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Original article

Functional Roles of Dnl4, DNA Ligase IV, in DNA Damage Stress in a Radiation-resistant Fungus, *Cryptococcus neoformans*

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ABSTRACT One of the significant biological effects of radiation is the genetic damage caused by DNA double strand breaks (DSBs), which can lead to mutations or cell death. To repair DNA damage, living things contain diverse and elaborate DNA repair systems, including homologous recombination (HR) and non-homologous end joining (NHEJ) that have been extensively studied across species, from yeast to humans. *Cryptococcus* has been utilized as a model organism in the studies of radiation response and DNA damage repair due to its radiation-resistance. This study focuses on the identification and characterization of the *DNL4* gene, encoding DNA ligase IV in the NHEJ pathway, in *C. neoformans*. We found that expression level of *DNL4* was induced in response to DNA damage stress and was dependent on the Rad53-Bdr1 pathway. Both deletions of *DNL4* and *RAD51* rendered strains more susceptible to radiation than each mutant, indicating that HR and NHEJ play redundant roles in DNA damage response. In addition, *DNL4* was also increased under H_2O_2 or tBOOH treatment although its deletion did not affect resistance to them. Perturbation of *DNL4* increased microevolution, suggesting its potential role in genetic stability. However, *dnl4*Δ mutant exhibited WT-levels of hyphal growth production. Taken together, *C. neoformans* Dnl4 plays roles in DNA damage response and microevolution.

Key words: DNA ligase IV, DNA damage response, Microevolution, *Cryptococcus*, Radiation

1. Introduction

Radiation therapy for cancer treatment and sterilization are practical examples that exploit the fact that radiation affects the DNA of living organisms, leading to cell death. The types and complexity of DNA damage caused by radiation depend on the linear energy transfer (LET) of the radiation [1]. Low LET γ-radiation induces approximately 1,300 base modifications, 1,000 single-strand breaks (SSBs), and 20 to 40 double-strand breaks (DSBs) per cell per gray (Gy) [2]. In contrast, high LET radiation results in more clustering of DNA damage, wherein two or more lesions occur within one or two helical turns of the DNA. This phenomenon, known as clustered DNA damage, is more pronounced with high LET compared to low LET radiation [3]. The DSB clustered damage includes DSBs accompanied by base lesions, whereas non-DSB clustered damages include SSBs and base lesions in close proximity. As the LET of radiation increases, more complex types of DNA damage occur, which are repaired more slowly [4].

Non-homologous end joining (NHEJ) and homologous recombination (HR) are two major mechanisms to repair DNA double strand breaks. The selection between HR and NHEJ pathways depends on the phase of the cell cycle during which DNA double strand breaks is sustained. Given that HR requires an intact template to restore the DNA damage, it is largely restricted in S/G2 phases of the cell cycle and is generally considered an error-free during DNA repair process [5]. On the other hand, as NHEJ simply rejoins resections between DNA ends with no sequence homology, it is dominantly operated throughout the cell cycle process and is an error-prone during DNA repair process [6]. For these reasons, the HR and NHEJ are considered as the slow and fast process of DSB repair, respectively [7].

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Although the HR and NHEJ systems for double-strand break (DSB) repair have been extensively characterized in mammalian cells, fungal model systems are also considered valuable experimental platforms for studying these pathways due to their genetic tractability. In the model yeast *Saccharomyces cerevisiae*, many proteins from the Rad52 epistasis group (including *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RDH54*/*TDI1*, *RAD55*, *RAD57*, *RAD59*, *MRE11*, and *XRS2*) are involved in homologous recombination [8]. During the DSB repair process mediated by HR, a series of diverse biological responses occur. Briefly, HR is initiated by the nucleolytic degradation of the 5'-terminated strand at the DSB site, a process known as end resection. Subsequently, the 3'-single-stranded DNA (ssDNA) overhangs generated during end resection are bound by the ssDNA-binding protein replication protein A (RPA), which protects ssDNA from nucleolytic degradation; RPA is later replaced by Rad51 recombinase [9]. The filament formation occurs when Rad51 interacts with the mediator Rad52 at the ssDNA overhangs to facilitate homology search. Once a homologous region is identified, the 3'-ssDNA invades a homologous DNA sequence, a process mediated by Rad54, and is subsequently extended through DNA synthesis. Finally, ligation of the broken DNA occurs following DNA synthesis [10]. In contrast, the NHEJ pathway primarily involves Ku70/Ku80 heterodimers and DNA ligase IV. Upon DSB formation, both Ku70 and Ku80 assemble at the broken DNA ends to protect them from nucleolytic activity and to recruit additional repair proteins [11]. DNA ligase IV (Dnl4), which interacts with Lif1 through its BRCT domains, is essential for the ligation of DNA during NHEJ [12].

