

Mitochondrial DNA Ligase Is Dispensable for the Viability of Cultured Cells but Essential for mtDNA Maintenance*

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Background: Nonviability of cells lacking mitochondrial DNA ligase suggests essential function of this enzyme.

Results: We report the isolation of viable *Lig3*^{-/-} cells, which lack mtDNA.

Conclusion: The lethality of the *Lig3* knock-out is mediated by the ρ^0 phenotype.

Significance: This is definitive proof that the essential function of LIG3 in mitochondria is limited to DNA transactions.

Multiple lines of evidence support the notion that DNA ligase III (LIG3), the only DNA ligase found in mitochondria, is essential for viability in both whole organisms and in cultured cells. Previous attempts to generate cells devoid of mitochondrial DNA ligase failed. Here, we report, for the first time, the derivation of viable LIG3-deficient mouse embryonic fibroblasts. These cells lack mtDNA and are auxotrophic for uridine and pyruvate, which may explain the apparent lethality of the *Lig3* knock-out observed in cultured cells in previous studies. Cells with severely reduced expression of LIG3 maintain normal mtDNA copy number and respiration but show reduced viability in the face of alkylating and oxidative damage, increased mtDNA degradation in response to oxidative damage, and slow recovery from mtDNA depletion. Our findings clarify the cellular role of LIG3 and establish that the loss of viability in LIG3-deficient cells is conditional and secondary to the ρ^0 phenotype.

In mammalian cells, there are two DNA-containing organelles, the nucleus and the mitochondrion. Human mitochondrial DNA (mtDNA) is a 16,568-bp circular molecule (1) crucial for proper mitochondrial function and cellular ATP production. It encodes 13 proteins as follows: 11 polypeptide subunits of the mitochondrial electron transport chain complexes I, III, and IV and two subunits of the ATP synthase, complex V. These polypeptides are encoded using a genetic code distinct from that used in the nucleus and therefore require a separate translational apparatus, some components of which (22 tRNAs and 2 rRNAs) also are encoded in mtDNA (2). Therefore, it comes as no surprise that the loss of mtDNA integrity either through mutation or depletion may lead to serious, often fatal diseases (3, 4). The mitochondrial electron transport chain is a major cellular source for the production of reactive oxygen species. Although the exact rates of mitochondrial reactive oxygen

species production *in vivo* remain controversial due to technical difficulties associated with their quantitation (5, 6), it is well established that reactive oxygen species can damage mtDNA (7). To deal with oxidative and other types of DNA damage, mitochondria possess an array of DNA repair mechanisms (8), of which the most active and the best studied is base excision repair (BER)² (9–11).

DNA ligase activity is crucial for both DNA replication and repair (12). In BER, the ligase catalyzes the last step in the series, nick sealing (13). Of the three mammalian DNA ligases, Lig1, LIG3, and Lig4, only Lig3 has been documented to be present in mitochondria, where it functions in both DNA repair and replication (14, 15). The mitochondrial isoform is transcribed from the same gene as the nuclear isoform and is generated by alternative translation initiation using an upstream in-frame ATG codon. The resulting N-terminal extension encodes an amphipathic helix, which serves as the mitochondrial matrix targeting sequence (MTS) (16, 17).

Consistent with the essential role played by LIG3 in the maintenance of mtDNA, the whole body *Lig3* knock-out is embryonically lethal, and *Lig3*-null mouse embryos die ~8.5 days post-coitum (18). Mice with a tissue-specific *Lig3* knock-out in the nervous system do not survive past postnatal day 20, and death of these animals is preceded by a reduction in mtDNA copy number (19). Similarly, mice with *Lig3* ablation in muscle die abruptly between 3.5 and 4.5 weeks of age, due to defects in cardiac, but not skeletal, muscle (19). Repeated attempts to culture *Lig3*^{-/-} fibroblasts from knock-out embryos proved unsuccessful, whereas fibroblast cultures from similarly aged wild type (WT) embryos were readily established (18). Similarly, *Lig3*-null mouse embryonic stem cells do not survive, unless cells are preemptively complemented with mitochondrially targeted DNA ligase (20). In the latter case, even DNA ligases as evolutionarily distant from LIG3 as the ATP-dependent *Chlorella* virus ligase and the NAD⁺-depen-

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² The abbreviations used are: BER, base excision repair; DSB, double strand break; EtBr, ethidium bromide; Lig3, DNA ligase III; MEF, mouse embryonic fibroblasts; MMS, methyl methanesulfonate; MTS, matrix targeting sequence; QSBN, quantitative Southern blotting under the nondenaturing conditions; QSBA, quantitative Southern blotting under alkaline (denaturing) conditions; SSB, single strand break; qPCR, quantitative PCR; h, human.

TABLE 1
Oligonucleotides used to generate shRNA for hLig3

Name	Sequence
hLig3-1F	GATCCGATCTTCTTTCCACAAACCTTCAAGAGAGGTTTGTGGAAAGAAGATCTTTTTTGGAAA
hLig3-1R	AGCTTTTCCAAAAAAGATCTTCTTTCCACAAACCTTCTTGAAGGTTTGTGGAAAGAAGATCG
hLig3-2F	GATCCGAACTGTGCCTATTCCGAATTCAGAGATTTCGGAATAGGCACAGTTCTTTTTTGGAAA
hLig3-2R	AGCTTTTCCAAAAAAGAACTGTGCCTATTCCGAATCTCTTGAATTCGGAATAGGCACAGTTCCG
hLig3-3F	GATCCGCATCACTGGCGTGATGTAATTCAGAGATTACATCACGCCAGTGATGTTTTTGGAAA
hLig3-3R	AGCTTTTCCAAAAAATCATCACTGGCGTGATGTAATCTCTTGAATTCATCACGCCAGTGATGCG
hLig3-4F	GATCCGACAATTCAGCCAGTGGTCTTCAAGAGAGACCCTGGCTGAATTTGCTTTTTTGGAAA
hLig3-4R	AGCTTTTCCAAAAAAGACAATTCAGCCAGTGGTCTCTTGAAGACCCTGGCTGAATTTGCTG
hLig3-5F	GATCCGTTTCAGCCAGTGGTTCAGAAATTCAGAGATTCTGACCACTGGCTGAATTTTTTGGAAA
hLig3-5R	AGCTTTTCCAAAAAATTCAGCCAGTGGTTCAGAAATCTCTTGAATTTCTGACCACTGGCTGAACG
hLig3-6F	GATCCACAGATCTGCTTTCATGGACTTCAAGAGAGTCCATGAAGCAGATCTGTTTTTTTGGAAA
hLig3-6R	AGCTTTTCCAAAAAAGAGATCTGCTTTCATGGACTCTCTTGAAGTCCATGAAGCAGATCTGTG

dent *ligA* of *Escherichia coli* were able to substitute for LIG3. Finally, the loss of *Lig3* was also reported to be lethal in chicken DT40 cells, unless preemptively complemented with mitochondrially targeted ligase (21). Provided that LIG3 is not involved in mitochondrial processes other than mtDNA replication and repair, the expected outcome of the *Lig3* knock-out is a loss of mtDNA. This explains the lethality of the *Lig3* knock-out in animal models but not in cultured cells. Cells lacking mtDNA (ρ^0 cells) are viable in appropriately formulated media and can be obtained with reasonable ease through chronic treatment of cultures with DNA intercalators such as ethidium bromide (EtBr) (22, 23), ditercalinium chloride (24–26), or by targeting a restriction endonuclease to mitochondria (27). Therefore, the previously observed lethality of the *Lig3* knock-out in cultured cells may suggest a possible novel role for the LIG3. Here, in contrast, we demonstrate that LIG3 does not play an indispensable role in cultured cells and that viable *Lig3*^{-/-} mouse embryonic fibroblasts (MEFs) can be readily engineered and maintained in culture. Furthermore, we show that LIG3 is present in mitochondria in amounts exceeding those required for mtDNA maintenance under routine cell culture conditions.

EXPERIMENTAL PROCEDURES

Cell Growth and Treatment—Unless specified otherwise, all cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 50 μ g/ml gentamycin, 50 μ g/ml uridine, and 1 mM sodium pyruvate in a humidified atmosphere containing 5% CO₂ at 37 °C.

