Human REV3 DNA Polymerase Zeta Localizes to Mitochondria and Protects the Mitochondrial Genome

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Abstract

To date, mitochondrial DNA polymerase γ (POLG) is the only polymerase known to be present in mammalian mitochondria. A dogma in the mitochondria field is that there is no other polymerase present in the mitochondria of mammalian cells. Here we demonstrate localization of REV3 DNA polymerase in the mammalian mitochondria. We demonstrate localization of REV3 in the mitochondria of mammalian tissue as well as cell lines. REV3 associates with POLG and mitochondrial DNA and protects the mitochondrial genome from DNA damage. Inactivation of Rev3 leads to reduced mitochondrial membrane potential, reduced OXPHOS activity, and increased glucose consumption. Conversely, inhibition of the OXPHOS increases expression of Rev3. Rev3 expression is increased in human primary breast tumors and breast cancer cell lines. Inactivation of Rev3 decreases cell migration and invasion, and localization of Rev3 in mitochondria increases survival and the invasive potential of cancer cells. Taken together, we demonstrate that REV3 functions in mammalian mitochondria and that mitochondrial REV3 is associated with the tumorigenic potential of cells.

Introduction

Mitochondria are involved in energy metabolism, cell proliferation, cell growth, apoptosis, and other cellular regulatory mechanisms. Mitochondria contain their own DNA, which encodes 13 essential components of the respiratory chain and is replicated continuously in dividing cells and in postmitotic tissues. Failure to preserve the genetic integrity of the mitochondrial genome during replication results in depletion, deletion, or mutation of mitochondrial DNA (mtDNA), which impairs oxidative phosphorylation (OXPHOS) and causes cellular dysfunctions and diseases [1–4]. Mitochondrial DNA polymerase γ (POLG), the only polymerase...
described to date in human mitochondria, is the key protein involved in mtDNA replication and repair [5–7]. Mutations in Polg1 are associated with depletion of mtDNA, mitochondrial malfunction, aging, carcinogenesis, and various other diseases [7,8]. However, the mechanisms by which mitochondria ensure stability and integrity of their own genome remain to be elucidated.

The capacity of a cell to duplicate its nuclear and mitochondrial genome in an error-free manner is necessary for maintaining homeostasis and limiting the risk for cancer and other diseases. The high fidelity of genome duplication is largely accredited to the proofreading capabilities of DNA polymerases delta and epsilon, which ensure that a correct nucleotide is incorporated at each step during DNA replication. But when these polymerases encounter an altered template due to covalent adducts and/or distortions in the secondary structure of DNA, the fidelity and processivity become limiting, and the replication fork stalls due to inability of these polymerases to accommodate bulky lesions or deformed structures within the DNA template. To overcome these conditions, cells have evolved a mechanism to promote replication despite such an altered template, a process known as translesion DNA synthesis (TLS). TLS bypasses the lesion and avoids the stall in the replication fork. After such adjustment to damage, the DNA can be repaired by the cell.

TLS is mediated by specialized DNA polymerases [9], including DNA polymerase zeta (Pol zeta, catalytic subunit REV3L). As a TLS polymerase, Pol zeta lacks the characteristic proofreading activity present in other B-family DNA polymerases [10]. Its two main subunits are REV3L, the catalytic subunit, and REV7, the structural subunit. Although REV3L alone is capable of polymerization, association of REV3L and REV7 is associated with stabilization of Pol zeta [11].

The human Rev3L gene encodes a ~350 kDa protein (REV3L) containing a large C-terminal DNA polymerase subunit. Human Rev3L (hereafter Rev3) is ubiquitously expressed in tissues and is involved in the bypass of many types of DNA damage induced by ultraviolet (UV) radiation [12–14] and chemical damaging agents [13]. Deletion of Rev3 is embryonically lethal [15,16], whereas over-expression of Rev3 leads to increased spontaneous mutations [17]. Rev3 is required for efficient replication of the common fragile site during the G2/M phase, and the resulting fragile site instability in Rev3 knockout mice may be associated with cell death during embryonic development [18]. Spontaneous chromosomal instability is present in Rev3-deleted mouse fibroblasts and Rev3-deleted cell lines [19,20]. Recent reports support the essential role of Rev3 in proliferation of normal mammalian cells [12,21]. Although expression of REV3 is necessary for normal physiology of cells, over-expression of REV3 is associated with breast cancers and brain gliomas [22,23]. Further, depletion of Rev3 sensitizes mouse B-cell lymphomas, lung adenocarcinoma, and human brain gliomas to cisplatin [23–25].

We have earlier reported that REV3 localizes to mitochondria in Saccharomyces cerevisiae yeast cells and participate in mtDNA mutagenesis [26]. In this report, we provide evidence that human REV3 also localizes to mitochondria and that inactivation of Rev3 leads to mitochondrial dysfunction.

