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Genetic interactions between *HNT3*/Aprataxin and *RAD27*/FEN1 suggest parallel pathways for 5′ end processing during base excision repair

James M. Daley[a], **Thomas E. Wilson**[b], and **Dindial Ramotar**[a,*]

[a]Centre de Recherche, Hôpital Maisonneuve-Rosemont, Université de Montréal, Montréal, QC H1T 2M4, Canada

[b]Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109, USA

Abstract

Mutations in Aprataxin cause the neurodegenerative syndrome ataxia oculomotor apraxia type 1. Aprataxin catalyzes removal of adenosine monophosphate (AMP) from the 5′ end of a DNA strand, which results from an aborted attempt to ligate a strand break containing a damaged end. To gain insight into which DNA lesions are substrates for Aprataxin action *in vivo*, we deleted the *Saccharomyces cerevisiae HNT3* gene, which encodes the Aprataxin homolog, in combination with known DNA repair genes. While *hnt3Δ* single mutants were not sensitive to DNA damaging agents, loss of *HNT3* caused synergistic sensitivity to H_2O_2 in backgrounds that accumulate strand breaks with blocked termini, including *apn1Δ apn2Δ tpp1Δ* and *ntg1Δ ntg2Δ ogg1Δ*. Loss of *HNT3* in *rad27Δ* cells, which are deficient in long-patch base excision repair (LP-BER), resulted in synergistic sensitivity to H_2O_2 and MMS, indicating that Hnt3 and LP-BER provide parallel pathways for processing 5′ AMPs. Loss of *HNT3* also increased the sister chromatid exchange frequency. Surprisingly, *HNT3* deletion partially rescued H_2O_2 sensitivity in recombination-deficient *rad51Δ* and *rad52Δ* cells, suggesting that Hnt3 promotes formation of a repair intermediate that is resolved by recombination.

Keywords

Aprataxin; DNA repair; Oxidative DNA damage; Ataxia oculomotor apraxia type 1; Base excision repair; DNA ligase

1. Introduction

In all living cells, genome integrity is constantly challenged by DNA damaging agents including reactive oxygen species generated during metabolism, ultraviolet light from the sun, ionizing radiation, and various environmental chemicals. A variety of DNA repair pathways exist to repair the diverse lesions caused by these agents. DNA ligases are a common feature of all DNA repair pathways, performing the final nick-sealing step in repair to restore the integrity of the chromosome. In eukaryotes, DNA ligation is initiated when

*Corresponding author. Tel.: +1 514 252 3400x4684; fax: +1 514 252 3430. dramotar.hmr@ssss.gouv.qc.ca (D. Ramotar).

Conflict of interest
There is no conflict of interest.

Appendix A. Supplementary data
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adenosine monophosphate (AMP) from ATP is covalently linked to a lysine residue in the active site of the ligase. AMP is then transferred to the 5′ phosphate of the DNA strand break, and nucleophilic attack by the adjacent 3′ hydroxyl breaks the 5′ pyrophosphate bond, sealing the break and releasing AMP [1]. DNA damaging agents often create single- and double-strand breaks with blocked ends that must be processed prior to ligation [2–6]. An end with a damaged 3′ terminus adjacent to a 5′ phosphate may be a substrate for premature attempted ligation, in which the absence of a 3′ hydroxyl causes the reaction to stall prior to the final step, leaving an adenylated 5′ phosphate.

Aprataxin reverses abortive ligation by removing the 5′ AMP, creating a 5′ phosphate and effectively resetting the break for a future ligation attempt [7]. Aprataxin was identified as the gene mutated in ataxia oculomotor apraxia type 1 (AOA1), a spinocerebellar ataxia that shares characteristics with known DNA repair disorders such as ataxia telangiectasia (AT), ataxia telangiectasia-like disorder (ATLD), and spinocerebellar ataxia with axonal neuropathy 1 (SCAN1), caused by mutations in ATM, MRE11, and TDP1, respectively [8,9]. Aprataxin is amember of the histidine triad (HIT) superfamily of nucleotide hydrolases and transferases, named for their characteristic HψHψHψψ catalytic motif, where ψ is a hydrophobic amino acid [9]. It also contains an N-terminal forkhead associated (FHA) domain, which is thought to mediate interactions with the ligase cofactors XRCC1 and XRCC4, and a C-terminal zinc finger domain implicated in DNA binding [10–12]. In addition to removing 5′ AMPs, Aprataxin has also been reported to possess 3′ processing activity [13]. This activity is only observed at higher enzyme concentrations, however, suggesting that 5′ AMP removal is the primary function of Aprataxin.

While the biochemical activity of Aprataxin has been well-characterized *in vitro*, how the protein functions in the context of DNA repair *in vivo* is less clear. Whether cells lacking Aprataxin are sensitive to DNA damaging agents is controversial, indicating that redundant mechanisms may cooperate to repair abortive ligation intermediates [14–17]. Recent reports in which Aprataxin has been inactivated in addition to other repair deficiencies have attempted to address this issue. Caldecott and co-workers showed that treatment of Aprataxin null cells with aphidicolin, which inhibits DNA synthesis in long-patch base excision repair (LP-BER), causes a delay in repair of H_2O_2- and MMS-induced damage [16]. Cells mutant for both Aprataxin and Tdp1, a 3′ processing enzyme, showed a similar phenotype [18]. These data support the idea that Aprataxin cooperates with other DNA repair mechanisms in processing 5′ AMPs.

We sought to investigate the role of Aprataxin *in vivo* using the yeast *Saccharomyces cerevisiae* as a model system, which allowed for rapid construction of mutants in multiple DNA repair pathways. Aprataxin-like proteins exist in lower eukaryotes, and of the HIT family members present in yeast, only Hnt3 can remove 5′ AMPs *in vitro*, suggesting that it is the functional homolog of Aprataxin [7]. Here, we tested multiple DNA damaging agents and did not observe sensitivity in *hnt3Δ* single mutants, but deletion of *HNT3* in strains defective for 3′ end processing (*apn1Δ apn2Δ tpp1Δ*), LPBER (*rad27Δ*), and glycosylase/lyase activity (*ntg1Δ ntg2Δ ogg1Δ*) caused synergistic sensitivity to agents that cause single-strand breaks. The pattern of sensitivity in these strains allowed us to identify potential repair intermediates where abortive ligation is predicted to occur. Mutation of conserved residues in the Hnt3 HIT and Zn-finger domains enhanced H_2O_2 sensitivity in the *rad27Δ* background as much as *HNT3* deletion, and human Aprataxin was able to substitute for Hnt3 in yeast. We also report increased sister chromatid exchange in *hnt3Δ* cells. *HNT3* deletion unexpectedly rescued theH_2O_2 sensitivity of recombination mutants *rad51Δ* and *rad52Δ*, but enhanced H_2O_2 sensitivity in the *rad50Δ* background. To explain this, we propose that Hnt3 might promote recombinational repair of strand breaks with 5′ AMP ends. Loss of Hnt3

could rescue *rad51Δ* and *rad52Δ* H_2O_2 sensitivity by preventing formation of a toxic intermediate that must be resolved by recombination.

2. Materials and methods

2.1. Yeast strains

S. cerevisiae strains were isogenic derivatives of the previously described wild-type strains YW388 (*MATa-inc ade2Δ 0 his3Δ 200 leu2 met15Δ 0 trp1Δ 63 ura3Δ 0*) and YW465 (*MATα ade2Δ 0 his3Δ 200 leu2 met15Δ 0 trp1Δ 63 ura3Δ 0*) [19]. YW778 (*apn1Δ*::*HIS3 apn2Δ*::*kanXM4 tpp1Δ*::*MET15*), YW1715 (*rad27Δ*::*KanMX4 carrying pTW572*) and YW1807 (YW389 carrying pTW572) were previously described [20,21]. All strains created for this study are listed in Supplemental Table 1. Deletions were introduced via the one-step gene replacement technique [22]. JDY102 (*hnt3-H130A*) and JDY103 (*hnt3-CCYC(188:191)AAYA*) were constructed by replacing *URA3* in JDY76 using a tailed PCR product containing the mutant allele, and the mutations were confirmed by sequencing. YW1621 was created by amplifying the *HIS3* sister chromatid exchange allele at the can1 locus (obtained from Mike Fasullo) with flanking primers and transforming the PCR product into YW465 [23].

