PAGE, tested for the presence of NEIL2 (with rabbit polyclonal antibody (Ab); see Ref. 5) and PNKP (rabbit polyclonal Ab; a gift from Dr. Michael Weinfeld), and tested for the purity of the fractions by successive Western blotting using nuclear- (RNA Pol II, Santa Cruz Biotechnology), cytoplasm- (lactate dehydrogenase, Santa Cruz Biotechnology), and mitochondrion (70-kDa subunit of complex II, Molecular Probes)-specific antibodies.

#### Immunofluorescence

Human neuroblastoma SH-SY5Y cells were grown on microscope coverslips, fixed in 4% paraformaldehyde in PBS, blocked with 10% goat serum for 60 min, and incubated with primary Ab for NEIL2, PNKP (rabbit poly-
clonal), 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 reagent with DAPI (Invitrogen). Images were taken using a Nikon Plan Fluor 60× 0.5–1.25 oil objective mounted on a Nikon Eclipse TE2000 confocal microscope equipped with a Hamamatsu EM-CCD camera. The image processing was limited to contrast enhancement.

**Chromatin Immunoprecipitation (ChIP) and Re-ChIP Assay**—Cells were cultured in DMEM containing 10% FBS. ChIP analysis was performed using a chromatin immunoprecipitation assay kit (Upstate Cell Signaling Solutions, Millipore, Temecula, CA) according to 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; lysed in 200 µl of 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% SDS with a protease inhibitor mixture; and sonicated to generate 32 ~400-bp-long DNA fragments. The supernatants were 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 precleared supernatant was then incubated overnight at 4 °C with Ab to FLAG, PNKP, or RNA polymerase II (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% Triton-X-100, 1 mM EDTA, and 0.1% SDS); a high salt wash buffer (same as low salt wash buffer except containing 500 mM NaCl); LiCl wash buffer; and a buffer containing 20 mM Tris-HCl, pH 8.0 and 1 mM EDTA. 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% Triton X-100, and protease inhibitors) and then subjected to further immunoprecipitation with the second Ab. Immunocomplexes were again extracted from the beads with elution buffer. The cross-linking of the eluted immunocomplexes was then reversed with 500 mM NaCl at 65 °C for 4 h, and DNA was isolated from the eluates with proteinase K treatment followed by phenol extraction and ethanol precipitation. The purified DNA was subjected to PCR (30 cycles), and the PCR products were then resolved in a 2.0% agarose gel and visualized by ethidium bromide staining.

**In Situ Proximity Ligation Assay (PLA)**—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; a gift from Dr. Michael Weinfeld) and Polγ (anti-POLG1; rabbit polyclonal; Agrisera AB, Sweden) and subjected to PLAs using the Duolink PLA kit from OLink Bioscience (Uppsala, Sweden) performed according to the manufacturer’s instructions. The nuclei were counterstained with DAPI, and the PLA signals were visualized in a fluorescence microscope (Olympus) at 20× magnification.

**Expression and Purification of Recombinant Proteins**—Recombinant NEIL2, PNKP, LigIII, and Polγ were purified as described previously (7, 35–39), and the purified proteins were stored in 50% glycerol-containing PBS at −20 °C.

**Analysis of DNA Glycosylase Activity of NEIL2 and 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 containing PBS at −20 °C.

