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Exo1 phosphorylation status controls the hydroxyurea sensitivity of cells lacking the Pol32 subunit of DNA polymerases delta and zeta

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Abstract

Exo1 belongs to the Rad2 family of structure-specific nucleases and possesses 5'-3' exonuclease activity on double-stranded DNA substrates. Exo1 interacts physically with the DNA mismatch repair (MMR) proteins Msh2 and Mlh1 and is involved in the excision of the mispaired nucleotide. Independent of its role in MMR, Exo1 contributes to long-range resection of DNA double-strand break (DSB) ends to facilitate their repair by homologous recombination (HR), and was recently identified as a component of error-free DNA damage tolerance pathways. Here we show that Exo1 activity increases the hydroxyurea sensitivity of cells lacking Pol32, a subunit of DNA polymerases δ and ζ . Both, phospho-mimicking and dephospho-mimicking *exo1* mutants act as hypermorphs, as evidenced by an increase in HU sensitivity of *pol32* cells, suggesting that they are trapped in an active form and that phosphorylation of Exo1 at residues S372, S567, S587, S692 is necessary, but insufficient, for the accurate regulation of Exo1 activity at stalled replication forks. In contrast, neither phosphorylation status is important for Exo1's role in MMR or in the suppression of genome instability in cells lacking Sgs1 helicase. This ability of an *EXO1* deletion to suppress the HU hypersensitivity of *pol32* cells is in contrast to the negative genetic interaction between deletions of *EXO1* and *POL32* in MMS-treated cells as well as the role of *EXO1* in DNA-damage treated *rad53* and *mec1* mutants.

Keywords

Pol32; Exo1; Exo1 phosphorylation; gross-chromosomal rearrangements; hydroxyurea hypersensitivity

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Introduction

Exo1 belongs to the Rad2-family of structure-specific nucleases and possesses 5' to 3' exonuclease activity on double-stranded DNA substrates as well as flap-endonuclease activity [1, 2]. Exo1 has a catalytic role during the excision step of DNA mismatch repair (MMR) and has been shown to physically interact with the MMR proteins Msh2 and Mlh1 in yeast and humans [1, 3-8]. Although missense mutations in *EXO1* from cancer patients have been shown to reduce binding to Msh2 *in vitro*, and disruption of *EXO1* function has been associated with somatic hypermutation and development of cancer in older mice, a causative association between *EXO1* mutations and hereditary nonpolyposis colorectal cancer (HNPCC), which is caused by MMR defects, remains unclear [9-15].

Independent of its role in MMR, *EXO1* plays a role in the resection of DNA double-strand breaks (DSBs). Formation of long, single-stranded 3' overhangs is necessary for Rad51 filament formation and non-mutagenic repair of DSBs by homologous recombination (HR) [16]. Resection appears to be a two-step reaction that commits repair of the DSB to the HR pathway. The Mre11/Rad50/Xrs2 complex binds first to the unprocessed DSB and, with help from Sae2 endonuclease, resects a short stretch. In the second step, long-range 5' to 3' resection is accomplished by Sgs1 and Dna2, which, respectively, contribute 3' to 5' helicase and endonuclease activities to the reaction [17, 18]. In addition to Sgs1/Dna2, Exo1 can carry out long-range resection by removing nucleotides from the 5'-ended strand [17-19]. Yeast cells that lack both Sgs1 and Exo1 exhibit severely retarded long-range resection and accumulate spontaneous chromosomal rearrangements at an extraordinarily high rate [20, 21]. Analysis of rearranged chromosomes in *sgs1 exo1* mutants where a DSB was induced by endonucleolytic cleavage revealed that incompletely processed DNA breaks become substrates for aberrant repair, predominantly *de novo* telomere addition and, to a lesser extent, break-induced replication [22, 23].

Recently, *EXO1* was also identified in a genetic screen for components of the error-free DNA damage tolerance (DDT) pathway, which is engaged when the replisome encounters a blocking DNA lesion in the template strand [24]. As opposed to the error-prone pathway where a translesion DNA polymerase is recruited to mono-ubiquitinated proliferating cell nuclear antigen (PCNA) to replicate across a lesion, in the error-free pathway the replicative DNA polymerase is thought to switch from the damaged template to the undamaged sister chromatid in a HR-dependent reaction, also referred to as template switching. Exo1 is thought to promote the HR-dependent steps in error-free DDT by extending the ssDNA gap behind the stalled replication fork [24]. Exo1-dependent ssDNA formation may also allow accumulation of enough replication protein A (RPA) at stalled forks to enhance the DNA-damage signal sufficiently for 9-1-1/Mec1-dependent checkpoint activation [25, 26]. In addition to bypassing replication blocking lesions in the template strand, error-free DDT can be specifically induced by deleting *POL32*, which encodes a noncatalytic subunit of DNA polymerase δ [27]. Through physical interaction with the polymerase clamp PCNA, Pol32 increases the processivity of DNA polymerase δ and the efficiency of DNA synthesis [28]. The DNA polymerase δ subunits Pol32 and Pol31 also function as subunits of DNA polymerase ζ , whose catalytic core is made up of Rev3 and Rev7 [29-31]. DNA polymerase ζ is conserved in eukaryotes and plays a critical role in translesion DNA synthesis and

mutagenesis [32-34]. Although cells that lack Pol32 are viable, they experience replication stress that leads to phosphorylation of the checkpoint kinase Rad53, S phase delay, accumulation in G2/M, and hypersensitivity to DNA damaging agents [24, 27, 35].

