

Determining the components and activity of DNA double-strand break repair pathways in the filamentous fungus *Magnaporthe oryzae*

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Abstract

Magnaporthe oryzae, the causal agent of rice blast disease, is an important model pathogen given its worldwide distribution, threat to agriculture across a range of monocot hosts, and varied aggressiveness among strains. While significant progress has been made in understanding the genetics of host-range and virulence, it is still unclear what mechanisms give rise to novel DNA variation given *M. oryzae*'s mainly asexual reproduction. Previous research from our lab and others indicates that at least four DNA double strand break (DSB) repair pathways are active in *M. oryzae*, including classical non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), single-strand annealing (SSA), and homologous recombination (HR). While DNA DSB repair is well studied in some eukaryotes, mainly mammals and yeasts, there is not a full description of the genetic requirements, and most importantly, the DNA DSB repair outcomes for each repair pathway in filamentous fungi, especially related to MMEJ and SSA. In this study we utilized a reverse genetics approach to identify the genetic requirements of previously characterized eukaryotic DNA DSB repair homologs, through the creation of gene deletion strains and characterizing repair outcomes in these genetic mutants. By disabling DNA end-resection at DSB sites in $\Delta mre11$, we determined that NHEJ repair resulted in mainly small INDELs that caused deletions of less than 9 base pairs (bp) and insertions of fewer than 5 bp, used minimal microhomology (MH) for repair, included tandem duplications in ~35% of repairs, and resulted in only a single deletion greater than 15 bp. Conversely, by disabling NHEJ and long-range end-resection mediated by Exo1, we specifically identified an MMEJ-like repair profile with much larger INDELs that ranged from -145 bp to -21 bp, and used longer MH, 3 bp to 6 bp, during repair. This study further investigated the genetic requirements of the individual DNA DSB repair pathways in *M. oryzae*. Deletion of Mre11

impaired end-resection, highlighting its vital role in homology-based repair mechanisms.

Hypersensitivity of $\Delta mre11$ to genotoxins confirmed Mre11's role in end-resection based repair, while insensitivity of $\Delta ku80$ to the tested genotoxins indicated that NHEJ was dispensable for repair of induced DSB. The absence of MMEJ repair in the $\Delta pol4$ repair profiles indicated Pol4's function as an MMEJ polymerase. Lig4 was shown to be crucial for NHEJ, as $\Delta lig4$ showed increased end-resection-based repair compared to WT. These findings underscore the essential roles of Mre11, Pol4, and Lig4 in their respective DSB repair pathways as well as provide the foundations for continued research into the DNA DSB repair pathways of *M. oryzae*.

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Introduction

Rice blast disease, caused by the haploid, ascomycete fungus *Magnaporthe oryzae*, significantly affects global agriculture and poses a substantial threat to food security through its impact on staple crops like rice and wheat. According to recent studies, rice blast disease is responsible for over 30% of rice crop losses in the United States alone (Nalley et al., 2016). As a result, *M. oryzae* has been recognized as a formidable fungal plant-pathogen, prompting its use as a model organism for genetic and molecular studies (Valent, 1990).

The lifecycle of *M. oryzae* begins with the production of conidia from necrotic lesions on *Oryza sativa* leaves under humid conditions. These conidia are then spread to nearby plants through dewdrop splash, wind, or rain (Ou, 1984; Talbot, 2003; Wilson & Talbot, 2009). Once a conidium lands on a leaf, it sticks to the leaf surface and forms a germ tube (Hamer et al., 1988). This germ tube then undergoes differentiation into an appressorium, which is used to generate a massive amount of turgor pressure to force a penetration peg through the rice cuticle (Bourett & Howard, 1990; de Jong et al., 1997; Foster et al., 2017). Once inside, *M. oryzae* releases an onslaught of effectors to suppress the host's immune responses and manipulate host biology to facilitate invasion, colonization, and ultimately the production of conidia to continue the cycle (Sakulkoo et al., 2018). This strategic use of effectors is crucial for the reproductive success of every plant-pathogen. Therefore, understanding how *M. oryzae* generates genetic diversity in these effector genes is essential, as it enables the pathogen to continually evade the co-evolving resistance genes of its host. One way to uncover how genetic diversity in virulence genes such as effectors are generated, not only within the species, but also in related, primarily asexual, haploid filamentous fungi, is by investigating the mechanisms of DNA double-strand break (DSB) repair in *M. oryzae*.

Background

Previous research from our lab and others indicates the activity of at least four DNA DSB repair pathways in *M. oryzae*, including non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), single-strand annealing (SSA), and homologous recombination (HR) (Huang et al., 2022). This was determined by using CRISPR-Cas gene editing to delete the Ku80 subunit of the Ku70/Ku80 heterodimer (Ku) in the wild-type O-137 strain (WT), as well as to target the *FKBP12* locus in the $\Delta ku80$ background. When FKBP12 was edited in the $\Delta ku80$ background, the resulting DSB repair profiles obtained from individually edited colonies changed drastically from being predominately repaired by NHEJ in WT, to 0% NHEJ, 100% MMEJ-like repair in $\Delta ku80$, indicating the presence of the MMEJ pathway and Ku80's end-protection function in NHEJ. In another test, 1 kb of homology to the FKBP12 locus was added to either end of the hygromycin resistance gene was transformed into both WT and $\Delta ku80$. This allowed for transformants to be selected on both FK506 and hygromycin, where hygromycin resistance indicated repair by HR. It was found that ~40% of DSB repair in WT was done by HR, whereas HR accounted for 97% of DSB repair in $\Delta ku80$. This indicated the presence of the HR pathway in *M. oryzae* and the increase of end-resection-based pathways due to $\Delta ku80$ and its loss of NHEJ. Evidence for SSA in *M. oryzae* comes from multiple independent instances in which CRISPR gene editing has resulted in extensive end-resectioning up to 17 kb between two transposable elements (TEs) and the deletion of all sequences between them, including one of the TEs. Similar observations have been made in the protozoan parasite *Leishmania* as well, supporting the activity of SSA in *M. oryzae* (W.-W. Zhang & Matlashewski, 2019). Additionally, PCR followed by the absence of a band after gel electrophoresis, indicating large deletions, suggests that sequences repaired by SSA increased to around 30% in the $\Delta ku80$

background, compared to WT, which was approximately 5%. This was expected, as the end-resection pathways were much more active in $\Delta ku80$.

DNA DSB repair pathways in eukaryotes, such as *M. oryzae*, can be largely grouped into two categories: those that protect the DNA ends, such as the NHEJ pathway, and those that require end-resection, including the MMEJ, SSA, and HR pathways (Table 1). Understanding these mechanisms and the proteins involved is crucial for explaining how *M. oryzae* balances genome stability with genetic variation, particularly under the selective pressures of host responses. NHEJ and HR are known to be relatively error-free and therefore are involved in maintaining genomic integrity, whereas more mutagenic pathways like MMEJ and SSA are known to create larger mutations and contribute to significant chromosomal rearrangements (Delacôte & Lopez, 2008). These more mutagenic pathways might possibly be contributing to the pathogen's aggressiveness, adaptability, and pathogenicity through the creation of novel genetic variation. However, DSB repair pathways in filamentous fungi are not well characterized. This is particularly true for the MMEJ pathway in *M. oryzae*, which lacks a fully functional homolog of the mammalian MMEJ polymerase, Pol θ . Furthermore, the genetic requirements of the SSA pathway are not well understood in any eukaryote, with the specific polymerases and ligases involved still unidentified (Bhargava et al., 2016). This gap highlights the need for a deeper understanding of how these mutagenic pathways function in filamentous fungi, where they are hypothesized to play a pivotal role in generating sequence diversity in genes related to plant-pathogen interactions.

NHEJ is described as an end-protection pathway due to the activity of the Ku70/Ku80 (Ku) heterodimer, which binds to free double-stranded DNA ends formed after a DSB and blocks end-resection nucleases such as exonuclease 1 (Exo1) and Mre11 (Krasner et al., 2015;

Walker et al., 2001). When blunt DNA ends are made, Lig4 reconnects them without any sequence loss, however, if there are missing base pairs (bps), small deletions may occur (Chang et al., 2017; Pannunzio et al., 2018). If there are 5' overhangs, they are chewed back by nucleases to form blunt DNA prior to ligation. For DNA with 3' overhangs, these are processed by Pol μ and Pol δ to prepare them for ligation by Lig4, often by generating short sequences of complementary sequences for annealing with the opposite 3' strand (Nick McElhinny et al., 2005).

All end-resection pathways, including MMEJ, SSA, and HR, begin with 5' end-resection by the MRN complex, which consists of 3 core proteins, Mre11, Rad50, and Nbs1 (Symington, 2014). Following a DSB and blockage of free ends by Ku, the endonuclease activity of Mre11, makes a nick up to 300 bp away from the DSB, enabling 3'–5' end-resection toward the DSB by Mre11. Following short-range end-resection and insufficient microhomology (MH) for MMEJ, Exo1 starts long-range end-resection of DNA ends. Single-stranded DNA (ssDNA) tails are coated with Replication Protein A (RPA) to prevent their degradation and premature annealing of MH (Garcia et al., 2011; Mateos-Gomez et al., 2016).

Together, Mre11 and Exo1 cooperate in chewing back the DNA to expose regions needed for the annealing of complementary sequences on opposing ssDNA tails (Bennardo et al., 2008). The end-resection pathways can be further divided into short-range end-resection leading to MMEJ and long-range end-resection, mediated by Exo1, leading to SSA and HR (Garcia et al., 2011).

MMEJ, also called alternative end-joining, is a repair pathway that, unlike NHEJ, relies on the presence of ssDNA tails, which contain sequences that are complementary between the two broken strands, termed MH, for repair. As previously stated, MH from both ends is exposed

by MRN, then annealed together, after which a flap endonuclease cleaves the 3' flaps containing non-complementary bases (Bai et al., 2021). Next, fill-in synthesis is done by the polymerase, Pol θ , in mammals, and polymerases Pol δ and Pol λ (Pol 3 and Pol 4) in *Saccharomyces cerevisiae* (Kent et al., 2015; Meyer et al., 2015). The following ligation step is done by Lig3 in mammals and the Lig1 homolog in *S. cerevisiae*, CDC9, since *S. cerevisiae* lacks a Lig3 homolog (Simsek et al., 2011).

MMEJ is considered error-prone by many because it often leads to deletions or insertions at the repair site, significantly larger than indels caused by NHEJ, which are largely caused by end-resection, the loss of nucleotides between two microhomologous sequences, including one of the microhomologous sequences itself, and templated synthesis caused by error-prone polymerases (McVey & Lee, 2008).

SSA is mechanistically similar to MMEJ, but the key difference is that the repetitive sequences flanking the DSB in SSA are much longer, meaning that they require extensive long-range end-resectioning to uncover (Bhargava et al., 2016). Additionally, in SSA, the annealing of the exposed complementary ssDNA is facilitated by the Rad52 protein in cooperation with the helicase domain of Pol θ , or the independent protein HelQ, which is involved in other end-resection-based pathways as well (Anand et al., 2022; Bennardo et al., 2008). Next, Rad52 binds to the ssDNA and facilitates the annealing of complementary sequences on both strands (Mortensen et al., 1996). The annealed strands are then processed by a flap nuclease to remove any unpaired flaps, any gaps are filled by an unknown polymerase, and the nicks are sealed by an unknown ligase. While SSA efficiently repairs DSBs, it does so at the cost of significant genetic information loss between the repeats, similar to MMEJ but on a much larger scale.

Consequently, SSA is considered highly mutagenic (Bhargava et al., 2016; Hanscom & McVey, 2020).

HR is the most accurate DSB repair pathway since it uses highly homologous sequences with > 100 bp MH, a sister chromatid, or a homologous chromosome as a template for error-free, template-base repair (Shrivastav et al., 2008). The use of a sister chromatid or homologous chromosomes limits interchromosomal HR to the S and G2 cell cycle phases in haploid organisms like *M. oryzae*, whereas intrachromosomal HR, in which HR occurs within the same chromosome, is likely active during all cell cycle phases (Fabre, 1978). The HR pathway starts with Exo1-mediated long-range end-resection, as previously stated, producing ssDNA that RPA then coats. Rad52 then displaces RPA, enabling Rad51 to form a nucleoprotein filament essential for locating and invading a homologous DNA sequence for precise, error-free repair (Bonilla et al., 2020).

Together, these diverse DSB repair pathways not only display the complexity of DNA repair processes in eukaryotic cells but also highlight the sophisticated mechanisms that organisms use to maintain genetic stability and adaptability across generations, particularly in the context of pathogenic species like *Magnaporthe oryzae*, where such diversity underpins pathogenicity and survival. Prior research into DNA DSB repair in humans and yeast indicates significant gaps in our understanding, particularly in how these mechanisms might function in *M. oryzae*. The advent of CRISPR-Cas technology has opened new avenues for studying molecular pathways, and as such, will be used to identify MMEJ components in the absence of a functional Pol θ , as well as SSA polymerases and ligases, both which remain uncharacterized. It is hypothesized that, as is observed in yeast, Pol 3 and Pol 4 play significant roles as polymerases in MMEJ in *M. oryzae* (Meyer et al., 2015). To test this hypothesis, this research will use a

reverse genetics approach to identify the genetic requirements of previously determined DNA DSB repair homologs, which we will then use to characterize the outcomes of DNA DSB repair pathways in *M. oryzae*, with a focus on understanding the role of MMEJ in the absence of Pol θ . Through CRISPR-Cas technology, this study seeks to unravel the complexities of DSB repair in *M. oryzae*, offering new perspectives on the pathogen's ability to adapt and evolve, and thereby informing possible strategies to effectively combat rice blast disease.

Methodology

Fungal Strain Preparation and incubation conditions

M. oryzae field isolate O-137 was used as the wild-type in this study. For long term storage of strains, a single spore was isolated using 1 mL of 0.25% glycerol solution to collect conidia from strains grown on OTA for 12 days. The spore solution was then transferred and spread on 2% water agar and allowed to germinate in a 28°C incubator in the dark overnight. Using a sharp scalpel, a box was cut around a single conidium, either before or after overnight incubation, and was transferred to OTA with 4 filter paper disks, each placed 2 cm from the center of the plate. This plate was then kept in a growth chamber set at 25 °C under constant light for ~10 days. After the mycelium has grown over the filter paper disks and produced conidia, the filter disks were removed, placed in a petri dish, conidia side facing up, and desiccated for 1-2 months, after which they were transferred to sterile coin envelopes and stored at -20°C. Oatmeal agar (OTA) plates were made by mixing 72.5 g Oatmeal Agar powder (Sigma Aldrich, Cat# O3506) to 1 L distilled water and autoclaving for 15 minutes at 121°C. After individual plates were poured and cooled, they were inoculated with spore filter stocks and grown at 25 °C under constant light (white and black light) for 2 weeks, or longer for slow growing strains.

Guide RNA Design, Guide DNA synthesis, In-Vitro Transcription and LbCas12a Ribonucleoprotein Assembly for Orthologous Gene Deletion

Knocking out genes in *M. oryzae* was done using the CRISPR-Cas12a ribonucleoprotein (RNP) based gene editing protocol (Huang & Cook, 2022). In this study, homologous DNA DSB repair genes, previously identified in both mammals and *S. cerevisiae*, were targeted for deletion by first using BLAST to find the orthologous genes in *M. oryzae*. The orthologous genes of

interest used for BLAST and their protein sequences can be downloaded from UniProt (for human genes) or the Saccharomyces Genome Database (for genes in *S. cerevisiae*) (Table 2). Using these protein sequences, BLASTp was performed against the *Pyricularia oryzae* 70-15 reference genome on the FungiDB website to search for conservation of the protein sequence. Once an orthologous gene was found in the 70-15 reference genome, the DNA sequence of this gene was downloaded and BLASTn was performed against the *M. oryzae* O-137 genome to check for DNA sequence conservation. The homologous gene sequence in 70-15 was then used for LbCas12a guide DNA design, which was done by searching for two LbCas12a Protospacer Adjacent Motif (PAM) sequences (5'-TTTV-3') on either end of the gene and selecting the 23 bp target sequence directly downstream of the PAM. The reverse complements of the target sequences were concatenated to the 5' end of the reverse complemented LbCas12a mature direct repeat to create the target specific reverse oligo. The target specific reverse oligo, as well as all other PCR primers, was then ordered from Integrated DNA Technologies (IDT) as a 25 nmole product for use in guide DNA synthesis. Using a thermocycler set to an initial denaturation at 95°C for 30 seconds, followed by 35 cycles of denaturation at 95°C for 10 seconds, annealing at 57°C for 10 seconds, extension at 72°C for 10 seconds, a final extension at 72°C for 2 minutes, and a holding step at 4°C for 2 minutes, the target specific reverse oligo was annealed with a universal Cas12 forward primer, containing a T7 promoter and complementary LbCas12a direct repeat, to create the guide DNA template needed for gRNA synthesis. The CRISPR guide data and DNA oligos used to create the KOs in this project are available in Supplemental Table 1. The synthesized gDNA template was then in-vitro transcribed using the HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, Cat# E2040S) to synthesize the gRNA in an overnight (12-20 hour) reaction. The next day, RNase free DNase I (New England Biolabs, Cat#

M0303S) was added to each tube and incubated at 37°C for 15 minutes to remove the DNA template. This was followed by purification of the synthesized gRNA using the Monarch RNA Cleanup Kit (New England Biolabs, Cat# T2050L) and subsequent quantification with a Qubit 3 spectrophotometer. To assemble the LbCas12 RNP complexes, 5 µg of purified LbCas12a protein (New England Biolabs, Cat# M0653T) was incubated with 0.5 µg of purified gRNA at 25°C for 15 minutes. The RNP needs to be used immediately and cannot be stored for future use.

Preparation of Donor DNA

Bialaphos resistance (BAR) donor DNA was prepared using a plasmid template, pBV9, and G418 resistance donor DNA was prepared using a plasmid template, pFGL921 (Kankanala et al., 2007; W. Zhang et al., 2021). Phusion High-Fidelity DNA Polymerase (New England Biolabs, CAT# M0530L) was used to amplify the donor DNA sequences from these plasmids with primer pairs that incorporate MH for the target genes to improve targeted gene insertion (Supplemental Table 3). Specifically, the target gene-specific donor DNA was amplified as follows: 10 µL of 5x Phusion HF Buffer, 1 µL of 10 mM dNTPs, 2.5 µL of each 10 µM forward and reverse primer, 0.5 µL of Phusion DNA Polymerase, 1 µL of pFGL821 (approximately 5 ng/µL), and 32.5 µL of water were mixed to achieve a total volume of 50 µL. The PCR cycling conditions included an initial denaturation at 98°C for 30 seconds, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 1 minute. The next step was a final extension at 72°C for 10 minutes and a subsequent hold at 4°C for 2 minutes. The gene specific donor DNA was then purified using the Wizard PCR Clean-Up System (Promega, CAT# A9282) and quantified with a Qubit 3 spectrophotometer for

use in protoplast transformation. For each donor DNA, approximately 3000 ng of purified DNA was used per transformation.

Protoplast Preparation

For transformation, protoplasts were generated from liquid cultures. Liquid cultures were started from mycelial plugs taken from the growing colony edge of 1–2-week-old OTA plates and transferred to liquid complete media (liquid CM) and grown at 28 °C, 120 RPM for 3-4 days. The mycelium was collected by filtering mycelia with a sterile glass funnel (Fisher Scientific, Cat# FB6015865) and a milk filter disk (KenAG) that was formed into a cone, stapled, and autoclaved. The milk filter disk containing the wet mycelia was blotted with blotting paper (No source, CAT# n/a) to remove excess liquid CM, with attention having been taken to not over-dry the mycelia so that it could still be resuspended in-solution. The collected mycelia was then added to 0.22 µm filter sterilized (Midwest Scientific, CAT# SFPES033022SI) protoplast lysing solution. The protoplast lysing solution was made by adding 25 mg of Yatalase (Takara Bio, CAT# T017) and 1.2 g of VinoTaste Pro (Bucher Vaslin) to 30 mL of an osmotic lysis buffer. The osmotic lysis buffer was made from 18 mL of 1M (NH₄)₂SO₄, 3 mL of 0.5M maleic acid and 9 mL of H₂O). The mycelium was incubated in the protoplast lysis solution for 2.5-3 hours in an incubator shaker set at 30°C, 70-80 rpm to degrade fungal cell walls and release single-celled protoplasts. Following digestion, the solution containing protoplasts was added to a pre-chilled 50 mL tube, pelleted using centrifugation at 4°C, 5000 rpm for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 5 mL 1x STC. Only a single centrifugation step was performed, in contrast to the protocol used in Huang et al. 2021, as it was found that the second round of centrifugation did not improve protoplast purity and could be

harmful due to the delicate nature of protoplasts. The protoplast concentration was determined using a hemocytometer and adjusted to a concentration between 8×10^6 to 5×10^7 protoplasts/mL with 1x STC as the diluent. A volume of 200 μ L to 250 μ L of the resuspended protoplast solution, containing 1.6×10^6 to 1.25×10^7 total conidia, was then aliquoted into 50 mL tubes.

PEG-Mediated Transformation

In the PEG-Mediated transformation process for making KOs, two RNP complexes and 3000 ng of donor DNA were added to a 50 mL tube containing 200 μ L to 250 μ L of the resuspended protoplast solution. In PEG-Mediated FKBP12 editing, one RNP complex, either Cas9 or Cas12, and no donor DNA, since FKBP12 can be directly used for FK506 selection in *M. oryzae*, was added to a 50 mL tube containing 200 μ L to 250 μ L of the resuspended protoplast solution (Huang et al., 2022; Kankanala et al., 2007; Marroquin-Guzman & Wilson, 2015; W. Zhang et al., 2021). To the 50 mL tube, 1 mL of 60% w/v PEG 4000 was added to temporarily increase the permeability of the cell membrane and allow the RNP(s), and optionally donor DNA, into the protoplasts. Subsequently, 5 mL of sterile liquid TB3 (made by adding 200 g of sucrose, 6 g of yeast extract, and 6 g of casamino acids in up to 1 L of H₂O) was added to the 50 mL tube and incubated in a shaking incubator set to 90 rpm at 28°C overnight (12-20 hours). Transformed cells were plated on one of the following selection plates depending on the experiment: 1) for BAR selection, defined complex medium (DCM) was prepared and 50 mg/mL bialaphos was added to 45 mL DCM for 100 μ g/mL in the first layer, and repeated for 200 μ g/mL in the second layer; 2) for G418 selection, the protoplasts were plated in 45 mL TB3 containing 300 μ g/mL of G418, and a second layer of TB3 containing 600 μ g/mL was poured on top; 3) for FK506 selection, the protoplasts were plated in 45 mL of TB3 containing 1 μ g/mL

FK506. After one week, antibiotic-resistant colonies were individually transferred onto 60 × 15 mm plates (Midwest Scientific, Cat# 901) containing the same media and antibiotic concentrations, with the sole change being that TB3 medium was substituted for CM.

