Drug-Sensitive DNA Polymerase $\delta$ Reveals a Role for Mismatch Repair in Checkpoint Activation in Yeast

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ABSTRACT We have used a novel method to activate the DNA damage S-phase checkpoint response in Saccharomyces cerevisiae to slow lagging-strand DNA replication by exposing cells expressing a drug-sensitive DNA polymerase $\delta$ (L612M-DNA pol $\delta$) to the inhibitory drug phosphonoacetic acid (PAA). PAA-treated pol3-L612M cells arrest as large-budded cells with a single nucleus in the bud neck. This arrest requires all of the components of the S-phase DNA damage checkpoint: Mec1, Rad9, the DNA damage clamp Ddc1-Rad17-Mec3, and the Rad24-dependent clamp loader, but does not depend on Mrc1, which acts as the signaling adapter for the replication checkpoint. In addition to the above components, a fully functional mismatch repair system, including Exo1, is required to activate the S-phase damage checkpoint and for cells to survive drug exposure. We propose that mismatch repair activity produces persisting single-stranded DNA gaps in PAA-treated pol3-L612M cells that are required to increase DNA damage above the threshold needed for checkpoint activation. Our studies have important implications for understanding how cells avoid inappropriate checkpoint activation because of normal discontinuities in lagging-strand replication and identify a role for mismatch repair in checkpoint activation that is needed to maintain genome integrity.

EUkaryotic cells have the ability to detect DNA damage and stalled replication forks, and, if problems are found, checkpoints are triggered to control cell cycle progression, induce DNA repair enzymes, stabilize replication forks, remodel chromatin, modulate gene expression, and many other activities. Coordinated action of these processes prevents cell death while preserving genome integrity; however, if surveillance and repair activities are not successful, the resulting genomic instability produces cell death or is mutagenic and even potentially carcinogenic in human cells (Hartwell and Kastan 1994; Myung et al. 2001; Nyberg et al. 2002; Brown et al. 2003). Given the multitude of genotoxic lesions that can be produced by endogenous and exogenous agents and the formation of aberrant DNA structures that can occur during normal DNA replication (Tourière and Pasero 2007), it is not surprising that S-phase checkpoint activation and responses are complex.

Briefly, checkpoint pathways are protein kinase cascades that begin with sensing DNA damage in the context of single-stranded DNA (ssDNA) that is coated with ssDNA binding protein (RPA) (Figure 1). The PCNA-like Ddc1-Rad17-Mec3 clamp complex (the Saccharomyces cerevisiae 9-1-1 DNA damage clamp) is loaded near DNA damage by the Rad24-dependent clamp loader, but does not depend on Mrc1, which acts as the signaling adapter for the replication checkpoint. In addition to the above components, a fully functional mismatch repair system, including Exo1, is required to activate the S-phase damage checkpoint and for cells to survive drug exposure. We propose that mismatch repair activity produces persisting single-stranded DNA gaps in PAA-treated pol3-L612M cells that are required to increase DNA damage above the threshold needed for checkpoint activation. Our studies have important implications for understanding how cells avoid inappropriate checkpoint activation because of normal discontinuities in lagging-strand replication and identify a role for mismatch repair in checkpoint activation that is needed to maintain genome integrity.

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Rad53 phosphorylation (Figure 1) is a key step in the signal transduction cascade (Tercero and Diffley 2001; Nyberg et al. 2002). Once activated, Rad53 phosphorylates protein targets including Dun1, which in turn phosphorylates proteins that lead to the up-regulation of deoxynucleoside triphosphate (dNTP) synthesis and the transcription of DNA repair genes (Huang et al. 1998; Bashkirov et al. 2000; Zhao and Rothstein 2002; Ahnesorg and Jackson 2007). Rad53 also acts either alone or in combination with Chk1 to arrest cells at G2/M, which prevents the premature segregation of damaged or broken chromosomes (reviewed in Hsieh and Yamane 2008; Branzei and Foiani 2009; Schleker et al. 2009).

Traditionally, the favored experimental approach to invoke the S-phase DNA damage checkpoint response is to treat cells with a high dose of a DNA-damaging agent. While studies using a variety of chemicals and radiation treatments have provided a wealth of information on checkpoint pathways, controversy remains due, in part, to the different types of genotoxic agents used, varying dosages, and the wide range of lesions generated from even a single type of treatment. One controversy concerns the role of mismatch repair (MMR) in checkpoint activation. In the “signaling” model, MMR complexes are proposed to bind to DNA damage and form a protein scaffold that recruits sensor and effector checkpoint kinases, which facilitates phosphorylation and checkpoint activation (Brown et al. 2003; Wang and Qin 2003; Adamson et al. 2005; Yoshioka et al. 2006). For the “processing” model, MMR DNA degradation activity is proposed to generate persisting ssDNA gaps that produce DSBs when encountered by advancing replication forks (Mojas et al. 2007). These proposed roles of MMR in checkpoint activation, however, are paradoxical since checkpoint activation is a mechanism generally thought to help cells survive DNA damage, but the absence of MMR activity often renders mammalian cells less sensitive to killing by several DNA-damaging agents (reviewed by Jiricny 2006; Hsieh and Yamane 2008). The MMR status of cancer cells is important for therapeutic treatments because the absence of MMR can confer resistance (reviewed by Karran 2001; Hsieh and Yamane 2008).