2.2. Survival curves and spotting assays

For survival curves, cells growing exponentially in YPAD media were washed twice with water, resuspended in 20 mM potassium phosphate buffer (pH 7.5) at an OD_{600} of 0.500, and exposed to varying concentrations of H_2O_2, MMS or bleomycin at 30 °C with shaking for 1 h. Survival was determined by plating serial dilutions to synthetic complete plates. In spotting assays, overnight YPAD cultures were diluted to an OD_{600} of 0.500 and 10-fold serial dilutions were spotted to YPD plates containing the indicated concentration of the agent. Briefly, cultures were diluted to 10,000 cells/ml in YPAD containing the indicated drug concentration. After 28 h of growth, survival was determined by plating to nonselective media. At this time, untreated wild-type cultures were nearing the end of the exponential growth phase.

2.3. Sister chromatid exchange frequency

A previously described *HIS3*-based assay was used to measure the sister chromatid exchange frequency [23]. Cells were either mock-treated or treated with H_2O_2 as described above and plated to synthetic complete media or media lacking histidine. The SCE frequency was calculated as the fraction of cells that grow on plates lacking histidine.

2.4. Construction of Aprataxin and Hnt3 expression plasmids

HNT3 or human *APTX* coding sequences were amplified from wild-type yeast genomic DNA or a lambda library of human cDNA [24], respectively, using primers with tails homologous to pTW438. The PCR products were then gap repaired into the 2μ plasmid pTW438 digested with HindIII (NEB) in the *rad27Δ hnt3Δ* yeast strain JDY107. This produced plasmids expressing C-terminal NLS-Myc tagged Hnt3 or Aprataxin from the *ADH1* promoter. The plasmids were sequenced to verify the lack of mutations. Sequencing was done by the McGill University and Genome Quebec Innovation Centre. Primer sequences are available upon request.

2.5. NHEJ oligonucleotide-modified plasmid assay

OMPs were created as previously described [25]. Briefly, two pairs of annealed oligonucleotides designed to restore the *ADE2* coding sequence were ligated onto the ends of pTW423 digested with BglII and XhoI. Plasmids were purified by agarose gel

electrophoresis and the concentration was quantified by UV spectrometry. For DSBs with 3′ hydroxyls, 5′ phosphates were added to OMPs with T4 polynucleotide kinase (NEB) followed by a second round of purification. For construction of DSBs containing 3′ phosphates, oligonucleotides were synthesized with 5′ phosphates to prevent removal of 3′ phosphates by the 3′ phosphatase activity of T4 polynucleotide kinase. Oligonucleotide ligation was monitored by primer extension, and 70–90% of the plasmid was typically ligated (data not shown). All strains used in the NHEJ assays carried pTW572, a *HIS3*-marked *RNH35* overexpression plasmid that complements the Okazaki fragment processing defects and reduces the toxicity of the transformation procedure in *rad27Δ* cells [21,26]. For joints with compatible overhangs (Fig. 6A), 100 ng OMP was co-transformed with 100 ng of the ClaI-digested (Fig. 6) or uncut (Supplemental Fig. 1) control plasmid pTW571 [21]. For gapped joints (Fig. 6B), which are joined less efficiently, 600 ng OMP was used.

3. Results

3.1. Deletion of HNT3 increases sensitivity to H_2O_2 and MMS when 3′ processing is impaired

Like Aprataxin, Hnt3 can process stalled ligation intermediates *in vitro* [7]. As a starting point for our analysis, we hypothesized that abortive ligation might occur at BER intermediates that contain 5′ phosphates, but cannot be ligated due to a damaged 3′ terminus or base. Models for three such intermediates are shown in Fig. 1. To determine whether Hnt3 is indeed important for processing of these substrates *in vivo*, we constructed an *hnt3Δ* strain and examined sensitivity to the DNA damaging agents H_2O_2 and MMS. H_2O_2 creates strand breaks with blocked 3′ termini, such as 3′ phosphates, which might undergo abortive ligation if a 5′ phosphate is present (Fig. 1A). MMS produces alkylated bases, which are rapidly processed by glycosylases to yield AP sites [27]. AP sites are cleaved by the AP endonucleases Apn1 and Apn2, forming strand breaks with 3′ hydroxyls and 5′ deoxyribosephosphates (dRPs) [28,29]. Despite the presence of a 3′ hydroxyl and a 5′ phosphate, this intermediate has been shown to undergo abortive ligation *in vitro* because it lacks an intact base [12] (Fig. 1B). As shown in Figs. 2A, 3B and E, *hnt3Δ* cells were not sensitive to either H_2O_2 or MMS, in agreement with recent reports showing wild-type levels of repair in Aprataxin-deficient human cells exposed to these agents [15,16].

The lack of sensitivity in *hnt3Δ* cells might be explained by rapid action of DNA repair enzymes on blocked 3′ ends. To test this idea, we deleted *HNT3* in an *apn1Δ apn2Δ tpp1Δ* strain, in which 3′ end processing is strongly compromised due to the lack of 3′ phosphodiesterase activities of Apn1 and Apn2 and 3′ phosphatase activity of Tpp1 [20]. This strain would be expected to accumulate persistent strand breaks with blocked 3′ termini upon treatment with H_2O_2 (Fig. 1A). The *apn1Δ apn2Δ tpp1Δ* strain was highly sensitive to H_2O_2, in agreement with previous results, and this sensitivity was exacerbated upon additional deletion of *HNT3* (Fig. 2A) [20]. The effect was relatively modest, however.Wereasoned that loss of the ability to remove 5′ AMPs might not have a strong effect because the cells in which abortive ligation occurs are already likely to die as a result of their 3′ processing defect. We therefore investigated whether *HNT3* deletion would instead have a greater effect in the *apn1Δ apn2Δ* double mutant, which possesses functional Tpp1 and retains the ability to process 3′ phosphates. Indeed, loss of *HNT3* sensitized these cells to H_2O_2 (Fig. 2B). This was especially evident at the highest dose, where the triple mutant was 18-fold more sensitive than the double (Fig. 2B). In agreement with previous data, *apn1Δ apn2Δ tpp1Δ* cells were sensitive to bleomycin, another agent that causes strand breaks with blocked 3′ termini, but loss of *HNT3* had no additional effect (Fig. 2C) [20]. This implies that a specific type of damage induced by H_2O_2 but not bleomycin is a substrate for abortive ligation.

Aprataxin has been shown to possess 3′ processing activity in addition to its 5′ AMP removal activity, but whether yeast Hnt3 also has this activity is unknown [13]. To further investigate whether loss of this activity might explain the enhanced H_2O_2 sensitivity observed in 3′ processing-deficient strains, we introduced an Hnt3 overexpression plasmid into *apn1Δ apn2Δ tpp1Δ* cells. If Hnt3 is indeed a 3′ processing enzyme, overproducing it might restore resistance to H_2O_2 in this strain. This was not the case, however, as the Hnt3 plasmid had no effect compared to the vector alone (Fig. 2D). This result is consistent with the 5′ AMP processing activity of Hnt3 primarily accounting for the phenotypes we observed.