DNA Extraction and PCR Genotyping for Knockout Confirmation

Potentially transformed colonies growing on selection were selected for DNA extraction. For DNA extraction, an approximately 1 cm² mycelial plug was collected from the plate grown culture and moved to an impact resistant homogenization tube containing 1.0 mm silica beads (BioSpec, Cat #11079110z), 400 µL of 1M KCl, and 400 µL of chloroform. Samples were homogenized in a Bead Ruptor Elite (OMNI international, Cat#19-2141E) bead mill, using power level 4 and 6 cycles with 30 seconds of bead-beating followed by 10 seconds of dwell time. Samples were centrifuged at 13,200 rpm for 10 minutes, 200 µL of supernatant was transferred to new tubes, 120 µL of isopropanol was added, and incubated for 20 minutes to allow for DNA precipitation. The tubes were placed back into a microtube centrifuge set to 13,200 rpm and ran for 10 minutes to pellet the DNA. The isopropanol supernatant was discarded and replaced with 500 µL of 75% ethanol, then centrifugation was repeated. The supernatant was then discarded, and any remaining ethanol was allowed to evaporate before adding 40 µL of sterilized distilled H₂O to dissolve the DNA pellet. The PCR genotyping process used the described extracted DNA and gene specific primer pairs to determine if the gene of interest was successfully removed (Supplemental Table 2). If gel electrophoresis of the gene specific PCR product indicated that the gene of interest was successfully removed (no gene specific band), then another PCR, one at minimum, was used to verify that the antibiotic resistance gene was successfully inserted into each gene of interest. Specifically, PCR was used

to amplify a region spanning from the genomic DNA to the antibiotic resistance gene and vice versa, termed “Up” or “Down”, indicating the upstream or downstream PCR for each gene of interest, as well as PCR spanning across the gene of interest, which either used the forward “Up” and reverse “Down” primers or a specifically designed “Across” primer pair (Supplemental Table 2). If the gel electrophoresis results indicated that the gene of interest was deleted, and the antibiotic resistance gene was successfully inserted, then the resistant colony which the DNA came from was confirmed as a KO. Each strain identified to be a KO was single spored, as described earlier, for long-term storage and subsequent characterization.

Characterizing FKBP12 repair outcomes in Knockout backgrounds

To assess how KOs of specific DNA DSB repair genes impact DNA DSB repair, both CRISPR-Cas9 and Cas12a RNPs were used to edit the *M. oryzae* ortholog of the yeast *FRP1* gene, encoding the FK506/rapamycin-binding protein FKBP12. Since only FKBP12 mutants were able to grow on complete media containing 1 µg/mL of the drug FK506, donor DNA did not have to be added to allow for the selection of mutant sequences, which might influence DSB repair (Huang & Cook, 2022b; Marroquin-Guzman & Wilson, 2015). Colonies resistant to FK506 were collected for DNA extraction and PCR using primer pairs to amplify the gene coding for Actin (MGG_03982) were used to confirm successful DNA extraction. Then, the FKBP12 locus was PCR amplified and samples that produced a PCR product were sent for Sanger sequencing (McLabs). Samples of DNA that produced the expected Actin PCR product (574 bp) during gel electrophoresis, yet produced no band from the FKBP12 locus amplicon, were interpreted to have a deletion of one or more of the FKBP12 locus primer binding sites resulting from CRISPR editing and subsequent DSB repair of the FKBP12 locus. The Sanger

sequence trace files of the FKBP12 locus from PCR-positive transformants were aligned against the wild-type FKBP12 locus sequence using Benchling, then analyzed to determine their DNA mutation profile. The mutation data that was collected included INDEL size in bp, any insertions over 50 bp, the number of times the unique mutation profile was recovered for a given knockout background, any MH used to repair the DSB, and tandem duplications. The Mann-Whitney U test was then used to determine significant differences in INDEL sizes between WT and other DSB repair gene KOs. To identify MH, the flanking regions of a deletion were examined for short, repeated DNA sequences in the WT sequence, termed MH. If only one microhomologous sequence was found in the region post-deletion, it was determined that the sequence between the two microhomologous sequences, including one of the microhomologous sequences, had been deleted. This left a single microhomologous sequence, indicative of repair by MMEJ. Identification of tandem duplications used the same alignment approach but involved looking for the entire inserted sequence directly adjacent to the insertion. These data were collected and correlated with DSB repair in the absence of the gene of interest and associated DSB repair pathway that it was hypothesized to be involved in, to determine the gene's placement in a particular DSB repair pathway.

Genotoxic Stress Assay

To confirm the genetic requirements of individual DSB repair pathways, growth tests of conidia in the presence of genotoxic agents, which cause DSBs among other types of damage, were used. Two concentrations of hydroxyurea (HU) (Thermo Fisher Scientific, Cat# A10831.06) were used in CM for growth tests, a low and high concentrations of 5 and 10 mM, respectively, based on previous research in the budding yeast, *Naumovozuma castellii*, which

used 50–100 mM hydroxyurea (Itriago et al., 2023). The low and high concentrations of bleomycin (BLM)(Thermo Scientific Chemicals, Cat# AAJ60727MCR) used in the CM plates were 0.25 and 0.5 $\mu\text{g/mL}$, respectively, also based on research in the budding yeast *N. castellii* (Itriago et al., 2023). Conidia were collected from 7 to 14-day-old OTA plates of various KO strains using 1 mL of 0.25% gelatin. The concentration of the conidial suspension was then determined using a hemocytometer and adjusted to 70,000 cells/mL. A two-fold serial dilution was performed, which resulted in the first 15 μL sample containing ~1050 cells, ~525 cells in sample 2, ~ 263 spores in sample 3, and ~132 spores in sample 4. Up to 6 KO genotype serial dilutions were added to a single plate. After plating all serial dilutions, the plate was wrapped with parafilm and stored in a 28°C incubator in the dark for 3 days, then pictures were taken to record the sensitivity of KO strains to each genotoxin treatment. Sensitivity to each genotoxin was determined by comparing the growth of a KO strain when grown on media containing the genotoxin against its growth on CM containing no genotoxin.

Assaying Radial Growth, Conidia Production and Colony Morphology in Knockout Strains

To assess the growth rate, conidia production, and colony morphology in various DNA DSB repair KO genotypes, OTA plates were first started using spore filter stocks obtained from single sporing of KOs. These plates were allowed to grow for 10 days in an incubator at 28°C under continuous light (white and black lights), after which two 5 mm diameter plugs transferred to individual OTA plates. These plates were grown for 12 more days, or until the colonies covered them completely. Photographs were taken on days 5 and 10 to document any distinct morphological characteristics of the KO colonies. Radial growth measurements were taken every other day with a ruler for 12 days or until the colonies filled the plate. Growth rates of each KO

strain were quantified by plotting these radial growth measurements over the 12-day period.

After the 12 days, spore production in each KO genotype was quantified by harvesting spores with 1 mL of 0.25% gelatin solution and counting them with a hemocytometer. The results were displayed in a line graph.

Results

Critical Role of Mre11 in End-Resection

Previous evidence for the activity of the main DNA DSB Repair pathways, NHEJ, MMEJ, SSA, and HR, were indirectly observed in *M. oryzae* in the absence of Ku80 (Huang et al., 2022). The DSB repair genes that were targeted in this study included key proteins involved in the regulation of downstream end-protection or end-resection pathways, as well as ligases and polymerases potentially involved in NHEJ and MMEJ (Table 2). Protein sequences for previously characterized DNA DSB repair genes in mammals and *S. cerevisiae* were found and BLASTp was used to identify their orthologs in *M. oryzae*. The orthologous DNA DSB repair genes of interest were effectively deleted from *M. oryzae* using two Cas9 or Cas12a RNPs flanking the coding sequence. A selectable marker was added during the transformation along with the RNPs to allow for selection of gene deletion mutants, which was then followed by DNA extraction and confirmation of target gene deletion and selectable marker insertion was determined by PCR genotyping. The genes involved in initiating end protection and end-resection pathways targeted for deletion included Ku80 in the previously created $\Delta ku80$ strain, Exo1 made in the $\Delta ku80$ background ($\Delta ku80 \Delta exo1$), and Mre11 ($\Delta mre11$). Ku80 is part of the Ku heterodimer, which binds to free DNA ends following a DSB, blocking end-resection nucleases and promoting NHEJ (Decottignies, 2007; Mimitou & Symington, 2010). To confirm that the MMEJ-like repair profiles previously seen in the $\Delta ku80$ strain were actually done by MMEJ, NHEJ was first disabled by the $\Delta ku80$ strain, then the long-range end-resection, including SSA and HR, were disabled by knocking out Exo1 in the $\Delta ku80$ background (Krasner et al., 2015; Truong et al., 2013). This effectively isolated MMEJ and allowed for direct characterization of MMEJ-dependent repair profiles. To confirm that the NHEJ-like repair

profiles previously seen in the WT strain were actually done by NHEJ, a KO strain of the *Mre11* endo/exonuclease, $\Delta mre11$, was created. The successful deletions of $\Delta ku80\Delta exo1$ and $\Delta mre11$ were confirmed via PCR genotyping, which verified the absence of the target gene and the integration of the selectable marker (Supplemental Fig. 3 and 4).

To understand the roles of gene deletion mutants in DNA DSB repair in *M. oryzae*, the FPR1 gene, which encodes the FK506-binding protein FKBP12, was edited using a single Cas12a RNP in each KO background (Huang et al., 2022). Transformants from FKBP12 editing could be selected on plates containing FK506 as FKBP12 mutants are insensitive to the drug FK506 (Marroquin-Guzman & Wilson, 2015; Yu et al., 2014). The FK506 resistant transformants were then subjected to DNA extraction, and amplification across the FKBP12 locus. As reported previously, some recovered transformants failed to produce a PCR amplified product due to extensive end-resection prior to repair (Huang et al., 2022). To ensure that the lack of PCR amplification was not due to inadequate DNA extraction, a product across the actin gene coding sequence was amplified. Transformant DNA that produced a successful actin PCR product but did not show amplification at the FKBP12 locus were termed PCR-negative. Cas12a RNP editing of FKBP12 in the WT O-137 strain resulted in a PCR-negative frequency of approximately 5% (2/42) (Fig. 1a). In comparison, when NHEJ was disabled in the $\Delta ku80$ strain, significantly higher PCR-negative frequencies were produced (~39%, 12/31, Mann Whitney U test, two-sided, p -value < 0.001) (Fig. 1a). This increase in PCR-negative FKBP12 mutants in the $\Delta ku80$ is consistent with the Ku's role in protecting DNA DSB ends and indicates that PCR-negative mutants are the result of extensive DNA end-resection normally suppressed by Ku. Similar to $\Delta ku80$, the $\Delta ku80\Delta exo1$ strain also produced a significantly higher PCR-negative frequency than WT (~41%, 13/32, Mann Whitney U test, two-sided, p -value < 0.001) (Fig. 1a).

The high PCR-negative frequency observed in $\Delta ku80\Delta exo1$ was unexpected because PCR-negative DNA samples were hypothesized to result from long-range end-resection and the use of substantial homology, such as by the SSA or HR pathways (Huang et al., 2022). To understand the role that end-resection plays in DSB repair in *M. oryzae*, a deletion strain of the Mre11 endo/exonuclease gene was made, termed $\Delta mre11$. Editing the FKBP12 locus in $\Delta mre11$ resulted in a PCR-negative frequency that was statistically similar to WT (~1%, 1/96, Mann Whitney U test, two-sided, p -value > 0.05) (Fig. 1a). This low PCR-negative frequency supports the hypothesis that PCR-negatives are the result of end-resection-based DSB repair, and that Mre11 initiates this end-resection as observed in other eukaryotes (Anand et al., 2016).

Together, the low PCR-negative frequency in $\Delta mre11$ and the high PCR-negative frequency in $\Delta ku80$ reinforces the idea that NHEJ, which involves very limited end-resection, was the predominant pathway used to repair Cas12a RNP induced DSBs in WT *M. oryzae*. It was concluded that PCR-negatives at the FKBP12 locus were likely due to large deletions created during end-resection-based DSB repair, as the FKBP12 locus PCR primer sites were located approximately 600 bp upstream and downstream of the Cas cut site. It is unlikely that NHEJ or MMEJ DNA DSB repair, which results in up to 14 bp deletions in NHEJ or up to 300 bp deletions in MMEJ, would result in DNA deletions large enough to create PCR-negatives (Garcia et al., 2011). The extensive resection observed in PCR-negative transformants instead suggests the involvement of long-range resection active during SSA or HR. Notably, the high PCR-negative frequency observed in the $\Delta ku80$ strain suggests that Ku80 is required for protecting DNA ends from premature end-resection. The unexpected high PCR-negative frequency in the $\Delta ku80\Delta exo1$ strain indicates that despite the absence of Exo1, backup end-resection nucleases, likely DNA2, can fill the role of Exo1 in long-range end-resection,

suggesting a functional redundancy within the long-range end-resection pathway (Zhu et al., 2008). Conversely, the similarity in PCR-negative frequencies between the $\Delta mre11$ strain and WT underlines the importance of Mre11 in initiating end-resection-based pathways.

FKBP12 Repair Profiles Produced in DSB Repair Mutant Backgrounds Highlights Mutagenicity of MMEJ

To further explore the effect of DSB repair across various knockout backgrounds, PCR-positive FKBP12 locus amplicons from independent transformants were Sanger sequenced and analyzed in each KO background. For this, the FKBP12 sequencing results from each independent transformant were compared to the WT sequence, and the recovered INDEL size and MH usage were recorded. The DSB repair profile for WT mainly resulted in INDELs with an average size of -3.6 bp, ranging from -37 to 5 bp (Fig. 1b, c, d). For this research, small INDELs were defined as being up to -9 bp deletions and up to 5 bp insertions. Using this definition, it was determined that small INDELs accounted for ~75% of repair events in WT, 51.4 % of which used ≤ 2 bp MH, indicating minimal MH usage (Fig. 1b, c, d). Based on the frequency of INDELs, minimal MH occurrence, and recovery of tandem duplications, the majority of recovered mutants were indicative of NHEJ mediated DSB repair. In contrast, DSBs in the $\Delta ku80$ strain were repaired with significantly larger INDELs (0% small INDELs), with an average size of -25 bp, and ranged from -88 bp to -13 bp (Mann Whitney U test, two-sided, p-value ≤ 0.0001) (Fig. 1b, c, e). Repair in $\Delta ku80$ had prevalent MH usage, where ~93% of repairs used ≥ 3 bp MH and 7% used 1-2 bp MH (Fig. 1b, c, e). These results were consistent with MMEJ-like repair and demonstrated that Ku80 suppresses end-resection-based repair, indicating that non-NHEJ repair is more mutagenic. The analyzed DSBs in $\Delta ku80\Delta exo1$ showed similar results to $\Delta ku80$, which had significantly larger INDELs compared to WT (0% small INDELs),

with an average size of -50.7 bp, and ranged from -145 bp to -21 bp (Mann Whitney U test, two-sided, $p\text{-value} \leq 0.0001$) (Fig. 1c). There was also prevalent MH usage (100% of repairs used ≥ 3 bp MH (Fig. 1b). The PCR-positive repair profiles in the $\Delta ku80\Delta exo1$ mutant should be the result of MMEJ, which is consistent with the overall profiles observed. In the $\Delta mre11$ strain, small INDELs accounted for $\sim 95\%$ of repair events, averaging -2.1 bp that ranged from -9 bp to 5 bp (Mann Whitney U test, two-sided, $p\text{-value} > 0.05$) (Fig. 1c). Also, there was minimal MH usage, as only 7.7% of repairs used ≥ 3 bp MH, and again, the presence of tandem duplications was observed (Fig. 1b, c, g). Therefore, the repair profiles in $\Delta mre11$ are indicative of NHEJ-like repair and confirm that these major proteins are active and function similarly to other eukaryotes. Also, re-interpreting the WT repair profile considering the mutant results suggests that NHEJ is the predominant pathway functioning during Cas12a RNP editing of FKBP12, with low PCR-negative transformants, small INDELs, and occasional tandem duplication insertions.

Pol 4 contributes to MMEJ repair

Efficient gap filling and synthesis of MH by polymerases are essential for faithful repair of DNA DSBs, as polymerase deficiencies within various pathways often result in genomic instability (Brambati et al., 2020; Brown et al., 2011). In *M. oryzae*, the key polymerases that were targeted for deletion include Pol θ , Pol 3, Pol 4, and Pol μ (Table 2). Notably, the Pol θ homolog in *M. oryzae* lacks the polymerase domain needed for gap filling during MMEJ in mammals but retains the helicase domain (Black et al., 2019; Huang et al., 2022). Because of this, Pol 3 and Pol 4 were targeted due to their proposed roles as gap-filling polymerases in the MMEJ pathway of *S. cerevisiae* (Meyer et al., 2015). Additionally, Pol 4 as well as Pol μ were targeted due to their function as NHEJ polymerases in mammals, where they are largely involved

in gap filling and the creation of MH, respectively (Bertocci et al., 2006; Brown et al., 2011; Nick McElhinny et al., 2005). Therefore, the described function of polymerases, such as Pol 4, vary between mammals and *S. cerevisiae* requiring further investigation.

To explore the roles of these polymerases in DSB repair, $\Delta ku80\Delta polq$ and $\Delta pol4$ were successfully created, but KO strains of Pol 3, Pol μ , and a Pol θ single KO were not obtained (Supplemental Fig. 5, 8, 9, 10, 11). The FKBP12 editing scheme was again used to characterize repair in the mutants. The PCR-negative frequency for $\Delta ku80\Delta polq$ was similar to $\Delta ku80$, but significantly different from WT (~40%, 7/16, Mann Whitney U test, two-sided, p-value ≤ 0.001) (Fig. 2a). The high PCR-negative frequency in $\Delta ku80\Delta polq$ is consistent with repair in the absence of Ku80 and elevated long-range end-resection. The PCR-negative frequency for $\Delta pol4$ was similar to WT (~6%, 1/16, Mann Whitney U test, two-sided, p-value > 0.05) (Fig. 2a). To determine the unique Cas12 repair profiles found in $\Delta ku80\Delta polq$ and $\Delta pol4$, all PCR-positive FKBP12 locus amplicons were sequenced. Repair in the $\Delta ku80\Delta polq$ strain showed significantly larger INDELs compared to WT (0% small INDELs), averaging at -62 bp and ranging from -145 bp to -21 bp (Mann Whitney U test, two-sided, p-value ≤ 0.0001) (Fig 2c). The repair profile also showed increased MH usage, where 87.5% of repair events used ≥ 3 bp MH (Fig. 2b, d). These repair results are consistent with MMEJ-like repair, as expected in the $\Delta ku80$, further suggesting that Pol θ is not an MMEJ polymerase in *M. oryzae*. The $\Delta pol4$ strain showed small INDELs, similar to those observed in WT (Mann Whitney U test, two-sided, p-value > 0.05), accounting for ~70% of repair events (Fig. 2c). The average small INDEL size was -4.5 bp and ranged from -9 bp to 1 bp (Fig 2c). The repair profiles showed minimal MH usage, 42.1% of repair events used ≤ 2 bp MH, with only 10% of repair events using 3 bp MH (Fig 2b, e).

A major goal of this research was to understand the genetic requirements and outcomes of MMEJ repair specifically as the pathway is underexplored in filamentous fungi. Since the majority of repair events from Cas12a editing of FKBP12 in WT O-137 resulted in NHEJ repair, it was difficult to assess any impact that KOs had on MMEJ repair. We previously observed that FKBP12 editing using Cas9 RNP instead of a Cas12 RNP increased the frequency of MMEJ repair pathway choice in WT O-137 (Huang et al., 2022). Based on this, the FKBP12 editing was repeated using the Cas9 RNP to edit FKBP12 in WT O-137 and $\Delta pol4$. The FKBP12 Cas9 editing in WT O-137 led to a significantly more PCR-negatives (39%, 9/23, Mann Whitney U test, two-sided, $p\text{-value} \leq 0.001$) compared to Cas12a editing, confirming previously published data that Cas9 tends to create larger deletions compared to Cas12a (Fig. 3f). Likewise, Cas9 editing in $\Delta pol4$ resulted in a PCR-negative frequency that was similar to what was observed from Cas9 editing in WT (~42%, 10/24, Mann Whitney U test, two-sided, $p\text{-value} > 0.05$) (Fig. 2f). The PCR-positive transformants obtained from FKBP12 editing in both genotypes were then sequenced to determine their repair profile. Cas9 repair in the WT strain showed larger INDELs compared to Cas12 editing in WT (Mann Whitney U test, two-sided, $p\text{-value} \leq 0.01$), average size was -20.2 bp and ranged from -152 bp to 69 bp (Fig. 2h), and small INDELs accounted for only ~33.3% of repair events (Fig. 2g, h, i). Likewise, the DSB repair from Cas9 editing showed increased MH usage, 33.3% of repair events used 2 bp MH and 25% of repair events used ≥ 3 bp MH (Fig. 2g). These results provided strong evidence that Cas9 induced DSBs more frequently resulted in MMEJ and end-resection-based repair patterns while Cas12a resulted in predominately NHEJ repair profiles. Cas9 editing of FKBP12 in the $\Delta pol4$ strain, however, resulted in ~85% of repair events being categorized as small INDELs that averaged -1 bp and ranged from -4 bp to 1 bp, significantly smaller than what was observed from Cas9 editing in

WT (Mann Whitney U test, two-sided, p -value ≤ 0.01) (Fig. 2h). Cas9 editing in $\Delta pol4$ also used minimal MH, 77% of repair events used ≤ 2 bp MH and no repair used ≥ 3 bp MH (Fig. 2g, h, j). These results indicate that Pol 4 is a positive contributor to MMEJ, as when a Cas9 RNP was used to edit FKBP12 in the $\Delta pol4$ background, the frequency of MMEJ-like repair was significantly reduced. It should also be noted that PCR-negatives were not impacted by $\Delta pol4$, further suggesting that MMEJ is a separate mechanism than the one producing PCR-negative mutations.

Lig4 is required for NHEJ repair in *M. oryzae*

Efficient ligation of nicks and joining of DNA ends is essential for maintaining genomic stability following a DSB (Ferguson et al., 2000). To explore the roles of these ligases in DSB repair, KO strains for Lig1b and Lig4 were created, while the Lig1a KO strain was not able to be made (Supplemental Fig. 1, 2, 6). Notably, no Lig3 homolog was found in either *M. oryzae* or *S. cerevisiae*, despite its function as the MMEJ ligase in mammals.

The FKBP12 editing strategy used to assess DSB repair in other strains was applied to characterize DSB repair without Lig1b and Lig4, respectively. The PCR-negative frequency for $\Delta lig1b$ was similar to WT (13%, 5/39, Mann Whitney U test, two-sided, p -value > 0.05), while $\Delta lig4$ showed a significantly higher PCR-negative frequency ($\sim 65\%$, 15/23, Mann Whitney U test, two-sided, p -value ≤ 0.0001) (Fig. 3a). In other eukaryotic systems, Lig4 is the ligase required for NHEJ, and the increase in PCR-negatives in the $\Delta lig4$ are consistent with its conserved function in NHEJ. Subsequent sequencing of PCR-positive FKBP12 locus amplicons from $\Delta lig1b$ resulted in NHEJ-like repair, characterized by small INDELs that accounted for $\sim 80\%$ of repair events, similar to those observed in WT (Mann Whitney U test, two-sided, p -

value > 0.05), with an average small INDEL size of -2.1 bp that ranged from -8 bp to 4 bp (Fig. 3b, c, d). The repairs also showed minimal MH usage (~42% of repairs used ≤ 2 bp) and the presence of tandem duplications (Fig. 3b, c, d). Only two PCR-positive repair profiles were successfully collected from $\Delta lig4$, which exhibited MMEJ-like repair indicated by large deletions (-273 bp and -88 bp) and relatively long MH (10 bp and 5 bp). The repair pattern in $\Delta lig4$ indicated a shift towards end-resection-based pathways (0% small INDELs), quite similar to repair observed in $\Delta ku80$. However, it is important to note that only a few FKBP12 mutants were recovered, a large majority of which were PCR-negative, so more replicates are needed to fully characterize DSB repair in this KO. The drastically higher PCR-negative frequency in $\Delta lig4$ underscores Lig4's essential role as the only NHEJ ligase, therefore contributing to genomic stability by limiting end-resection.