Cryptococcus neoformans is an opportunistic human fungal pathogen, alongside *Candida albicans* and *Aspergillus fumigatus* that primarily infects immunocompromised individuals [13]. In addition to its pathogenic traits, *C. neoformans* demonstrates the ability to survive exposure to high levels of ionizing radiation [14]. Our previous studies have revealed that *C. neoformans* employs a variety of intracellular responses to overcome high doses of radiation, including the induction of DNA repair genes and oxidative stress response genes, a reduction in ergosterol content, and an increase in the expression levels of molecular chaperones [14]. Notably, the Rad53-Bdr1 pathway plays a crucial role in regulating the DNA damage response by controlling the expression levels of DNA repair genes [15]. Recently, our group discovered that HR and NHEJ pathways collaboratively regulate both DNA damage response and oxidative stress. Furthermore, these pathways are also implicated in maintaining genome stability [16]. However, the role of DNA ligase IV (Dnl4), a key component of the NHEJ pathway, remains elusive.

In this study, we identified *DNL4* and characterize regulation of expression in response to DNA damage or oxidative stresses through qRT-PCR and roles in DNA damage and oxidative stresses via phenotypic analyses using *dnl4*Δ and *rad51*Δ *dnl4*Δ mutants. Furthermore, we demonstrated role of *DNL4* in genome stability through phenotypic change of microevolution.

2. Materials and Methods

2.1. Construction of strains

Genetic information for gene disruption was obtained from the FungiDB (https://fungidb.org/fungidb/). Gene deletion mutants were generated using double joint PCR (DJ-PCR) strategies in the *Cryptococcus neoformans* var. *grubii* background [17,18]. For construction of *dnl4*Δ mutants, the 5'- and 3'-flanking regions of the *DNL4* were amplified by PCR using H99 genomic DNA as a template, with J1220/J1221 and J1222/J1223 primer pairs. The *NAT* (nourseothricin resistant marker) was amplified by PCR with M13Fe and M13Re primers using a pNAT plasmid. In the second round PCR, 5'- and 3'-flaking regions of *DNL4* with *NAT*-split marker were amplified with J1220/NSR and J1223/NSL primers. To construct *rad51*Δ *dnl4*Δ double mutants, primer pairs J1373/J1374 and J1375/J1376 were used for the amplification of the 5'- and 3'-flanking regions of *RAD51*, with H99 genomic DNA as a template. The M13Fe and M13Re primers were used to amplify the neomycin resistant marker (*NEO*) as a pNEO plasmid. In the second round PCR, 5'- and 3'-flaking regions of *RAD51* with *NEO*-split marker were amplified with J1373/NEO-NSR and J1376/NEO-NSL primers. The *DNL4* and *RAD51* disruption cassettes with dominant selection marker were introduced into H99 and *dnl4*Δ mutant, respectively, by biolistic transformation, as previously described [18]. Stable transformants were selected on YPD medium containing nourseothricin (100 μg/mL) or neomycin (100 μg/mL), and initially screened by diagnostic PCR with B79 and SO primers. Next, to demonstrate correct genotypes of *dnl4*Δ and *rad51*Δ *dnl4*Δ strains, we performed Southern blot analysis, as previously described [19,20]. Primer information was described in the Table S1.

2.2. Total RNA isolation, cDNA synthesis and qRT-PCR analysis

To assess whether the expression levels of *DNL4* were regulated by the Rad53-Bdr1 pathway, we isolated total RNA from the WT (H99), *rad53*Δ, and *bdr1*Δ mutant with or without radiation exposure. Strains were cultured in 20 mL of liquid YPD medium for 16 h at 30°C. Next, the grown cells were inoculated into 100 mL of fresh YPD medium and adjusted to OD_{600} = 0.2. Then, the cells were further incubated until OD_{600} reached approximately 0.6~0.7. Fifty milliliters of cells were pelleted for the zero-time sample and the remaining cells were exposed to γ-radiation (0.5 kGy for 1 h). After radiation exposure, 50 mL of the cells was further incubated at 30°C for 0.5 h. To determine whether *DNL4* expression was induced in response to DNA damage or oxidative stress, total RNA was isolated from WT as follows. The WT strains were grown in 20 mL of YPD medium for 16 h at 30°C in a shaking incubator. The cells were synchronized with OD_{600} 0.2 into 150 mL of fresh YPD medium and further incubated until the OD_{600} reached approximately 0.6~0.7. After incubation, the 50 mL of cells were sampled as a basal sample, and the remaining cells were treated with the indicated concentration of DNA damage inducing agents or oxidative stress inducing agents. The cells were further incubated and sampled at the indicated time points and put into a liquid nitrogen until lyophilizing.

