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A mutation in *EXO1* defines separable roles in DNA mismatch repair and post-replication repair

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Abstract

Replication forks stall at DNA lesions or as a result of an unfavorable replicative environment. These fork stalling events have been associated with recombination and gross chromosomal rearrangements. Recombination and fork bypass pathways are the mechanisms accountable for restart of stalled forks. An important lesion bypass mechanism is the highly conserved post-replication repair (PRR) pathway that is composed of error-prone translesion and error-free bypass branches. *EXO1* codes for a Rad2p family member nuclease that has been implicated in a multitude of eukaryotic DNA metabolic pathways that include DNA repair, recombination, replication, and telomere integrity. In this report, we show *EXO1* functions in the *MMS2* error-free branch of the PRR pathway independent of the role of *EXO1* in DNA mismatch repair (MMR). Consistent with the idea that *EXO1* functions independently in two separate pathways, we defined a domain of Exo1p required for PRR distinct from those required for interaction with MMR proteins. We then generated a point mutant *exo1* allele that was defective for the function of Exo1p in MMR due to disrupted interaction with Mlh1p, but still functional for PRR. Lastly, by using a compound *exo1* mutant that was defective for interaction with Mlh1p and deficient for nuclease activity, we provide further evidence that Exo1p plays both structural and catalytic roles during MMR.

Keywords

DNA mismatch repair; EXO1; DNA nuclease; DNA damage and post-replication repair

1. Introduction

Cells expend a great deal of energy and enlist a large number of gene products to faithfully duplicate their genomes [1,2]. S-phase replication checkpoints are cellular failsafe mechanisms

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that arrest cells or slow S-phase progression allowing for the repair, recovery and if these are not possible, the bypass of lesions that prevent duplication of the genome. Replication forks stall because of encounters with upstream lesions or low dNTP pools. Pathways to overcome fork stalling are highly conserved across species. When checkpoints, fork repair or fork bypass processes are defective, stalled replication forks can generate anomalous structures [1]. These recombinogenic structures have been proposed to account for genomic rearrangements at fragile sites and for genomic instability in cancer cells [3]. In fact, human cancers have been shown to have defects in genes for checkpoints, fork repair or fork bypass proteins [3]. Moreover, recent studies have suggested that these replication response pathways serve as the earliest barrier to tumorigenesis [4,5].

Post-replication repair (PRR) is a eukaryotic pathway that facilitates the bypass or tolerance of fork stalling events but does not "repair" these lesions [6-8]. The pathway is defined by the *RAD6-RAD18* heterodimer, which encodes a ubiquitin-conjugating and ubiquitin-ligase enzyme complex. This pathway has been shown to consist of minimally three genetic branches: a checkpoint sub-pathway [9]; an error-free sub-pathway; and an error-prone translesion synthesis sub-pathway [10]. Genetic defects in these sub-pathways can result in increased sensitivity to DNA damaging agents, growth deficiencies and effects on spontaneous and induced mutagenesis [11].

Exo1p is a member of the Rad2 family of structure-specific nucleases which based on in vitro studies possess 5′->3′ exonuclease and 5′-flap endonuclease activities [12,13]. First isolated as a nuclease activity induced during meiosis in fission yeast [14], Exo1p has since been implicated in multiple DNA metabolic pathways that include DNA repair, recombination, replication, and telomere integrity [12,13]. *EXO1* involvement in these multiple pathways makes it a logical target for mutation during oncogenesis; supported by the tumor prone phenotype of a mouse model [15]. Exo1p has been best characterized by its structural and catalytic role during DNA mismatch repair (MMR) mutation avoidance [16-19]. However, based on the *exo1Δ* mutational spectra and genetic interaction with *REV3*, we previously hypothesized that *EXO1* participates in at least one MMR-independent mutation avoidance pathway [12]. In agreement with our supposition, recent studies have identified a potential role for *EXO1* in the maintenance and repair of stalled replication forks [20-22].

Here, we report on the role of *EXO1* in the tolerance to the fork stalling lesion(s) produced from low doses of methylmethane sulfonate (MMS) and the MMR dependency of this function. Based on our findings, we propose that *EXO1* functions in the *MMS2* error-free branch of the PRR pathway, independent of the role of *EXO1* in MMR. Consistent with the idea that *EXO1* functions in two separate genome stability pathways, we defined a domain of Exo1p required for PRR distinct from those required for interaction with MMR proteins. Finally, we generated an *exo1* missense mutant that was functional for PRR, but defective for MMR.

2. Materials and methods

2.1. Strains and media

E. coli strain DH-10B was used for plasmid construction and amplification. *S. cerevisiae* strains used in this study are described in Table 1. Bacterial and yeast strains were grown under conditions described previously [19]. Yeast transformations were performed by the polyethylene glycol-lithium acetate method [23].

The genomic *exo1* point mutant strains [19] (Table 1) were created with a two-step recombination procedure as described previously (for *exo1-D173A*) and as below (for *exo1-FF447AA*). Targeting construct YIp-exo1-FF477AA was digested with *Xma*I and transformed into appropriate strains as described previously [19]. We screened for the mutant alleles using

the PCR oligos: EXO1-999.S, 5′-CGACGACGATATAGATCACCAC-3′; and EXO1-1501.AS, 5′-CACTCAGGTTGTCGTCATCCTC-3′. The exo1-FF4477AA mutation creates a second *Alu*I site in this interval. All point mutants were also confirmed by sequencing using oligo EXO1-1230.S, 5′-CATCCATAGTCTAAGACAAGCGG-3′.

All other yeast deletion mutants were created using PCR generated disruption amplicons from strains provided by the *Saccharomyces* Genome Deletion Project.

2.2. Plasmid construction

All DNA manipulations were performed using standard molecular biology procedures [24]. DNA sequencing was performed at OHSU Microbiology and Immunology core sequencing facility with an ABI automated sequencer.

(i) Targeting vectors—YIp-exo1-FF447AA was created as follows. A *Bam*HI-*Xma*I fragment of *EXO1* containing the *FF447AA* mutation taken from *pJAS-exo1-FF447AA* was cloned into YIp-EXO1 and replaced the wildtype *Bam*HI-*Xma*I segment. The correct clone was confirmed by sequencing.

(ii) Expression plasmids—The construction of pJAS-EXO1-FLAG and nuclease deficient variants and pJAS-RAD27 were described previously [19]. The *pJAS-exo1-FF447AA* and *pJAS-exo1-D173A,-FF447AA* constructs were made as follows.

The *exo1-FF4477AA* allele was created using the Quikchange™ Site-Directed Mutagenesis Kit (Stratagene) on pJAS-EXO1-FLAG using the following oligos: exo1-FF447AA.S, 5′-GGATACAAGAAGCAAAGCTGCTAATAAACCCTCCATG-3′; and exo1-FF447AA.AS, 5′-CATGGAGGGTTTATTAGCAGCTTTGCTTCTTGTATCC-3′. The region between unique internal *Bam*HI and *Xma*I sites was sequenced for the *FF447AA* allele and to exclude any second site mutations. The desired *Bam*HI- *Xma*I fragment was cloned back into the parent *pJAS-EXO1-FLAG* and *pJAS-exo1-D173A-FLAG* to produce *pJAS-exo1-FF447AA-FLAG* and *pJAS-exo1-D173A,-FF447AA-FLAG*, respectively.

2.3. Mutation rates assays

Measurement of mutation rates were performed as stated previously [19]. Briefly, strains were streak purified, individual colonies grown to saturation in YPD or -Trp, then various dilutions plated onto complete synthetic media (CSM), -Thr and + Canavanine (+CAN) [60 μg/ml] plates and colonies counted after 2-3 days growth at 30°C. Rates were determined as previously described. Statistical analysis was performed using Prism version 2a software (GraphPad Software Inc.).

Patch assays were performed by streak purifying strains, individual colonies patched onto YPD or –Trp plates, grown to confluence at 30°C, then replica plated onto -Thr or -Thr -Trp plates and colonies counted after 2-3 days growth at 30°C.

