Role of tyrosyl-DNA phosphodiesterase (TDP1) in mitochondria

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Human tyrosyl-DNA phosphodiesterase (TDP1) hydrolyzes the phosphodiester bond at a DNA 3′-end linked to a tyrosyl moiety and has been implicated in the repair of topoisomerase I (Top1)-DNA covalent complexes. TDP1 can also hydrolyze other 3′-end DNA alterations including 3′-phosphoglycolate and 3′-abasic sites, and exhibits 3′-nucleosidase activity indicating it may function as a general 3′-end-processing DNA repair enzyme. Here, using laser confocal microscopy, subcellular fractionation and biochemical analyses we demonstrate that a fraction of the TDP1 encoded by the nuclear TDP1 gene localizes to mitochondria. We also show that mitochondrial base excision repair depends on TDP1 activity and provide evidence that TDP1 is required for efficient repair of oxidative damage in mitochondrial DNA. Together, our findings provide evidence for TDP1 as a novel mitochondrial enzyme.

DNA repair | ligase III | oxidative DNA damage | topoisomerase I | mitochondrial BER

Mitochondrial DNA (mtDNA) is an essential component of eukaryotic cells because it encodes a critical subset of mitochondrial proteins for the production of cellular ATP. Each mitochondrion contains 4–6 copies of the double-stranded circular 16 kilobase long mitochondrial genome. MtDNA needs to be tightly preserved because more than 93% of the mitochondrial genome has to be accurately transcribed into 13 individual messenger RNAs coding for the essential mitochondrial proteins that are part of the mitochondrial electron transport chain (oxidative phosphorylation) (1). MtDNA also encodes 2 mitochondrial-specific ribosomal RNAs and 22 transfer RNAs that are essential for protein synthesis inside mitochondria. Mitochondria go through alternative rounds of fission and fusion to maintain mitochondrial integrity and mtDNA copy number (2). Because of the proximity of reactive oxygen species (ROS) generated by the mitochondrial oxidative phosphorylation chain, mtDNA is potentially exposed to oxidative DNA damage (3, 4). Accumulation of mtDNA damage has been involved in neurodegenerative disorders (Parkinson, Alzheimer's, and Huntington diseases), myopathies and diabetes, and has been associated with aging, cancer, and age-related degenerative disorders (5, 6).

Mitochondria are dependent on the nucleus for all the mitochondrial proteins necessary for mtDNA replication, repair, and maintenance (6, 7). Noticeably, some nuclear genes encode proteins exclusively for the mitochondria such as polymerase gamma (POLγ), mitochondrial helicase (TWINKLE), transcription factor A (TFAM) (8, 9), and vertebrate mitochondrial topoisomerase IB (Top1mt) (10, 11). Moreover, the mitochondrial genome does not encode DNA repair proteins, and is thus completely dependent on proteins encoded in the nucleus for its repair and integrity. The corresponding genes encode proteins for both nuclear and mitochondrial repair (12–14). Yet, the full spectrum of nuclear DNA repair proteins involved in mtDNA repair is not entirely known.

MtDNA repair primarily uses base excision repair (BER) and lacks nucleotide excision repair (6, 13, 15). However, mismatch repair (16) and DNA double-strand break (DSB) end joining (17–19) activities have been reported in mitochondria. Recently, several reports have focused on the existence of long-patch BER (LP-BER) by the flap endonucleases FEN-1 and DNA2 in mitochondria (13, 20–23). ROS can also generate 3′-deoxyribose residues that are oxidized, unsaturated or fragmentary (e.g., 3′-phosphoglycolates and 3′-phosphate). The DNA glycosylases with associated AP-lyase activity would generate 2,3-unsaturated deoxyribose as an indirect product of DNA oxidation (24). Abasic endonuclease 1 (APE1) is the a well characterized enzyme in mammals for the repair of 3′-phosphoglycolate esters (3′-PG) during oxidative DNA damage (25–27). Recently, APE1 has been shown to be localized both in nuclei and mitochondria (14, 28). However, the 3′-PG removal activity of APE1 is highly selective and APE1 is relatively ineffective when the 3′-PG is in single-stranded DNA, at 3′-overhangs or at blunt or recessed 3′-ends of DSBs (27, 29–32). Furthermore, APE1 is not able to remove topoisomerase-DNA complexes (33, 34), which can be trapped by the endogenous lesions generated by ROS (oxidized bases, abasic sites, and strand breaks) (13, 35–37).

