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Crucial roles for DNA ligase III in mitochondria but not in XRCC1-dependent repair

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Abstract

Mammalian cells have 3 ATP-dependent DNA ligases, which are required for DNA replication and repair[1]. Homologs of ligase I (Lig1) and ligase IV (Lig4) are ubiquitous in eukarya, whereas ligase III (Lig3), which has nuclear and mitochondrial forms, appears to be restricted to vertebrates. Lig3 is implicated in various DNA repair pathways with its partner protein XRCC1[1]. Deletion of *Lig3* results in early embryonic lethality in mice, as well as apparent cellular lethality[2], which has precluded definitive characterization of Lig3 function. Here we used pre-emptive complementation to determine the viability requirement for Lig3 in mammalian cells and its requirement in DNA repair. Various forms of Lig3 were introduced stably into mouse embryonic stem (ES) cells containing a conditional allele of *Lig3* that could be deleted with Cre recombinase.

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With this approach, we find that the mitochondrial, but not nuclear, Lig3 is required for cellular viability. Although the catalytic function of Lig3 is required, the zinc finger (ZnF) and BRCT domains of Lig3 are not. Remarkably, the viability requirement for Lig3 can be circumvented by targeting Lig1 to the mitochondria or expressing *Chlorella* virus DNA ligase, the minimal eukaryal nick-sealing enzyme[3], or *Escherichia coli* LigA, an NAD+-dependent ligase[1]. *Lig3* null cells are not sensitive to several DNA damaging agents that sensitize XRCC1-deficient cells[4,5,6]. Our results establish a role for Lig3 in mitochondria, but distinguish it from its interacting protein XRCC1.

Biochemical and cell biological experiments implicate the nuclear Lig3-XRCC1 complex in single-strand break repair, short patch base excision repair, and nucleotide excision repair[1]. Lig3 and XRCC1 interact via C-terminal BRCT domains found in each protein[7]. This interaction is important for the stability of Lig3[7] and the recruitment of Lig3 to DNA damage foci[8]. Purified Lig3-XRCC1 is proficient at nick sealing *in vitro*[9], and the complex associates with several other proteins involved in single-strand break repair, including Parp1[10], aprataxin, and TDP1[1].

Lig3 also has a mitochondrial form due to an alternative translation start site, which results in a mitochondrial leader sequence (MLS)[11]. Mammals differ in this respect from budding yeast, where the Lig1 homolog, Cdc9, is the mitochondrial DNA ligase[12]. In mitochondria, Lig3 appears to act independently of XRCC1, as XRCC1 is not present in this organelle[13]. Disruption of the *Lig3* gene, like *Xrcc1*, results in early embryonic lethality in the mouse[2,5], and *Lig3* null cell lines could not be established from these animals[2]. The similar timing of lethality of *Lig3* and *Xrcc1* null embryos suggests that death could result from similar phenotypic consequences related to Lig3 nuclear functions in DNA repair. Alternatively, or in addition, the mitochondrial function of Lig3 may be critical for survival.

To determine whether *Lig3* is an essential gene due to its nuclear and/or mitochondrial function, we developed a pre-emptive complementation strategy in mouse ES cells (Fig 1a). A $Lig3^{KO/cKOneo+}$ cell line was constructed which contains one conditional *Lig3* allele with an intronic *Neo* selection marker and *LoxP* sites flanking exons 6 and 14 and a second allele in which these exons were already removed by Cre recombinase (Fig. 1a; Supplementary Fig. 1). These exons encode part of the DNA binding domain and the catalytic core of the protein. Cre recombinase was expressed in the $Lig3^{KO/cKOneo+}$ cells, and 145 clones were replica-plated in media with or without G418. No G418 sensitive clones (i.e., $Lig3^{KO/KO}$) were obtained (Fig. 1b), consistent with the requirement for Lig3 for cellular viability. We then stably integrated transgenes expressing wild-type, mitochondrial, or nuclear Lig3; the nuclear (NucLig3) version lacked the MLS, and the mitochondrial (MtLig3) version contained the MLS but was mutated at the nuclear translation initiation site (M88T) (Fig. 1c; Supplementary Fig. 2). GFP fusions of these proteins were also tested (Supplementary Fig. 3a).

To determine which *Lig3* transgenes permit the survival of cells deleted for endogenous *Lig3*, Cre recombinase was used to transform $Lig3^{KO/cKOneo+}$ cells to $Lig3^{KO/KO}$ cells. A large fraction of the post-Cre clones expressing wild-type Lig3 or MtLig3 were G418 sensitive (34 to 50%), whereas no G418 sensitive clones were obtained with NucLig3 (Fig.

1b; Supplementary Fig. 4). We confirmed that G418 sensitive cells were *Lig3*$^{KO/KO}$ (Fig 1a) and that endogenous Lig3 was no longer present, with the only Lig3 present in the cells expressed from the transgene (Fig. 1d). Thus, cellular viability requires mitochondrial Lig3. To determine whether DNA ligase activity was essential for cell survival, we introduced a K508V mutation that abolishes ligase adenylylation and nick-sealing[1] into MtLig3. No G418 sensitive clones were derived from 4 independent transgenic cell lines (Fig. 1b), demonstrating that the requirement for mitochondrial Lig3 depends on its ligase activity.

BRCT domains are frequently involved in protein-protein interactions, and the BRCT domain of Lig3 is known to interact with XRCC1[7]. However, as XRCC1 is not found in mitochondria[13], the role of the BRCT domain for mitochondrial function of Lig3 is uncertain. Loss of the BRCT domain had no effect on the presence of Lig3 in mitochondria (Lig3-ΔBRCT and MtLig3-ΔBRCT; Supplementary Fig. 3a; data not shown). *Lig3*$^{KO/KO}$ clones expressing Lig3-ΔBRCT or MtLig3-ΔBRCT (Fig. 2a) were recovered as a substantial fraction of clones (39 to 49%; Fig. 2b), indicating that the BRCT domain is not required for viability. Thus, MtLig3 does not have a partner protein bound to its BRCT domain that is essential for its function.