Mouse Embryonic Fibroblasts—Cre-TM mice (JAX 004682, B6.Cg-Tg(CAG-cre/Esr1)5Amc/J) were intercrossed with *Lig3*^{LoxP} mice (19) to provide tamoxifen-inducible cre expression driven from the actin promoter. *Lig3*^{Cre-TM} mouse embryonic fibroblast (MEF) cell cultures were prepared from E13.5 embryonic mesenchyme. Embryonic tissue was minced using dissection scissors, trypsinized, filtered through a sterile 40- μ m sieve, and resuspended in DMEM supplemented with 10% fetal bovine serum (v/v), 1 \times GlutaMAX, 100 units/ml penicillin, 100 μ g/ml streptomycin, and β -mercaptoethanol. Cells were allowed to proliferate in T-25 tissue culture flasks (Falcon) at 37 °C in a humidified CO₂-regulated (5%) incubator and were split at a 1:4 dilution when the monolayer was confluent (24 h post-isolation). MEFs were immortalized with retroviral construct 3315, a derivative of pSF91 (28) which encodes SV40 large T antigen (Fig. 1A), and selected depending upon their

ability to form colonies. One clone was selected for its superior growth characteristics and designated Cre4. This clone was further modified by introducing a Tet-On transactivator with retrovirus rv2641 (29), thus producing a clone designated 4B6. To a loss of mtDNA, Cre4 cells were treated with 5 μ g/ml EtBr for 3 weeks and cloned. One of the clones (clone 11) demonstrated an absence of mtDNA as determined by both PCR and Southern blotting (data not shown) and was used in subsequent experiments.

Lig3 knockdown in HeLa cells was performed using pSilencerTM 5.1-H1 Retro (Invitrogen). Six shRNA constructs were generated by cloning annealed complementary oligonucleotides (Table 1) into BamHI-HindIII digested vector. Retroviral supernatants were generated using Phoenix-Ampho cells as described earlier (29). HeLa cells were infected with retroviral supernatants overnight in the presence of 10 μ g/ml Polybrene and selected with 3 μ g/ml puromycin.

Quantitative Southern Blotting—Quantitative Southern blotting under alkaline (denaturing) conditions (QSBA) was performed essentially as described earlier (30). Quantitative Southern Blotting under the nondenaturing conditions (QSBN) was performed similarly, except that there was no alkaline pretreatment of DNA samples, and no NaOH was included in the loading dye, agarose gel, or the electrophoresis buffer (electrophoresis was performed using TBE buffer). BamHI-digested total human DNA was separated in appropriately formulated 0.6% agarose gels overnight. After blotting, the membrane was cut at the level of the 9-kb band of λ /HindIII marker. The top portion was then hybridized with the mtDNA probe (detects a 16,569-bp fragment), and the lower portion was hybridized with the 18 S rDNA probe (5,102-bp fragment). After hybridization, membranes were exposed to an imaging screen to determine band intensity. The number of pixels per band was determined by encompassing bands with identical rectangular regions of interest and subtracting the background.

The DNA break frequency per analyzed DNA fragment (16,569 bp for mtDNA and 5,102 bp for nDNA) was determined using the Poisson expression ($s = -\ln P_0$, where s is the number of breaks per fragment, and P_0 is the fraction of fragments free of breaks). Break frequency was expressed as a number of breaks per 10,000 bp. The percent mtDNA degradation was determined by means of QSBN as % = $(C - 6h)/C \times 100\%$, where C is intensity of the band in control and $6h$ is intensity of the band at 6 h.

Low Levels of Lig3 Support Normal mtDNA Copy Number

TABLE 2

Primers and probes used for quantitation of mtDNA copy number by TaqMan qPCR

Human mtDNA F2198 R2353 hMito probe3:	AAAGCGTTCAAGCTCAACACCCAC TAATCTGACGCAGGCTTATGCGGA 6-FAM/TGAACCTCCT/ZEN/CACACCCAATTGGACCA/IABkFQ
Human nDNA hTertTaqManF hTertTaqManR hTertTaqManProbe	CAAGCACTTCCTCTACTCCTC TGGAACCCAGAAAGATGGTC TEX615/CACGAGCCTCCGAGCGCCAG/IABkRQ
Mouse mtDNA rtF-mtDNA rtR-mtDNA mtDNA-probe	ACTTCTAACTAAAAGAATTACAGC TAGACGAGTTGATTCATAAAATTG 6-FAM/CCCGAAACC/ZEN/AAACGAGCTACCT/IabFQ
Mouse nDNA rtF-mTert rtR-mTert mTret-probe	CCTCAAGCATTACCTCTTCTTTG CCAAGGACCTGCTCGATGAC TEX613-Y/ACCACCTCTCTGACTCCAGCCA/IabRQ

The frequency of double strand breaks (DSB) was determined using QSB; the combined frequency of DSB and single-strand breaks (SSB) was determined using QSBA, and the frequency of SSB was determined by subtracting the frequency of DSB from the combined frequency of SSB and DSB.

Determination of mtDNA Copy Number—mtDNA copy number was estimated by QSB as the ratio of the intensities of the bands corresponding to mtDNA and 18 S DNA. Precise determination of mtDNA copy number was performed using TaqMan qPCR with the primers and probes listed in the Table 2. For human cells, simplex qPCR was employed, and for mouse cells duplex qPCR was employed. To generate a standard curve for both human and mouse DNA, a separate calibrator plasmid containing cloned nuclear and mitochondrial targets was used.

Isolation of mitochondrion-enriched fractions was performed according to Pallotti and Lenaz (31), with some modifications. Briefly, cells were collected by centrifugation, washed with PBS, and frozen until isolation. For isolation, cells were thawed, resuspended in buffer containing 0.21 M mannitol, 70 mM sucrose, 10 mM KH_2PO_4 , 5 mM HEPES, 0.5 mM EGTA, 1× Halt protease inhibitor mixture (Thermo Fisher Scientific, Waltham, MA), pH 7.4, and disrupted by five passages through a 27-gauge needle. Cell debris was removed by centrifugation for 5 min at $1,200 \times g$, 4 °C, and mitochondria were recovered from the supernatant by centrifugation for 5 min at $13,000 \times g$, 4 °C.

Clonogenic Assays—For clonogenic assays, cells were collected by trypsinization, counted using a Coulter counter, plated at a density of 250 cells per 60-mm dish, and allowed to attach overnight. The next day medium was removed; cells were washed once with PBS and exposed to various concentrations of either H_2O_2 or MMS in HBSS for 30 min at 37 °C in an atmosphere of 5% CO_2 . After treatment, the medium was replaced, and plates were left in the incubator to allow for the formation of colonies. Upon formation of colonies, they were washed with PBS, fixed with methanol/acetic acid (3:1), stained with hematoxylin, washed with deionized water, dried, and counted. Plating efficiency was expressed as percentage of untreated control. A survival and proliferation assay was performed essentially as the clonogenic assays, except experimental plates were trypsinized and cells counted, instead of being fixed and stained.

Mitochondrial membrane potential was measured with JC-1. The cells were plated in 12-well plates, allowed to attach overnight, and incubated with 5 μM JC-1 in DMEM for 10 min at 37 °C in an atmosphere of 5% CO_2 . After treatment, cells were trypsinized and immediately subjected to flow cytometry on FAC-SCantoII (BD Biosciences). Membrane potential was expressed as a ratio of fluorescence intensities in the phycoerythrin and FITC channels.

Mitochondrial respiration in whole attached cells was measured with the help of an XF-24 extracellular flux analyzer (Seahorse Biosciences, Billerica, MA) according to the manufacturer's recommendations and expressed as picomoles/min/ μg of protein. ATP-linked respiration was determined with the help of oligomycin (5 μM); maximal respiration was induced with carbonyl cyanide *m*-chlorophenyl hydrazine (1 μM), and non-mitochondrial respiration was determined after injection of rotenone and antimycin A (1 μM each). Transient mitochondrial DNA depletion was achieved by treating cells for 3 days with 5 $\mu\text{g}/\text{ml}$ EtBr.

Genotyping ρ^0 Cells—To test for a ρ^0 genotype, total DNA was amplified with two pairs of primers (duplex reaction). Primers 4f (GCCTACACCCAGAAGATTTTCAT) and 4r (AGACAGTTGGACCCTCGTTTAG) were used to amplify 1,000 bp of mouse mtDNA between coordinates 1,075 and 2074. Primers mPolGchrF4 (ACTGGATGGATATCAGCAGTGCCA) and mPolGchrR4 (ATGTGTTCTGTGCCTCTGTCAGGT) were used to amplify the 629-bp fragment encompassing intron 17 and parts of exons 17 and 18 of the *PolG* gene, which encodes mitochondrial DNA polymerase γ . PCR was performed using the following parameters: denaturation at 95 °C for 1 min and then 35 cycles of 94 °C (10 s), 55 °C for 20 s, 68 °C for 1 min, and a final extension of 68 °C for 3 min.