**TABLE 1**

| DNA substrate and primer sequences used in the study | F, forward; R, reverse. |
|-----------------------------------------------|------------------------|
| **A. DNA substrate sequence**                 |                        |
| 5'-OHU-containing bubble (X:5-OHU)            |                        |
| ![5'-OHU-containing bubble](image)            |                        |
| 5'-OHU-containing duplex (X:5-OHU)            |                        |
| ![5'-OHU-containing duplex](image)           |                        |
| Primer-template substrate for DNA polymerase activity |                |
| 5′-GCTTACGTTGGAATGTAGTACACCTGTAGCGAGGTCCGGAG - 25 nt | |
| **B. Primer sequences used for ChIP assay** |                        |
| MT-CO2                                        |                        |
| F: 5′-GAGAGACTATTAAGATTTGATGTTG-3′            |                        |
| R: 5′-GAATGATCACAAGAATCTGGGTAAT-3′            |                        |
| MT-CO-3                                       |                        |
| F: 5′-CTGAGCTCACCATAAGTCTAAGTAG-3′           |                        |
| R: 5′-GATGTTTGGAATGTAAAGTGAAAT-3′            |                        |
| β-actin                                       |                        |
| F: 5′-GATGACGTTGCGATCGTCT-3′                  |                        |
| R: 5′-CTTGAATCTGGTTGCGTA-3′                   |                        |
| **C. Primer sequences used for PCR amplification assay** |                        |
| MT-8.9kb                                      |                        |
| F: 5′-TCTAAGCCTTCTTATTCCAGGCCA3′              |                        |
| R: 5′-TTTCATCATGGGAGAGTTGGAGTGG3′             |                        |
| MT-211bp                                      |                        |
| F: 5′-CCCCCAAGGACCACTAACCATACACCA-3′         |                        |
| R: 5′-CCCCCAAAACGACCACTAACCATACACCA-3′       |                        |

**TABLE 1**

| DNA substrate and primer sequences used in the study | F, forward; R, reverse. |
|-----------------------------------------------|------------------------|
| **A. DNA substrate sequence**                 |                        |
| 5'-OHU-containing bubble (X:5-OHU)            |                        |
| ![5'-OHU-containing bubble](image)            |                        |
| 5'-OHU-containing duplex (X:5-OHU)            |                        |
| ![5'-OHU-containing duplex](image)           |                        |
| Primer-template substrate for DNA polymerase activity |                |
| 5′-GCTTACGTTGGAATGTAGTACACCTGTAGCGAGGTCCGGAG - 25 nt | |
| **B. Primer sequences used for ChIP assay** |                        |
| MT-CO2                                        |                        |
| F: 5′-GAGAGACTATTAAGATTTGATGTTG-3′            |                        |
| R: 5′-GAATGATCACAAGAATCTGGGTAAT-3′            |                        |
| MT-CO-3                                       |                        |
| F: 5′-CTGAGCTCACCATAAGTCTAAGTAG-3′           |                        |
| R: 5′-GATGTTTGGAATGTAAAGTGAAAT-3′            |                        |
| β-actin                                       |                        |
| F: 5′-GATGACGTTGCGATCGTCT-3′                  |                        |
| R: 5′-CTTGAATCTGGTTGCGTA-3′                   |                        |
| **C. Primer sequences used for PCR amplification assay** |                        |
| MT-8.9kb                                      |                        |
| F: 5′-TCTAAGCCTTCTTATTCCAGGCCA3′              |                        |
| R: 5′-TTTCATCATGGGAGAGTTGGAGTGG3′             |                        |
| MT-211bp                                      |                        |
| F: 5′-CCCCCAAGGACCACTAACCATACACCA-3′         |                        |
| R: 5′-CCCCCAAAACGACCACTAACCATACACCA-3′       |                        |
Role of NEIL2 and PNKP in Mitochondrial Genome Repair

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, pH 7.5, 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 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 unlabeled dNTPs, and 10 μM [α-32P]dNTPs (the concentration of the corresponding cold dNTP was lowered to 15 μM unless specified otherwise) 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 (see Fig. 6B). Briefly, pUC19CPD plasmid, which contains two recognition sequences for the restriction enzyme N.BstNBI 32 nt apart (41), was completely digested with N.BstNBI. The plasmid was partially denatured by heating at 65 °C for 10 min to remove the 32-nt oligo (5′-GGCG GAT ATT AAT GTG 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 Dynabeads (Invitrogen). The resulting gapped plasmid was extracted with phenol/chloroform and ethanol-precipitated to remove the N.BstNBI. The plasmid was circularly closed with T4 DNA ligase. The covalently closed Form-I plasmid containing a 5′-P and 3′-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 mt Genome by PCR Amplification Assay—Mitochondrial genome-specific semiquantitative 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 8.9-kb 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 a 211-bp fragment using specific primers (Table 1). Unrepaired oxidized bases in DNA from NEIL2-depleted cells were measured by digestion with Fpg/endonuclease III to generate strand breaks before PCR analysis (43).