2.4. Two-hybrid Analysis

Protein-protein interactions were assessed using the two-hybrid technique as described previously [18]. Four independently generated pGAD-exo1-FF447AA “prey” clones were tested. “Bait” and “prey” plasmids were transformed into L40, growth on –TRP –URA plates served as a control, while growth on –TRP –URA –HIS plates indicated “bait”-“prey” interaction. L40 has a second chromosomal lexA-GAL4A reporter system, *URA3*::(*lexAop*)$_8$-*lac*Z. β-galactosidase assays were performed on –TRP –URA –HIS plates as described [25]. Reactions were placed at 30°C until desired blue color development was achieved.

2.5. Methylmethane Sulfonate Epistasis Analysis

Designated single and double mutant combinations were grown overnight to saturation in YPD, serially diluted (1:5), dilutions spotted onto YPD and YPD containing 0.0005-0.0175% methylmethane sulfonate (MMS) plates with a 48-prong replicator and allowed to grow at 30° C for 3-4 days.

2.6. Plasmid Loss Assay

To assess synthetic lethality of *exo1* mutations in combination with *rad27Δ*, we performed the assay as described previously 2-3 times per experiment [24].

3. Results

3.1. Epistasis analysis defines a role for EXO1 in the MMS2 error-free branch of PRR that is MMR-independent

Treatment of *S. cerevisiae* with low doses of MMS has been used to assess the function of the intra-S-phase checkpoint and replication fork restart/bypass processes [26-29]. We performed epistasis analysis for sensitivity to low doses of MMS between *EXO1* and genes known to be involved in PRR or replication fork bypass. The *rad6Δ* and *rad18Δ* mutations, the founding genes of the PRR pathway, displayed an epistatic relationship to *exo1Δ* (Figure 1A) [11], suggesting that *EXO1* functioned in PRR for MMS tolerance. As mentioned previously, PRR is composed of at least three genetically distinct sub-pathways: a checkpoint pathway; an error-free pathway; and an error-prone pathway, defined by the genes *RAD9* [9], *MMS2* [30] and *REV3* [10,31,32], respectively. We found that *mms2Δ* was epistatic to *exo1Δ*, but that both *rad9Δ* and *rev3Δ* were synergistic with *exo1Δ* (see Figure 1B-D) by epistasis analysis for MMS sensitivity. These data suggested that *EXO1* functions in the *MMS2* error-free sub-pathway of PRR and is redundant to the *RAD9* checkpoint and *REV3* error-prone sub-pathways. *RAD5* has been mapped to the *MMS2*-error free branch by both genetic and protein interaction data [33-35]. Interestingly, we were unable to generate an *exo1Δ rad5Δ* double mutant suggestive of a synthetically lethal interaction (see Figure 1A).

EXO1 was first isolated in a screen for recombination gene products. Recombination plays a role in replication restart of stalled forks independent of the PRR pathway and possibly some facet of the *MMS2* error-free bypass pathway of PRR [36]. Consistent with a role of *EXO1* in recombinational bypass of stalled replication forks, the recombination mutants *rad52Δ* and *rad51Δ* demonstrated a epistatic relationship to *exo1Δ* for MMS sensitivity (see Supplemental Figure S1A).

MGS1 encodes for a DNA-dependent ATPase with ssDNA annealing activities that has been suggested to compete with the PRR pathway for resolution of fork stalling lesions [6,37,38]. *RAD30* has been implicated in an error-free form of translesion synthesis [39,40] that some have placed in the PRR pathway [41]. Neither of these genes demonstrated any sensitivity to low dose MMS as single mutants. Nor did *MGS1* or *RAD30* have any additive or synergist genetic interaction with *EXO1* (see Figure 1D), consistent with our data that *EXO1* functions in the *MMS2* error-free sub-pathway of PRR.

Finally, this role of *EXO1* in MMS tolerance was independent of the role of *EXO1* in MMR. Figure 1C and supplemental Figure S1B showed that canonical MMR genes *msh2Δ* and *pms1Δ* did not display any sensitivity to low dose MMS consistent with a lack of role in PRR. Similarly, neither *msh2Δ* or *pms1Δ* interacted with *exo1Δ* as double mutants. Consistent with published reports for mutation avoidance [18] and PRR [36], *MSH2* or *PMS1* did not interact with *REV3* in our MMS sensitivity assays. Taken together, our epistasis analyses suggested

EXO1 functions in the *MMS2* error-free branch of the PRR pathway in response to low dose MMS and that this role is independent of MMR.

3.2. Mapping separate Exo1p "PRR" and "MMR" domains

Structure-function studies have delineated modular domains of Exo1p (see Figure 2A) [12, 19,42-45]. Functionally, Exo1p can be split into NH_2- and COOH-terminal halves, required for nuclease and protein-protein interaction activities [19,42,45], respectively. To identify an Exo1p domain necessary for MMS tolerance we performed complementation assays with progressively larger COOH –terminal deletion *exo1* mutants (see Figure 2A-B). To increase the sensitivity of the assay we performed complementation in the *exo1Δ rev3Δ* double mutant which demonstrates synergistic MMS sensitivity. We have previously shown that many of the functions of Exo1p require active nuclease activity [19], therefore deletions of the NH_2-terminal half were not likely to be fruitful. Figure 2B shows that residues 1-438 of Exo1p are required for full complementation of the MMS sensitive *exo1Δ* phenotype or full activity in PRR (see Figure 2A for summary).

In conjunction with examining the Exo1p domains required for PRR in response to MMS, we also performed complementation studies with these *exo1* deletion mutants for MMR mutator phenotype. The mutation reporter *hom3-10* reports reversions that occur primarily from a single nucleotide deletion in a homonucleotide run of seven T-A base pairs, and has been shown to be a sensitive marker of MMR activity *in vivo* [18,46]. Similar to our MMS complementation experiments, we used the double mutant *pms1-61 exo1Δ* for complementation to improve the sensitivity of our assay. Our complementation data suggested that the COOH-terminal MMR interaction domains of Exo1p are critical for full MMR activity (see Table 2 and Figure 2A for summary). As summarized in Figure 2A, the domains required for PRR (MMS tolerance) and MMR were distinct from one another. Consistent with this idea, we defined an *exo1* deletion (Table 2) that was functional for PRR, but not MMR (Exo1p deletion mutant #4).

3.3. The exo1-FF447AA allele is defective for MMR-dependent mutation avoidance

As suggested by the MMR complementation data for *EXO1* above, interactions between Exo1p and MMR components are important for full MMR activity, but not PRR. The MutLα heterodimer composed of Mlh1p and Pms1p is a required component in MMR that interacts with Exo1p via the Mlh1p protomer [18,43]. We hypothesized that we could create a more subtle allele of *EXO1* that would be defective in MMR, but not PRR by mutating the Mlh1p minimal binding motif in Exo1p [47]: 445-RSKFF-448 to 445-RSKAA-448 (designated as *exo1-FF447AA* for the remainder of this manuscript). We validated that exo1p-FF447AA no longer interacted with Mlh1p by yeast two-hybrid (see Figure 3). However, this *exo1* mutant was still capable of interaction with Msh2p, another required component of MMR, by yeast two-hybrid (Figure 3). Retention of Msh2p interaction would seem to rule out that gross disruption of the Exo1p COOH-terminal tertiary structure or increased turnover was the reason for the lack of an exo1p-FF447AA-Mlh1p interaction (Figure 3).

To validate the failure of exo1p-FF447AA to interact with Mlh1p *in vivo*, we made use of the dominant negative allele, *exo1-D173A*, which when overexpressed in a hypomorphic MMR defective background resulted in a synergistic MMR defect using the *hom3-10* reporter (Table 3) [19]. We chose to use the *pms1-E61A* allele because the role of *EXO1* in MMR can be more readily appreciated with the use of hypomorphic alleles of *mlh1* or *pms1*, which demonstrate synergistic defects in MMR when combined with *exo1* mutants [18,19]. As demonstrated by Table 3, the *exo1-FF447AA* allele was able to complement the defect of *exo1Δ* using the "general" mutation reporter *CAN1*, which reports a wide variety of mutational events including small frameshift insertion/deletions, nucleotide substitutions and large deletions. However, the compound *exo1-D173A, -FF447AA* mutant was no longer able to exert a dominant negative

effect, per the MMR-specific *hom3-10* reporter, as compared to the *exo1-D173A* allele when expressed in the background of a weak *pms1* defective allele. Taken together these data suggest that the protein encoded by *exo1-FF447AA* is stable and functional for MMR-independent mutation avoidance, but is not capable of interaction with the MutLα heterodimer, consistent with our two-hybrid data above.