When replication forks stall due to exposure to the DNA damaging agents hydroxyurea (HU) or methyl methanesulfonate (MMS), which deplete the dNTP pool and generate base adducts in the template strand, respectively, activation of the replication checkpoint helps to maintain the activity of the replisome, possibly through phosphorylation, that permits fork restart after withdrawal of the genotoxin [36-38]. Although Exo1 has been found at these stalled forks the presence of the intact replisome is thought to prevent it from acting on the nascent strands [26]. In cells that lack a functional replication checkpoint, components of the replisome, most notably DNA polymerase α and the replicative helicase Mcm2-7, have been found to dissociate from stalled forks, which might make the ends of the nascent strands vulnerable to degradation by nucleases, including Exo1 [39-41]. Nascent strand degradation may contribute to the destabilization of stalled replication forks in checkpoint mutants and, possibly, provide sufficiently long ssDNA regions for recombination [26, 42, 43]. A recent comparison of the composition of replisomes in checkpoint-deficient cells and wildtype cells also raised the possibility that the inability to restart stalled forks in checkpoint-deficient cells may be due to a lack of phosphorylation of replisome components and accessory factors rather than their loss from the replication fork [44, 45]. Exo1 has been found to be phosphorylated in a Mec1-dependent manner upon treatment of cells with HU or MMS [38, 46]. In cells with uncapped telomeres (*cdc13-1*), four phosphorylated serine residues were identified in the C-terminal half of Exo1 [47, 48]. Although the effect of mutating these serine residues on the viability of the *cdc13-1* mutant was small, it hinted that DNA-damage induced phosphorylation inhibits Exo1 activity.

To better understand Exo1 function in the DNA-damage response and the regulation of these functions by phosphorylation, we have determined the effect of defects in DNA repair and the replication stress response on DNA-damage sensitivity and accumulation of mutations in the *exo1* mutant and cells harboring alleles that encode phospho-mimicking and dephospho-mimicking mutants of Exo1.

Materials and Methods

Yeast strains and media

Yeast strains used in DNA-damage sensitivity and mutation assays were all derived from KHSY802 (*MATa*, *ura3-52*, *trp1* 63, *his3* 200, *leu2* 1, *lys2Bgl*, *hom3-10*, *ade2* 1, *ade8*, *hxt13*: *URA3*). Gene deletions were carried out as described [49] using HR-mediated integration of a selectable cassette. Mutants containing more than one gene deletion or mutation were obtained by random spore isolation from diploids heterozygous for the desired mutations [50]. Residues S372, S567, S587, and S692 of Exo1 were changed to alanine (*exo1-SA*) or aspartic acid (*exo1-SD*) and residues E150 and D173 to aspartic acid and alanine, respectively (*exo1-ND*, *exo1-ND-SA*, *exo1-ND-SD*) by site-directed mutagenesis of the *EXO1* ORF in plasmid pKHS610. pKHS610 was generated by inserting *EXO1* into pXP320 [51] by gap-repair using yeast strain KHSY2331 (*lig4*: *kanMX6*) to promote HR-mediated insertion. The resulting plasmids with mutant *exo1* alleles were

pKHS611 (*exo1-SD*), pKHS612 (*exo1-SA*), pKHS613 (*exo1-ND-SA*), and pKHS614 (*exo1-ND-SD*). The *exo1* alleles, including a HIS3MX6 cassette were amplified from the appropriate plasmids and integrated at the chromosomal *EXO1* locus in KHSY2338 (*exo1::KANMX6*) using the lithium acetate method [49]. Yeast strains used in this study are listed in Supplemental Table S1.

Yeast was grown at 30°C in yeast extract (10 g/l), peptone (20 g/l), dextrose (20 g/l), media (YPD) with or without Bacto agar, or in synthetic complete (SC) media (yeast nitrogen base 6.7 g/l, dextrose 20 g/l) supplemented with the appropriate amino acid mix.

Gross-chromosomal rearrangement (GCR) assay

GCR rates were determined by fluctuation analysis by taking the median rate of at least 15 cultures from at least two isolates [52, 53] and are shown with 95% confidence intervals [54]. Cells with GCRs were identified by their resistance to canavanine and 5-fluoro-orotic acid (Can^r 5-FOA r), which is indicative of simultaneous inactivation of *CAN1* and *URA3* on chromosome V. Selective media for the GCR assay was prepared as previously described [55].

Fluctuation assays and *CAN1* mutation spectrum analysis

Forward mutation rates were determined by fluctuation analysis by method of the median as previously described [53, 56] and are shown with 95% confidence intervals [54]. Five cultures from at least two different isolates were grown overnight in 3-5 ml of YPD media. Dilutions were plated on YPD agar plates to determine the viable cell count, and either 500 μl or 1000 μl were plated on synthetic media supplemented with 60 $\mu\text{g/ml}$ canavanine, but lacking arginine (SC-ARG/canavanine) to select for *can1* mutants. To select for Thr^+ (*hom3-10*) revertants, 250 μl - 4000 μl were plated on synthetic media lacking threonine. Semi-quantitative assessment of accumulation of *CAN1* mutations was performed by a patch assay. Briefly, three independent colonies of each mutant were spread over approximately 1-inch squares on YPD agar or YPD agar containing 10 mM HU. Patches were allowed to grow for five days at 30°C and replica plated onto SC-ARG/canavanine. Formation of papillae was documented each day for four days of incubation at 30°C. To determine the *CAN1* mutation spectrum, the *CAN1* open reading frame (ORF) and 50 bp upstream as well as 43 bp downstream of the ORF were amplified by PCR from canavanine-resistant colonies and sequenced.