**Materials and Methods**

**Construction of expression vectors for REV3 localization and confocal microscopy**

The REV3 mitochondrial localization signal (MLS) and nuclear localization signal (NLS) were predicted by use of MitoProt II and PredictNLS Online software, respectively. The Rev3MLS and Rev3NLS fragments were produced by PCR on pcDNA3.1+Flag, which contained a full-length Rev3 gene (a gift from Dr. Yoshiki Murakumo). The PCR products were cloned into a
pEGFP-N2 plasmid (Clontech, Palo Alto, CA). The primers for Rev3MLS were Xho1-5' GATCTCGAGGATGGTTATGGACAGCAGCC and BamH1-5' GGTTGATCCGGCGTTTTCGGAA CTTGACAGC, and, for Rev3NLS, were Xho1-5' CAGATCTCGAGATCCCATGGAAATTGGTGAA and BamH1-5' GGTTGATCCCTCACTCAGCA CCAGACTGAGA. Transfection of NIH 3T3 cells was accomplished with Fugene HD (Roche, Indianapolis, IN). Similarly, the full length Rev3-pcDNA3.1+Flag vector was transfected in HEK293 cells (HEK293 cells have very low level of endogenous REV3 expression) using Fugene HD reagent (Roche). Full length REV3 expressing cells were used for immunodetection of REV3 using antiFlag-M2 antibody (Sigma-Aldrich, St. Louis, MO). Cell staining was performed with Mitotracker (Molecular Probes, Eugene, OR) and 4', 6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA). Confocal pictures were taken with a Leica confocal instrument. Analysis of the pictures was carried out with ImageJ software (NIH, http://rsbweb.nih.gov/ij/).

Construction of expression vectors containing mutant Rev3 cDNA

A Rev3 mutant was constructed by removing, by use of XbaI, a 3.6-kb fragment that contained the NLS from the full-length Rev3. This mutant was named pcDNA3.1+Flag Rev3+MLS-NLS (Rev3 with MLS but without NLS). A second mutant, pcDNA3.1+Flag Rev3-MLS+NLS (Rev3 with NLS but without MLS), was designed to remove the MLS. The full-length Rev3 plasmid was digested with EcoRI and AleI, and a 1.75-kb fragment including the MLS was removed. PCR was used to produce a 1.225-kb fragment without the MLS but with EcoRI and AleI at the 5' and the 3' ends. The PCR product was placed into the backbone of the digested Rev3 plasmid so that a 0.425-kb fragment with the MLS was knocked out. The Rev3+MLS-NLS and the full-length Rev3 were transfected into HEK293 cells, and stable clones were selected.

Gene expression analyses

Rev3+/+ and Rev3−/− cells were used for analysis of expression of Polg1, Polg2 and Cox II genes. Rev3+/+ cells exposed to 5 J/m2 of UV were used for Rev3 gene expression studies. Expression of Rev3 was analyzed in tetracycline inducible (Tet-on) human mammary epithelial breast cancer cell line MCF7 (MCF7) and Tet-On MCF7 Polg1 D1135A (MCF7 Polg1DN) cells after 12 days of induction with Doxycycline (1 μg/ml). Tet-On MCF7 Polg1DN cells were prepared as reported earlier [7]. Expression of Polg1 D1135A, a dominant negative mutation, leads to depletion of mitochondrial DNA [7]. Total RNA was isolated and reverse-transcribed using standard procedures. RT PCR was used to measure the expression levels of these genes. Beta-2-microglobulin (B2M) or β-actin was used as an internal control.

Rev3 knockdown by shRNA

The Rev3 shRNA was synthesized as 5'-TAGTAGTCTGCAGTCACTATCCTACTGGAAGCT TGCG GTGAGGATAGTGACTGCGGACTATTACATTTTTTTT-3' [27] in the pGPU6/GFP/Neo vector (GenePharma, Shanghai). The Rev3 shRNA and its control shRNA were transfected into HeLa cells using Fugene HD (Roche), and stable clones were selected. The RNA samples were extracted with Trizol (Invitrogen, Carlsbad, CA). cDNA synthesis was accomplished with SuperSctrip III kits (Invitrogen). Real-time PCR using SYBR Green (Invitrogen) was employed to identify Rev3 knockdown in the clones.

Mitochondrial membrane potential measurement

Mitochondrial membrane potential was measured by the fluorescence of tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes). Cells were incubated with 100 nM TMRE for 35
min, harvested, and suspended in PBS. The fluorescence of the cells was read on the FL2 channel of a Becton Dickinson FACScan Flow Cytometer (Franklin Lakes, NJ).

**ROS measurements**

Oxidation of dihydroethidium (DHE) (Molecular Probes) fluorescent probe was used to measure intracellular ROS. Cells were labeled with 10 μg/ml DHE for 40 min, harvested, and suspended in PBS. The fluorescence of the cells was read on the FL2 or FL1 channel of a Becton Dickinson FACScan Flow Cytometer.

**Mitochondrial OXPHOS enzyme activities**

Mitochondrial OXPHOS enzyme activities were measured on enhanced mitochondrial preparations as previously described [28,29]. Complex I activity was measured as the rate of NADH oxidation in 25 mM potassium phosphate (pH 7.2), 5 mM MgCl₂, 2.5 mg/ml BSA, 65 μM coenzyme Q₁, 2 μg/ml antimycin A, and 2 mM KCN. The rate of absorbance change at 340 nm with a 425 nm reference wavelength was measured for 2 min. To measure complex II activity, total cellular protein was stimulated with and without 20 mM succinate for 10 min at 30°C in 25 mM potassium phosphate (pH 7.2), 5 mM MgCl₂, 2.5 mg/ml BSA, and 2 mM KCN. Coenzyme Q₁ (65 μM), antimycin A (2 μg/ml), rotenone (2 μg/ml), and 2, 6-dichlorophenolindophenol (DCIP, 50 μM) were added to the reaction system, and the rate of reduction of DCIP was measured at 600 nm for 3 min. Complex III activity was measured with cytochrome c (III) (15 μM) in 25 mM potassium phosphate (pH 7.2), 5 mM MgCl₂, 2.5 mg/ml BSA, 2 mM KCN, 2 μg/ml rotenone, 0.5 mM n-dodecyl β-maltoside, and 35 μM coenzyme Q₂H₂. The rate of absorbance change at 550 nm with a 580 nm reference wavelength was read for 2 min. Complex IV activity was measured by the oxidation of cytochrome c (II) (15 μM) in 20 mM potassium phosphate (pH 7.0) and 0.5 mM n-dodecyl β-maltoside. The rate of absorbance change at 550 nm with a 580 nm reference wavelength was measured for 2 min.