We next examined the effect of the *exo1-FF447AA* allele when expressed from the native *EXO1* chromosomal locus. Previously we ascribed much of the *CAN1* mutator phenotype of an *exo1Δ* to a defect in a MMR-independent mutation avoidance pathway based both on mutational spectra data and suppression by deletion of *REV3* [12,19]. In agreement with the argument that the *exo1-FF447AA* mutant is functional for PRR or fork bypass, we did not observe an increased mutation rate at the *CAN1* mutation reporter (Table 4, strain PTY1300). We next analyzed the effect of the *exo1-FF447AA* mutant on the MMR mutator phenotype using the *hom3-10* reporter. As described previously, a singular defect in *EXO1* has little or no effect on MMR-dependent mutation avoidance as compared to the primary components *MLH1* or *PMS1* (Table 4). The lack of a strong MMR mutator phenotype is likely due to *EXO1* redundant activities [12]. As stated previously, we used hypomorphic alleles of *mlh1* or *pms1* to better evaluate the effect of *exo1-FF447AA* on MMR, because *mlh1-E31A* and *pms1-E61A* alleles demonstrate synergistic defects in MMR when combined with *exo1* mutants [18,19]. As shown in Table 4, *exo1-FF447AA* demonstrated synergistic defects in MMR with either *mlh1* (strain PTY223) or *pms1* (strain PTY224) hypomorph alleles. Taken together, these data suggest that the *exo1-FF447AA* mutant is defective for MMR function most likely due to loss of interaction with the MutLα heterodimer.

3.4. The exo1-FF447AA allele is proficient for PRR

We next examined the function of the *exo1-FF447AA* mutant for PRR, or fork bypass, by sensitivity to low dose MMS. As shown in Figure 4A-B, the *exo1-FF447AA* mutant was no more sensitive to low dose MMS than wildtype. In addition, the *exo1-FF447AA* mutant displayed no synergistic defects with either *rev3Δ* or *rad9Δ* mutations (see Figure 4A-B). As stated previously, *RAD5* has been mapped to the *MMS2*-error free branch [33-35] and we demonstrated above a synthetic interaction between *exo1Δ* and *rad5Δ* (Figure 1A). Consistent with our hypothesis that the *exo1-FF447AA* allele is only dysfunctional for MRR, we were able to generate a double *exo1-FF447AA rad5Δ* mutant (see Figure S2C). In summary, these data suggest that the *exo1-FF4477A* allele defines a mutation that separates the PRR and MMR functions of *EXO1*.

4. Discussion

In this study, we report genetic data suggesting that *EXO1* functions in the *MMS2* error-free branch of PRR in response to MMS. This novel function of *EXO1* in PRR was independent of MMR. By performing structure-function studies on Exo1p, we defined an NH_2-terminal fragment of Exo1p that was required for PRR. This fragment included regions of Exo1p not attributed previously to any known activities. Finally, using an allele of *EXO1* that encodes a protein incapable of binding to Mlh1p, we were able to genetically separate the function of *EXO1* in MMR from that in PRR.

Although the mechanism of fork bypass by the *MMS2* error-free branch of PRR is poorly defined, selection of which PRR branch is utilized during a fork stalling event is thought to occur via ubiquitin states of PCNA [48-50]. Briefly, upon S-phase DNA damage Rad6p-Rad18p mono-ubiquitinates PCNA on K164 promoting translesion synthesis or mutagenic fork bypass. Alternatively, Mms2-Ubc13p-Rad5p poly-ubiquitinates PCNA on K164 through further chain assembly on K63 of ubiquitin, which directs the stalled fork to error-free bypass. Recent data suggest that the *MMS2* error-free branch likely facilitates fork bypass using a

recombinational mechanism via copy choice or template switching. This same study also suggested that a smaller fraction of error-free fork bypass events was *RAD52*-dependent [36]. Our results (Figure 1A-D & Figure 4) showing that *mms2Δ* was epistatic to *exo1Δ* suggest that *EXO1* plays a role in the *MMS2*-dependent error-free bypass pathway. Given *EXO1*'s role in other recombinational pathways and its biochemical activity on recombination substrates, we hypothesize that the Exo1p nuclease functions are utilized to generate or resolve intermediates for copy choice or template switching bypass in the *MMS2*-sub-branch of PRR.

In addition to the role of *EXO1* in PRR proposed here, *EXO1* has been implicated to function in cell cycle checkpoint, fork maintenance and fork repair pathways [20-22,51,52]. *EXO1* has been shown to play subtle roles in end-processing for mitotic recombination and function redundantly with at least one other unidentified nuclease. This "other" redundant nuclease may be regulated by the Mre11p- Rad50p-Xrs2p (MRX) complex for recombination [51]. However, more recently the MRX complex and Exo1p have been implicated in the activation of DSB- and UV-induced Mec1p-dependent checkpoints [52]. The MRX complex and Exo1p were shown to collaborate in producing long ssDNA tails at DSB ends and promote Mec1p association with the DSBs. The long ssDNA tails produced by Exo1p can also revoke fork reversal by resecting newly synthesized chains and resolving the sister chromatid junctions that cause regression of collapsed replication forks in a *rad53*-deficient background [22]. Consistent with a role in replication fork maintenance, *EXO1* has been demonstrated to have a synthetic interaction with *SGS1* mutations [20]. The Sgs1p helicase is required for genome stability and is thought to be important for maintenance of stalled forks [20]. Thus, the requirement for Exo1p in the absence of Sgs1p suggests an important role for Exo1p in the maintenance, repair or restart of stalled replication forks. Finally, *EXO1* and *PSO2* appear to have overlapping roles in the processing of collapsed replication forks due to some forms of endogenous DNA damage and from nitrogen mustard induced interstrand cross-links [21].

Our inability to generate an *exo1Δ rad5Δ* double mutant seems at odds with a role of *EXO1* in the *MMS2*-sub-pathway of PRR. This can be explained by several studies suggesting a function of yeast *RAD5* beyond its activity in PRR. *RAD5* deficiency results in higher sensitivity to various types of DNA damage than deletion of either *MMS2* or *UBC13* [33]. Furthermore, *rad5Δ* mutants, relative to other PRR mutants, are highly sensitive to ionizing radiation, have elevated rates of spontaneous mitotic recombination, higher rates of gross chromosomal rearrangements, paradoxically increased stability of simple repetitive sequences and higher end-joining activity in a plasmid gap repair [53-56]. Similar to our findings, Chen *et. al.* recently isolated a separation of function mutant of *RAD5* for PRR and *MRE11-XRS2-RAD50*-dependent double strand break repair [57]. Given the data supporting multiple PRR-independent functions of *RAD5*, we believe the synthetic lethality between *exo1Δ* and *rad5Δ* uncovers a redundancy for an essential PRR-independent function or possibly a strain-dependent phenomenon.

Studies have suggested that Exo1p plays both catalytic and structural roles during MMR-mediated mutation avoidance [16,17,19]. Our results using nuclease-deficient, *exo1-D173A*, and Mlh1p-binding defective, *exo1- FF447AA*, *exo1* alleles further support both catalytic and structural roles, respectively, for Exo1p in MMR. Interestingly, the "structural-catalytic" double *exo1* mutant (*exo1-D173A, -FF447AA*) behaves like *exo1Δ*, as determined by synergistic interaction with weak *pms1* alleles (see Table 4; compare strain PTY204 to PTY225). Another interesting MMR-related finding is that the Mlh1-binding mutant exo1p-FF447AA (Figure 3) although still capable of interaction with Msh2p, was nevertheless defective in its MMR spellchecker function. These data suggest that a complex interplay between Exo1p and MutLα and MutSα is necessary for optimum MMR activity.