3.2. HNT3 deletion sensitizes ntg1Δ ntg2Δ ogg1Δ cells to H_2O_2, but not MMS

In the nucleotide incision repair (NIR) pathway, the endonuclease activity of Apn1 cleaves upstream of an oxidized base, leaving a strand break with a 3′ hydroxyl adjacent to a flap containing a 5′ phosphate and the damaged base, a potential substrate for abortive ligation (Fig. 1C) [30]. To test this possibility, we deleted *HNT3* in an *ntg1Δ ntg2Δ ogg1Δ* background. These cells lack the glycosylases that cleave the N-glycosidic bond of oxidized bases, forcing the cell to utilize the NIR pathway. The *ntg1Δ ntg2Δ ogg1Δ hnt3Δ* strain was indeed more sensitive to H_2O_2 than the *ntg1Δ ntg2Δ ogg1Δ* strain (Fig. 2E), supporting the idea that abortive ligation can occur at NIR intermediates.

Because the glycosylases also act as AP lyases, their deletion could drive repair of MMS-induced damage down the AP endonuclease pathway, leading to accumulation of 5′ dRPs adjacent to 3′ hydroxyls and increased dependence on Hnt3. The *ntg1Δ ntg2Δ ogg1Δ* strain was no more sensitive to MMS than the parent, and loss of *HNT3* had no further effect (data not shown). This result is consistent with the idea that the AP lyases process only a small fraction of AP sites *in vivo*, and the 5′ dRP ends created by AP endonucleases can be efficiently processed by LP-BER. In this scenario, the 3′ hydroxyl terminus serves as a primer for DNA synthesis, and the resulting 5′ flap containing the AMP could then be cleaved by the flap endonuclease Rad27, the homolog of human FEN1 [31] (Fig. 1).

3.3. Hnt3 cooperates with long-patch BER in repair of oxidation and alkylation damage

To directly test the possibility that Hnt3 and LP-BER act in parallel to process 5′ AMP-containing SSBs, we constructed *rad27Δ hnt3Δ* strains and treated these with H_2O_2 and MMS. Indeed, the double mutant was more sensitive to both agents than either single mutant (Fig. 3A, B and E), implying that abortive ligation can occur during processing of both H_2O_2- and MMS-induced lesions under conditions where 3′ processing is not impaired. While loss of *HNT3* caused robust sensitivity to H_2O_2 in a *rad27Δ* background (Fig. 3A), the effect was less pronounced with MMS when tested via both transient and chronic exposure (Fig. 3B and E). To test whether loss of *HNT3* affects activation of the DNA damage checkpoint, we measured Rad53 phosphorylation by Western blotting and cell cycle progression by FACS in wild-type, *hnt3Δ*, *rad27Δ* and *rad27Δ hnt3Δ* cells after H_2O_2 treatment. *rad27Δ* cells showed increased Rad53 phosphorylation and a prolonged S-phase, but *HNT3* deletion had no additional effects, indicating that checkpoint activation occurs normally in the absence of Hnt3 (data not shown).

To gain further insight into the types of lesions at which abortive ligation occurs, we treated *rad27Δ hnt3Δ* cells with a variety of DNA damaging agents. The double mutant was synthetic sensitive to *tert*-butyl hydroperoxide, an oxidizing agent thought to create damage similar to H_2O_2 (Fig. 3C) [32,33]. We did not observe synthetic sensitivity to bleomycin, 4-NQO, a UV-mimetic agent that produces base adducts, or the topoisomerase 1 poison camptothecin (Fig. 3D and E). These data suggest that these agents do not produce DNA

structures that undergo failed ligation at a substantial level, or that any such intermediates are rapidly processed even in the absence of *HNT3* and LP-BER.

3.4. Point mutations in the HNT3 catalytic and Zn-finger domains confer a null phenotype

Human Aprataxin contains an N-terminal FHA domain that mediates interactions with other DNA repair proteins, a central catalytic HIT domain, and a C-terminal zinc finger domain that is required for DNA binding [8,12]. The HIT and Zn-finger domains are conserved in *S. cerevisiae* Hnt3, but not the FHA domain. We introduced *HNT3* point mutations at the chromosomal locus to investigate the roles of these domains in DNA repair *in vivo*. In the HIT domain, histidine 130 was mutated to alanine. The corresponding mutation in Aprataxin eliminates catalytic activity of the enzyme *in vitro* [7]. Alanine substitutions were also introduced for conserved cysteines 188, 189 and 190, which presumably coordinate the Zn^{2+} ion in the Zn-finger. Analogous Aprataxin Znfinger mutations eliminate DNA binding [12]. In combination with *rad27Δ*, both *hnt3-H130A* and *hnt3-CCYC(188:191)AAYA* were as sensitive to H_2O_2 as *hnt3Δ* (Fig. 4A), indicating that the HIT and Zn-finger domains are required for Hnt3 function *in vivo*.

3.5. Human Aprataxin can complement hnt3Δ in yeast

To determine whether human Aprataxin can substitute for Hnt3 in yeast, we introduced a plasmid into the *rad27Δ hnt3Δ* strain in which C-terminal Myc-NLS-tagged Aprataxin is expressed from the *ADH1* promoter. An *HNT3* expression plasmid with the same tag was used as a control. Expression of Aprataxin restored the H_2O_2 resistance to a similar extent as *HNT3*, indicating that Aprataxin can substitute for Hnt3 *in vivo* (Fig. 4B). These data further establish that Hnt3 and Aprataxin are indeed homologs.

3.6. HNT3 deletion does not affect the mutation rate, but increases spontaneous and induced sister chromatid exchange

Loss of DNA repair genes often causes a mutator phenotype due to increased use of error-prone lesion bypass and backup repair mechanisms. We therefore used the canavanine forward mutation assay to measure the spontaneous mutation rate in *hnt3Δ* and *rad27Δ hnt3Δ* strains. As previously reported, the mutation rate was increased in *rad27Δ* yeast, but *HNT3* deletion had no further effect (Supplemental Table 2) [34]. In addition, the H_2O_2-induced mutation frequency was not affected by loss of *HNT3* (Supplemental Table 2). These data indicate that the mechanisms utilized to repair persistent adenylated strand breaks in the absence of *HNT3* and *RAD27* are not mutagenic.

SSBs with 5′ AMPs could be converted to DSBs upon collision with replication forks. Such DSBs would be expected to be repaired by recombination with the newly replicated intact sister chromatid. To test this hypothesis, we used a previously described system in which two partial fragments of *HIS3* are placed on chromosome IV, and full-length *HIS3* can only be restored by a sister chromatid exchange (SCE) event [23]. In this assay, the *hnt3Δ* strain spontaneously gave rise to 4.8-fold more His+ colonies than wild-type (Fig. 5A). Upon treatment with H_2O_2, the SCE frequency was higher in both wild-type and *hnt3Δ*, and as above, deletion of *HNT3* led to a 3.6-fold increase in His+ colonies (Fig. 5B). Therefore, loss of *HNT3* leads to higher levels of recombination, likely caused by persistent SSBs.

We next measured SCE events in *rad27Δ* and *apn1Δ apn2Δ tpp1Δ* strains. While the spontaneous and H_2O_2-induced SCE frequencies were elevated in both compared to wild-type, additional deletion of *hnt3Δ* had no further effect (Fig. 5A and B). Thus, loss of LP-BER or 3′ processing masks the *hnt3Δ* effect that is observed in a wild-type background (see Section 4). To gain further insight into the connection between Hnt3 and recombination, we attempted to quantify Rad52-GFP foci in wild-type, *hnt3Δ*, *rad27Δ* and *rad27Δ hnt3Δ* cells,

but found that H_2O_2 did not induce Rad52 foci even at lethal doses and after prolonged incubation (data not shown). Spontaneous Rad52 foci were visible in ~30% of *rad27Δ* cells as compared to ~1% of wild-type cells, but loss of *HNT3* had no further effect in either background (data not shown).

