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DNA Ligases I and III Support Nucleotide Excision Repair in DT40 Cells with Similar Efficiency

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ABSTRACT

In eukaryotic cells helix-distorting DNA lesions like cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone photoproducts (6-4 PPs) are efficiently removed by nucleotide excision repair (NER). NER is a multistep process where in the end, subsequent to replication over the gap, the remaining nick is sealed by a DNA ligase. Lig1 has been implicated as the major DNA ligase in NER. Recently, Lig3 has been implicated as a component of a NER subpathway that operates in dividing cells, but which becomes particularly important in non-dividing cells. Here, we use DT40 cells and powerful gene targeting approaches for generating DNA ligase mutants to examine the involvement and contribution of Lig1 and Lig3 in NER using cell survival measured by colony formation, and repair kinetics of CPD by immunofluorescence microscopy and immuno-slot-blotting. Our results demonstrate an impressive and previously undocumented potential of Lig3 to substitute for Lig1 in

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removing helix-distorting DNA lesions by NER in proliferating cells. We show for the first time in a clean genetic background a functional redundancy in NER between Lig1 and Lig3, which appears to be cell cycle independent and which is likely to contribute to the stability of vertebrate genomes.

INTRODUCTION

A variety of cytotoxic agents induce forms of base damage that alter the structure of the DNA molecule. Examples of this class of lesions are the cyclobutane pyrimidine dimers (CPDs) and the pyrimidine-pyrimidone (6-4) photoproducts (6-4 PPs) generated after exposure of cells to ultraviolet light (UV). Such lesions are efficiently removed from the DNA by nucleotide excision repair (NER) (1).

NER is a multistep process initiated by proteins, which recognize bulky alterations in the shape of the DNA double helix, or the stalling of an RNA polymerase at a DNA lesion. During NER, the DNA duplex around the lesion is opened and a single-stranded DNA segment containing the lesion is released. The single-strand DNA gap thus created is filled by DNA synthesis utilizing a DNA polymerase and PCNA, as well as the undamaged strand as template, and the remaining nick is finally sealed by a DNA ligase (2).

Two subpathways of NER have been characterized: Global genome repair (GGR) and transcription-coupled repair (TCR). They differ in the mechanism of recognition of the helix-distorting DNA damage, but initiate a common pathway to excise and replace the damage site.

GGR is initiated when the helix distorting lesion is recognized by XPC together with its partner hHR23B that recruits the TFIIH complex. Recruitment of XPA, RPA, and XPG follows to generate the pre-incision complex. Subsequently, the XPB and XPD DNA-helicase components of TFIIH unwind about 30 nucleotides (nt) of DNA that are stabilized by RPA and XPA, which in-turn help to position the endonucleases XPG and ERCC1-XPF that incise 3' and 5', respectively, of the lesion at the damaged strand (3).

TCR, on the other hand, is initiated by DNA-damage-mediated blockage of RNA-Pol II. Then CSB is recruited to the stalled RNA-Pol II followed by the recruitment of the pre-incision factors TFIIH, XPA, RPA, ERCC1-XPF, and XPG together with the CSA-CNS complex (3). After these distinct initial steps the repair process continues in a unified manner as follows.

The pre-incision complex is released, except of XPG and RPA, which then recruit the replication factors PCNA, RFC and the DNA polymerases δ, ε or κ. Subsequent to repair synthesis over the gap, the DNA is sealed by a DNA ligase (3). This final ligation step is the focus of the present report.

DNA ligases are versatile enzymes and most organisms have multiple ligases. It is widely believed that DNA ligases have dedicated functions in the various aspects of the DNA metabolism, such as joining of Okazaki fragments during replication, and DNA repair including NER, base excision repair

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(BER), single strand break repair (SSBR), or repair of DNA double strand breaks (DSBs) via non-homologous end joining (NHEJ) or homologous recombination repair (HRR) (4, 5).

Lig1 has an essential role in DNA replication and has been implicated in long-patch BER and NER (6-8). The involvement of Lig1 in NER is primarily based on the UV sensitivity of cells derived from the only known patient deficient in Lig1 (46BR) (9). 46BR cells show no obvious defects in proliferation, but show a marked defect in Okazaki fragment joining (10, 11) and a hypersensitivity to damaging agents, including alkylating agents, suggesting a function of Lig1 in NER (9). Furthermore, biochemical studies show that more strand breaks persist in UV-irradiated 46BR than in normal cells (12). However, other studies with 46BR cells show only marginally increased UV sensitivity; moreover, wild-type and Lig1 mutant mouse fibroblasts display no significant differences in their capacity to repair DNA damage induced by UV (13). These observations implicate Lig1 in NER, but also suggest that alternative mechanisms exist with considerable efficiency involving other DNA ligases.