A unique feature of Lig3 compared with the other mammalian DNA ligases is a ZnF at its N-terminus. The Lig3 ZnF interacts with Parp1[10], and this interaction is reported to be important for the association of Lig3 with mitochondrial DNA (mtDNA)[14]. Biochemical studies have shown that the ZnF promotes DNA nick recognition[15] and intermolecular ligation[16]. Nonetheless, *Lig3*$^{KO/KO}$ clones expressing Lig3-ΔZnF or MtLig3-ΔZnF (Fig. 2a) were efficiently recovered after Cre expression (37 to 38%; Fig. 2b).

Our results reveal that the catalytic activity of Lig3 is critical for cell survival, but that the ZnF and BRCT domains, which interact with various proteins, are dispensable, raising the question whether Lig3 itself is critical for mitochondrial function, or whether another DNA ligase would substitute. As the Lig1 homolog in yeast provides mitochondrial ligase function[12], we provided an MLS to murine Lig1 (Fig. 2a). GFP-tagged MtLig1, but not wild-type Lig1, was localized to mitochondria (Supplementary Fig. 3c), as expected. Stable *Lig3*$^{KO/cKOneo+}$ cell lines expressing MtLig1, but not wild-type Lig1, could be efficiently converted to *Lig3*$^{KO/KO}$ (35%; Fig. 2b). MtLig1 *Lig3*$^{KO/KO}$ clones were devoid of Lig3, and expressed instead a larger Lig1 protein due to the GFP tag (Fig. 3a). Thus, targeting Lig1 to mitochondria circumvents the viability requirement for Lig3, allowing the creation of *Lig3* null cells. In this way, the DNA ligase repertoire of mammalian cells is converted to that of yeast.

Given that ZnF and BRCT-truncated forms of Lig3 and MtLig1 could rescue *Lig3*$^{KO/KO}$ cells, we investigated their proficiency in mtDNA maintenance and repair. The mtDNA copy number was maintained as well (or better) in these cell lines as in wild-type Lig3-expressing cells (Fig. 2c), indicating that cells expressing these altered ligases are competent to replicate their mtDNA during continued passage. A long-range quantitative PCR assay[17] was performed to assess the mitochondrial base excision repair capacity of these cells in response to oxidative damage, and these altered ligases were similarly proficient in repairing mtDNA lesions compared to wild-type Lig3-expressing cells (Fig. 2d).

At 298 amino acids, *Chlorella* virus DNA ligase (ChVLig) is the smallest eukaryal ligase known, consisting solely of a catalytic core[3]. We expressed ChVLig and a modified form containing an MLS, MtChVLig (Fig. 2a), and found that expression of either allowed deletion of *Lig3* from the mouse genome (Fig. 2b). It is conceivable that ChVLig contains an internal sequence that allows translocation into mitochondria[18].

Thus, a minimal ATP-dependent ligase, devoid of auxiliary domains, rescues the survival of *Lig3* null mammalian cells. Further, Lig3 null cells rescued by MtChVLig were proficient at mtDNA maintenance (Fig. 2c) and repair (Fig. 2d).

Whereas ATP-dependent ligases are widespread, ligases that use NAD+ as a cofactor are usually restricted to bacteria[1]. *E. coli* DNA ligase, LigA, is NAD+-dependent and has a distinctive domain organization compared with mammalian ligases[1]. LigA and a modified form with an MLS (Fig. 2a) were expressed from transgenes, and like ChVLig, both forms were found to allow the survival of Lig3 null cells (Fig. 2b). Hence, there is no essential functional distinction between NAD+ and ATP-dependent ligases in the mammalian mitochondria, akin to swaps of NAD+ and ATP-dependent ligases performed in bacteria[19] and yeast[20].

We demonstrated that nuclear Lig3 is not required for cell survival, as MtLig1 $Lig3^{KO/KO}$ cells are null for *Lig3*. To impair nuclear localization of MtLig1, we removed the Lig1 nuclear localization signal, creating MtLig1-ΔNLS $Lig3^{KO/KO}$ cells (Fig. 2a,2b; Supplementary Fig. 3c). MtLig1-ΔNLS, like MtLig1, was expressed at a substantially lower level than endogenous Lig1 (Fig. 3a). As a complementary approach, we also created $Lig3^{KO/KO}$ cells expressing MtLig3-ΔBRCT-NES, whose interaction with XRCC1 is abrogated and which is excluded from the nucleus by addition of a potent nuclear export signal (NES)[21] (Fig. 2a,2b; Fig. 3a; Supplementary Fig. 3b).

To assess the nuclear role of Lig3, we tested the sensitivity of *Lig3* null ($Lig3^{KO/KO}$; MtLig1-ΔNLS) and nuclear Lig3-deficient ($Lig3^{KO/KO}$; MtLig3-ΔBRCT-NES) cells to a variety of DNA damaging agents. XRCC1-deficient cells are highly sensitive to alkylating agents like methyl methanesulfonate (MMS)[4,5,6]. If the interaction of XRCC1 with Lig3 is relevant to base excision repair, cells without nuclear Lig3 would also be expected to be sensitive to MMS; however, we found that these cells were no more sensitive than transgenic cells expressing wild-type Lig3 (Fig. 3b) or the parental cells (Supplementary Fig. 5). XRCC1-deficient cells are also sensitive to agents which produce DNA single and double-strand breaks, including hydrogen peroxide and ionizing radiation[4,5,6], and to ultraviolet radiation[22]. By contrast, we found that cells without nuclear Lig3 were not any more sensitive to these agents than control cells (Fig. 3c-e, Supplementary Fig. 5). Thus, Lig3 appears to be dispensable for nuclear DNA damage repair that requires XRCC1. Finally, we tested sensitivity to Parp inhibiton, which causes the accumulation of single-strand breaks[23], and found that nuclear Lig3 was also not required for resistance of cells to Parp inhibiton (Fig. 3f).