3.7. Loss of HNT3 partially rescues H_2O_2 sensitivity in rad51Δ and rad52Δ mutants, but increases the sensitivity of a rad50Δ strain

The observation that SCE is increased in *hnt3Δ* yeast suggests that recombination acts as an alternative mechanism for repair of 5′ adenylated strand breaks. To further test this hypothesis, we deleted *HNT3* in *rad50Δ*, *rad51Δ*, and *rad52Δ* mutants, in which different steps of recombination are impaired. Rad50 is part of the Mre11/Rad50/Xrs2 (MRX) complex, which has nuclease activity implicated in 5′ resection of DSBs and is also important for checkpoint activation [35–38]. After resection, Rad51 coats the newly generated 3′ ssDNA tails, promoting strand exchange [39]. Rad52 promotes pairing of complementary strands and is required for all forms of recombination [40]. We predicted that the double mutants would show increased sensitivity to H_2O_2, but this was only observed for the *rad50Δ hnt3Δ* strain (Fig. 5C). Surprisingly, *HNT3* deletion partially rescued sensitivity to H_2O_2 in the *rad51Δ* and *rad52Δ* backgrounds (Fig. 5D and E, respectively). These data imply that Hnt3 activity at replication-associated DSBs is toxic in the absence of Rad51 or Rad52. Parallel roles for Hnt3 and MRX-catalyzed resection in 5′ AMP removal could account for the synthetic sensitivity observed in *rad50Δ hnt3Δ* cells. To explain the rescue of *rad51Δ* and *rad52Δ*, we speculate that Hnt3 may act to promote recombination upstream of strand invasion and exchange catalyzed by Rad51 and Rad52 (see Section 4). Loss of *HNT3* had no effect on MMS sensitivity in *rad50Δ* or *rad52Δ* backgrounds (Fig. 5F), indicating that this phenomenon is specific to H_2O_2. Thus, Hnt3 may affect recombination mediated by H_2O_2-induced single-strand breaks but not MMS-induced AP sites.

3.8. Lack of evidence for Hnt3 involvement in nonhomologous end joining

The nonhomologous end joining (NHEJ) pathway has long been known to rejoin DSBs with damaged or mismatched termini, making it a likely scenario for abortive ligation [21,41]. The observation that Aprataxin interacts with XRCC4, which forms a stable complex with DNA ligase IV and is required for NHEJ, also suggests a role for Aprataxin in NHEJ [11]. To test this directly, we used oligonucleotide-modified plasmids (OMPs) to ask whether Hnt3 is required for NHEJ of DSBs that are likely substrates for failed ligation [25]. In this system, annealed oligonucleotides are ligated onto the ends of a linearized plasmid in an essential region of the *ADE2* gene, allowing selection for a specific joint. A control plasmid digested with ClaI within the *LEU2* gene is co-transformed with the OMP and is repaired by “simple religation” NHEJ [21]. Because this DSB does not require end processing and is not a substrate for abortive ligation, a reduction in the ratio of Ade+ to Leu+ colonies indicates a specific defect in processed NHEJ. We used this system to create a panel of DSBs with 4-base 3′ overhangs in which the annealed bases were kept constant. DSBs were constructed to contain 5′ phosphates adjacent to each possible combination of 3′ phosphates and 1-nt gaps (Fig. 6). Premature 5′ adenylation would be expected to occur only if the presence of a gap or 3′ phosphate causes an abortive ligation attempt. Since Rad27 has been implicated in NHEJ and functions in parallel with Hnt3 in BER, we tested *hnt3Δ*, *rad27Δ* and *hnt3Δ rad27Δ* strains in addition to wild-type and *pol4Δ* controls [21,42,43]. None of the strains we tested showed a significant defect at DSBs with no gaps, regardless of the presence of a 3′ phosphate (Fig. 6A). At gapped DSBs, the *rad27Δ* strain showed a partial defect when a 3′ phosphate was present, but loss of *HNT3* had no additional effect (Fig. 6B). Additive effects at processed joints were not observed when the experiment was repeated using a supercoiled plasmid control, although these data were complicated by the low and highly variable rate at

which *rad27Δ* strains are transformed with circular plasmids (Supplemental Fig. 1). These data indicate that DSBs with blocked termini can be efficiently rejoined in the absence of both Rad27 and Hnt3. Thus, while this does not rule out a role for Hnt3 in NHEJ, it shows that redundant mechanisms exist for 5′ end processing.

4. Discussion

The ability of Aprataxin to remove 5′ AMPs has been well-characterized *in vitro*, but how this activity functions in the context of DNA repair *in vivo* remains largely unknown [7,12,44]. To address this issue, we used yeast genetics to characterize the role of Hnt3, the homolog of Aprataxin, in DNA repair. We find that *HNT3* deletion increases sensitivity to DNA damaging agents in mutants that accumulate potential abortive ligation substrates. Hnt3 cooperates with Rad27-dependent LP-BER in repair of oxidative and alkylation damage, an idea supported by recent mammalian data [16]. Finally, we present genetic evidence that Hnt3 also affects recombinational repair of H_2O_2-induced damage.

4.1. Hnt3 and Rad27-dependent LP-BER provide parallel pathways for processing adenylated strand breaks

It was recently shown that repair of H_2O_2- and MMS-induced damage is delayed when long-patch DNA synthesis is inhibited by aphidicolin in cells lacking Aprataxin [16]. Our finding that *rad27Δ hnt3Δ* double mutant yeast show synergistic sensitivity to both of these agents (Fig. 3) extends and confirms this result. Together, these data support a model in which adenylated 5′ ends can be processed either via direct reversal by Aprataxin/Hnt3 or by flap cleavage in LP-BER (Fig. 1A). Overexpression of Hnt3 did not increase the H_2O_2 resistance above the level of a *rad27Δ* strain (Fig. 4), suggesting that Rad27 performs functions that cannot be complemented by Hnt3, such as cleavage of non-adenylated 5′ flaps. Our finding that *hnt3Δ* cells are not hypersensitive to H_2O_2 was unexpected given that extracts from *hnt3Δ* yeast cannot repair plasmids damaged by direct treatment with H_2O_2 [7]. A likely explanation for this discrepancy is that LP-BER is active *in vivo*, but a plasmid with numerous SSBs is probably a poor substrate for LPBER. A shattered plasmid may lack stably paired 3′ hydroxyl termini from which to initiate repair synthesis, and yeast extracts may not contain active DNA replication machinery required for long-patch synthesis.

4.2. Identification of DNA lesions that are substrates for abortive ligation in vivo

If the primary role of Aprataxin/Hnt3 is indeed processing of adenylated 5′ ends, synthetic sensitivity upon loss of Hnt3 should only occur when cells are treated with agents that induce DNA structures that undergo abortive ligation. Any strand break that has a 5′ phosphate but cannot be ligated due to the absence of a 3′ hydroxyl or an intact base could potentially be a failed ligation substrate. These could include 3′ phosphates, aldehydes and phosphoglycolates, as well as 5′ dRPs. Our genetic data implicate abortive ligation at single-strand breaks with damaged 3′ termini (Fig. 1A), strand breaks with 3′ hydroxyls and 5′ dRPs that occur as intermediates in AP site processing (Fig. 1B), and oxidized bases cleaved by Apn1 during NIR (Fig. 1C).

The *Schizosaccharomyces pombe* homolog of Aprataxin, SPCC18.09c, was recently identified in a screen for 4-NQO sensitivity, but we did not observe sensitivity to this agent in either the *hnt3Δ* single mutant or in combination with *rad27Δ* (Fig. 3B) [45]. Therefore, processing of 4-NQO damage may produce an intermediate that can undergo abortive ligation in *S. pombe* but not *S. cerevisiae*. The presence of the UV damage endonuclease Uve1 in *S. pombe* but not *S. cerevisiae* might explain this difference [46,47]. Uve1 incises 5′ of UV-induced photoproducts and bulky adducts created by 4-NQO, yielding a 3′ hydroxyl

adjacent to a flap containing the lesion and a 5′ phosphate, a potential substrate for abortive ligation.