Lig3 plays a central role in DSB repair via B-NHEJ (14-19) and is involved in the repair of DNA single-strand breaks (7, 20). Recently, Lig3 has been implicated as a component of a NER subpathway that operates in dividing cells, but which becomes particularly important in non-dividing cells (21). The latter study suggests the function of two distinct protein complexes in the gap filling step of NER: While, as expected, Lig1 and DNA polymerase ε are active in cycling cells, Xrcc1/Lig3 and DNA polymerase δ interact with NER components and actively perform ligation functions in non-dividing cells, although they remain active in cycling cells as well.

These observations support the notion that Lig3 can substitute for Lig1, but also suggest that when both ligases are present, Lig1 may be preferentially used during NER. DNA ligase IV (Lig4) is firmly implicated in the repair of DSBs by the DNA-PK-dependent pathway of NHEJ and does not seem to have detectable functions in other cellular DNA ligation reactions such as those required for NER.

The evidence summarized above suggests unexplored preferences and possible interplays between Lig1 and Lig3 in NER. Here, we use the chicken B cell line, DT40, and powerful targeting approaches to generate DNA ligase mutants allowing us, for the first time, to examine the involvement and contribution of Lig1 and Lig3 in NER in a clean genetic background. For this purpose, single and double mutants of *Lig1* and *Lig4,* as well as special knock-in mutants are used to study the functions of Lig1 and Lig3 in NER.

MATERIALS AND METHODS

Cell culture: The Lig1 deficient cell line 46BR has been established from a patient with *Lig1* mutations in both alleles (kindly provided by Dr. A. Lehman). One of the mutations in 46BR fibroblasts results in an inactivating Glu-566→Lys replacement within the active site of the enzyme. The mutation in the other, maternal allele, Arg-771→Trp, encodes for an enzyme with only 3–5% of the wild-type enzyme activity (9, 22). 46BR 1N originates from the 46BR fibroblasts and was

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generated by transfection with pSV3neo expressing SV40 T-antigen. 46BR 1N cells are immortalized, transformed and homozygous for the Arg-771→Trp *Lig1* mutation, which leads to normal protein levels, albeit with only 3% - 5% remaining activity. 46BR PBAHL cells have been generated by transfecting the Lig1 deficient 46BR 1N cells with a Lig1 expressing vector carrying the neo gene as selection marker. 46BR 1N and 46BR PBAHL cells were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS).

The PF20 (wild type) and the Lig1 deficient (PFL13) mouse cell lines are spontaneously immortalized mouse fibroblasts (kindly provided by Dr. D. Melton). They were derived from normal or Lig1 deficient mouse embryos - the latter obtained by removing exons 23-27 beyond the enzyme active site (13). PF20 and PFL13 were cultured in DMEM supplemented with 10% FBS and non-essential amino acids.

XPC-EB cells are human fibroblasts deficient in Xpc. XPC-EB cells were cultured in DMEM supplemented with 10% FBS.

For experiments, all adherent cells were maintained in the exponential phase of growth at 37°C in a humidified incubator, in an atmosphere of 5% CO_2 and 95% air.

DT40 cells were grown in D-MEM/F12 supplemented with 10% FBS, 1% chicken serum and 50 μM β-mercaptoethanol at 41ºC in a humidified incubator with 5% CO_2. All cell lines were maintained in the logarithmic phase of growth through routine sub-culturing. DNA ligase mutants analyzed here were derived from the DT40-Cre1 cell line and are described in greater detail in the following section and in Table 1 (see also (23, 24)). Xpa deficient mutants (kindly provided by Dr. S. Takeda) were generated on an alternative wild-type background by deleting amino acids Asp^{125}–Gln^{195} (25). DT40 cells have only a single Xpa gene, which is located on a sex chromosome (see: www.rg.med.kyoto-u.ac.jp/index-e.html for a description of the gene-disruption strategy).

Exposure of cells to ultraviolet (UV) light: Cells were exposed to UVC (Osram, HNS 25W OFR) in PBS in an open petri dish at a distance of 45 cm. Exposure was monitored using a UV-meter with a single sensor for UVC (Hönle, UV-METER with FS UVC D0 E110). After exposure, cells were returned to normal cell culture conditions and processed as required by the experimental protocol.

Colony Forming Assay: For all cell lines, cell survival after UVC exposure was determined using the colony forming assay. All cell lines used were plated in 5 ml growth medium appropriately diluted to obtain 100 – 200 colonies per dish. Dishes were incubated for 10 - 14 days at normal growth conditions and subsequently colonies were counted by eye. For evaluation of colony forming ability in DT40 cells, cells were seeded in medium containing 1.5% methylcellulose (MC) (Sigma, M0387).

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Immunofluorescence microscopy: For DT40 cells, approximately 0.3 x 10^6 cells were allowed to attach to ImmunoSelect Adhesion Slides (Squarix) for 15 min on ice. All cell lines used were fixed for 15 min with 2% paraformaldehyde and after washing with PBS permeabilized for 15 min in P-solution (0.5% Triton X-100 in 100 mM Tris, 50 mM EDTA). DNA was denatured for 30 min with 2 N HCl and cells were blocked with PBG solution (PBS, 0.5% BSA, 0.2% Gelatin) overnight at 4°C, or for 1 h at room temperature.