As the ZnF domain of Lig3 has been reported to be critical for its intermolecular ligation activity[16], we also investigated whether deletion of this domain in the context of an

otherwise wild-type Lig3 would impair resistance of cells to ionizing radiation. As with the other mutants, Lig3-ΔZnF *Lig3*$^{KO/KO}$ cells were no more sensitive than control cells (Fig. 3e).

XRCC1-deficient cells are notable for their high rate of spontaneous sister-chromatid exchange (SCE): both mouse and hamster XRCC1 mutants have ~10-fold higher SCE levels than control cells[4,5]. We examined spontaneous SCEs in MtLig1 *Lig3*$^{KO/KO}$ cells and found levels similar to control cells (Fig. 3g). Thus, the high level of SCEs found with XRCC1 deficiency is not recapitulated with Lig3 deficiency.

The lack of Lig3 in many model organisms has limited their use to study its function. In mouse, disruption of any of the DNA ligase genes leads to embryonic death, but the most severe phenotype occurs with Lig3 disruption[2,24,25]. Lig1 has been considered to be the replicative ligase[1,26], but the earlier death associated with Lig3 disruption, together with the inability to obtain viable Lig3 null cells, left open the possibility that Lig3 could have a critical role in nuclear DNA metabolism. The generation of viable and healthy *Lig3* null cells by providing a mitochondrial ligase conclusively rules out an essential role for Lig3 in the nucleus.

The well-documented interaction between Lig3 and XRCC1 had suggested that Lig3 would be critical for the same nuclear DNA repair pathways as XRCC1, similar to the Lig4-XRCC4 complex in DSB repair[1]. However, the lack of sensitivity of Lig3 null cells to the spectrum of DNA damaging agents that sensitize XRCC1-deficient cells, as shown here in proliferating cells and in the accompanying report in quiescent cells[27], together with a normal SCE frequency, provides strong evidence that Lig3 is not required for XRCC1-dependent nuclear DNA repair, pointing instead to a role for Lig1.

Our results demonstrate instead that *Lig3* is an essential gene because of its requirement in mitochondria. However, Lig3 can be replaced in mitochondria with Lig1, the mitochondrial ligase in lower eukaryotes, with an algal viral ligase consisting solely of a catalytic core, and even the NAD+-dependent *E. coli* LigA. Thus, these results attest to the requirement for a functional DNA ligase, which trumps even co-factor specificity. Why vertebrates developed a requirement for Lig3 is uncertain, but given our results, the additional domains found in Lig3 do not appear to be essential for mitochondrial function, including mtDNA maintenance or repair of oxidative damage. These results underscore a surprising plasticity that mammalian cells have in their mitochondrial DNA ligase requirement.

Methods Summary

Cell culture

To construct stable cell lines expressing various DNA ligases, 5×10^6 *Lig3*$^{KO/cKOneo+}$ cells were electroporated with 12 μg ligase expression vector at 800 V, 3 μF. Hygromycin resistant clones were picked after incubation for 10 days in 150 μg/μl hygromycin. Initial screening for exogenous ligase expression was performed by RT-PCR using specific primers, followed by Western blotting. For deletion of the endogenous *Lig3* allele, 5×10^6 cells were electroporated with 5 μg Cre recombinase vector at 250 V, 950 μF. Cells were

plated based on a serial dilution. After 7 days, colonies were picked and expanded, and then replica plated into two 96-well plates. One plate was cultured with 200 μg/μl G418, whereas the other plate was cultured in normal media. Clones that did not grow in G418, but grew in normal media, have converted the *Lig3*$^{cKOneo+}$ allele to a *Lig3*^{KO} allele. The genotype of these clones was confirmed by PCR.

Western Blotting

Whole cell extracts were prepared with Nonidet-P40 buffer and were run on a 7.5% (w/v) Tris-HCl SDS page gel, blotted, and then probed with Lig3 antibody clone 7 (BD Transduction Labs), which recognizes both the human and mouse Lig3 proteins, or Lig1 antibody N-13 (Santa Cruz). α-tubulin (Sigma) was used as a loading control.

Full methods accompany this paper.

Full Methods

DNA constructs

A vector containing wild-type human Lig3 cDNA (with both mitochondrial and nuclear translation initiation sites), a gift from Dr. K.W. Caldecott (University of Sussex, Brighton, UK), was digested with NheI and XbaI and subcloned into the NheI site of pCAGGS. As the cDNA contained a 51 bp linker located before the nuclear translation initiation site, it was modified by site directed mutagenesis to remove the linker, with the primers: 5'-GTGGCCCCTGTGAGATGGCTGAGCAACG-3' and 5'-CGTTGCTCAGCCATCTCACAGGGGCCAC-3', to restore an unmodified Lig3 sequence, creating pCAGGS-Lig3. A Pgk-hygromycin resistance gene was inserted at the PsiI site to create pCAGGS-Lig3-hyg. MtLig3 was generated by using site directed mutagenesis to generate a M88T mutation in pCAGGS-Lig3-hyg using the primers: 5'GAGAGGCCCCTGTGAGACCGCTGAGCA-3' and 5'GAGAGGATCCCTAGCAGGGAGCTACCAGTCTC-3'. For NucLig3, amino acids 1 to 87 were deleted by introducing NotI and BamHI sites into pCAGGS-Lig3-hyg via PCR using the primers: 5'-GCATGCGGCCGCCTGTGAGATGGCTGAGCAACGGT-3', 5'-GGATGGATCCCTAGCAGGGAGCTACCAGTC-3'. For the ΔBRCT mutation, amino acids 934 to 1009 were deleted by introducing NheI and MfeI sites via PCR using the primers: 5'-GGCCGCTAGCGGGCAGCTATATGTCTTTGGCTTTCAAGAT-3' and 5'-GAGACAATTGTTACTATACCTTTGTTTGGCACAGCGTC-3'. The ΔZnF mutation was generated by deleting amino acids 89 to 258 using site directed mutagenesis with primers 5'-TGGCCCCTGTGAGATGAAGGACTGTCTGCTAC-3' and 5'-GTAGCAGACAGTCCTTCATCTCACAGGGGCCA-3'. For GFP tagging of the Mt-tagged constructs, SacII and AgeI sites were introduced and stop codons of the full length or ΔBRCT proteins were converted into alanine codons by PCR and cloned in frame into SacII and AgeI sites of pEGFP-N1 (Clontech). PCR primers for full length were 5'-ACGGTACCGCGGCAGCTATATGTCTTTGG-3'and 5'-ACGGTACCGCGGCAGCTATATGTCTTTGG-3', and for ΔBRCT were 5'-ACGGTACCGCGGCAGCTATATGTCTTTGG-3' and 5'-GGCGACCGGTGGTACCTTTGTTTGGCACAGCG-3'.