Genotyping Lig3 Allele—To genotype the *Lig3* allele, the following primers were used: Lig3- Δ , CAGTCGACGAGATGGCTCAGTGGTTAAGAGC; Lig3-5, GATGCGGCCCGCAGCCAAGTGTGAATATACAGC; and Lig3-8, CAGTCGACAGGGAGCTTGGGACGGATGC.

Lig3-5/Lig3-8 pair amplifies the 555-bp fragment of the *Lig3*^{loxP/loxP} allele, and no amplification was observed with the *Lig3*^{-/-} allele. Conversely, Lig3-5/Lig3- Δ pair amplifies a 560-bp fragment of the *Lig3*^{-/-} allele, and no amplification was observed with *Lig3*^{loxP/loxP} allele. PCR was performed using the

following parameters: denaturation at 95 °C for 1 min and then 35 cycles of 94 °C (10 s), 55 °C for 20 s, 68 °C for 1 min, and final extension of 68 °C for 3 min.

Detection of the transduced human *Lig3* cDNA in mouse cells was performed with primers hLig3cDNAf (TGGCCACA-AAGTCTTCTCCAGTGA) and hLig3cDNAr (CGTGCGTG-GCTGAAGTCATATCAA). These primers amplify the 209-bp fragment using the same cycling parameters as above.

Western Blotting—For Western blot analysis, whole cell or mitochondrial lysates were prepared by lysis in a buffer containing 10 mM Tris, 0.5% SDS, 1× Halt protease inhibitor mixture, pH 8.0. Protein was quantitated by either the Bradford or BCA assay.

Confocal Microscopy—For confocal microscopy, cells were plated into 35-mm glass bottom MatTek dishes, allowed to attach overnight, and imaged with a Nikon AIR confocal microscope using a ×60 water immersion objective.

Statistical Analysis—Pairwise comparisons were performed using the two-tailed unpaired Student's *t* test assuming unequal variances.

RESULTS

Steady-state Levels of LIG3 Are Excessive for the Maintenance of mtDNA Copy Number—To evaluate the effect of reduced cellular LIG3 levels on mtDNA maintenance, six HeLa cell lines expressing shRNA constructs directed at different regions of the *Lig3* mRNA were established (Fig. 1). Constructs 2842-1 and 2842-6 caused a modest knockdown, and construct 2842-2 initiated very efficient knockdown, as estimated by Western blotting. Little or no LIG3 signal was detected by Western blotting of whole cell extracts of WT and HeLa cells transduced with the 2842-2 construct (hereafter designated KD cells) (Fig. 2A). Therefore, to estimate the actual efficiency of the knockdown, Western blotting was performed on serial dilutions of the mitochondrial fraction of WT cells versus the undiluted mitochondrial fraction of KD cells. Using this approach, a 90% knockdown of LIG3 in the mitochondrial fraction of KD cells was estimated (Fig. 2B). Surprisingly, mtDNA copy number determined in KD cells by either QSBN (Fig. 2, C and D) or by TaqMan qPCR (Fig. 2E) failed to reveal any correlation between levels of *Lig3* expression and mtDNA copy number indicating that as little as 10% of the actual LIG3 levels may be sufficient to maintain normal mtDNA copy number in HeLa cells.

Reduction of LIG3 Levels Increases Susceptibility of Cells to Alkylating and Oxidative Damage—To evaluate the effect of *Lig3* knockdown on mitochondrial physiology, mitochondrial membrane potential and respiration were measured in WT and KD cells. Neither membrane potential nor basal, ATP-linked, maximal, or nonmitochondrial respiration differed significantly between the two cell types (Fig. 3, A and B). However, there was a slight decrease in the viability of the KD cells as compared with their WT counterparts in response to treatment with DNA-damaging agents, methyl methanesulfonate (MMS) and H₂O₂ (Fig. 3, C and D). This reduction was significant only at higher doses of MMS (Fig. 3C).

Lig3 Knockdown Enhances mtDNA Degradation in Response to Oxidative Damage—To study the effect of reduced LIG3 levels on mtDNA damage and repair, WT and KD HeLa cells

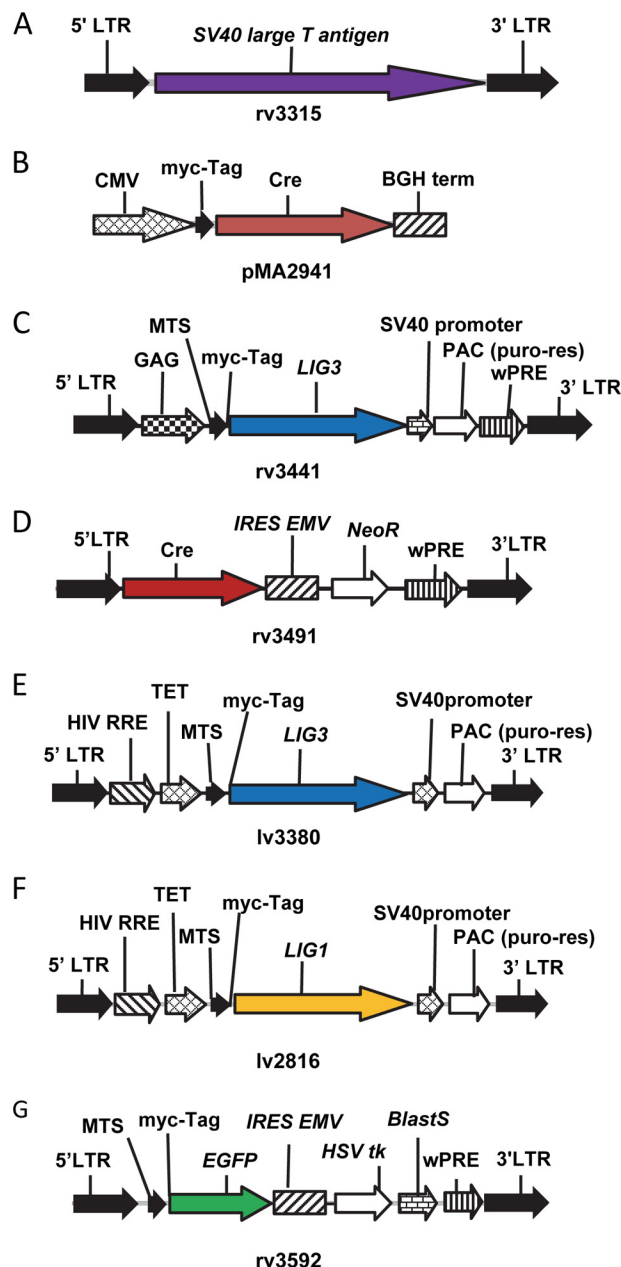


FIGURE 1. DNA constructs used in the study. A, retrovirus rv3315 encodes SV40 large T antigen driven by murine embryonic stem cell virus long terminal repeat (*LTR*) promoter. No antibiotic resistance marker is present. B, plasmid pMA2941 encodes CMV promoter-driven Myc-tagged cre recombinase gene. C, retrovirus rv3441 encodes Moloney murine leukemia virus LTR promoter-driven mitochondrially targeted LIG3. The gene's native MTS and an initiating codon were removed and replaced with MTS from human ornithine transcarbamylase followed by a Myc tag. D, retrovirus rv3491 encodes LTR promoter-driven cre recombinase whose expression is transcriptionally linked (through an internal ribosome entry site, *IRES EMV*) to the expression of the G418 resistance gene. E, lentivirus lv3380 encodes doxycycline-inducible promoter (TET)-driven mitochondrially targeted LIG3 as in C.

were exposed to the S_n2 alkylating agent MMS (32) or to H₂O₂, and mtDNA repair was followed with QSBa and QSBn as described under “Experimental Procedures.” At the concentrations used, MMS induced strand breaks with similar frequency in mtDNA of both WT and KD cells (Fig. 4, A and C). A vast majority of those breaks (>90%) were SSB. In contrast, H₂O₂ induced more strand breaks in mtDNA of KD