Total RNA was isolated using Trizol reagent (EasyBlue) as previously described [21] and further purified using RNeasy spin column (Qiagen) according to the manufacturer's protocol. Next, the RNA concentration was measured by Nanodrop and cDNA was synthesized using the PrimeScript™ 1st strand cDNA Synthesis Kit (Takara Bio) with 5 μg of RNA as a template. The qRT-PCR was performed with *DNL4* primers listed in a Table S1 using the CFX96 real-time PCR detection system (Bio-Rad) and *ACT1* expression was used as an internal control. The relative *DNL4* expression was analyzed with the $2^{-\Delta\Delta Ct}$ method [22] and the data were illustrated with Prism 5.0. The statistical significance of difference was determined using one-way of ANOVA with Bonferroni's multiple comparison test (GraphPad Software Inc.).

2.3. Stress susceptibility test

Strains, described in Table S2, were cultured in liquid YPD medium at 30°C for 16 h. The grown cells were then serially diluted (1 to 10^4 dilutions) in distilled water and spotted onto a solid YPD medium containing the indicated concentration of chemical agents [DNA damage stress: 4-nitroquinoline N-oxide (4-NQO), bleomycin, methylmethane sulfonate (MMS), and cisplatin; Oxidative stress: hydrogen peroxide, menadione, and *tert*-butyl hydroperoxide]. For radiation and UV-C exposure, the serially diluted cells were spotted, and then were exposure to the indicated dose of radiation and UV-C. The plates were further incubated at 30°C for 1-3 days, with daily photographs taken.

2.4. Phenotypic characterization of microevolution of *dnl4*Δ and *rad51*Δ *dnl4*Δ strains

To demonstrate whether Dnl4 affects phenotypic changes during repeated passages, we streaked single colonies of the WT, *rad51*Δ, and *dnl4*Δ strains every 3 days for 2 month (approximately more than 600 generations) on YPD plates at 30°C, previously described [16]. Every 300 passaged, we monitored phenotypic changes in the seven independent passage strains from each original strain.

2.5. Mating analysis

For mating analysis, *C. neoformans* strains were cultured in liquid YPD medium for 16 h. The strains were washed with sterilized distilled water and then equal concentration of *MAT*α and *MAT***a** cells (10^7 cells/mL) were mixed and then spotted onto V8 mating medium. The plates were incubated in the dark at room temperature for 2 weeks. Filament formation were monitored and photographed weekly using an Olympus BX51 microscope equipped with a SPOT Insight digital camera (Diagnostic Instrument Inc.).

3. Results

3.1. Identification of the *DNL4* gene in *C. neoformans*

To identify a *DNL4* ortholog in *C. neoformans,* we performed BLAST searches (blastp) with the *S. cerevisiae* Dnl4 protein sequence as a query. We found that *C. neoformans* has one gene (CNAG_07573) with a high similarity (score: 275, e-value: 1e-76). Protein domain analysis showed that CNAG_07573 contains domains involved in ATP-dependent DNA ligase domain (PF01068), ATP-dependent DNA ligase C-terminal domain (PF04679), and BRCT domain (PF16589), as to those in *S. cerevisiae* Dnl4 (Fig. 1A). Therefore, CNAG_07573 was designated Dnl4 in *C. neoformans*.