Human tyrosyl-DNA phosphodiesterase (TDP1) typically hydrolyzes the phosphodiester bond between a tyrosyl moiety and a DNA 3′-end (33, 34). TDP1 was originally discovered in yeast (34) and has been implicated in the repair of stalled Top1-DNA covalent complexes (38, 39). The ability of TDP1 to resolve 3′-phosphotyrosyl linkages is consistent with its role in protecting cells against Top1-DNA lesions (40–43). Homozygous mutation of TDP1 causes spinocerebellar ataxia with axonal neuropathy (SCAN1), an autosomal recessive neurodegenerative syndrome (44). Cells from SCAN1 patients are hypersensitive to the specific Top1 poison camptothecin and accumulate elevated Top1-associated DNA breaks in response to camptothecin (39, 43, 45).

TDP1 activity is not limited to the removal of Top1 adducts. TDP1 can also process other 3′-DNA end blocking groups: 3′-abasic sites and 3′-phosphoglycolates (45–49). Accordingly, TDP1-deficient cells are deficient in the removal of 3′-phosphoglycolate and are hypersensitive to bleomycin in addition to their hypersensitivity to camptothecin (39, 41–43, 49). TDP1 also possesses a limited DNA and RNA 3′-nucleosidase activity in which a single nucleoside is removed from the 3′-hydroxyl end of the substrate (45). Thus, TDP1 may function to remove a variety of adducts from 3′-DNA ends during DNA repair (50). Yeast TDP1 has also been shown to process Top2-DNA adducts (51).

In this study, we employed Immunofluorescence staining, cellular fractionation, Western blotting analysis, and biochemical assays to demonstrate the presence of TDP1 in mitochondria and its function in repairing mtDNA.

Results

Human TDP1 Localizes to the Mitochondria. While performing immunofluorescence staining with TDP1, we noted the presence of TDP1 not only in the nuclei but also as a punctuate pattern within the cytoplasm (Fig. 1*A*). This observation was consistent with

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the recent observation that the TDP1 protein is prominently expressed in the cytoplasm of some neurons (41). The existence of cytoplasmic TDP1 raised the possibility that TDP1 localizes to the mitochondria and participates in mtDNA repair. To determine whether TDP1 localizes to the mitochondria, we costained cells with the mitochondrion-specific dye MitoTracker red. MCF7 cells were utilized for those studies because they contain a relatively large cytoplasm that allows high resolution of the mitochondria with limited interference from nuclear staining. Upon immunofluorescence, we observed significant colocalization of TDP1 with MitoTracker red (Fig. 1*A*), suggesting the presence of a substantial fraction of TDP1 in mitochondria. To test this observation, we examined the expression of exogenous TDP1-RED fusion protein under confocal microscopy. Fig. 1*B* demonstrates colocalization of the TDP1-RED fluorescence pattern with the mitochondrial matrix-targeted protein (YFP-mito). Together, these immunofluorescence data indicate that TDP1 localizes to both the nucleus and mitochondria.

We next performed cellular fractionation and Western blotting to confirm the immunofluorescence findings. Mitochondrial and nuclear fractions revealed the presence of TDP1 as a single band with similar molecular weight (69-kDa) both in the nuclear and mitochondrial fraction (Fig. 2*A*). We have previously described that siRNA knockdown of TDP1 abrogated the TDP1 signal in Western blotting, confirming the specificity of the TDP1 antibody (40). It is unlikely that the mitochondrial TDP1 signal was the result of nuclear contamination because nuclear Top1 and Lamin B, which are abundant nuclear proteins, were undetectable in the mitochondrial fraction (Fig. 2*A*). The relative purity of the mitochondrial and nuclear fractions was further assessed by analysis of COX IV and TFAM, which were only detectable in the mitochondrial fractions (Fig. 2*A*).

To further confirm that TDP1 is present inside mitochondria rather than adventitiously associated with the outer surface of the organelle, we performed proteinase K protection assays. Proteinase K treatment of purified mitochondria has often been used to assess the mitochondrial localization of a protein (22, 52). As shown in Fig. 2*B*, we observed a significant fraction of TDP1 resistant to proteinase K-treatment (Fig. 2*B*, lane 2). Furthermore, upon addition of Triton X-100 (to disrupt the mitochondrial membrane), TDP1 in the mitochondrial fraction was fully proteinase K-sensitive (Fig. 2*B*, compare lanes 2 and 3), demonstrating the mitochondrial localization of TDP1.

