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A Curious New Role for MRN in *Schizosaccharomyces pombe* Non-Homologous End-Joining

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Abstract

Chromosomal breaks can be healed by several repair processes, including one called Non-Homologous End-Joining (NHEJ) where the two broken ends are ligated together with a loss of 0–5 bp of DNA. The protein requirements for NHEJ of cut DNA ends in the budding yeast *Saccharomyces cerevisiae* include its version of the Mre11-Rad50-Nbs1 (MRN) complex. In contrast, the fission yeast *Schizosaccharomyces pombe* and mammalian cells do not require MRN for this process. Recent work in *S. pombe* used transposon excision to generate breaks that were capped by DNA hairpins, which must be opened to produce ligatable ends. Repair in *S. pombe* was through an NHEJ reaction that now requires MRN. Surprisingly, wild type cells and MRN mutants that lack nuclease activity showed the same levels of excision. These genetic results suggest that MRN recruits an unknown hairpin-opening nuclease for this unusual NHEJ reaction.

Keywords

MRN; MRX; NHEJ; *Schizosaccharomyces*; *Saccharomyces*; transposon

DNA double-strand breaks (DSBs) in eukaryotic chromosomes are frequently generated by both the organism itself and damage from environmental insults. Failure to repair these mitotic DSBs can activate DNA damage checkpoints, resulting in a cell cycle pause, permanent arrest or death depending on the organism or cell type (Featherstone and Jackson 1999; Harper and Elledge 2007; Harrison and Haber 2006). In meiotic cells, programmed DSBs are key intermediates that are processed to drive recombination between homologs and the appropriate segregation of chromosomes (e.g. (Cromie and Smith 2008; Cromie and Smith 2007)). In mitotic cells, DSBs can occur due to errors in DNA replication, from the exposure to radiation or chemicals such as chemotherapeutic agents or from unusual DNA structures (Kondo *et al.* 2010; Rastogi *et al.* 2010; Wang and Vasquez 2009). Programmed DSBs also occur in cells of the vertebrate immune system, where rearrangements of genes for immunoglobulins and T-cell receptors created DSBs capped by covalently closed hairpins which are then joined to form functional genes (Gellert 2002). These hairpins, which effectively turn the chromosome into a palindrome, are a potential source of genomic

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instability from humans to yeast, as they can form through a variety of mechanisms and their processing can result in chromosomal translocations and rearrangements (Aplan 2006; Lieber 2016; Tanaka and Yao 2009). For example, palindromic A–T rich repeats on human chromosomes 11 and 22 cause frequent chromosomal translocations in human germ cells (Kato *et al.* 2006; Kurahashi and Emanuel 2001; Shaikh *et al.* 2001). The rearrangement of immunoglobulin and T-cell receptor can lead to errors in the form of specific chromosomal translocations in lymphomas and leukemias (Lieber 2016). Inverted repeats and trinucleotide repeats can extrude palindromic hairpin structures from the chromosome during DNA replication (Lewis and Cote 2006; Lobachev *et al.* 2002; Lobachev *et al.* 2007; Sundararajan *et al.* 2010) or after a resection of a nearby DSB produces a single-stranded DNA that can hybridize to itself (Darmon *et al.* 2010; Deng *et al.* 2015; Lobachev *et al.* 2002; Mizuno *et al.* 2009). Repair of DSBs and processing of palindromes have long been realized to be important biological functions and have therefore been studied extensively for many years.

Repair is generally accomplished by either of two mechanisms: error-free homologous recombination (HR) and error-prone end-joining or annealing (EJ)(Fig. 1A)(Symington and Gautier 2011). In HR, the existing DSB is resected to produce 3′ overhangs that can invade and copy information from homologous sequences (Jain and Cooper 2010; Shrivastav *et al.* 2008; Symington and Gautier 2011). End-joining mechanisms do not involve a copying mechanism to restore genetic information, but join the two ends of the DSB together after losing different amounts of DNA sequences. Non-Homologous End-Joining (NHEJ) joins ends with very little sequence loss (e.g. 0–5 bp (McVey and Lee 2008)), hence the DSB is subject to little or no resection. Microhomology-Mediated End-Joining (MMEJ) uses resection of the 5′ strand of each side of the DSB (e.g. 10–30 bp) to expose short (~3–5 bp) stretches of homology that allows the two ends to be held together, processed and ligated to produce a fusion with small mutations and a more extended deletion than NHEJ. Single-Strand Annealing (SSA) involves more extensive 5′ strand degradation to expose larger stretches of homology (e.g. repeated sequences in the genome) that can anneal to produce a larger deletion than MMEJ and leaves a hybrid sequence where the two repeats are joined. These processes and their genetic requirements have been well-worked out in *S. cerevisiae*, revealing key gene products in each process that are conserved from yeasts to humans (McVey and Lee 2008).