Hydroxyurea (HU) and methyl methanesulfonate (MMS) sensitivity assays

Cultures of single colonies grown overnight at 30°C in YPD liquid media were adjusted to $\text{OD}_{600} = 0.2$ and grown at 30°C to $\text{OD}_{600} = 0.5$. Ten-fold serial dilutions were spotted on YPD, and YPD supplemented with the indicated concentrations of MMS and HU. Colony formation was documented each day for 5 days of incubation at 30°C.

Results

Functional interactions between Exo1, DNA repair and DNA-damage response

Exo1 and Sgs1/Dna2 act in parallel in long-range 5' end resection during DSB repair by HR [18]. When both pathways are disrupted (*sgs1 exo1*) the accumulation of gross-chromosomal rearrangements (GCRs) increases synergistically to 450-fold over the rate of the *sgs1* single mutant [20, 21]. Here we report that this rapid accumulation of GCRs in the *sgs1 exo1* mutant can be further accelerated by increasing replication stress either by impairing DNA polymerase δ (*pol32*) or by failure to complete repair (*apn1*, *rad1*) or bypass (*rev3*, *pol32*) of spontaneous DNA lesions (Table 1). Disruption of these activities in the *exo1* single mutant (Table 2), but not in the *sgs1* single mutant (Table S2), also caused significant increases in genome instability, with the strongest synergistic increases caused by the *rev3* and *pol32* mutations. Deletion of *TEL1* also caused a significant increase in GCR accumulation in the *sgs1 exo1* mutant, but to a lesser extent than the 28-fold increase observed upon deletion of *MEC3* [20], suggesting that the Mec3-Ddc1-Rad17-mediated replication stress response plays a significantly greater role in the suppression of genome instability in the *sgs1 exo1* mutant than the Tel1-mediated checkpoint response to DNA breaks.

The small, but significant, increases in genome instability in the *sgs1 exo1* mutant upon deletion of either *TEL1*, *REV3* or *APN1* were accompanied by increases in sensitivity to hydroxyurea (HU), which impedes replisome progression by depleting the dNTP pool, whereas no such increase was observed upon deletion of *RAD1* (Figure 1 A-D). Combining *sgs1* and *pol32* mutations caused a clear fitness defect on rich media, which was not further exacerbated by deletion of *EXO1* (Figure 1 E). However, deletion of *EXO1* appeared to slightly suppress the HU hypersensitivity of the *sgs1 pol32* mutant, but not its hypersensitivity to methyl methanesulfonate (MMS), which impairs replisome progression by generating DNA lesions (e.g., base adducts) in the template strand (Figure 1 E). Since insensitivity to HU (200 mM) and sensitivity to MMS (0.05%) are characteristic of an *exo1* mutant [20], we next evaluated the HU sensitivity of *sgs1* and *pol32* single mutants in the presence and the absence of *EXO1*. We increased the HU concentration from 10 mM to 55 mM - a level at which *sgs1* and *pol32* mutants display significantly higher HU sensitivity than the wildtype strain. We found that the *exo1* mutation suppressed the HU hypersensitivity of the *pol32* mutant to a level comparable to the wildtype strain, but had no effect on the *sgs1* mutant (Figure 1 F). Disrupting the nuclease activity of Exo1 (*exo1-ND*) achieved the same level of suppression of HU hypersensitivity in the *pol32* mutant as deletion of *EXO1* (Figure 2 A). Disruption of Exo1, however, had no effect on the viability of *pol32* cells treated with high concentrations of HU (200 mM) (Figure 2 B). These findings suggest that under low replication stress Exo1-dependent events, most likely ssDNA formation at stalled replication forks, are the major contributor to the inviability of *pol32* cells, whereas under conditions when DNA synthesis is grossly impeded by high concentrations of HU Exo1-independent lesions, such as DNA breaks, are the major cause of *pol32* inviability.

In contrast to HU, Exo1 was not found to be toxic to MMS-treated *pol32* cells at the tested concentrations. In fact, when the *pol32* mutant was exposed to MMS we observed the opposite response: while *exo1*, *exo1-ND* and *pol32* single mutants grew almost like wildtype cells on 0.01% MMS, combining the *pol32* mutation with *exo1* or *exo1-ND* mutations increased MMS sensitivity synergistically (Figure 2 A). This is consistent with the recent finding that *EXO1* is required for the bypass of MMS-induced DNA lesions by error-free DNA-damage tolerance pathways [24].