Exo1p is an interesting nuclease that appears to have multiple independent roles in several DNA metabolic processes important for genome duplication and stability (see Figure S3 and Table 5). In this report, we have uncovered an additional function of *EXO1* as a component in the *MMS2*-error free branch of PRR independent of its role in MMR. The findings reported here and those of others strengthen the assertion that *EXO1* is truly a "multi-tasking" nuclease [12]. However, similar to the function of *EXO1* in MMR [12], the role of *EXO1* in PRR will be somewhat difficult to assess because a singular defect in *EXO1* is apparently masked by redundant gene functions within the PRR pathway. Further studies of *EXO1* in both the MMR and PRR pathways will likely require the use of specific *exo1* alleles such as those described here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Branzei D, Foiani M. The DNA damage response during DNA replication. Curr Opin Cell Biol 2005;17:568–575. [PubMed: 16226452]
2. Waga S, Stillman B. The DNA replication fork in eukaryotic cells. Annu Rev Biochem 1998;67:721–751. [PubMed: 9759502]
3. Kolodner RD, Putnam CD, Myung K. Maintenance of genome stability in Saccharomyces cerevisiae. Science 2002;297:552–557. [PubMed: 12142524]
4. Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C, Orntoft T, Lukas J, Bartek J. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature 2005;434:864–870. [PubMed: 15829956]
5. Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, Ditullio RA Jr, Kastrinakis NG, Levy B, Kletsas D, Yoneta A, Herlyn M, Kittas C, Halazonetis TD. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature 2005;434:907–913. [PubMed: 15829965]
6. Barbour L, Xiao W. Regulation of alternative replication bypass pathways at stalled replication forks and its effects on genome stability: a yeast model. Mutat Res 2003;532:137–155. [PubMed: 14643434]
7. Lawrence CW. Following the RAD6 pathway. DNA Repair (Amst). 2007
8. Lawrence C. The RAD6 DNA repair pathway in Saccharomyces cerevisiae: what does it do, and how does it do it? Bioessays 1994;16:253–258. [PubMed: 8031302]
9. Barbour L, Ball LG, Zhang K, Xiao W. DNA Damage Checkpoints Are Involved in Postreplication Repair. Genetics 2006;174:1789–1800. [PubMed: 17057245]
10. Xiao W, Chow BL, Broomfield S, Hanna M. The Saccharomyces cerevisiae RAD6 group is composed of an error-prone and two error-free postreplication repair pathways. Genetics 2000;155:1633–1641. [PubMed: 10924462]
11. Friedberg, EC.; Walker, GC.; Siede, W.; Wood, RD.; Schultz, RA.; Ellenberger, T., editors. DNA Repair and Mutagenesis. ASM Press; Washington, DC: 2006.
12. Tran PT, Erdeniz N, Symington LS, Liskay RM. EXO1-A multi-tasking eukaryotic nuclease. DNA Repair (Amst) 2004;3:1549–1559. [PubMed: 15474417]
13. Liberti SE, Rasmussen LJ. Is hEXO1 a cancer predisposing gene? Mol Cancer Res 2004;2:427–432. [PubMed: 15328369]
14. Szankasi P, Smith GR. A DNA exonuclease induced during meiosis of Schizosaccharomyces pombe. J Biol Chem 1992;267:3014–3023. [PubMed: 1737756]

15. Wei K, Clark AB, Wong E, Kane MF, Mazur DJ, Parris T, Kolas NK, Russell R, Hou H Jr, Kneitz B, Yang G, Kunkel TA, Kolodner RD, Cohen PE, Edelmann W. Inactivation of Exonuclease 1 in mice results in DNA mismatch repair defects, increased cancer susceptibility, and male and female sterility. Genes Dev 2003;17:603–614. [PubMed: 12629043]
16. Sokolsky T, Alani E. EXO1 and MSH6 are high-copy suppressors of conditional mutations in the MSH2 mismatch repair gene of Saccharomyces cerevisiae. Genetics 2000;155:589–599. [PubMed: 10835383]
17. Amin NS, Nguyen MN, Oh S, Kolodner RD. exo1-Dependent mutator mutations: model system for studying functional interactions in mismatch repair. Mol Cell Biol 2001;21:5142–5155. [PubMed: 11438669]
18. Tran PT, Simon JA, Liskay RM. Interactions of Exo1p with components of MutLalpha in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 2001;98:9760–9765. [PubMed: 11481425]
19. Tran PT, Erdeniz N, Dudley S, Liskay RM. Characterization of nuclease-dependent functions of Exo1p in Saccharomyces cerevisiae. DNA Repair (Amst) 2002;1:895–912. [PubMed: 12531018]
20. Ooi SL, Shoemaker DD, Boeke JD. DNA helicase gene interaction network defined using synthetic lethality analyzed by microarray. Nat Genet 2003;35:277–286. [PubMed: 14566339]
21. Barber LJ, Ward TA, Hartley JA, McHugh PJ. DNA interstrand cross-link repair in the Saccharomyces cerevisiae cell cycle: overlapping roles for PSO2 (SNM1) with MutS factors and EXO1 during S phase. Mol Cell Biol 2005;25:2297–2309. [PubMed: 15743825]
22. Cotta-Ramusino C, Fachinetti D, Lucca C, Doksani Y, Lopes M, Sogo J, Foiani M. Exo1 processes stalled replication forks and counteracts fork reversal in checkpoint-defective cells. Mol Cell 2005;17:153–159. [PubMed: 15629726]
23. Gietz RD, Schiestl RH. Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. Yeast 1991;7:253–263. [PubMed: 1882550]
24. Fritsch, TMEF.; Sambrook, J., editors. Molecular Cloning. Cold Spring Harbor Laboratory; Cold Spring Harbor: 1982.
25. Welz-Voegele C, Stone JE, Tran PT, Kearney HM, Liskay RM, Petes TD, Jinks-Robertson S. Alleles of the yeast Pms1 mismatch-repair gene that differentially affect recombination- and replication-related processes. Genetics 2002;162:1131–1145. [PubMed: 12454061]
26. Myung K, Kolodner RD. Suppression of genome instability by redundant S-phase checkpoint pathways in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 2002;99:4500–4507. [PubMed: 11917116]
27. Frei C, Gasser SM. The yeast Sgs1p helicase acts upstream of Rad53p in the DNA replication checkpoint and colocalizes with Rad53p in S-phase-specific foci. Genes Dev 2000;14:81–96. [PubMed: 10640278]
28. Sidorova JM, Breeden LL. Rad53-dependent phosphorylation of Swi6 and down-regulation of CLN1 and CLN2 transcription occur in response to DNA damage in Saccharomyces cerevisiae. Genes Dev 1997;11:3032–3045. [PubMed: 9367985]
29. Paulovich AG, Hartwell LH. A checkpoint regulates the rate of progression through S phase in S. cerevisiae in response to DNA damage. Cell 1995;82:841–847. [PubMed: 7671311]
30. Broomfield S, Chow BL, Xiao W. MMS2, encoding a ubiquitin-conjugating-enzyme-like protein, is a member of the yeast error-free postreplication repair pathway. Proc Natl Acad Sci U S A 1998;95:5678–5683. [PubMed: 9576943]
31. Morrison A, Christensen RB, Alley J, Beck AK, Bernstine EG, Lemontt JF, Lawrence CW. REV3, a Saccharomyces cerevisiae gene whose function is required for induced mutagenesis, is predicted to encode a nonessential DNA polymerase. J Bacteriol 1989;171:5659–5667. [PubMed: 2676986]
32. Nelson JR, Lawrence CW, Hinkle DC. Thymine-thymine dimer bypass by yeast DNA polymerase zeta. Science 1996;272:1646–1649. [PubMed: 8658138]
33. Ulrich HD, Jentsch S. Two RING finger proteins mediate cooperation between ubiquitin-conjugating enzymes in DNA repair. Embo J 2000;19:3388–3397. [PubMed: 10880451]
34. Ulrich HD. The srs2 suppressor of UV sensitivity acts specifically on the RAD5- and MMS2-dependent branch of the RAD6 pathway. Nucleic Acids Res 2001;29:3487–3494. [PubMed: 11522817]