Most DNA repair proteins are not required for normal growth or conidiation

To understand how DNA DSB repair proteins impact normal fungal growth and development, radial growth rates and conidia production were assayed across the different KO strains. The results indicated that the majority of strains, except for $\Delta mre11$ and $\Delta ku80\Delta exo1$, exhibited a normal growth rate and production of conidia compared to the WT (Fig. 4). Specifically, WT showed a radial growth rate of approximately 0.4 cm/day and produced $\sim 3 \times 10^8$ conidia from a single 12-day-old OTA plate (Fig. 5, 6). The $\Delta ku80$ strain maintained a radial growth rate similar to WT but it showed a slight decrease in conidia production. Significant growth deficiencies were observed in $\Delta mre11$ (Mann Whitney U test, two-sided, p-value ≤ 0.05). $\Delta mre11$ grew at approximately half the rate of WT and conidia production was reduced to ~9% of the total conidia produced in WT. These findings suggest that while most DNA DSB repair

proteins were not essential for maintaining normal fungal growth and conidiation in *M. oryzae*, Mre11 was proven essential, as knocking out Mre11 severely impacted both the growth rate and reproductive capacity compared to WT.

Mre11 is required for DSB Repair outside of the G1 cell-cycle phase

To explore the roles of different DNA repair proteins throughout the cell cycle, we examined responses of WT and KO strains to genotoxic agents which induce DSBs and cell cycle stalling. Known pathways, such as NHEJ and HR, primarily function during specific cell cycle phases. NHEJ is largely used before DNA synthesis, whereas after DNA synthesis, HR is preferred for error-free template-based repair (Delacôte & Lopez, 2008). More recent data suggests that MMEJ is active mainly during mitosis (Brambati et al., 2023). To understand how the cell cycle controls DNA DSB repair choice in *M. oryzae*, WT and mutant strains were grown on media containing various genotoxic drug treatments. Each drug impacts distinct aspects of DNA damage and repair, providing insights into the repair mechanisms active at various phases of the cell cycle. For example, bleomycin (BLM) causes DSBs, causing cell cycle stalling at the G1/S and G2/M checkpoints, whereas hydroxyurea (HU) inhibits DNA synthesis, causing DSBs and cell cycle stalling at the G1/S checkpoint (Baus et al., 2003; Suzuki et al., 1970). To assess sensitivity to genotoxins, conidia were harvested from a 10-day-old OTA plate of each strain and adjusted to a concentration of 70,000 conidia/mL. Conidia were used, as opposed to vegetative mycelia, as conidia have been shown to be locked in the stationary G₀ phase until germination (Bittner-Eddy et al., 1994). A two-fold serial dilution was performed to create 15 µL spots containing approximately 1000, 500, 250, and 125 conidia each. These 15µL spots were plated on CM with high and low concentrations of the genotoxins BLM and HU, as well as on CM

without genotoxins. These conidia were allowed to germinate and grow over 3 days, after which pictures were taken to record their sensitivity to genotoxins (Fig. 7). The concentrations used were based on previous literature and were chosen to identify the maximum concentration where WT growth was unaffected while truly sensitive KO strains showed no growth (Itriago et al., 2023). WT conidia consistently exhibited insensitivity to all genotoxins, indicating its ability to repair DSBs during genotoxin treatments. Similarly, the $\Delta ku80$ strain, in which NHEJ is disabled, also showed insensitivity to genotoxin treatments at all concentrations tested. This indicates that disruption of the NHEJ pathway does not significantly affect the cell's ability to deal with DSBs induced by genotoxic stress at the G1/S (HU and BLM) and G2/M checkpoints (BLM). The $\Delta ku80\Delta exo1$ double knockout, which lacks Ku80 (needed for NHEJ) and Exo1 (crucial for both HR and SSA), displayed consistent growth deficiencies, possibly caused in part by its ~0.5x reduction in growth rate. Although growth in $\Delta ku80\Delta exo1$ conidia appeared to be inhibited in the presence of genotoxins, it was difficult to fully assess this effect due to the strain's overall poor growth, even on CM without genotoxins. Strikingly, $\Delta mre11$ showed hypersensitivity to all genotoxins tested, regardless of the concentration, highlighting the critical role of Mre11-dependent end-resection pathways in DNA DSB repair at the G1/S and G2/M checkpoints (Fig. 7, 8).

The genotoxin sensitivity results from WT, $\Delta ku80$, and $\Delta mre11$ were used as a baseline for determining sensitivity, and therefore pathway involvement, of other KO strains. The polymerase KOs, $\Delta ku80\Delta polq$ and $\Delta pol4$, showed no growth defects when grown on CM without genotoxins and showed insensitivity to most genotoxins. $\Delta ku80\Delta polq$ showed a significant sensitivity to the low concentration of BLM, and $\Delta pol4$ showed significant sensitivity to low HU (Mann Whitney U test, two-sided, p-value < 0.05 for both). The ligase 1, CDC9

homolog, KO strain, $\Delta lig1b$, showed insensitivity to all treatments other than the low concentration of HU, which was significantly different than WT (Mann Whitney U test, two-sided, p -value < 0.05)(Fig. 7, 8). Genotoxin sensitivity for $\Delta lig4$ has yet to be determined.

Additional Studies Required to Determine Effects of $\Delta ku80\Delta lig4$ on DSB Repair

To determine any role of Lig4 in MMEJ, Lig4 was knocked out in the $\Delta ku80$ background (Supplemental Fig. 7). So far, ten FKBP12 edited colonies have been collected from $\Delta ku80\Delta lig4$. Of those, nine colonies were PCR-negative for the FKBP12 locus (1/10; 90% PCR-negative) and the 1 PCR-positive sample resulted in a single SNP located in the FKBP12 locus, which allowed for FK506 selection. The $\Delta ku80\Delta lig4$ strain needs more replicates to determine its effect on DSB repair, growth rate, conidia production, and genotoxin sensitivity.

$\Delta ku80\Delta rad51$ Displays MMEJ Repair and Reduced Growth, Further Studies Needed

Rad51 nucleoprotein filament formation has been shown essential for the key steps of HR, including the search for homologous sequences and the strand invasion necessary for error-free template-based DNA repair (Bonilla et al., 2020). To determine whether PCR-negatives at the FKBP12 locus were being caused by either SSA or error-prone HR pathways, $\Delta ku80\Delta rad51$ was made and FKBP12 was edited with a Cas12a RNP (Supplemental Fig. 12). In theory, this effectively disabled both high-fidelity pathways, NHEJ and HR, and isolated the mutagenic MMEJ and SSA pathways. More replicates are needed, however the PCR-negative frequency of 27% (3/11) observed from a single replicate of Cas12a editing, was significantly different than WT (Mann Whitney U test, two-sided, p -value ≤ 0.05) and similar to $\Delta ku80$ (Mann Whitney U

test, two-sided, p-value > 0.05). This high PCR-negative frequency indicates that the large deletions causing PCR-negatives is independent of Rad51 and suggests that they are likely dependent on Rad52 and its involvement in mutagenic pathways like SSA. *Δku80Δrad51* showed MMEJ-like repair, as indicated by the presence of the most frequent repair profile in the *Δku80* background, which used 4 bp MH and a 21 bp deletion and is consistent with repair in the absence of Ku80. This KO also exhibited reduced growth rate, similar to *Δmre11* and *Δku80Δexo1*, suggesting that the growth rate reduction in *Δku80Δrad51*, *Δmre11*, and *Δku80Δexo1* might be caused by Rad51, Mre11, and Exo1's shared function in promoting HR. One replicate of a genotoxic stress assaying was performed in *Δku80Δrad51*, in which the strain showed insensitivity to HU, but hypersensitivity to BLM at all tested concentrations. Insensitivity to HU, yet sensitivity to BLM indicates that Rad51 was required for repair when stalled at the G2/M checkpoint, consistent with the requirement of HR in late S/G2 phase. Insensitivity to HU in *Δku80Δrad51* suggests that DSBs can be repaired by DSB repair pathways other than NHEJ and HR when stalled at the G1/S checkpoint, possibly by MMEJ or SSA (Mathiasen & Lisby, 2014). Overall, more replicates are needed to determine the KO's effect on DSB repair, growth rate, conidia production, and genotoxin sensitivity.

Discussion

NHEJ is the Primary DSB Repair Mechanism from Cas12a editing in *M. oryzae*

It was determined that NHEJ was the primary pathway for repairing Cas12a RNP induced cuts by comparing the FKBP12 repair profiles of both WT and $\Delta mre11$ strains, in which NHEJ is proposed to be the only active DSB repair pathway. In the WT O-137 strain, Cas12a editing of FKBP12 results in a PCR-negative frequency of approximately 5%, consistent with a low dependence on end-resection pathways in *M. oryzae* as well as in filamentous fungi as a whole (Ninomiya et al., 2004; Villalba et al., 2008). $\Delta mre11$ showed an overwhelming majority of NHEJ repairs, which is consistent with all end-resection pathways having been disabled. NHEJ is crucial for maintaining genome stability by preventing large genomic deletions, as indicated by PCR-negatives, instead generating small deletions up to 9 bp long, insertions up to 5 bp, and the usage of ≤ 3 bp of MH. This suggests that NHEJ is not central during mitotic repair of DSBs, highlighting a reliance on end-resection-based repair mechanisms during cell division.

Fungal MMEJ is Functionally Distinct from Mammalian TMEJ

A key finding from our research into *M. oryzae*'s DNA DSB repair mechanisms is the functional distinction of its MMEJ pathway from the Pol θ -mediated end-joining (TMEJ) pathway observed in mammals. Notably, *M. oryzae* possesses a truncated version of Pol θ , lacking the catalytic domain crucial for Pol θ polymerase function in mammalian TMEJ (Black et al., 2019). This truncation indicates that an alternative MMEJ polymerase produced the MMEJ-like repair profiles previously observed. One hypothesis is that *M. oryzae* relies on Pol 3 and Pol 4 for MMEJ, similar to *S. cerevisiae* (Meyer et al., 2015). However, unlike *S. cerevisiae*, which completely lacks a Pol θ homolog, *M. oryzae* retains the SF2 helicase domain and a Rad51

interacting motif (Ceccaldi et al., 2015). These features are also found in the related HelQ protein, which plays a crucial role in end-resection-based pathways by displacing RPA to allow for annealing of MH and bridging broken DNA strands following a DSB (Anand et al., 2022; Schaub et al., 2022). Although the full impact of the Pol θ helicase has not been fully explored in *M. oryzae*, it is possible that its role in stripping RPA to promote MH annealing in mammals is conserved in *M. oryzae* (Kamp et al., 2021; Mateos-Gomez et al., 2017). This capability could be important in haploid pathogens like *M. oryzae*, where alternative repair mechanisms are crucial for survival and adaptation in the absence of a second chromosome set for error-free template-based HR repair.

Cas12 RNP editing of FKBP12 in the $\Delta ku80\Delta polq$ strain resulted in a PCR-negative frequency similar to that of $\Delta ku80$ and different from WT, consistent with an increased reliance on end-resection-based pathways in the absence of Ku80. The $\Delta ku80\Delta polq$ strain exhibited MMEJ-like DSB repair profiles, characterized by substantial MH usage and larger deletions, suggesting that the absence of Pol θ 's helicase domain does not affect end-resection and homology-driven repair, although more replicates are needed to fully confirm this. While Cas12a editing in $\Delta ku80\Delta polq$ revealed a dependency on end-resection pathways characterized by MMEJ-like repair, editing FKBP12 in $\Delta pol4$ resulted in a WT-like repair profile, characterized by minimal use of MH, small INDELs, and a low PCR-negative frequency. Conversely, Cas9 editing of FKBP12 in WT O-137 consistently resulted in repair by MMEJ and larger deletions, whereas Cas9 editing in $\Delta pol4$ resulted in minimal use of MH and small INDELs relative to Cas9 editing in WT, thereby confirming Pol 4's involvement in MMEJ.

Comparative Insights and Evolutionary Considerations

The involvement of Pol 4 in the MMEJ pathway of *M. oryzae* suggests that the polymerases used in the MMEJ pathway of *S. cerevisiae*, Pol 3 and Pol 4, might both be conserved, but a Pol 3 KO would be needed to confirm this (Meyer et al., 2015). It is also important to note that MMEJ in *M. oryzae* might also be functionally distinct from MMEJ in *S. cerevisiae* due to the activity of the Pol θ helicase homolog. This variable presence of the Pol θ helicase among eukaryotes may indicate functional differences related to end-bridging of non-complementary sequences during DNA repair. Specifically, the absence of a Pol θ helicase in *S. cerevisiae* indicates that end-bridging of long homologous ends are only done by the MRX complex. In comparison, the presence of the Pol θ helicase domain, such as in mammals and many filamentous fungi like *M. oryzae*, indicates that end-bridging can be done by both the Pol θ helicase or by the MRX complex, providing an extra layer of protection for preventing large deletions which would result in PCR-negatives (Mojumdar et al., 2019; Schaub et al., 2022).

Future Directions for characterizing MMEJ and SSA

An in-depth investigation into the interplay between the Pol θ helicase/HelQ and the potential MMEJ polymerases Pol 3 and Pol 4 offers a promising avenue for future research on end-resection-based DSB repair in filamentous fungi. Future studies could focus on determining how these components collaborate to maintain genomic stability and create genomic variation, particularly in the context of adaptation and survival strategies in haploid filamentous fungal plant pathogens. Such research could illuminate novel aspects of fungal biology and provide insights into the evolutionary dynamics of DNA repair mechanisms.

The high PCR-negative frequency observed in the $\Delta ku80\Delta exo1$ strain revealed that long-range end-resection is still operational, possibly due to the presence of the backup nuclease DNA2, rather than the MMEJ pathway causing large deletions (Zheng et al., 2020). Once DNA2 and Exo1 are both knocked out, PCR-positive results are expected for all DNA samples collected, fully isolating the MMEJ pathway.

Determining the effect of $\Delta lig1b$ on DSB repair, specifically MMEJ, could be more easily observed by using Cas9 RNP to edit FKBP12, as this was previously shown to increase the amount of MMEJ repair relative to Cas12 editing in WT. Alternatively, knocking out Lig1b in the $\Delta ku80$ background could increase the relative amount of MMEJ. Additionally, since $\Delta lig1b$ showed insensitivity to HU and BLM, conducting a genotoxic stress assay with the drug nocodazole might reveal hypersensitivity in $\Delta lig1b$ due to the involvement of the *S. cerevisiae* Lig1b homolog, CDC9, in yeast MMEJ. Nocodazole prevents mitotic exit by binding to tubulin and disrupting the mitotic spindle, causing cell cycle arrest in the mitosis phase, where MMEJ is most active (Brambati et al., 2023; Feng et al., 2011).

Overall, a more in-depth exploration into the specific roles and interactions between DSB repair proteins is needed, especially for the mutagenic MMEJ and SSA pathways. This research could reveal potential targets for developing new pathogen management strategies. For example, by inhibiting MMEJ and SSA, it might be possible to reduce the variation that leads to fungicide resistance and develop more effective fungicides.

Isolating Cell-Cycle-Specific DNA DSB Repair Pathways in *Magnaporthe oryzae* through Genotoxic Stress Assays

To test the function of proteins in various DSB repair pathways, multiple DSB inducing chemicals were used to stall the cell cycle at specific stages of growth. For instance, HU was used to stall the cell cycle at the G1/S check point. It did this by inhibiting ribonucleotide reductase, preventing the synthesis of dNTPs needed for DNA synthesis, leading to polymerase stalling and the collapse of replication forks which resulted in DSBs (Krakoff et al., 1968; Musiałek & Rybaczek, 2021; Petermann et al., 2010). BLM directly created both SSBs and DSBs by removing thymine from dsDNA through the hydrolysis of the N-glycosidic bonds, stalling cell cycle progression at the end of the S-phase and the early half of the G2-phase in which HR is most active (Baus et al., 2003; Müller & Zahn, 1977; Suzuki et al., 1970).

By plating cells on complete media containing these genotoxins, individual DNA DSB repair pathways can be isolated and used to infer the function and pathway placement of various gene KOs based on hypersensitivity to these drugs. $\Delta mre11$ and $\Delta ku80\Delta rad51$ were sensitive to genotoxins, but no other strains were, including $\Delta ku80$, indicating that NHEJ is dispensable for the repair of HU and BLM induced DSBs, whereas HR is required.

Critical Functions of Mre11 in Mitosis

The hypersensitivity of the $\Delta mre11$ strain to the genotoxic stressors BLM and HU underscores the essential role of Mre11 in maintaining genomic stability throughout cell division. Mre11's involvement in genotoxin associated DSB repair demonstrates the intricate connection between DNA DSB repair mechanisms and the cell cycle, affirming its essential,

evolutionarily conserved function in safeguarding *M. oryzae*'s viability and ensuring accurate genetic transmission.

In future work, we will validate the genotoxin sensitivity observed in the $\Delta mre11$ strain by complementing it with the original Mre11 gene flanked by 1 kb of homology on either end to facilitate HR-mediated repair. Additionally, introducing a variant of Mre11 fused with UltraID would enable proximity-dependent biotinylation of all DNA DSB repair proteins interacting with MRE11 (Zhao et al., 2021). This approach will precisely identify all repair proteins involved in DNA DSB repair pathways and provide a deeper understanding of their functions.

Conclusion

This research sheds light on the DNA DSB repair mechanisms in the rice blast fungus *M. oryzae*, revealing significant functional distinctions in its MMEJ pathway compared to both mammalian TMEJ and *S. cerevisiae* MMEJ. Our findings highlight the critical roles of the Ku70/80 heterodimer in protecting DNA ends and the Mre11 endo/exonuclease in initiating end-resection. These mechanisms are crucial for maintaining genomic stability in NHEJ and HR, and more mutagenic pathways like MMEJ and SSA are thought to contribute significantly to genetic variation within haploid filamentous fungal pathogens. This research could potentially lead to the development of new pathogen management strategies by inhibiting the use of mutagenic repair pathways like MMEJ and SSA. Reducing the genetic variation that leads to fungicide resistance and the development of virulence genes could be a promising strategy for future pathogen control.

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Table 1. Proposed DNA DSB Repair Pathways and Targeted Genes

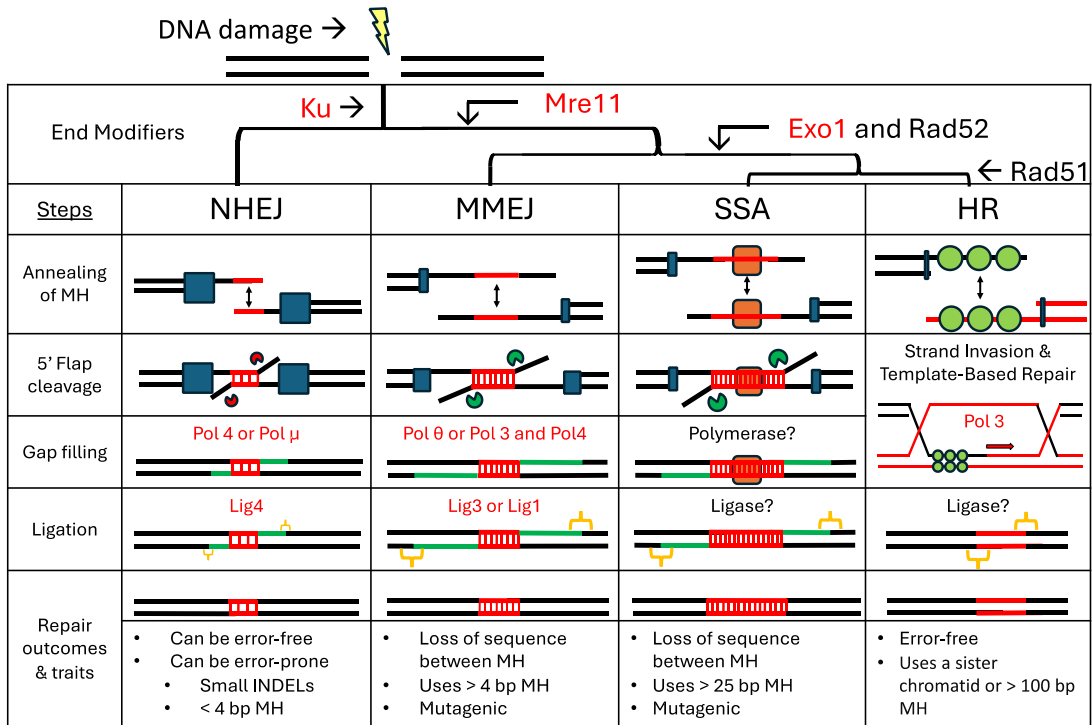
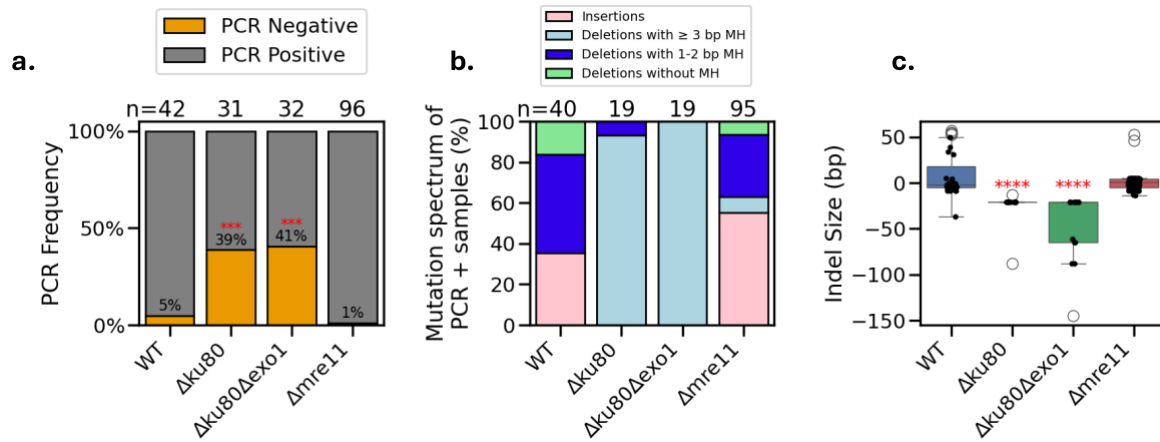


Table 2. Targeted DSB Repair Genes, Proposed Pathways, and KO Strains

Category	Protein	Mammals	<i>S. cerevisiae</i>	<i>M. oryzae</i>	Single KO	KO in $\Delta ku80$
End modification	Ku80	NHEJ			✓	
	Mre11	End-resection			✓	
	Exo1	End-resection				✓
Polymerases	Pol θ	MMEJ	No homolog			✓
	Pol 4/λ	NHEJ	NHEJ/MMEJ	NHEJ ?/ MMEJ	✓	✓
	Pol 3/δ	MMEJ/HR		?		
	Pol μ	NHEJ	No homolog	?		
Ligases	Lig1A	MMEJ		?		
	Lig1B	MMEJ		MMEJ ?	✓	
	Lig3	MMEJ	No homolog			
	Lig4	NHEJ			✓	✓