Phosphorylation-site mutants of Exo1 are hypermorphic in HU-treated *pol32* cells

In yeast cells with a defective telomere capping complex (*cdc13-1*), the C-terminal half of Exo1 has been found to be phosphorylated in a Mec1-dependent manner at four dispersed sites (S372, S567, S587, S692) [47]. To determine how phosphorylation contributes to the adverse effect of Exo1 on HU-treated *pol32* cells we crossed *pol32* cells with *exo1* mutants that either mimicked constitutive Exo1 phosphorylation (*exo1-SD*) or were refractory to phosphorylation at these four sites (*exo1-SA*). Although we expected that mutating the phosphorylation sites would affect Exo1 activity and therefore affect the viability of *pol32* cells on HU we were surprised to find that both alleles, *exo1-SA* and *exo1-SD*, led to increases in HU hypersensitivity that clearly exceeded that of the *pol32* mutant with an *EXO1* wildtype allele (Figure 2 A). Since the nuclease activity of Exo1 appears to sensitize *pol32* cells to HU, the strong synergistic increases in HU sensitivity in the *pol32 exo1-SA* and *pol32 exo1-SD* mutants could indicate that the *exo1-SA* and *exo1-SD* alleles code for hyperactive nucleases. To test this, we inactivated the nuclease activity of the *exo1-SA* and *exo1-SD* mutants by introducing E150D and D173A mutations. Consistent with our hypothesis we observed suppression of HU hypersensitivity for both alleles (Figure 2 C). To determine if the *exo1-SA* and the *exo1-SD* alleles were equally toxic to HU-treated *pol32* cells we lowered the HU concentration to a level at which the *pol32* mutant was no more sensitive than wildtype (20 mM) and compared the growth of *exo1-SA pol32* and *exo1-SD pol32* cells. We observed that the dephospho-mimicking *exo1-SA* allele was slightly more damaging than the phospho-mimicking *exo1-SD* allele (Figure 2 D). This difference disappeared at higher HU concentrations (> 40 mM).

In contrast to the *exo1* mutation, expression of the *exo1-SA* and *exo1-SD* alleles had no effect on the viability of *pol32* cells exposed to MMS (Figure 2 A). As expected from the phenotype of *exo1-ND pol32* cells, mutation of the nuclease domain of *exo1-SA* or *exo1-SD* alleles in *pol32* cells caused a synergistic increase in MMS-hypersensitivity (Figure 2 C).

Since Pol32 not only plays a major role as the processivity factor for DNA polymerase δ , but also functions as a subunit of the translesion DNA polymerase ζ we wanted to determine if the effect of the *pol32* mutation on HU and MMS sensitivity could be due to disruption of Pol ζ activity. We found that deletion of *REV3*, which encodes the catalytic subunit of Pol ζ did not cause an increase in sensitivity to 55 mM HU, a concentration to which the *pol32* mutant is hypersensitive (Figure 2 E), or 100 mM HU (Supplemental Figure S1). Opposite to the effect in the *pol32* mutant, deletion of *EXO1* had no effect on the HU sensitivity of the *rev3* mutant at 55 mM HU and increased its sensitivity when exposed to 100 mM HU

(Figure 2 E, Supplemental Figure S1). Also in contrast to the *pol32* mutant, the *exo1-SA* and *exo1-SD* alleles had no effect on the HU sensitivity of the *rev3* mutant, even at 100 mM HU (Supplemental Figure S1). These differences between *rev3* and *pol32* mutations suggest that the genetic interactions between *pol32* and *exo1* mutations on HU are largely caused by the role of Pol32 as a subunit of Pol δ . On 0.01% MMS, deletion of *REV3* did not increase the sensitivity of the *exo1* or *pol32* mutant, but led to a further increase in the sensitivity of the *pol32 exo1* double mutant, suggesting that Pol32, Exo1 and Rev3 play at least partially redundant roles in the response to MMS (Figure 2 A, E).

Similar to the *rev3* mutant, we observed no difference in the HU sensitivity between *sgs1* or *tell1* mutants expressing the *exo1-SA* or the *exo1-SD* allele or the *EXO1* wildtype allele (Supplemental Figure S1). Using the gross-chromosomal rearrangement (GCR) assay [55, 57], which measures the rate of accumulating viable outcomes of chromosome break repair, we found that the loss of Exo1 activity in untreated *pol32* cells caused a significant (8-fold) increase in chromosomal instability whereas expression of the *exo1-SA* or *exo1-SD* allele had no effect (Table 3). These results mirror the role of *EXO1* in MMS-treated *pol32* cells. Although, *EXO1* is the strongest known suppressor of spontaneous chromosome rearrangements in *sgs1* cells [20, 21], the four phospho-sites are also dispensable for this suppressor function (Table 3).

Mismatch repair activity partially contributes to Exo1 toxicity in *pol32* cells

In addition to its role in DSB processing, Exo1 performs a late step in MMR, most likely degradation of the mismatch-containing nascent strand, and binds to the MMR factors Msh2 and Mlh1 through its C-terminus [1, 58]. Thus, one way in which Exo1 could localize to replicating DNA is through interaction with the MMR machinery, whose subunits Msh6 and Msh3 interact with the replication machinery by binding to PCNA [59, 60]. Once there, Exo1 is thought to be able to degrade nascent DNA strands by acting on the 5' ends of Okazaki fragments [61, 62]. In addition to these pre-existing 5' ends, the MMR factor MutL α is thought to make incisions in the nascent strand [63]. Disruption of the MMR pathway by deletion of *MSH2* would disrupt one avenue for recruitment of Exo1 to replicating DNA and prevent the formation of MutL α -dependent strand nicks for Exo1 entry and increased ssDNA formation. To test the possibility that MMR factors contribute to Exo1 toxicity in HU-treated *pol32* cells, we assessed the HU sensitivity of *pol32 msh2* cells expressing either *exo1 EXO1*, *exo1-SA* or *exo1-SD* alleles. We observed that a *msh2* mutation had no effect on the HU sensitivity of the *pol32* and *pol32 exo1-SA* mutants, but suppressed the HU sensitivity of the *pol32 exo1-SD* mutant (Figure 3 A). These results show that MMR activity contributes significantly to the hypertoxicity of the phospho-mimicking *exo1-SD* allele in *pol32* cells. It also suggests that MMR-independent functions of Exo1 are mostly responsible for the toxicity of the dephospho-mimicking *exo1-SA* allele in HU-treated *pol32* cells. That the impact of Exo1 activity in the *pol32* mutant exceeds its role in MMR is also suggested by the different responses of the *exo1 pol32* and *msh2 pol32* mutants to MMS; whereas the *exo1 pol32* mutant shows an increase in MMS sensitivity compared to the single mutants, sensitivity of the *msh2 pol32* mutant is indistinguishable from that of the *pol32* single mutant (Figure 3 B). Additionally, *exo1*,