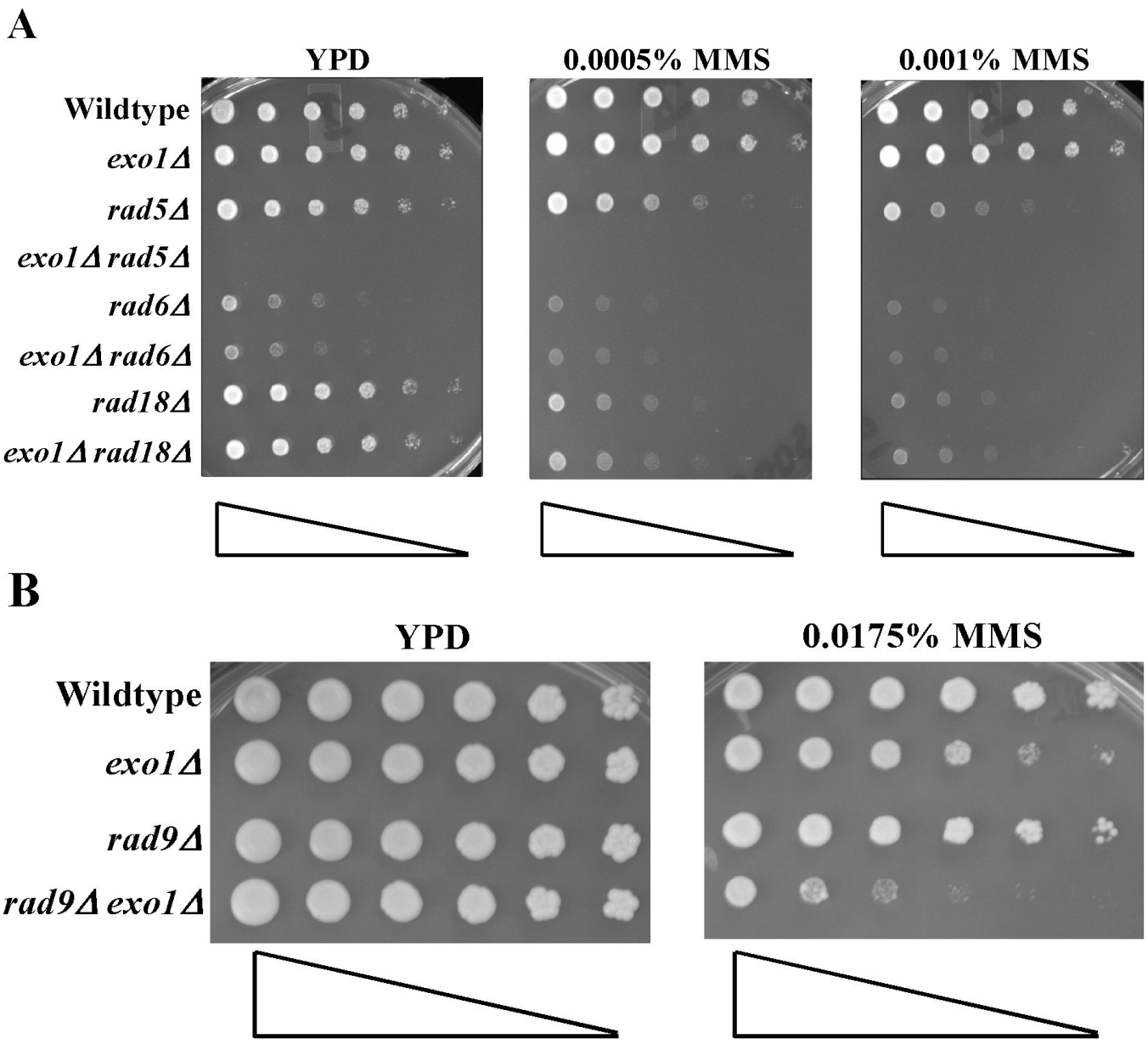
35. Broomfield S, Xiao W. Suppression of genetic defects within the RAD6 pathway by srs2 is specific for error-free post-replication repair but not for damage-induced mutagenesis. Nucleic Acids Res 2002;30:732–739. [PubMed: 11809886]
36. Zhang H, Lawrence CW. The error-free component of the RAD6/RAD18 DNA damage tolerance pathway of budding yeast employs sister-strand recombination. Proc Natl Acad Sci U S A 2005;102:15954–15959. [PubMed: 16247017]
37. Hishida T, Ohno T, Iwasaki H, Shinagawa H. Saccharomyces cerevisiae MGS1 is essential in strains deficient in the RAD6-dependent DNA damage tolerance pathway. Embo J 2002;21:2019–2029. [PubMed: 11953321]
38. Kawabe Y, Branzei D, Hayashi T, Suzuki H, Masuko T, Onoda F, Heo SJ, Ikeda H, Shimamoto A, Furuichi Y, Seki M, Enomoto T. A novel protein interacts with the Werner's syndrome gene product physically and functionally. J Biol Chem 2001;276:20364–20369. [PubMed: 11301316]
39. Johnson RE, Prakash S, Prakash L. Requirement of DNA polymerase activity of yeast Rad30 protein for its biological function. J Biol Chem 1999;274:15975–15977. [PubMed: 10347143]
40. Johnson RE, Prakash S, Prakash L. Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Poleta. Science 1999;283:1001–1004. [PubMed: 9974380]
41. McDonald JP, Levine AS, Woodgate R. The Saccharomyces cerevisiae RAD30 gene, a homologue of Escherichia coli dinB and umuC, is DNA damage inducible and functions in a novel error-free postreplication repair mechanism. Genetics 1997;147:1557–1568. [PubMed: 9409821]
42. Schmutte C, Sadoff MM, Shim KS, Acharya S, Fishel R. The interaction of DNA mismatch repair proteins with human exonuclease I. J Biol Chem 2001;276:33011–33018. [PubMed: 11427529]
43. Nielsen FC, Jager AC, Lutzen A, Bundgaard JR, Rasmussen LJ. Characterization of human exonuclease 1 in complex with mismatch repair proteins, subcellular localization and association with PCNA. Oncogene 2004;23:1457–1468. [PubMed: 14676842]
44. Tishkoff DX, Boerger AL, Bertrand P, Filosi N, Gaida GM, Kane MF, Kolodner RD. Identification and characterization of Saccharomyces cerevisiae EXO1, a gene encoding an exonuclease that interacts with MSH2. Proc Natl Acad Sci U S A 1997;94:7487–7492. [PubMed: 9207118]
45. Lee BI, Wilson DM 3rd. The RAD2 domain of human exonuclease 1 exhibits 5′ to 3′ exonuclease and flap structure-specific endonuclease activities. J Biol Chem 1999;274:37763–37769. [PubMed: 10608837]
46. Chen C, Merrill BJ, Lau PJ, Holm C, Kolodner RD. Saccharomyces cerevisiae pol30 (proliferating cell nuclear antigen) mutations impair replication fidelity and mismatch repair. Mol Cell Biol 1999;19:7801–7815. [PubMed: 10523669]
47. Gellon L, Werner M, Boiteux S. Ntg2p, a Saccharomyces cerevisiae DNA N-glycosylase/apurinic or apyrimidinic lyase involved in base excision repair of oxidative DNA damage, interacts with the DNA mismatch repair protein Mlh1p. Identification of a Mlh1p binding motif. J Biol Chem 2002;277:29963–29972. [PubMed: 12042306]
48. Hoege C, Pfander B, Moldovan GL, Pyrowolakis G, Jentsch S. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature 2002;419:135–141. [PubMed: 12226657]
49. Ulrich HD. The RAD6 pathway: control of DNA damage bypass and mutagenesis by ubiquitin and SUMO. Chembiochem 2005;6:1735–1743. [PubMed: 16142820]
50. Stelter P, Ulrich HD. Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. Nature 2003;425:188–191. [PubMed: 12968183]
51. Moreau S, Morgan EA, Symington LS. Overlapping functions of the Saccharomyces cerevisiae Mre11, Exo1 and Rad27 nucleases in DNA metabolism. Genetics 2001;159:1423–1433. [PubMed: 11779786]
52. Nakada D, Hirano Y, Sugimoto K. Requirement of the Mre11 complex and exonuclease 1 for activation of the Mec1 signaling pathway. Mol Cell Biol 2004;24:10016–10025. [PubMed: 15509802]
53. Johnson RE, Henderson ST, Petes TD, Prakash S, Bankmann M, Prakash L. Saccharomyces cerevisiae RAD5-encoded DNA repair protein contains DNA helicase and zinc-binding sequence motifs and affects the stability of simple repetitive sequences in the genome. Mol Cell Biol 1992;12:3807–3818. [PubMed: 1324406]