**Western blot analyses**

Cells were lysed in RIPA lysis buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1 mM PMSF; 1 mM EDTA; 1% Triton x-100; 1% sodium deoxycholate; and 0.1% SDS) with addition of Protease Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA) and Phosphatase Inhibitor Cocktail I (Sigma–Aldrich). Nuclear, cytoplasmic and mitochondrial fractions were prepared as described earlier [30]. Fifteen to thirty microgram protein was size fractionated on a 12% sodium dodecyl sulfate—polyacrylamide (SDS-PAGE) gel (7.5% SDS-PAGE was used for REV3 western blot), and transferred onto a polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA) using the wet transfer system and blocked in 5% skim milk in PBST for 1 h. A premixed cocktail (7.2 μg/ml) containing primary monoclonal antibodies against subunits of OXPHOS complexes (Mitosciences, Eugene, OR) was used to detect representative subunits from OXPHOS complex I, II, III and V. For detection of OXPHOS complex IV subunit COX II, an antibody from Life Technologies (Grand Island, NY) was used. Rabbit polyclonal primary antibodies against POLG1 and POLG2 (gifts from Dr. William C. Copeland, NIEHS) and Pol zeta (REV3L, Santa Cruz Biotechnology, Santa Cruz, CA, Cat# sc-48814) were used to detect the expression of these proteins. α-Tubulin, Lamin B1, and Tom20 (Santa Cruz Biotechnology) antibodies were used as markers for cytoplasmic, nuclear, and mitochondrial protein fractions, respectively. HRP-conjugated secondary antibodies (Vector Laboratories) and ECL reagent kits (GE Healthcare Biosciences, Pittsburg, PA) were used for film development.
Glucose consumption

Rev3+/+ and Rev3-/- cells were plated in 6-well dishes and, at 24 h after seeding, fresh growth media was added. Every subsequent 24 h, the total cell numbers were counted with a hemocytometer. Glucose in media samples was measured with a OneTouch Ultra LifeScan Glucometer (Milpitas, CA). Glucose consumption was calculated as (glucose in fresh media—glucose in media sample)/cells per well.

Cell survival assay

HEK293 cells containing full-length Rev3, Rev3+MLS-NLS, or a control vector clone were seeded into 60-mm dishes and cultured overnight. The cells were treated with 7.5 J/m² of ultraviolet (UV) light. The cells were trypsinized and then seeded into six-well plates at 2000 cells per well. The control cells were seeded at 500 cells per well. The cells were cultured for 10 days and then fixed with methanol. The colonies were stained with 0.01% coomassie blue (in 10% methanol and 10% acetic acid) for 15 min. Cell colonies with at least 50 cells were counted under a stereo microscope, and the surviving fractions were calculated.

Cell-culture migration assay

Cell-culture migration assay was carried out as described earlier [31]. Briefly, stable HeLa cell clones of Rev3 shRNA or control shRNA, seeded into six-well plates, reached confluence on the next day. The cells were wounded with yellow pipet tips. Pictures of the wounded lines at the same positions were taken at 0 and 72 h after wounding.

Matrigel invasion assay

The Matrigel invasion assay was accomplished with BD BioCoat Matrigel Invasion Chambers (Cat. 354480, BD Biosciences, San Jose, CA). HEK293 stable clones of Rev3 were seeded as 1x10⁵ cells per chamber; HeLa stable clones of Rev3 shRNA or the control shRNA were seeded as 0.5 x10⁵ cells per chamber. DMEM media with 10% FBS was used as the chemoattractant. Cells were allowed to migrate for 24 h, and then the membranes were stained with the Diff-Quik Stain Set (Dade Behring, Newark, DE). The invading cells were counted in 6 views per membrane under a microscope at 20X magnification. Cell counts were averaged and statistically analyzed.

Mitochondrial DNA damage and mtDNA content analysis

To induce mtDNA damage, Rev3+/+ and Rev3−/− mouse embryonic fibroblast cells were treated with 2.5 or 5 J/m² of UV radiation by a Stratalinker UV Crosslinker 2400 (Stratagene, La Jolla, CA). UV-irradiated cells were harvested at 6 and 24 h post-treatment, and their DNA was isolated with QIAamp DNA mini kits (Qiagen, Valencia, CA). The mitochondrial DNA content was analyzed by real-time PCR by absolute quantification with the following primers: mMitoF: 5’-CTAGAAACCCCGAAACCAAA-3’, mMitoR: 5’-CCAGCTATCACAAGGCTC GT-3’, mB2MF: 5’-ATGGGAAGCCGAACATACTG-3’, and mB2MR: 5’-CAGTCTCAGTGGGGGTG AAT -3’. B2M was used as an internal control.

