EXTRA VIEW

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Disconnecting XRCC1 and DNA ligase III

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DNA strand break repair is essential for the prevention of multiple human diseases, particularly those which feature neuropathology. To further understand the pathogenesis of these syndromes, we recently developed animal models in which the DNA single-strand break repair (SSBR) components, XRCC1 and DNA Ligase III (LIG3), were inactivated in the developing nervous system. Although biochemical evidence suggests that inactivation of XRCC1 and LIG3 should share common biological defects, we found profound phenotypic differences between these two models, implying distinct biological roles for XRCC1 and LIG3 during DNA repair. Rather than a key role in nuclear DNA repair, we found LIG3 function was central to mitochondrial DNA maintenance. Instead, our data indicate that DNA Ligase 1 is the main DNA ligase for XRCC1-mediated DNA repair. These studies refine our understanding of DNA SSBR and the etiology of neurological disease.

Key words: DNA repair, nervous system, neurodegeneration, DNA ligase III, DNA damage, XRCC1, mitochondria, mtDNA

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Introduction

Normal cellular metabolic processes in mammals result in the production of reactive oxygen species (ROS). Although oxygen consumption is essential for life, the ROS byproduct is a potential cellular genotoxin, as ROS-mediated attack of DNA generates direct DNA single-strand breaks (SSBs), and, accordingly, DNA SSBs are one of the most common DNA lesions that occur within the cell.[1,2] The substantial oxygen requirement for maintenance of the CNS renders neurons especially sensitive to DNA damage from the oxidative effects of metabolic ROS.[3-5] Consequently, neurons require rapid and efficient DNA strand-break surveillance and repair mechanisms to deal with these types of lesions.

Repairing DNA Single-Strand Breaks

DNA SSBs can include a multitude of 3'- and 5'-end-modifications, which must be processed by specific DNA end-processing enzymes prior to gap-filling and resealing of the DNA backbone.[6-8] DNA SSBs can occur directly to DNA via ROS-induced deoxyribose breakdown or abortive topoisomerase 1 (Top1)-DNA intermediates (Top1-3'-DNA) that form Top1-SSBs upon collision with RNA polymerase II or due to proximal secondary DNA damage.[9-12] Indirect DNA SSBs can also occur as an intermediate of base excision repair (BER) due to enzymatic incision at an apurinic-apyrimidinic (AP) site by APE1 or DNA glycoslase.[9]

DNA SSBs are repaired utilizing a dedicated repair pathway (**Fig. 1**) that centrally involves XRCC1 and components of the BER pathway.[9,13] Detection of direct SSBs is mediated by poly(ADP-ribose) polymerase (PARP), which acts to post-translationally modify the XRCC1 scaffolding protein for recruitment to the DNA break site. XRCC1 is a key factor for DNA SSBR, as it orchestrates a variety of enzymes involved in DNA end-processing (TDP1, APTX, APE1, PKNP), DNA Polβ-mediated short-patch gap-filling (single-nucleotide insertion) and DNA nick sealing (ligation), generally considered to be via XRCC1-bound DNA ligase IIIα (LIG3). However, in some instances, DNA nick gap-filling may continue for 2–12 nucleotides