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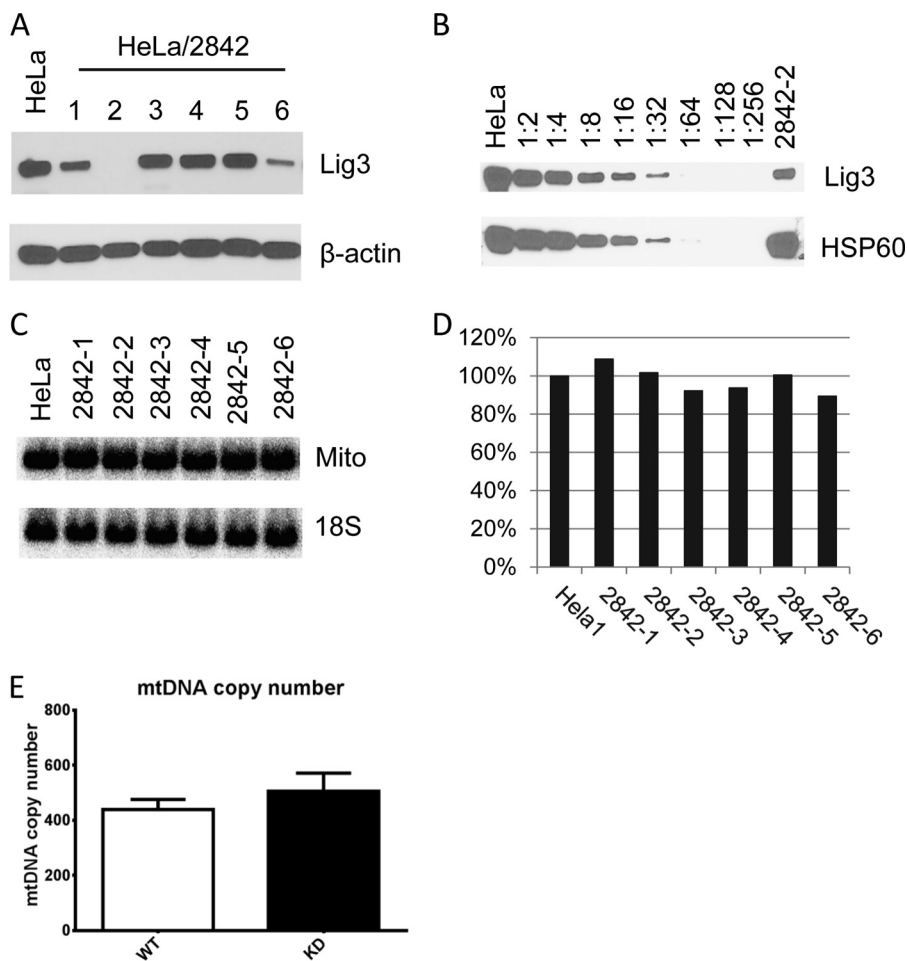


FIGURE 2. Effect of Lig3 knockdown on mtDNA copy number. Six shRNA constructs (2842-1 through 6) were generated, and their effects on Lig3 expression and mtDNA copy number were studied. *A*, Western blots of whole cell lysates from HeLa cells infected with shRNA constructs 2842-1 through 6. β -Actin, loading control. *B*, Western blotting of serial dilutions of the mitochondrial lysate of the WT HeLa cells and undiluted mitochondrial lysate of HeLa/2842-2. Mitochondrial HSP60, loading control. *C*, QSNB of the total DNA from HeLa cells and cells infected with knockdown constructs. Total DNA was digested with BamHI and processed as described under "Experimental Procedures." Mito, mitochondria. *D*, quantitation of the QSNB in *C*. *E*, quantitation of mtDNA copy number in WT HeLa cells and HeLa/2842-2 by TaqMan qPCR. Error bars, S.E., $n = 3$.

cells. A majority of those were also SSBs (Fig. 4, *B* and *C*). Of note, H_2O_2 , but not MMS, stimulated mtDNA degradation in KD cells, even though MMS induced twice as many strand breaks (Fig. 4*D*).

LIG3 Is Dispensable for the Viability of MEFs—Previous studies reported that mitochondrial, but not nuclear, LIG3 is required for viability of MEFs (18), mouse embryonic stem cells (20), and chicken DT40 cells (21). The lethality of the *Lig3*^{-/-} phenotype observed in those studies leaves open the possibility that mitochondrial DNA ligase activity was involved in an essential process other than mtDNA transactions. To resolve this issue, Cre4 cells were transfected with plasmid pMA2941 (Fig. 1*B*), which encodes cre recombinase, and selected for resistance to G418 in medium supplemented with uridine and pyruvate. Some of the resulting clones grew poorly and demonstrated the expected deletion in *Lig3* and a concomitant loss of mtDNA (Fig. 5*A*). To see whether the loss of mtDNA can be rescued by expressing LIG3 in mitochondria, Cre4 cells were simultaneously infected with two retroviruses, rv3441 and rv3491 (Fig. 1, *C* and *D*). rv3441 encodes LIG3 from which the native MTS and initiating codon for the nuclear isoform were removed and replaced with the MTS from human ornithine

transcarbamylase (33) followed by a Myc tag. rv3491 encodes cre recombinase. Co-infection with these two viruses allowed simultaneous cre-mediated excision and complementation of the deletion with LIG3 encoded by the second virus. As expected, mitochondrial LIG3 prevented the loss of mtDNA in Cre4/3441/3491 cells. Additional experiments demonstrated that in agreement with a previous report (20), neither nuclear LIG3 nor catalytically inactive mitochondrial LIG3 was able to rescue the loss of mtDNA, although mitochondrially targeted LIG1 prevented mtDNA loss (Fig. 5, *B* and *D*, and results not shown). Western blotting with monoclonal antibodies directed against LIG3 protein confirmed the loss of LIG3 expression in Cre4/2941 ρ^0 , but not in Cre4#11 ρ^0 cells, which were derived by treatment with EtBr rather than by introducing a deletion into the *Lig3* allele. However, no LIG3 expression was detected in whole cell lysates from the Cre4/3441/3491 cells, even though these cells did not exhibit any reduction in mtDNA copy number as compared with parental Cre4 cells (Fig. 5, *C* and *E*). Nevertheless, LIG3 was detectable in the mitochondrial fraction from these cells, although its level of expression was only ~3% of that found in WT mitochondria (Fig. 5*F*). The loss of mtDNA was accompanied by shortening and swelling of

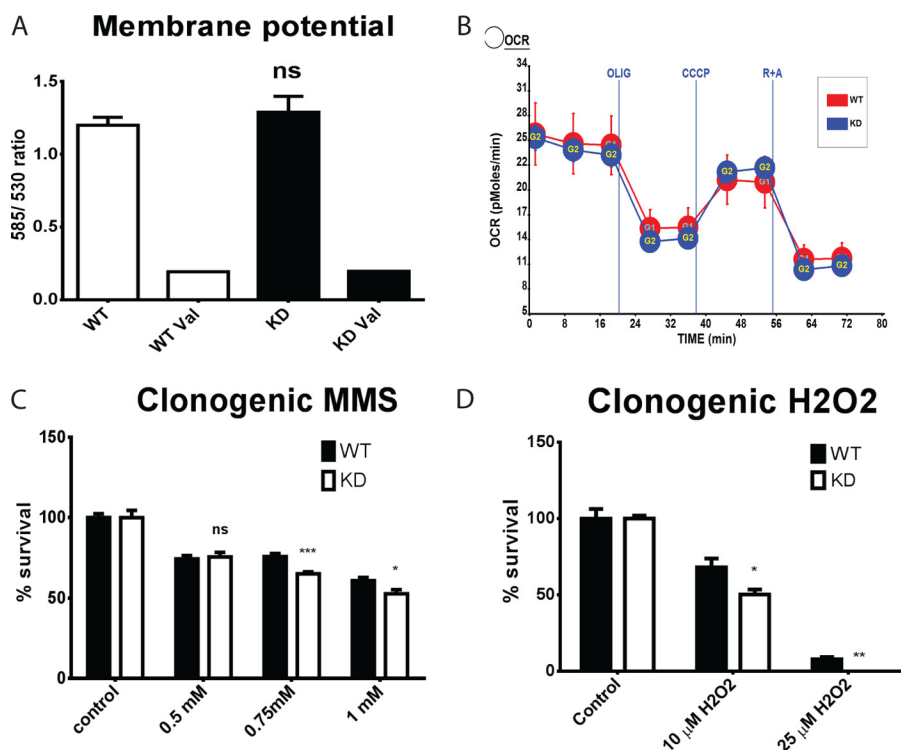


FIGURE 3. **Effect of Lig3 knockdown on physiological responses of the HeLa cells and DNA repair.** *A*, effect of Lig3 knockdown on mitochondrial membrane potential as measured by JC-1 fluorescence. *Val*, 0.2 μ M valinomycin (ionophore). *B*, effect of Lig3 knockdown on cellular respiration as measured by an XF-24 analyzer. Oxygen consumption is expressed in picomoles of O₂/min/ μ g of protein. *C* and *D*, effect of Lig3 knockdown on susceptibility to DNA-damaging agents MMS (*C*) and hydrogen peroxide (H₂O₂, *D*) as determined by clonogenic assay. Two-tailed Student's *t* test assuming unequal variance; *n* = 5 or 6. *ns*, not significant; *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001.