For analysis of mtDNA damage, HEK293 cells containing full-length Rev3, Rev3-MLS, or control vector were treated with 2.5 J/m² of UV light and cultured for 6 h, and DNA was isolated from irradiated cells by use of QIAamp DNA mini kits (Qiagen). mtDNA damage was assessed by amplifying long (L) and short (S) fragments of mtDNA with the following primers: L-F: 5’-CACACGAGAAACCAACCTCA-3’, L-R: 5’-CTATGGCTGAGGGAGTCG AGCAG-3’, S-F: 5’-TCCAATCT ATGAGACCCCA-3’, and S-R: 5’-TGAGGCTTTAGATCGCTTT-3’, as described earlier [32].
RT PCR after treatment of cells with mitochondrial inhibitors

143B parental cells were treated with the mitochondrial enzyme complex inhibitors rotenone (20 μM), TTFA (25 μM), antimycin (10 μM), and KCN (10 mM) for 2 h, which block complex I, II-UQ, III, and IV, respectively. The cells were collected and total RNA was isolated, and cDNA was synthesized by following standard procedures. RT PCR was performed to analyze the expression of Rev3.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed with Rev3+/+ and Rev3−/− cells using SimpleChip Enzymatic Chromatin IP Kits (Cell Signaling, Danvers, MA), as suggested by the manufacturer. In brief, Rev3+/+ and Rev3−/− cells (~5 x 10⁸), grown in 100-mm tissue culture dishes, were cross-linked with 1% formaldehyde, digested with micrococcal nuclease, and sonicated. Soluble chromatin was collected and incubated overnight at 4°C on a rotating platform with a antibody against DNA polymerase zeta (gift from Dr. Christopher W. Lawrence, University of Rochester, and Santa Cruz Biotechnology), which detects REV3. The DNA was recovered and subjected to real-time PCR analysis with mouse D-loop and Cox II primers. These primers, used for end point real-time PCR amplification by the SYBR green method (Bio-Rad Laboratories, Hercules, CA), were as follows: D-Loop forward primer, 5'-CAGTGGCATGCACCCAGGGAA-3', and reverse primer, 5'-GCATGC CCCTTTTAGCCTTGGCA-3'; Cox II forward primer 5'-CAGTGCCATGGGACCCAGGGAA-3', and reverse primer 5'-GCATGCCCCCTTTTAGCCTTGGCA-3'. Amplification of chromatin before immunoprecipitation at a dilution of 1:50 was used as a positive control (input); ChIP with rabbit serum IgG served as a negative control. The assays were accomplished in three replicates. Agarose gel electrophoresis of PCR-amplified products for ChIP DNA and input DNA samples were used to represent the results.

Co-immunoprecipitation (Co-IP) assay

Co-IP assays were accomplished with Rev3+/+ and 143B cells with or without UV exposure. Co-IP with Rev3−/− cells were used as negative control. In brief, cells in 100-mm tissue culture dishes were grown for 6 h after treatment with 5 J/m² of UV light. Untreated cells were used as controls. For immunoprecipitation, cells were lysed for 8 min on ice with lysis buffer (20 mM Tris-HCl, pH 8; 150 mM NaCl; 0.1% NP-40; and 1X Protease Inhibitor (Thermo Fisher Scientific, Waltham, MA) and the lysate was centrifuged at 4800 rpm for 10 min. Supernatant protein contents were measured with protein assay kits (Bio-Rad Laboratories). Supernatant (250 μg protein) was pre-cleared with Magnetic Protein A Dynabeads (Invitrogen; 35 μL beads/mL lysate) for 1 h and then used for Co-IP with 10 μg of Pol zeta primary antibody. The lysate was incubated with antibody overnight on a rotator at 4°C. Magnetic Protein A Dynabeads were added, and the preparations were incubated for 1 h on a rotator at 4°C. Beads were washed in lysis buffer (five times) and captured with a magnetic separator (Qiagen). Proteins were eluted in SDS sample buffer at 65°C for 10 min and with frequent vortexing. The protein samples were separated by SDS-PAGE on a 10% polyacrylamide gel and electroblotted onto a PVDF membrane. The blots were incubated with specific primary rabbit polyclonal antibodies against POLG1 and POLG2 (gift from Dr. William C. Copeland, NIEHS) and detected with an HRP-conjugated secondary antibody (Vector Laboratories). Generic ECL reagent kits (GE Healthcare Biosciences) were used for film development.

Rev3 expression in breast tumors

All experiments were approved by the Roswell Park Cancer Institute Institutional Review Board, permit number I92106. Consent from patients was not needed, as anonymous tissue
samples were used for study. Normal and matched breast tumor RNA samples were obtained from the biorepository resource facility of the Roswell Park Cancer Institute and provided to us under IRB-approved permit number I92106. RNA (1 μg) was reverse transcribed, and the gene expression of Rev3 was analyzed by RT PCR.

Statistical analyses
All statistical analyses were performed with Sigma Plot 11.0 software (Systat Software, San Jose, CA). Data were compared using two-tailed Student’s t-tests.

Results
Human REV3 localizes to mitochondria
In our previous study conducted with yeast, we demonstrated that REV3 functions in mitochondria [26]. Using MitoProt II software, we have now identified, at the N-terminal region of the human REV3 protein, a mitochondrial localization signal (MLS) that facilitates translocation of this protein to mitochondria. We analyzed two known isoforms of human REV3 synthesized from the same Rev3 gene via the use of two alternative translation initiation (ATI) sites. Human Rev3 mRNA contains two AUGs. Translation initiation from the first AUG synthesizes a long isoform of REV3 (3130 amino acid; ~352kDa; NP_002903.3), and initiation from the second AUG generates a small isoform of REV3 (3052 amino acid; ~343kDa; AAG09402.1). The import of proteins into the mitochondria is frequently dependent on an N-terminal, positively charged amphipathic α-helix, which functions as an MLS [33]. Our analyses for MLS sequence using MitoProt II revealed that short isoform of REV3 contains a 107-amino acid long putative MLS sequence at the N-terminus of the protein (76.9% confidence, MitoProt II). The long isoform of the REV3 protein contains an additional 78 amino acid sequence at the N-terminus (Fig 1A).