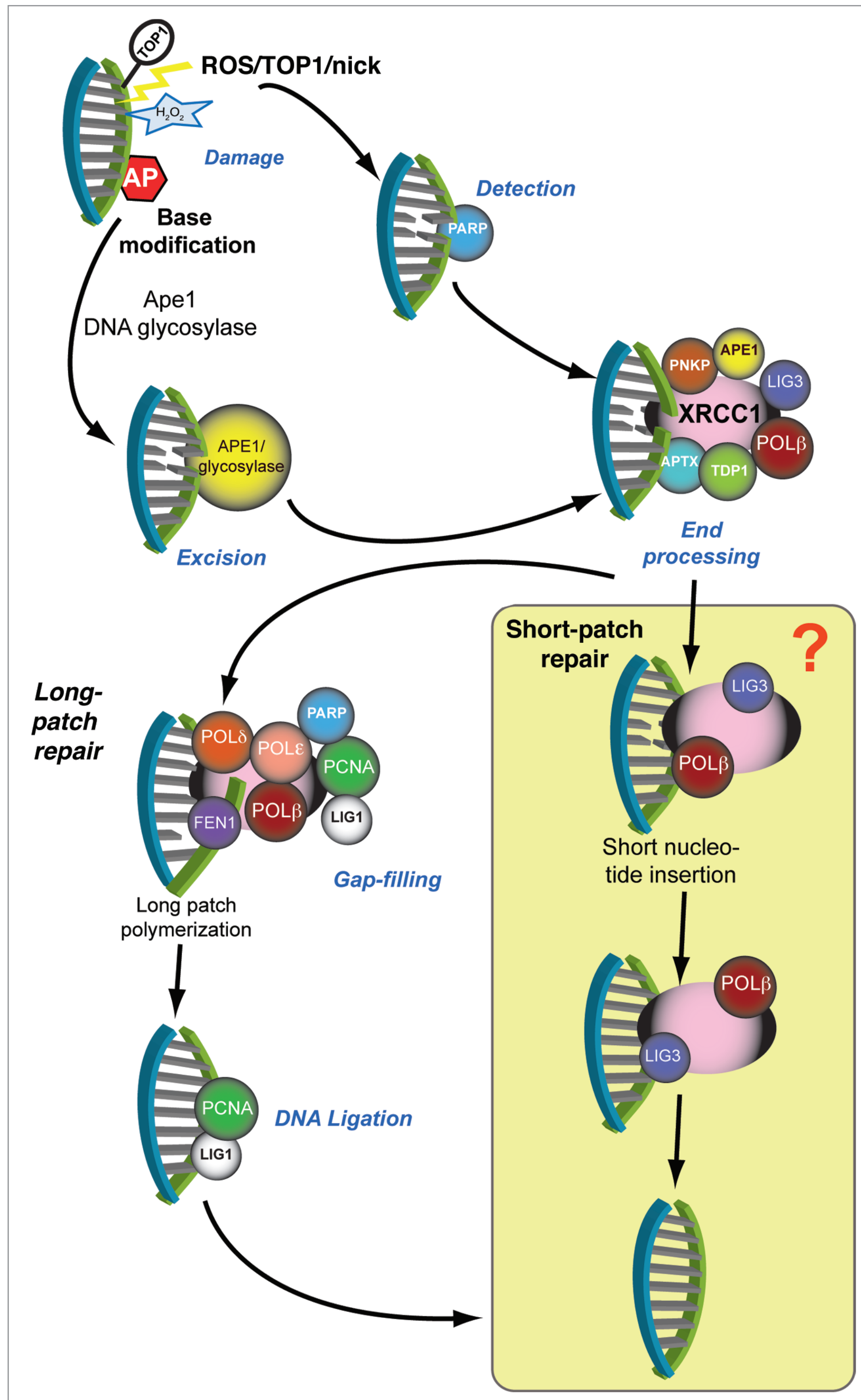

Figure 1. For figure legend, see page 2271.

(long-patch polymerization) via Polβ/δ/ε, thereby requiring the Flap endonuclease 1 (FEN1) to remove the resultant displaced nucleotides followed by DNA ligation via PCNA-bound DNA ligase 1 (LIG1).[6,14]

Human syndromes that feature pronounced neuropathology can arise from defects in end-processing enzymes.[6,15-17] For example, spinocerebellar ataxia with axonal neuropathy (SCAN1) and ataxia with oculomotor apraxia 1 (AOA1) are syndromes that are associated with defective single-strand break repair (SSBR) and feature ataxia linked to cerebellar degeneration and neuropathy.[18,19] SCAN1 results