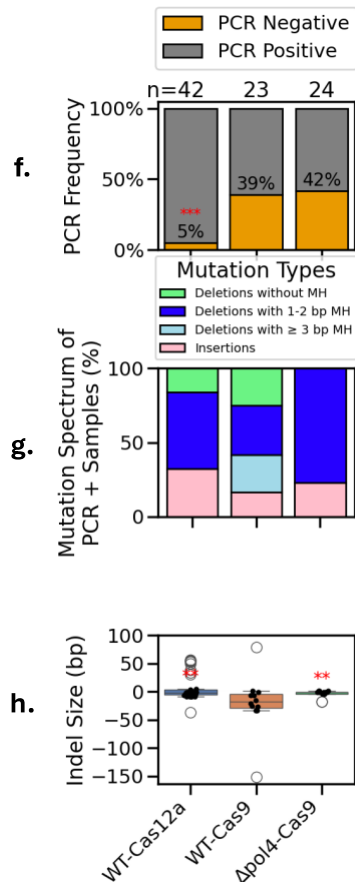


d. Cas12a FKBP12-guide2 in wild-type O-137 (reps 1-3)				INDEL Size (bp)	Repair Profile Frequency	MH
PAM Target Seq CUT TTTCTTCTTCAGGTTTCCTTCGTTGGGCAGGGGCGACTTC				WT reference sequence		
TTTCTTCTTCAGGTT-----TGGGCAGGGGCGACTTC				-37	1/37	TG
TTTCTTCTTCAGGTTTCG-----CGTGGGCAGGGGCGACTTC				-9	5/37	CG
TTTCTTCTTCAGGTTTCGAC-----TGGGCAGGGGCGACTTC				-7	1/37	T
TTTCTTCTTCAGGTTTCGACT-----GTGGGCAGGGGCGACTTC				-7	1/37	
TTTCTTCTTCAGGTTTCGACT-----GTGGGCAGGGGCGACTTC				-5	2/37	
TTTCTTCTTCAGGTTTCGA-----CGTGGGCAGGGGCGACTTC				-5	3/37	C
TTTCTTCTTCAGGTTTCG-----TTCCGTGGGCAGGGGCGACTTC				-4	2/37	
TTTCTTCTTCAGGTTTCGACT-----CGTGGGCAGGGGCGACTTC				-4	1/37	T
TTTCTTCTTCAGGTTTCGACTCT-----TGGGCAGGGGCGACTTC				-4	1/37	C
TTTCTTCTTCAGGTTTCGA--CTTCCGTGGGCAGGGGCGACTTC				-2	2/37	CT
TTTCTTCTTCAGGTTTCGACTCT--CGTGGGCAGGGGCGACTTC				-2	1/37	
TTTCTTCTTCAGGTTTCGACTC--TCCGTGGGCAGGGGCGACTTC				-1	3/37	T
TTTCTTCTTCAGGTTTCGACTCTT--CGTGGGCAGGGGCGACTTC				-1	2/37	C
TTTCTTCTTCAGGTTTCGACTCTTCCGTGGGCAGGGGCGACTTC				1	2/37	C
TTTCTTCTTCAGGTTTCGACTCTTCCGTGGGCAGGGGCGACTTC				4	1/37	CTTC
TTTCTTCTTCAGGTTTCGACTCTTCCGTGGGCAGGGGCGACTTC				5	1/37	TTCCG
TTTCTTCTTCAGGTTTCGACTCTT ⁺³¹ CGTGGGCAGGGGCGACTTC				31	1/37	
TTTCTTCTTCAGGTTTCGACTCTT ⁺³⁴ CGTGGGCAGGGGCGACTTC				34	1/37	
TTTCTTCTTCAGGTTTCGACTCTT ⁺³⁹ CGTGGGCAGGGGCGACTTC				39	1/37	
TTTCTTCTTCAGGTTTCGACTCTT ⁺⁵⁰ CGTGGGCAGGGGCGACTTC				50	1/37	
TTTCTTCTTCAGGTTTCGACTCTT ⁺⁵³ CGTGGGCAGGGGCGACTTC				53	1/37	
TTTCTTCTTCAGGTTTCGACTCTT ⁺⁵⁴ CGTGGGCAGGGGCGACTTC				54	1/37	
TTTCTTCTTCAGGTTTCGACTCTT ⁺⁵⁵ CGTGGGCAGGGGCGACTTC				55	1/37	
TTTCTTCTTCAGGTTTCGACTCTT ⁺⁵⁷ CGTGGGCAGGGGCGACTTC				57	1/37	
e. Cas12a FKBP12-guide2 in Δku80 (summary of reps 1-2)						
-----TCGGT				-88	1/15	TCGGT
TTTCTTCTT-----CAGGGGCGACTTC				-21	9/15	CAGG
TTTCTTCTTCAGGTT-----CGACTTC				-21	4/15	CGACT
TTTCTTCTTCAG-----GTGGGCAGGGGCGACTTC				-13	1/15	GT
f. Cas12a FKBP12-guide2 in Δku80Δexo1 (rep #1)						
-----CGACCC				-145	1/13	CGACCC
-----TCGGT				-88	2/13	TCGGT
-----GACTTC				-65	2/13	GACTT
-----GGGCGACTTC				-61	1/13	GGGCG
TTTCTTCTTCAGGTT-----CGACTTC				-21	3/13	CGACT
TTTCTTCTT-----CAGGGGCGACTTC				-21	4/13	CAGG

g. Cas12a FKBP12-guide2 in Δ mre11 (reps 1-3)	INDEL Size (bp)	Repair Profile Frequency	MH
PAM Target Seq CUT TTTCTTCTTCAGGTTTCGACTC TTCCG TGGGCAGGGGCGACTTC	WT reference sequence		
TTTCT-----TCTTC	-14	1/78	TCTTC
TTTCTTCTTCAG-----G	-13	1/78	GT
TTTCTTCTTCAGGTT-----CG	-9	9/78	CG
TTTCTTCTTCAGG-----TTC	-8	5/78	TTC
TTTCTTCTTCAGGT-----TTCCG	-7	2/78	T
TTTCTTCTTCAGGTTTCGAC-----T	-7	1/78	T
TTTCTTCTTCAGGTTTCGACT-----CT	-5	2/78	CT
TTTCTTCTTCAGGTTTCGA-----CCG	-5	1/78	C
TTTCTTCTTCAGGTTTCG-----TTCCG	-4	1/78	
TTTCTTCTTCAGGTTTCGA-----CTCCG	-2	6/78	CT
TTTCTTCTTCAGGTTTCGACTC-----TCCG	-1	2/78	T
TTTCTTCTTCAGGTTTCGACT-----TTCCG	-1	4/78	
TTTCTTCTTCAGGTTTCGACTCTCCCG	1	2/78	C
TTTCTTCTTCAGGTTTCGACTCTTTCCG	1	11/78	T
TTTCTTCTTCAGGTTTCGACTCTTCCCG	2	2/78	CG
TTTCTTCTTCAGGTTTCGACTCTTCCCG	2	2/78	CC
TTTCTTCTTCAGGTTTCGACTCTTTCCG	2	2/78	TT
TTTCTTCTTCAGGTTTCGACTCTTCCCTC	4	11/78	CTTC
TTTCTTCTTCAGGTTTCGACTCTTCCGTTCCG	5	11/78	TTCCG
TTTCTTCTTCAGGTTTCGACTCTT ⁺⁴⁶ CCG	46	1/78	
TTTCTTCTTCAGGTTTCGACTCTT ⁺⁵³ CCG	53	1/78	

Figure 1. FKBP12 Editing Outcomes in End Modification Knockout Strains

(a) Mutation frequencies of FKBP12 locus PCR-negative and PCR-positive results in Cas12a-edited FK506^R transformants in the wild-type O-137 (WT), Δ ku80, and Δ mre11 backgrounds. PCR analysis of FKBP12 locus detects large deletions (>600bp) resulting in PCR-negative outcomes. Data represents average results across multiple replicates. The number of independent transformants collected is shown above each bar plot. The number of independent transformants per background, x, is listed above each box plot as $n = x$. (b) The mutation frequency spectrum of PCR-positive Δ fkbp12 mutants in each background strain. (c) Size of deletions and insertions from PCR-positive Δ fkbp12 mutants in each background strain. Deletions were considered negative values, while insertions were treated as positive values. In figures a and c, the asterisks denote the level of statistical significance, where “*” indicates a p-value ≤ 0.05 , “**” indicates a p-value ≤ 0.01 , “***” indicates a p-value ≤ 0.001 , and “****” indicates a p-value ≤ 0.0001 (Mann Whitney test, two-sided). Source data are provided as sanger sequence traces received from McLabs. (d) DNA DSB repair profiles from three replicates of Cas12a FKBP12-guide2 editing in the WT O-137 background. PAM, target, and cut sequences are identified with bold text and different colors as indicated. Black triangles indicate the nuclease site for Cas12a. Letters in blue boxes highlight microhomology at the repair junction. Bolded and underlined letters indicate tandem duplications at the repair junction. The INDEL size (bp), frequency of each unique repair profile, and any microhomology used for DSB repair, is listed in the right panel. (e) DNA DSB repair profiles from two replicates of Cas12a FKBP12-guide2 editing in the Δ ku80 background. (f) DNA DSB repair profiles from one replicate of Cas12a FKBP12-guide2 editing in the Δ ku80 Δ exo1 background. (g) DNA DSB repair profiles from three replicates of Cas12a FKBP12-guide2 editing in the Δ mre11 background.



i. Cas9 FKBP12-guide1 in wild-type O-137 (rep 1)	INDEL Size (bp)	Repair Profile Freq.	MH
Target Seq CUT PAM TGGGACGAGG GGTGT CGTTTCCATGAA AGTTGGC GAGAAGGCCA	WT reference sequence		
-----	-152	1/12	TCT
TGGGACGAGGGTGTCTGTTTCCA-----	-34	1/12	TG
-----GGCGAGAAGGCCA	-33	1/12	GGC
TGGGA-----CGAGAAGGCCA	-27	1/12	CGAG
TGG-----TGGCGAGAAGGCCA	-26	1/12	
TGGGACGAGGGT-----GAGAAGGCCA	-21	1/12	G
TGGGACGAGGG-----AGTTGGCGAGAAGGCCA	-15	1/12	
TGGGACGAGGGTGTCTGTTTCCA-----TCGCGAGAAGGCCA	-7	1/12	TG
TGGGACGAGGGTGTCTGTTT-----AAGTTGGCGAGAAGGCCA	-6	1/12	
TGGGACGAGGGTGTCTGTTTCCATG-----AAGTTGGCGAGAAGGCCA	-1	1/12	A
TGGGACGAGGGTGTCTGTTTCCATGA A AGTTGGCGAGAAGGCCA	1	1/12	A
TGGGACGAGGGTGTCTGTTTCCATGA A ⁺⁷⁹ GTTGGCGAGAAGGCCA	79	1/12	
j. Cas9 FKBP12-guide1 in Δ <i>pol4</i> (rep 1)			
TGGGACGAGGG-----TCGCGAGAAGGCCA	-18	1/13	TG
TGGGACGAGGGTGT-----CCAGAAGGCCA	-18	1/13	CG
TGGGACGAGGGTGTCTGTTTCC-----AAGTTGGCGAGAAGGCCA	-4	2/13	A
TGGGACGAGGGTGTCTGTTTCCATG-----AAGTTGGCGAGAAGGCCA	-1	6/13	A
TGGGACGAGGGTGTCTGTTTCCATGA A AAGTTGGCGAGAAGGCC	1	3/13	A

Figure 2. FKBP12 Editing Outcomes in Polymerase Knockout Strains

(a) Mutation frequencies of FKBP12 locus PCR-negative and PCR-positive results in Cas12a-edited FK506^R transformants in the $\Delta ku80\Delta polq$, and $\Delta pol4$ backgrounds. PCR analysis of FKBP12 locus detects large deletions (>600bp) resulting in PCR-negative outcomes. Data represents average results across multiple replicates. The number of independent transformants collected is shown above each bar plot. The number of independent transformants per background, x , is listed above each box plot as $n = x$. (b) The mutation frequency spectrum of PCR-positive $\Delta fkbp12$ mutants in each background strain. (c) Size of deletions and insertions from PCR-positive $\Delta fkbp12$ mutants in each background. Deletions were considered negative values, while insertions were treated as positive values. In figures a and c, the asterisks denote the level of statistical significance, where “*” indicates a p-value ≤ 0.05 , “**” indicates a p-value ≤ 0.01 , “***” indicates a p-value ≤ 0.001 , and “****” indicates a p-value ≤ 0.0001 . Source data are provided as sanger sequence traces received from McLabs. (d) Sanger sequencing results used for DSB repair profile analysis from Cas12a FKBP12-guide2 editing in the $\Delta ku80\Delta polq$ background. PAM and target sequences are identified with bold text and different colors as indicated. Black triangles indicate the nuclease site for Cas12a. Letters in blue boxes highlight microhomology at the repair junction. Bolded and underlined letters indicate tandem duplications at the repair junction. The INDEL size (bp), frequency of each unique repair profile, and any microhomology used for DSB repair, is listed in the right panel. (e) Sanger sequencing results used for DSB repair profile analysis from Cas12a FKBP12-guide1 editing in the $\Delta pol4$ background. (f) Mutation frequencies of FKBP12 locus PCR-negative and PCR-positive results in Cas9-edited FK506^R transformants in the WT O-137 and $\Delta pol4$ backgrounds. (g) The mutation frequency spectrum of PCR-positive $\Delta fkbp12$ mutants in each background strain. (h) Size of deletions and insertions from PCR-positive $\Delta fkbp12$ mutants in each background. Deletions were considered negative values, while insertions were treated as positive values. (i) Sanger sequencing results used for DSB repair profile analysis from Cas9 FKBP12-guide1 editing in the WT O-137 background. (j) Sanger sequencing results used for DSB repair profile analysis from Cas9 FKBP12-guide1 editing in the $\Delta pol4$ background.

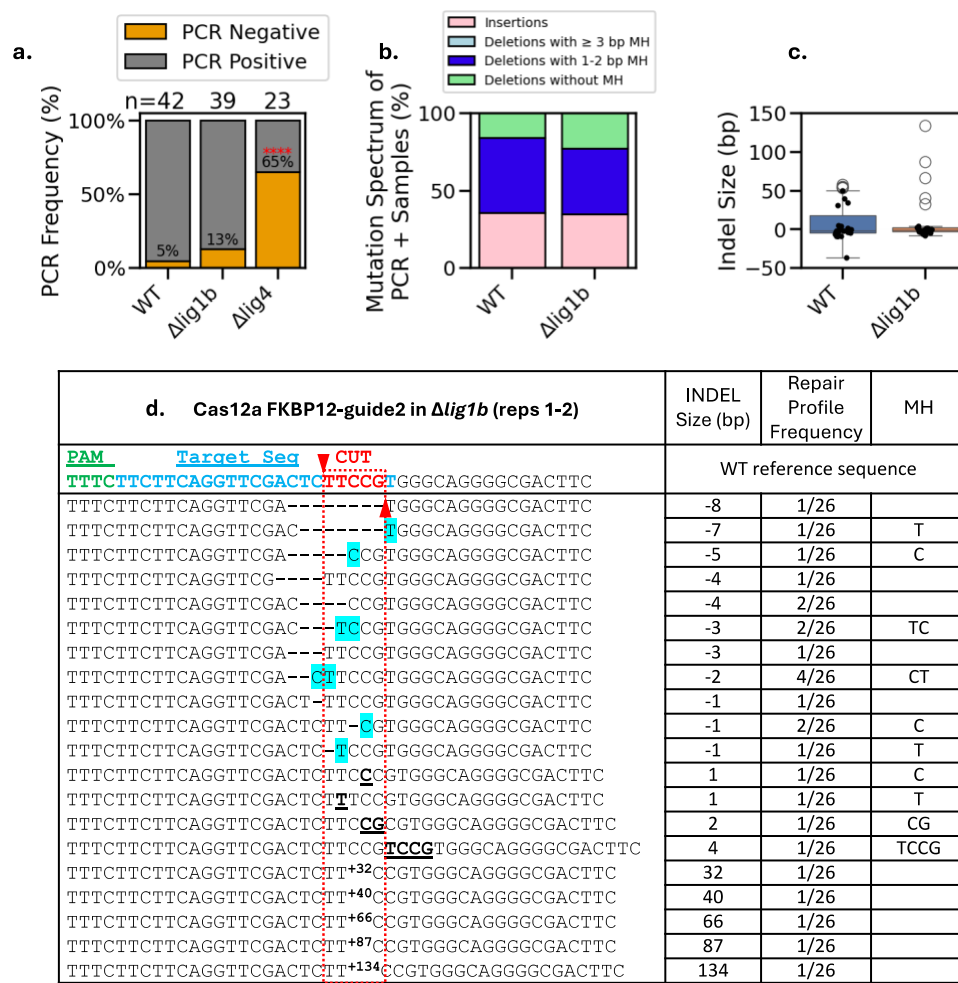


Figure 3. FKBP12 Editing Outcomes in Ligase Knockout Strains

(a) Mutation frequencies of FKBP12 locus PCR-negative and PCR-positive results in Cas12a-edited FK506^R transformants in the wild-type O-137, *Δlig1b*, and *Δlig4* backgrounds. PCR analysis of FKBP12 locus detects large deletions (>600bp) resulting in PCR-negative outcomes. Data represents average results across multiple replicates. The number of independent transformants collected is shown above each bar plot. The number of independent transformants per background, *x*, is listed above each box plot as *n* = *x*.

(b) The mutation frequency spectrum of PCR-positive *Δfkbp12* mutants in each background strain. (c) Size of deletions and insertions from PCR-positive *Δfkbp12* mutants in each background. Deletions were considered negative values, while insertions were treated as positive values. In figures a and c, the asterisks denote the level of statistical significance, where “*” indicates a *p*-value ≤ 0.05, “**” indicates a *p*-value ≤ 0.01, “***” indicates a *p*-value ≤ 0.001, and “****” indicates a *p*-value ≤ 0.0001 (Mann Whitney test, two-sided). Source data are available as sanger sequence traces received from McLabs. (d) Sanger sequencing results used for DSB repair profile analysis from Cas12a FKBP12-guide2 editing in the *Δlig1b* background. PAM, target, and cut sequences are identified with bold text and different colors as indicated. Black triangles indicate the nuclease site for Cas12a. Letters in blue boxes highlight microhomology at the repair junction. Bolded and underlined letters indicate tandem duplications at the repair junction. The INDEL size (bp), frequency of each unique repair profile, and any microhomology used for DSB repair, is listed in the right panel.

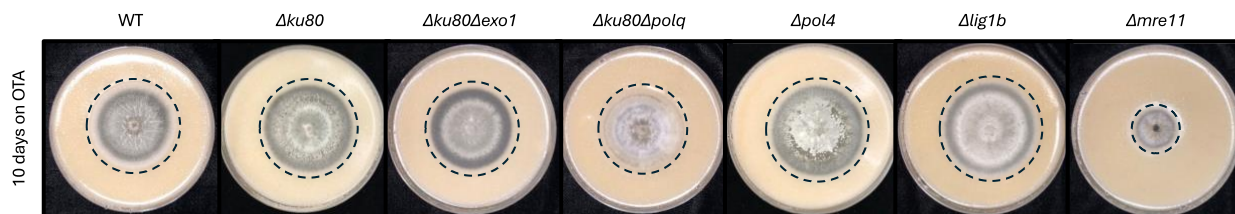


Figure 4. Mre11 is required for normal in vitro fungal growth

This figure shows the radial growth of different KO strains compared to WT on CM plates. *Δmre11* exhibits noticeably reduced growth, indicating the critical role of Mre11 in normal fungal growth. Data was collected by transferring two 5 mm diameter plugs from 10-day-old OTA plates to individual OTA plates, which were then grown for an additional 12 days. Growth was documented with photographs taken on days 5 and 10.

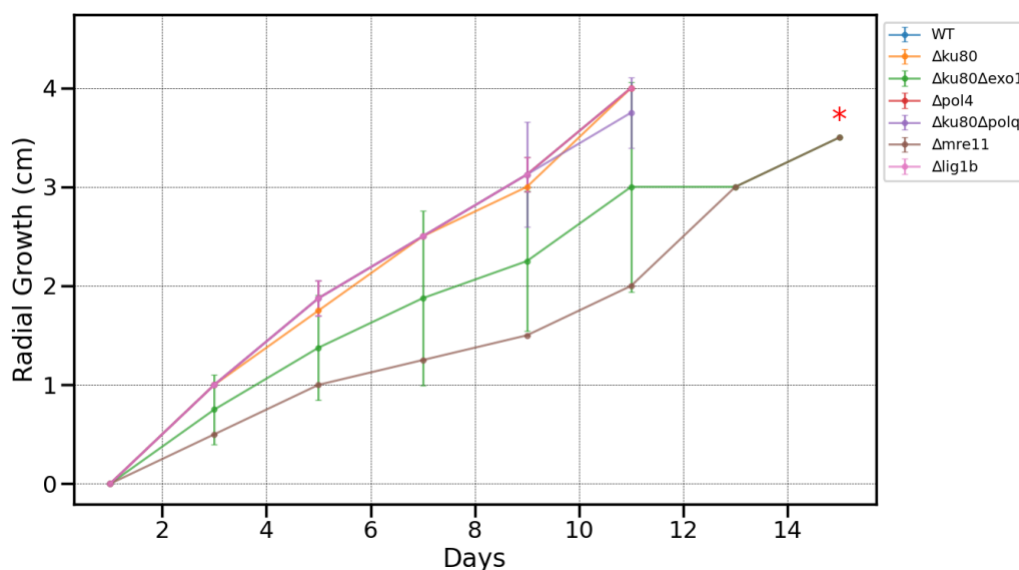


Figure 5. Significantly slower growth rate consistently observed in *Δmre11*

The radial growth rates over a period of 14 days were measured for various strains in two independent replicates, then plotted. *Δmre11* and *Δku80Δexo1* show reduced growth rates compared to WT, highlighting their growth deficiencies. Data was collected by measuring radial growth with a ruler every other day for 12 days or until the colonies covered the plates. These measurements were then plotted to quantify growth rates. Error bars indicate variance in observed growth rates between replicates. The asterisk denotes the level of statistical significance, where “*” indicates a p-value ≤ 0.05 (Mann Whitney test, two-sided).