but not *msh2*, caused a statistically significant increase in the accumulation of GCRs in the *pol32* mutant (Table 2).

Since the four phosphorylation sites are located in the C-terminal half of Exo1 where Mlh1 and Msh2 have been reported to bind we tested if the *exo1-SA* and *exo1-SD* alleles were associated with a mutator phenotype. Three single colonies of wildtype, *pol32*, or *sgs1* cells with either *EXO1*, *exo1*, *exo1-SA* or *exo1-SD* alleles were grown into thick, 1-inch squares on nonselective rich media (YPD) and replica-plated onto synthetic media containing canavanine to select for clones that had spontaneously acquired inactivating mutations in the *CAN1* gene. In contrast to *exo1*, neither the *exo1-SA* nor the *exo1-SD* mutation affected the *CAN1* mutation rate in wildtype, *sgs1* or *pol32* cells (Figure 4 A, Supplemental Figure S2), mirroring the lack of effect of these *exo1* alleles on GCR accumulation (Table 3). A more accurate determination of the *CAN1* mutation rate using a fluctuation assay confirmed the previously reported weak mutator phenotype of the *exo1* cells [1], but did not reveal statistically significant differences between the *exo1 pol32* mutant and the single mutants, or the *exo1-SA pol32* or *exo1-SD pol32* mutants and the single mutants (Figure 4 C). These findings indicate that the phosphorylation site mutations do not affect the function of Exo1 in mutation avoidance at the *CAN1* locus. However, when we determined the mutation rate of the *hom3-10* allele (Thr⁻), which detects mutations that revert a +1T insertion in a mononucleotide (T₁₀) run, we observed a synergistic (24-fold) increase in Thr⁺ reversion events in the *pol32 exo1* mutant (Figure 4 C), consistent with a previously reported synergistic mutation rate increase in the *lys2-10A* assay, which detects mutations that revert +1A insertions [64]. In the *hom3-10* assay, we also detected a small (2.8-fold), but significant increase of Thr⁺ revertants in the *exo1-SD* mutant; but no synergistic interaction when the *exo1-SD* and *pol32* mutations were combined (Figure 4 C). Finally, growing the cells in the presence of 10 mM HU before replica-plating onto canavanine media did not lead to noticeable changes in mutation rates, except in the *exo1-SA pol32* mutant, which exhibited a markedly reduced number of papillae compared to the single mutants (Figure 4 B). The slow growth of *pol32 exo1-SA* cells even in the presence of a very low concentration of HU (Figure 2 C) is the most likely cause of the lower number of Can^r papillae in this mutant.

When we analyzed the *CAN1* mutation spectrum in Can^r clones (Supplemental Table S3) we found that the majority (89.5%) of mutations in the *exo1* mutant were base substitutions and small (< 3 bp) insertions/deletions, whereas larger insertions/deletions ranging from 18-72 bp dominated (52.6%) in *pol32* mutant. Upon deletion of *EXO1* in the *pol32* mutant these large deletions decreased to 20% whereas small insertions/deletions increased (40%), with a similar fraction of base substitutions (30%) as in the *pol32* mutant (36.8%). We also identified rare complex rearrangements that consist of large deletions (8-233 bp) that contain small insertions (2-36 bp).

Discussion

In wildtype cells, the nuclease activity of Exo1 is important for 5' end resection of DSB ends during HR, for the excision step of MMR and for HR-mediated template switching during error-free bypass of DNA lesions. Exo1 has also been shown to counteract fork reversal in

checkpoint-deficient cells and is thought to promote template switching by contributing to the extension of ssDNA behind stalled replication forks [25-27, 65-67]. We have shown here that Exo1 nuclease activity is toxic to HU-treated *pol32* cells and that this toxicity can be further increased by mutating four serine residues in the C-terminal tail of Exo1 that have previously been shown to be phosphorylated in response to telomere uncapping [47]. That the viability of HU-treated *pol32* cells could be rescued by disruption of the nuclease activity of Exo1 suggests that phosphorylated Exo1, which forms in response to HU [46], is still a potent nuclease, but at a level that is compatible with normal growth of HU-treated wildtype cells. Only when replication fork stalling due to HU treatment is combined with another event that independently impairs DNA synthesis (*pol32*), do Exo1 nuclease-dependent events, most likely ssDNA formation, reach toxic levels. This could suggest that there is a balance between the speed of ssDNA formation at replication forks and the speed of DNA synthesis, which does not allow ssDNA to accumulate at deleterious levels. If the speed of DNA synthesis is reduced (HU treatment), this balance may be restored by reducing Exo1 activity through Mec1-dependent phosphorylation of Exo1. However, this reduced level of Exo1 activity might be damaging if DNA synthesis is inhibited further (e.g., HU-treatment of the *pol32* mutant) and DNA resection exceeds DNA synthesis. That Exo1 phosphorylation also occurs in MMS-treated cells [38], which depend on Exo1 activity for viability, also supports a model in which phosphorylation reduces, rather than deactivates, Exo1 nuclease function. This model is also consistent with the finding that the C-terminal tail of Exo1, where the phosphorylation sites are located, is dispensable for normal function of Exo1 in MMS-treated cells [20] and the finding in this study that expression of *exo1-SA* and *exo1-SD* alleles has no effect on the viability of MMS-treated wildtype or *pol32* cells, leading us to suggest that downregulation of Exo1 activity by phosphorylation is tolerated in MMS-treated cells, but not necessary.