For visualization of CPD's, slides were incubated for 90 min at room temperature with anti-CPD mouse monoclonal antibody (TDM-2, Cosmobio) diluted 1:1000 in PBG solution. Slides were washed once in PBS and an anti-mouse IgG antibody, conjugated with AlexaFluor488 (Invitrogen), was added for 60 min at room temperature at a 1:500 dilution in PBG solution. Cell nuclei were counterstained for 30 min with 2 µg/ml 4',6-diamidin-2-phenylindol (DAPI), 0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$, 0.05% Triton X-100 and washed once with PBS. Coverslips were mounted on slides using Prolong-Gold Antifade (Invitrogen). Samples were scanned with a 40× objective in an automated analysis station equipped with a fluorescence microscope (Axio Imager Z2, Zeiss) and controlled by the Metafer software (MetaSystems). On average, 4000 cells per sample were scored and analyzed using Metafer software. The DAPI signal intensity was used to analyze CPD signal throughout the cell cycle.

Immuno-slot-blotting: Genomic DNA was isolated using NucleoSpin tissue kit (MN). For each sample 500 ng extracted DNA was denatured in 100 µl TE buffer for 10 min at 95°C on ice and diluted with 100 µl ice-cold 2 M ammonium acetate. Nitrocellulose membrane (MN) was soaked in 1 M ammonium acetate and 200 µl sample was applied onto the membrane through a slot blot apparatus. Membrane was soaked in 5 x SSC for 5 min, washed with H_2O, dried, heated for 2 h at 80°C and blocked with PBG solution for 1h at room temperature. Membrane was incubated with the same anti-CPD mouse monoclonal antibody (TDM-2, Cosmobio) diluted 1:4000 in PBG solution for 1 h at room temperature. The primary antibody was detected with anti-mouse HRP-linked secondary antibody (Cell Signalling) applied for 1 h at room temperature at a 1:20000 dilution in PBG solution and visualized by SuperSignal™ West Femto Maximum Sensitivity Substrate (Life Technologies) using a VersaDoc Imaging Station (Bio-Rad). All immuno slot-blot analyses were performed in triplicate.

RESULTS

Targeting strategies to generate required mutants

To investigate individual functions and functional overlap between Lig1 and Lig3 in NER, we applied in DT40 a comprehensive gene targeting strategy allowing the generation of a unique set of mutants. Constitutive and conditional knockouts were developed and combined with knock-ins of different DNA ligases in an effort to study the ligation requirements of NER in a clean genetic background. To minimize the risk of residual DNA ligase activity in knockouts, or of dominant negative effects from residual, truncated protein expression, our targeting vectors were designed to delete a substantial portion of the catalytic core including the enzyme active site of *Lig1*, *Lig3*, and *Lig4* (red lines in Figure 1A) (26, 14). Two forms of Lig3 are shown in Figure 1A, nuclear and mitochondrial. They are

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generated from the same gene transcript using two alternative translation-start-codons. The longer polypeptide is endowed with a mitochondria targeting sequence (MTS) that ensures its efficient transport into this organelle. Lig3 is the only ligase of mitochondria and essential for their function. Lethality associated with knockout of Lig3 in vertebrates derives from its essential role in mitochondria function. We included Lig4 in our targeting strategy in order to generate single-ligase systems that allow conclusive answers regarding the function of each DNA ligase in NER.

The first goal of our study was the generation of mutants expressing only Lig3 (Figure 1B). Therefore, we consecutively inactivated the alleles of Lig1 and Lig4. First, one *Lig1* allele was targeted using the vector *pLig1Bsr,* to generate a *Lig1*$^{+/-}$ mutant. Subsequently, the second *LIG1* allele was disrupted with the *pLig1Puro* targeting vector to generate the *Lig1*$^{-/-}$ mutant (Figure 1B) (26). *Lig1*$^{-/-}$ cells grew normally indicating that the remaining ligases efficiently support DNA replication (26).

In the second step of our targeting approach, we inactivated *Lig4* in the *Lig1*$^{-/-}$ background (Figure 1B). The first vector *pLig4Bsr* was designed to delete the entire coding sequence of one *Lig4* allele, whereas the second vector *pLig4Puro4* was designed to delete amino acids 185-614 of the second allele, while inserting an in frame stop codon after codon 184 (Figure 1B) (26). The double *Lig1*$^{-/-}$*Lig4*$^{-/-}$ mutant grows only slightly slower than wt cells, using Lig3 as sole DNA ligase for all its DNA replication functions (26, 14). This mutant was essential for the studies described below.