Figure 1 (See opposite page). DNA single-strand break repair. Resolution of DNA single-strand breaks involves specialized factors that process and repair specific types of breaks. Direct DNA breaks (which may arise from radiation, oxidation or abortive Top1-DNA intermediates) undergo detection by PARP, which signals the breaksite through poly-ADP ribosylation of XRCC1 and DNA end-processing factors. The formation of ADP-ribose polymers on these proteins serves to augment affinity and facilitate protein complex recruitment to the DNA breaksite. Damaged 5′- and 3′-end DNA termini undergo end-modification by DNA processing factors to generate 5′-end phosphate and 3′-end hydroxyl termini, which allows DNA polymerase to replace the missing nucleotide(s) and DNA ligase to restore integrity of the DNA strand backbone. DNA short-patch repair involves integration of a single nucleotide via DNA polymerase β into the damaged strand and DNA ligation by XRCC1-bound LIG3. Indirect DNA breaks arise from enzymatic excision of apurinic-apyrimidinic (AP) bases (which can occur from base alkylation) via APE1 or DNA glycosylases. Although this substrate can undergo XRCC1-mediated end-processing, followed by DNA short-patch repair, certain classes of damage undergo long-patch DNA repair, whereby DNA polymerase δ and ε generate an extended nucleotide polymer (2–12 nucleotides) to replace DNA bases at and proximal to the DNA break site. Flap-endonuclease 1 (FEN1) cleaves the extraneous DNA flap, and the intact DNA backbone is ligated via PCNA-bound LIG1. The shaded box represents the aspect of SSBR involving LIG3, which is now less clear based on new data[44,65] discussed in this paper.

from disruption of tyrosyl-DNA phosphodiesterase 1 (TDP1), an enzyme that is required for the end-processing of 3'-phosphoglycolate and 3'-DNA-Top1 moieties.[20,21] Lymphoblastoid cells derived from SCAN1 individuals are defective in their ability to repair DNA breaks after DNA SSB-inducing agents, such as peroxide (H_2O_2) or the Top1 poison camptothecin (CPT), while DNA double-strand break repair (DSBR) responses are normal.[20,22] Mice deficient in Tdp1 (*Tdp1*$^{-/-}$) show cerebellar hypoplasia and defective DNA SSBR responses to similar SSB-inducing agents.[21] In AOA1, mutations in aprataxin (APTX), a nucleotide hydrolase, causes defects in the removal of 5'-adenylate-DNA intermediates that arise due to abortive DNA ligation reactions.[23,24] AOA1 lymphoblastoid cells and neural cells from Aptx-deficient mice (*Aptx*$^{-/-}$) are defective in DNA short-patch repair.[23-25] More recently, individuals with mutations in polynucleotide kinase 3'-phosphatase (PNKP) have been shown to develop microcephaly with early-onset seizures and developmental delay (a syndrome termed MCSZ).[26] Cell lines from MCSZ patients are defective in their ability to repair peroxide- or CPT-induced DNA damage. Similarly, human A549 cells deficient in PNKP display genomic instability and sensitivity to a variety of DNA damaging agents.[27] Collectively, these syndromes highlight the importance of SSB resolution in avoiding neuropathology. Nonetheless, the mechanistic contribution of SSBR during neurogenesis and the maintenance of neural homeostasis remain ill-defined.

XRCC1-Dependent SSBR is Neuroprotective In Vivo

Understanding the roles of the SSBR/BER pathway in the mammalian nervous system requires the establishment of tractable models. However, germline inactivation of key SSBR factors in the mouse have not yielded insight, as they do not show overt neurodegeneration (as is the case in *Aptx*$^{-/-}$ and *Tdp1*$^{-/-}$ mice) or they result in early embryonic lethality (*Xrcc1*$^{-/-}$, *Ape1*$^{-/-}$, *Pol*β$^{-/-}$, *Lig1*$^{-/-}$ and *Lig3*$^{-/-}$ mice).[28-34] Therefore to understand the etiology of SSBR deficiency as it relates to neurodegenerative disease, we produced a conditional *Xrcc1* allele using *Nestin-cre* to drive *Xrcc1* deletion in the developing murine nervous system and generated *Xrcc1*$^{Nes\text{-}cre}$ mice.