TDP1 Activities in Mitochondrial Extracts. The presence of TDP1 in the mitochondria prompted us to test tyrosyl-DNA-phosphodiesterase enzymatic activity in mitochondrial extracts. TDP1 activity was examined using gel-based assays (33). TDP1 catalyzes the hydrolysis of a 5′ end ^{32}P-labeled nucleopeptide substrate (14-Y) to a product with a 3′-phosphate (14-P) with increased electrophoretic mobility (Fig. 2*C–E*) (34, 46, 53). Fig. 2*D* shows TDP1 activity both in the nuclear and in the mitochondrial extracts. To demonstrate that the nuclear-encoded TDP1 gene is responsible

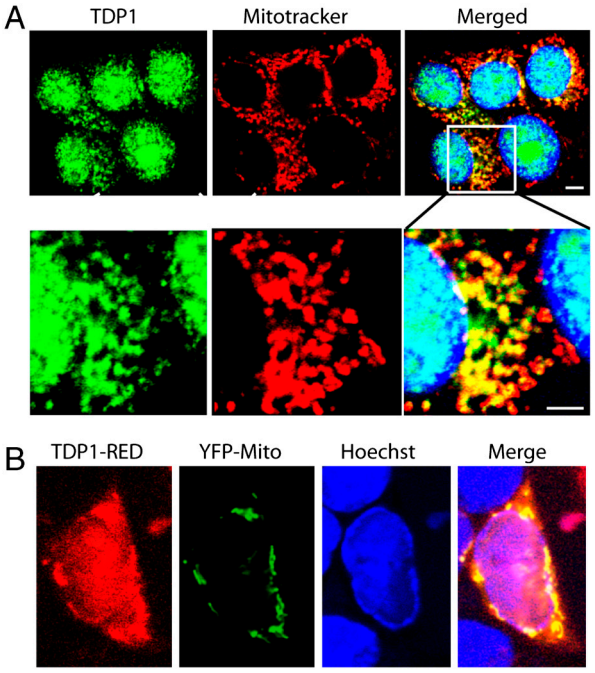

Fig. 1. TDP1 localizes to the mitochondria. (*A*) Immunofluorescence images showing colocalization of TDP1 (green) and mitochondria (labeled with MitoTracker red) in MCF-7 breast carcinoma cells. Nuclei were stained with DAPI (blue). (*Upper*) Cellular distribution of TDP1 at lower magnification. The boxed area is shown magnified below (*Lower*) to better show colocalization of TDP1 and mitochondria. (*B*) Localization of exogenous TDP1 by live cell microscopy. MCF7 cells were cotransfected with a red fluorescence construct of TDP1 (*pTDP1-DsRED*) and with mitochondrial matrix-targeted protein (*pYFP-mito*) and the fluorescence pattern was observed under confocal microscopy. Exogenous TDP1 and mito-targeted protein are shown in red and green, respectively. Nuclei were detected by Hoechst staining (blue). Colocalization of TDP1-RED and YFP-mito indicates TDP1 localization in mitochondria.

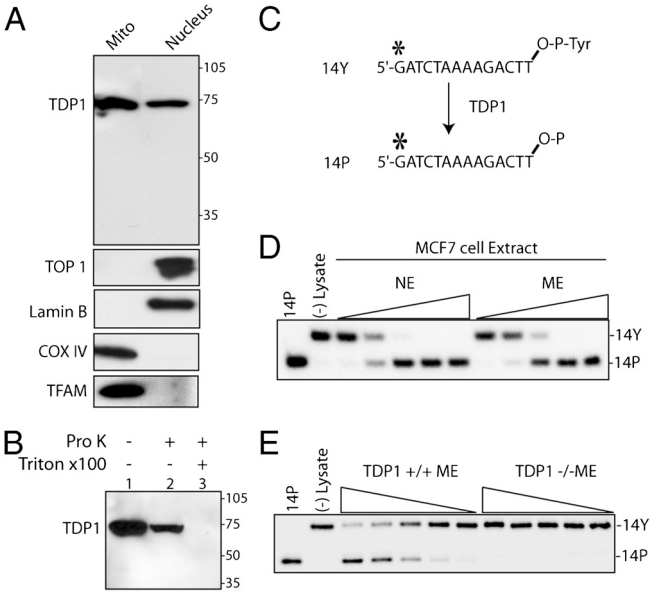

Fig. 2. TDP1 activities in mitochondrial extracts. (*A*) Western blotting of TDP1 in mitochondrial extract (ME), and nuclear extract (NE) from MCF-7 cells. Fifty micrograms of ME, and NE were resolved in SDS-PAGE. The presence of TDP1, nuclear Top1, lamin B, COX IV or TFAM was determined by Western blotting using antibodies to corresponding proteins. COX IV or TFAM were used as positive mitochondrial markers. Lamin B and nuclear Top1 were used as a nuclear markers. TDP1 is present in both NE and ME. (*B*) Protease insensitivity of mitochondrial TDP1. Isolated mitochondria from MCF7 cells were treated with proteinase K (ProK) in the presence or absence of Triton-X-100 as indicated at the top of the figure. Blots were developed using antibody against TDP1. (*C*) Schematic representation of the TDP1 biochemical assays using single-stranded oligopeptide 14Y. ^{32}P-Radiolabeling (*) was at the 5′ terminus of the oligopeptide. TDP1 catalyzes the hydrolysis of the 3′-phosphotyrosine bond and converts 14Y to an oligonucleotide with 3′-phosphate, 14P. (*D*) Representative gel showing TDP1 activity in nuclear extract (NE) and mitochondrial extract (ME) from MCF7 cells. Same extracts were used as shown in (*A*). NE and ME were adjusted to give identical protein concentrations (2 μg/μL). Serial dilutions (3-fold) were tested in TDP1 biochemical assay. (*E*) Mitochondrial tyrosyl-DNA phosphodiesterase activity is TDP1-dependent. ME were generated from TDP1+/+ and TDP1−/− MEFs cells and adjusted to give identical protein concentrations (1.5 μg/μL). Serial dilutions (3-fold) were tested in the TDP1 biochemical assay. 14P: oligonucleotide marker corresponding to the expected product that runs quicker than the corresponding tyrosyl oligonucleotide substrate (14Y) in a denaturing PAGE.