In addition to removing 5′ AMPs, Aprataxin has been reported to process 3′ phosphate and 3′ phosphoglycolate ends [13]. Processing of adenylated 5′ ends by Aprataxin is much more efficient, however, likely indicating that this is its physiological function [7]. Whether Hnt3 has 3′ processing activity is currently unknown. Although many of the phenotypes we observed could be explained by 3′ processing defects, we favor the interpretation that impaired 5′ AMP processing is principally responsible for these data, since overexpression of Hnt3 had no effect on the H_2O_2 resistance of 3′ processing-deficient *apn1Δ apn2Δ tpp1Δ* cells (Fig. 2D). The observations that *apn1Δ tpp1Δ rad1Δ* yeast are slow growing and *apn1Δ apn2Δ rad1Δ* cells are inviable due to accumulation of 3′ blocking lesions also argue against Hnt3 providing additional 3′ processing activity in yeast [48].

4.3. HNT3 deletion increases the SCE frequency in wild-type but not repair-deficient backgrounds

hnt3Δ Yeast showed increased spontaneous and H_2O_2-induced SCEs, but this effect was masked in *rad27Δ* and *apn1Δ apn2Δ tpp1Δ* backgrounds (Fig. 5A and B). The SCE frequency in both of these backgrounds is increased relative to wild-type (Fig. 5A and B), likely the result of persistent SSBs stalled at 5′ or 3′ processing steps, respectively. We reasoned that *HNT3* deletion may not further increase the SCE frequency in these strains if Hnt3 acts at a subset of strand breaks that can be repaired by BER in a wild-type background, but are unrepairable except by recombination in the absence of 5′ flap endonuclease or 3′ processing activities. Such breaks are therefore committed to recombinational repair regardless of whether abortive ligation occurs, explaining why *HNT3* deletion does not further increase the SCE frequency in these backgrounds. The observations that *RAD52* deletion is synthetic lethal with both *rad27Δ* and *apn1Δ apn2Δ tpp1Δ* support this idea [20,34].

4.4. Genetic interactions between HNT3 and recombination genes

The inviability of *rad52Δ rad27Δ* and *rad52Δ apn1Δ apn2Δ tpp1Δ* yeast implies that recombination serves as a backup pathway for repair of oxidative DNA damage, likely after SSBs are converted to DSBs by replication fork collision [20,34]. We therefore predicted that *HNT3* deletion would sensitize recombination mutants to oxidizing agents such as H_2O_2. Our observation that loss of *HNT3* partially rescued *rad51Δ* and *rad52Δ* yeast while sensitizing *rad50Δ* cells to H_2O_2 was therefore surprising (Fig. 5C–E). This difference may be explained by the step in recombination affected by each mutation. Rad50 is part of the Mre11-Rad50-Xrs2 (MRX) complex, which is involved in 5′ resection of DSBs, the first step in recombination and a likely mechanism by which the 5′ AMP could be removed [35–37]. Thus, synthetic sensitivity in *hnt3Δ rad50Δ* yeast may be explained by the loss of two parallel pathways for 5′ AMP removal. After resection generates 3′ ssDNA tails, Rad51 and Rad52 catalyze strand invasion and exchange with the template duplex [49]. Removal of the 5′ AMP by Hnt3 might commit the DSB to repair by recombination, perhaps by generating a DNA structure that requires the activity of Rad51 and Rad52 for resolution. In this scenario, *HNT3* deletion would rescue *rad51Δ* and *rad52Δ* cells by preventing formation of this intermediate. Alternative mechanisms of 5′ AMP removal might allow recombination-independent pathways such as post-replication repair or NHEJ to resolve the damage. While we were unable to identify a requirement for Hnt3 in NHEJ (Fig. 6), it remains possible that Hnt3 participates in NHEJ in parallel with unidentified nucleases, or at specific DSB structures that were not included in our analysis. Future work will focus on identifying and characterizing novel biochemical activities of Hnt3 that might provide a more detailed mechanistic explanation for these data.

In summary, the data presented here establish a role for Hnt3 in processing abortive ligation intermediates in parallel with Rad27-dependent LP-BER. We identify multiple BER intermediates including SSBs with blocked 3′ ends and 5′ dRPs formed after AP site cleavage as potential abortive ligation substrates *in vivo*, and we provide genetic evidence that Hnt3 affects downstream DSB repair by affecting recombination. This work establishes roles for Hnt3 in multiple DNA repair pathways *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AOA1	ataxia oculomotor apraxia type 1
AMP	adenosine monophosphate
BER	base excision repair
AT	ataxia telangiectasia
ATLD	ataxia telangiectasia-like disorder
SCAN1	spinocerebellar ataxia with axonal neuropathy 1
HIT	histidine triad
NHEJ	nonhomologous end joining
MMS	methylmethane sulfonate
4-NQO	4-nitroquinoline-1-oxide
SCE	sister chromatid exchange
MRX	Mre11-Rad50-Xrs2
dRP	deoxyribosephosphate
SSB	single-strand break
DSB	double-strand break

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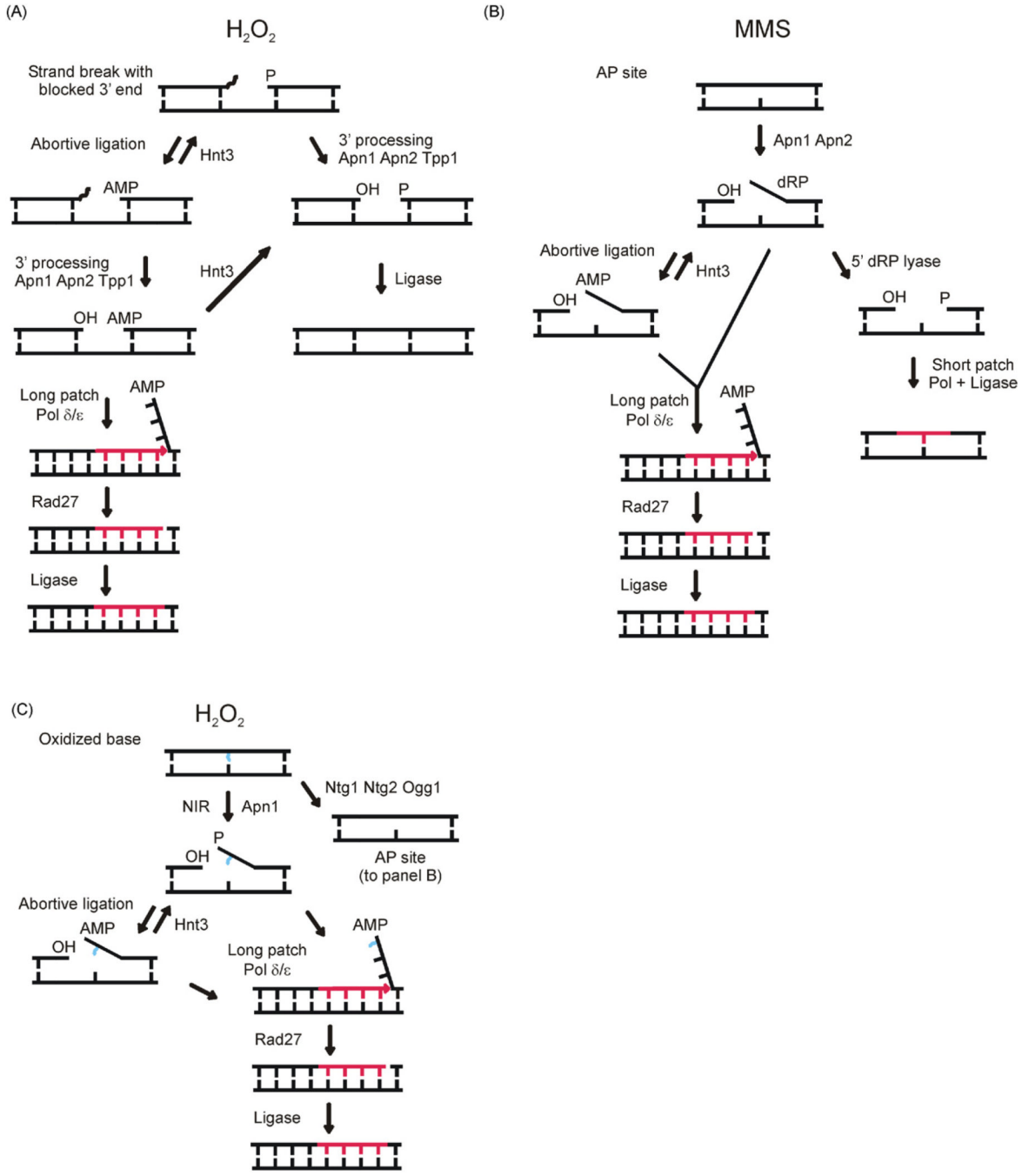