As in HU-treated *pol32* cells, endogenous Exo1 activity was previously shown to inhibit proliferation of the *cdc13-1* mutant [47]. In these cells, which accumulate uncapped telomeres, a phospho-mimicking *exo1* allele led to a minor increase in cell viability whereas a dephospho-mimicking allele had no effect. Although the effect was subtle, it suggested that phosphorylation inhibits Exo1 activity. Unlike in the *cdc13-1* mutant, however, we did not observe a rescue of cell viability upon expression of the phospho-mimicking *exo1-SD* allele in HU-treated *pol32* cells. Instead, *exo1-SD* and *exo1-SA* inhibited proliferation more strongly than wildtype *EXO1*. For both alleles this negative effect was dependent on the nuclease activity, suggesting that *exo1-SA* and *exo1-SD* encode functional exonucleases that are trapped in an active state. The hyperactivity of both alleles further suggests that both, phosphorylated and the non-phosphorylated forms of Exo1, and possibly the ability to convert one form into the other, are necessary for the proper response of *pol32* cells to HU. Although Pol32 functions as a subunit of Pol δ and Pol ζ the different effects of *pol32* and *rev3* mutations, the latter of which disrupts the catalytic activity of Pol ζ suggest that the observed genetic interactions between *pol32* and *exo1* mutations on hydroxyurea are dominated by the role of Pol32 as a processivity factor of Pol δ .

The inability to phosphorylate serine residues 372, 567, 587 and 692 could explain the unrestrained activity of the *exo1-SA* mutant, which appears to exceed that of the *exo1-SD*

mutant slightly. Whereas hypertoxicity of *exo1-SA* suggests that phosphorylation of the four serine residues is necessary for the proper regulation of Exo1 activity, hypertoxicity of the *exo1-SD* mutant could suggest that it is insufficient. That the aspartic acid substitutions in the *exo1-SD* mutant, which mimic the negative charge of phosphorylated serine residues, do not inactivate, even partially, Exo1, argues that irreversible negative charges at residues 372, 567, 587, and 692 may interfere with additional events that are required for the downregulation of Exo1 activity at stalled replication forks, such as additional phosphorylation events or conformational changes. In addition to phosphorylation, the activity of Exo1 could be affected by binding to proteins that modulate its activity through conformational changes, recruitment to DNA substrates, subcellular relocalization, or sequestration. The proteins that are currently known to bind to yeast Exo1 are the MMR factors Msh2 and Mlh1 [1], the 14-3-3 proteins Bmh1 and Bmh2 [46], and Mec3, a subunit of the Ddc1/Rad17/Mec3 (9-1-1) checkpoint clamp [24]. All could affect Exo1 activity. First, we show here that deletion of *MSH2* significantly suppresses the detrimental effects of the hyperactive *exo1-SD* allele, suggesting that constitutive phosphorylation of the Exo1 C-terminus might lead to a dysregulation of its physical interaction with Msh2. MMR factors, which travel with the replisome via interaction with PCNA [59, 60], could be involved in recruiting Exo1 to replication forks and provide additional 5' ends for Exo1 to resect, thereby contributing to increased Exo1-dependent ssDNA formation at stalled replication forks. Recent evidence in yeast indeed suggests a role for MMR activity and Exo1 in checkpoint activation through promoting ssDNA formation at stalled replication forks and DNA repair-induced gaps [68, 69]. Second, 14-3-3 proteins can modulate protein activity through affecting access to substrates by changing a protein's subcellular localization, by inducing conformational changes that either increase or decrease activity, or by bridging interaction with another protein [70]. Although the vast majority of 14-3-3 proteins interact with phosphorylated targets, the question whether Bmh1/2 interacts with a phosphorylated or a unphosphorylated form of Exo1 is currently unresolved, as is the molecular mechanism by which Bmh1/2 affects Exo1 activity [46]. Third, as Exo1 phosphorylation occurs in a Mec1-dependent manner in response to HU and MMS [38, 46], it is plausible that phosphorylation-dependent binding of Exo1 to the Mec3/Ddc1/Rad17 checkpoint clamp, which loads onto the ds/ssDNA junctions at ssDNA gaps, could control Exo1 activity during the DNA-damage response. Whereas Rad27 activity is strongly stimulated through physical interaction with PCNA [71-73], it is not known if Exo1 activity at replication forks is regulated by another protein. The interaction of Exo1 with the Mec3 checkpoint clamp, however, could provide such regulation during the DNA damage response - analogous to the interaction between Rad27 and PCNA during DNA replication.