RESULTS

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

FIGURE 1. Identification of NEIL2 and PNKP in mitochondria. A, a 5′-[32P]-labeled 51-mer oligo (5-OHU-B11; Table 1A) was used for a 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 NEIL2 (20 fmol) as a positive control. C, Western analysis of NEIL2 depletion in HEK293 cell extracts (10 μg; lane 1), Western blot analysis of cytosolic, nuclear, and mt fractions from HEK293 cells. Abs specific for RNAP II, lactate dehydrogenase (LDH), and the 70-kDa 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. D, Western blot analysis of cytosolic, nuclear, and mt fractions from HEK293 cells. Abs specific for RNAP II, lactate dehydrogenase (LDH), and the 70-kDa 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 references. S, 32P-labeled 3′-P-containing oligo substrate; P, released phosphate; siRNA-C, siRNA control.
Role of NEIL2 and PNKP in Mitochondrial Genome Repair

trapped complex formation using ME from HEK293 cells with a
5-OHU-containing bubble substrate (5-OHU-B11). These gly-
cosylases 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 mito-
chondria has already been reported without detailed charac-
terization (27). To provide evidence that NEIL2 also contrib-
utes 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 versus
NEIL2-depleted cells (siRNA-mediated; Fig. 1C). Fig. 1D
shows an ~50% decrease in activity with ME from NEIL2-de-
pleted cells compared with control (lane 2 versus lane 3). The mitochondrial presence of NEIL2 was further confirmed by
Western analysis of the ME from HEK293 cells using anti-
NEIL2 Ab (Fig. 1E and Ref. 5). We have shown previously that
NEIL-initiated repair in the nucleus utilizes PNKP, not AP
endonuclease 1, 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 recom-
binant NEIL2 and PNKP (10 ng). Quantitation of the band
intensities on the blots indicated that 30 µg of ME contained
~20 ng of PNKP and ~4 ng of NEIL2. Our data thus suggest
that PNKP is a relatively abundant DNA repair protein in mito-
chondria. 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
tried 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, specific for the cytosolic fraction,
was absent in the mitochondrial and nuclear fractions. An
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 the 70-kDa subunit of complex II.

To further confirm that NEIL2 and PNKP localize to the
mitochondria in human cells, we co-stained SH-SY5Y cells with
the mitochondrion-specific MT-CO2 Ab and Ab 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 Mitochondrial Genes**—To
further confirm the association of NEIL2 and PNKP with the
mtDNA, we carried out 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) (sup-
plemental Fig. S1), 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, panels i and ii). Lack of any amplification of
mt genes in IP using anti-RNAP II Ab or IgG served as controls
(Fig. 3A, panel iii, upper panel). As expected, amplification of
Role of NEIL2 and PNKP in Mitochondrial Genome Repair

nuclear β-actin gene fragment was observed in the RNAP II IP (Fig. 3A, panel iii, lower panel). In the re-ChIP assay, we subjected the cross-linked chromatin fraction to a first IP with anti-FLAG and PNKP Ab separately and prepared them for a second immunopulldown (re-ChIP) with anti-Pol Ab or IgG. Amplification was observed for NEIL2-Pol sequential immunoprecipitation (lane 3) but not in the case of IgG (lane 2), indicating the specific association of NEIL2 and Pol on the mt genome (Fig. 3B). Similarly, we demonstrated an association between PNKP and Pol on the mt genome (Fig. 3B, lane 5).