Neuraxis-wide Xrcc1 deficiency led to pronounced neurological dysfunction that was characterized by the loss of cerebellar interneurons and hippocampal dysfunction associated with a progressive seizure-like phenotype.[35] In vivo and in vitro analyses of *Xrcc1*$^{Nes\text{-}cre}$ mice revealed that widespread DNA damage accumulated throughout the nervous system. Alkali comet analysis of *Xrcc1*$^{Nes\text{-}cre}$ neurons and astrocytes showed deficient DNA repair in response to a variety of DNA SSB-inducing agents, including ionizing radiation (IR), H_2O_2, CPT and methyl methanesulphonate (MMS, an alkylating agent). Xrcc1 deficiency sensitized Pax2-immunopositive cerebellar interneuron progenitors to undergo DNA damage-induced, p53-mediated cell cycle arrest, resulting in a failure to populate the cerebellar molecular layer with basket, stellate and Golgi interneurons.[35] Furthermore, elsewhere in the cerebellum, Xrcc1-deficiency resulted in progressive DNA damage-induced p53-mediated granule neuron apoptosis and disrupted hippocampal homeostasis, likely causing the observed seizures in these mice. The data from the *Xrcc1*$^{Nes\text{-}cre}$ mouse have augmented our understanding of the biology of SSBR/BER and are relevant to understanding the etiology and neuropathology associated with defective DNA SSBR responses.

XRCC1 and LIG3 Are Tightly Associated in the Cell Nucleus

Efficient DNA SSBR requires XRCC1-mediated assembly of the repair machinery, and the final step in DNA nick repair requires a DNA ligase to reseal the DNA backbone. Because XRCC1 and LIG3 are tightly associated, this ligase has been assumed to fulfill this requirement. XRCC1 and LIG3 can be co-immunoprecipitated from whole cell extracts, and recombinant XRCC1 and LIG3 form a strong interaction that is resistant to high salt concentrations.[36-39] Furthermore, *Xrcc1* and *Lig3* germline knockout mice die at a similar early embryonic stage.[32,33] LIG3 protein levels and activity have also been shown to be dependent on Xrcc1 protein expression, as *Xrcc1*$^{Nes\text{-}cre}$ CNS neural tissue and Xrcc1-deficient Chinese hamster ovary (CHO) cell lines (EM7, EM9, EM11-C11 and EM-C12) have markedly reduced LIG3 protein levels and defective DNA substrate ligation activity.[36,39,40] Given the close biochemical relationship between XRCC1 and LIG3, the substantial DNA repair deficiency after XRCC1 inactivation and the essential requirement for ligation as a final step during repair, LIG3 activity is potentially critical during XRCC1-mediated SSBR.

LIG3 is Also Present in the Mitochondria

A mitochondrial isoform of *LIG3* (*mtLIG3*) is also present in the cell.