On the basis of this prediction, the corresponding nucleotide sequence for these 107-amino acids, Rev3MLS was produced by PCR. The PCR product was cloned into a pEGFP-N2 plasmid and transfected into NIH3T3 cells. Mitotracker and DAPI dyes were used to locate the mitochondrial and nuclear compartments, respectively (Fig 1B). Merged images show the localization of passenger protein GFP in mitochondria. These analyses suggest that the N-terminal 107-amino acid sequence of short isoform of REV3 contains an active mitochondrial localization signal that has the ability to direct localization of human REV3 into mitochondria. Full length protein expression of REV3 in mitochondria was also detected by immunofluorescence in Rev3-pcDNA3.1Flag transfected HEK239 cells using anti-Flag-M2 antibody (S1 Fig).

We performed Western blot analyses of extracts prepared from proteinase K (0.014%)-treated mitochondria isolated from HEK293 cells (HEK293 cells have very low level of endogenous REV3 expression) transfected with pEGFP-N2 vector or pEGFP-N2-Rev3MLS expressing the fusion protein. Fig 1C shows a single band of ~40 kDa REV3_MLS_GFP protein only in cells transfected with the Rev3MLS construct. Proteinase K treatment confirmed that Rev3MLS localizes the passenger protein GFP inside the mitochondria, not to the mitochondrial outer membrane. The membrane was stripped and re-probed with antibody against COX II antibody (20 kDa), an authentic mitochondrial protein (Fig 1C). To further confirm the localization of REV3 in mitochondria, we performed Western blots with cytoplasmic, nuclear, and mitochondrial protein fractions from mouse primary organ heart as well as from the human breast epithelial cell line, MDA-MB-231. We detected presence of both long and short isoforms of REV3 in cytoplasm and nuclear fractions while only short isoform of REV3 localized to mitochondria in both mouse and human samples (Fig 1D). Specificity of the REV3 antibody was confirmed by Western blot with protein samples from Rev3+/+ and Rev3-/- cells and Rev3 shRNA
transfected HeLa cells (S2 Fig). The presence of full-length REV3 protein in mitochondria indicates towards a possible role of this DNA polymerase in mitochondria.

Analyses performed with PredictNLS software predicted the presence of a nuclear localization signal (NLS) in both isoforms of human REV3 (Fig 1A), and we confirmed the capacity of this predicted NLS region for translocation of GFP to nucleus (Fig 1E).

**Rev3 inactivation inhibits mitochondrial functions**

The above observations suggest that REV3 is localized to mitochondria. Since OXPHOS is the major metabolic pathway in mitochondria, whether the presence of REV3 in mitochondria is indispensable for mitochondrial functions was assessed. Rev3+/+ and Rev3−/− mouse embryonic fibroblasts (MEFs) (gifts from Dr. Richard D. Wood) were used to measure endogenous expression levels of mitochondrial genome-encoded gene *Cox II*. In these cells, changes in enzymatic activities and protein expression of OXPHOS complexes I-V in the presence or absence of REV3 were measured, and the effect of inactivation of Rev3 on mitochondrial membrane potential and intracellular ROS levels was examined.

For OXPHOS complex IV, which has three mitochondrial-encoded proteins, lower expression of COX II, a mitochondrial-encoded complex IV protein (Fig 2A), lower enzymatic activity (Fig 2C), and the lower expression of mitochondrial genome-encoded gene *Cox II* (Fig 2B) in Rev3−/− cells relative to Rev3+/+ cells indicated a role of REV3 in regulation of mitochondrial genes/genome and OXPHOS functions. There were no significant changes in the activities of other complexes of mitochondrial OXPHOS in Rev3−/− cells relative to Rev3+/+ cells (data not shown).
Since mitochondrial OXPHOS is the main source for production of ATP, cells with compromised OXPHOS switch their metabolism towards glycolysis [34]. Therefore, the glucose consumption was measured in Rev3+/+ and Rev3-/- MEFs. The higher glucose consumption rate in Rev3-/- cells (Fig 2D) indicates a compromised OXPHOS system and altered mitochondrial metabolism in these cells. Mitochondrial membrane potential, an indicator of the capacity of the cells to pump hydrogen ions across the inner membrane during energy production by OXPHOS, was also decreased in Rev3-/- cells (Fig 2E). Treatment with carbonyl cyanide m-chlorophenyl hydrazine (CCCP), an inhibitor of OXPHOS, decreased the membrane potential in Rev3-/- cells relative to Rev3+/+ cells (Fig 2E). Mitochondria produce reactive oxygen species (ROS) during oxidative phosphorylation [29]. ROS production in the form of superoxide radicals was low in Rev3-/- cells (Fig 2F). Further, after treatment with the mitochondrial inhibitor antimycin A, there was less ROS production in Rev3-/- cells relative to Rev3+/+ cells (Fig 2F).