54. Liefshitz B, Steinlauf R, Friedl A, Eckardt-Schupp F, Kupiec M. Genetic interactions between mutants of the 'error-prone' repair group of Saccharomyces cerevisiae and their effect on recombination and mutagenesis. Mutat Res 1998;407:135–145. [PubMed: 9637242]

55. Friedl AA, Liefshitz B, Steinlauf R, Kupiec M. Deletion of the SRS2 gene suppresses elevated recombination and DNA damage sensitivity in rad5 and rad18 mutants of Saccharomyces cerevisiae. Mutat Res 2001;486:137–146. [PubMed: 11425518]

56. Smith S, Hwang JY, Banerjee S, Majeed A, Gupta A, Myung K. Mutator genes for suppression of gross chromosomal rearrangements identified by a genome-wide screening in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 2004;101:9039–9044. [PubMed: 15184655]

57. Chen S, Davies AA, Sagan D, Ulrich HD. The RING finger ATPase Rad5p of Saccharomyces cerevisiae contributes to DNA double-strand break repair in a ubiquitin-independent manner. Nucleic Acids Res 2005;33:5878–5886. [PubMed: 16224103]



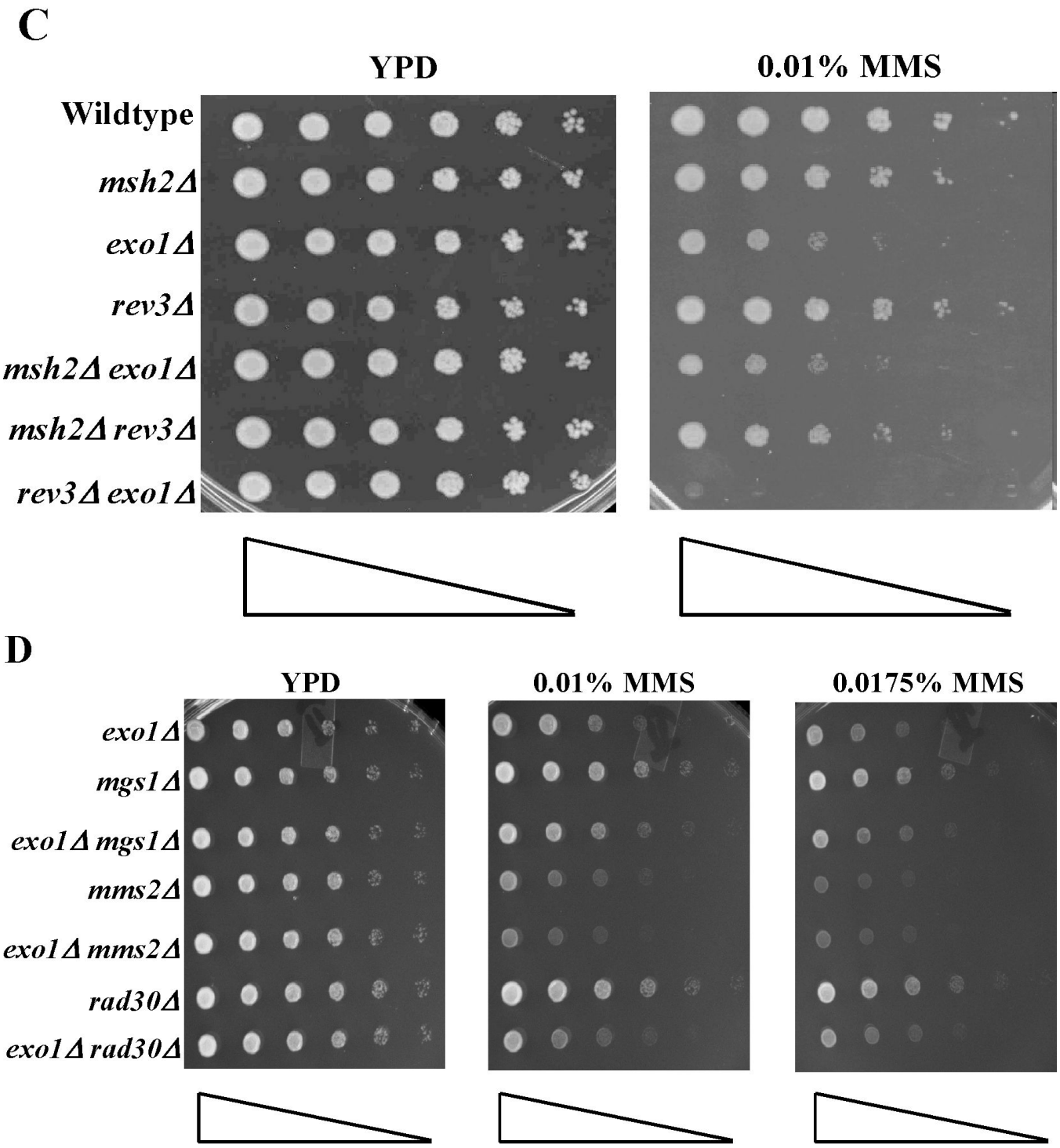
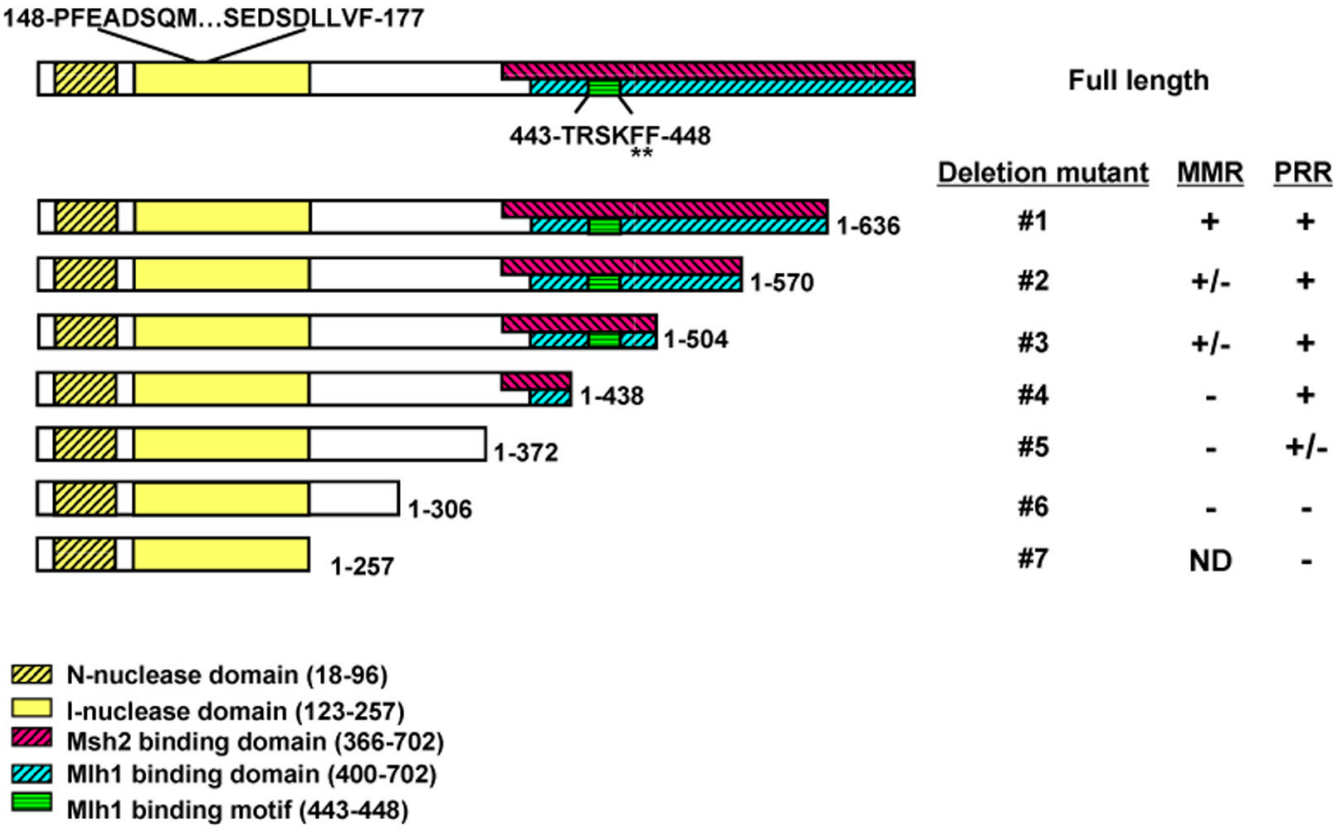


Figure 1.