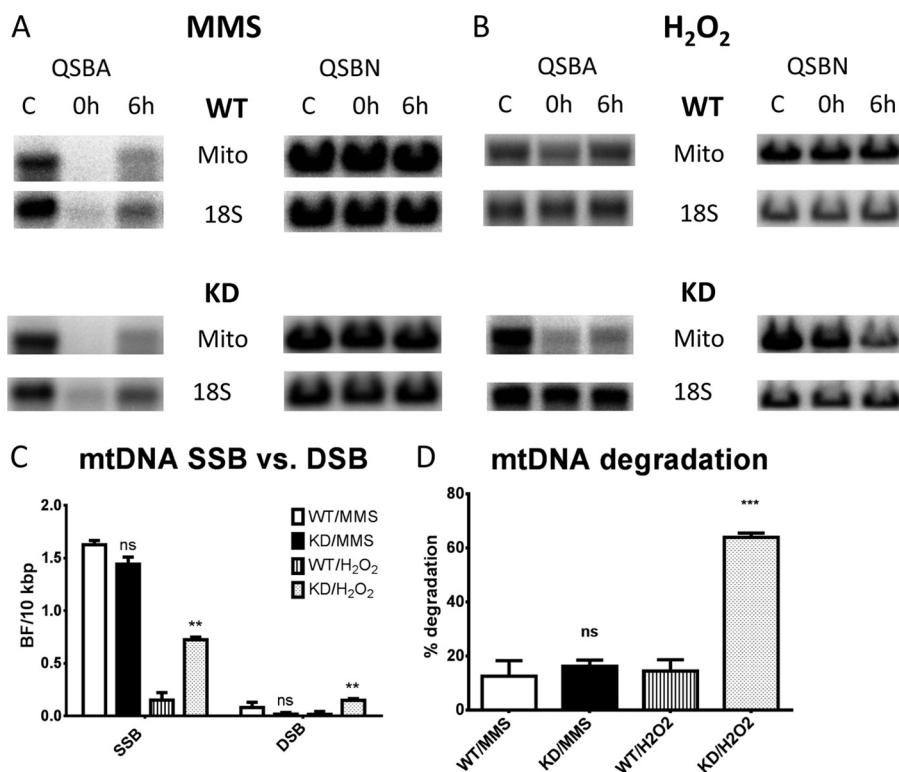


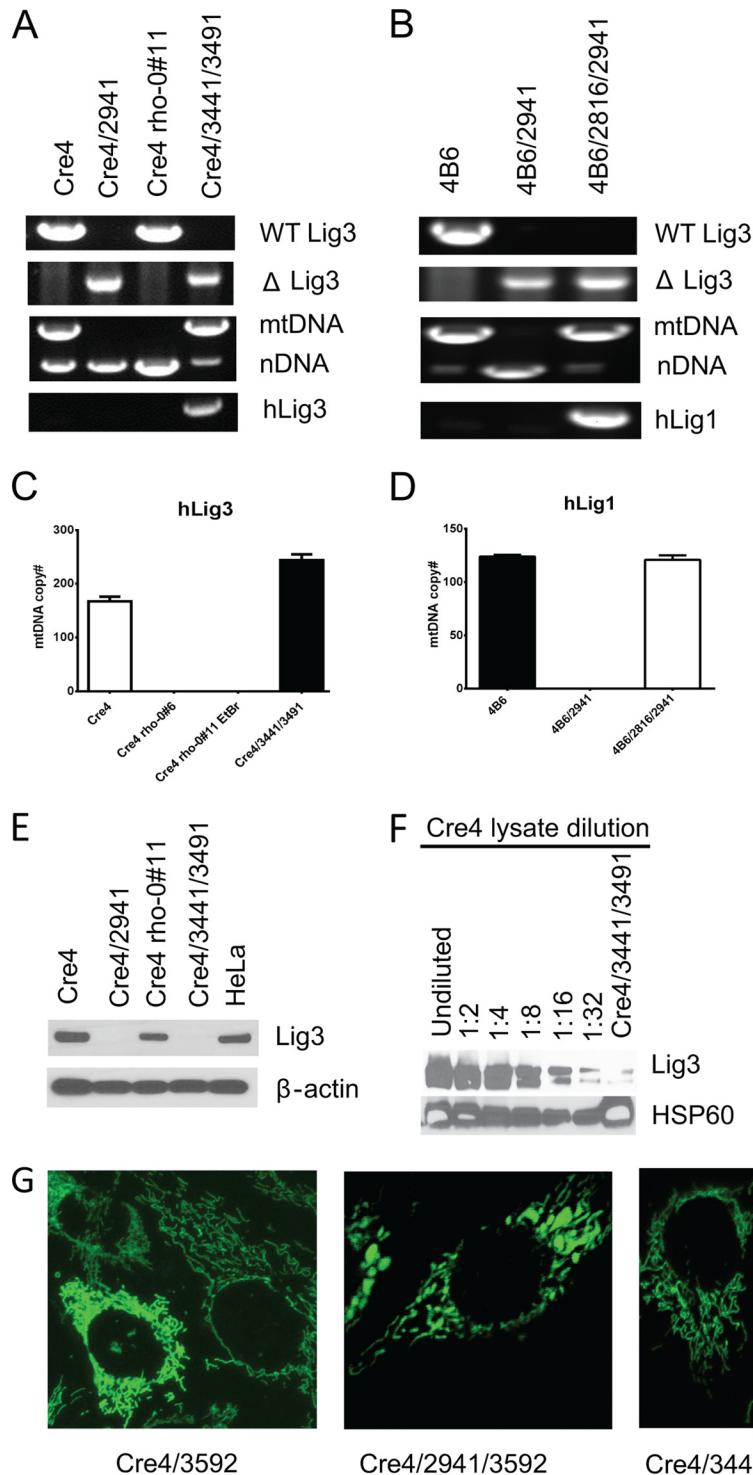
FIGURE 4. **Effect of LIG3 knockdown on mtDNA repair.** *A* and *B*, WT and KD cells were treated with either 2 mM MMS (*A*) or 100 μ M H₂O₂ (*B*) for 30 min at 37 °C in the humidified atmosphere of 5% CO₂. Total cellular DNA was extracted either immediately after the treatment (0 h) or after 6 h of recovery in fresh DMEM (6 h), digested with BamHI, and subjected to QSBA and QSNB. *C*, frequencies of SSB and DSB induced in mtDNA by MMS and H₂O₂ as measured immediately after treatment (0-h time point). *D*, % mtDNA degradation as determined by QSNB at the 6-h time point. Error bars, S.E., *n* = 4. *C* and *D*, two-tailed Student's *t* test assuming unequal variance, WT versus KD. *ns*, not significant; *Mito*, mitochondria; **, *p* < 0.01; ***, *p* < 0.001.

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mitochondria, which was not observed in Cre4/3441/3491 cells, in which loss of mtDNA was prevented by complementation with hLIG3 (Fig. 5G).