For other constructs with GFP fusions (NucLig3, Lig3, ΔZnF and K508V), plasmids were digested with PmlI and ligated into the vector backbone of MtLig3-GFP using the same enzyme. The MAPKK nuclear export signal (NES)[21] was fused to the C terminus of GFP via PCR using the primers 5'-GCCCCCTCAGCCAGTACCAAGAA-3' and 5'GGCCAATTGGCCTTATTACTGCTGCTCGTCCAGCTCCAGCTCCTCCAGCTT CTTTTGGAGGTCCACGAGATTCTTGTACAGCTCGTCCAT-3'. Mouse Lig1 cDNA (Invitrogen) was amplified with primers introducing KpnI and AgeI sites and changing a stop codon into an alanine codon; this fragment was cloned in frame into the KpnI and AgeI sites of pEGFP-N1. The Lig3 MLS was amplified with the following primers 5'-GGCGAATTCTATATGTCTTTGGCTTTCAAGATCTTCTTTC-3'and 5'-ATTGGTACCCCTCACAGGGGCCACTGCAG-3' and cloned into the EcoRI and KpnI sites of Lig1-GFP-hyg vector. The *Chlorella* virus DNA ligase coding region was amplified with ChV-NheI and ChV-MfeI primers and cloned into the NheI and MfeI sites of the pCAGGS-Lig1-Hyg vector. ChV-NheI: 5'-GCCGCTAGCACCATGGCAATCACAAAGCCATT-3', ChV-MfeI: 5'-GCCCAATTGTTAACGGTCTTCCTCGTGAC-3'. The *Escherichia coli* DNA ligase coding region was amplified with LigA-NheI and LigA-MfeI primers and cloned into the NheI and MfeI sites of the pCAGGS-Lig1-Hyg vector. LigA-NheI: 5'-GCCGCTAGCACCATGGAATCAATCGAACAACAA -3', LigA-MfeI: 5'-GCCCAATTGTCAGCTACCCAGCAAACG -3'.

RT-PCR

Hygromycin resistant clones were screened by RT-PCR using primers specific to human Lig3. A primer pair was used with the forward primer to the pCAGGS backbone and the reverse primer to exon 3 of human Lig3, resulting in a size difference for mitochondrial and nuclear forms (Supplementary Fig. 3): pCAGRTfw 5'-CAACGTGCTGGTTATTGTGC-3', hLig3Rv 5'-ACAGCTTTCTTCTTTGGTGTACCT-3'. A similar strategy was used for Lig1, with primers pCAGRTfw and Lig1RT_RV (5'-ACCGCTGAGCAACGGTTCT-3'), for *Chlorella* virus DNA ligase, with primers pCAGRTfw and chlRTPCR-RV1 (5'-CAGCACTTGTGGTGTCTTGAA-3') and, for *Escherichia coli* DNA ligase, with primers pCAGRTfw and LigARTPCR-RV1 (5'-CCTGCACACGTTTGTTGAAA -3'). RNA was isolated using RNeasy Mini Kit (Qiagen) and cDNA was generated by SuperScript III First-strand Synthesis system (Invitrogen).

Genotyping

Genomic DNA was isolated using the Genelute Mammalian Genomic DNA Miniprep Kit (Sigma). Each primer was named for the location on the genomic DNA (e.g., Int5-6Fw means that the primer is at the intron between exons 5 and 6). Primer pairs used for genotyping are as follows: Exon 5Fw and Neo2Rv (primer pair a in Fig. 1a): 5'-GGCTTTCACGGTGATGTGTA-3' and 5'-TCTGGATTCATCGACTGTGG-3', using an annealing temperature of 62°C; Int5-6Fw and Int16-17Rv (primer pair b in Fig. 1a): 5'-CGGGTGTAGGGAGGTCATAA-3', 5'-GAAGGAAGAGGTCTCCAGCA-3', using an annealing temperature of 62°C; Int10-11Fw and Int11-12Rv (primer pair c in Fig. 1a): 5'-CACTAAACGTGGCAGAGCAA-3', 5'-CCAGCCCAGACTACAGCTTC-3', using anannealing temperature of 62°C; Int5-6Fw2 and Int5-6Rv (Supplementary Fig. 1d): 5'-

GCCAAGTGTGAATATACAGC-3' and 5'-CAGGGAGCTTGGGACGGATGC-3', using an annealing temperature of 64°C; Int5-6 and Int16-17(Supplementary Fig. 1d): 5'-CGGGTGTAGGGAGGTCATAA-3' and 5'-GAAGGAAGAGGTCTCCAGCA-3', using an annealing temperature of 64°C.