The ability of an *EXO1* deletion to rescue *pol32* cells treated with a low dose of HU (55 mM), but not those exposed to a high dose of HU (200 mM) might be due to a difference in the types of DNA lesions and their quantity induced by low and high doses of HU. For instance, deletion of *EXO1* may have a positive effect on viability by preventing excessive ssDNA formation at slowed or stalled forks, which are likely to dominate in *pol32* cells exposed to low HU concentrations. In contrast, deletion of *EXO1* is unlikely to prevent the formation DNA breaks, which may be more typical in cells exposed to high HU concentrations. Similarly, the different extent by which the dephospho-mimicking and

phospho-mimicking *exo1* alleles affect the viability of the *cdc13-1* mutant [47] and HU-treated *pol32* cells could indicate that the DNA-damage response is differently regulated for different regions of the genome. Specifically, differences in the sets of proteins and DNA structures found at telomeres versus those found at replicating DNA could cause differences in the regulation of Exo1 activity and the extent of DNA resection at those sites. In an *cdc13-1* mutant, for instance, Exo1 is responsible for the majority of new ssDNA formation at the unprotected telomeres and for the initiation of Mec1-dependent checkpoint activation [74, 75] reviewed in [76], whereas Exo1 appears to be redundant with other nuclease functions during 5' end resection elsewhere in the genome, most notably in the processing of DSB ends during HR [18] and degradation of the mismatch-containing strand in MMR [1]. Finally, the differences between the effect of the *exo1* phosphorylation mutants in *pol32* and *cdc13-1* mutants could also suggest that phosphorylation is more important for regulating Exo1 activity at slowed and stalled replication forks than at uncapped telomeres.

Although in the absence of Exo1 excessive ssDNA formation and loss of cell viability could be prevented during increased fork stalling, other aberrant structures may still form due to lack of resection by Exo1, leading to genome instability. This is supported by the synergistic increase in the GCR rate in the *exo1 pol32* mutant. Lack of resection in the absence of Exo1 could also provide an explanation for the rare complex mutations we observed at the *CAN1* locus, which appear to be a combination of deletion and insertion events. They could, for example, arise if ends of the nascent strands dissociate from their templates, misanneal in a chickenfoot structure and then dissociate after limited DNA synthesis to reanneal with their template strands.

Our findings are consistent with the report in cells with uncapped telomeres that phosphorylation inhibits Exo1 activity, but they do, on their own, not exclude the possibility that phosphorylation upregulates Exo1 activity at stalled replication forks. Upregulation of Exo1 activity by phosphorylation is consistent with our finding that disruption of the nuclease activity of Exo1 rescues the viability of HU-treated *pol32* cells. It would also provide an explanation for the hypertoxicity of the phospho-mimicking *exo1*-SD mutant in HU-treated *pol32* cells and the normal function of the allele in MMS-treated *pol32* cells, whose viability depends on functional Exo1. The hypertoxicity of the *exo1*-SA mutant, which is refractory to phosphorylation at the previously identified four serine residues, could be explained if activating phosphorylation events shift to other residues. Since the C-terminal half of Exo1 is predicted to be unstructured [20], other serine residues in this 331-residue region should be easily accessible to phosphorylation. Indeed, Western blot analysis has shown that the *exo1*-SA mutant can still be phosphorylated in the presence of DNA damage [47]. Additional analyses of Exo1 post-translational modifications will therefore be needed to determine if Exo1 activity is regulated differently in response to varying DNA damage-inducing agents, genetic mutations, or types of DNA lesions, and how these modifications affect the binding of Exo1 to proteins that might modulate its activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Exo1 nuclease activity is toxic to HU-treated *pol32* cells
- Exo1 phosphorylation site mutations increase HU sensitivity of *pol32* cells
- *msh2* suppresses HU sensitivity of *pol32* cells carrying the *exo1-SD* allele
- Exo1 phosphosite mutations do not affect genome stability in *sgs1* cells

Figure 1.

Effect of DNA repair and checkpoint mutations on DNA-damage sensitivity of *sgs1*, *exo1* and *sgs1 exo1* cells. (A – D) At 10 mM HU, the *tel1*, *rad1*, *rev3* and *apn1* mutations do not affect the viability of *sgs1* and *exo1* mutants, but inhibit growth of the *sgs1 exo1* mutant. (E) The *exo1* mutation suppresses the hypersensitivity of the *pol32 sgs1* mutant to HU, but not to MMS. (F) Hypersensitivity of the *pol32* mutant to higher concentrations of HU (55 mM) is suppressed by *exo1*.

Figure 2.

Sensitivity of *pol32* cells to HU and MMS is differently affected by nuclease-dead and phosphorylation site mutants of *EXO1*. (A) Toxicity of Exo1 in HU-treated *pol32* cells is due to the nuclease activity of Exo1. *exo1* mutants with four serine residues substituted with either alanine (*exo1-SA*, dephospho-mimic) or aspartic acid (*exo1-SD*, phospho-mimic) are more toxic to HU-treated *pol32* cells than wildtype Exo1. In MMS-treated *pol32* cells, deletion of Exo1 activity causes a decrease of viability whereas the phospho-site mutants of Exo1 have no effect. (B) Deletion of *EXO1* does not improve viability of *pol32* cells exposed to a high concentration of HU (C) The hypertoxicity of *exo1-SA* and *exo1-SD* in HU-treated *pol32* cells depends on the nuclease activity of Exo1. (D) The dephospho-mimicking *exo1-SA* allele is more toxic to HU-treated *pol32* cells than the phospho-mimicking *exo1-SD* allele. (E) The *rev3* mutation does not affect the sensitivity of the *exo1 pol32* mutant to 55 mM HU, but increases its sensitivity to 0.01% MMS. Unlike the

exo1 mutation, the *rev3* mutation is not a major suppressor of the hypersensitivity of the *pol32* mutant to 55 mM HU.