To further confirm the association of NEIL2 or PNKP with Pol, we used an in situ PLA in which the close physical association of two proteins is visualized by a fluorescent signal (Olink Bioscience). This is a relatively new technique to study the 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 (Pol). A species-specific second 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-Pol and PNKP-Pol in MEs from control and PNKP-depleted cells using an oligo substrate containing an SSB with 3′-32P as described under “Experimental Procedures” (Fig. 5B). The 3′-phosphatase activity of PNKP would release the 3′-32P, which was analyzed by separation by denaturing PAGE. Robust 3′-phosphatase activity was observed in ME from cells expressing control miRNA (Fig. 5B, lanes 6 and 7); however, ME from PNKP-depleted cells had only a residual 20–30% 3′-phosphatase activity (lanes 4 and 5) compared with 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—To provide direct evidence for the role of PNKP 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 quantitative reverse transcription PCR (Fig. 5A, right panel, lane 2). We then examined the 3′-phosphatase activity in MEs from control and PNKP-depleted cells using an oligo substrate containing an SSB with 3′-32P as described under “Experimental Procedures” (Fig. 5B). The 3′-phosphatase activity of PNKP would release the 3′-32P, which was analyzed by separation by denaturing PAGE. Robust 3′-phosphatase activity was observed in ME from cells expressing control miRNA (Fig. 5B, lanes 6 and 7); however, ME from PNKP-depleted cells had only a residual 20–30% 3′-phosphatase activity (lanes 4 and 5) compared with 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—To provide direct evidence for the role of PNKP 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 quantitative reverse transcription PCR (Fig. 5A, right panel, lane 2). We then examined the 3′-phosphatase activity in MEs from control and PNKP-depleted cells using an oligo substrate containing an SSB with 3′-32P as described under “Experimental Procedures” (Fig. 5B). The 3′-phosphatase activity of PNKP would release the 3′-32P, which was analyzed by separation by denaturing PAGE. Robust 3′-phosphatase activity was observed in ME from cells expressing control miRNA (Fig. 5B, lanes 6 and 7); however, ME from PNKP-depleted cells had only a residual 20–30% 3′-phosphatase activity (lanes 4 and 5) compared with 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—To provide direct evidence for the role of PNKP 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 quantitative reverse transcription PCR (Fig. 5A, right panel, lane 2). We then examined the 3′-phosphatase activity in MEs from control and PNKP-depleted cells using an oligo substrate containing an SSB with 3′-32P as described under “Experimental Procedures” (Fig. 5B). The 3′-phosphatase activity of PNKP would release the 3′-32P, which was analyzed by separation by denaturing PAGE. Robust 3′-phosphatase activity was observed in ME from cells expressing control miRNA (Fig. 5B, lanes 6 and 7); however, ME from PNKP-depleted cells had only a residual 20–30% 3′-phosphatase activity (lanes 4 and 5) compared with 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—To provide direct evidence for the role of PNKP 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 quantitative reverse transcription PCR (Fig. 5A, right panel, lane 2). We then examined the 3′-phosphatase activity in MEs from control and PNKP-depleted cells using an oligo substrate containing an SSB with 3′-32P as described under “Experimental Procedures” (Fig. 5B). The 3′-phosphatase activity of PNKP would release the 3′-32P, which was analyzed by separation by denaturing PAGE. Robust 3′-phosphatase activity was observed in ME from cells expressing control miRNA (Fig. 5B, lanes 6 and 7); however, ME from PNKP-depleted cells had only a residual 20–30% 3′-phosphatase activity (lanes 4 and 5) compared with 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—To provide direct evidence for the role of PNKP 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 quantitative reverse transcription PCR (Fig. 5A, right panel, lane 2). We then examined the 3′-phosphatase activity in MEs from control and PNKP-depleted cells using an oligo substrate containing an SSB with 3′-32P as described under “Experimental Procedures” (Fig. 5B). The 3′-phosphatase activity of PNKP would release the 3′-32P, which was analyzed by separation by denaturing PAGE. Robust 3′-phosphatase activity was observed in ME from cells expressing control miRNA (Fig. 5B, lanes 6 and 7); however, ME from PNKP-depleted cells had only a residual 20–30% 3′-phosphatase activity (lanes 4 and 5) compared with 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—To provide direct evidence for the role of PNKP 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 quantitative reverse transcription PCR (Fig. 5A, right panel, lane 2). We then examined the 3′-phosphatase activity in MEs from control and PNKP-depleted cells using an oligo substrate containing an SSB with 3′-32P as described under “Experimental Procedures” (Fig. 5B). The 3′-phosphatase activity of PNKP would release the 3′-32P, which was analyzed by separation by denaturing PAGE. Robust 3′-phosphatase activity was observed in ME from cells expressing control miRNA (Fig. 5B, lanes 6 and 7); however, ME from PNKP-depleted cells had only a residual 20–30% 3′-phosphatase activity (lanes 4 and 5) compared with 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—To provide direct evidence for the role of PNKP 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 quantitative reverse transcription PCR (Fig. 5A, right panel, lane 2). We then examined the 3′-phosphatase activity in MEs from control and PNKP-depleted cells using an oligo substrate containing an SSB with 3′-32P as described under “Experimental Procedures” (Fig. 5B). The 3′-phosphatase activity of PNKP would release the 3′-32P, which was analyzed by separation by denaturing PAGE. Robust 3′-phosphatase activity was observed in ME from cells expressing control miRNA (Fig. 5B, lanes 6 and 7); however, ME from PNKP-depleted cells had only a residual 20–30% 3′-phosphatase activity (lanes 4 and 5) compared with 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—To provide direct evidence for the role of PNKP 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 quantitative reverse transcription PCR (Fig. 5A, right panel, lane 2). We then examined the 3′-phosphatase activity in MEs from control and PNKP-depleted cells using an oligo substrate containing an SSB with 3′-32P as described under “Experimental Procedures” (Fig. 5B). The 3′-phosphatase activity of PNKP would release the 3′-32P, which was analyzed by separation by denaturing PAGE. Robust 3′-phosphatase activity was observed in ME from cells expressing control miRNA (Fig. 5B, lanes 6 and 7); however, ME from PNKP-depleted cells had only a residual 20–30% 3′-phosphatase activity (lanes 4 and 5) compared with that from control miRNA-treated cells, clearly indicating that PNKP was the major 3′-phosphatase activity in the ME.
PNKP plays a major role in repair of oxidized bases in the mitochondrial genome.