Microscopy

The subcellular localization of the various GFP fusion constructs was checked by Mitotracker Red CMXRos (Invitrogen) and Hoechst 33342 (Invitrogen) to stain mitochondria and nucleus, respectively. DNA constructs were transiently transfected with Lipofectamine 2000 (Invitrogen). After incubating cells with Opti-MEM (Invitrogen) containing 10 nM Mitotracker Red CMXRos and 2.5 μM Hoechst 33342 for 20 min at 37°C, cells were monitored with a Zeiss LSM 510 META laser scanning microscope.

qPCR mtDNA repair assay

1×10^6 ES cells with the indicated genotypes were plated on 6-cm^2 plates. After 16 h, cells were cultured with 6.25 ml, 175 μM hydrogen peroxide for 15 min and then cultured with conditioned medium for 1.5 h. mtDNA copy number and mtDNA repair were determined by a long-range quantitative PCR assay[17]. Basically, DNA was extracted from pellets of 1×10ˆ6 cells with the DNeasy Blood and Tissue kit (Qiagen) by a QIAcube automated DNA extraction robot (Qiagen). Initial DNA concentration was measured using Picogreen dsDNA binding agent (Invitrogen) and a DNA standard curve. Total mouse genomic DNA at an approximate final concentration of 4.5 ng/μL was then digested with with HaeII (New England Biosciences) for 1 hr at 37°C in a reaction mixture containing1X NEBuffer 4, 1X BSA, and 20 U undiluted HaeII enzyme. HaeII linearizes the mouse mtDNA by digesting once (2604) near the D-loop region. Linearization of mitochondrial DNA ensures efficient amplification and allows accurate determination of mtDNA copy number. After digestion, DNA concentration was measured with Picogreen and an appropriate volume was directly removed from the digest to use for qPCR, with less than 5% variability in DNA concentration between samples.

The qPCR reaction was performed with the GeneAmp XL PCR kit (Applied Biosystems) as follows: 10-15 ng total DNA, in a reaction mix of 50 μL, with 1X buffer, 100 ng/μL BSA, dNTPs at 200 μM each nt, 1.2 mM $MgO(Ac)_2$, and 20 pmoles for each of two primers. Primer pairs for a 10 kb fragment of mtDNA (long) and for a 117 bp fragment of mtDNA (short) were used for calculating mtDNA damage and mtDNA copy number, respectively. Primer sequences are as described previously[17]. DNA polymerase was added at a concentration of 1 U/reaction. A 50% control and a "no template" blank were used to ensure that the assay was within quantitative range and free of contamination, respectively. PCR products were quantified using fluorescence-blank measurements from the Picogreen dsDNA binding agent. PCR products from the long fragment were normalized to the short fragment to account for the effect of differing mtDNA copy number on amplification of the long fragment.

Sister chromatid exchange

5×10^6 ES cells with the indicated genotypes were plated on 10-cm^2 plates. After 24 h, cells were cultured with 10 μM bromodeoxyuridine for 20 h (approximately two cell cycle periods) and pulsed with 0.03 μg/ml Colcemid for the final 30 min. The cells were collected by centrifugation and exposed to 0.075 mM KCl hypotonic solution for 30 min at 25°C. The cells were washed twice with the fixative (methanol:acetic acid = 3:1) and suspended in a small volume of the fixative. The cell suspension was dropped onto ice-cold glass slides and air-dried at 60°C for 2 h. Two days later, slides were incubated with 1 μg/ml Hoechst 33258 in Sorensen's phosphate buffer (38 mM KH_2PO_4, 60mM Na_2HPO_4, pH 7.0) for 10 min, rinsed with 2X SSC buffer (300 mM NaCl, 30 mM $Na_3C_6H_5O_7$, pH 7.0) and then overlaid with coverslips. Slides were exposed to black light (λ = 352 nm) at a distance of 1 cm for 20 min. After removal of coverslips, the slides were incubated in 2X SSC at 60°C for 2 h and then stained with 4% (v/v) Giemsa solution in Sorensen's buffer for 10 min, rinsed in water, and air-dried. A two-tailed unpaired *t*-test was used to analyze the data.

Drug Sensitivity assays

2×10^3 ES-cells per well were seeded in 24-well plates in duplicate. After 24 h, cells were incubated with various drugs at the indicated concentrations (Fig. 3a) for 24 h in ES cell medium, except hydrogen peroxide which was for 1 h. For irradiation, plates were exposed to the X-ray source from an X-RAD 225C apparatus at a rate of 687 cGy/minute. Six days later cells were fixed with a solution of 12.5% (v/v) acetic acid, 12.5% (v/v) methanol for 15 min and then stained with 1% (w/v) crystal violet. Afterwards, stained cells were treated with 0.1% SDS in methanol and the absorbance was measured at 595 nm. Each point in the plots was the average of 2 experiments where each experiment had a duplicate and is a percentage of the absorbance from untreated ES cells. For *Lig3* null cells expressing the Lig3 ΔZNF-GFP, MtLig3-ΔBRCT-GFP-NES and MtLig1 GFP transgenes, two independent null clones were used for each. For the colony formation assays (Fig. 3b), 2×10^3 ES cells were plated in 10-cm^2 plates and exposed to ionizing radiation or ultraviolet radiation (UVC). Eight days later, surviving colonies were fixed with methanol and stained with 3% (v/v) Giemsa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Ellenberger T, Tomkinson AE. Eukaryotic DNA ligases: structural and functional insights. Annu Rev Biochem. 2008; 77:313–338. [PubMed: 18518823]
2. Puebla-Osorio N, Lacey DB, Alt FW, Zhu C. Early embryonic lethality due to targeted inactivation of DNA ligase III. Mol Cell Biol. 2006; 26:3935–3941. [PubMed: 16648486]