Lig3^{-/-} Cells Are Dependent on Uridine and Pyruvate for Their Survival and Growth—It has been demonstrated that loss of mitochondrial DNA induced by means other than deletion of the *Lig3* (34) leads to the dependence of the resulting cell lines on media supplementation with uridine and pyruvate for survival and growth. Therefore, the Cre4 cell line as well as its

derivatives Cre4/2941 (*Lig3*^{-/-}), Cre4 ρ^0 #11 (obtained by EtBr treatment), and Cre4/3441/3491 were exposed to DMEM with and without uridine and pyruvate. Within 24 h of exposure to the medium without uridine and pyruvate, Cre4/2941 cells stopped their growth, and cell death was clearly detectable within 48 h (Fig. 6A). Cre4 ρ^0 #11 cells also died in media without uridine or pyruvate, although with somewhat slower kinetics. Both cell lines survived and grew in media containing uridine and pyruvate. Cre4 and Cre4/3441/3491, cell lines that



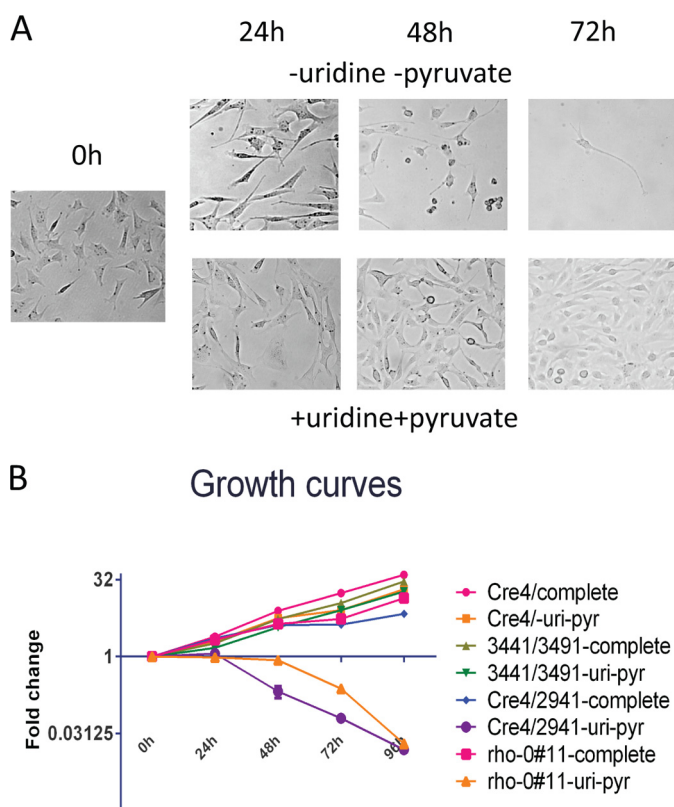


FIGURE 6. $Lig3^{-/-}$ cells are dependent on uridine and pyruvate for their survival and growth. *A*, representative microscopic field views of Cre4/2941 cells exposed to media with and without uridine and pyruvate for 0 h (immediately after media change) and 24, 48, and 72 h after media change. *Top panels*, DMEM without uridine or pyruvate supplementation. *Bottom panels*, DMEM supplemented with uridine and pyruvate. Cells proliferate in media supplemented with uridine and pyruvate and die in unsupplemented media. *B*, growth curves of Cre4, Cre4/2941, Cre4 ρ^0 #11, and Cre4/3441/3491 in media with or without uridine and pyruvate. Note that neither Cre4 ρ^0 #11 (ρ^0 due to EtBr treatment) nor Cre4/2941 (ρ^0 due to $Lig3$ deletion) survive in media without uridine or pyruvate.

contained mtDNA, grew regardless of supplementation with uridine and pyruvate (Fig. 6*B*).

Low Levels of Lig3 Support Normal mtDNA Replication and Cellular Respiration—To exclude positional effects of gene integration and to better evaluate cellular requirements for $LIG3$ under different conditions, we deleted $Lig3$ in MEFs that were engineered for doxycycline-inducible expression of the $LIG3$ with a retrovirally encoded reverse tetracycline transactivator (4B6, see under “Experimental Procedures”) and lentivirus 3380, which encodes Myc-tagged human $LIG3$ (Fig. 1*E*). After confirming proper deletion in the endogenous $LIG3$ gene

and introduction of the $LIG3$, we observed that mtDNA was present in cells even in the absence of $LIG3$ induction (Fig. 7*A*). Moreover, induction did not alter mtDNA copy number (Fig. 7*B*), even though $LIG3$ was undetectable in whole cell lysates from uninduced cells, as determined by Western blotting with antibodies directed against either $LIG3$ or the Myc tag (Fig. 7*C*). Apparently, low levels of “leaky” $LIG3$ expression were sufficient to support mtDNA replication. Indeed, Western blotting of serial dilutions of the mitochondrial fraction from the induced cells side-by-side with the undiluted mitochondrial fraction from uninduced cells revealed that $LIG3$ content of mitochondria in uninduced cells was less than 1% of that in induced cells (Fig. 7*D*). These dramatically reduced levels of $Lig3$ supported normal mtDNA replication and respiration (Fig. 7, *B* and *F*). Similarly, mitochondrial levels of $LIG3$ in the WT 4B6 cells were more than 100-fold higher than those in uninduced 4B6/3380/2941 cells (Fig. 7*E*). Even though mtDNA copy number in uninduced 4B6/3380/2941 cells was lower than that in the WT 4B6 cells, this difference is not likely to be due to the difference in the $Lig3$ expression. Indeed, both induced and uninduced 4B6/3380/2941 cells have reduced mtDNA copy number as compared with the WT 4B6 cells, even though $LIG3$ levels in the WT 4B6 and induced 4B6/3380/2941 cells are comparable (Fig. 7, *B–E*).

Low Levels of $LIG3$ Increase Susceptibility to Alkylating and Oxidative Damage, Increase mtDNA Degradation in Response to Oxidative Stress, and Delay Recovery of mtDNA Levels after Depletion— $LIG3$, the only mitochondrial DNA ligase, catalyzes the final step in both SSB repair and BER in mitochondria. Because mtDNA integrity has been implicated in cell viability (35–42), it is conceivable that reduced $LIG3$ levels may sensitize cells to agents like MMS and H_2O_2 , which produce lesions in mtDNA that are repaired by either BER or SSB repair pathways. Indeed, uninduced cells, which expressed less than 1% of $LIG3$ as compared with induced cells, were more sensitive to both MMS and H_2O_2 (Fig. 8, *A* and *B*). Moreover, they demonstrated more DSB and mtDNA degradation in response to oxidative stress (Fig. 8, *C* and *D*). $LIG3$ is also the only ligase available for the replication of mtDNA. Therefore, we examined the effect of reduced $LIG3$ expression on the recovery of mtDNA copy number after depletion with EtBr. After 3 days of exposure to 5 μ g/ml EtBr, mtDNA copy number declined to similar levels in both induced and uninduced cells. However, after the removal of EtBr, the dynamics of mtDNA copy number per cell differed significantly between cells with high and low $LIG3$ lev-

FIGURE 5. $Lig3$ is dispensable for the viability of MEFs. *A*, PCR genotyping demonstrates the presence of the WT $Lig3$ allele in Cre4 and Cre4 ρ -0#11 (EtBr-derived ρ^0) cell lines, whereas a deleted allele is present in the Cre4/2941 and Cre4/3441/3491 cell lines due to Cre-mediated floxing out. Deleted $Lig3$ allele in Cre4/2941 leads to a loss of mtDNA. However, mtDNA is present in Cre4/3441/3491 despite deletion in the $Lig3$ allele due to complementation with human $Lig3$ encoded by rv3441. This complementing $hLig3$ gene is detectable by PCR. WT $Lig3$ primers amplify the loxP/loxP allele but not the deleted allele. Conversely, $\Delta Lig3$ primers amplify deleted $Lig3^{-/-}$ allele but not the WT allele. mtDNA and nDNA primers amplify, in the same tube, mitochondrial and nuclear DNA, respectively. $hLig3$ primers amplify cDNA for the human $Lig3$ but not the endogenous mouse gene. *B*, PCR genotyping of the original 4B6 cell line, 4B6 cells in which deletion of the $Lig3$ allele was induced with Cre recombinase (4B6/2941), and 4B6 cells complemented with doxycycline-inducible mitochondrially targeted human $Lig1$ gene prior to excision of the endogenous $Lig3$ allele (4B6/2816/2941). See Fig. 1*F* for the map of lv2816. *C* and *D*, quantitation of mtDNA copy number in the same cell lines by real time TaqMan qPCR. Mitochondrially targeted $hLig1$ supports normal levels of mtDNA in 4B6/2816/2941 even without doxycycline induction due to the “leaky” expression from the Tet promoter. *Error bars*, S.E., $n \geq 3$. *E*, Western blotting of the whole cell lysates of the same cell lines. Note that $Lig3$ expression is not detectable in the Cre4/3441/3491 cell line even though antibodies can detect both mouse (Cre4) and human (HeLa) proteins and even though this cell line normally maintains mtDNA, which suggests low expression. *F*, reduction in the $Lig3$ expression levels in Cre4/3441/3491 cells was estimated by Western blotting of the mitochondrial fractions of the Cre4/3441/3491 cells versus dilutions of the mitochondrial fraction of the Cre4 cells. *G*, the loss of mtDNA induces morphological changes in mitochondria. Cre4 cells (WT), Cre4/2941(ρ^0 , $\Delta Lig3$), and Cre4/3441/3491 ($\Delta Lig3$, complemented) were infected with rv3591 (Fig. 1*G*), which encodes mitochondrially targeted enhanced GFP and imaged by confocal microscopy.