for the mitochondrial TDP1 activity, similar experiments were performed with mitochondrial extracts (ME) from TDP1 knockout (TDP1−/−) fibroblasts (41). Fig. 2*E* shows that ME from the TDP1−/− cells fails to hydrolyze the 14-Y substrate to the 14-P product, consistent with the conclusion that the TDP1 protein encoded in the nucleus is responsible for the TDP1 activity in mitochondria. Taken together, these results reveal the presence of functional TDP1 in mitochondria.

Mitochondrial Base Excision Repair Is Dependent on both TDP1 and DNA Ligase III. Because ligase III is known to interact directly with TDP1 (43) and to be present in mitochondria (12), we tested the potential role of TDP1 along with Ligase III in mitochondrial BER. To that effect, we incubated a radiolabeled double-stranded nicked DNA substrate with ME (Fig. 3). The mitochondrial extract readily converted the tyrosyl substrate to the expected 14-P product (Fig. 3*B*, lane 3; also see Fig. 2*C*). The minor

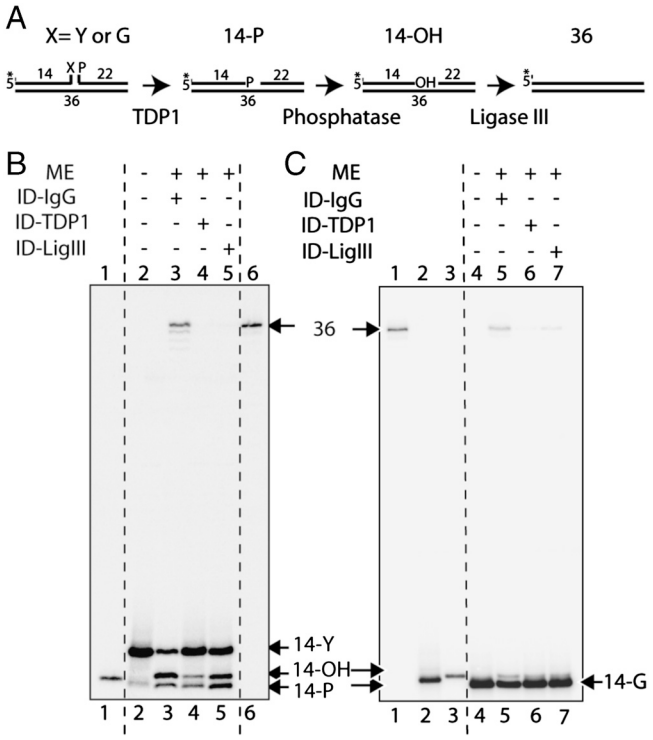

Fig. 3. Mitochondrial base excision repair is dependent on both TDP1 and DNA Ligase III. (*A*) Schematic representation of the in vitro repair assays. The nicked DNA substrate was generated by annealing the 5′-end radiolabeled 14-Y oligopeptide or 14-G oligonucleotide with a 36-mer bottom strand and a 22-mer top strand. TDP1 catalyzes the conversion of 3′-phosphotyrosine (14-Y) or 3′-phosphoglycolate (14-G) to an oligonucleotide with 3′-phosphate (14-P), which is further processed by a phosphatase to the 3′-hydroxyl product (14-OH). DNA Ligase III generates the final repair product (36). TDP1- and Ligase III-mediated repair activities in mitochondrial extract (ME) from MCF-7 cells. (*B*) Two microgram ME immunodepleted (ID) with control IgG (ID-IgG, lane 3) or TDP1 (ID-TDP1, lane 4) or Ligase III (ID-LigIII, lane 5) was incubated with the double-stranded nicked DNA substrates containing a 3′-phosphotyrosine (14-Y) (*A*, *Upper*). Lane 2 corresponds to the DNA substrates containing a 3′-phosphotyrosine (14-Y) without (–) ME, as indicated. Reactions were carried out at 25 °C for 30 min. Positions of the [γ-^{32}P]-labeled markers corresponding to repair product 36 (lane 6) and repair intermediates (14-P and 14-OH, lane 1) are indicated. *C*) Three microgram ME immunodepleted (ID) with control IgG (ID-IgG, *lane 5*) or TDP1 (ID-TDP1, lane 6) or Ligase III (ID-LigIII, lane 7) was incubated with the double-stranded nicked DNA substrates containing a 3′-phosphoglycolate (14-G). Lane 4 corresponds to the DNA substrates containing a 3′- phosphoglycolate (14-G) without ME. Reactions were carried out at 25 °C for 2 h. Positions of the [γ-^{32}P]-labeled markers corresponding to the repair product 36 (lane 1) and repair intermediates (14-P, lane 2 and 14-OH, lane 3) are indicated.