We report here a new approach to probe the role of MMR in the activation of the S-phase DNA damage checkpoint in *S. cerevisiae* by using a drug-sensitive DNA polymerase (pol) δ (Li et al. 2005). The yeast L612M-DNA pol δ, which was modeled on the bacteriophage T4 L412M-DNA pol (Reha-Krantz et al. 1993; Reha-Krantz and Nonay 1994), has a Leu-to-Met substitution in the conserved motif A sequence in the polymerase active center. Both the phage T4 and yeast mutant DNA pols are sensitive to the antiviral drug phosphonoacetic acid (PAA) (Reha-Krantz et al. 1993; P. Burgers, personal communication). Mounting evidence suggests that there is a division of labor at DNA replication forks with DNA pol ε performing leading-strand replication (Pursell et al. 2007) and DNA pol δ serving as the lagging-strand DNA pol (Nick McElhinny et al. 2008); thus, PAA is expected to inhibit lagging-strand replication in cells expressing the L612M-DNA pol δ (pol3-L612M cells). All other DNA pol δ functions in DNA repair and recombination will also be compromised in the presence of PAA.

PAA-treated pol3-L612M cells arrest with the classic dumbbell morphology—a large mother cell and an attached daughter bud of similar size with a single nucleus located in the bud neck (Figure 2A) as observed for yeast treated with DNA-
damaging agents and for temperature-sensitive DNA pol δ (cdc2) cells at the nonpermissive temperature (Weinert and Hartwell 1993). A fully functional MMR system is required to maintain viability of pol3-L612M cells exposed to PAA (Li et al. 2005). pol3-L612M cells lacking any component of MMR except for Msh3, but including Exo1, are killed by very low concentrations of PAA (≤0.2 mg/ml). Although a large increase in mutations may be suspected of causing cell death, error catastrophe is unlikely in the case of EXO1 deficiency because only a modest increase in mutations is observed (Li et al. 2005).

Here we tested the hypothesis that the severe PAA sensitivity of MMR-defective pol3-L612M cells is because MMR is needed to assist activation of an S-phase checkpoint.

Materials and Methods

Media, strains, and the synthetic genetic array screen

Cells were grown in either glucose or galactose standard synthetic media supplemented with amino acids; the type of sugar used did not affect PAA sensitivity. PAA gradients were made as described previously (Li et al. 2005). We find that spotting a constant number of cells (50–100) across a gradient is more useful for determining chemical sensitivity than assays in which serial dilutions of cells are spotted on a chemical-containing plate because high cell concentrations mask sensitivity.

All strains are described in Table 1. Gene inactivation was done by replacement of an endogenous wild-type gene with the kanMX cassette in POL3/pol3-L612M heterozygous diploids. Haploids containing the desired mutations were identified by tetrad dissection or by random spore analysis. All gene replacements were verified by PCR and the DNA pol δ gene was sequenced to confirm the presence of the pol3-L612M allele.

The pol3-L612M query strain Y6295 was constructed for the synthetic genetic array (SGA) analysis by transforming the YS563 starter strain (MATa can1Δ::MEF1pr-HIS3 lyp1Δ urac3Δ0 leu2Δ0 his3Δ1 met5Δ0) with the URA3 YIP-5 pol3-L612M plasmid (Li et al. 2005). The transformant had a truncated pol3 gene and a full-length pol3-L612M gene as confirmed by DNA sequencing. The linked URA3 marker was retained to select for pol3-L612M double mutants. The SGA screen was performed in triplicate as described (Tong et al. 2001, 2004). The final step was to test the double-mutant array strains on plates with and without PAA. The PAA sensitivity of all double mutants was confirmed (Supporting Information, Figure S1, Figure S2, and Figure S3).

DNA labeling, cell morphology, cell viability

Because fungi do not have a nucleoside salvage pathway, yeast were engineered to express the human nucleoside transporter and herpes virus thymidine kinase from the GAL1-10 promoter (Vernis et al. 2003), which allows [3H]thymidine to be taken up by yeast cells and phosphorylated to [3H]dTMP. [3H]dTMP is then further phosphorylated by cellular enzymes to [3H]dTTP, which can be used in DNA replication. A total of 20 μCi/ml [3H]thymidine was added to the labeling cultures at the start of the experiment. To measure [3H]dTMP incorporation into DNA, 0.2-ml samples of labeled cells were mixed with 0.2 ml of yeast carrier cells (10⁹ cells/ml) plus 0.7 ml 20% trichloroacetic acid (TCA). The cells were pelleted and the supernatant was removed by aspiration. The cells were then suspended in 0.5 ml 0.1 M NaOH, and then 0.5 ml of 15% TCA was added to reprecipitate acid-insoluble molecules including DNA. Suspensions were filtered through GF/C filters (Whatman) to capture DNA, and the dried filters were counted in scintillation fluor.

To estimate the amount of DNA replication in PAA-arrested pol3-L612M cells, we first determined the amount of label in a single cell. pol3-L612M cells were evenly labeled with [3H]dTMP in the absence of PAA. The amount of label per dumbbell was then determined for fully arrested pol3-L612M cells (4 hr exposure to 1 mg/ml PAA). Since nearly all of the arrested cells are large-budded cells, the amount of label per arrested cell was divided by the amount of label expected for two genomes; this value was ~70%.

In most experiments, early exponential-phase cells were synchronized by adding α-factor to a final concentration of 5 μg/ml and incubating for 2.5 hr. The cells were then pelleted by centrifugation, resuspended in fresh medium, pelleted again, and then resuspended.

Cell morphology was monitored by microscopic examination of cells throughout the experiment. Cell viability was determined by comparing the cell titer from counting cells in a hemocytometer to the number of cells that can form visible colonies in 3 days.