Low Levels of Lig3 Support Normal mtDNA Copy Number

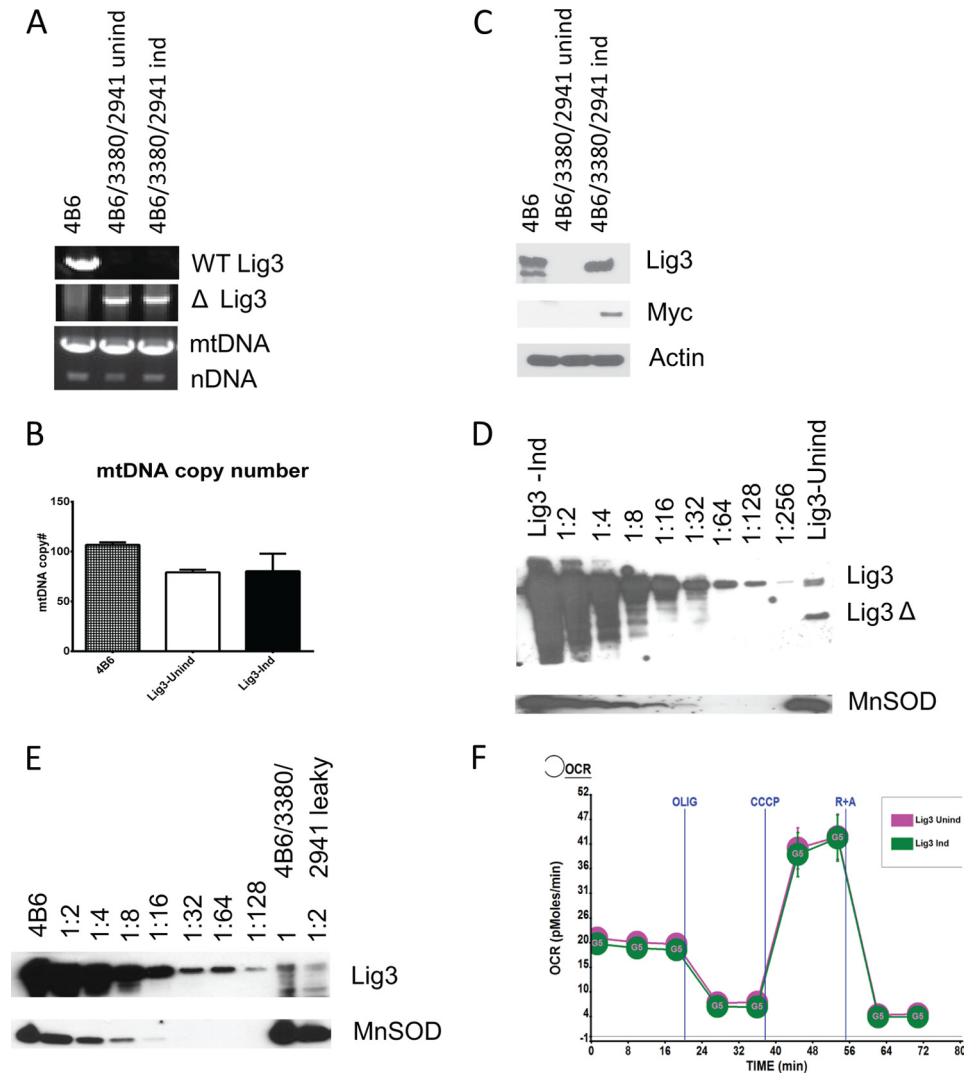


FIGURE 7. Low levels of Lig3 are sufficient to support normal mtDNA replication and cellular respiration. *A*, PCR genotyping of the 4B6 cells and their derivative obtained by simultaneous deletion and complementation of the Lig3. Note the presence of mtDNA in both induced (*Ind*) and uninduced (*Unind*) cells. *B*, quantitation of the mtDNA copy number in the 4B6 cell line and its deleted/complemented derivative (4B6/3380/2941) grown in the presence (*Ind*) or absence (*Unind*) of doxycycline (inducer). *C*, Lig3 expression in the 4B6 cell line and its deleted/complemented derivative 4B6/3380/2941, induced (*Ind*) and uninduced (*Unind*). Note that Lig3 in rv3380 is tagged with Myc tag. *D*, estimation of the relative levels of the Lig3 in induced and uninduced 4B6 cells with deletion/complementation. *MnSOD*, loading control. *E*, relative levels of the mitochondrial Lig3 in the WT 4B6 cells versus uninduced/complemented (4B6/3380/2941) cells. *F*, mitochondrial respiratory profiles of the induced and uninduced Lig3 deleted/complemented 4B6 cells. Labels as in Fig. 3*B*. Oxygen consumption expressed in picomoles of O₂/min/μg of protein. Error bars, S.E.; OCR, oxygen consumption rate.

els. In cells with high LIG3 expression, mtDNA copy number remained stable for the first 24 h after the removal of EtBr, and then began to rise quickly, essentially restoring mtDNA copy number within 4 days. In contrast, in uninduced cells, mtDNA copy number continued to drop for the first 24 h after EtBr withdrawal, and then remained stable for another 48 h. Only 3 days after EtBr removal did mtDNA copy number began to rise in these cells, and even then at a slower rate than in induced cells (Fig. 8*E*). Cells with low levels of LIG3 grew somewhat faster than their induced counterparts during depletion. This trend continued for the first 24 h after EtBr removal, but then the growth rate of induced cells surpassed that of uninduced cells (Fig. 8*F*). Examination of the total mtDNA copy number in the population (an extrapolated product of cell number and mtDNA copy number per cell) revealed that for the first 24 h after the removal of EtBr, the total mtDNA copy number in the

population of uninduced cells continued to decline, indicating mtDNA degradation. In contrast, the level of mtDNA in the population of induced cells remained stable during this period. Beyond 24 h of EtBr removal, the total mtDNA copy number in both populations grew, although it grew faster in the population of induced cells (Fig. 8, *G* and *H*).

DISCUSSION

Experimental evidence collected so far points to the possibility that LIG3 is essential for cellular viability due to the role it plays in mitochondria (12). Indeed, previous attempts to isolate viable cells from whole body knock-out embryos (18), as well as attempts to inactivate both *Lig3* alleles in mouse embryonic stem cells (20) or in DT40 cells (21), all failed. Here, we report the generation, *in vitro*, of viable MEFs that carry a deletion in both alleles of *Lig3*. These cells are completely devoid of