Fig. 1.

Potential roles for Hnt3 in processing stalled ligation intermediates after (A) H_2O_2-induced SSBs, (B) AP sites induced by MMS, and (C) oxidative base damage repaired by NIR.

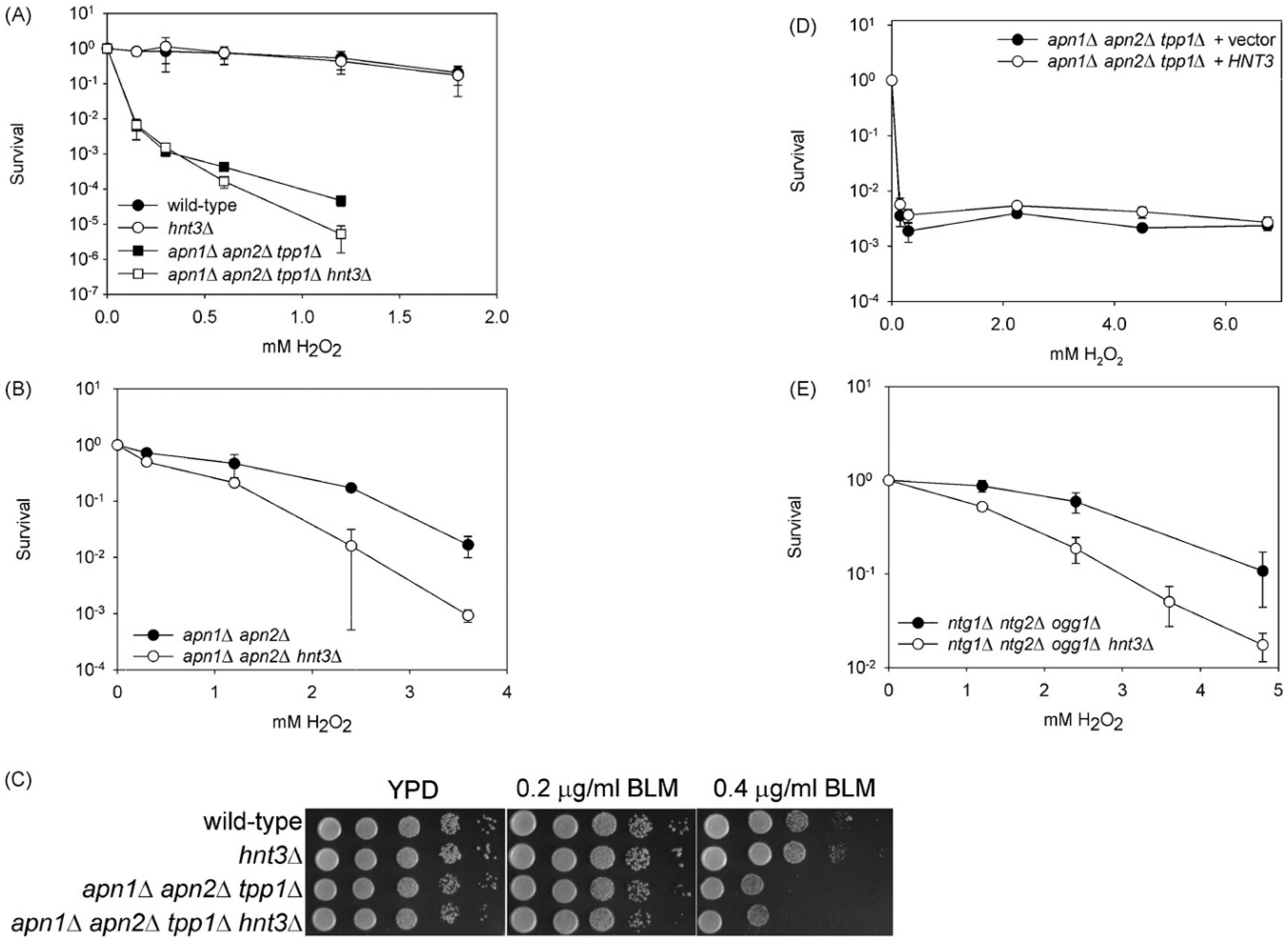

Fig. 2.

HNT3 deletion causes synthetic sensitivity to various DNA damaging agents in the *apn1Δ apn2Δ tpp1Δ* and *ntg1 ntg2 ogg1* backgrounds. (A), (B), (D) and (E) Cells were treated with H_2O_2 for 1 h at the indicated concentrations, and survival was determined relative to the untreated control as described in Section 2. Curves represent the mean±standard deviation of three independent experiments for each strain. (C) 10-fold serial dilutions of cells were spotted to YPD plates containing the indicated concentration of bleomycin.