**OXPHOS inhibition increases Rev3 expression**

Since Rev3 inactivation altered mitochondrial functions, we examined whether mitochondrial dysfunction similarly affects expression of the Rev3 gene. For this, mRNA expression of Rev3 in rho° cells (mtDNA deficient) was measured and compared with Rev3 expression in parental
cells (Fig 3A). mRNA expression of Rev3 was higher in rho° cells relative to parental cells (Fig 3A). Increased expression of Rev3 in rho° cells suggests a role of Rev3 in metabolism of mtDNA (Fig 3A). Similarly, Rev3 expression was also assessed in cells in which OXPHOS was inhibited by specific inhibitors of the OXPHOS complexes (Fig 3A). There was increase in Rev3 mRNA expression after treatment with each inhibitor (Fig 3A).

REV3 associates with mitochondrial DNA Polymerase γ

We show localization of human REV3 in mitochondria and DNA polymerase γ is known to be present in mitochondria [35,36]. We determined, by immunoprecipitation experiments, if REV3 associates with mitochondrial polymerase γ in mitochondria. This was accomplished with protein samples from Rev3+/+ and 143B cells exposed or unexposed to UV (Fig 3B). Positive Western blots with both subunits (POLG1 and POLG2) of polymerase γ with precipitated protein samples from both unexposed and UV-exposed Rev3+/+ and 143B cells using REV antibody suggests an association of REV3 with polymerase γ in mitochondria (Fig 3B).

Since REV3 associates with polymerase γ, we determined if inhibition of polymerase γ affects expression of Rev3 by use of cells expressing the tetracycline-inducible, dominant negative mutant POLG1 (MCF7 Polg1 DN) described earlier [7]. Earlier, we have shown depletion of mtDNA content in MCF-7 cells by induction of this dominant negative mutant of POLG1 [7].
After induction of the dominant negative POLG1 with doxycycline (dox, 1 μg/ml), there was induction of Rev3 mRNA and protein expression (Fig 3C and 3D).

**REV3 protects cells from mtDNA damage**

Polymerase γ is involved in mtDNA synthesis and its integrity. Therefore, we examined if REV3 is involved in maintenance of mtDNA integrity. REV3+/+ MEFs were exposed to UV (5 J/m²), and mRNA expression of Rev3 was examined after 6 and 12 h of UV exposure. In UV-exposed Rev3+/+ MEFs but not in unexposed MEFs (data not shown), there was a time-dependent increase in the mRNA expression of Rev3 (Fig 4A). The mRNA expression status of Polg1 and Polg2 in Rev3+/+ and Rev3−/− cells was also examined by RT PCR. There was higher expression of both Polg1 and Polg2 in Rev3−/− cells relative to Rev3+/+ cells (Fig 4B). To confirm the binding of REV3 to mtDNA, chromatin immunoprecipitation (ChIP) assays were accomplished with Rev3−/− cells exposed and not exposed to UV (Fig 4C). PCR amplification of immunoprecipitated mtDNA from two different regions, the D loop (non-coding regulatory
region) and COX II (coding region), indicated binding of REV3 to mtDNA (Fig 4C). PCR amplification of both COX II and D loop region in both UV-exposed and unexposed cells indicate that REV3 binds to mtDNA.

To further elucidate the functional importance of REV3 in mitochondria, mtDNA damage was measured after 6 h of UV exposure of cells expressing full-length REV3 or REV3-MLS (REV3 without MLS). There was more mtDNA damage in cells in which REV3 does not localize to mitochondria relative to cells expressing full-length REV3 (Fig 4D). The mtDNA content in Rev3+/+ and Rev3−/− MEFs with and without UV exposure was also measured. The mtDNA content recovered in Rev3+/+ cells after 6 h of UV exposure (5 J/m²) and further increased after 24 h of exposure, whereas, in Rev3−/− cells, UV exposure decreased the mtDNA content compared with unexposed Rev3−/− cells after 6 h; the levels either remained unchanged or only slightly recovered after 24 h of exposure (Fig 4E).

REV3 is over-expressed in primary human breast tumors and breast cancer cell lines

An imbalance in the expression of polymerase zeta, due either to a decrease in expression or over-expression, is associated with increased genomic instability [37,38]. Thus, a balance in the expression of Pol zeta is necessary for genomic integrity. Although REV3 is involved in genomic stability, its role in human cancers is unclear [9,39–41].

To assess the expression status of the Rev3 in human breast cancer, expression of Rev3 in the non-neoplastic human breast epithelial cell line, MCF-12A, and in 10 human breast epithelial cancer cell lines was determined by RT PCR (Fig 5A). In most of the breast cancer cell lines, there was higher expression of Rev3 relative to that in MCF-12A cells (Fig 5A). mRNA expression of Rev3 was also measured in 10 sets of primary breast tumors and matched normal breast tissues derived from the same patients (Fig 5B). Of 10 cases, Rev3 mRNA expression was higher in 7 breast tumor samples, similar in 2 samples and was lower in one sample relative to their normal breast tissue counterparts (Fig 5A).

Mitochondrial REV3 contributes to tumorigenic properties

Human REV3 contains both an NLS and an MLS and is present in cytoplasm, nuclei, and mitochondria (Fig 1). To define the role of mitochondrial REV3 in tumorigenesis, full-length Rev3 or Rev3 with MLS but without NLS (Rev3+MLS-NLS) were expressed in HEK293 cells (cells with a very low level of endogenous Rev3 expression). The effect of an increase in expression of full-length REV3 or mitochondrial REV3 on cell survival was determined. HEK293 cells expressing full-length Rev3 and HEK293 cells expressing Rev3+MLS-NLS were exposed to 7.5 J/m² of UV, as described in the Materials and Methods. After that 2000 UV-exposed cells were plated and allowed to form clones for 10 days, the survival fractions were calculated. Increased expression of Rev3 or Rev3+MLS-NLS increased survival of HEK293 cells after UV exposure (Fig 5C). Increased expression of Rev3 or Rev3+MLS-NLS also increased Matrigel invasion of HEK293 cells (Fig 5D).