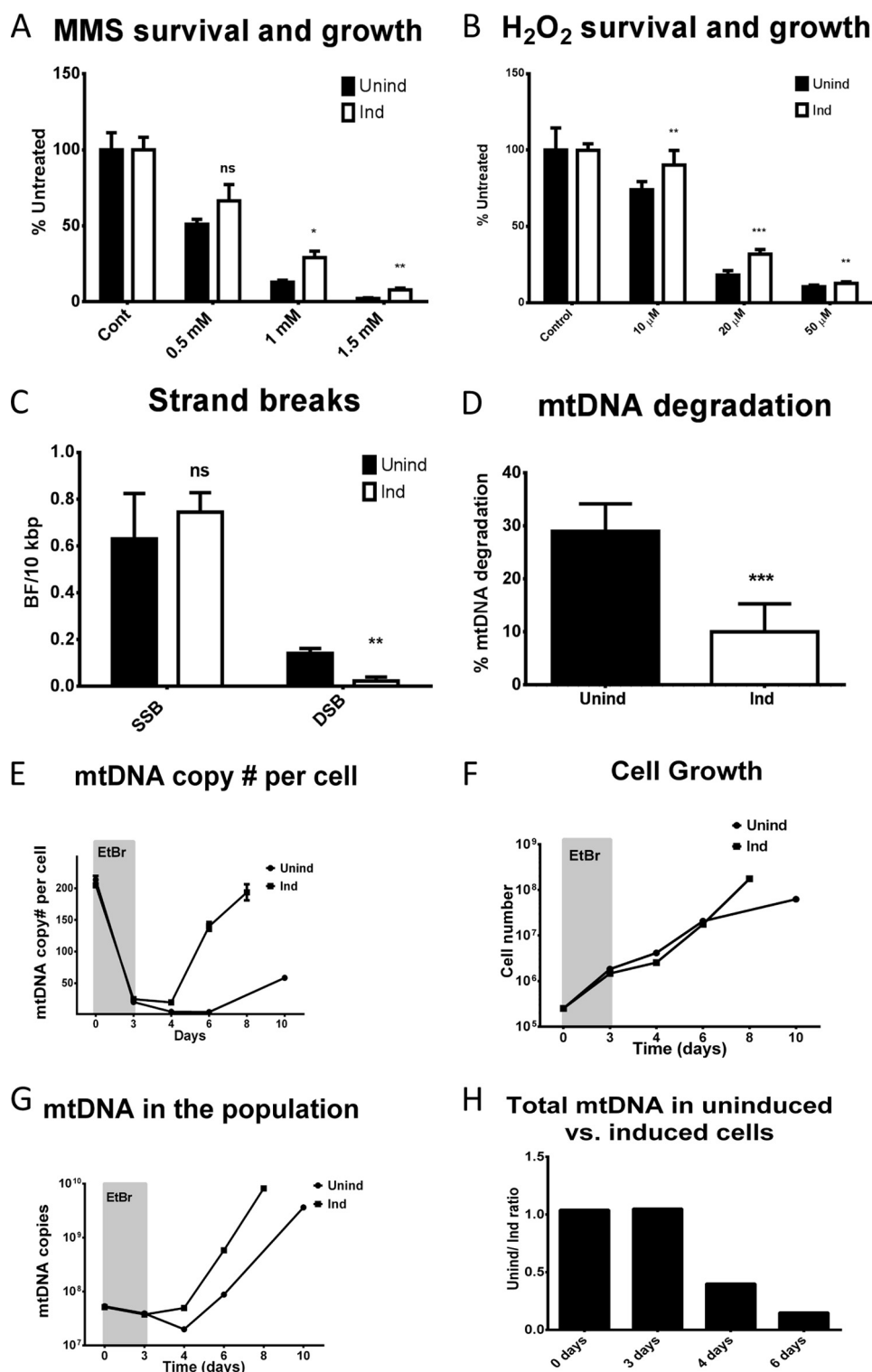


FIGURE 8. Low levels of Lig3 increase susceptibility to alkylating and oxidative damage, increase mtDNA degradation in response to oxidative stress, and delay recovery of mtDNA levels after depletion. *A* and *B*, survival and growth of induced and uninduced *Lig3* deleted/complemented 4B6 cells (4B6/3380/2941) in response to alkylating and oxidative damage, respectively. *Error bars*, S.E., $n = 6$, two-tailed Student's *t* test assuming unequal variance. *Cont*, control. *C* and *D*, frequency of strand breaks and extent of mtDNA degradation, respectively, in induced and uninduced 4B6/3380/2941 cells in response to treatment with H₂O₂. *Error bars*, S.E., $n = 3$ or 4, two-tailed Student's *t* test assuming unequal variance. *ns*, not significant, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *E–H*, dynamics of mtDNA copy number per cell, cell growth, dynamics of the total mtDNA copy number in the population, and the ratios of the total mtDNA copy numbers in the populations of induced and uninduced cells at different time points during depletion-repletion, respectively. Induced (*Ind*) and uninduced (*Unind*) cells were subjected to 3 days of mtDNA depletion with 5 μ g/ml EtBr (grayed areas on the graphs) followed by release. *Error bars*, S.E.; $n = 3$ for 0-h time point and $n = 5$ for remaining time points. Aliquots of cells were withdrawn at different time points, followed by determination of the total cell number as well as mtDNA copy number per cell. Note that induced and uninduced cells have similar starting mtDNA copy numbers and deplete with similar kinetics, yet show different repopulation kinetics upon release.

Low Levels of Lig3 Support Normal mtDNA Copy Number

mtDNA and die, as do other ρ^0 cells (34), unless the medium is supplemented with uridine and pyruvate. This may explain why previous attempts to isolate viable *Lig3*^{-/-} cells were unsuccessful (18–21). Consistent with previous findings (20), the loss of mtDNA was readily prevented by introducing, simultaneously with *Lig3* deletion, a retrovirus-encoded *LIG3* or *LIG1* that was engineered to localize exclusively to mitochondria, but not by introducing an enzymatically inactive mitochondrial or a nuclear *LIG3*.

Unexpectedly, both HeLa and MEF cells severely depleted of *LIG3* were able maintain normal mtDNA copy number, membrane potential, and respiration. This indicates that steady-state levels of *LIG3* are excessive for routine mtDNA maintenance and contradicts a previous report that the decrease in *LIG3* level leads to a reduction in mtDNA copy number (43). The reason for this discrepancy is not clear, but it is worth noting that in a previous study an observed molecular weight of *LIG3* (approximately 80 kDa) was considerably less than predicted (113 kDa, UniProtKB P49916) and that when quantitating, no attempt was made to normalize mtDNA to nDNA.

After depletion with EtBr, cells with reduced *LIG3* expression demonstrated slow growth, an extended period of mtDNA degradation, a delayed recovery of mtDNA copy number, and a slower mtDNA replication. Taken together, these findings independently factor in on the debate pertaining to the mechanism of mtDNA replication, which has remained the subject of controversy for over a decade. Two models have been proposed as follows: the strand-asynchronous “Clayton model” in which replication is initiated at the origin of the heavy strand and proceeds $\frac{2}{3}$ of the mtDNA circle prior to exposing the origin of the light strand and initiating replication of the second strand (44). The strand-asynchronous model does not involve synthesis (and hence ligation) of Okazaki fragments and therefore predicts a minimum requirement for *LIG3*. The alternative strand-coupled model (45) involves a traditional replication fork with continuously synthesized leading strand and lagging strand synthesized through Okazaki fragments. This model has a greater reliance on *LIG3* activity because it necessitates ligation of Okazaki fragments. Our observations favor both strand-asynchronous and bimodal mechanisms for mtDNA replication over the strand-coupled model. The bimodal mechanism was first proposed (46) and later abandoned by the authors in favor of the strand-coupled model (45). It postulates that during routine maintenance, mtDNA replicates predominantly according to the strand-asynchronous mechanism, while during periods of recovery of mtDNA copy number from depletion, replication occurs predominantly according to the strand-coupled mechanism. Therefore, the bimodal model predicts only a minimal requirement for *LIG3* to circularize mtDNA after the completion of replication during routine maintenance and a higher reliance for mtDNA replication on *LIG3* during recovery from depletion due to the need to ligate Okazaki fragments in the lagging strand. We propose that the apparently excessive steady-state levels of *LIG3* observed in cells are maintained to efficiently cope with various eventualities, such as degradation of mtDNA due to oxidative damage (47). Such a loss would have to be compensated for by mtDNA replication, which requires higher levels of *LIG3*.

HeLa cells with reduced expression of *LIG3* showed increased mtDNA degradation in response to H₂O₂ but not to MMS. Similarly, reduced levels of *LIG3* were associated with increased H₂O₂-induced mtDNA degradation in MEFs. This may reflect the fact that H₂O₂ can induce DNA strand breaks directly, whereas MMS-induced strand breaks are predominantly indirect and are intermediates of BER. Also, oxidative damage to deoxyribose generates a distinct lesion, deoxyribonolactone, which may inhibit BER by suicide trapping of the DNA polymerase γ and require processing by long patch BER (8, 48). Overwhelming mitochondrial BER machinery with such unprocessed lesions in cells with reduced *LIG3* expression may lead to mtDNA degradation (49). In both HeLa cells and MEF, reduced levels of *LIG3* were associated with increased susceptibility to MMS and H₂O₂, agents that induce lesions repaired by BER. This observation is consistent with the essential role played by *LIG3* in the mitochondrial BER.

Overall, our observations indicate that mitochondrial levels of *LIG3* are excessive for routine mtDNA maintenance in cultured cells and that even though *LIG3* is essential *in vivo*, *Lig3*^{-/-} cells can be successfully generated and propagated *in vitro*. Also, they suggest that *LIG3* inhibitors can be used to sensitize cancer cells to chemotherapy with DNA-alkylating drugs or drugs that induce oxidative stress.

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