We next investigated the role of PNKP in mitochondrial SSBR using a plasmid substrate containing a single SSB with a 3’-P blocking group. SSBR repair was measured by incorporation of [α-32P]dCMP after processing of the 3’-P by PNKP. We first reconstituted SSBR repair using the recombinant mt BER components, PNKP, Poly, and LigIII (Fig. 6B, lane 2). As for oxidized BER, ME from control cells showed robust SSBR repair (Fig. 6B, lanes 3 and 4), and a marked decrease in repair was observed with ME from PNKP-depleted cells (lanes 5 and 6). Addition of recombinant PNKP (50 and 100 fmol) restored the SSBR repair in PNKP-depleted ME (lanes 7 and 8). Together, these data show that PNKP plays a critical role in both BER and SSBR in mitochondria.

Evidence for Role of NEIL2 and PNKP in mt Genome Maintenance—To provide direct evidence for the role of NEIL2 and PNKP 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 unrepair oxidized bases in NEIL2-depleted cells (Fig. 7), the isolated DNA was digested with Fpg and Nth (Escherichia 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 unrepair 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 unrepair SSBs in mtDNA of PNKP-depleted cells (Fig. 7) compared with their respective controls. Before analysis, the DNA was normalized for mt copy number by PCR of a 211-bp region of the mt genome (43). Taken together, these results strongly suggest an important role for 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-κB, p53, BRCA1, and AP-1) 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 extramitochondrial 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 coordinated 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 Pol. Furthermore, PNKP, Poly, 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 the role of PNKP 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
Role of NEIL2 and PNKP in Mitochondrial Genome Repair

proteins involved in this pathway, aprataxin and tyrosyl-DNA phosphodiesterase 1, are mutated in hereditary neurodegenerative diseases (58–60). Recent reports of the presence of tyrosyl-DNA phosphodiesterase 1 and aprataxin in mitochondria further underscore the importance of end-processing activity in mt genome maintenance (53, 61). Recently it has been shown that mutation in or lower levels of PNKP cause an autosomal recessive disease (denoted MCSZ; Ref. 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.