The second goal of our study was the generation of viable cells relying exclusively on Lig1 for all ligation requirement of their DNA metabolism (Figure 1C). For this purpose we consecutively inactivated Lig3 and Lig4, while rescuing the lethal phenotype of Lig3 knockout through the expression of human Lig1 endowed with a MTS (26, 14).

Lig3 targeting using conventional strategies readily generated *Lig3*$^{+/-}$ cells, but *Lig3*$^{-/-}$ mutants cannot be recovered and genetic studies are hampered by the lethality of *Lig3*$^{-/-}$ cells (26, 27). The lethality associated with *Lig3* knockout lead us to introduce the Cre/loxP system to generate conditional mutants. In the first step, we knocked-in two loxP sites into one allele of the endogenous *Lig3* gene using the vector *pLig3Lox3Bsr* (Figure 1C). In these *Lig3*$^{+/2loxP}$ cells the second allele was targeted using the conventional targeting vector *pLig3Gpt* to generate *Lig3*$^{-/2loxP}$ cells (Figure 1C). *Lig3*$^{-/2loxP}$ cells are viable but as a result of the single allele expression, *Lig3* mRNA is 50% reduced (14, 26). The remaining two loxP sites mediate upon further treatment with 4HT the removal of exons 6-9 of the *Lig3* gene, which encode the DNA binding domain and a part of the catalytic core including the enzyme active site (Figure 1A), to generate *Lig3*$^{-/-}$ cells which die within 4 days (26, 14). Next, we inactivated *Lig4* using conventional knockout strategies, as described above, in the *Lig3*$^{-/2loxP}$ genetic background to generate *Lig3*$^{-/2loxP}$*Lig4*$^{-/-}$ cells (Figure 1B). The inception of apoptosis at low levels of Lig3 in the 4HT treated conditional DT40 *Lig3* mutant hampers conclusive analysis of Lig1 function in NER. To overcome this limitation we rescued lethality of *Lig3*$^{-/-}$ cells by complementing the mitochondria defect responsible for the associated lethality. Indeed, it was shown that lethality of *Lig3*$^{-/-}$ mutants is rescued by expression of diverse DNA ligases, if endowed with a MTS (28, 29, 14, 26). For our experiments, we integrated therefore into *Lig3*$^{-/2loxP}$*Lig4*$^{-/-}$ cells with the help of the vector *pChr8Cdc9MtsHLig1Bsr* human mts-hLig1 to generate *Lig3*$^{-/2loxP}$*Lig4*$^{-/-}$*mts-hLig1* (Figure 1C).

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Treatment of the latter mutant with 4HT generates *Lig3*$^{-/-}$*Lig4*$^{-/-}$ cells rescued by mts-hLig1. This mutant expresses only the endogenous chicken Lig1 and the overexpressed mts-hLig1 (14). It represents the second essential mutant for the studies on NER reported here. *Lig3*$^{-/2loxP}$, *Lig3*$^{-/2loxP}$*Lig4*$^{-/-}$*mts-hLig1* and *Lig3*$^{-/-}$*Lig4*$^{-/-}$*mts-hLig1* mutants grow with kinetics similar to wt cells. Only the growth of *Lig3*$^{-/2loxP}$*Lig4*$^{-/-}$ cells is slightly decreased, similar to the mono ligase mutant *Lig4*$^{-/-}$ (14). Table 1 summarizes the DT40 mutants used in this study and their phenotypes.

Lig1 deficiency has no effect on cell sensitivity to UVC exposure

Wt DT40 cells are moderately sensitive to UVC and surprisingly the *Lig1*$^{-/-}$ mutant shows no detectable increase in this sensitivity (Fig. 2A). In contrast, an Xpa deficient DT40 mutant displays the expected hyper-sensitivity to UVC (Fig. 2A). The wt cells used to derive the Xpa mutant show sensitivity to UVC similar to the wt used to derive the ligase mutants (Suppl. Figure 1). This suggests that Lig1 is not essential for NER in DT40 cells. Evidently, in the absence of Lig1, the remaining DNA ligases Lig3 and Lig4 are capable of efficiently substituting for all NER-related ligation requirements of the cells.

To confirm these results in a different species, we measured clonogenic survival after exposure to UVC of the Lig1 deficient MEFs, PFL13, and their corresponding wt counterpart, PF20. Again, both cell lines are moderately sensitive to UVC and show no detectable differences in their radiosensitivity to killing (Fig. 2B). Similar results were previously reported (13) and demonstrate a non-essential role for Lig1 in NER in the mouse system as well.

Similar results were also obtained when investigating the Lig1 deficient human fibroblasts, 46BR 1N, and their corrected counterparts, 46BR PBAHL. Although these cells are overall more resistant to UVC than mouse or chicken cells, the sensitivity of the Lig1 mutant is indistinguishable to that of the corrected cell line. A human fibroblast cell line defective in Xpc, which was used as a positive control, shows the expected hyper-sensitivity to UVC (Fig. 2C). Collectively, the results in three species demonstrate a non-essential role for Lig1 in NER and suggest that other DNA ligases, with Lig3 as the most likely candidate, fully support the ligation requirements of NER.