A conundrum in the evolutionary conservation of these processes is exhibited with NHEJ, which requires the DNA end-binding protein Ku, DNA ligase 4 and the Mre11-Rad50-Xrs2 (MRX) complex in *S. cerevisiae* (Moore and Haber 1996; Zhang and Paull 2005), but only Ku and DNA ligase 4 in *S. pombe* and mammals (Manolis *et al.* 2001; Rass *et al.* 2009; Xie *et al.* 2009). The Mre11 and Rad50 components have clear homologs in bacteria, archaea and eukaryotes, while the Xrs2 subunit is replaced with the Nbs1 protein in other organisms such as mammals and *S. pombe* (Carney *et al.* 1998; Ueno *et al.* 2003). MRN/MRX is one of the first complexes to bind DSBs and processes them by initiating resection of the 5′ strand, a process required for HR and MMEJ (McVey and Lee 2008; Mimitou and Symington 2008; Zhu *et al.* 2008). Mre11 has both endo- and exonuclease activities that are important for this process, and one of the properties of the Rad50 subunit is to stimulate these activities (Nicolette *et al.* 2010; Paull 2010). Rad50 also possesses coiled-coiled and Zn²⁺ hook domains that that can help tether MRN/MRX complexes and their bound DNA ends together

(Williams *et al.* 2007). The Xrs2 or Nbs1 subunits help recruit DNA damage checkpoint kinases such as Tel1 or ATM to DSBs to pause or arrest the cell cycle (Falck *et al.* 2005; Nakada *et al.* 2003; You *et al.* 2005). MRN/MRX nuclease activities also play a key role in meiosis, where the programmed DSBs contain a DNA-peptide conjugate that must be removed to allow further processing (Daley and Wilson 2005; Hartsuiker *et al.* 2009; Keeney 2008; Milman *et al.* 2009). This peptide removal property has been proposed to remove the Topoisomerase II-DNA adducts that form after treatment of mammalian cells with etoposide (Aparicio *et al.* 2016; Hoa *et al.* 2016), indicating a role for MRN/MRX in processing non-ligatable DSBs so they can be repaired by other cellular enzymes (Fig. 1A top right). These properties of MRX, and its associated nuclease Sae2, have been examined for the processing of hairpins. Both genetic and biochemical evidence suggest that MRX-Sae2 can open hairpin ends in *S. cerevisiae* to produce a ligatable DNA end for HR or EJ (Deng *et al.* 2015; Lengsfeld *et al.* 2007; Lobachev *et al.* 2002; Rattray *et al.* 2001; Rattray *et al.* 2005). The *S. cerevisiae* requirement for MRN/MRX in the NHEJ of DSBs generated by nucleases is dispensable in both *S. pombe* and mice (Fig. 1A top left)(Manolis *et al.* 2001; Rass *et al.* 2009; Xie *et al.* 2009), and the reason for this difference is unknown. MRN/MRX is therefore a multi-functional complex with roles in DNA lesion removal, HR and MMEJ, but its specific roles in NHEJ in different species remain to be established.