Acknowledgments—We acknowledge the generosity of Drs. Michael Weinfeld and John Hays for giving us PNKP Abs (both mouse monoclonal and rabbit polyclonal) and pUC19CPD plasmid, respectively, and Dr. Chandrasekha Yallampalli for allowing us to use the fluorescence microscope. We thank Dr. David Konkel for carefully editing the manuscript.

REFERENCES
1. Breen, A. P., and Murphy, J. A. (1995) Reactions of oxyl radicals with DNA. Free Radic. Biol. Med. 18, 1033–1077
2. Cadet, J., and Brannock, C. (1998) Free radicals and the pathobiology of brain dopamine systems. Neurochem. Int. 32, 117–131
3. Hegde, M. L., Hazra, T. K., and Mitra, S. (2008) Early steps in the DNA base excision/single-strand interruption repair pathway in mammalian cells. Cell Res. 18, 27–47
4. Dodson, M. L., Michaels, M. L., and Lloyd, R. S. (1994) Unified catalytic mechanism for DNA glycosylases. J. Biol. Chem. 269, 32709–32712
5. Banerjee, D., Mandal, S. M., Das, A., Hegde, M. L., Das, S., Bhakat, K. K., Boldogh, I., Sarkan, P. S., Mitra, S., and Hazra, T. K. (2011) Preferential repair of oxidized base damage in the transfected genes of mammalian cells. J. Biol. Chem. 286, 6006–6016
6. Dou, H., Theriot, C. A., Das, A., Hegde, M. L., Matsumoto, Y., Boldogh, I., Hazra, T. K., Bhakat, K. K., and Mitra, S. (2008) Interaction of the human DNA glycosylase NEIL1 with proliferating cell nuclear antigen. The potential for replication-associated repair of oxidized bases in mammalian genomes. J. Biol. Chem. 283, 3130–3140
7. Wiedheordl, L., Leppard, J. B., Kedar, P., Karimi-Busheri, F., Rasoulif-Nia, A., Weinfeld, M., Tomkinson, A. E., Izeni, T., Prasad, R., Wilson, S. H., Mitra, S., and Hazra, T. K. (2004) AP endonuclease-independent DNA base excision repair in human cells. Mol. Cell. 15, 209–220
8. Das, A., Wiedheordl, 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) NEIL2-initiated, APE-independent repair of oxidized bases in DNA: evidence for a repair complex in human cells. DNA Repair 5, 1439–1448
9. Das, S., Chattopadhyay, R., Bhakat, K. K., Boldogh, I., Kehboh, K., Prasad, R., Wilson, S. H., and Hazra, T. K. (2007) Stimulation of NEIL2-mediated oxidized base excision repair via YB-1 interaction during oxidative stress. J. Biol. Chem. 282, 28474–28484
10. Stucki, M., Pascucci, B., Parlati, E., Fortini, P., Wilson, S. H., Hübscher, U., and Dogliotti, E. (1998) Mammalian base excision repair by DNA polymerases α and ε. Oncogene 17, 835–843
11. Giloni, L., Takeshita, M., Johnson, F., Iden, C., and Grollman, A. P. (1981) Bleomycin-induced strand-scission of DNA. Mechanism of deoxyribonuclease cleavage. J. Biol. Chem. 256, 8608–8615
12. Henner, W. D., Grunberg, S. M., and Haseltine, W. A. (1983) Enzyme action at 3′ termini of ionizing radiation-induced DNA strand breaks. J. Biol. Chem. 258, 15198–15205
13. Weinfeld, M., Mani, R. S., Abdou, I., Acyrtuyo, R. D., and Glover, J. N. (2011) Tidying up loose ends: the role of polynucleotide kinase/phosphatase in DNA strand break repair. Trends Biochem. Sci. 36, 262–271
14. Caldecott, K. W. (2001) Mammalian DNA single-strand break repair: an X-rayed affair. BioEssays 23, 447–455
15. Caldecott, K. W. (2008) Single-strand break repair and genetic disease. Nat. Rev. Genet. 9, 619–631
16. Illani, A., Ramotar, D., Slack, C., Ong, C., Yang, X. M., Scherer, S. W., and Lasko, D. D. (1999) Molecular cloning of the human gene, PNKP, encoding a polynucleotide kinase 3′-phosphatase and evidence for its role in repair of DNA strand breaks caused by oxidative damage. J. Biol. Chem. 274, 24176–24186
17. Karimi-Busheri, F., Daly, G., Robins, P., Canas, B., Pappin, D. J., Spouros, J., Miller, G. G., Fakhrai, H., Davis, E. M., Le Beau, M. M., and Weinfeld, M. (1999) Molecular characterization of a human DNA kinase. J. Biol. Chem. 274, 24187–24194
18. Barry, M. A., and Eastman, A. (1993) Identification of deoxyribonucleoside II as an endonuclease involved in apoptosis. Arch. Biochem. Biophys. 300, 440–450
19. Pohjanpelto, P., and Hölttä, E. (1996) Phosphorylation of Okazaki-like DNA fragments in mammalian cells and role of polynucleotides in the processing of this DNA. EMBO J. 15, 1193–1200
20. Mandavilli, B. S., Santos, J. H., and Van Houten, B. (2002) Mitochondrial DNA repair and aging. Mutat. Res. 509, 127–151
21. Richter, C., Park, J. W., and Ames, B. N. (1988) Normal oxidative damage to mitochondrial and nuclear DNA is extensive. Proc. Natl. Acad. Sci. U.S.A. 85, 6465–6467
22. Kaneo, M., and Inoue, F. (1998) The sensitivity to DNA single strand breakage in mitochondria, but not in nuclei, of Chinese hamster V79 and variant cells correlates with their cellular sensitivity to hydrogen peroxide. Toxicol. Lett. 99, 15–22
23. Wallace, D. C. (2002) Animal models for mitochondrial disease. Methods
Role of NEIL2 and PNKP in Mitochondrial Genome Repair