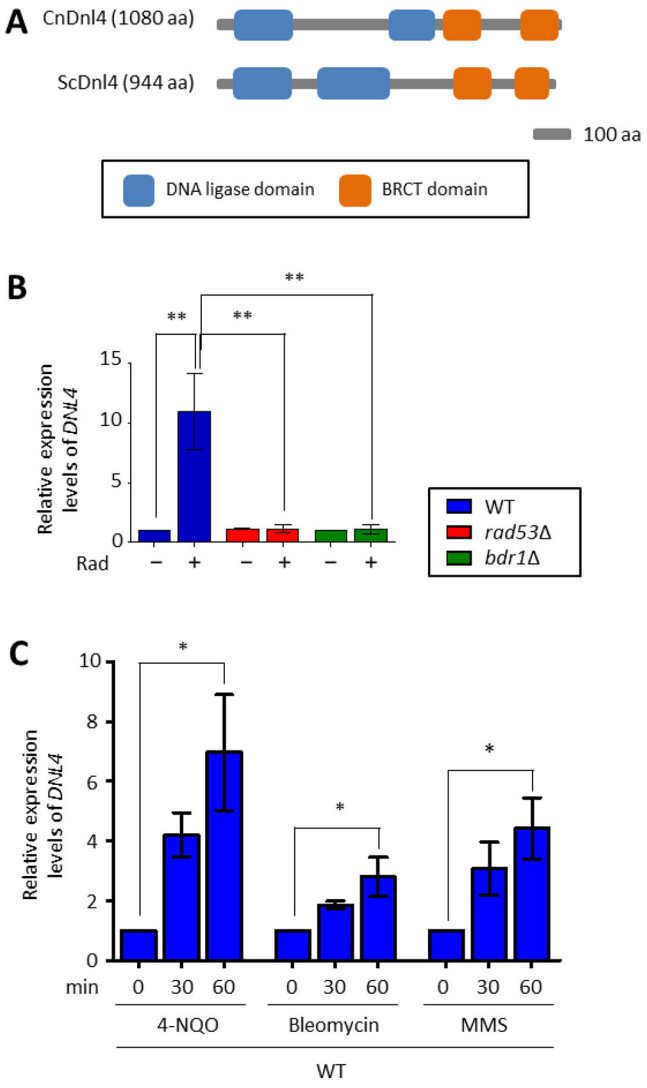

Fig. 1. Expression levels of *DNL4* in response to DNA damage stress. (A) Domain analysis of Dnl4 proteins in *C. neoformans* and *S. cerevisiae*. (B) Expressions of *DNL4* were controlled by Rad53-Bdr1 pathway. The qRT-PCR analysis was executed with *DNL4*-specific primers with cDNA synthesized from WT, *rad53*Δ, and *bdr1*Δ mutants with or without γ-radiation (0.5 kGy). (C) Expression levels of *DNL4* in response to DNA damage inducers. The qRT-PCR analysis was performed with *DNL4*-specific primers with cDNA synthesized from WT treated with the indicated concentration of 4-NQO (0.1 μg/mL), MMS (0.02%), and bleomycin (2 μg/mL) for 1 h. The qRT-PCR was performed with three independent samples. Statistical change of *DNL4* expression was analyzed using the Bonferroni's multiple comparison test with Prism software (*$p<0.05$ and **$p<0.01$).

3.2. *DNL4* expression is differently regulated by DNA damage stress

Our previous study revealed that expression levels of *KU70* and *KU80* are regulated by Rad53-Bdr1 pathway [16], we measured expression levels of *DNL4* in WT (H99), *rad53*Δ, and *bdr1*Δ mutants. Similar to *KU70* and *KU80* expression, *DNL4* expression was highly increased in WT after radiation exposure whereas that was not induced in both *rad53*Δ and *bdr1*Δ mutants after radiation exposure (Fig. 1B). Next, we further demonstrated whether the expression of *DNL4* is also induced in response to diverse types of DNA damage stress [4-NQO: a DNA damage inducer through the production of reactive oxygen species; MMS: an inducer of DNA alkylation; and Bleomycin: an inducer of DNA double strand breaks]. Although expression abundance was less than radiation exposure, *DNL4* gene was gradually induced under diverse types of DNA damage stress inducers (Fig. 1C). Taken together, *Cryptococcus DNL4* was also increased in response to DNA damage stress in a Rad53-Bdr1 pathway like other NHEJ components such as *KU70* and *KU80*.