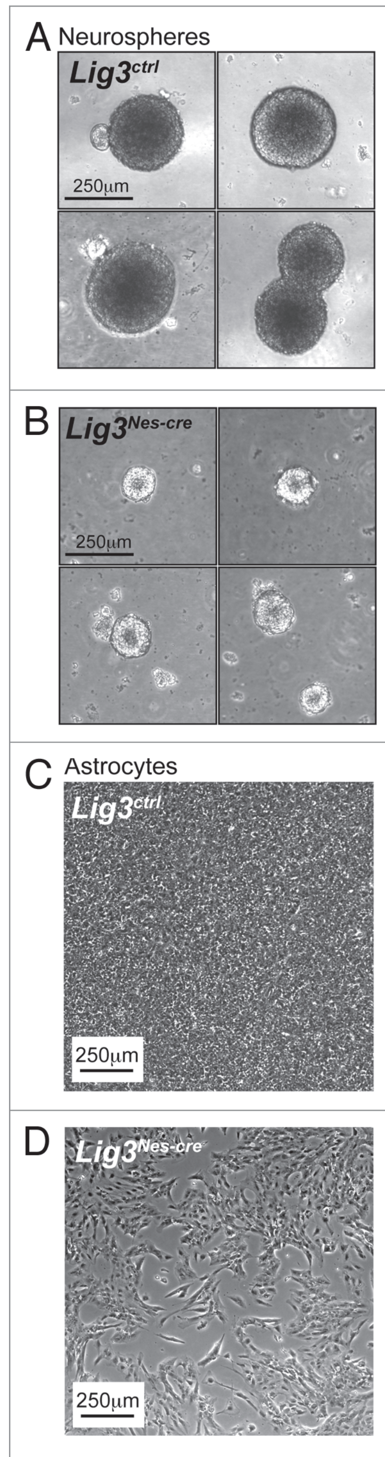

Figure 2. Primary *Lig3*$^{Nes\text{-}cre}$ neural cell lines have growth defects. Photomicrographs of primary neurospheres derived from (A) *Lig3*^{ctrl} and (B) *Lig3*$^{Nes\text{-}cre}$ neural stem cells isolated from E14.5 mouse embryos. *Lig3*$^{Nes\text{-}cre}$ neurospheres are significantly smaller and display reduced cellularity compared to WT (*Lig3*^{ctrl}) counterparts. (C) Primary astrocytes isolated from WT P3 mice form a tight confluent monolayer five days post-isolation.
(D) In contrast, *Lig3*$^{Nes\text{-}cre}$ astrocytes cultures are growth delayed.

MtLIG3 is encoded from the single nuclear *LIG3* gene via an alternate 5'-exon encoding an N-terminal mitochondrial targeting sequence.[41] Notably, mitochondrial extracts derived from Xrcc1-deficient EM9 CHO cells display intact ligase activity, while BER-dependent ligase activity is absent in the corresponding nuclear extracts.[42] Antisense-mediated reduction of mtLIG3 in HT1080 cells compromised mitochondrial DNA (mtDNA) integrity, resulting in reduced mtDNA levels and increased mtDNA nicks.[43] Thus, LIG3 can function in both the nucleus and mitochondria, but its stability is only dependent upon XRCC1 in the nucleus. This suggests that LIG3 functions in both nuclear DNA repair and in maintaining mitochondrial integrity.

LIG3 Inactivation Results in Neuropathology Distinct to XRCC1 Loss

Given the DNA damage-associated neuropathology present in the *Xrcc1*$^{Nes\text{-}cre}$ mice, we reasoned that much of the damage was a failure to repair strand breaks due to Lig3 loss. To establish if this was the situation and to determine the DNA repair role of Lig3, we generated a conditional *Lig3* allele and inactivated this gene throughout the developing nervous system using *Nestin-cre*.[44] We noted striking biological differences between *Lig3*$^{Nes\text{-}cre}$ and *Xrcc1*$^{Nes\text{-}cre}$ mice. Compared to *Xrcc1*$^{Nes\text{-}cre}$ mice that developed a seizure-like phenotype by three months of age, *Lig3*$^{Nes\text{-}cre}$ mice became profoundly ataxic and growth retarded by two weeks of age and failed to survive beyond postnatal day 20. Additionally, the Lig3-deficient cerebellum was substantially smaller than its Xrcc1-deficient counterpart and was characterized by pronounced developmental abnormalities associated with extensive granule cell loss. However, in contrast to Xrcc1 loss, the *Lig3*$^{Nes\text{-}cre}$ molecular layer displayed a normal complement of cerebellar interneurons. This indicates that the Xrcc1-mediated DNA repair pathway that prevents damage accumulation in the interneuron progenitors, leading to failed neurogenesis, occurs independently of Lig3. Furthermore, unlike *Xrcc1*$^{Nes\text{-}cre}$ mice, we found that cortical and cerebellar *Lig3*$^{Nes\text{-}cre}$ oligodendrocytes numbers were markedly reduced. Primary astrocytes or neurospheres derived from *Lig3*$^{Nes\text{-}cre}$ tissues display pronounced cell growth defects compared to wild-type (WT) and *Xrcc1*$^{Nes\text{-}cre}$ counterparts (**Fig. 2**). These data highlight the differing neuropathological bases for the observed phenotypes in these mice, and, despite the close biochemical relationship between XRCC1 and LIG3, the biological roles for these factors within the CNS differ greatly.