Mol. Biol. 197, 3–54

24. Copeland, W. C. (2010) Understanding heterogeneous diseases in mtDNA maintenance. Methods 51, 363

25. de Souza-Pinto, N. C., Wilson, D. M., 3rd, Stovnsner, T. V., and Bohr, V. A. (2008) Mitochondrial DNA base excision repair and neurodegeneration. DNA Repair 7, 1098–1109

26. Bohr, V. A. (2002) Repair of oxidative DNA damage in nuclear and mitochondrial DNA, and some changes with aging in mammalian cells. Free Radic. Biol. Med. 32, 804–812

27. Hu, J., de Souza-Pinto, N. C., Haraguchi, K., Hogue, B. A., Jaruga, P., Greenberg, M. M., Dizdaroglu, M., and Bohr, V. A. (2005) Repair of formamidopyrimidines in DNA involves different glycosylases: role of the OGG1, NTH1, and NEIL1 enzymes. J. Biol. Chem. 280, 40544–40551

28. Chattopadhyay, R., Wiederhold, L., Szczesny, B., Boldogh, I., Hazra, T. K., Izumi, T., and Mitra, S. (2006) Identification and characterization of mitochondrial abasic (AP)-endonuclease in mammalian cells. Nucleic Acids Res. 34, 2628–2636

29. Langley, M. J., Prasad, R., Srivastava, D. K., Wilson, S. H., and Copeland, W. C. (2010) Understanding heterogeneous diseases in mtDNA maintenance. Methods 51, 363

30. Bohr, V. A. (2002) Repair of oxidative DNA damage in nuclear and mitochondrial DNA, and some changes with aging in mammalian cells. Free Radic. Biol. Med. 32, 804–812

31. Dickins, R. A., Hemann, M. T., Zilfou, J. T., Simpson, D. R., Ibarra, I., Dickins, R. A., Hemann, M. T., Zilfou, J. T., Simpson, D. R., Ibarra, I., Patrick, M. E., Adcock, P. M., Gomez, T. M., Altekruse, S. F., Holland, P. W., Cleary, M. A., Elledge, S. J., and Hannon, G. J. (2005) Second-generation shRNA libraries covering the mouse and human genomes. Proc. Natl. Acad. Sci. U.S.A. 102, 9890–9895