Western blots

Standard procedures were followed. Cells were synchronized with α-factor and then exposed to PAA for various times. Protein extracts were prepared by the TCA method. Briefly, 2 × 10⁸ cells were washed first with water and then 20% TCA. The cell pellets were suspended in 100 μl 20% TCA and stored at −20°C. Extracts were prepared by mixing an equal volume of glass beads with the TCA-treated cells and then by vigorous vortexing until the majority of the cells were broken as determined by microscopic examination. The broken cells were transferred to a fresh tube, and the glass beads were washed twice in 100 μl of 5% TCA. Protein was pelleted by centrifugation and resuspended in 100 μl Laemli buffer and neutralized by adding 50 μl of 2 M Tris. The sample was then boiled for 3 min and centrifuged. The supernatant, which is the protein sample, was then transferred to a fresh tube. Protein concentration was determined by SDS gel analysis. Rad53 phosphorylation was revealed by goat polyclonal antibodies to yeast Rad53 (Santacruz catalog no. sc6749) and rabbit anti-goat secondary antibodies conjugated with horseradish peroxidase (HRP) (Sigma catalog no. A5420). H2A phosphorylation was revealed by rabbit polyclonal antibodies to yeast phosphorylated H2A.
(phosphorylated Ser129) (Abcam catalog no. ab15083) and goat anti-rabbit IgG HRP (Santacruz catalog no. sc2004).

**Gross chromosomal rearrangement rates**

Gross chromosomal rearrangement (GCR) rates were determined as described (Motegi and Myung 2007). Deletion of the left arm of chromosome V, which contains the endogenous **CAN1** gene and the **URA3** gene inserted into the nearby **HXT13** gene, was detected by plating cells on plates containing both canavanine and 5-FOA. Parallel cultures in synthetic dextrose medium were grown either with or without 1 mg/ml PAA starting with \(\sim 0.7 \times 10^5\) cells or with a single 3-day colony. The pH of the PAA-containing medium was adjusted to 4.5 with NaOH. PAA-containing cultures were grown for 20 hr with shaking and then pelleted and resuspended in 10 ml YPD and grown overnight. Cultures without PAA were grown overnight. Cells were concentrated 10-fold and titered to determine the total number of cells and the number of cells that could grow on plates containing both canavanine and 5-FOA. Under these conditions, we could not detect any CanR 5-FOA-resistant mutants in cultures of wild-type or **pol3-L612M** cells not exposed to PAA or in cultures of wild-type

<table>
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<th>Strains</th>
<th>Genotype</th>
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<td>Amy Tong, C. Boone</td>
</tr>
<tr>
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<td>R. Kolodner</td>
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<td>SY125 rad51::kanMX</td>
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<tr>
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<td>SY243 pol3-L612M</td>
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**Table 1. S. cerevisiae strains used in this study.**
cells exposed to PAA, but we assume that the GCR rate is the low reported rate of $0.3 \times 10^{-9}$ as previously reported (Motegi and Myung 2007). As a control, we determined the GCR rate for $\text{xrs2}$ cells and observed a rate of $190 \times 10^{-9}$, which is similar to the reported rate (Chen and Kolodner 1999) (Table 2). Rates were determined as described (Motegi and Myung 2007); the median rate is reported.

## Results

### PAA-treated pol3-L612M cells arrest growth and DNA replication

PAA-treated pol3-L612M cells arrested as large-budded (swollen) cells with the mother and a similarly sized daughter sharing an undivided nucleus (Figure 2A). To examine the progress of DNA replication following addition of PAA, cells were engineered to incorporate $[3H]$dTMP into newly replicated DNA as described in Materials and Methods. In the absence of PAA, the doubling time of pol3-L612M cells in galactose medium was $\sim 2.5$ hr (Figure 2B, solid circles); DNA replication, as measured by $[3H]$dTMP incorporation, paralleled cell growth (Figure 2C, solid circles). In contrast, cell division ceased after 1 hr in the presence of 1 mg/ml PAA (Figure 2B, open circles), but DNA replication continued for $\sim 3$ hr (Figure 2C, open circles). About 70% of the nuclear genome of PAA-arrested pol3-L612M cells was replicated as determined by comparing the amount of $[3H]$dTMP-labeled DNA per cell for cells not exposed to PAA to the amount of label per cell in cells exposed to PAA (see Materials and Methods). A similar value was observed by Conrad and Newlon (1983).

In contrast to pol3-L612M cells, wild-type (POL3) cells did not arrest in the presence of PAA, but progressed through the cell cycle at a slower doubling rate of $\sim 3.5$ hr compared to $\sim 2.5$ hr in the absence of PAA (data not shown). Thus, PAA slows growth of wild-type cells, but triggers cell cycle arrest in pol3-L612M cells.

### PAA-triggered cell cycle arrest in pol3-L612M cells depends on the DNA damage S-phase checkpoint

Both the replication and DNA damage S-phase checkpoints arrest cell cycle progression (Figure 1). The two checkpoints

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**Table 2 GCR rates for pol3-L612M cells exposed to PAA**

<table>
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<th>Relevant genotype</th>
<th>Strain</th>
<th>GCR rates ($\times 10^9$)</th>
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<tbody>
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<td>Wild type</td>
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<td>$0.3$ (1)$^{a,b}$</td>
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<tr>
<td>pol3-L612M</td>
<td>SY120</td>
<td>$0.3$ (1) $6$ (20)</td>
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<tr>
<td>$\text{xrs2 POL3}$</td>
<td>SY180</td>
<td>190 (633) $\text{ND}^{c}$</td>
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<td>$\text{xrs2 pol3-L612M}$</td>
<td>SY185</td>
<td>160 (533) $\text{ND}^{c}$</td>
</tr>
<tr>
<td>rad51 POL3</td>
<td>SY243</td>
<td>0.8$^{f}$ (3) $1.0$ (3)</td>
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<tr>
<td>rad51 pol3-L612M</td>
<td>SY242</td>
<td>1.0 (3) $2.7$ (9)</td>
</tr>
</tbody>
</table>

All strains are isogenic with the wild-type strain. GCR rates were determined as described in Materials and Methods.