We recently explored the repair of a non-ligatable end by EJ in *S. pombe* using the excision of *hAT* transposon as a model system (Li *et al.* 2017). The *hAT* transposons excise to leave a DSB capped by covalently closed DNA hairpins, much like ends of intermediates in mammalian V(D)J recombination or structures formed by palindromes, inverted repeats and trinucleotide repeats (Lewis and Cote 2006; Lieber *et al.* 2004; Lobachev *et al.* 2007; Sundararajan *et al.* 2010; Zhou *et al.* 2004). The excision reaction only requires transposase, which cleaves the top strand at the border of the genomic DNA and the transposon to leave a 3' OH that subsequently reacts with the bottom strand to produce a hairpin-capped genomic DNA end and excision of the transposon (see Zhou *et al.* 2004). Repair of these hairpin-capped DSBs then relies on the cellular machinery (Fig. 1B). An EJ reaction is required to produce the repaired genomic DNA without the transposon, which can be analyzed because the sequences that border the transposon are known and can be examined post-excision. An earlier study with the *hAT* transposon family member *Ac/Ds* showed that excision could occur in *S. cerevisiae* via a hybrid pathway: Ku, MRX and DNA ligase 4 were required for efficient excision, as with NHEJ, but the repair products showed the deletions and mutations consistent with MMEJ, which was surprising as MMEJ is independent of Ku (Yu *et al.* 2004)(Fig. 1B). We found that this unusual reaction was not recapitulated in *S. pombe*, which instead heals these excision events using standard NHEJ in a reaction that now requires MRN (Li *et al.* 2017)(Fig. 1B).

Our assays followed excision of the *Hermes* transposon using a semi-quantitative PCR assay that would amplify the excised loci but not the loci containing the transposon. The majority of excision events showed the 0–5 bp loss expected for NHEJ, with 0 to 5 nucleotide mutations at each event. Excision was eliminated by loss of Ku, DNA ligase 4 and MRN, and the simultaneous loss of the genes for DNA ligase 4 and Mre11 gave the same results as expected for all components acting in the same pathway. Thus, similar to the different

pathways for NHEJ repair of cut ends in *S. cerevisiae* and *S. pombe*, the pathways that repair non-ligatable hairpins was also different.

To address what activity was required to open the hairpins to allow ligation, we “rounded up the usual suspects” (Curtiz 1943) according to previous evidence in the literature, tested them genetically for a role in excision, but could not identify a hairpin opening activity. Briefly, Williams et al. (Williams *et al.* 2008) had created *S. pombe* Mre11 mutants that disrupt dimerization of MRN complexes or disrupt Mre11 endo- and exonuclease activities. While mutations in the dimerization domain reduced excision, the nuclease mutants had little effect. MRN can interact with another nuclease called Ctp1 (CtIP in mammals and Sae2 in *S. cerevisiae*), and deleting the gene for Ctp1 did reduce excision. However, removing Ctp1 from the Mre11 nuclease mutant gave excision levels equivalent to wild type cells, indicating that neither Ctp1 nor Mre11 had the genetic properties of the hairpin opening activity. The final candidate was Pso2, a nuclease that can cleave hairpins in vitro and has limited homology to *Artemis* which cleaves the hairpins formed during V(D)J rearrangement (Tiefenbach and Junop 2012). Deleting the gene for Pso2 from the Mre11 nuclease mutant lacking Ctp1 made no difference to excision frequency, suggesting that the hairpin opening activity is not Mre11, Ctp1 or Pso2.

What do these surprising genetic results tell us? First, NHEJ of the non-ligatable hairpin-capped ends in *S. pombe* requires MRN. The similar requirements for NHEJ repair of cut ends in *S. pombe* and mammals suggest that these results for non-ligatable ends may apply to humans as well. Second, MRN, and to some extent Ctp1, are required for the hairpin opening activity to work, suggesting that this complex may help recruit the actual opening activity (Fig. 1C). Third, *S. cerevisiae* shows more extensive nucleolytic degradation during repair of these lesions than *S. pombe*, which may reflect an increased level of nucleolytic processing in general compared to other eukaryotes. This interpretation is consistent with the high levels of homologous recombination in *S. cerevisiae*, which have made it one of the best unicellular molecular genetic systems available. Extending these types of studies to mammalian systems will illuminate specific molecular mechanistic details that may be exploited for novel therapeutic approaches.