Lig3 efficiently supports NER

To conclusively determine that the ligase operating in Lig1 deficient cells is Lig3, we tested the sensitivity to UVC of the *Lig4*$^{-/-}$ mutant, as well as the *Lig1*$^{-/-}$*Lig4*$^{-/-}$ double knockout mutant. The results summarized in Figure 3A show an indistinguishable sensitivity to UVC of both mutants in comparison to the wt cells. These results show that Lig4 has no essential and exclusive function in NER, and formally demonstrate that Lig3 alone can proficiently cover for all ligation requirements of NER.

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Lig1 alone efficiently supports ligation during NER

It has been suggested that in the presence of Lig1 and Lig3, each ligase is assigned to different NER subpathways showing distinct cell cycle, or cell-growth-state specificities (21). The results presented above for Lig3 show that it efficiently substitutes Lig1 in all pathways it is involved, while operating with similar efficiency. To test this possibility also for Lig1, we used the above described *Lig3*$^{-/-}$*Lig4*$^{-/-}$ *mts-hLig1* mutant, which as explained above covers all its DNA ligation needs using Lig1 as the sole ligase. *Lig3*$^{-/-}$*Lig4*$^{-/-}$*mts-hLig1* cells show sensitivity to UVC indistinguishable from that of the wt cells (Figure 3B). This observation confirms the efficient operation of Lig1 in all NER subpathways.

The mono-ligase mutants process CPDs with wild type efficiency

To further underpin the observations of functional flexibility between Lig1 and Lig3 in NER using cell killing as endpoint, we examined repair capacity in the same mutants using the removal of UVC-generated CPDs as endpoint. Efficient repair of CPD by NER is indicated by the decay of CPD staining-intensity after exposure to UVC. Figure 4A shows the CPD repair kinetics after exposure to 25 J/m^2 UVC of the different mutants, plotted as median CPD signal intensity as a function of time. The kinetics of CPD removal in the *Lig1*$^{-/-}$, *Lig4*$^{-/-}$, and *Lig1*$^{-/-}$*Lig4*$^{-/-}$ mutants are similar to the wt cells (Figure 3A), suggesting a similar repair capacity in all of them. Notably, the *Lig3*$^{-/-}$*Lig4*$^{-/-}$*mts-hLig1* mutant that exclusively relies on Lig1, also shows efficiency of CPD removal similar to the wt cells (Figure 3A).

All DT40 cells examined above show a spike in the removal kinetics at 3 h after exposure. To investigate whether this response reflects a DT40-cell-specific effect, we carried out similar experiments using human fibroblasts (46BR PBAHL, the Lig1 mutant 46BR 1N, and the Xpc deficient XPC-EB). The results in Figure 4B show similar repair efficiency in normal and Lig1 deficient cells confirming thus the results obtained with DT40 cells. Xpc deficient cells show a strong deficiency in CPD removal as expected, particularly at early time points, confirming thus that the methodology employed can detect NER repair defects.

Also an immuno-slot-blot assay using DT40 wt DNA for CPD detection shows similar patterns of CPD repair with a spike at 3 h after exposure to UVC (Suppl. Figure 2), although the magnitude of the spike is reduced compared to the results shown in Figure 4A.

We conclude therefore that the observed peak reflects an inherent peculiarity of the method and refrained from further attempts to characterize its origins, as it does not interfere in any way with conclusions we make on the basis of the results obtained. Collectively, the results confirm that also when the actual repair of UVC induced lesions is followed, Lig1 and Lig3 are equally capable of carrying out the required ligation functions.

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DISCUSSION

The results presented above allow a conclusive analysis of the functions of Lig1 and Lig3 in NER through the use of unique mutants generated in the chicken DT40 cell system (14). The results obtained when analyzing either cell survival or the kinetics of CPD-removal from the DNA after UVC exposure clearly demonstrate full flexibility in the utilization of either DNA ligase.

Specifically, we report a normal sensitivity to UVC of the double *Lig1*$^{-/-}$*Lig4*$^{-/-}$ mutant, which contrasts the hyper-sensitivity of the Xpa deficient mutant (Figure 2A) (25). In the double *Lig1*$^{-/-}$*Lig4*$^{-/-}$ mutant Lig3 is the sole remaining DNA ligase, unequivocally showing not only contribution but actually full complementation of NER by Lig3. Similar conclusions are drawn from experiments measuring the actual removal of UVC-induced CPD using immunofluorescence approaches in the same mutant. We conclude therefore that Lig3 can be recruited to UV damage sites not only in quiescent (21) but also in actively proliferating cells.

It remains possible of course that when both DNA ligases are present, a recruitment hierarchy exists that is different for different lesions and stages of cellular growth. It is relevant to also consider that when Lig3 utilization is analyzed by knockdown, the associated mitochondria-initiated toxicity may mask or distort the actual effects of Lig3 on DNA repair.