3.3. A minor role of Dnl4-mediated NHEJ in DNA damage response

Given that *DNL4* was increased in response to radiation and DNA damage inducing agents, we decided to characterize role of Dnl4 in DNA damage response in *C. neoformans*. In the end, we generated two independent *dnl4*Δ mutants through biolistic transformation, and confirmed correct genotype through Southern blot analysis (Fig. 2A and B). In accordance with the other NHEJ mutants such as *ku70*Δ and *ku80*Δ mutants, *dnl4*Δ mutants showed susceptibility to bleomycin. However, *dnl4*Δ mutants exhibited WT-levels of resistance in response to radiation, 4-NQO, cisplatin, MMS, and UV-C (Fig. 2C). This result indicates that Dnl4-NHEJ pathway plays a minor role in DNA damage response compared to that of HR. Since our previous study reveals that both deletion of *KU70*/*KU80* and *RAD51* renders strains more susceptible to radiation and UV-C than deletion of each gene, we constructed two independent *rad51*Δ *dnl4*Δ double mutants through biolistic transformation (Fig. 2D and E), and performed phenotypic analyses using them. Similarly, *rad51*Δ *dnl4*Δ double mutants were more susceptible to radiation, cisplatin, and UV-C than *rad51*Δ mutant. Furthermore, *rad51*Δ *dnl4*Δ double mutants exhibited similar levels of resistance in response to bleomycin, 4-NQO, and MMS as *rad51*Δ mutant (Fig. 2F). Taken together, Dnl4-mediated NHEJ played redundant roles in DNA damage response with HR in *C. neoformans*.

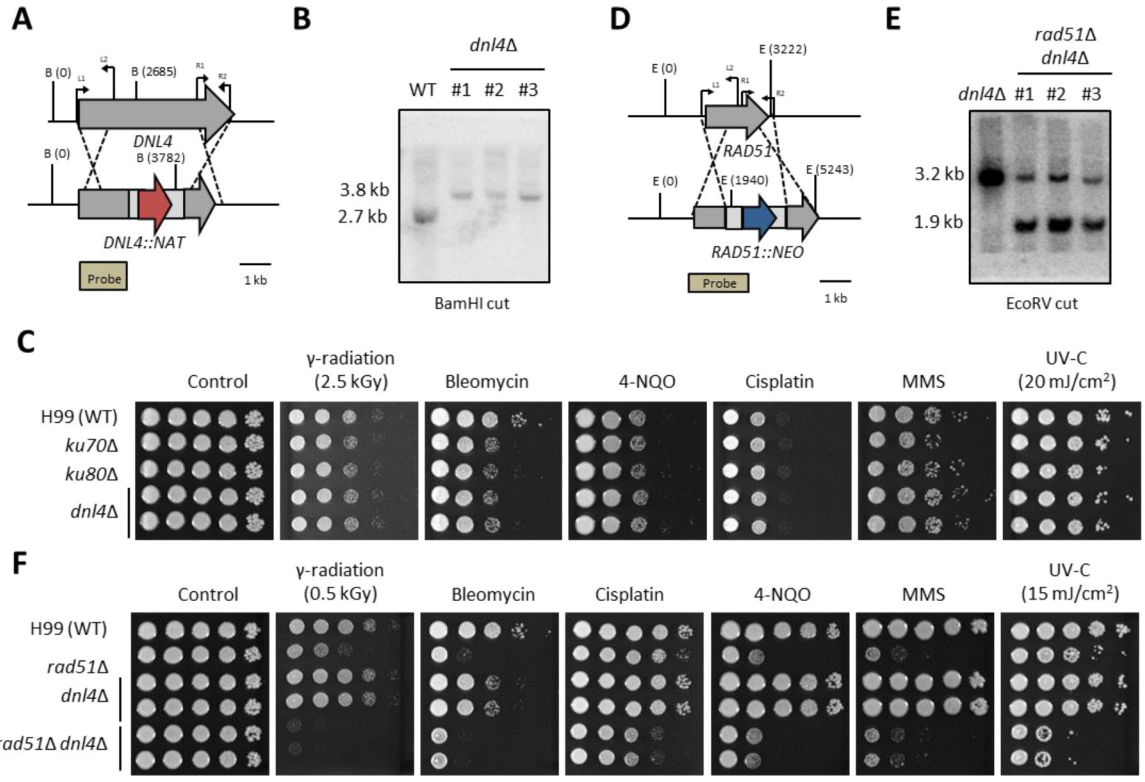

Fig. 2. The role of Dnl4 in DNA damage stress in *C. neoformans*. (A and D) Diagrams for deletions of *DNL4* with *NAT* marker (A) and *RAD51* with *NEO* marker (D). (B and E) Verification of correct genotypes of the strains. The membrane was hybridized with the *DNL4* or *RAD51* gene-specific probes, washed, and developed. (C and F) The WT, *ku70*Δ, *ku80*Δ, *rad51*Δ, *dnl4*Δ and *rad51*Δ *dnl4*Δ strains were cultured in liquid YPD medium at 30°C for 16 h. Next, the strains were 10-fold serially diluted and spotted on the YPD medium containing DNA damage inducing agents. For γ-radiation and UV-C exposure, the 10-fold serially diluted and spotted on the YPD medium and then the plates were exposed to the indicated dose of γ-radiation and UV-C. Next, the plates were incubated at 30°C for 3 days.