32. Boehr, V. A. (2002) Repair of oxidative DNA damage in nuclear and mitochondrial DNA, and some changes with aging in mammalian cells. Free Radic. Biol. Med. 32, 804–812

33. Taylor, R. M., Whitehouse, C. J., and Caldecott, K. W. (2000) The DNA damage response in mammalian cells. Nat. Genet. 24, 2423–2429

34. Scovassi, A. I. (2004) Mitochondrial poly(ADP-ribosylation): from old controversy to new perspectives. J. Biol. Chem. 279, 107–117

35. Haar, N., Zhou, M., Guo, Z., Hu, L., Qian, L., Dai, H., Qiu, J., Yakubovskaya, E., Bogenhagen, D. F., Demple, B., and Shen, B. (2008) Human DNA2 is a mitochondrial nuclease/helicase for efficient processing of DNA replication and repair intermediates. Mol. Cell 32, 325–336

36. Szczesny, B., Tann, A. W., Longley, M. J., Copeland, W. C., and Mitra, S. (2008) Long patch base excision repair in mammalian mitochondrial genomes. J. Biol. Chem. 283, 26349–26356

37. Taylor, R. M., Whitehouse, C. J., and Caldecott, K. W. (2000) The DNA damage response in mammalian cells. Nat. Genet. 24, 2423–2429

38. Poter, T., Ross, F., Samson, M., Mari, M., Hofman, P., and Peyron, J. F. (2001) IκB-α, the NF-κB inhibitory subunit, interacts with ANT, the mitochondrial ATP/ADP translocator. J. Biol. Chem. 276, 21317–21324

39. Date, H., Onodera, O., Tanaka, H., Iwabuchi, K., Uekawa, K., Igarashi, S., Iwama, H., Sekijima, Y., Kawachi, I., Takiyama, Y., Nishizawa, M., Fukushima, K., Hiroi, T., Yuasa, T., Awaya, Y., Sakai, T., Takahashi, T., Nagatomo, H., Sekijima, Y., Kawachi, I., Takiyama, Y., Nishizawa, M., Fukushima, K., Hiroi, T., Yuasa, T., Awaya, Y., Sakai, T., Takahashi, T., Nagatomo, H., Sekijima, Y., Kawachi, I., Takiyama, Y., Nishizawa, M., Fukushima, K., Hiroi, T., Yuasa, T., Awaya, Y., Sakai, T., Takahashi, T., Nagatomo, H., Sekijima, Y., Kawachi, I., Takiyama, Y., Nishizawa, M., Fukushima, K., Hiroi, T., Yuasa, T., Awaya, Y., Sakai, T., Takahashi, T., Nagatomo, H., Sekijima, Y., Kawachi, I., Takiyama, Y., Nishizawa, M.
strong, D., Mao, Y., Quiocio, F. A., Roa, B. B., Nakagawa, M., Stockton, D. W., and Lupski, J. R. (2002) Mutation of TDP1, encoding a topoisomerase I-dependent DNA damage repair enzyme, in spinocerebellar ataxia with axonal neuropathy. Nat. Genet. 32, 267–272

61. Sykora, P., Croteau, D. L., Bohr, V. A., and Wilson, D. M., 3rd. (2011) Aprataxin localizes to mitochondria and preserves mitochondrial function. Proc. Natl. Acad. Sci. U.S.A. 108, 7437–7442

62. Shen, J., Gilmore, E. C., Marshall, C. A., Haddadin, M., Reynolds, J. L., Eyaid, W., Bodell, A., Barry, B., Gleason, D., Allen, K., Ganesh, V. S., Chang, B. S., Grix, A., Hill, R. S., Topcu, M., Caldecott, K. W., Barkovich, A. J., and Walsh, C. A. (2010) Mutations in PNKP cause microcephaly, seizures and defects in DNA repair. Nat. Genet. 42, 245–249