As removing the candidates for the biochemically-identified hairpin opening activity did not block transposon excision, one remaining question is whether the hairpin opening is a new, unknown protein or a repurposed existing activity from another process (e.g. HR, restarting of replication forks, RNA processing). We propose genetic and proteomic screens to answer this question. The genetic screen uses a marker gene whose promoter is disrupted by a *Hermes* insertion (Fig. 2A)(Li 2015). As *Hermes* excision by NHEJ leaves the surrounding sequence intact, NHEJ repair after hairpin opening should restore gene expression in cells expressing transposase. Mutants that reduce or eliminate hairpin opening as well as mutations in Ku, MRN or DNA ligase 4 should fail to produce cells with restored gene expression. The advances in labeling protein “neighbors” and the increasing sensitivity of mass spectrometry may provide a proteomic approach. The recently developed APEX2 technology allows biotinylation of proteins in close proximity to the APEX2 enzyme over a 1-minute time course (Fig. 2B). The biotin-conjugated proteins can then be purified using streptavidin beads and identified by mass spectrometry (Hung *et al.* 2016; Lam *et al.* 2015).

Adaptation of this technology to chromatin in cells that can be induced to produce large amounts of a site-specific endonuclease or transposase may allow the kinetic analysis of protein recruitment to DSBs with or without hairpins. This proteomics approach would be exciting for both testing our current picture of DSB detection and processing, as well as revealing the difference between ligatable and non-ligatable ends.

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Figure 1.

Types of DSB repair and conundrum of eukaryotic NHEJ. **A.** Double-strand breaks created by endonucleases (HO or I-SceI) or by radiation or DNA damaging drugs can be processed to enter the “error free” homologous recombination pathway (HR) or the “error prone” End-Joining pathway (EJ) that includes NHEJ, MMEJ and SSA. NHEJ is the pathway that requires the DNA end binding Ku heterodimer. Oddly, repair of endonuclease-generated ends requires Ku and DNA ligase 4 in *S. pombe* and mammals but requires Ku, DNA ligase 4 and MRX in *S. cerevisiae* (see text). **B.** Generation of a non-ligatable DSB by *hAT* transposon excision. The *hAT* transposons excise to leave hairpin-capped ends that must be processed prior to end-joining to produce a transposon-free locus. In *S. pombe*, this excision occurs predominantly by NHEJ and now requires MRN, but not Mre11 nuclease activities. In contrast, the *S. cerevisiae* excision reaction occurs by a hybrid pathway that requires Ku but produces MMEJ products (see text). **C.** A speculative model to explain the repair of DNA hairpins by NHEJ. The relative order of MRN vs. Ku binding and hairpin cleavage is unknown, so both possibilities are depicted. As the obvious candidate nucleases for hairpin opening did not affect the frequency of NHEJ, MRN and Ctp1 are proposed to help recruit the DNA hairpin opening activity to allow subsequent end-joining. Identifying the hairpin opening activity will help reveal how much more we have to learn about the metabolism of DSBs.

Figure 2.

Possible genetic and proteomic approaches to identify the hairpin opening activity. **A.** The *Hermes* transposon is placed between the *S. cerevisiae HIS3*⁺ gene and the CaMV35 promoter to reduce expression in an *S. pombe his5*⁻ strain (as described in (Li 2015)). The *HIS3* gene is chosen because it complements *his5* and the encoded enzyme can be inhibited by 3-amino triazole (Brennan and Struhl 1980). Conditions can be established where cells lacking the transposon can grow but cells that retain the transposon cannot. Low frequency excision can be monitored by patching ~10⁶ cells and looking for the outgrowth of *His*⁺ cells (papillae). Mutants reducing NHEJ and hairpin opening are expected to produce many fewer *His*⁺ cells. A screen of deletion or random mutants may reveal the in vivo hairpin opening activity. **B.** A fusion protein containing a DNA binding domain and APEX2 (an

engineered ascorbate oxidase (Lam *et al.* 2015)) is tethered to a unique site in the genome (red box) adjacent to an inducible DSB or *Hermes* insertion. Pre-loading cells with the membrane-permeable reagent biotin-phenol followed by a 1-minute treatment with H₂O₂ produces a short-lived biotin-phenoxy radical that can modify proteins near the tethered APEX2. These biotinylated proteins can then be purified using streptavidin beads and identified using mass spectrometry (Hung *et al.* 2016). Induction of the site-specific endonuclease or transposase prior to H₂O₂ treatment would allow the labeling of proteins at the DSB.