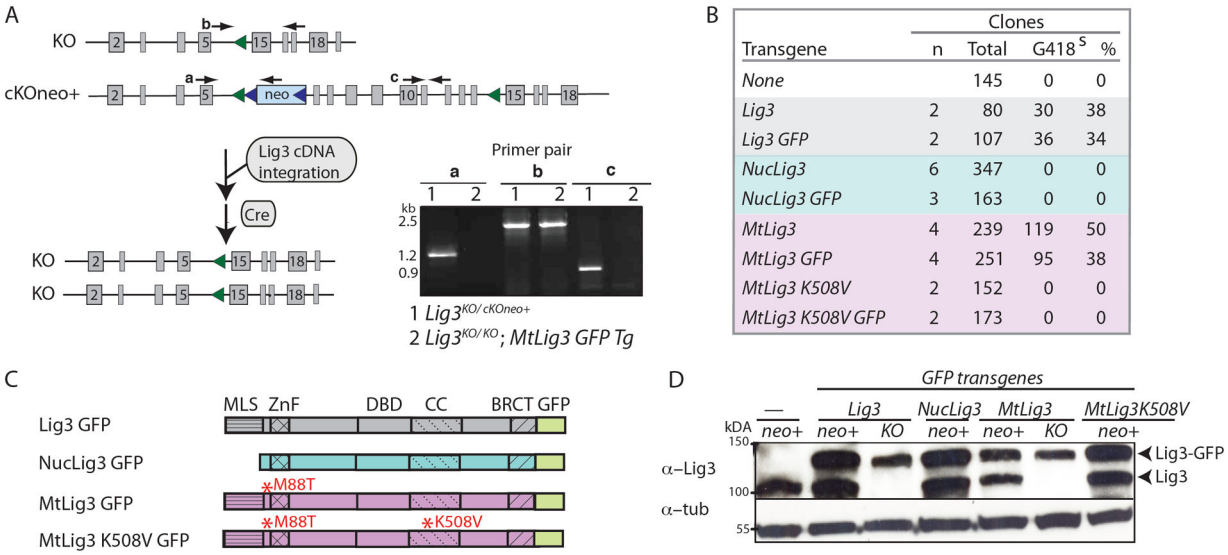
3. Ho CK, Van Etten JL, Shuman S. Characterization of an ATP-dependent DNA ligase encoded by Chlorella virus PBCV-1. J Virol. 1997; 71:1931–1937. [PubMed: 9032324]
4. Thompson LH, Brookman KW, Jones NJ, Allen SA, Carrano AV. Molecular cloning of the human XRCC1 gene, which corrects defective DNA strand break repair and sister chromatid exchange. Mol Cell Biol. 1990; 10:6160–6171. [PubMed: 2247054]
5. Tebbs RS, et al. Requirement for the Xrcc1 DNA base excision repair gene during early mouse development. Dev Biol. 1999; 208:513–529. [PubMed: 10191063]
6. Lee Y, et al. The genesis of cerebellar interneurons and the prevention of neural DNA damage require XRCC1. Nat Neurosci. 2009; 12:973–980. [PubMed: 19633665]
7. Caldecott KW, McKeown CK, Tucker JD, Ljungquist S, Thompson LH. An interaction between the mammalian DNA repair protein XRCC1 and DNA ligase III. Mol Cell Biol. 1994; 14:68–76. [PubMed: 8264637]
8. Mortusewicz O, Rothbauer U, Cardoso MC, Leonhardt H. Differential recruitment of DNA Ligase I and III to DNA repair sites. Nucleic Acids Res. 2006; 34:3523–3532. [PubMed: 16855289]
9. Chen X, et al. Distinct kinetics of human DNA ligases I, IIIalpha, IIIbeta, and IV reveal direct DNA sensing ability and differential physiological functions in DNA repair. DNA Repair (Amst). 2009; 8:961–968. [PubMed: 19589734]
10. Leppard JB, Dong Z, Mackey ZB, Tomkinson AE. Physical and functional interaction between DNA ligase IIIalpha and poly(ADP-Ribose) polymerase 1 in DNA single-strand break repair. Mol Cell Biol. 2003; 23:5919–5927. [PubMed: 12897160]
11. Lakshmipathy U, Campbell C. The human DNA ligase III gene encodes nuclear and mitochondrial proteins. Mol Cell Biol. 1999; 19:3869–3876. [PubMed: 10207110]
12. Willer M, Rainey M, Pullen T, Stirling CJ. The yeast CDC9 gene encodes both a nuclear and a mitochondrial form of DNA ligase I. Curr Biol. 1999; 9:1085–1094. [PubMed: 10531002]
13. Lakshmipathy U, Campbell C. Mitochondrial DNA ligase III function is independent of Xrcc1. Nucleic Acids Res. 2000; 28:3880–3886. [PubMed: 11024166]
14. Rossi MN, et al. Mitochondrial localization of PARP-1 requires interaction with mitofilin and is involved in the maintenance of mitochondrial DNA integrity. J Biol Chem. 2009; 284:31616–31624. [PubMed: 19762472]
15. Mackey ZB, et al. DNA ligase III is recruited to DNA strand breaks by a zinc finger motif homologous to that of poly(ADP-ribose) polymerase. Identification of two functionally distinct DNA binding regions within DNA ligase III. J Biol Chem. 1999; 274:21679–21687. [PubMed: 10419478]
16. Cotner-Gohara E, Kim IK, Tomkinson AE, Ellenberger T. Two DNA-binding and nick recognition modules in human DNA ligase III. J Biol Chem. 2008; 283:10764–10772. [PubMed: 18238776]
17. Santos JH, Meyer JN, Mandavilli BS, Van Houten B. Quantitative PCR-based measurement of nuclear and mitochondrial DNA damage and repair in mammalian cells. Methods Mol Biol. 2006; 314:183–199. [PubMed: 16673882]
18. Neupert W, Herrmann JM. Translocation of proteins into mitochondria. Annu Rev Biochem. 2007; 76:723–749. [PubMed: 17263664]
19. Park UE, Olivera BM, Hughes KT, Roth JR, Hillyard DR. DNA ligase and the pyridine nucleotide cycle in Salmonella typhimurium. J Bacteriol. 1989; 171:2173–2180. [PubMed: 2649488]
20. Sriskanda V, Schwer B, Ho CK, Shuman S. Mutational analysis of Escherichia coli DNA ligase identifies amino acids required for nick-ligation in vitro and for in vivo complementation of the growth of yeast cells deleted for CDC9 and LIG4. Nucleic Acids Res. 1999; 27:3953–3963. [PubMed: 10497258]
21. Henderson BR, Eleftheriou A. A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals. Exp Cell Res. 2000; 256:213–224. [PubMed: 10739668]
22. Moser J, et al. Sealing of chromosomal DNA nicks during nucleotide excision repair requires XRCC1 and DNA ligase III alpha in a cell-cycle-specific manner. Mol Cell. 2007; 27:311–323. [PubMed: 17643379]
23. Farmer H, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature. 2005; 434:917–921. [PubMed: 15829967]