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Figure 3.

Effect of DNA mismatch repair on DNA damage sensitivity of *pol32* and *exo1* mutants.

(A) Deletion of *MSH2* does not affect the HU hypersensitivity of the *pol32* mutant or the *pol32 exo1-SA* mutant, but suppresses the hypertoxicity of the phospho-mimic *exo1-SD* allele in HU-treated *pol32* cells. (B) In contrast to inactivation of *EXO1* (*exo1*, *exo1-ND*), deletion of *MSH2* does not increase the sensitivity of the *pol32* mutant to 0.01% MMS.

Figure 4.

Effect of phosphorylation site mutations on the function of Exo1 function in mutation avoidance. Patches were grown from individual colonies of the indicated mutants to allow accumulation of mutations at the *CAN1* locus. Patches were then replica-plated onto media supplemented with canavanine to select Can^r mutants. The *exo1-SA* and *exo1-SD* alleles do not exhibit a mutator phenotype when grown in the absence of HU (A) or presence of 10 mM HU (B) prior to replica-plating onto canavanine media (see Supplemental Figure S2 for the *exo1-SD* allele). (C) Compared to the single mutants, the *exo1 pol32* mutant exhibits a synergistic (24-fold) increase in Thr^r reversion events (*hom3-10* allele), but no increase in the accumulation of Can^r mutants (*CAN1* locus). Whereas the *exo1-SA* allele exhibits a

wildtype mutation rate, the *exoI-SD* allele causes a small (2.8-fold), but significant, increase in the accumulation of Thr⁺ revertants.

Table 1
Gross-chromosomal rearrangements (GCRs) in the *sgs1 exo1* mutant

Relevant Genotype	GCR Rate (Can ^r 5-FOA ^r ×10 ⁻¹⁰)	95% CI ^a (Can ^r 5-FOA ^r ×10 ⁻¹⁰)	Fold increase over <i>sgs1 exo1</i>
wildtype	1.1	< 1 – 6.2	–
<i>exo1</i>	14	7-28	–
<i>sgs1</i>	89	57-177	–
<i>exo1 sgs1</i>	40,500	31,000-49,800	–
<i>exo1 sgs1 rev3</i>	176,000	148,000-210,000	4.4
<i>exo1 sgs1 pol32</i>	141,000	107,000-166,000	3.5
<i>exo1 sgs1 tell</i>	140,000	126,000-174,000	3.4
<i>exo1 sgs1 rad1</i>	96,600	74,500-180,000	2.4
<i>exo1 sgs1 apn1</i>	78,500	57,000-131,000	1.9

^a95% confidence intervals (CI) were calculated according to reference [54]

Table 2
***EXO1*-dependent suppression of gross-chromosomal rearrangements (GCRs) in DNA repair and DNA-damage checkpoint mutants**

Relevant Genotype	GCR Rate ^a (Can ^r 5-FOA ^r × 10 ⁻¹⁰)	95% CI ^b (Can ^r 5-FOA ^r × 10 ⁻¹⁰)	Increase over single mutant ^c
wildtype	1.1	< 1 – 6.2	
<i>exo1</i>	14	7-28	
<i>rad1</i>	10	< 9-23	
<i>exo1 rad1</i>	45	30-66	3.2
<i>apn1</i>	19	14-41	
<i>exo1 apn1</i>	64	42-224	3.3
<i>tel1</i>	2	1.8-2.2	
<i>exo1 tel1</i>	95	46-180	6.7
<i>pol32</i>	20	15-26	
<i>exo1 pol32</i>	173	94-273	8.6
<i>rev3</i>	13	8-20	
<i>exo1 rev3</i>	205	127-304	14.6
<i>msh2</i>	40	n.d.	
<i>msh2 pol32</i>	41	25-102	1

^aGCR rates for *msh2* and *tel1* are from references [77,78].

^b95% confidence intervals (CI) were calculated according to reference [54]; n.d. not determined.

^cThe increase in the GCR rate of the double mutant was calculated by dividing the GCR rate of the double mutant by the higher GCR rate of the two single mutants.

Table 3

Effect of nuclease-dead and phosphosite mutations in *EXO1* on the accumulation of gross-chromosomal rearrangements.

Relevant Genotype	GCR Rate (Can ^r 5-FOA ^r × 10 ⁻¹⁰)	95% CI ^a (Can ^r 5-FOA ^r × 10 ⁻¹⁰)
wildtype	1.1	< 1-6.2
<i>exo1</i>	14	7-28
<i>exo1-ND</i>	58	12-100
<i>exo1-ND sgs1</i>	69,000	45,000-79,000
<i>exo1 sgs1</i>	40,500	31,000-50,000
<i>pol32</i>	20	15-26
<i>exo1-ND pol32</i>	270	110-650
<i>exo1 pol32</i>	170	94-270
<i>exo1-SA</i>	23	16-85
<i>exo1-SA sgs1</i>	33	<12-119
<i>exo1-SD sgs1</i>	43	<22-140
<i>exo1-SA pol32</i>	<20	<17-33
<i>exo1-SD pol32</i>	<8	<7-19

^a 95% confidence intervals (CI) were calculated according to reference [54].