35-mer product observed in Fig. 3*B*, lane 3 corresponds to a minor ligation product resulting from ligation following a 1 base removal either from the 3′- or the 5′-end of the DNA substrate. We also observed a 3′-OH product (14-OH) resulting from hydrolysis of the 3′-phosphate (Fig. 3*B*, lane 3), and a 36-mer final repair product (Fig. 3*B*, lane 3). Immunodepletion of TDP1 largely diminished the removal of 3′-phosphotyrosyl substrate (14-Y) and also abrogated the final 36-mer-repair product (Fig. 3*B*, lane 4). Ligase III immunodepletion only abrogated the production of the final 36-mer repair product (Fig. 3*B*, lane 5), consistent with the presence of Ligase III activity in mitochondria (12, 54).

Because oxidative fragmentation of DNA sugars generates 3′-phosphate and 3′-phosphoglycolate moieties (24) and TDP1 is known to process such 3′-blocking groups in cells (47, 55), we tested the ability of ME to process a 3′-phosphoglycolate substrate (14-G). As shown in Fig. 3*C*, ME converted the 14-G substrate to the expected 14-P product. The 14-P migrates closely with the 14-G (Fig. 3*C*, compare lane 2 with lane 4) due its negative charge. Subsequent conversion to the 14-OH product results from hydrolysis of the 3′-phosphate (Fig. 3*C*, lane 5). Upon repair, the 14-OH product is finally converted to 36-mer final product (Fig. 3*C*, lane 5). Immunodepletion of TDP1 diminished the processing of the 3′-phosphoglycolate substrate (14-G) to the 3′-OH product (14-OH) indicating less conversion of 14-G to 3′-phosphate substrate (14-P), and also abrogated the final 36-mer product (Fig. 3*C*, lane 6), suggesting that TDP1 participates in the processing of 3′-phosphoglycolate in mitochondria. Ligase III immunodepletion diminished the production of the final 36-mer repair product (Fig. 3*C*, lane 7), consistent with the Ligase III activity in mitochondria (12, 54). The small residual 36-mer ligated product in Fig. 3*C*, lane 7, may be due to use of higher mitochondrial extract concentration to obtain 3′-phosphoglycolate processing. Taken together our data indicate mitochondrial BER is dependent on both TDP1 and DNA Ligase III activity.

TDP1 Is Required for Efficient Repair of mtDNA Oxidative Damage In Vivo. To investigate the biological significance of TDP1 in mtDNA repair, we evaluated mtDNA damage in TDP1−/− mouse embryonic fibrblasts (MEFs) (41) and its wild-type counterpart. The cells were challenged with H_2O_2 to introduce oxidative DNA lesions and followed at later times to monitor DNA repair. MtDNA damage was assessed by a gene-specific long-range quantitative PCR assays (LR-PCR) in which base lesions, abasic sites, or strand breaks interfere with the amplification of long mtDNA segments (56). This assay has proven particularly useful in examining mtDNA damage and repair kinetics after oxidative DNA damage (22, 25, 56). The relative PCR amplification of a long 10,000 bases mtDNA fragment was normalized to that of a short (117 bases) control PCR fragment. Because of the short size of the 117 base fragment, the frequency of mtDNA damage is expected to be approximately 100-fold less than in the 10 kb fragment. Thus, the short PCR fragment could be used to normalize the mtDNA input from different samples. In response to given H_2O_2 (Fig. 4*A* and *B*), significantly more oxidative damage accumulated in the mtDNA of TDP1−/− cells compared to TDP1+/+ cells. Time course experiments also showed that the amount of oxidative mtDNA damage was more pronounced immediately after the H_2O_2 treatment in TDP1−/− cells, and that the repair of mtDNA was slower in TDP1−/− cells (Fig. 4*C* and *D*). These results demonstrate that TDP1 deficiency impairs the repair of oxidative damage in mtDNA.