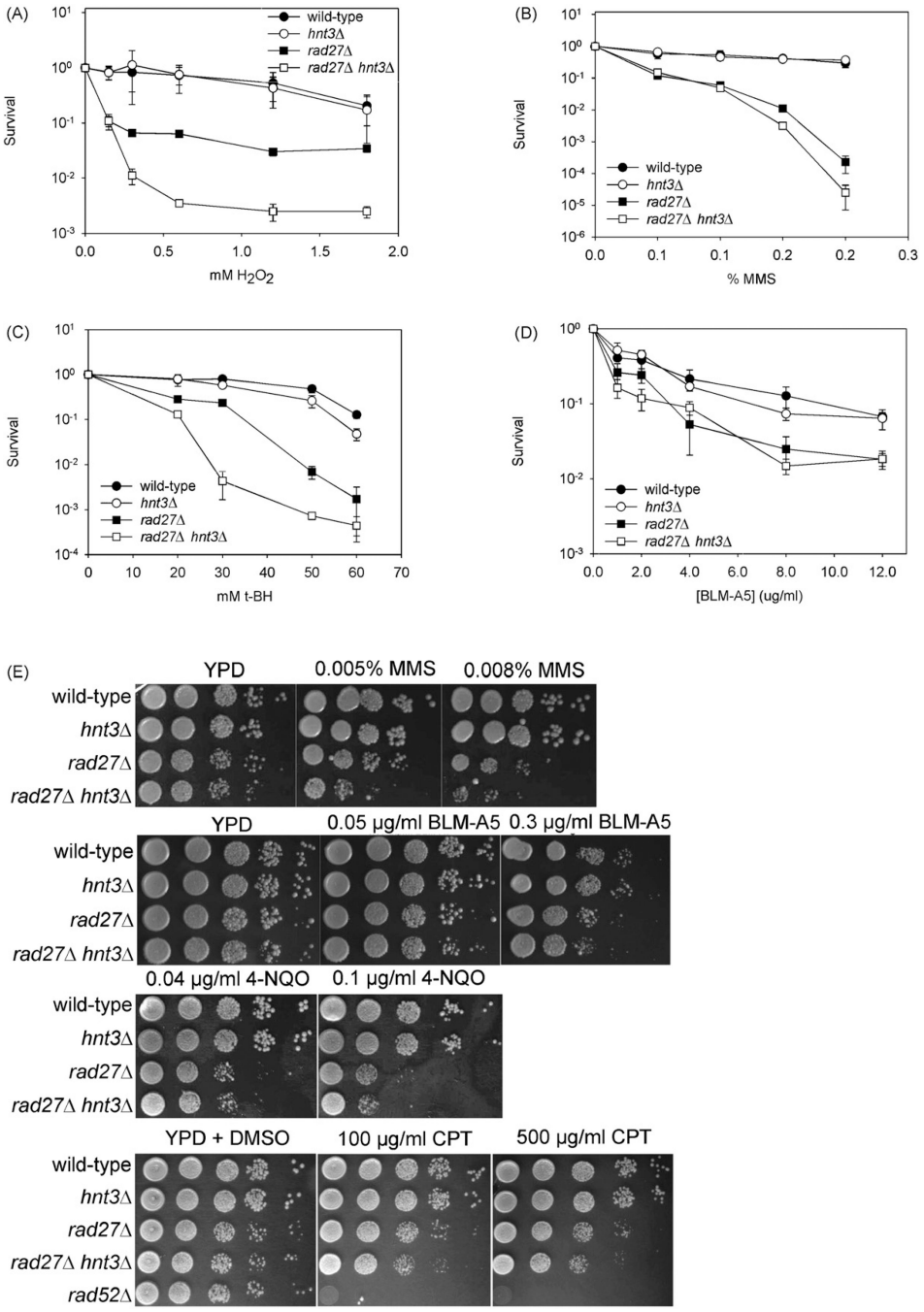

Fig. 3.

rad27Δ hnt3Δ strains show synergistic sensitivity to H_2O_2, t-BH, and MMS, but not other DNA damaging agents. Survival after (A) H_2O_2, (B) MMS, (C) t-BH and (D) bleomycin A5 treatment was determined as in Fig. 2A. (E) Cells were spotted to YPD plates containing the indicated concentration of the agent.

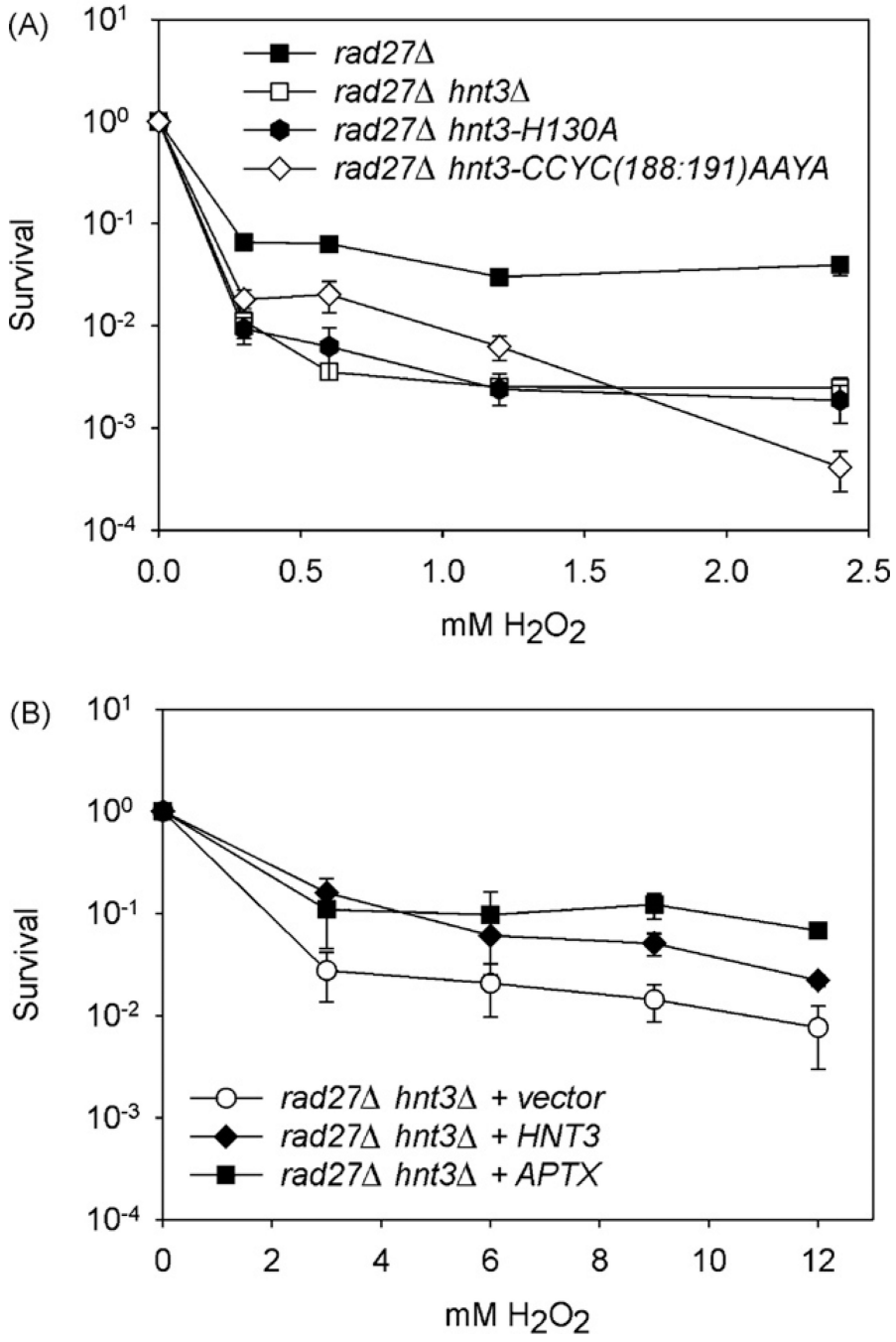

Fig. 4.

Hnt3 point mutations and complementation with human Aprataxin. (A) Point mutations in the Hnt3 HIT and Zn-finger domains cause a null phenotype. Survival after H_2O_2 treatment was determined as in Fig. 2A. (B) Expression of Myc-NLS-tagged human Aprataxin from a plasmid complements *HNT3* deletion. Survival after H_2O_2 treatment was determined as in Fig. 2A, except the strains were grown in synthetic complete media lacking uracil to maintain selection for the plasmid.