24. Bentley D, et al. DNA ligase I is required for fetal liver erythropoiesis but is not essential for mammalian cell viability. Nat Genet. 1996; 13:489–491. [PubMed: 8696349]

25. Frank KM, et al. Late embryonic lethality and impaired V(D)J recombination in mice lacking DNA ligase IV. Nature. 1998; 396:173–177. [PubMed: 9823897]

26. Barnes DE, Tomkinson AE, Lehmann AR, Webster AD, Lindahl T. Mutations in the DNA ligase I gene of an individual with immunodeficiencies and cellular hypersensitivity to DNA-damaging agents. Cell. 1992; 69:495–503. [PubMed: 1581963]

27. Gao Y, et al. DNA ligase III is essential for mitochondrial DNA integrity but not nuclear DNA repair. Nature. in press.



Transgene	n	Clones Total	G418S	%
None		145	0	0
Lig3	2	80	30	38
Lig3 GFP	2	107	36	34
NucLig3	6	347	0	0
NucLig3 GFP	3	163	0	0
MtLig3	4	239	119	50
MtLig3 GFP	4	251	95	38
MtLig3 K508V	2	152	0	0
MtLig3 K508V GFP	2	173	0	0

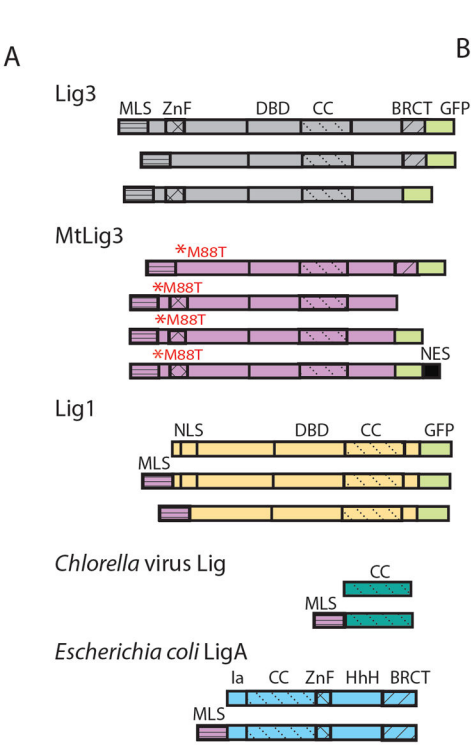
Figure 1. Mitochondrial Lig3 activity is critical for cellular viability

(a) Pre-emptive complementation strategy for *Lig3* deletion. $Lig3^{KO/KO}$ clones were identified by their lack of growth in G418 (*neo*–). PCR confirmed the genotype, as indicated.

(b) Mitochondrial Lig3 activity is critical for cellular viability. n, number of independently-derived Lig3 transgenic cell lines analyzed; Total, total number of post-Cre colonies analyzed by replica plating with and without G418; $G418^S$, G418 sensitive; %, ratio of $G418^S$ to Total.

(c) Lig3 proteins tested for pre-emptive complementation. MLS, mitochondrial leader sequence; ZnF, zinc finger; BRCT, BRCA1 C-terminal related domain; DBD, DNA-binding domain; CC, catalytic core; *M88T, mutation of the nuclear translation initiation site.

(d) Western blot analysis showing the loss of endogenous Lig3 in $Lig3^{KO/KO}$ clones stably expressing GFP-tagged Lig3 or MtLig3. Lig3 is 105 kDa, whereas the GFP fusions are ~135 kDa. *neo*+, $Lig3^{KO/cKOneo+}$; *KO*, $Lig3^{KO/KO}$; α-tub, α-tubulin.



B

Transgene	n	Clones Total	G418^S	%
Lig3 GFP	2	107	36	34
Lig3 ΔZnF GFP	2	96	37	38
Lig3 ΔBRCT GFP	2	89	35	39
MtLig3 ΔZnF GFP	2	116	43	37
MtLig3 ΔBRCT	4	192	95	49
MtLig3 ΔBRCT GFP	3	156	65	42
MtLig3 ΔBRCT GFP NES	2	110	42	38
Lig1 GFP	2	110	0	0
MtLig1 GFP	2	174	61	35
MtLig1 ΔNLS GFP	2	130	51	39
ChVLig	2	102	41	40
MtChVLig	2	78	34	44
EcoLigA	3	96	53	55
MtEcoLigA	3	96	62	65