Rev3 in HeLa cells (cells with a detectable endogenous level of Rev3) was inactivated with two shRNAs specific to Rev3. These Rev3-knockdown cells were used for assays of Matrigel invasion and cell-culture migration. Lower invasion (Fig 5E) and migration (Fig 5F) after shRNA-mediated knockdown of Rev3 support a tumorigenic role of Rev3.

Discussion

Present study identified for the first time that human REV3, the catalytic subunit of DNA polymerase zeta localizes to mitochondria and affect mtDNA metabolism. Using MitoProt II
software, we identified a mitochondrial localization signal (MLS) at the N-terminal of REV3 protein that helps in translocation of this protein to mitochondria. We analyzed two known isoforms of REV3 synthesized from the same Rev3 gene via the use of two ATI sites. Presence of ATI sites is one of the gene regulatory mechanisms that diversifies the mammalian proteome and has earlier been reported to be associated with generation of N-terminal protein variants destined to locate to different compartments of the cell [33,42,43]. Use of ATI site to expose a cryptic MLS, located within the coding sequence has earlier been reported for other genes [33]. On the basis of our analyses, we cloned the corresponding cDNA sequence to 107 amino acid long N-terminal region from short isoform of Rev3 into GFP containing vector and confirmed the ability of this MLS in translocation of GFP into mitochondrial matrix by fluorescent microscopy (Fig 1B), and by western blot with mitochondrial protein fractions (Fig 1C).

In our previous report, we cloned a sequence corresponding to the N-terminal 1–100 amino acids of human REV3 in frame with GFP in the pEGFP-N2 plasmid and evaluated the capacity of this N-terminal region of REV3 for localization of GFP to mitochondria [26], but we were...
not able to demonstrate the localization of human REV3 into mitochondria. In that study, the putative MLS was identified from the longer isoform of human REV3 [26]. The longer isoform of human REV3 (NP_002903.3) contains extra 78 amino acid long sequence at the N-terminal before the MLS sequence (Fig 1A). Thus, the first 100 amino acid sequence from the longer isoform of human REV3, used in our previous study, lacked an active MLS [26]. Our intense analyses about the active MLS in human REV3 in the present study, confirmed the presence of an active MLS only in the shorter isoform of REV3 synthesized from an ATI site (Fig 1A). Our immunofluorescence analysis of REV3 confirmed localization of full length REV3 in mitochondria (S1 Fig). Western blot analyses of cytoplasmic, nuclear, and mitochondrial fractions from mouse heart as well as human cells indicate that both long (~352 kDa) and short (~343 kDa) isoforms of REV3 are present in the cytoplasm and nuclear fractions while only short isoform of REV3 localizes to mitochondria (Fig 1D). How the presence of an additional 78 amino acid sequence at the N-terminal of the long isoform of human REV3 affects the translocation of this isoform to mitochondria is not yet known.

Our analyses revealed presence of nuclear localization signal (NLS) in both isoforms of REV3 (Fig 1A). We confirmed the ability of this predicted NLS region in translocation of GFP to the nucleus (Fig 1E). To examine effects of REV3 on mitochondrial functions, we used Rev3+/+ and Rev3−/− MEFs with p53−/− background [38], and vice versa, i.e., to observe the effects of alterations in mitochondrial OXPHOS on the REV3 expression, we used 143B rho− (mtDNA deficient) cells as well as specific inhibitors of mitochondrial OXPHOS complexes. Decrease in the expression of mitochondrial genome-encoded gene Cox II, mtOXPHOS complex IV expression and its enzymatic activity in Rev3−/− cells compared to Rev3+/+ cells indicated towards a possible role of REV3 in the maintenance of mitochondrial genome and thus mtOXPHOS functions (Fig 2A, 2B and 2C). Increased glucose consumption rate in Rev3−/− cells (Fig 2D) further points towards compromised OXPHOS system and altered mitochondrial metabolism towards glycolysis, an effect known as Warburg effect [34]. Decreased mitochondrial membrane potential and mitochondrial ROS producing ability in Rev3−/− cells compared to Rev3+/+ cells after treatment with CCCP and Antimycin A, respectively, further suggested a role of REV3 in the regulation of mitochondrial functions (Fig 2E and 2F). Increased Rev3 mRNA expression in 143B cells treated with mitochondrial inhibitors of OXPHOS complexes indicates a compensatory response and also indicates that REV3 might be involved in the maintenance of OXPHOS system (Fig 3A). Increased expression of Rev3 mRNA in mtDNA deficient 143B rho− cells compared to 143B cells (Fig 3A) again suggests that REV3 expression is linked to mitochondrial function. Overall, these results indicate that REV3 functions in mitochondria and that its inactivation leads to mitochondrial dysfunction.

To date, polymerase γ is the only polymerase described in mitochondria from higher eukaryotes [35,36] but our chromatin immunoprecipitation (ChIP) analyses using mitochondrial fractions from Rev3+/+ cells suggest presence of REV3 in mitochondria (Fig 4C). Moreover, association of REV3 with polymerase γ (Fig 3B) demonstrate a cross talk between these two polymerases. Data showing binding of REV3 to two different regions; COX II (coding) and the D loop (non-coding) of mtDNA indicate that REV3 physically interacts with mtDNA irrespective of coding or non-coding region of mtDNA (Fig 4C). Increased binding of REV3 to D loop region of mtDNA, a region known to be more susceptible for DNA damage after UV exposure further indicates towards a possibility of involvement of REV3 in the maintenance of DNA integrity in mitochondria (Fig 4C).