Discussion

To our knowledge the present study provides unique evidence for the presence of TDP1 in mitochondria based on immunofluorescence localization and cellular fractionation experiments. It also shows 3′-tyrosyl phosphodiesterase activity in mitochondrial

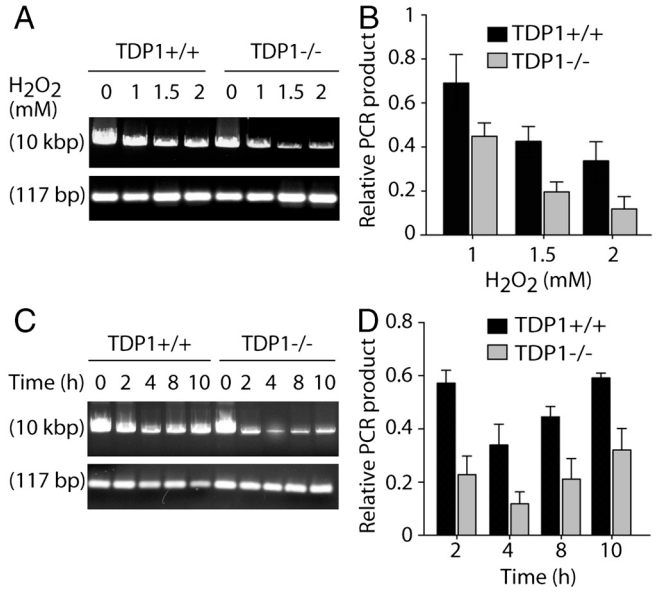

Fig. 4. Mitochondria from TDP1−/− cells are defective in repairing oxidative DNA damage. (*A*, *B*) Differential H_2O_2-induced mitochondrial DNA damage from TDP1+/+ and TDP1−/− cells. Long-range quantitative PCR was used to evaluate mtDNA damage. Panel A shows representative gels of long-range amplifications (10 kbp) and short-range amplifications (117 bp) of mtDNA after various doses of H_2O_2 for 4 h. The 117 bp fragment of mtDNA was amplified to monitor the copy number of the mitochondrial genome. (*B*) Quantitation of the relative PCR amplification of 10 kb mtDNA fragment normalized to mtDNA copy number. (*C*, *D*) Time course of H_2O_2-induced mtDNA damage in TDP1+/+ and TDP1−/− cells. Cells were treated with 2 mM H_2O_2 for the indicated time periods. (*C*) Representative gels showing PCR amplifications of 10 kbp and 117 bp mtDNA fragments. (*C*) Quantitation of the relative PCR amplification of 10 kb mtDNA fragment normalized to mtDNA copy number. Data represents the mean ± standard error of independent experiments.

extracts, which is related to the nuclear-encoded TDP1. Finally, the abnormal accumulation of oxidative mtDNA damage in cells lacking TDP1 demonstrates a role for TDP1 in the repair of mtDNA.

MtDNA repair is particularly critical because the mitochondrial genome is intronless and almost all of it (except for the D-loop regulatory region corresponding to approximately 7% of the mitochondrial genome) codes for essential oxidative phosphorylation proteins as well as mitochondrial mRNA and tRNA. This is remarkably different from the nuclear genome, where only 1% of the DNA is actually coding and most genes are interrupted by introns. Another reason for the importance of mtDNA repair stands from the fact that mtDNA resides in the mitochondrial matrix where oxygen is converted to water at the end of the oxidative phosphorylation chain with the potential of generating ROS. The known enzymatic activities of TDP1 are consistent with the types of lesions encountered in mtDNA. Indeed, TDP1 can process 3′-DNA ends by removing Top1 peptide adducts and 3′-deoxyribose adducts including phosphoglycolate (33, 45, 48, 50, 53). The formation of Top1-DNA adducts in mitochondria is plausible based upon the fact that mitochondria possess their own topoisomerase, Top1mt (10), which has recently been shown to form Top1-DNA adducts in mtDNA (11). TDP1 is far more efficient than APE1 in hydrolyzing Top1-DNA adducts (34). TDP1 is also a plausible candidate for removing 3′-phosphoglycolate (see Fig. 3*C*) and 3′-deoxyribose adducts, which can be produced by ROS either directly by attack of the deoxyribose backbone (24, 48, 55, 57) or indirectly after the processing of oxidized bases by DNA glycosylases with AP lyase activity (15, 16, 57). For instance, 8-oxoguanine-DNA glycosylase (OGG1) has an associated lyase activity that breaks the phosphodiester backbone 3′ to the abasic site with generation of 5′-phosphate and 3′-blocking groups (15). Until now, APE1 was the only known 3′-processing repair enzyme in mitochondria (14, 28). However, the 3′-exonuclease/phosphodiesterase activity of APE1 is relatively weak (26) and its tyrosyl-DNA phosphodiesterase activity is even weaker (34). Moreover, in contrast to TDP1, APE1 cannot remove phosphoglycolates on 3′-overhangs (47). A second protein with homology to APE1, as well as its bacterial ancestor exonuclease III, APE2, has been shown to localize to mitochondria (58), but its AP endonuclease function has not been substantiated (59). Thus, our findings indicate that, in addition to APE1, TDP1 can act as a 3′-end processing repair enzyme in mitochondria.