$^{a}$ We could not detect any Can$^R$ S-FOAR$^a$ mutants in the wild-type strain; thus the rate is $< 1 \times 10^{-9}$. The wild-type GCR rate reported in the literature is $\sim 0.3 \times 10^{-9}$ (Motegi and Myung 2007).

$^{b}$ Rates relative to the wild-type strain are given in parentheses and were calculated assuming that the wild-type GCR rate is $0.3 \times 10^{-9}$.

$^{c}$ The reported GCR rate for $\text{xrs2}$ cells, $220 \times 10^{-9}$ (Myung et al. 2001), is similar to the rate determined here.

$d$ Not determined.

$e$ GCR rates could not be measured in the presence of PAA because these strains are too PAA sensitive.

$^{f}$ The reported GCR rate for rad51 cells, $3.5 \times 10^{-9}$ (Chen and Kolodner 1999), is about fourfold higher than the rate observed in our studies.
share several components in common, but a distinguishing feature is the dependence of the DNA damage checkpoint on Rad9 and its independence on Mrc1, which is required to mediate the replication checkpoint at stalled replication forks. To determine which checkpoint components are required for the PAA-triggered arrest of pol3-L612M cells and to uncover other DNA pol δ interactions that affect PAA sensitivity, we carried out a SGA in which the pol3-L612M

Figure 3 Identification of S-phase checkpoint genes required to protect pol3-L612M cells from PAA. Cells of the indicated genotypes were spotted across a 0- to 1-mg/ml PAA gradient. Colonies arising from cells plated on medium without PAA are shown on the left edge of each PAA gradient plate. Photos of the gradient after 3 days (A) and 6 days (B) incubation are shown. Deletion of MRC1 increases the PAA sensitivity of pol3-L612M cells (slow growth, synthetic sick phenotype), but deletion of RAD24 produces synthetic lethality. Deletion of RAD24 in pol3-L612M cells prevents the arrest of cell growth that is observed for pol3-L612M cells treated with 1 mg/ml PAA (C) and the arrest of DNA replication (D) and produces a sharp decline in cell viability (E). In contrast, mrc1 pol3-L612M cells arrest cell growth in the presence of 1 mg/ml PAA (F, open squares) as observed for pol3-L612M cells (F, open circles). Normal growth was observed in the absence of PAA for mrc1 pol3-L612M cells (F, solid squares) and for pol3-L612M cells (F, solid circles). DNA replication arrest was also observed for mrc1 pol3-L612M cells (G, open squares) to almost the same level measured for pol3-L612M cells (G, open circles). DNA replication in mrc1 pol3-L612M cells in the absence of PAA (G, solid squares) paralleled cell growth. Although cell viability declined during PAA treatment of mrc1 pol3-L612M cells (H) compared to pol3-L612M cells that maintained ~90% viability throughout the experiment (horizontal line), this decline was not as severe as observed for rad24 pol3-L612M cells (E).
PAA concentrations as demonstrated for DDC1 concentration after 6 days (Figure 3B). In addition to notype because growth was observed only at the lowest PAA cells (Figure 3A). This is the killing or synthetic lethal phenotype of the same extent as observed for deletion of enhanced the slow growth or synthetically sick phenotype to phosphorylation was not observed.

Figure 4 PAA induces Rad53 phosphorylation in pol3-L612M cells. Rad53 phosphorylation, as indicated by the slower migrating bands in a denaturing protein gel (Rad53-P), was detected within 1 hr after the addition of 1 mg/ml PAA to a-factor-synchronized pol3-L612M cells in glucose medium (top). An increase in Rad53 phosphorylation was detected after 2 and 3 hr exposure to PAA. The length of PAA treatment (in hours) is indicated above each lane. Rad53 phosphorylation was also observed for asynchronized pol3-L612M cells treated with 1 mg/ml PAA after 5 hr, but not for wild-type cells (middle). Rad53 phosphorylation was also observed for asynchronized mrc1 pol3-L612M cells, but not for rad24 pol3-L612M cells (bottom). Rad53 phosphorylation is correlated with cell cycle progression. PAA-treated pol3-L612M and mrc1 pol3-L612M cells arrest as swollen dumbbell-shaped cells, and robust Rad53 phosphorylation is detected for these cells. In contrast, PAA-treated wild-type and rad24 pol3-L612M do not arrest cell cycle progression, and Rad53 phosphorylation was not observed.

Strain was crossed to ~4700 deletion mutants (Tong et al. 2001, 2004). The double mutants were placed into two groups on the basis of PAA sensitivity.

For one group, PAA increased the slow growth phenotype as demonstrated by mrc1 pol3-L612M cells. PAA sensitivity was measured by spotting cells on a plate with a gradient of PAA from 0 to 1 mg/ml. Increased PAA sensitivity was observed for the double-mutant mrc1 pol3-L612M compared to the single-mutant strains at 3 days (Figure 3A), but was less apparent after 6 days incubation (Figure 3B). Deletion of TOF1, CTF18, CTF8, or CTF4 in the pol3-L612M background enhanced the slow growth or synthetically sick phenotype to the same extent as observed for deletion of MRC1 (Figure S1).