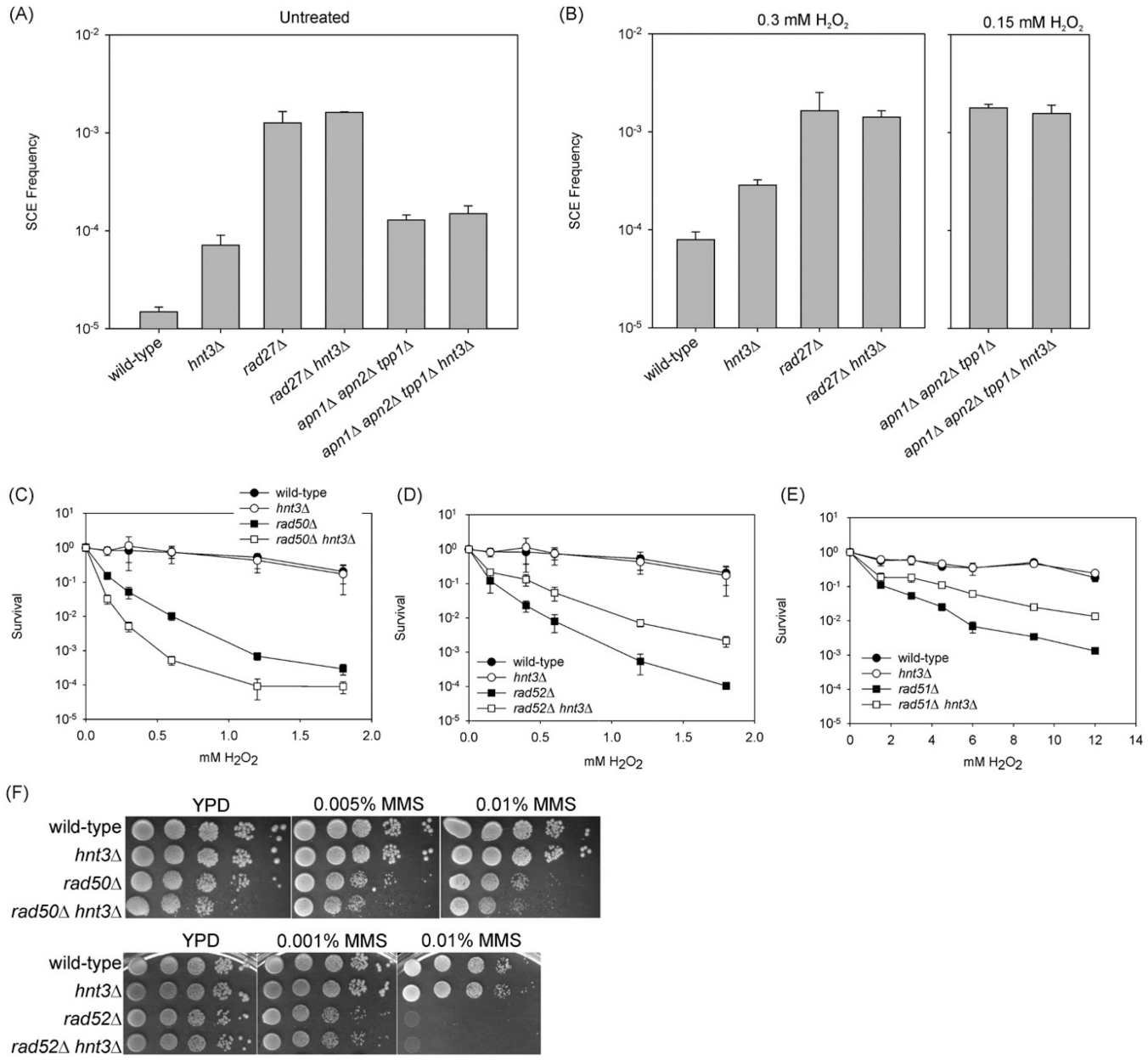

Fig. 5.

hnt3Δ cells show increased sister chromatid exchange, but this effect is lost in *rad27Δ* and *apn1Δ apn2Δ tpp1Δ* backgrounds. SCE frequencies were calculated as described in Section 2 after mock-treatment (A) or treatment with the indicated concentrations of H_2O_2 (B). (C)–(E) *HNT3* deletion sensitizes *rad50Δ* cells to H_2O_2, but rescues *rad51Δ* and *rad52Δ* strains. Survival after H_2O_2 treatment was determined as in Fig. 2A. (F) *HNT3* deletion does not affect MMS sensitivity in *rad50Δ*, *rad51Δ*, or *rad52Δ* backgrounds. Cells were spotted to YPD plates containing the indicated concentration of MMS.

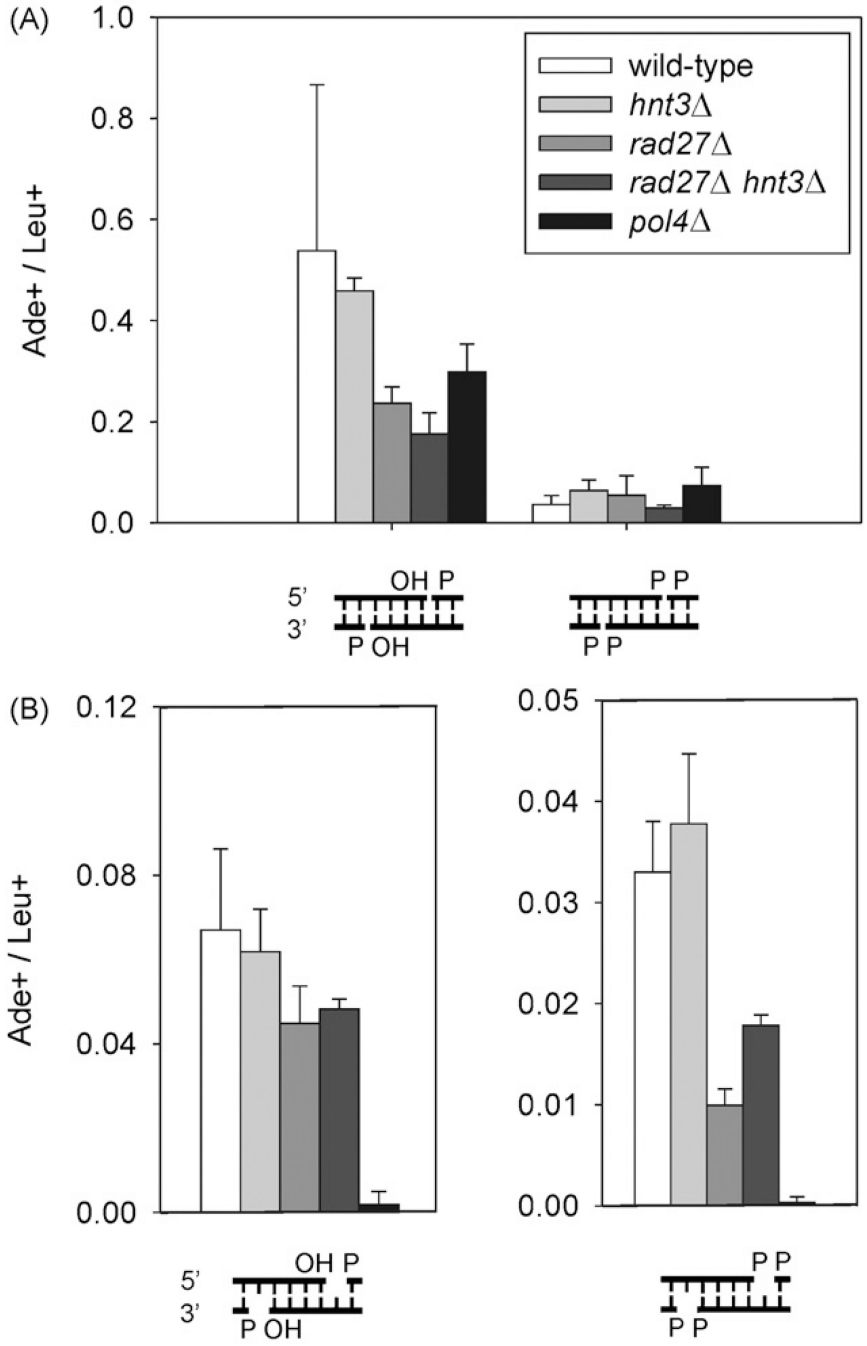

Fig. 6.

NHEJ of DSBs with ligation-blocking 3′ phosphates or gaps does not require Hnt3. (A) and (B) OMPs were used to create DSBs with the indicated structures (see Methods). Repair is expressed as the ratio of Ade+ colonies, which arise from accurate repair of the OMP, to Leu+ colonies, which result from co-transformation of a control plasmid digested with ClaI. A reduction in this ratio indicates a defect in processed NHEJ. Each bar represents the mean ±standard deviation from three independent transformations.