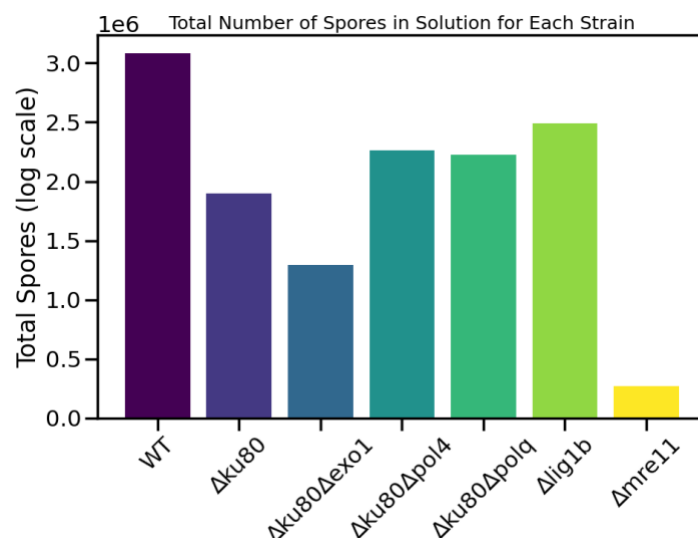


Figure 6. Conidia Production Decreases in $\Delta mre11$

This bar graph illustrates the total number of spores produced by each strain. $\Delta mre11$ shows a severe reduction in spore production, down to approximately 9% of WT levels, demonstrating its crucial role in growth and conidiation. A single replicate of data was collected by harvesting spores with 1 mL of 0.25% gelatin solution from 12-day-old OTA plates and counting them using a hemocytometer.

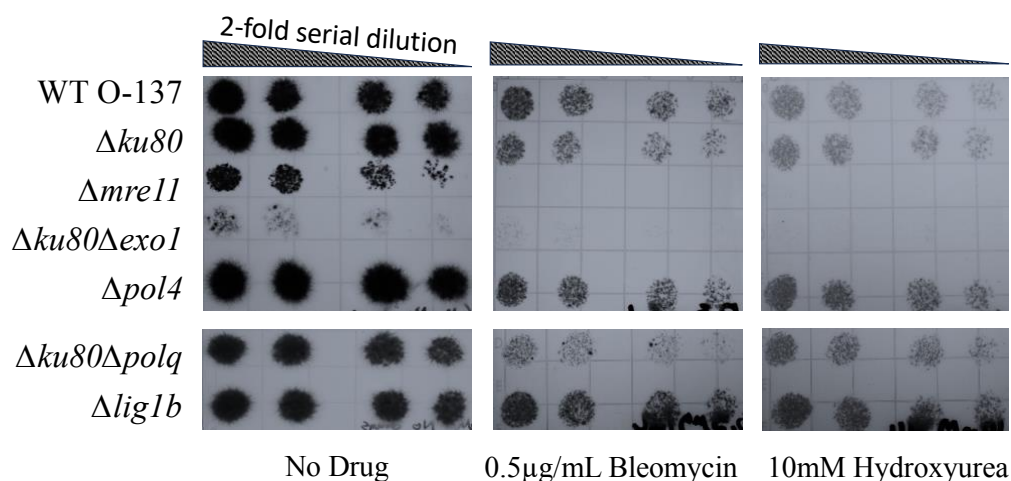


Figure 7. Hypersensitivity to Genotoxins Observed in $\Delta mre11$

Growth of different strains under no drug, low (0.5 μg/mL) bleomycin, and high (10 mM) hydroxyurea treatments. $\Delta mre11$ exhibits hypersensitivity to genotoxic agents, indicating its essential role in DNA DSB repair during the G1/S and G2/M phases. Data was collected by performing a two-fold serial dilution of conidia suspended in 0.25% glycerol, plating them on CM plates with and without genotoxins, incubating for 3 days at 28°C in the dark, and then photographing the growth for further quantitative analysis of genotoxin sensitivity results.

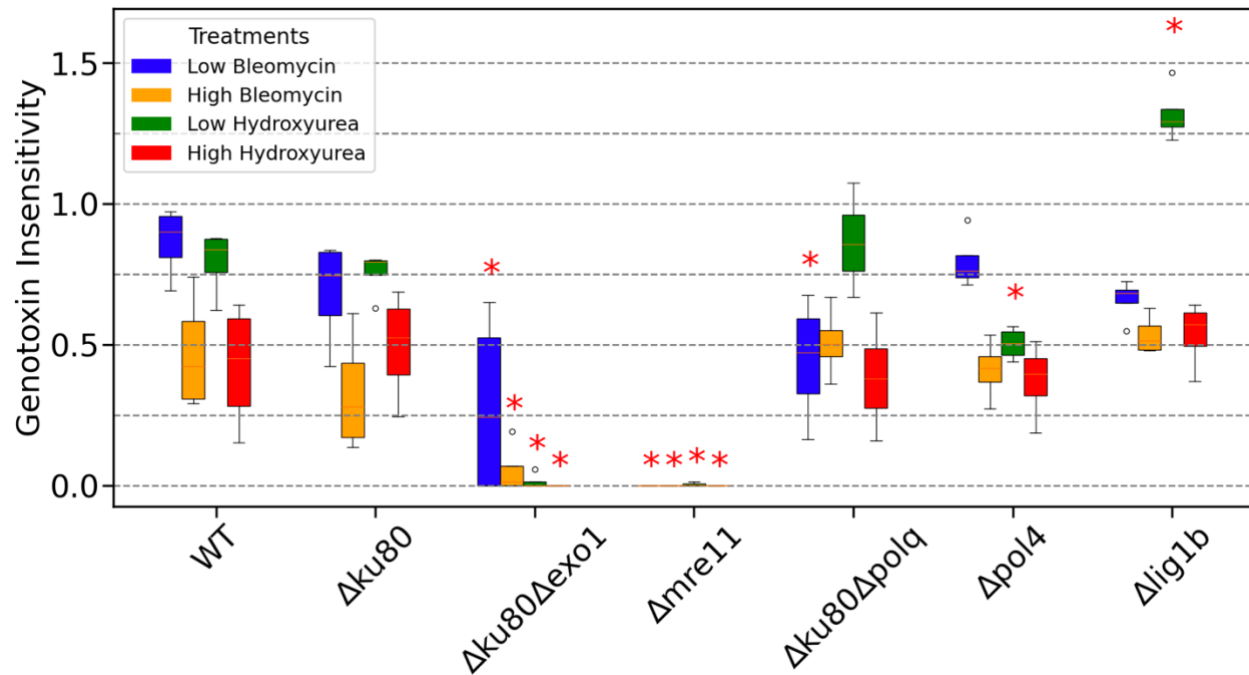


Figure 8. Quantified Genotoxin Sensitivity Results

The genotoxin sensitivity to low and high bleomycin (0.25 and 0.5 $\mu\text{g/mL}$ respectively) and hydroxyurea (5 and 10 mM respectively) treatments was calculated for each strain by dividing the average pixel density of serially diluted colonies grown on media containing genotoxins, by the average pixel density when serially diluted colonies were grown on media without genotoxins. A genotoxin insensitivity value of 1 indicates insensitivity, whereas a value of 0 indicates genotoxin sensitivity. The asterisks denote the level of statistical significance as indicated by Mann, where “*” indicates a p-value ≤ 0.05 , “**” indicates a p-value ≤ 0.01 , “***” indicates a p-value ≤ 0.001 , and “****” indicates a p-value ≤ 0.0001 (Mann Whitney test, two-sided).

Glossary of Terms

BAR (Bialaphos Resistance): A gene that provides resistance to the herbicide bialaphos, used as a selectable marker in genetic engineering experiments.

BLAST (Basic Local Alignment Search Tool): A bioinformatics tool used to compare an input sequence against a database of sequences to find regions of similarity.

Cas9: A specific Cas protein used in CRISPR gene editing to create precise blunt-ended cuts in DNA.

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats): A genome editing tool that uses a Cas endonuclease, guided by a single-guide RNA, to target DNA sequences near a PAM site and introduce precise double-strand breaks for targeted genetic modifications.

DSB (Double-Strand Break): A type of DNA damage where both strands of the DNA double helix are broken, often leading to mutations if not repaired properly.

End-Resection: The process of trimming the ends of a DNA double-strand break to create single-stranded DNA overhangs, which are necessary for certain repair mechanisms.

Exo1 (Exonuclease 1): A nuclease involved in long-range DNA end resection during homologous recombination (HR) and microhomology-mediated end joining (MMEJ).

FKBP12 (FK506 Binding Protein 12): A small protein that binds to immunosuppressant drugs like FK506 and rapamycin, involved in the TOR signaling pathway. It is used in genetic research as a selectable marker to confer drug resistance in transformed cells.

gRNA (Guide RNA): An RNA molecule that guides the Cas protein to a specific location in the DNA sequence for precise editing.

HR (Homologous Recombination): A DNA repair pathway that uses a homologous sequence as a template to accurately repair a double-strand break.

Ku80: A protein that is part of the Ku heterodimer, which plays a key role in the non-homologous end joining (NHEJ) repair pathway by protecting DNA ends from degradation.

LbCas12a: A specific Cas protein used in CRISPR gene editing to create precise staggered cuts with 5' overhangs in DNA.

Lig4 (DNA Ligase IV): A ligase enzyme crucial for the NHEJ pathway, responsible for ligating the ends of double-strand breaks without a homologous template.

MH (Microhomology): Short sequences of DNA (typically 2-20 base pairs) that are identical or nearly identical on both sides of a double-strand break. These sequences are used during

microhomology-mediated end joining (MMEJ) to align the DNA ends before they are joined together.

MMEJ (Microhomology-Mediated End Joining): An error-prone DNA repair pathway that uses small regions of microhomology (short, identical sequences) to align and repair DNA double-strand breaks.

MRN Complex: A protein complex composed of Mre11, Rad50, and Nbs1, which is involved in the detection and processing of DNA double-strand breaks.

Mre11: A key protein in the MRN complex with endonuclease and exonuclease activities, essential for DNA end resection and initiating DNA repair processes.

NHEJ (Non-Homologous End Joining): A DNA repair pathway that directly ligates the broken DNA ends without the need for a homologous template, often resulting in small insertions or deletions.

PAM (Protospacer Adjacent Motif): A short DNA sequence required for the Cas12a protein to bind and cut DNA in CRISPR-based gene editing.

Pol θ (Polymerase Theta): A DNA polymerase involved in the error-prone repair of double-strand breaks through the MMEJ pathway.

Pol4 (Pol IV): A polymerase in *Magnaporthe oryzae* proposed to play a role in MMEJ, potentially involved in gap filling during DNA repair.

Pol μ (Polymerase Mu): A DNA polymerase involved in the NHEJ pathway, especially in gap filling and creating microhomologies during DNA repair.

Protospacer Adjacent Motif (PAM): A specific DNA sequence that must be present adjacent to the target site for the CRISPR-Cas system to function. “NGG” for Cas9 and “TTTV” for LbCas12a, where “V” is any base other than “T”.

RNP (Ribonucleoprotein): A complex consisting of RNA and protein. In CRISPR-Cas systems, the RNP complex typically refers to the combination of the guide RNA (gRNA) and the Cas protein, which together facilitate targeted DNA editing.

RPA (Replication Protein A): A protein complex that binds to single-stranded DNA during replication and repair, protecting it from degradation and preventing annealing of microhomology.

SSA (Single-Strand Annealing): A relatively mutagenic DNA repair pathway that repairs double-strand breaks using large stretches of homology such as transposable elements, often resulting in large deletions.

Tandem Duplication: A type of mutation where a segment of DNA is duplicated and the copies are adjacent to each other in the genome.

T7 Promoter: A specific DNA sequence recognized by T7 RNA polymerase, used to initiate transcription in in vitro RNA synthesis.

Masters Report
Supplemental Information
Tomas McAnany
PCR Genotyping to Confirm KOs

General gene deletion KO overview

- Refer to materials and methods for full details.
- All gene deletion mutants were made using two sgRNAs flanking the gene to be deleted.
- Deletion mutants were made using RNPs as previously described (Huang, 2021, Star Protocols) using either Cas12 or Cas9. The method for each gene is labeled in the slides and guide sequence.

KO Overview

Only one band is needed to confirm gene KO

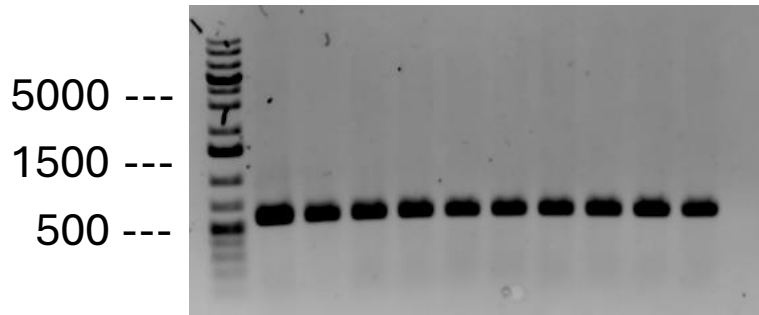


Target Gene	Transformation Date	Actin	Gene Specific	DonorUP	DonorDown	Across Target Gene	Singlesporing
Lig1A		Complete	WT band	NOT COMPLETED	NOT COMPLETED	NOT COMPLETED	NOT COMPLETED
Lig1B	6/7/2022	Complete	Complete	Complete	Complete	Not needed	Complete
Exo1 in $\Delta ku80$	3/3/2023	Complete	Complete	Complete	Not needed	Not needed	Complete
Mre11	3/3/2023	Complete	Complete	Complete	Not needed	Not needed	Complete
Pol4	1/11/2024	Complete	Complete	Not needed	Not needed	Complete	Complete
Lig4	1/11/2024	Complete	Complete	Not needed	Not needed	Complete	Complete
Lig4 in $\Delta ku80$	12/03/2023	Complete	Complete	Not needed	Not needed	Complete	Complete
PolQ	1/11/2024	Complete	WT band	NOT COMPLETED	NOT COMPLETED	NOT COMPLETED	NOT COMPLETED
PolQ in $\Delta ku80$	10/21/2022	Complete	Complete	Not complete	Not complete	Not complete	Complete
Pol3	3/2/2024	Complete	Complete	NOT COMPLETED	NOT COMPLETED	NOT COMPLETED	NOT COMPLETED
Pol μ	3/2/2024	Complete	WT band	NOT COMPLETED	NOT COMPLETED	NOT COMPLETED	NOT COMPLETED
Rad51 in $\Delta ku80$	3/3/2023	Complete	Complete	Complete	Complete	Not needed	Complete

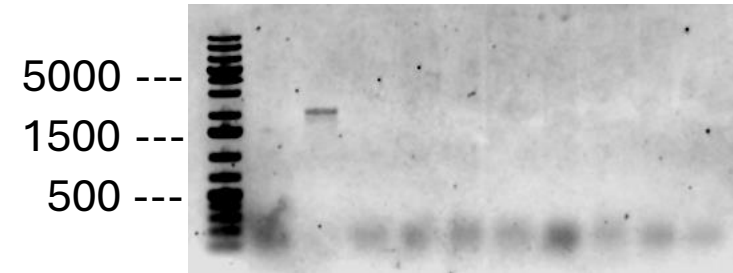
Supplemental Figure 1. PCR results for *Lig1A* editing

- 10 putative transformants were screened for *Lig1A* gene deletion.
- Transformation completed on 7/24/22

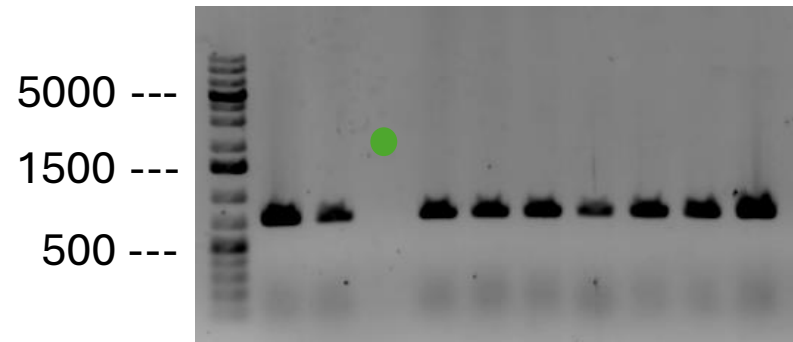
Actin Amplicon: 574 bp



Lig1A-Up Amplicon: ~2100 bp

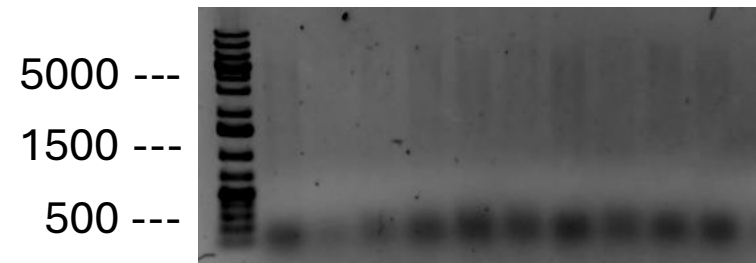


Lig1A Gene Specific Amplicon: 762 bp



● Putative *Lig1A* deletion mutation

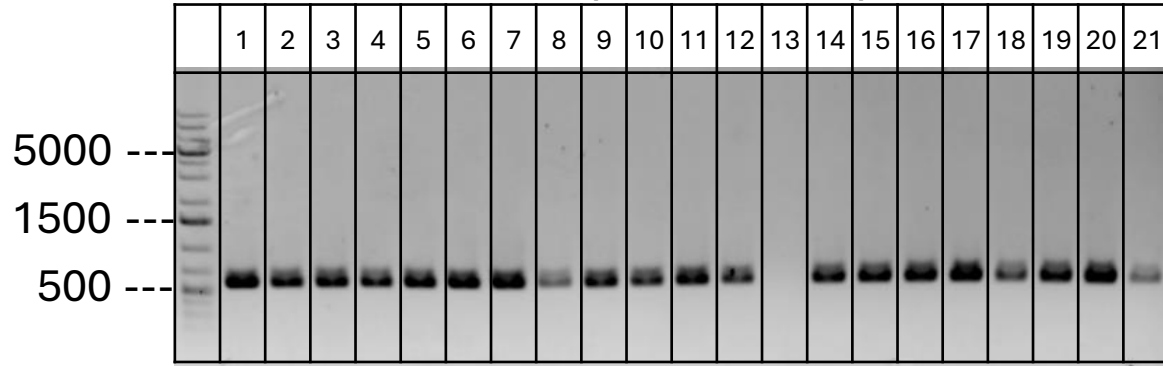
Lig1A-Down Amplicon: 157 bp



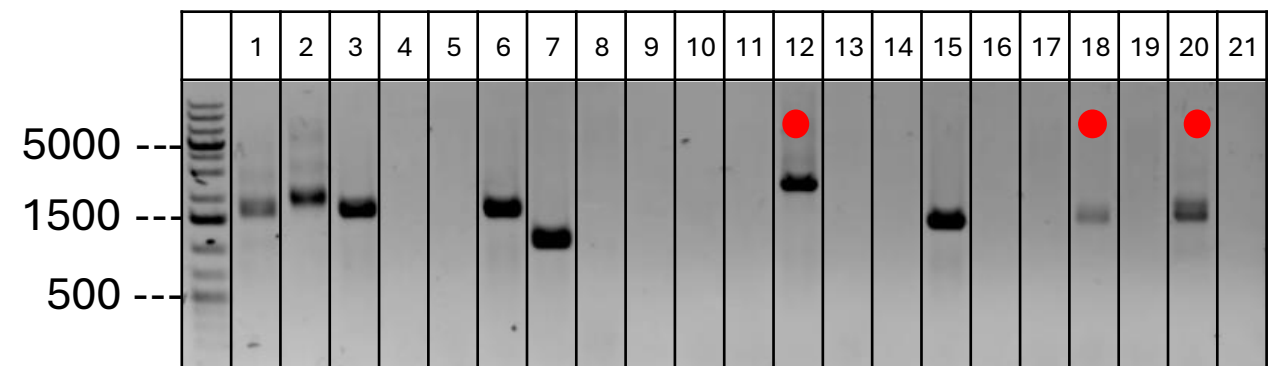
Supplemental Figure 2. PCR results for *Lig1B* editing

- 21 putative transformants were screened for *Lig1B* gene deletion.
- Transformation completed on 6/7/2022

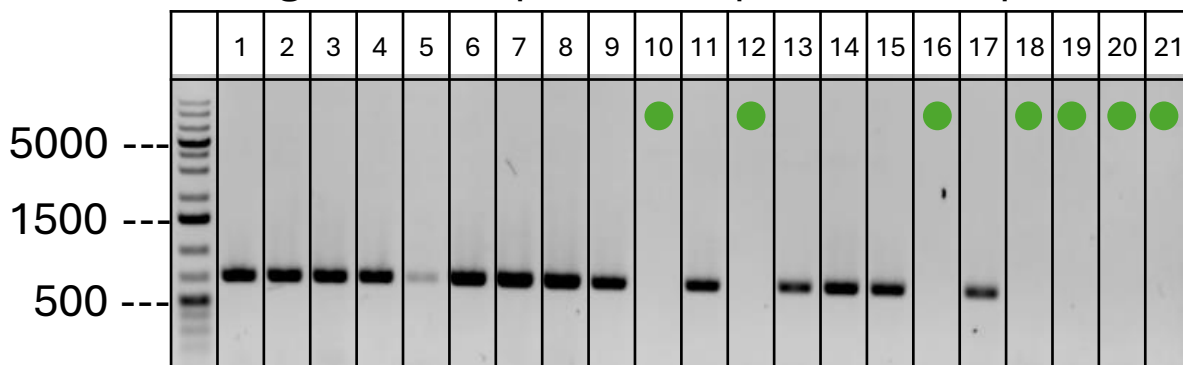
Actin Amplicon: 574 bp



Lig1B G418-Up Amplicon: 1441 bp

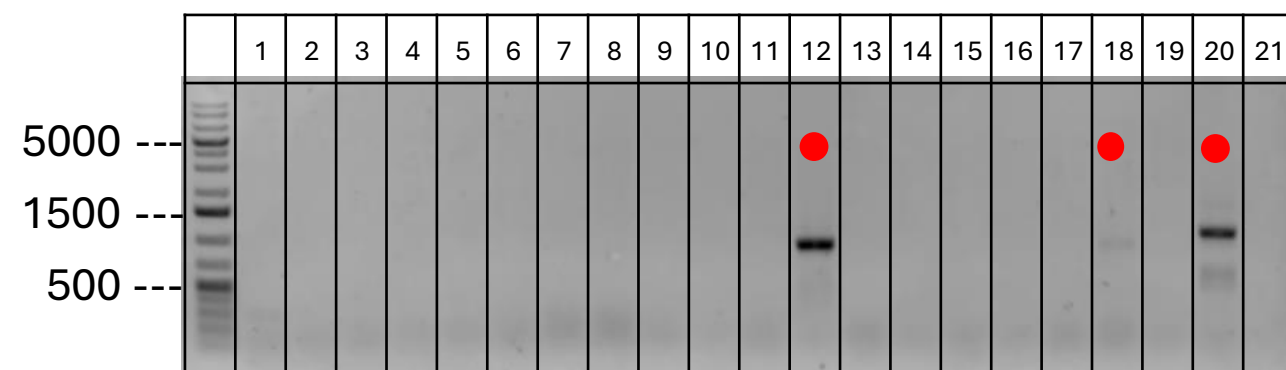


Lig1B Gene Specific Amplicon: 762 bp



● Putative *Lig1B* deletion mutation

Lig1B G418-Down Amplicon: 870 bp

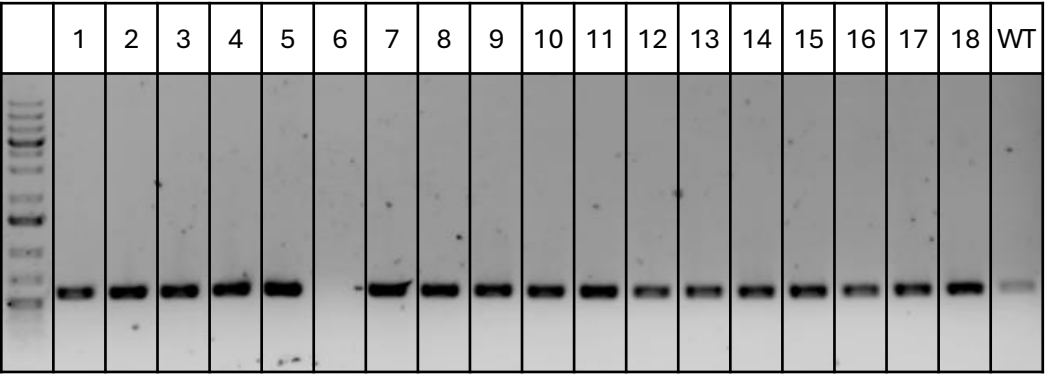


● Selected as $\Delta lig1b$ mutants for further analysis

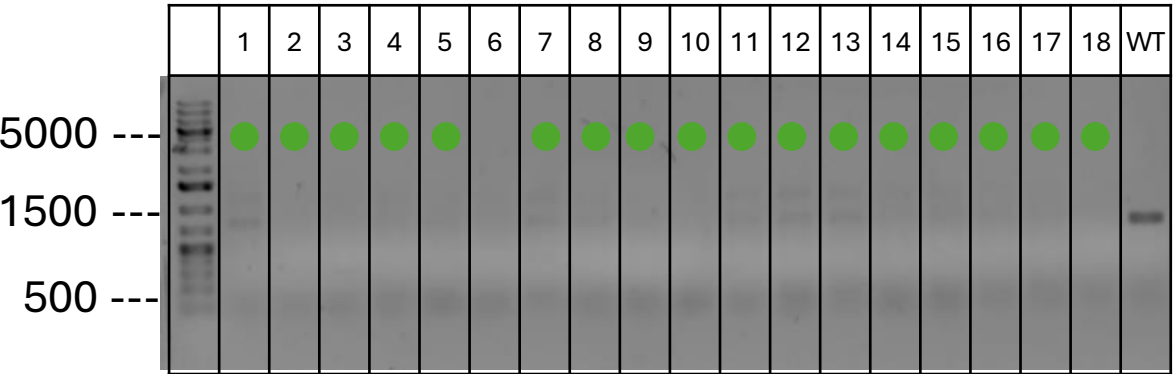
Supplemental Figure 3. PCR results for *Exo1* editing in $\Delta ku80$ back