How TDP1 is translocated into the mitochondrion remains unknown. For some proteins, mitochondrial targeting relies on canonical N-terminal mitochondrial targeting sequences (MTS) and cleavage by mitochondrial matrix peptidases (60). However, TDP1 has similar size both in the nuclear and mitochondrial fractions (Fig. 2*A*) and does not contain a MTS sequence (10, 60). This implies that TDP1, like many other proteins and transcription factors such as APE1, FEN1, NF-kB, p53, and BRCA1 (23) that act both in the nucleus and mitochondria, enter mitochondria despite their lack of canonical mitochondria targeting sequences, indicating the existence of still unknown mechanisms of intracellular trafficking (13, 14, 28, 61, 62).

MtDNA mutations and deletions are frequently observed in neurodegenerative disorders, myopathies, metabolic diseases, and cancers, where they contribute to altered energy metabolism, increased ROS, and attenuated apoptotic response (5). The role of TDP1 in protecting against oxidative DNA damage in mitochondria is evident from our present experiments showing defective repair of oxidative DNA damage in mitochondria from TDP1 −/− cells. This finding is consistent with previous reports indicating an involvement of TDP1 in protecting cells against oxidative DNA damage (41, 42, 63, 64), and with the impaired ability of TDP1-deficient cells to remove 3′-phosphoglycolate (48, 49, 55). Homozygous mutation of TDP1 causes SCAN1, a neurodegenerative syndrome associated with marked cerebellar atrophy and peripheral neuropathy (44). Age-related cerebellar atrophy has also been reported in TDP1−/− mice (42). Thus, one might speculate that the dependence of neurons on mitochondria for energy production and the high levels of ROS in neurons may render them particularly sensitive to mitochondrial defects and TDP1 deficiencies.

Materials and Methods

Cells. Cells were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% FCS (Life Technologies). The human breast cancer cell line (MCF-7) was obtained from the Developmental Therapeutics Program [National Cancer Institute (NCI), National Institutes of Health (NIH)]. TDP1+/+ and TDP1−/− primary MEFS cells were a gift from Cornelius F. Boerkoel.

Antibodies, Expression Constructs, and Transfections. Rabbit polyclonal TDP1 (Ab4166) and mouse polyclonal Ligase III (Ab587, mouse monoclonal), rabbit polyclonal mtTFAM (Ab47517), mouse monoclonal COX IV (mAb33985), and rabbit polyclonal Lamin B1 (Ab16048) antibodies were from Abcam. Mouse monoclonal C21 antibodies against nuclear Top1 was kind gift of Yung-Chi Cheng, Yale University. Secondary antibodies: HRP-conjugated anti-rabbit IgG or anti-mouse IgG were obtained from Santa Cruz Biotechnology. Human TDP1-DsRED fusion constructs were generated using mammalian expression vector pDsRED1-N1 (Clontech) by standard PCR techniques, using pET-His-TDP1 (containing full-length human TDP1) as template. YFP-Mito construct (protein targeted to mitochondrial matrix) was provided by Richard J. Youle (National Institutes of Neurological Disorders and Stroke, NIH). Plasmid DNAs were cotransfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Immunocytochemistry and Confocal Microscopy. Immunofluorescence staining and confocal microscopy were performed as described (40). Briefly, MCF-7

cells grown on chamber slides (Nalge Nunc International) were incubated in medium containing 100 nM Mitotracker Red 580 (Molecular Probe) at 37 °C for 30 min and fixed with 4% paraformaldehyde. Primary antibody against TDP1 was detected with anti-rabbit IgG secondary antibodies labeled with Alexa 488 (Invitrogen). Cells were mounted in antifade solution with DAPI (Vector Laboratories) and examined using a laser scanning confocal microscope (Zeiss LSM510) with a 63× oil objective.

For live cell microscopy, cells growing on chamber cover glass were cotransfected with TDP1-DsRED and YFP-mito fusion proteins and mounted on an incubation chamber filled with medium 24 h after transfection. Nuclei were stained with Hoechst 33342 (1 μg/mL PBS; Sigma). Fluorescent signals were accessed under laser confocal microscopy as described above. Images were collected and processed using the Zeiss AIM software and sized in Adobe Photoshop 7.0.