3.4. *DNL4* expression was induced under oxidative stress

Our previous study reveals that oxidative stress induced by hydrogen peroxide and *tert*-butyl hydroperoxide (tBOOH) increased expression levels of *KU70* and *KU80* genes [16]. In accordance with our previous study, *DNL4* expression was also induced in response to hydrogen peroxide and tBOOH, not menadione (Fig. 3A). This result indicates that *DNL4* is involved in oxidative response. To demonstrate whether Dnl4 is required for oxidative stress response, we performed survival assays using *dnl4*Δ and *rad51*Δ *dnl4*Δ strains under oxidative stress. Consistent with *DNL4* expression pattern against menadione, *dnl4*Δ mutants exhibited WT-levels of resistance under menadione treatment. Interestingly, although expression levels of *DNL4* were enhanced in response to H_2O_2 and tBOOH, *dnl4*Δ mutants did not show growth defect against to them (Fig. 3B).

3.5. The deletion of *DNL4* increased microevolution

Previous studies have shown that disruptions of DNA repair systems including mismatch repair (MMR), HR and NHEJ, lead to increased DNA mutations at high frequencies in *C. neoformans* [16,23]. The fact that deletion of *DNL4* renders cells more susceptible to DNA damage stress enables us to investigate whether Dnl4 is required for microevolution. To elucidate the role of Dnl4 in microevolution, we compared the phenotypic changes among the original WT, *dnl4*Δ and *rad51*Δ strains with those of 7 independent passage strains after approximately 600 generations in response to various stresses. We found that passaged strains from *dnl4*Δ and *rad51*Δ mutants showed different degrees of stress susceptibility in response to radiation, DNA damage stress inducers, and oxidative stress inducers whereas the passaged strains from WT exhibited WT-levels of stress resistance (Fig. 4). Taken together, the Dnl4-mediated NHEJ controled genome stability in *C. neoformans*.