We and others have demonstrated that Lig3 knockout lethality can be rescued by expression of a ligase endowed with a mitochondria-targeting signal (28, 29, 14). We explore this strategy in the present work to generate a DT40 mutant covering all its ligation requirement using representatives of the Lig1 family of DNA ligases. In this mutant, mitochondria function is rescued by expressing human Lig1 endowed with a mitochondrial targeting signal (mts-hLig1). This mutant gave us the unique opportunity of studying the roles and efficiency of Lig1 in all aspects of NER.

The *Lig3*$^{-/-}$*Lig4*$^{-/-}$*mts-hLig1* mutant clearly shows that cells relying solely on Lig1 are not hampered in their ability to carry out NER after exposure to UVC. It appears that in contrast to yeast, which lack Lig3 and where Cdc9 is exclusively involved in excision repair, both Lig3 and Lig1 participate in a flexible fashion in this repair pathway in vertebrates.

Xrcc1 is usually considered an obligatory protein partner of Lig3 (30). The two molecules are thought to operate as a complex, forming through their BRCT domains (31). Interestingly, recent reports uncouple the function of Lig3 from Xrcc1 for certain cellular functions (28, 29) and indicate that Xrcc1 may not be required, for example, for B-NHEJ (32). Since the chicken homolog of Xrcc1 remains uncharacterized it is not possible to contribute in this discourse at the present time.

Overall, our results demonstrate an impressive and previously undocumented potential of Lig3 to substitute for Lig1 in the removal of helix-distorting DNA lesions. Moreover, they show for the first time in a clean genetic background a functional redundancy between Lig1 and Lig3 in NER. It is intriguing that such relationships developed only after the evolutionary appearance of *Lig3,* as *Lig1*

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and *Lig4* family members have well separated functions in lower eukaryotes.

The functional flexibility of Lig3 is anticipated by the impressive substrate flexibility it shows (33). Together with our recent results on the function of Lig3 in semi-conservative DNA replication (26), and the role of Lig1 and Lig3 in B-NHEJ (14), the present findings expand the functional redundancy and flexibility of Lig1 and Lig3 to DNA replication, NER, and HRR (unpublished observations). Our results provide a solid rationale for explaining the previously reported lack of UVC sensitivity in mouse and human cells deficient in Lig1 (13).

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SUPPLEMENTARY INFORMATION

Additional Supplementary Information may be found in the online version of this article:

Figure S1: Cell survival by colony formation after UVC exposure of wt DT40 cells of two different origins. Wt DT40 *AID-/-IgL$_{dual}$* are used in this study for the generation of the DNA ligase mutants (26). Wt DT40 cells were used to generate the Xpa deficient mutants (25).

Figure S2: Repair kinetics of UVC-induced CPDs measured by immuno slot-blot in wt DT40 cells after exposure to 50 J/m$_2$ UVC.

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FIGURE CAPTIONS

Figure 1. A) Alignment of the domain structure of chicken DNA ligases. The red bars indicate regions deleted in the mutants generated. B) Approach to generate a DT40 mutant exclusively relying on Lig3 for DNA ligation functions. The steps and vectors followed to generate the indicated mutants are outlined. C) Approach to generate a mutant exclusively relying on Lig1 for DNA ligation functions. The steps and vectors followed to generate the indicated mutants are outlined.

Figure 2. A) Cell survival measured by colony formation of DT40 wt, *Lig1*$^{-/-}$ and *Xpa*$^{-}$ cells after exposure to increasing doses of UVC. B) Cell survival of MEF PF20 wild type and the Lig1-deficient PFL3 cells after exposure to increasing doses of UVC. C) Cell survival of human Lig1 deficient 46BR

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1N, Lig1 corrected 46BR PBAHL and Xpc deficient XPC-EB cells after exposure to increasing doses of UVC.

Figure 3. A) Cell survival of DT40 wt, *Lig4*$^{-/-}$ and double knockout *Lig1*$^{-/-}$*Lig4*$^{-/-}$ cells after exposure to increasing doses of UVC. B) Cell survival of DT40 wt and *Lig3*$^{-/-}$*Lig4*$^{-/-}$*mts-hLig1* cells after exposure to increasing doses of UVC.

Figure 4. A) Repair kinetics of UVC-induced CPD measured by immunofluorescence microscopy in DT40 wt, *Lig1*$^{-/-}$, *Lig4*$^{-/-}$, *Lig1*$^{-/-}$*Lig4*$^{-/-}$ and *Lig3*$^{-/-}$*Lig4*$^{-/-}$*mts-hLig1* cells after exposure to 25 J/m^2. B) As in A for human Lig1 deficient 46BR 1N, Lig1 corrected 46BR PBAHL and Xpc deficient XPC-EB cells after exposure to 25 J/m^2 UVC.