EXO1 acts in the *MMS2* error-free branch of PRR in response to MMS independent of MMR. Overnight saturated cultures were serially diluted (1:5), spotted on YPD plates and YPD plates with increasing concentrations of MMS using a 48-prong replicator and then incubated at 30 °C for 2-4 days. The *exo1Δ* mutation defines an MMS tolerance pathway that functions in the (A) *RAD6*/*RAD18* PRR pathway, but is redundant with the *REV3* error-prone branch and the

RAD9 checkpoint branch as the double (B) *exo1Δ rad9Δ* and (C) *exo1Δ rev3Δ* mutants are synergistically more sensitive than the single mutants alone. In contrast, (D) *EXO1* is hypostatic to *MMS2* and other components of the error-free branch of the PRR, but *EXO1* did not interact with pathways competing with PRR as defined by *MGS1* and *RAD30*. This *EXO1* DNA damage tolerance pathway is independent of MMR as there was no genetic interaction when (C) *msh2Δ* or (Figure S1B) *pms1Δ* mutants were examined.



Deletion mutant	MMR	PRR
#1	+	+
#2	+/-	+
#3	+/-	+
#4	-	+
#5	-	+/-
#6	-	-
#7	ND	-

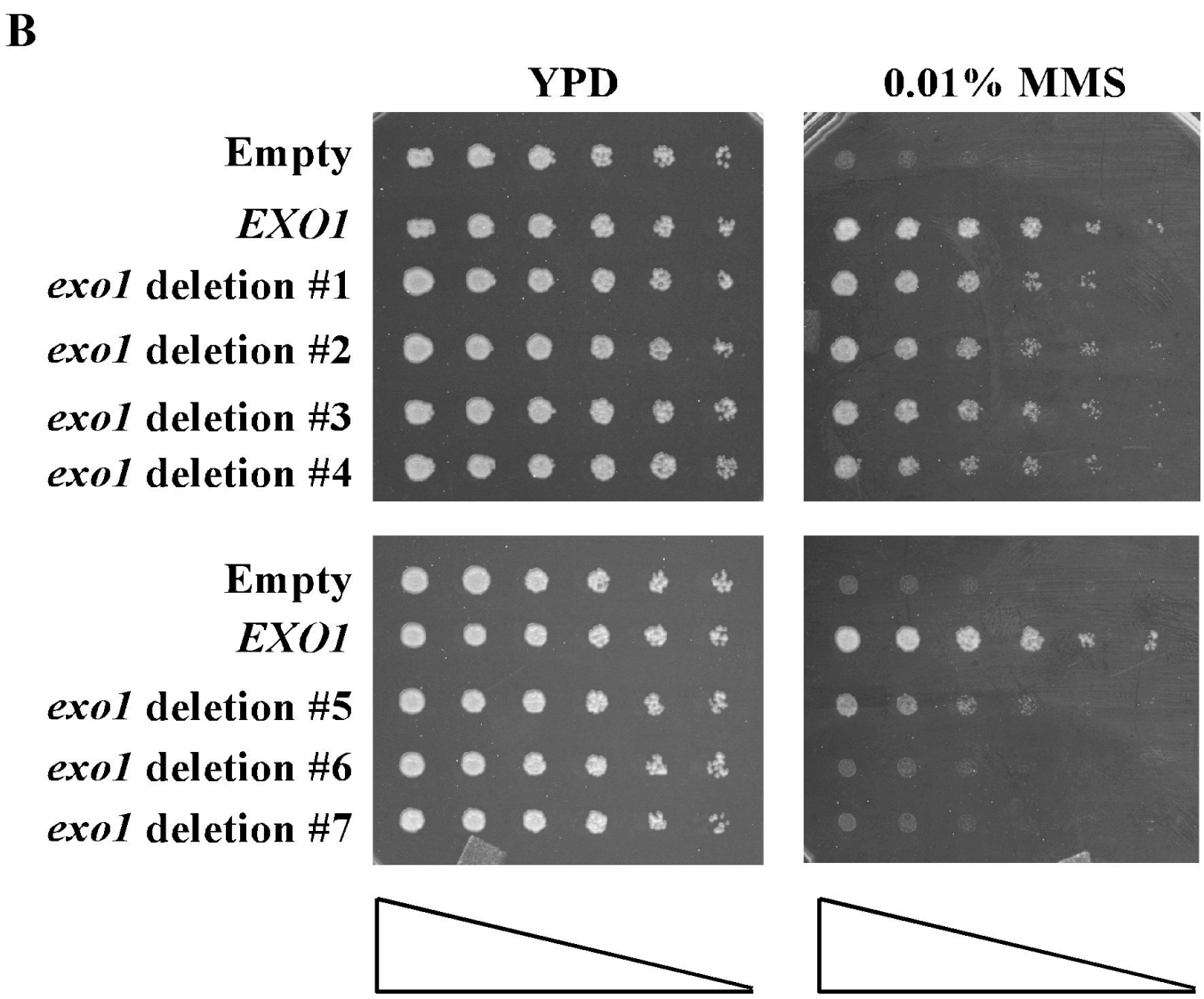


Figure 2.

Defining separate Exo1p PRR and MMR domains. (A) Schematic and summary of phenotypes for Exo1p deletion mutants tested by MMS sensitivity (PRR) and for MMR mutator as shown in Figure 2B and Table 2, respectively. Exo1p with functional domains and motifs as indicated by respective hatched boxes. Site-specific mutations experimentally examined are highlighted by asterisks as detailed in the text. This analysis defines Exo1p deletion mutant #4 (1-438 aa) as a separation of function mutant. (B) Complementation of MMS tolerance in an *exo1Δ rev3Δ* strain with the listed Exo1p deletion mutant constructs suggests that residues 1-438 are necessary for functional PRR. MMS sensitivity was performed as described previously in Figure 1.