Mitochondrial Lig3 Preserves mtDNA in the Murine CNS

Because of the differences between inactivation of Xrcc1 and Lig3 in the mouse nervous system, we considered that disruption of mtLig3, rather than nuclear Lig3, underpinned the striking *Lig3*$^{Nes\text{-}cre}$ phenotype. This notion is consistent with the broad spectrum of phenotypes found in human syndromes associated with mtDNA defects.[45-48] We also observed that media from *Lig3*$^{Nes\text{-}cre}$ primary astrocyte cultures quickly turned yellow, reflective of lactic acid accumulation in the media due to defective mitochondrial oxidative respiration. Subcellular analysis using mitotracker-Red and picogreen stains, combined with cellular oxygen consumption assays, confirmed that Lig3-deficient cells accumulated profound mitochondrial defects.[44] Analysis of *Lig3*$^{Nes\text{-}cre}$ tissues by electron microscopy (EM) and fluorescent immunostaining techniques also indicated substantial mitochondrial deficiency. These data demonstrated that, unlike *Xrcc1*$^{Nes\text{-}cre}$ mice, the pronounced neuropathology observed in *Lig3*$^{Nes\text{-}cre}$ mice was due to mitochondrial dysfunction. Furthermore, as mitochondrial Lig3 expression is independent of Xrcc1, the difference between the *Lig3*$^{Nes\text{-}cre}$ and *Xrcc1*$^{Nes\text{-}cre}$ mouse models highlights the mechanistic differences by which Lig3 and Xrcc1 promote and maintain neuronal homeostasis. As LIG3 is the only DNA ligase expressed in the mitochondria, its loss, especially in high oxygen-consumptive cells, such as neurons, would be dire, consistent with the neuropathology observed in *Lig3*$^{Nes\text{-}cre}$ mice.

While a detailed understanding of mtLIG3 function is unavailable, LIG3 is

likely to have critical roles in both mtDNA replication and repair. It is generally accepted that XRCC1-independent BER mediates repair of mtDNA nicks.[49-54] In addition to mtLIG3 as the only mitochondrial DNA ligase, DNA polymerase γ (Polγ) is the sole mitochondrial DNA polymerase and performs critical gap-filling functions that are normally attributed to DNA Pol β/δ/ε in nuclear DNA replication and SSBR.[55-57] Defective Polγ activity resulting from inherited hypomorphic mutations results in a broad spectrum of human diseases.[45] More recently, BER pathway components, including APTX, TDP1 and FEN1, have been identified in mitochondria,[58-60] further supporting the active requirement for BER in maintaining the integrity of mtDNA.[49-54]

Normal DNA SSBR activity in Lig3-deficient neural cells. While Lig3 loss clearly impacted mitochondrial homeostasis, its contribution to nuclear DNA repair was uncertain. To assess this aspect of Lig3 function, we performed DNA repair assays using primary cortical astrocyte cultures derived from *Lig3$^{Nes-cre}$* mice. These assays were done in parallel with primary cell lines derived from *Xrcc1$^{Nes-cre}$*, *Aptx$^{-/-}$* and *Tdp1$^{-/-}$* mice, lines previously shown to be defective in SSBR/BER.[21,23-25] Although Lig3 loss resulted in mitochondrial dysfunction and compromised cell growth, we were able to establish Lig3 primary astrocytes at early passage and grew these to quiescence for DNA repair studies. Surprisingly, Lig3-deficient cells displayed similar DNA repair activity to WT cells after a variety of genotoxins that induce different classes of DNA damage, including IR, H_2O_2, CPT, MMS. One clear exception was found after ultraviolet radiation (UV)-induced damage, which displayed a repair deficiency when compared to WT cells, although this repair defect was substantially less than after Xrcc1 loss.

These data were somewhat paradoxical, as Lig3 is considered important during SSBR repair.[9,61,62] It is likely that the reported studies linking Lig3 inactivation to repair deficiency may, in some cases, actually reflect Xrcc1 inactivation to promote Lig3 destabilization, as direct inactivation of LIG3 would compromise cellular viability because of mitochondrial dysfunction. Consistent with our data, normal repair is observed after oxidative damage when the XRCC1-LIG3 interaction is disabled[63] and knockdown of LIG3 via RNA interference results in repair deficiency after UV damage.[64] Furthermore, a companion study to our own used nuclear Lig3-deficient ES cells complemented with a mitochondrial version of Lig3 to maintain cellular viability and showed survival of these ES cells was similar to controls after an assortment of genotoxic agents.[65] These data strengthen the case that nuclear LIG3 is dispensable for survival after DNA damaging agents that require repair via the SSBR/BER pathway.