Table 1. List of mutants used with their genotypes and relevant phenotypes.

Cell Line	Feature	Viability	UV Sensitivity
wt	DT40 wt	viable	normal
***Xpa*$^{-}$**	*Xpa* knockout	viable	high
***Lig1*$^{-/-}$**	*Lig1* knockout	viable	normal
***Lig4*$^{-/-}$**	*Lig4* knockout	viable	normal
***Lig1*$^{-/-}$*Lig4*$^{-/-}$**	*Lig1* knockout; *Lig4* knockout	viable	normal
Lig3*$^{-/-}$*Lig4*$^{-/-}$ *mts-hLig1	*Lig3* knockout; *Lig4* knockout; overexpressing *mts-hLig1*	viable	normal

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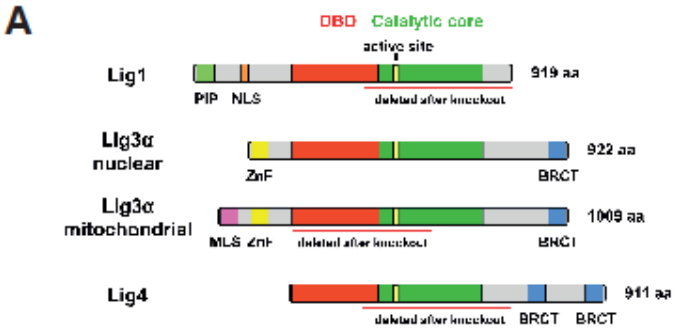