Ultraviolet radiations are known to damage cellular macromolecules. Both nuclear and mtDNA are susceptible to several types of damage caused by UV irradiation [44]. Thymine dimers and 6, 4 photoproducts are the predominant types of damage caused by UV radiation [45]. UV-induced thymine dimers have been shown to distort the nuclear and mtDNA
backbone [36,44]. While nuclear DNA damage gets repaired due to presence of a battery of DNA repair enzymes including TLS DNA polymerase, mitochondria are known to lack nuclear-encoded DNA repair enzymes that can repair UV-induced mtDNA damage and thus inherently become more prone to the accumulation of mtDNA damage. Although presence of nuclear encoded base excision repair enzymes in mitochondria has been reported [46,47], yet there is clear lack of evidence that can support presence of TLS DNA polymerase activity in mammalian mitochondria. Lack of polymerase zeta has been shown to confer extreme UV sensitivity [12]. REV3 is not only required to function as DNA damage tolerance system but has also been shown to be required for maintaining genomic integrity and proliferation in normally proliferating cells [12,21].

Increased expression of Rev3 after UV exposure (Fig 4A) and recovery of mtDNA content only in Rev3+/+ cells but not in Rev3−/− cells indicates towards the involvement of REV3 in the maintenance of mtDNA after UV exposure (Fig 4E). Increased mRNA expression of Polg1 and Polg2 in the absence of Rev3 (Fig 4B) and similarly increased mRNA and protein expression of REV3 in presence of a dominant negative POLG1 allele (Fig 3C and 3D) suggest that both polymerases (polymerase γ and REV3) compensate functions of each other in mitochondria and like polymerase γ, REV3 might also be involved in the maintenance of mtDNA (Fig 4D and 4E). A recent report in yeast system also support our results that Pol zeta reduces mitochondrial mutability caused by pathological mutations in Polg1 gene [48].

Recent evidences support that REV3 alters oncogenic potential of the cells [9,39–41]. Studies have shown effects of REV3 depletion on the cancer cell growth in vitro [19,40]. However, dilemma still exists whether REV3 is oncogenic or tumor suppressor in nature. To examine whether Rev3 behaves as an oncogene or tumor suppressor gene, we cloned and over expressed full length Rev3 as well as Rev3 containing MLS but without NLS (Rev3+MLS-NLS). Greater cell survival and invasion in case of expression of Rev3+MLS-NLS indicate that mitochondrial REV3 is predominantly responsible for the higher tumorigenicity of the cells (Fig 5C and 5D).

Decreased matrigel invasion as well as cell migration capacity of the cells after shRNA-mediated knocked down of Rev3 further strengthen the observations that support the role of Rev3 in development of cancer (Fig 5E and 5F). Association of inhibition of human Rev3 with less mutagenic properties has been shown earlier [49,50]. Our results indicate that REV3 protects against mtDNA damage (Fig 4D), that its inactivation leads to the Warburg effect (Fig 2D), and that higher mitochondrial expression is associated with increased tumorigenicity of the cells (Fig 5C and 5D).

Increased expression of Rev3 in human breast epithelial cancer cell lines compared to non-neoplastic human breast epithelial cells MCF-12A, and in human breast tumors compared to normal breast tissues further qualifies Rev3 as a gene associated with tumorigenesis (Fig 5A and 5B). DNA repair and cell cycle checkpoint mechanisms are frequently abrogated in cancer cells and extent of endogenous DNA damage is higher in tumor tissues [51]. REV3 is a TLS enzyme and helps in maintaining genomic integrity but TLS is a mutagenic process as it often incorporates incorrect nucleotides [52,53]. Thus increased expression of Rev3 in breast cancer cells and tumor tissues might help carcinogenic processes in two ways, either by acting as a protective mechanism against DNA damage that provide survival advantage to the tumor cells by keeping DNA damage lower than the threshold and/or by inducing mutagenesis during translesion DNA synthesis. Therefore, a balance in the expression of REV3 is required to maintain cellular homeostasis. Overall, our results provide evidences that mammalian REV3 localizes to mitochondria and maintain the integrity of mitochondrial genome. REV3 contributes to tumorigenic potential of cells, however, further experiments are needed to evaluate whether nuclear and mitochondrial REV3 have different effects on tumorigenic activity.
Supporting Information

S1 Fig. Immunofluorescence analysis to show the localization of full length REV3 protein in HEK293 cells. A construct containing full length Rev3 and a Flag tag (a gift from Dr. Yoshiki Murakumo) was transfected and anti Flag-M2 antibody was used to detect the localization of full length REV3 in mitochondria. (TIF)

S2 Fig. (A) Western blot with protein samples from REV3+/+ and REV3−/− cells to show the specificity of REV3 antibody (Santa Cruz Biotechnology, Cat # sc-48814) used in this study. (B) Western blot showing specificity of REV3 antibody (Santa Cruz Biotechnology, Cat # sc-48814) as well as Rev3 shRNA-mediated knockdown of REV3 in HeLa cells. (TIF)

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Author Contributions

Conceived and designed the experiments: KKS BS. Performed the experiments: BS XL KMO AV. Analyzed the data: BS KKS. Contributed reagents/materials/analysis tools: PL. Wrote the paper: BS KKS.

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