For the second group, cell death was observed at very low PAA concentrations as demonstrated for rad24 pol3-L612M cells (Figure 3A). This is the killing or synthetic lethal phenotype because growth was observed only at the lowest PAA concentration after 6 days (Figure 3B). In addition to RAD24, this group includes the DNA damage clamp genes DDC1, RAD17, and MEC3 as well as RAD9 and MEC1 (Figure S2 and Figure S3). The synthetically sick and lethal phenotype was observed only in the presence of the pol3-L612M allele (Figure 3, A and B; Figure S1; Figure S2; Figure S3). The stronger dependence of PAA-treated pol3-L612M cells on RAD9 function compared to MRC1 indicates that survival is dependent on the DNA damage checkpoint.

Two methods were used to confirm the presence or absence of S-phase checkpoint activation. If an S-phase checkpoint is not activated, DNA replication is expected to continue unabated in the presence of PAA as observed for the human ataxia telangiectasia mutant or related ATM/ATR cells in which replication continues in the presence of DNA damage (Painter and Young 1980). rad24 pol3-L612M cells failed to arrest cell growth in the presence of PAA (Figure 3C, open triangles) or to curb DNA replication, as measured by [3H]dTMP incorporation (Figure 3D) in contrast to pol3-L612M cells in which DNA replication plateaued at a low level after 3 hr (Figure 2C). Cell viability declined sharply; <10% of rad24 pol3-L612M cells were viable after 5 hr exposure to PAA (Figure 3E). rad24 pol3-L612M cells also did not produce the high numbers of large-budded cells that were observed for arrested pol3-L612M cells in the presence of PAA. The continued cell cycle progression and DNA replication observed for PAA-treated rad24 pol3-L612M cells suggests that the severe PAA sensitivity of these cells is caused by failure to activate an S-phase checkpoint.

In contrast, mrc1 pol3-L612M cells (open squares) arrested growth as large-budded cells in response to PAA as observed for pol3-L612M cells (open circles) (Figure 3F). This experiment was done with asynchronous cells, but the doubling times for pol3-L612M (solid circles) and mrc1 pol-L612M (solid squares) cells in the absence of PAA were ~2.5–2.7 hr (Figure 3F) as observed with synchronous cells (Figure 2B). The doubling time of mrc1 pol3-L612M cells as measured by incorporation of [3H]dTMP was ~2.7 hr (Figure 3G, solid squares). Cell viability declined gradually to ~37% after 32 hr of continuous exposure to PAA (Figure 3H). The slow decline in the viability of mrc1 pol3-L612M cells is in contrast to the sharp decline observed for rad24 pol3-L612M cells exposed to PAA (Figure 3E). Thus, the slow growth/synthetically sick phenotype observed for mrc1 pol3-L612M cells is due to a combination of apparent checkpoint activation and to a loss of viability upon prolonged exposure to PAA.

A second test for checkpoint activation was to measure Rad53 phosphorylation in PAA-treated pol3-L612M cells. Rad53 phosphorylation is a key indicator of checkpoint activation (Figure 1). Rad53 phosphorylation was detected in pol3-L612M cells after 1 hr treatment of synchronized cells with 1 mg/ml PAA in glucose medium in which the doubling time is ~90 min; phosphorylation increased further after 2 and 3 hr exposure (Figure 4, top). Rad53 phosphorylation was also observed for mrc1 pol3-L612M cells (Figure 4, bottom), which is consistent with the PAA-induced cell cycle arrest observed for these cells and for the cessation of DNA replication (Figure 3, F and G). Little Rad53 phosphorylation, however, was detected for rad24 pol3-L612M cells (Figure 4,
bottom), which is consistent with the absence of cell cycle arrest observed for these cells (Figure 3, C and D). Rad53 phosphorylation was also not detected for wild-type cells exposed to PAA (Figure 4, middle). Note that these later experiments were done with asynchronized cells, but swollen dumbbell-shaped cells quickly accumulated for PAA-treated pol3-L612M and mrc1 pol3-L612M cells as expected for checkpoint activation, but they did not accumulate for PAA-treated wild-type or rad24 pol3-L612M cells, and cell growth did not arrest.

**pol3-L612M cells exposed to PAA require DSB repair**

We next examined the possibility that DSBs were produced in pol3-L612M cells exposed to PAA. Deletion of the recombinase (RAD51) or RAD52 genes, which are required for homologous recombination, increases the PAA sensitivity of pol3-L612M cells (Li et al. 2005). More severe PAA sensitivity was observed by deletion of genes that encode components of the yeast MRX complex (Mre11-Rad50-Xrs2) (Figure 5), but less than that detected for deletion of genes required for the DNA damage checkpoint such as RAD24 (Figure 3, A and B). The PAA sensitivity of pol3-L612M cells in the absence of MRX components may reflect the role of these proteins in checkpoint signaling as well as in DSB repair since the DNA damage checkpoint can be initiated but not sustained in the absence of the MRX complex (D’Amours and Jackson 2001). PAA-treated xrs2 pol3-L612M cells arrested initially as swollen dumbbell-shaped cells as observed for pol3-L612M cells, but viability was just 42% by 5 hr and many abnormal multi-budded cells started to accumulate that were not observed for pol3-L612M cells. Viability declined to 25% by 10 hr and was just 8% by 24 hr (data not shown).

We also tested the possibility that DSBs produced by exposure of pol3-L612M cells to PAA increased GCRs. An isogenic set of strains (Table 1) was constructed with a GCR reporter (Chen and Kolodner 1999; Myung et al. 2001; Motegi and Myung 2007). GCRs are detected in the reporter by deletion of the nonessential end of the left arm of chromosome V, which contains the endogenous CAN1 gene and the nearby URA3 gene that was inserted into the nonessential HXT13 gene. The major class of GCRs observed by the simultaneous loss of the CAN1 and URA3 genes is terminal deletions of the end of chromosome V that are healed by de novo telomere addition (Motegi and Myung 2007). A 20-fold increase in the GCR rate was observed for pol3-L612M cells exposed to PAA (Table 2), which suggests that PAA treatment induces DSBs and that repair is not completely error free.