3.6. *Dnl4* was dispensable for sexual reproduction

Previous studies reveal that the deletion of *LIG4* (*DNL4*) im-

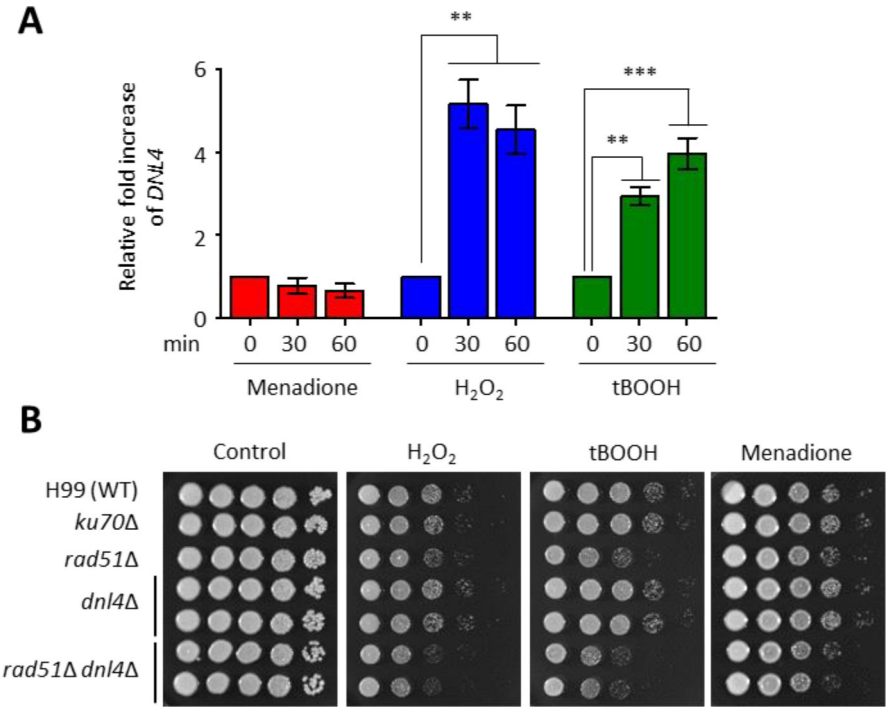

Fig. 3. The role of Dnl4 in oxidative stress in *C. neoformans*. (A) Expression of *DNL4* gene in response to oxidative stress. The qRT-PCR analysis was performed with *DNL4*-specific primers with cDNA synthesized from WT treated with the indicated concentration of menadione (0.02 mM), hydrogen peroxide (2.5 mM), and *tert*-butyl peroxide (tBOOH; 1 mM) for 1 h. The qRT-PCR was performed with three independent samples. Statistical change of *DNL4* expression was analyzed using the Bonferroni's multiple comparison test with Prism software (**$p<0.01$ and ***$p<0.001$). (B) *C. neoformans* strains were cultured in liquid YPD medium at 30°C for 16 h. The grown cells were serially diluted (1 to 10^4) and then spotted on a YPD plates containing the oxidative stress agents (hydrogen peroxide: 2.5 mM; tBOOH: 1 mM; menadione: 0.02 mM). Strains were further incubated at 30°C for 3 days.

pairs production of germination [24]. To demonstrate whether Dnl4 affects sexual differentiation in *C. neoformans*, we performed mating assay. Our result showed that *dnl4*Δ mutants crossing with KN99, which is *MAT***a** strain of *C. neoformans*, exhibited similar levels of filamentation formation to WT (H99) crossing with KN99 (Fig. 5). Therefore, Dnl4 was not required for sexual differentiation in *C. neoformans*.

4. Discussion

In this study, we demonstrated that the role of *C. neoformans* Dnl4 (CnDnl4) in DNA damage and oxidative stresses, microevolution and sexual differentiation. We found that CnDnl4 was required for DNA damage response and microevolution, but not oxidative stress and sexual differentiation.

Although Dnl4, which are also known for Lig4 in eukaryotes, are evolutionarily conserved, the role of its protein in viability and DNA damage response and hyphal growth are divergent depending on species. In mice, DNA ligase IV deficiency leads to embryonic lethality via the p53 pathway [25]. However, *Lig4* deficient *Drosophila* strains are viable even they are hypersensitive to ionizing radiation [26]. Moreover, to date, there have been not yet reported that a deficiency in DNA ligase IV in fungi leads to decreased viability. In DNA damage response, *Candida* species *lig4*Δ mutants show WT-levels of resistance in DNA damage stress [24,27]. However, *lig4*Δ mutants in *Aspergillus oryzae, Neurospora crassa* and *Monascus ruber* show increased MMS susceptibility compared to WT [28-30]. Furthermore, *lig4*Δ mutant in *Schizosaccharomyces japonicas* exhibited reduced growth in response to UV light. Although deletion of *DNL4* did not affect growth defect under MMS treatment in *C. neoformans*, the *dnl4*Δ mutant exhibited susceptibility in response to bleomycin and *rad51*Δ *dnl4*Δ double mutants showed more susceptible than each *rad51*Δ and *dnl4*Δ mutant. In case of hyphal growth, disruptions of *LIG4* (*DNL4*) result in decreased hyphal growth in *S. japonicus* and *C. albicans* [31]. Contrary to *S. japonicas* and *C. albicans*, deletion of *LIG4* does not affect hyphal growth in *M. ruber* [30]. Taken together, the