- 18 putative transformants were screened for *Exo1* gene deletion in $\Delta ku80$ background
- Transformation completed on 3/3/2023

Actin Amplicon: 574 bp

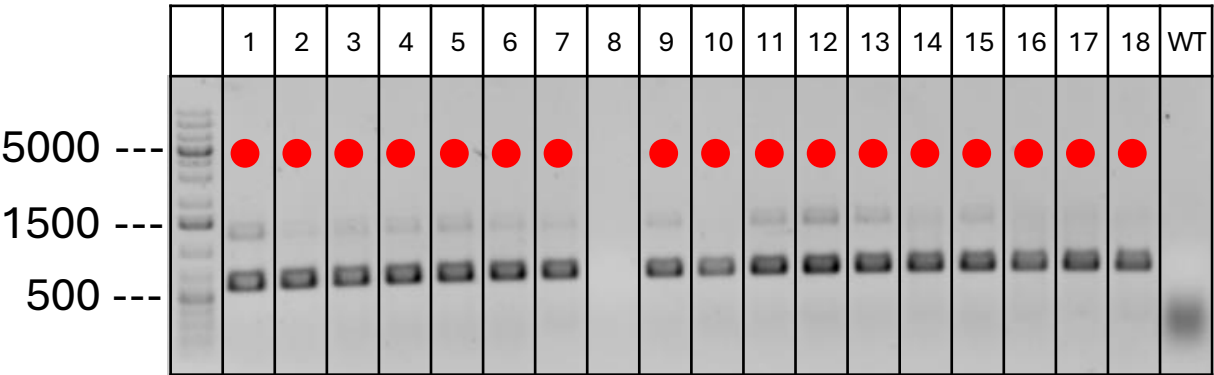


Exo1 Gene Specific Amplicon: 646 bp



● Putative *Exo1* deletion mutation

Exo1 BAR-Up Amplicon: 606 bp

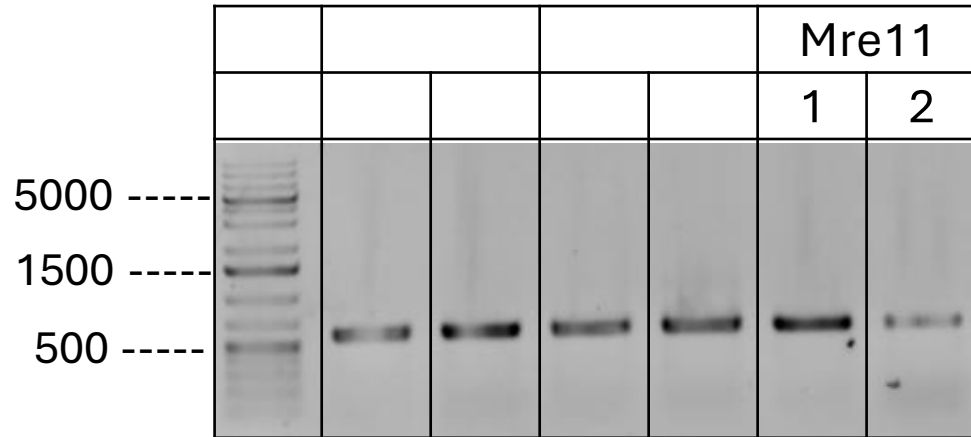


● Selected as $\Delta ku80 \Delta exo1$ mutants for further analysis

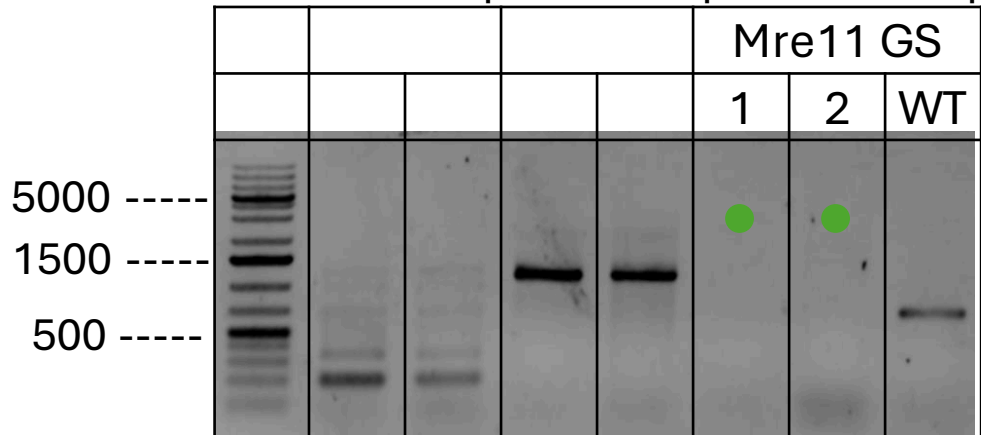
Supplemental Figure 4. PCR results for *Mre11* editing

- 2 putative transformants were screened for *Mre11* gene deletion.
- Transformation completed on 3/3/2023

Actin Amplicon: 574 bp

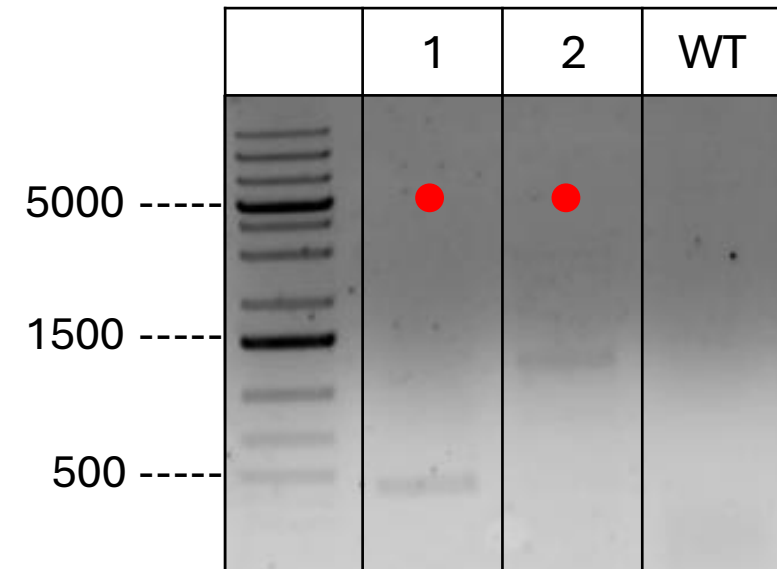


Mre11 Gene Specific Amplicon: 686 bp



● Putative *Mre11* deletion mutation

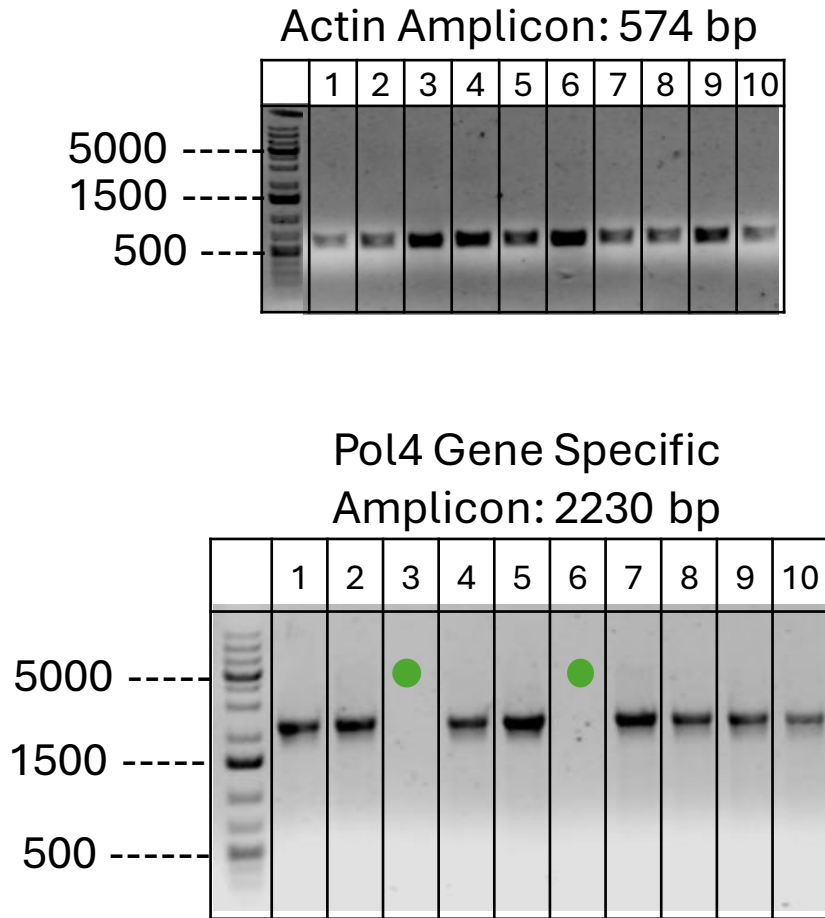
Mre11 BAR-Up Amplicon: 434 bp



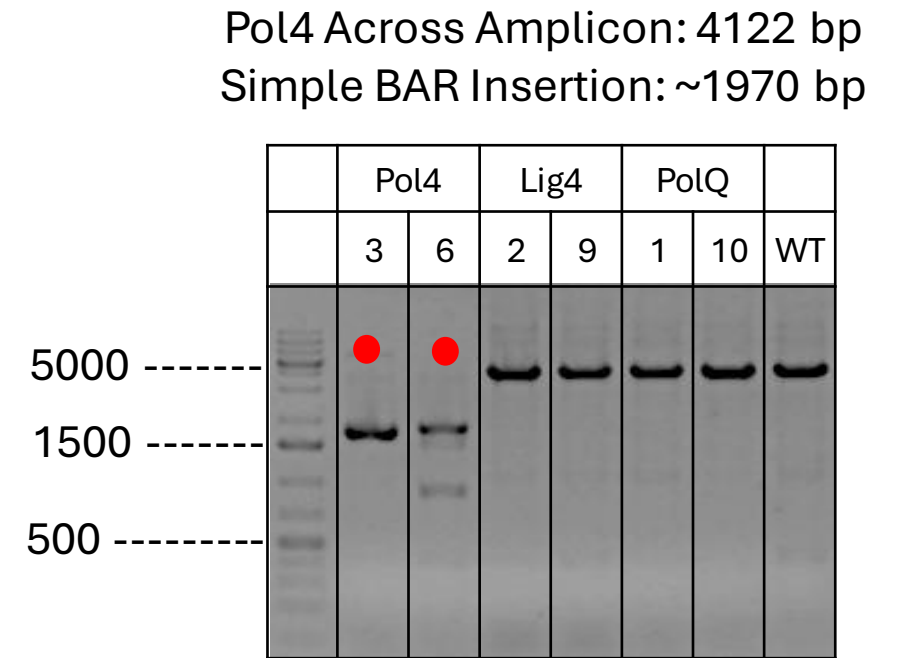
● Selected as $\Delta mre11$ mutants for further analysis

Supplemental Figure 5. PCR results for *Pol4* editing

- Transformation completed on 1/11/2024
- 10 putative transformants were screened for *Pol4* gene deletion.



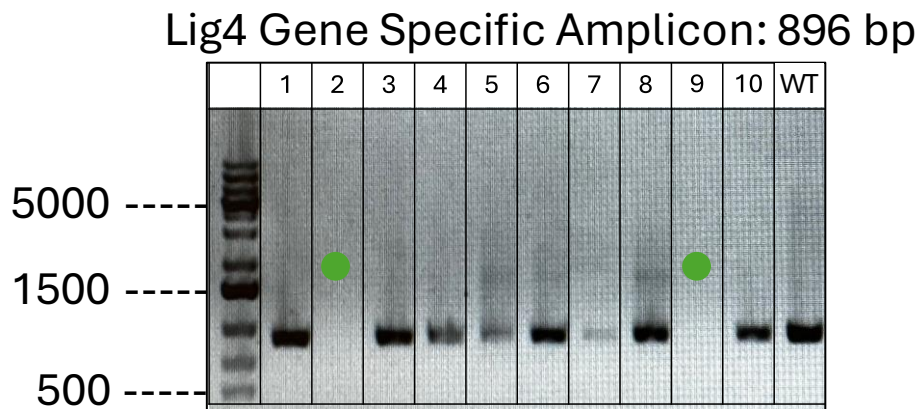
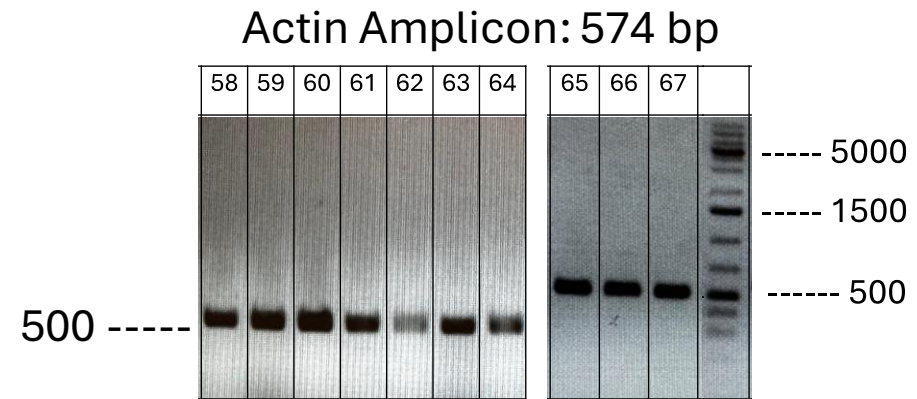
● Putative *Pol4* deletion mutation



● Selected as $\Delta pol4$ mutants for further analysis

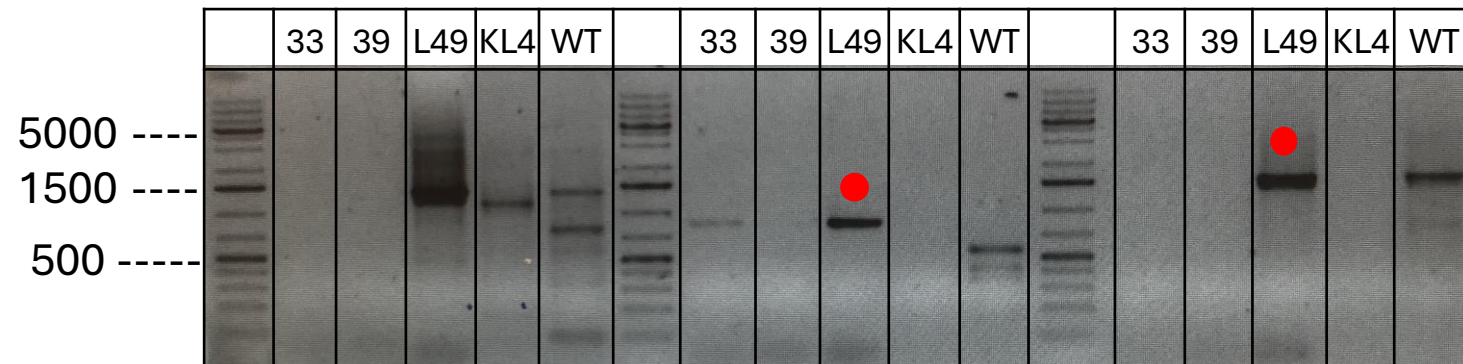
Supplemental Figure 6. PCR results for *Lig4* editing

- 10 putative transformants were screened for *Lig4* gene deletion.
- Transformation completed on 1/11/2024



● Putative *Lig4* deletion mutation

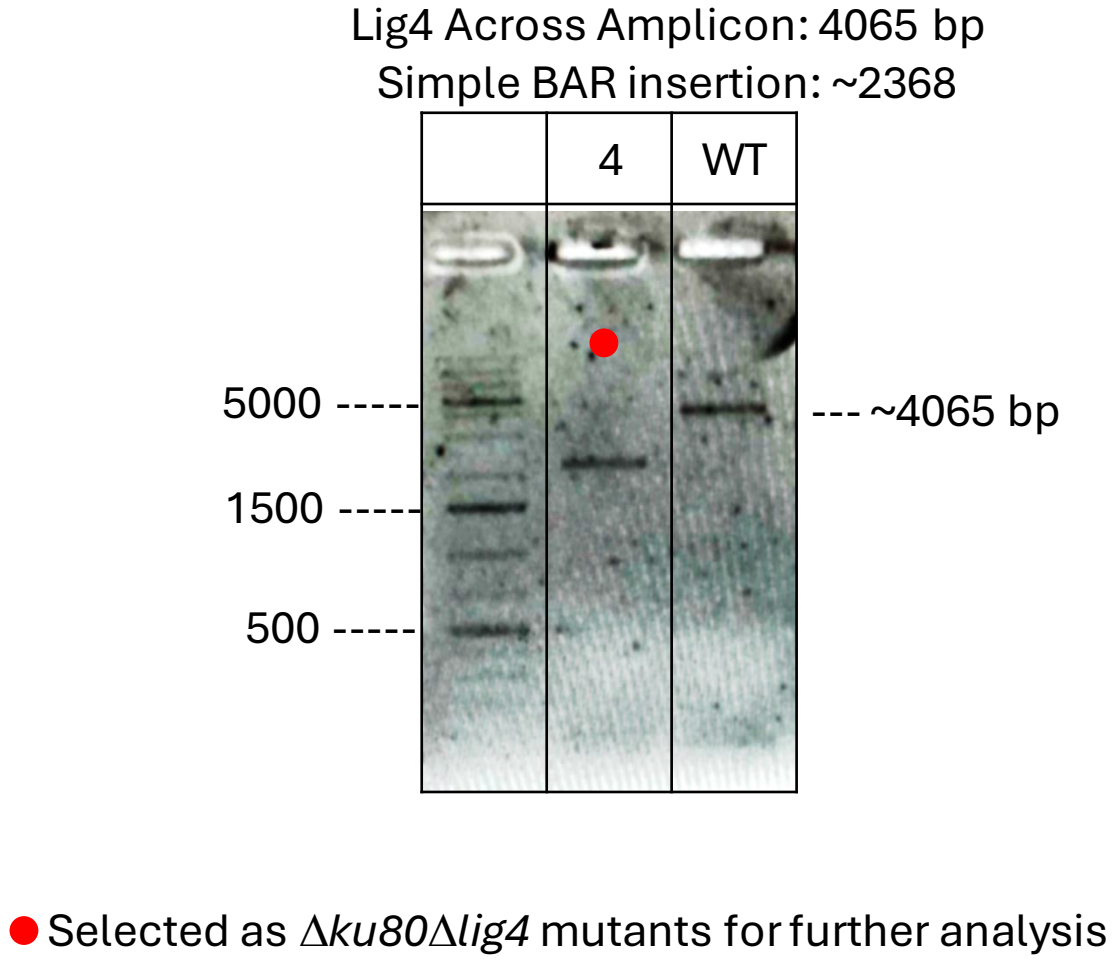
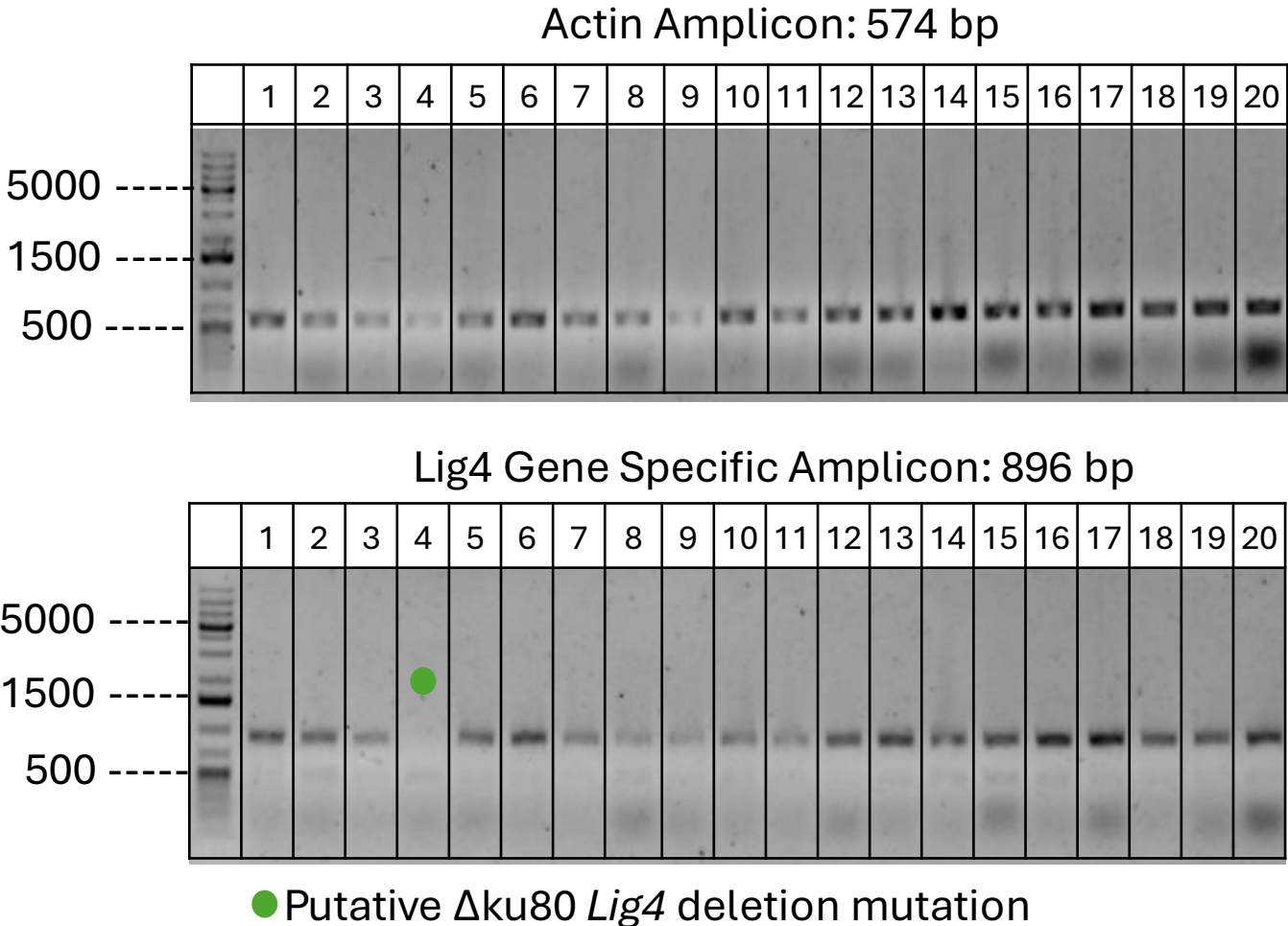
Lig4-BAR Up inverted Amplicon: ~1371 bp
Lig4-BAR Down inverted Amplicon: ~876 bp
Lig4-Across (BAR-F Up/R Down) WT Amplicon: 4068 bp
Lig4-Across (BAR-F Up/R Down) Simple BAR Insertion: ~2371 bp