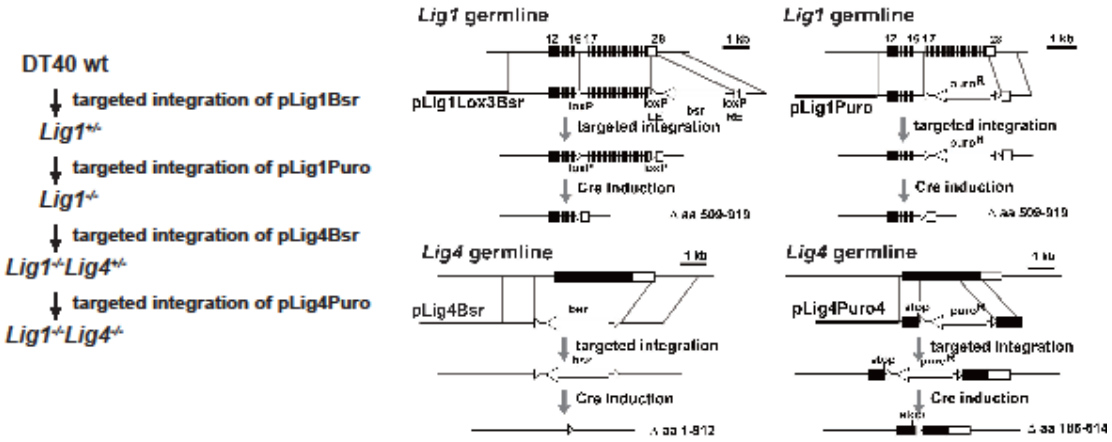

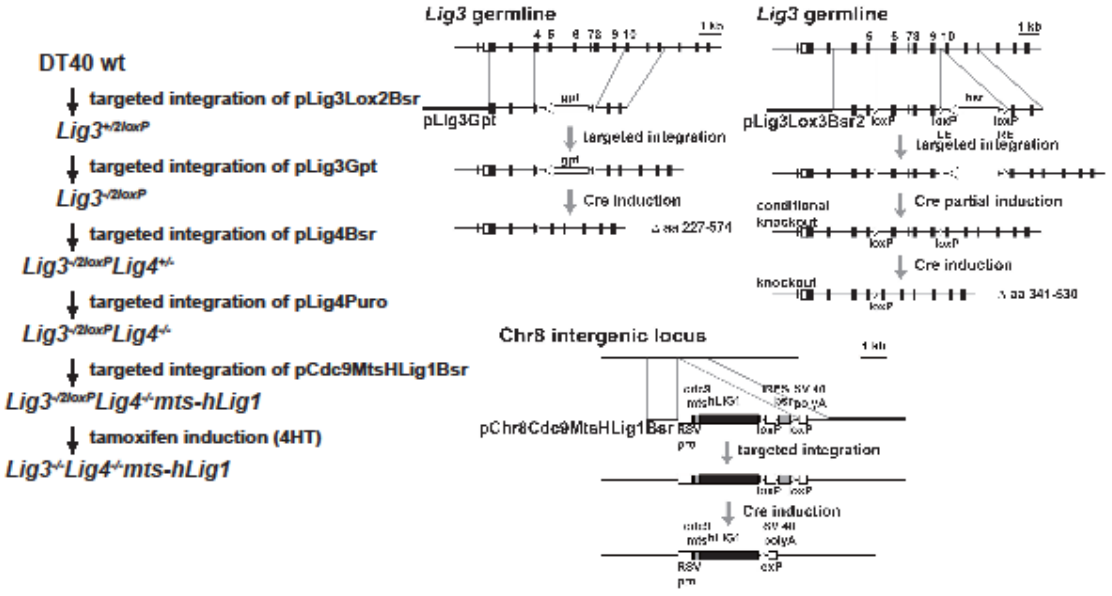

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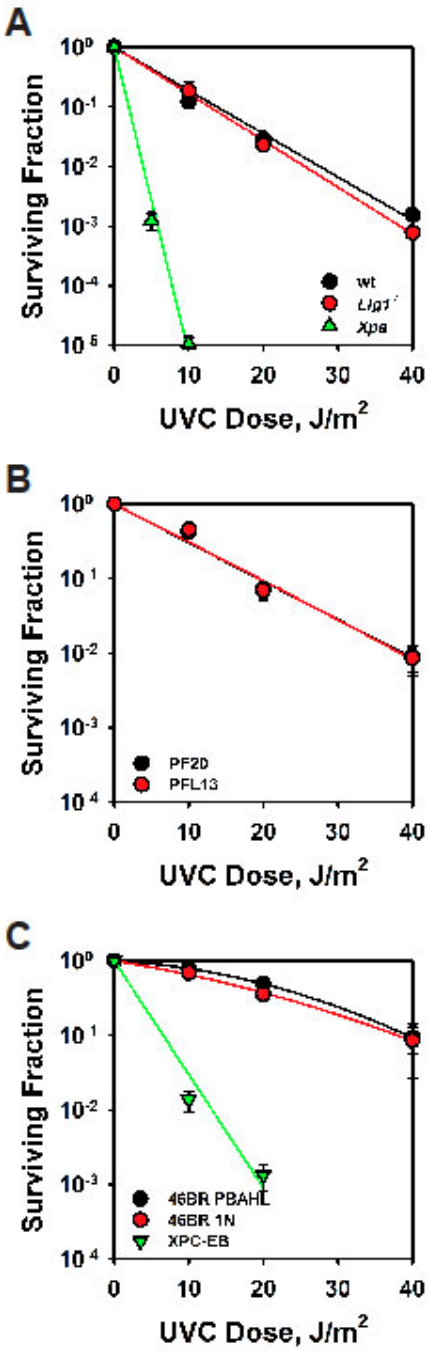

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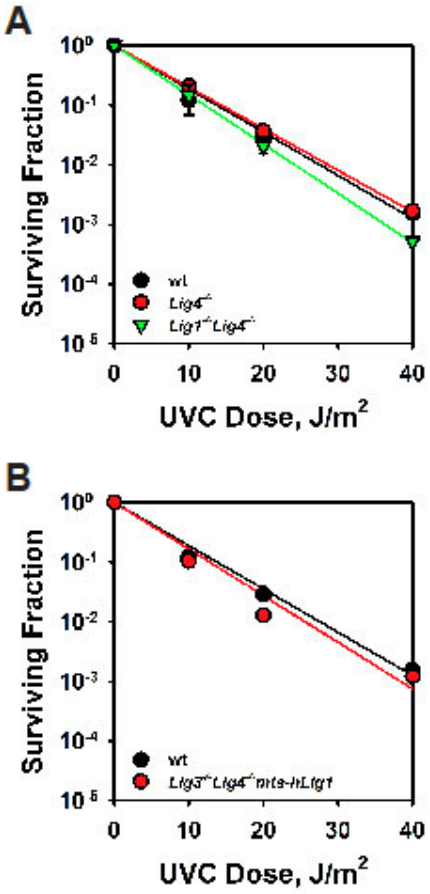

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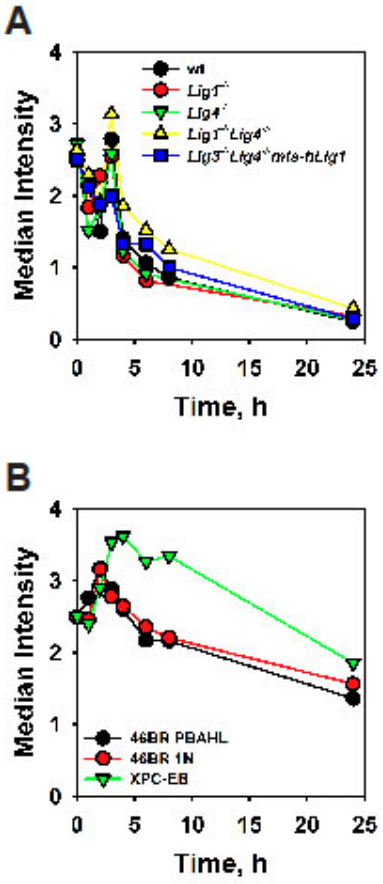

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