**Role of mismatch repair in protecting pol3-L612M cells exposed to PAA**

We reported previously that deletion of MSH2, MLH1, PMS1, MSH6, or EXO1 (but not MSH3) markedly increases the PAA sensitivity of pol3-L612M cells (Li et al. 2005). The dependence of pol3-L612M cells on MMR was confirmed here (Figure 6A). To avoid complications produced by the high genetic instability of completely MMR-deficient pol3-L612M cells, msh6 pol3-L612M and exo1 pol3-L612M cells were examined. These cells are nearly as PAA sensitive as completely MMR-deficient pol3-L612M cells, but are 80–100% viable in the absence of PAA, and only a small increase in mutations was observed for the exo1 pol3-L612M strain (Li et al. 2005). Cell growth and DNA replication were monitored as described above; PAA-treated msh6 pol3-L612M and exo1 pol3-L612M cells did not arrest cell growth or curb DNA replication as observed for checkpoint-deficient rad24 pol3-L612M cells (Figure 3, C and D). Cell viability declined for PAA-treated msh6 pol3-L612M cells (Figure 6B) at a slower rate than observed for rad24 pol3-L612M cells (Figure 3E), but <5% cells were viable after 26 hr exposure, which accounts for the observed PAA-induced killing/synthetic lethality phenotype. A similar pattern of PAA sensitivity was observed for exo1 pol3-L612M cells (Li et al. 2005).

Little if any Rad53 phosphorylation was observed for synchronized msh6 pol3-L612M cells over a 3 hr exposure to 1 mg/ml PAA (Figure 6C, top) under the same conditions in which extensive Rad53 phosphorylation was observed in pol3-L612M cells after just 1 hr (Figure 4). For synchronized...
was observed for response to DNA damage (Figure 1). H2A phosphorylation phosphorylated histone H2A, which is a well-characterized a more sensitive test to detect DNA damage: antibodies to only/C24 pol3-L612M served for pol3-L612M. Despite the possibility that cannot be replaced by other DNA polymerases (L. Rehakranti and Md. Siddique, unpublished data). Thus, we attempted to detect DSBs in PAA-treated exo1 pol3-L612M or exo1 pol3-L612M cells from PAA. DNA damage treatments sufficient to activate the S-phase damage response on their own could not be used because of the essential roles of DNA pol δ in recombination and repair, which are compromised in PAA-treated pol3-L612M cells. For example, we have observed that PAA increases the UV sensitivity of pol3-L612M cells, which indicates a role for DNA pol δ in the gap-filling step of nucleotide excision repair that cannot be replaced by other DNA polymerases (L. Rehakranti and Md. Siddique, unpublished data). Thus, we tested if a low concentration of HU (10 mM) could substitute for MMR. Partial rescue of the PAA sensitivity of msh6 pol3-L612M and exo1 pol3-L612M cells was observed on a 1-mg/ml PAA gradient plate; colony formation was

ex1 pol3-L612M cells, no increase in Rad53 phosphorylation was visible after 1 hr, but by 3 hr a small amount of Rad53 phosphorylation was detected at ~20% the amount observed for PAA-treated pol3-L612M cells (Figure 6C, lower panels); however, there was much less of the most highly phosphorylated Rad53, which has the slowest rate of migration. The low amount of Rad53 phosphorylation for PAA-treated exo1 pol3-L612M cells may reflect the ability of other exo1 pol3-L612M cells to substitute for Exo1 in MMR; other possibilities are suggested in the Discussion. Despite the small amount of Rad53 phosphorylation observed, PAA-treated exo1 pol3-L612M did not arrest cell growth as observed for pol3-L612M cells.

**H2A phosphorylation is produced in response to PAA for MMR-proficient pol3-L612M cells but not for msh6 pol3-L612M or exo1 pol3-L612M cells**

We attempted to detect DSBs in PAA-treated pol3-L612M cells by using a fluorescent protein–Rad52 construct to detect DSB repair foci (Lisby et al. 2003). If DSBs are responsible for activation of the DNA damage S-phase checkpoint, then Rad52 foci are expected to be visible in most of the PAA-treated, MMR-proficient pol3-L612M cells. Although MRX deficiency strongly increases the PAA sensitivity of pol3-L612M cells (Figure 5), Rad52 foci were observed in only ~20% of PAA-treated pol3-L612M cells. Thus, we tried a more sensitive test to detect DNA damage: antibodies to phosphorylated histone H2A, which is a well-characterized response to DNA damage (Figure 1). H2A phosphorylation was observed for α-factor-synchronized pol3-L612M cells after just 1 hr exposure to 1 mg/ml PAA in glucose medium, which did not increase after 2 and 3 hr exposure (Figure 7). In contrast, little H2A phosphorylation was observed for msh6 pol3-L612M or exo1 pol3-L612M cells even after 3 hr exposure to PAA (Figure 7). The appearance of phosphorylated Rad53 (Figure 4) and H2A (Figure 7) in pol3-L612M cells just 1 hr after exposure to PAA indicates that DNA damage and checkpoint activation occur in the first S phase.