● Selected as Δ *lig4* mutants for further analysis

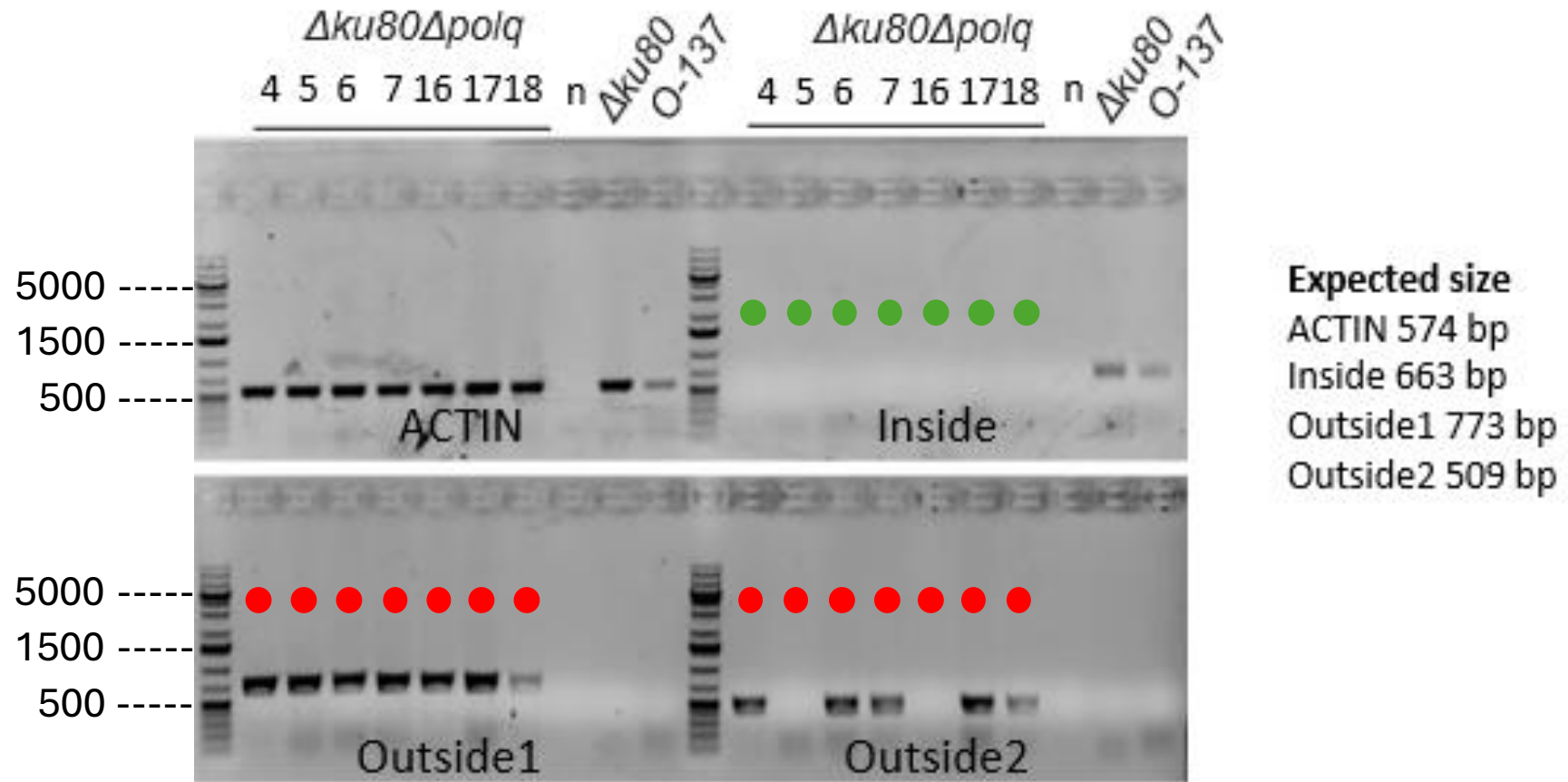
Supplemental Figure 7. PCR results for *Lig4* editing in $\Delta ku80$ background

- 23 putative transformants were screened for *Lig4* gene deletion in the $\Delta ku80$.
- Transformation completed on 12/03/23



Supplemental Figure 9. PCR results for *PolQ* editing in $\Delta ku80$ background

- 18 putative transformants were screened for *PolQ* gene deletion in $\Delta ku80$ background.
- Transformation completed on 10/21/2022
- Jun performed the PCR reactions below on 11/16/2022



● Putative *PolQ* deletion mutation

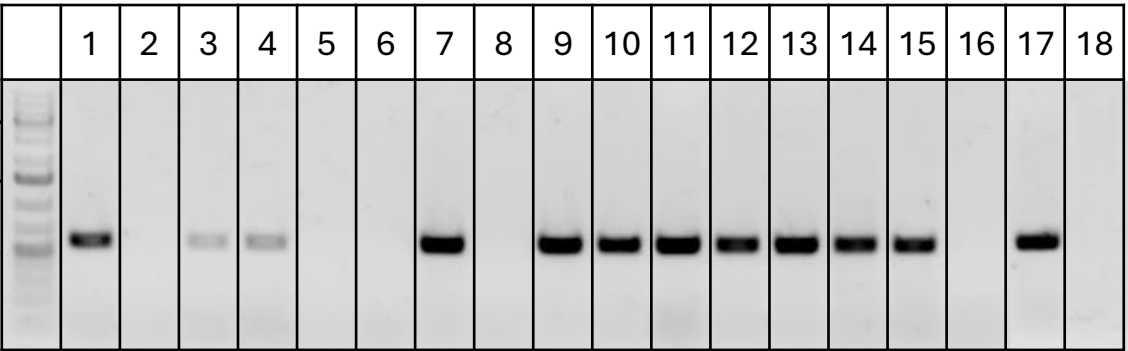
● Selected as $\Delta ku80\Delta polq$ mutants for further analysis

Supplemental Figure 10. PCR results for *Pol3* editing

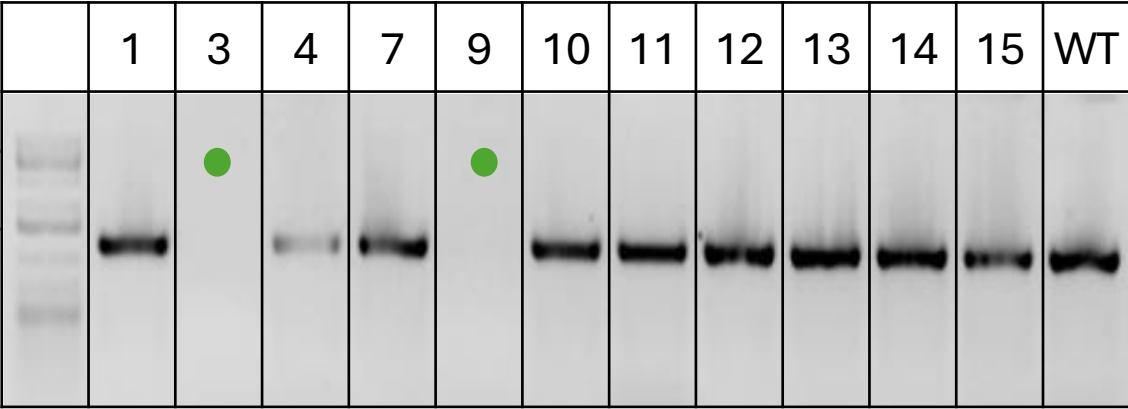
- 18 putative transformants were screened for *Pol3* gene deletion.
- Transformation completed on 3/2/2024

Actin PCR

Expected: 574 bp



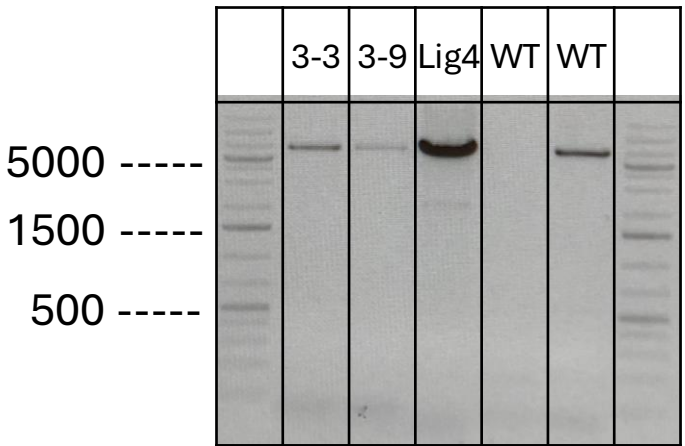
Pol3 Gene Specific Amplicon: 1172 bp



● Putative *Pol3* deletion mutation

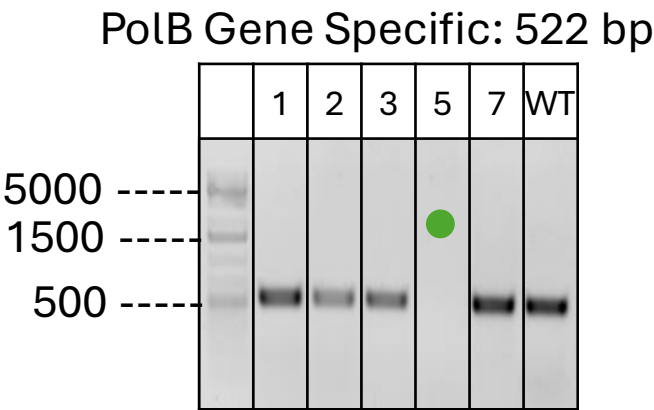
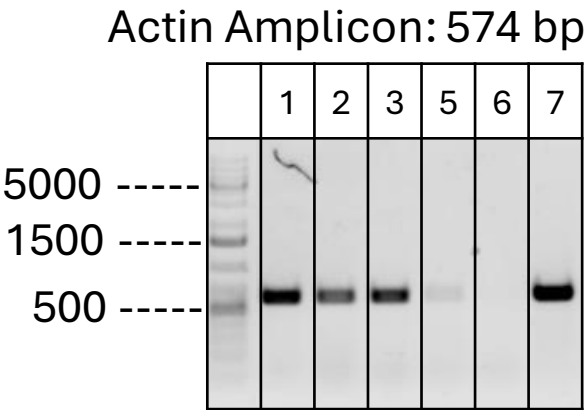
Pol3- Across: ~6300 bp

Pol3-Across: ~3400 bp for simple BAR ins.

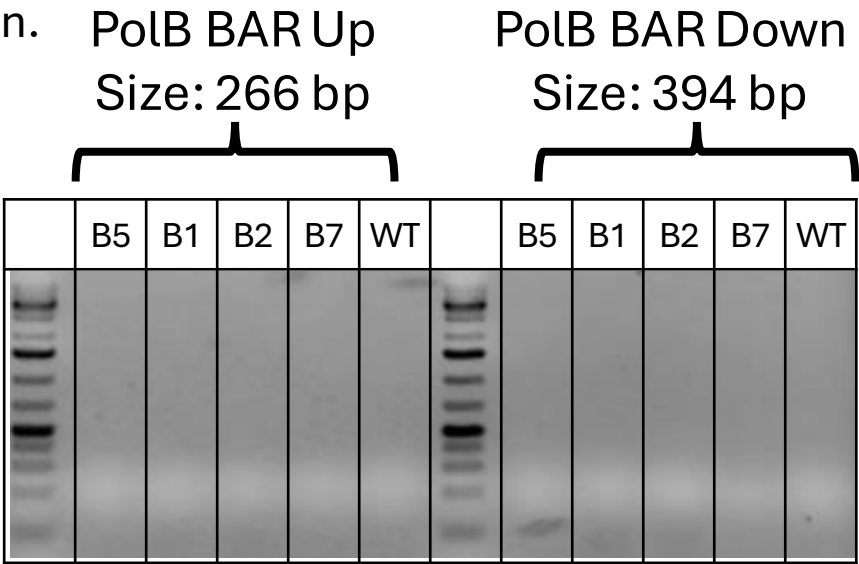


Supplemental Figure 11. PCR results for *Polμ* editing

- 6 putative transformants were screened for *Polμ* gene deletion.
- Transformation completed on 3/2/2024

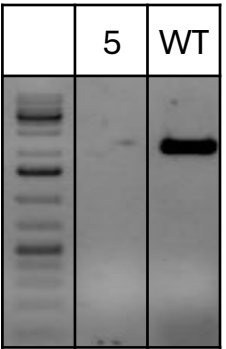


● Putative *PolB* deletion mutation

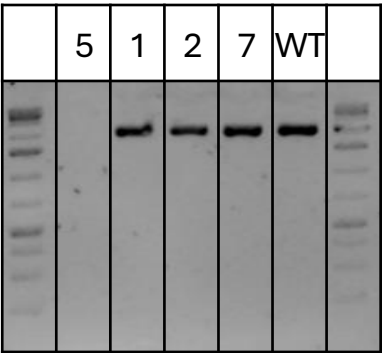


PolB Across Amplicon: 2232 bp

Attempt #1

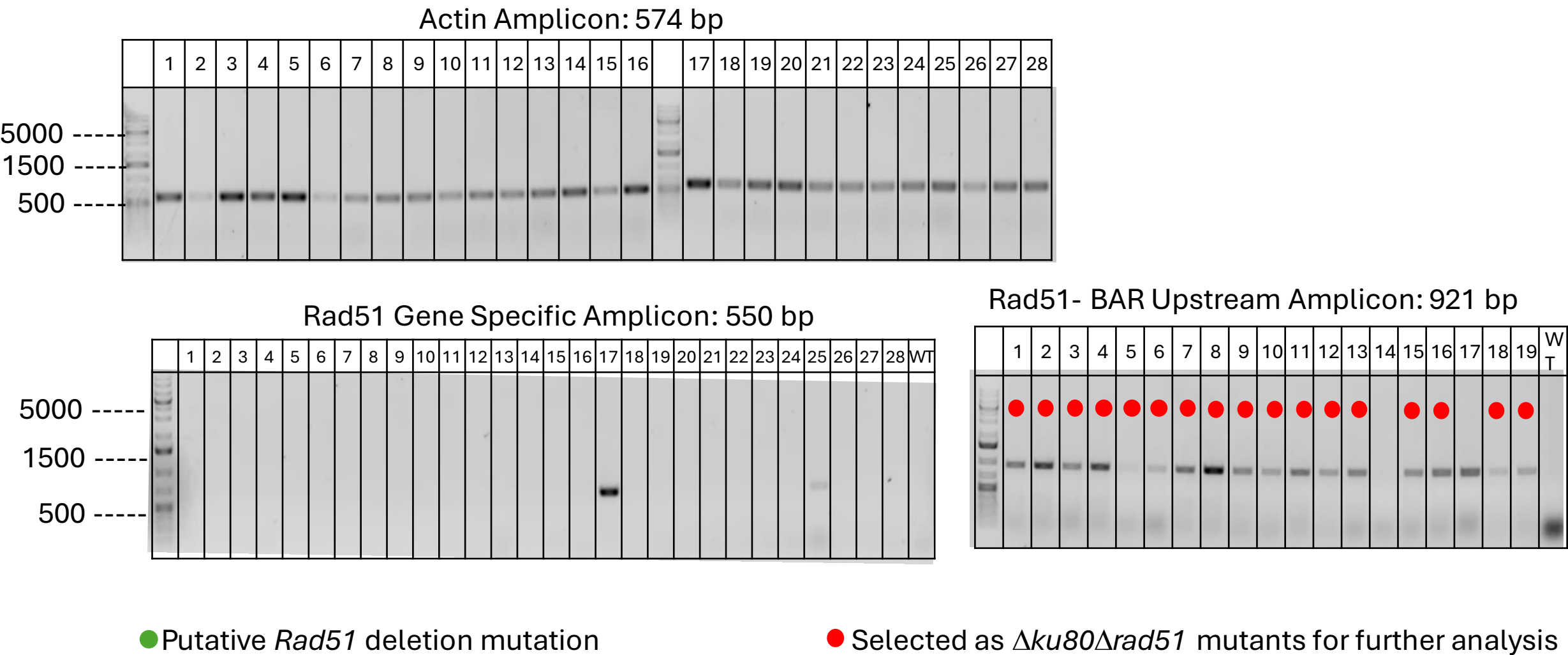


Attempt #2



Supplemental Figure 12. PCR results for *Rad51* editing in $\Delta ku80$

- 28 putative transformants were screened for *Rad51* gene deletion.
- Transformation completed on 3/3/2023



Supplemental Table 1: Target and ordered sequences to make RNPs for *M. oryzae* gene deletions

Guide Name	Target Sequence without PAM (TTTV for Cas12, NGG for Cas9)	Ordered oligo
Cas9-lig1b_guide1R	GTGTGCCATACATTGATGTC	TTCTAATACGACTCACTATAGTGTGCCATACATTGATGTCGTTT TAGAGCTAGA
Cas9-lig1b_guide2R	CATGCTATATTACGGGGCTC	TTCTAATACGACTCACTATAGCATGCTATATTACGGGGCTCGTTT TAGAGCTAGA
Cas9_fkbp12_guide1R	GGTGTGCGTTTCCATGAAAGT	TTCTAATACGACTCACTATAGggtgtcgtttccatgaaagtGTTT TAGAGCTAGA
LbCas12a_pol3_guide1R-1	AGATGCCAAGCATAACAACACCG	CGGTGTTGTTATGCTTGGCATCTatctacacttagtagaaattA
LbCas12a_pol3_guide2R-1	GGAGTTGGTCAGGGTCCCAGGGG	CCCCTGGGACCCTGACCAACTCCatctacacttagtagaaattA
LbCas12a_pol3_guide1R-2	AGCTGGAAGGTGTTGCGGAGATG	CATCTCCGCAACACCTTCCAGCTatctacacttagtagaaattA
LbCas12a_pol3_guide2R-2	CCAGATGGCAGCCCTATCAAAGT	ACTTTGATAGGGCTGCCATCTGGatctacacttagtagaaattA
LbCas12a_polq_guide1R	GGGGGAATCAAACCTCAAGCCGAG	CTCGGCTTGAGTTTGATTCCCCCatctacacttagtagaaattA
LbCas12a_polq_guide2R	AGGCCACGCCGACCTCTACAGCA	TGCTGTAGAGGTCGGCGTGGCCTatctacacttagtagaaattA
LbCas12a_exo1_guide1R	GGCTCGGGGAGACGATGAGAAAG	CTTCTCATCGTCTCCCCGAGCCatctacacttagtagaaattA
LbCas12a_exo1_guide2R	GAGATTCTGGCTCATTCTCCCGA	TGGGGAGAATGAGCCAGAATCTCatctacacttagtagaaattA
LbCas12a_mre11_guide1R	CCGAAGACTGCGAATAACCTGGT	ACCAGGTTATTCGCAGTCTTCGGatctacacttagtagaaattA
LbCas12a_mre11_guide2R	CTGCTCATAGTTGCCAGCATTGG	CCAATGCTGGCAACTATGAGCAGatctacacttagtagaaattA
LbCas12a_pol4_guide1R	AAGCTCTAGATGCCGTTTGGGAT	ATCCCAAACGGCATCTAGAGCTTatctacacttagtagaaattA
LbCas12a_pol4_guide2R	AAAGATCCTCCTTTTCATCGCTTC	GAAGCGATGAAAGGAGGATCTTTatctacacttagtagaaattA
LbCas12a_lig4_guide1R-1	CATTGACCTCTCAACTGATCACC	GGTGATCAGTTGAGAGGTCAATGatctacacttagtagaaattA
LbCas12a_lig4_guide2R-1	TGCTGGCAATCGAAGACGGCCAT	GCTATCCAGAAACTCGTCGACATatctacacttagtagaaattA
LbCas12a_lig4_guide1R-2	AAGGCCTCTTTCTTGGA CTTGAC	GACTTCTCCCAAGCCAGCATCACatctacacttagtagaaattA
LbCas12a_lig4_guide2R-2	TTGGACTTGTAGTCTGATGCCTA	GACATCAAAGCTGTCTGCGTCCatctacacttagtagaaattA
LbCas12a_lig1a_guide1R-1	CAATGTTGCGGTAACGACGTTGG	CCAACGTCGTTACGCGAACATTGatctacacttagtagaaattA
LbCas12a_lig1a_guide2R-1	TATCTAGGTGGTTATGTGCTGTC	GACACGACATAACCACCTAGATAatctacacttagtagaaattA
LbCas12a_lig1a_guide1R-2	TATGGTTCTAGCGACTTCACAAG	CTTGTGAAGTCGCTAGAACCATAatctacacttagtagaaattA
LbCas12a_lig1a_guide2R-2	TGCAACCTGTCTGCTCGATGTTG	CAACATCGAGCAGACAGGTTGCAatctacacttagtagaaattA
LbCas12a-rad51guide1R	CTCCAACATCCGCTGGGTGTGT	ACACACCCAGGCGGATGTTGGAGatctacacttagtagaaattA
LbCas12a-rad51guide2R	TGACAGTCCATGTCTACCAGAGA	TCTCTGGTAGACATGGACTGTCAatctacacttagtagaaattA
LbCas12a_polb_guide1R	CTTCTCCAATTCTTCCGCGTTAG	CTAACCGCGGAAGAATTGGAGAAgatctacacttagtagaaattA
LbCas12a_polb_guide2R	AGGGCCTGAAATTGGAATGGCGG	CCGCCATTCCAATTTCAGGCCCTatctacacttagtagaaattA
LbCas12a_fkbp12_guide2R	TTCTTCAGGTTGACTCTTCCGT	ACGGAAGAGTCGAACCTGAAGAAatctacacttagtagaaattA
LbCas12a-crRNA universal primer		CCCTAATACGACTCACTATAGGTAATTTCTACTAAGTGTAGAT