Preparation of Mitochondria and Proteinase K Protection Assay. Mitochondria were prepared as described (11, 65). Briefly, cell pellets were suspended in 10 mM NaCl, 1.5 mM $CaCl_2$, and 10 mM Tris-HCl (pH 7.5) at 25 °C for 5 min. Following osmotic shock, cells were homogenized using a glass Dounce homogenizer and mixed with stabilizing buffer [2 M sucrose, 35 mM EDTA, and 50 mM Tris-HCl (pH 7.5) at 25 °C]. Cell lysates were centrifuged at 750 × *g* for 5 min to remove nuclei and cell debris. Mitochondria were spun down from the supernatant at 10,000 × *g* for 20 min, washed thrice with MT buffer [250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, and 20 mM Hepes-KOH (pH 7.4) at 25 °C], and resuspended in MT buffer.

Proteinase K protection assays were performed as described (22, 52). Briefly, equal aliquots of freshly prepared mitochondria (from MCF7 cells) were treated with 0.5 mg/mL proteinase K (Sigma), in the presence or absence of Triton-X-100 (1% final concentration; Sigma) for 15 min at 25 °C. Proteinase K activity was stopped with the addition of 2 volumes of 20% trichloroacetic acid at 0 °C for 20 min. Protein pellets were washed once with ice-cold acetone, resuspended in 2× Laemmli sample buffer, and evaluated by Western blotting as indicated below.

Immunodepletion and Immunoblotting. Freshly prepared mitochondria were lysed in 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 0.4% NP40, 0.5 mM dithiothreitol with protease and phosphatase inhibitors. ME were obtained by centrifugation at 15,000 × *g* at 4 °C for 20 min. Thirty microgram of ME were incubated overnight at 4 °C with indicated antibodies (5–6 μg) with protein A/G-PLUS-Agarose beads. After a brief centrifugation at 4,000 × *g*, supernatants were used for repair assays. Immunoblotting were done by standard procedures, and immunoreactivity was detected by ECL chemiluminescence reaction (Amersham).

Oligonucleotides and Preparation of DNA Substrates. The N14Y or 14G oligonucleotide (5′-GATCTAAAAGACTT**Y** or **G**-3′), which contains a 3′-phosphotyrosine (**Y**) or a 3′-phosphoglycolate (**G**) were synthesized by Midland Certified Reagents Company. All of the other DNA oligonucleotides were synthesized by Integrated DNA Technologies.

The N14Y or 14G oligonucleotide was 5′-end labeled using T4 polynucleotide kinase and [γ-^{32}P] ATP. Unincorporated radioactive nucleotides were removed using a mini Quick Spin Oligo column (Roche Diagnostics) after inactivation of the kinase by heating for 5 min at 95 °C. For construction of double-stranded nicked DNA substrate, the 5′-end radiolabeled N14Y or 14G oligonucleotides was annealed separately with the 22-mer (5′-pGGAAAAATTTTAAAAAAGATC-3′) and the 36-mer (5′-GATCTTTTTTAAAAATTTTCCAAGTCTTTTAGATC-3′) by heating for 5 min at 95 °C and slowly cooling to 25 °C.

TDP1 Activity and In Vitro Repair Assays. One nanomolar of the 5′-end radiolabeled N14Y substrate was incubated with either nuclear or mitochondrial extract for 30 min at 25 °C in a reaction buffer containing 1× PBS, 80 mM KCl, and 0.01% Tween-20.

For the repair assays, 1 nm of the 5′-end radiolabeled double-stranded DNA substrate containing a nick with a 3′-phosphotyrosine or 3′-phosphoglycolate was incubated separately with ME at 25 °C in reaction buffer containing 25 mM Tris-HCl (pH 8), 150 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA, 1 mM DTT, and 1 mM ATP.

Reactions were terminated by the addition of two volumes of gel loading buffer (96% (v/v) formamide, 10 mM EDTA, 1% (w/v) xylene cyanol and 1% (w/v) bromophenol blue). The samples were subsequently heated for 5 min at 95 °C and subjected to 20% sequencing gel electrophoresis.

Mitochondrial DNA Damage and Quantitative PCR Analysis. To compare the levels of mtDNA damage and repair in TDP1+/+ or TDP1−/− MEF cells treated with H_2O_2, quantitative PCR (56) used the following primers to amplify a 10-kb fragment of mtDNA: 5′-GCCAGCCTGACCCATAGCCATAATAT-3′ (sense primer) and 5′-GAGAGATTTTATGGGTGTAATGCGG-3′ (antisense primer). A small 117 base pair mtDNA fragment was also amplified for normalization: 5′-CCCAGCTACTACCATCATTCAAGT-3′ (sense primer) and 5′-GATGGTTTGGGAGATTGGTTGATGT-3′ (antisense primer). The relative PCR product was normalized to the copy number of the mitochondrial genome.

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