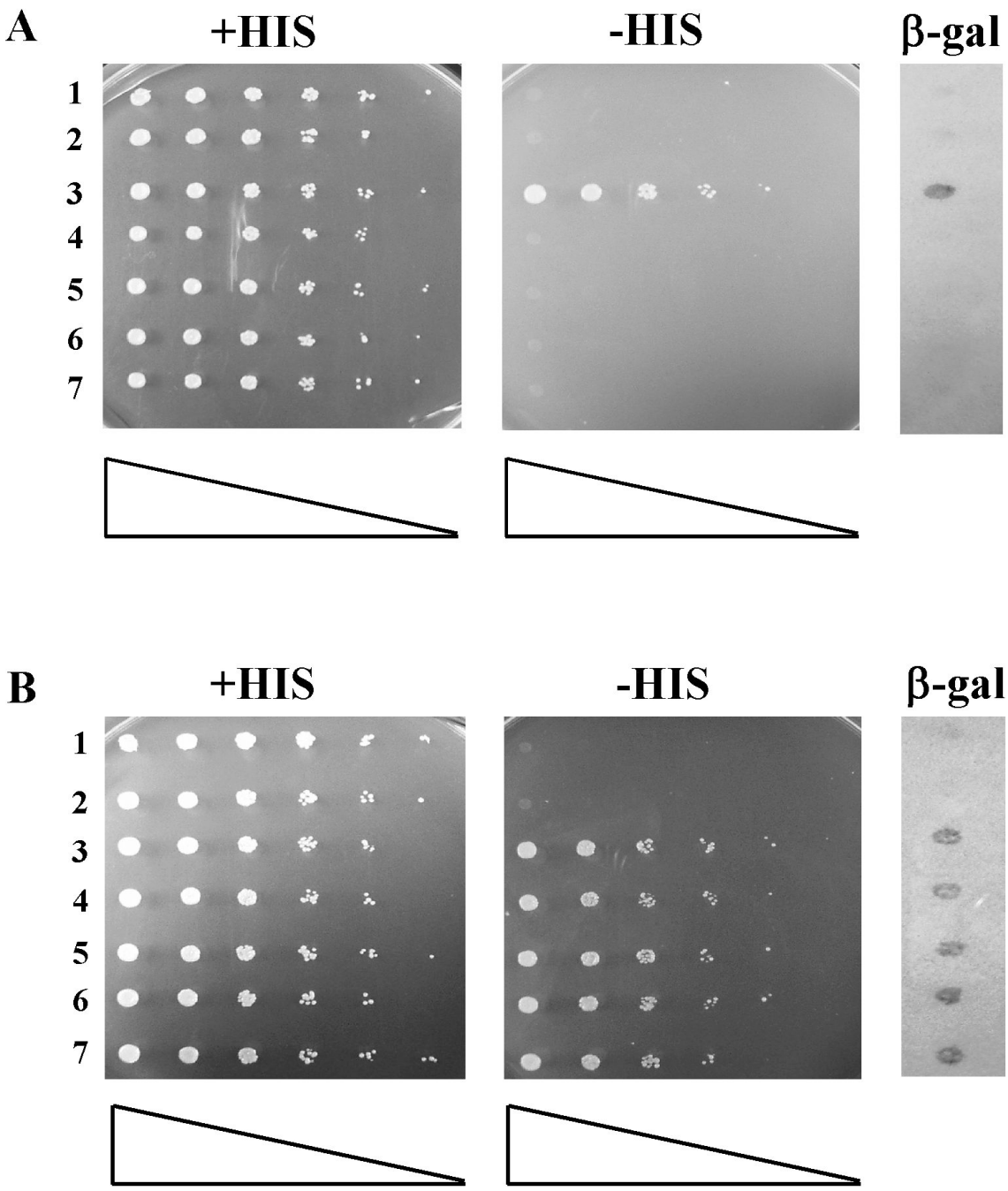


Figure 3.

The FF->AA mutation prevents Exo1p from interacting with Mlh1p, but not Msh2p by yeast two-hybrid. Strains with designated bait-prey sets were grown in nonselective media (-TRP -URA) to saturation, serially diluted (1:5), spotted on the indicated plates using a 48-prong replicator and then incubated at 30 °C for 3 days. Strains were assayed for β-gal activity by lift assays as described in the Materials and Methods. Growth on –HIS plates and blue color development on the β -gal assay indicates interaction between the bait and prey fusion proteins. (A) Mlh1p-LexAp bait and Exo1p-Gad4p prey combinations demonstrate that the *exo1-FF447AA* mutation prevents Exo1p-Gad4p interaction with Mlh1p-LexAp. Lanes: 1, pBTM (empty bait control) + pGAD (empty prey control); 2, pBTM-MLH1 + pGAD; 3, pBTM-MLH1

+ pGAD-EXO1; 4, pBTM-MLH1 + pGAD-exo1-FF447AA # 1; 5, pBTM-MLH1 + pGAD-exo1-FF447AA # 5; 6, pBTM-MLH1 + pGAD-exo1-FF447AA # 6; and 7, pBTM-MLH1 + pGAD-exo1-FF447AA # 10. (B) Msh2p-LexAp bait and Exo1p-Gad4p prey combinations demonstrate that the *exo1-FF447AA* mutation does not prevent Exo1p-Gad4p interaction with Msh2p-LexAp. Lanes: 1, pBTM (control) + pGAD (control); 2, pBTM-MSH2 + pGAD; 3, pBTM- MSH2 + pGAD-EXO1; 4, pBTM- MSH2 + pGAD-exo1-FF447AA # 1; 5, pBTM-MSH2 + pGAD-exo1-FF447AA # 5; 6, pBTM- MSH2 + pGAD-exo1-FF447AA # 6; and 7, pBTM- MSH2 + pGAD-exo1-FF447AA # 10. Clones pGAD-exo1-FF447AA #1, 5, 6 & 10 represent four independently generated “prey” constructs.

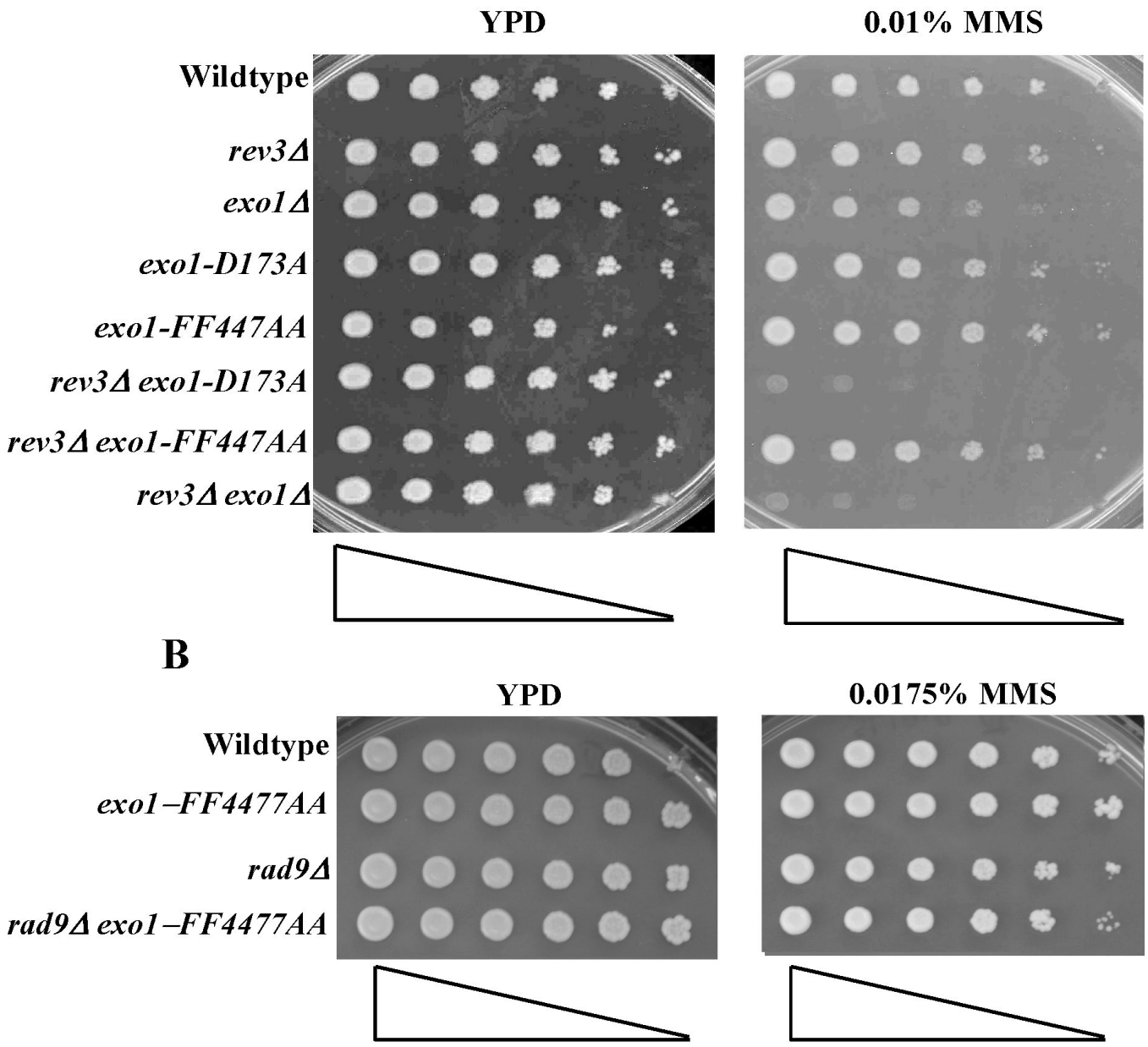


Figure 4.

The *exo1-FF447AA* mutant is functional for PRR. Overnight saturated cultures were serially diluted (1:5), spotted on YPD plates and YPD plates with increasing concentrations of MMS using a 48-prong replicator and then incubated at 30 °C for 2 days. The *exo1-FF447AA* mutation shows no defect in the *PRR* pathway as this mutant is as resistant to MMS as wildtype and does not show any genetic interaction with the redundant (A) *REV3*- or (B) *RAD9*-dependent branches of PRR.