**Can HU rescue MMR-deficient pol3-L612M cells from PAA?**

If the role of MMR in activating the S-phase DNA damage checkpoint in PAA-treated pol3-L612M cells is to increase the level of checkpoint-inducing DNA damage, then another method for creating damage is expected to protect msh6 pol3-L612M and exo1 pol3-L612M cells from PAA. DNA damage treatments sufficient to activate the S-phase damage response on their own could not be used because of the essential roles of DNA pol δ in recombination and repair, which are compromised in PAA-treated pol3-L612M cells. For example, we have observed that PAA increases the UV sensitivity of pol3-L612M cells, which indicates a role for DNA pol δ in the gap-filling step of nucleotide excision repair that cannot be replaced by other DNA polymerases (L. Rehakranti and Md. Siddique, unpublished data). Thus, we tested if a low concentration of HU (10 mM) could substitute for MMR. Partial rescue of the PAA sensitivity of msh6 pol3-L612M and exo1 pol3-L612M cells was observed on a 1-mg/ml PAA gradient plate; colony formation was

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**Figure 6** MMR activity is required to protect pol3-L612M cells from PAA. Cells of the indicated genotypes were spotted across a 0- to 1-mg/ml PAA gradient. (A) Colonies arising from cells plated on medium without PAA are shown on the left edge of each PAA gradient plate. Photos of the gradient after 3 and 7 days incubation are shown. A synthetic lethal phenotype was observed for msh6 pol3-L612M and exo1 pol3-L612M cells at higher PAA concentrations in the gradient. Visible microcolonies were detected for msh6 pol3-L612M and exo1 pol3-L612M cells, but microcolonies at the higher end of the PAA gradient did not enlarge after further incubation, and viable cells could not be recovered by restreaking on medium without PAA. Large, PAA-resistant colonies were also observed. PAA resistance is due to a suppressor mutation that encodes a second amino acid substitution in the POL3/DNA pol δ gene (Li 2004). Viability of PAA-treated msh6 pol3-L612M cells declined in the presence of PAA (B) and also for exo1 pol3-L612M cells (data not shown). Rad53 phosphorylation was severely compromised in msh6 pol3-L612M and exo1 pol3-L612M cells (C). Synchronized msh6 pol3-L612M and exo1 pol3-L612M cells were exposed to PAA from 0 to 3 hr. There was a small amount of Rad53 phosphorylation observed, PAA-treated exo1 pol3-L612M did not arrest cell growth as observed for pol3-L612M cells.
observed in the presence of 10 mM HU (Figure 8, bottom) at higher PAA concentrations than observed in the absence (Figure 8, top). HU may be acting in several ways. For example, HU could increase the length of ssDNA gaps in the lagging strand and/or uncouple leading- and lagging-strand replication; however, in all scenarios, it is likely that ssDNA is increased.

**DUN1 is required for pol3-L612M cells to survive PAA**

If the S-phase DNA damage checkpoint is required for pol3-L612M cells to survive PAA, then downstream targets of Rad53 phosphorylation such as Dun1 are also expected to be required for viability. As expected, dun1 pol3-L612M cells are as PAA sensitive as rad24 pol3-L612M cells in which Rad53 is not activated (Li 2004). Thus, one or more Dun1-regulated activities is required for pol3-L612M cells to survive PAA. We have verified by the SGA screen that one Dun1 phosphorylation target—Rad55, which functions in DSB repair, is needed for pol3-L612M cells to survive PAA. Deletion of SML1, however, did not affect the PAA sensitivity of pol3-L612M cells (Figure S3). Sml1, an inhibitor of ribonucleotide reductase, is phosphorylated by activated Dun1, which relieves inhibition of ribonucleotide reductase and restarts dNTP synthesis (Chabes et al. 2003). If increased dNTP synthesis alone were sufficient to rescue PAA sensitivity, then deletion of SML1 would be expected to decrease the PAA sensitivity of pol3-L612M cells. As this was not observed, other Dun1 and damage checkpoint targets, likely in addition to inactivation of Sml1, are required for pol3-L612M cells to survive PAA.

**POL32 is required for pol3-L612M cells to survive PAA**

Deletion of the POL32 gene, which encodes the DNA pol δ PCNA-binding protein (Johansson et al. 2004), produced severe PAA sensitivity; no growth was detected even at the lowest PAA concentrations tested (Figure S2). Although POL32 is not essential in S. cerevisiae, a slow growth phenotype is observed in its absence and POL32 is essential in other eukaryotes. The strong dependence of pol3-L612M cells on Pol32 in the presence of PAA suggests that PCNA tethering of DNA pol δ at replication forks and/or at other sites of DNA pol δ action is required for viability. A role in maintaining the stability of stalled replication forks is another possibility because deletion of POL32 also increases HU sensitivity (Parsons et al. 2004).

**Discussion**

PAA treatment of pol3-L612M cells activates the DNA damage S-phase checkpoint. Survival requires all components of the DNA damage checkpoint, including Mec1 (the yeast ATR Ser/Thr kinase), the signaling adapter Rad9, and the PCNA-like DNA damage clamp (Ddc1-Rad17-Mec3) and damage clamp loader (Rad24) complex (Figure 3; Figure S2 and Figure S3). pol3-L612M cells deficient in any of these proteins are highly sensitive to PAA and, as shown for rad24 pol3-L612M cells, fail to activate the DNA damage checkpoint as demonstrated by the failure of cells to arrest cell cycle progression, to curb DNA replication and to phosphorylate Rad53 and by subsequent loss of cell viability (Figures 2–4). pol3-L612M cells have less dependence on Mrc1, the checkpoint adapter for stalled replication forks (Figure 3).

An important finding is that while DNA damage as detected by phosphorylated H2A is observed for PAA-treated pol3-L612M cells after 1 hr, only a little phosphorylated H2A is observed for PAA-treated msh6 pol3-L612M or exo1 pol3-L612M cells after 3 hr (Figure 7). Note that phosphorylated H2A is a well-known marker for DSBs, but there is a possibility that phosphorylated H2A is also produced in response to ssDNA. Detection of phosphorylated H2A is
correlated with Rad53 phosphorylation, which is readily detected for PAA-treated pol3-L612M cells after 1 hr (Figure 4), but not for PAA-treated msh6 pol3-L612M cells even after 3 hr (Figure 6C, top) and only a small amount is observed for exo1 pol3-L612M cells (Figure 6C, bottom left).