LIG1 is Important for DNA SSBR

As discussed above, DNA repair assays of Lig3-deficient primary neural cells suggest that Lig3 is generally dispensable for Xrcc1-mediated repair. Three mammalian DNA ligases have been identified: LIG1, LIG3 and LIG4.[62] While LIG3 has generally been considered to be the primary SSBR ligase because it associates with XRCC1, a proportion of DNA SSBs undergo repair that utilizes a variation on SSBR/BER termed long-patch repair, wherein PCNA-bound LIG1 performs the final DNA nick resealing event.[66,67] LIG1 also functions during DNA replication in ligating Okazaki fragments within the lagging strand at DNA replication forks.[68] LIG4 functions in concert with XRCC4 as a key factor during non-homologous end-joining of DNA DSBs[69] and is not considered important for SSBR.[66] Thus, a central question arising from our studies on Lig3 inactivation is what ligase is critical for SSBR/BER? To address this, we used lentiviral-delivered shRNAs to knockdown Lig1, Lig3 and Lig4, both individually and in combination, for DNA repair studies in quiescent primary mouse embryonic fibroblasts. We achieved greater than 90% target knockdown of each ligase and measured DNA repair in these cells after a variety of DNA damage-inducing agents. Our analysis showed that like *Lig3$^{Nes-cre}$* cells, shLig3 (Lig3-deficient) cells had DNA repair rates similar to control cells. Lig4 loss had minimal impact on SSBR activity, as previously reported.[66] However, our analyses showed that Lig1 was the key functional SSBR ligase in vivo, as shLig1 cells accumulated/retained the highest proportion of DNA breaks compared to control, shLig3 and shLig4 cells. Nevertheless, compared to the repair deficiency of *Tdp1$^{-/-}$* quiescent cells after IR, H_2O_2 and MMS treatment, Lig1 inactivation did not result in the same magnitude of DNA repair deficiency. However, cells deficient for both Lig1 and Lig3 (shLig1/shLig3) accumulated/retained more DNA breaks than shLig1 cells alone and at levels comparable to Tdp1-deficient cells, thereby indicating that Lig1 and Lig3 coordinate the full breadth of DNA end-ligation events in SSBR. Thus, this approach provided the identification of a functional contribution for Lig3 during nuclear DNA SSBR.

Biological Roles for LIG1 and LIG3 in DNA SSB Repair?

The data outlined above serves to focus on the relative in vivo contribution for the mammalian ligases during specific types of DNA repair. However, we still do not know the specific biological roles of nuclear LIG3 in the context of DNA repair and, from a disease perspective, its role in neural homeostasis. As the *Lig3$^{Nes-cre}$* mice succumb to mitochondrial defects around two weeks of age, this mouse model is not suited to address this question. There is a great deal of speculation suggesting that inefficient SSBR leads to age-related neurological decline;[14,16] however, the lack of tractable biological models has prevented any sort of definitive study addressing this question. One potentially useful model to address nuclear Lig3 function would involve generating a mouse that exclusively expresses mitochondrial Lig3. This would overcome the deleterious effects of mtLig3-deficiency attributed to the *Lig3$^{Nes-cre}$* mouse and facilitate analysis of the consequences of nuclear Lig3 deficiency.

Another related question is whether conditional loss of Lig1 alone or together with nuclear Lig3 phenocopies the neurological defects observed in *Xrcc1$^{Nes-cre}$* mice. A similar approach in studying Lig1 in the context of the CNS in vivo would be useful (i.e., generation of *Lig1$^{Nes-cre}$* mice), as Lig1 is required for cellular viability[70]

and Lig1-deficient mice develop hematopoietic defects and die during embryonic development.[28,34]

Should *Lig1*$^{Nes\text{-}cre}$ mice share common neuropathology with *Xrcc1*$^{Nes\text{-}cre}$ mice, it would support a direct biochemical relationship between XRCC1 and LIG1 through protein-protein interaction or via PCNA or DNA Polβ.[67] Whatever the situation, generation of a conditional *Lig1* allele will facilitate understanding the relative roles during tissue homeostasis and DNA repair of the three mammalian ligases.

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