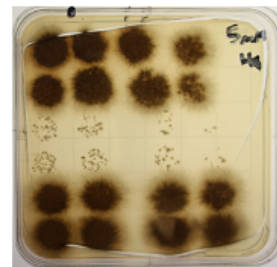
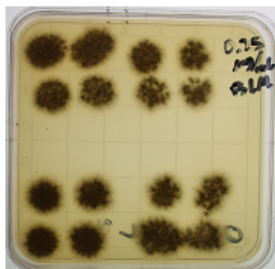
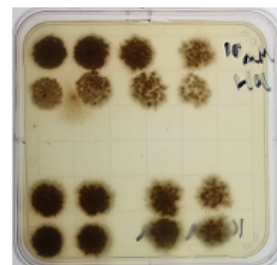
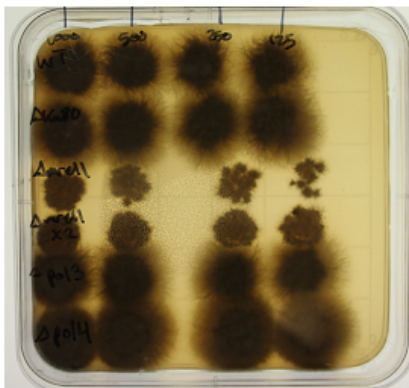
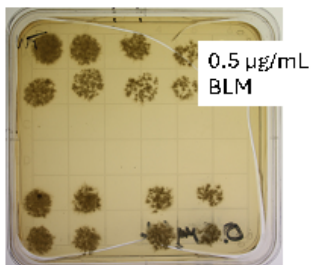
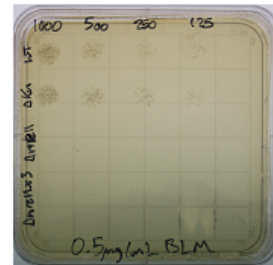
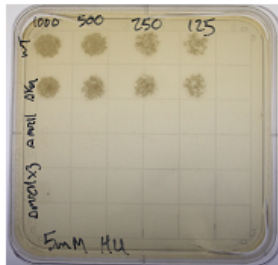
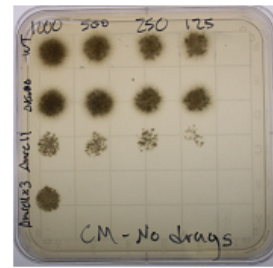
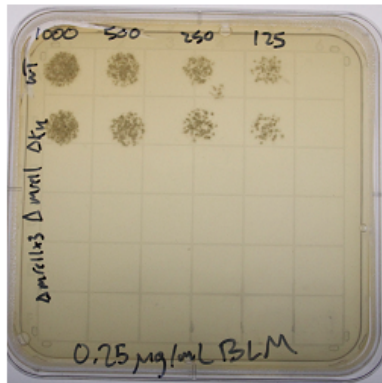
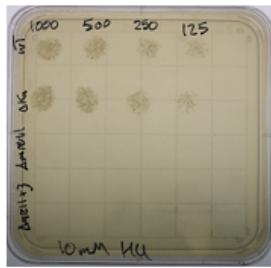
Supplemental Table 2: Oligo sequences used for gene deletion mutant PCR genotyping			
Primer Name	Sequence	Amplicon Size	
Actin_Gene_Specific_F	CCGTGACTTGACCGACTACCTGA	574	
Actin_Gene_Specific_R	AGAGCGAGGCGAGAATGGAAC		
Exo1_Gene_Specific_F	CAACCCCATCACAAAGGAGC	646	
Exo1_Gene_Specific_R	TGTCGAGCTTAGTGAACCGT		
Exo1_BAR_Up_F	GGCAGGTAGACAGGGATGAT	606	
Exo1_BAR_Up_R	GGGAGGAAGGGCGAACTTAA		
Exo1_BAR_Down_F	GCACCATCGTCAACCACTAC	835	
Exo1_BAR_Down_R	TCACAGTACCACAGGCACAC		
FKBP12_Down_F	CTCACCAGCGATGTCTACCT	350	
FKBP12_Down_R	AAGGGTCAAGACTATACAGAAGTA		
FKBP12_Gene_Specific_F	GCAGTAGCTGGTGCTGTTTG	1242	
FKBP12_Gene_Specific_R	CCTCTATACCTATGTCCCTTGTC		
FKBP12_Up_F	TGTGGCTTCATTGTTGGTTT	498	
FKBP12_Up_R	CGATGTCTGTATTTACGGGACT		
Ku80_Gene_Specific_F	CTTGAAGACGGCGAGGAATT	530	
Ku80_Gene_Specific_R	CCAGTGGCGGAAACTGATAG		
Ku80_HYG_Up_F	TGTTGATTTGGATCTGGCTAA	?	did not use
Ku80_HYG_Up_R	ATACCGTAAAGCACGAGGAA		
Ku80_HYG_Down_F	ATGAACTGCAAGACGAGGCA	?	did not use
Ku80_HYG_Down_R	GCGTTGTGAAGATACCGATGAC		
Lig1A_Gene_Specific_F	AATAAGGAACCAGATGACCAAGAG	743	
Lig1A_Gene_Specific_R	GACTTGACAGCAACAAGACCTAAA		
Lig1A_BAR_Up_F	TGGATGCTTCGGAGTGACAA	386	
Lig1A_BAR_Up_R	CTACTGCTACAAGTGGGGCT		
Lig1A_BAR_Down_F	TGCCCCGTACCGAGATTTAGTT	157	
Lig1A_BAR_Down_R	AAAAGGAAGGATGAGCGACCGA		
Lig1A_Across_F	ATGACCCACTACAACCCCTG	4368	
Lig1A_Across_R	TGGACTTTTACCGTGCCAAG		
Lig1B_Gene_Specific_F	CGCCACATCTAGCCAAGTCT	762	
Lig1B_Gene_Specific_R	GTTTGTAGCCAGCCAGACCT		
Lig1B_G418_F_Up	AAAACCAAATCAGGAAACTGCGA	1441	
Lig1B_G418_R_Up	ATCAGCCATGATGGATACTTTCTC		
Lig1B_G418_F_Down	AGGATCTCTGTATCTCACCTT	870	
Lig1B_G418_R_Down	AGTTCTACACTGGCAAGTACATCG		
Lig1B_Across_F	ATTGTGGGGAGGGAAAGAGG	4111	
Lig1B_Across_R	CTAGAGGGTAGCGTGGACTG		
Lig4_Gene_Specific_F	CCTCACTCACCAACTTTGC	896	
Lig4_Gene_Specific_R	TCCATCCATTGCCCCTCTGT		
Lig4_BAR_Up_F	AGTCTTGGGTAGTCTGGCAC	1024	
Lig4_BAR_Up_R	TAAAGGGAGGAAGGGCGAAC		
Lig4_BAR_Down_F	GCACCATCGTCAACCACTAC	667	
Lig4_BAR_Down_R	TGGCAAGTGAAAGGGATGAAG		
Lig4_Across_F	GAGTGGTTGGTTAGTGTGTGA	4065	
Lig4_Across_R	GCAACTCCCCATTTCTTCCC		
Mre11_Gene_Specific_F	CGAAAAGGTCCCGCTGAAAA	687	
Mre11_Gene_Specific_R	TCGTCTGTCTCAAGTGCCAT		
Mre11_BAR_Up_F	AAGAACTTCCTTCCCTCCCG	434	
Mre11_BAR_Up_R	TAAAGGGAGGAAGGGCGAAC		
Mre11_BAR_Down_F	GCACCATCGTCAACCACTAC	990	
Mre11_BAR_Down_R	CTCAACCACAGGCGTTTCTC		

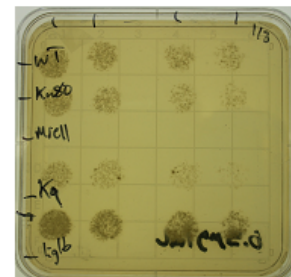
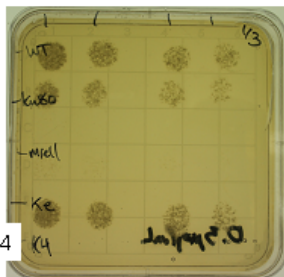
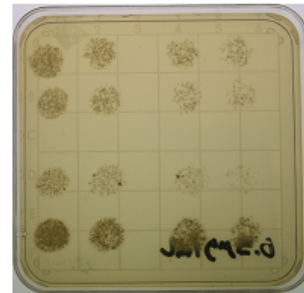
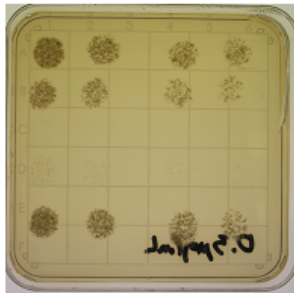
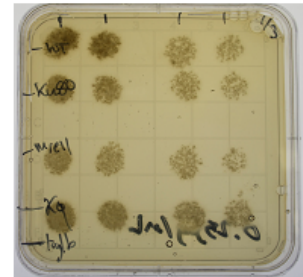
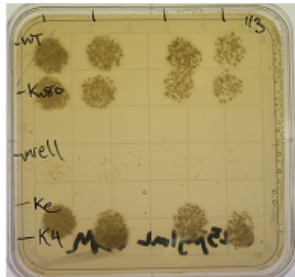
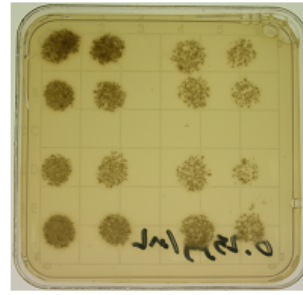
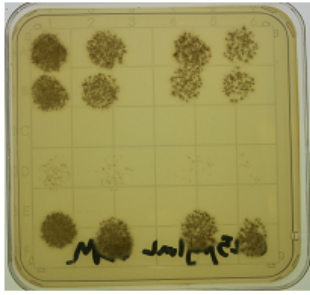
Pol3_Gene_Specific_F	AGACCAACATCGCACAAACAC	1172	
Pol3_Gene_Specific_R	GCACCTGTGACCTAGACAT		
Pol3_BAR_Up_F	AGGCGAGCGACCCAATATAA	850	
Pol3_BAR_Up_R	GTAGTGGTTGACGATGGTGC		
Pol3_BAR_Down_F	TCTACACCCACCTGCTGAAG	687	
Pol3_BAR_Down_R	CCTTTTGTGAGGGAGGAGGT		
Pol3_HYG_Up_F (Pol3_BAR_Up_F)	AGGCGAGCGACCCAATATAA	1428	
Pol3_HYG_Up_R	ACTCCCCAATGTCAAGCACT		
Pol3_HYG_Down_F	CGTGGTTGGCTTGTATGGAG	1112	
Pol3_HYG_Down_R (Pol3_BAR_Down_R)	CCTTTTGTGAGGGAGGAGGT		
Pol3_Across_F (Pol3_BAR_Up_F)	AGGCGAGCGACCCAATATAA	4769	
Pol3_Across_R (Pol3_BAR_Down_R)	CCTTTTGTGAGGGAGGAGGT		
Pol3_Across_F	GCAAGTTGGAGGGCGATTTT	4314	
Pol3_Across_R	GGCGTTTATCAGTTGGCACA		
Pol4_Gene_Specific_F	ATGCGTTCGGTCACTTCAAC	2228	
Pol4_Gene_Specific_R	CATCCGTCACCTTCTCCCTA		
Pol4_BAR_Up_F	GATAACCGCTGTGAAGTGCC	614	
Pol4_BAR_Up_R	GGAAGTTGACCGTGCTTGTC		
Pol4_BAR_Down_F	GCACCATCGTCAACCACTAC	823	
Pol4_BAR_Down_R	GGCAATGTTTTCCCGAGAGG		
Pol4_HYG_Up_F	GATAACCGCTGTGAAGTGCC	761	
Pol4_HYG_Up_R	GTCTCGTTCCTGTCTGCTA		
Pol4_HYG_Down_F	GCAAAGTGTGATGGACGACA	1224	
Pol4_HYG_Down_R	TGGGAAAGACTCTGGGCAAT		
Pol4_Across_F	TGGGATGGGTTGTCTTTGGT	4122	
Pol4_Across_R	AGTAGTGCTGCTCTGAGTGG		
PolB_Gene_Specific_F	ACACCACAACCCCTACAACA	522	
PolB_Gene_Specific_R	CTATATGCGTCGTCAAGCCG		
PolB_Up_F	TCTGCTTCTCTGACTCCG	426	
PolB_Up_R	CTACTGCTACAAGTGGGGCT		
PolB_Down_F	TCTACACCCACCTGCTGAAG	455	
PolB_Down_R	GCCCATTTCTGTTGTTGTGC		
PolB_Across_F (PolB_Up_F)	TCTGCTTCTCTGACTCCG	2232	
PolB_Across_R (PolB_Down_R)	GCCCATTTCTGTTGTTGTGC		
PolQ_Gene_Specific_F	ACTCGTCGCCGATGTTCTTA	1130	
PolQ_Gene_Specific_R	ACTGGACGGTATGCGGTTTC		
PolQ_BAR_Up_F	TCAATGACCTCTGGACGCCACCTT	773	
PolQ_BAR_Up_R	TCGGCGACGAGCCAGGGATA		
PolQ_BAR_Down_F	AACTGGCATGACGTGGGTTT	509	
PolQ_BAR_Down_R	GGGAGGTGGTGGGAGTATAAGA		
PolQ_Across_F (PolQ_Up_F)	TCAATGACCTCTGGACGCCACCTT	4115	
PolQ_Across_R (PolQ_Down_R)	GGGAGGTGGTGGGAGTATAAGA		
Rad51_Gene_Specific-F	ACCACACCCTGTCAATAGCA	710	
Rad51_Gene_Specific-R	GCGACCTTCTTCAGACTCA		
Rad51_BAR_Up-F	CCCCTTGGCCACTTCCGATTC	921	
Rad51_BAR_Up-R	AGCCCGATGACAGCGACCAC		
Rad51_BAR_Down-F	GCCCTTCTCCCTTTATTTT	845	
Rad51_BAR_Down-R	CAAAGTCCACTGTTACGTCCAT		

Supplemental Table 3: Oligo sequences used for synthesizing Donor DNA with flanking MH specific to target gene

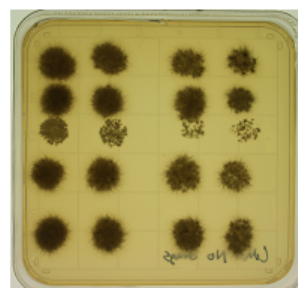
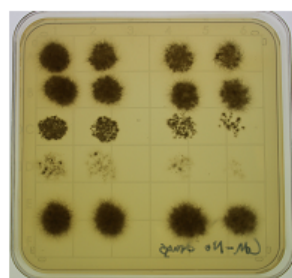
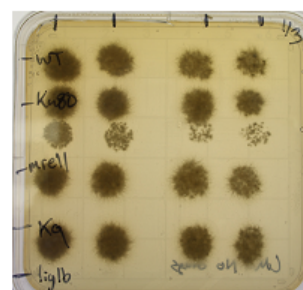
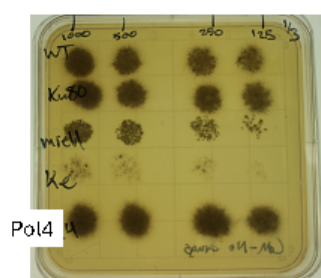
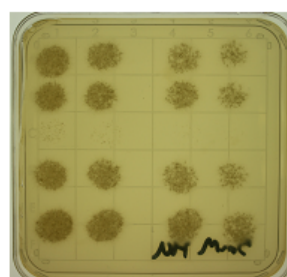
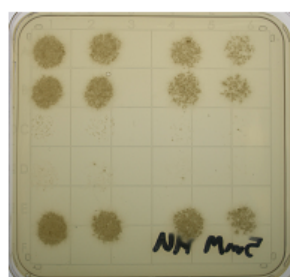
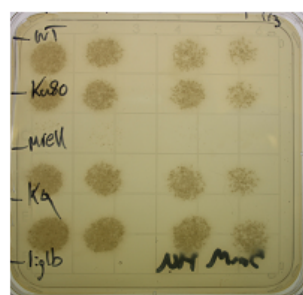
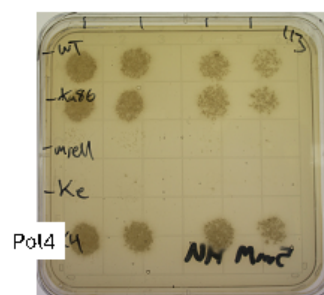
Primer Name	Sequence
BAR_Pol3_ampF	TGGCGTCACGGTCAATTTCCGACGCGTGTCTGTTAATTGATCGACAGAAGATGATATTGAAGG
BAR_Pol3_ampR	GGAATAGAATTTCTTTGAAACACGCTGCTAGGAGCCAAGATAAcTAAATCTCGGTGACGGGC
BAR_Pol4_ampF	GCGGCCCCGCCGCGGCTGCCTAGGAGACGGGCATTAATGCGGATTCAATTCGACAGAAGATGATATTGAAGG
BAR_Pol4_ampR	TTTGTACAAAGCAACCCGACGTCGCCCTGGCCGTATGGGTTTAGCGTGTTAAcTAAATCTCGGTGACGGGC
BAR_Rad24_ampF	TCTGCGGGATATCGACCAGCAGCGATCGAGCAGCAAATGCTCCAATCAACTCGACAGAAGATGATATTGAAGG
BAR_Rad24_ampR	CGTATCTCAGTTTCTGTCTTTGGAAGTCTCCAAAACACACCTCCTCACTAAcTAAATCTCGGTGACGGGC
BAR_Exo1_ampF	AAGTAAATCGCGTCCTTGACAGCCAGTGAATTTTTCAATTTACCTGATGTCGACAGAAGATGATATTGAAGG
BAR_Exo1_ampR	TGATGATGACAGAACAACAAAACCTTTGACGCTGAGTGGCGTCTATACAATAAcTAAATCTCGGTGACGGGC
BAR_Mre11_ampF	CTCAACTCTGTGACCGTCATTATTTCTGCCAACTGGGAATGGATCTGAATCGACAGAAGATGATATTGAAGG
BAR_Mre11_ampR	GCCCCACTGCCTACCTGCCCCCTAGGTATTCTGCTTGTTACCCCTTACTTAAcTAAATCTCGGTGACGGGC
BAR_Rad52_ampF	ttatccgctactcatgtgcaaatagctgacgcagctctgctcctcccgcaTCGACAGAAGATGATATTGAAGg
BAR_Rad52_ampR	cattgtccccccccctccggcacgtcatcctcagagttacgattggccTAAcTAAATCTCGGTGACGGGC
BAR_Polq_ampF	CTCCGAATCGTGGAGAGCGCGGACGACGGTGTGTTTTCGCTTCGAACAAGGTCGACAGAAGATGATATTGAAGg
BAR_Polq_ampR	GGTCGACAATGCCCTAACGCACATACATCCCTGAAAGCACAGACCTTCATTAACCTAAATCTCGGTGACGGGC
BAR_Lig1A_ampF	AATTAAGAATCAACGCGCCGGGGCGCGTCACTTGATCTCTCGACAGAAGATGATATTGAAGG
BAR_Lig1A_ampR	GCAGAGTGTTGGTCGCCAAGGACACGCTCGACTTGGCCAATAAcTAAATCTCGGTGACGGGC
BAR_PolB_ampF	ctttcctcacaaacaccgaacgacttatcttcaaaggTCGACAGAAGATGATATTGAAGG
BAR_PolB_ampR	accatgatttgaattacacagttgttacaatgtacgctcTAAcTAAATCTCGGTGACGGGC
BAR_R51_ampF	TtcactacttcacgagcttgtgctcccgaaagcgataagactgccaaaTCGACAGAAGATGATATTGAAGG
BAR_R51_ampR	tgtaaacagtaaaaaagacaaggttcatgaaaaaaatattcgaacactTAAcTAAATCTCGGTGACGGGC

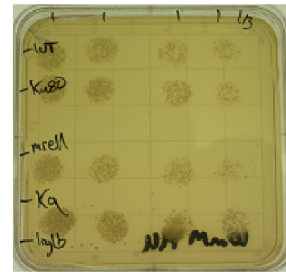
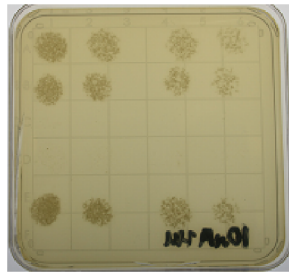
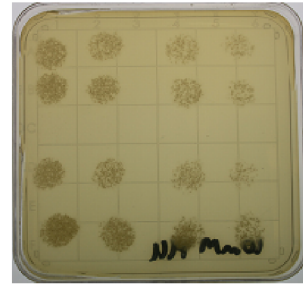
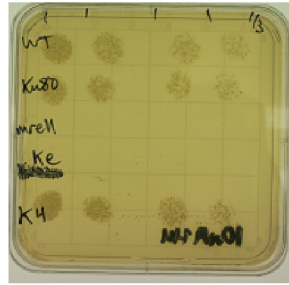
Genotoxic Stress Assay Results



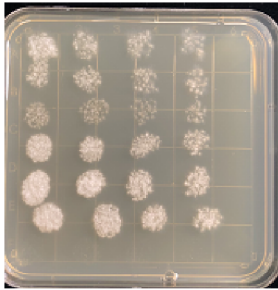


Pol4

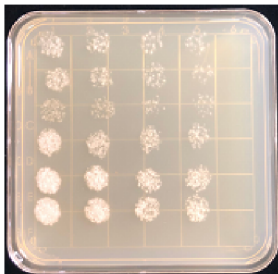
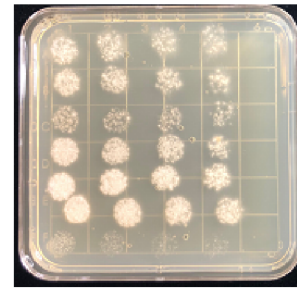




0.25 $\mu\text{g/mL}$ BLM

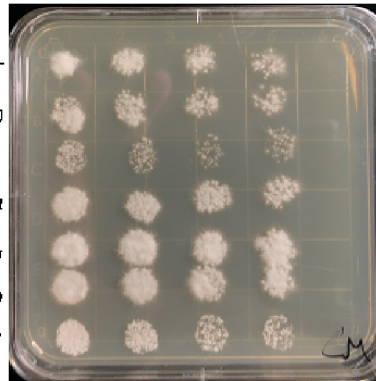


5 mM HU



0.5 $\mu\text{g/mL}$ BLM

WT
 Δku80
 $\Delta\text{ku80}\Delta\text{exo1}$
 Δpoi4
 $\Delta\text{ku80}\Delta\text{poi4}$
 Δlig1b
 Δmre11



10 mM HU