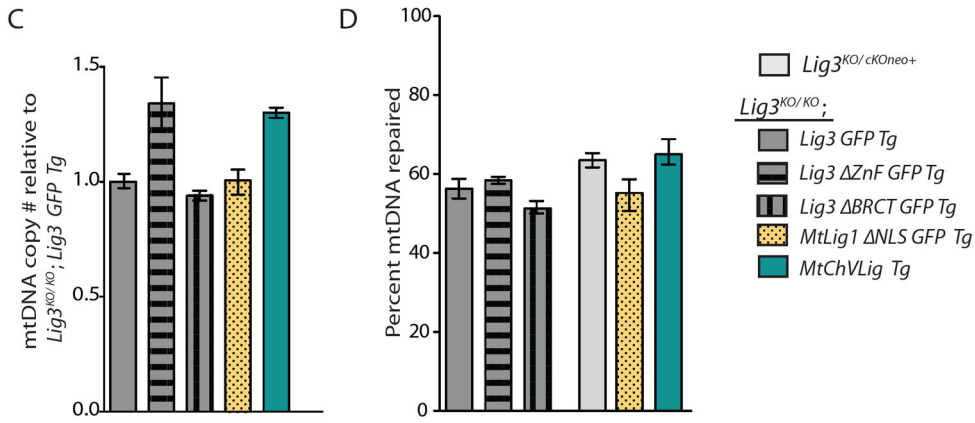


Figure 2. Mitochondrial DNA ligase activity can be provided by a variety of DNA ligases

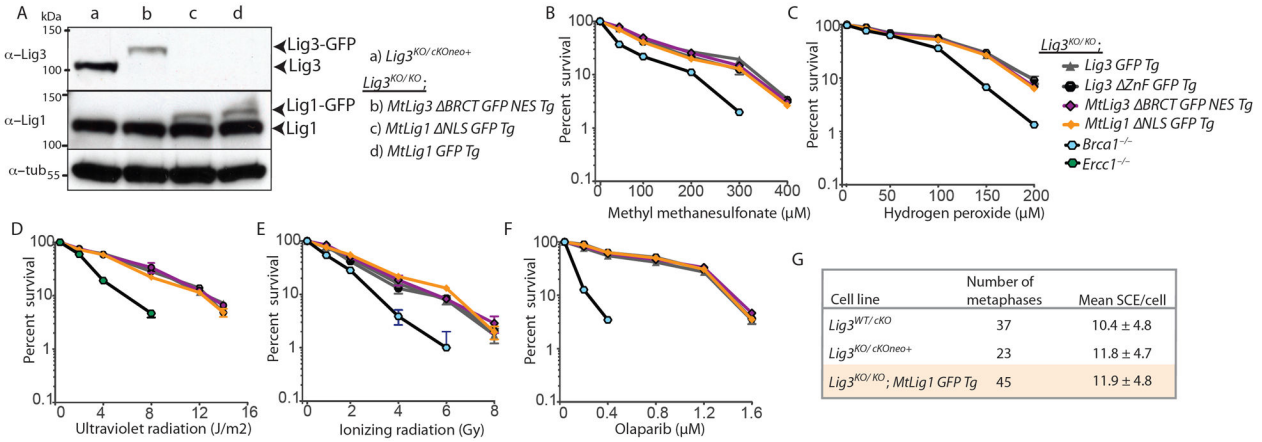
(a) DNA ligases tested for pre-emptive complementation of *Lig3$^{KO/KO}$* cells. ΔZnF, deletion of Lig3 amino acids 89 to 258; ΔBRCT, deletion of Lig3 amino acids 934 to 1009; ΔNLS, deletion of Lig1 amino acids 135 to147; NES, nuclear export signal from MAPKK[21]; Mt, presence of Lig3 MLS; NLS, nuclear localization signal; HhH, helix-hairpin-helix.

(b) Mitochondrial DNA ligase activity can be provided by a variety of DNA ligases. ChVLig and EcoLigA presumably enter mitochondria without the requirement for an MLS.

(c) Cells expressing exogenous DNA ligases are competent to replicate and maintain mtDNA. mtDNA copy number was quantified by qPCR by amplifying a 117 bp fragment from mtDNA. Values are presented relative to levels of mtDNA in *Lig3$^{KO/KO}$*; *Lig3 GFP Tg*

cells. Data represent the mean of two biological repeats each determined twice by qPCR +/- SEM.

(d) Cells expressing exogenous DNA ligases showed similar capacities for mtDNA repair after oxidative damage. Cells were treated with 175 μM H_2O_2 for 15 min and allowed to recover for 1.5 h. To measure repair, a 10 kb mtDNA fragment was amplified following damage and quantified by qPCR. Values were normalized to the amplification of a 117 bp mtDNA fragment. Percent repair is the amount of damage remaining after 1.5 h recovery divided by the initial damage. There was no significant difference between cell lines expressing wild-type Lig3 (parental cells and transgene rescued cells) and the other ligase forms. For cells expressing MitLig1-ΔNLS, and MtChVLig two transgenic cell lines were analyzed. Data represent the mean of 2-4 determinations on multiple clones with each qPCR performed twice +/- SEM.



Cell line	Number of metaphases	Mean SCE/cell
$Lig3^{WT/cKO}$	37	10.4 ± 4.8
$Lig3^{KO/cKOneo+}$	23	11.8 ± 4.7
$Lig3^{KO/KO}$; MtLig1 GFP Tg	45	11.9 ± 4.8

Figure 3. Loss of Lig3 is not associated with sensitivity to several DNA damaging agents or with increased sister-chromatid exchange

(a) Western blot analysis showing the loss of endogenous Lig3 protein in $Lig3^{KO/KO}$ cells with the indicated transgenes.

(b-f) Sensitivity of $Lig3^{KO/KO}$ cell lines to the indicated DNA damaging agents was measured using colony formation assays. $Brca1^{-/-}$ and $Ercc1^{-/-}$ cells are only shown on graphs when they are sensitive. For each cell line and agent, n = 4 and error bars = SEM; error bars in some cases are smaller than the symbol.

(g) SCE analysis. The range of SCEs was between 5 and 21 per metaphase for each cell line. For cells expressing MtLig1, two transgenic cell lines were analyzed. The differences between the cell lines are not significant using a two-tailed unpaired *t*-test. Values are presented with 1 SD from the mean.