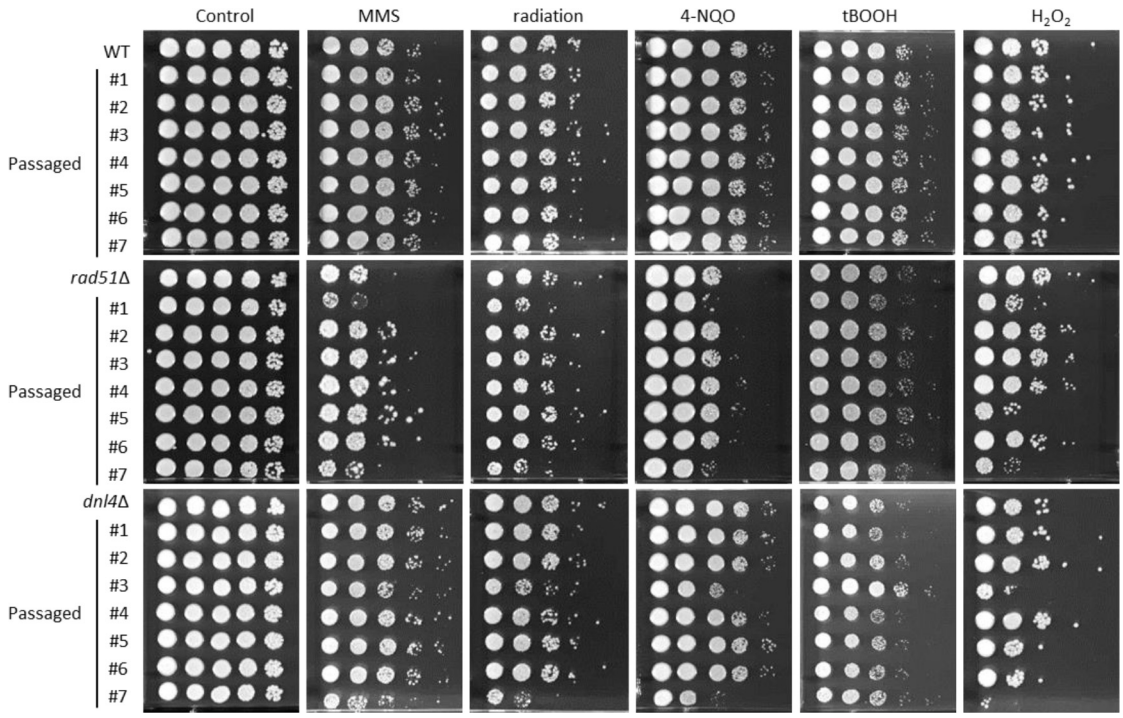

Fig. 4. Deletion of *DNL4* increased the DNA mutation frequency. The phenotypic changes of original strains and their corresponding passaged strains (more 600 generations) were compared to diverse stresses. Each strain was cultured in a liquid YPD medium 30°C for 16 h. Next, the grown cells were 10-folded serially diluted and then spotted on a YPD medium containing DNA damage or oxidative stress inducers. The cells were further incubated at 30°C for 3 days.

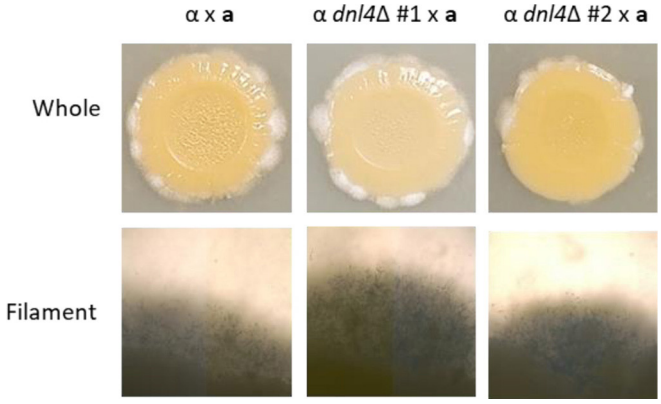

Fig. 5. Dnl4 was not required for sexual differentiation of *C. neoformans*. Serotype A *MAT*α and *MAT***a** strains were co-cultured V8 medium (pH 5.0) for 2 weeks at room temperature in the dark.

role of Dnl4 in viability, DNA damage stress and hyphal growth in species-and damage type-dependent manners.

During the NHEJ process in *S. cerevisiae*, Dnl4 interacts with Lif1 through two BRCT domain and its interaction affects stability and activity of Dnl4 [32,33]. However, studies on the regulation of *DNL4* expression in response to environmental changes remain insufficient in fungi. Our present study revealed that *DNL4* was highly induced in response to DNA damage stress and treatment of certain oxidative stress inducers. Interestingly, the finding that Lif1 (XRCC4 in human) homolog was not found in *C. neoformans* genome suggests that regulation of Dnl4 appears to be divergent from that of Dnl4/Lig4 in other eukaryotes.

DNA repair system has been evolved to prevent genetic alteration for preservation of living things. Therefore, the loss of its function results in the accumulation of DNA sequence changes, whether they are evolutionarily advantageous or detrimental. Recent studies have revealed that loss of DNA repair systems including HR, NHEJ and mismatch repair system (MMR) has led to increase microevolution in the fungal pathogens [16,23]. The experimental result that the passaged strains of *dnl4*Δ mutant showed phenotypic change compared to its parent strain in this study also supported the previous studies. Paradoxically, deletion of *DNL4* increased gene targeting efficiency for homologous recombination compared to the WT in diverse fungi [34]. The previous studies that when the *DNL4* is deleted, HR efficiency tends to increase regardless of the phenotype in response

to DNA damage stress indicate that the role of Dnl4-dependent HR in the DNA damage stress response appears to be distinct from the Dnl4-dependent HR mechanism involved in gene integration. Considering this point, further studies are needed to validate the aforementioned hypothesis.

Conflicts of Interests

Authors declare no competing interests.

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