The dependency of PAA-treated pol3-L612M cells on Exo1 for survival suggests that ssDNA gaps produced by MMR are required for checkpoint activation; however, Exo1 is reported to play several roles, including a structural role in stabilizing MMR complexes that is independent of its enzymatic role (Amin et al. 2001). Outside of MMR, Exo1 has many functions, including processing and stabilizing stalled replication forks, controlling DNA damage checkpoint activation, and in recombination (Tran et al. 2004; Cotta-Ramusino et al. 2005; Segurado and Diffley 2008). Furthermore, Exo1 is reported to function in checkpoint activation as part of the nucleotide excision repair pathway (Giannattasio et al. 2010). Note that DNA replication by DNA pol δ is also involved in these processes. Thus, the severe PAA sensitivity of PAA-treated exo1 pol3-L612M cells may indicate the need of Exo1 function and/or the combined need of Exo1 and DNA pol δ function in multiple pathways. The low level of Rad53 phosphorylation detected after 3 hr exposure of exo1 pol3-L612M cells to PAA (Figure 6C) may be caused by DNA damage arising because of the absence of Exo1 in several different processes, but with regard to the PAA sensitivity of pol3-L612M cells, Exo1 function is epistatic to Msh2 (Li et al. 2005), and, thus, Msh2 and Exo1 are working in the same pathway.

We propose that MMR creates ssDNA gaps that persist in PAA-treated pol3-L612M cells (Figure 9). DNA pol δ is responsible for resynthesis of MMR-created ssDNA gaps (Kadyrov et al. 2007), but since PAA inhibits the L612MDNA pol δ, gap filling will be slowed. This proposal is supported by the observation that a low dose of HU, which is expected to provide an additional source of ssDNA, can partially substitute for MMR activity (Figure 8). These data together suggest that MMR is required to increase ssDNA/DSBs above a threshold that is needed to activate the S-phase DNA damage checkpoint.

A threshold amount of ssDNA is required to activate S-phase checkpoints to prevent damage-like DNA structures present during normal DNA replication from accidentally triggering checkpoint activation (Shimada et al. 2002). This reasoning certainly applies to lagging-strand replication, in which individual Okazaki fragments are primed, elongated by DNA pol δ, and eventually joined together in reactions that also require DNA pol δ (Jin et al. 2004); these steps will be compromised in PAA-treated pol3-L612M cells. We propose that lagging-strand replication is not monitored for ssDNA gaps or nicks that exist during the maturation of Okazaki fragments because these strand discontinuities are a normal aspect of lagging-strand DNA replication; however, there are likely more gaps and nicks in the lagging strand of PAA-treated pol3-L612M cells than wild-type cells, and, more importantly, the gaps/nicks are predicted to heal slowly. Experiments presented here suggest that lagging-strand discontinuities fall under the radar of checkpoint surveillance, while larger gaps produced by MMR activity and DSBs are detected. S-phase checkpoint activation for PAA-treated pol3-L612M cells is lifesaving because cell cycle arrest provides more time and additional resources (dNTPs, DSB repair enzymes) for the drug-sensitive L612M-DNA pol δ to fully complete lagging-strand replication and to repair DSBs before the cells enter mitosis.

We examined the possibility that hard-to-replicate DNA sequences are likely to be even slower to replicate in PAA-treated pol3-L612M cells. Previous studies suggest that unreplicated regions like the ribosomal DNA (rDNA) locus are not detected by S-phase checkpoint sensors and, as a consequence, do not prevent premature cell cycle progression into mitosis (Torres-Rosell et al. 2007). Pulsed-field gel electrophoresis analysis of chromosomes from PAA-treated pol3-L612M cells, however, did not reveal problems with replication of rDNA (data not shown). Thus, the most reasonable causes of cell death in PAA-treated pol3-L612M cells in the absence of checkpoint activation are unrepaird DSBs and persisting strand discontinuities in the lagging strand that produce more DSBs when encountered by advancing replication forks in the next cycle of DNA replication.

Even in the presence of checkpoint activation, DSBs are a problem for pol3-L612M cells as demonstrated by the increased PAA sensitivity observed when the genes required for DSB repair—RAD51, RAD52, XRS2, or MRE11—are deleted (Figure 5). The slow growth/synthetic sickness phenotype that was observed for pol3-L612M cells deleted for MRC1, TOF1, CTF4, CTF18, or CTF8 (Figure 3; Figure S1)
is also likely related to the function of these genes in the sister-chromatid cohesion that is required for recombination repair of DSBs (Xu et al. 2004). Furthermore, since DNA pol δ is required for the replication phase of homologous recombination (Maloisel et al. 2008), PAA inhibition of the L612M-DNA pol δ is expected to adversely affect recombination if reactions initiated are slow to be completed. This could explain the 20-fold increase in the GCR rate detected for PAA-treated pol3-L612M cells (Table 2).

In conclusion, we have provided additional evidence from studies of PAA-treated pol3-L612M cells for the proposal that a threshold level of DNA damage is required to activate the S-phase DNA damage checkpoint in yeast. Lagging-strand replication problems produced by drug inhibition of the L612M-DNA pol δ do not appear to provide sufficient DNA damage to trigger checkpoint activation even though continued cell cycle progression results in cell death. MMR activity, however, increases DNA damage within 1 hr of PAA exposure, as demonstrated by phosphorylated H2A (Figure 7). In a related observation, Exo1 independent of MMR is observed in noncycling cells to enlarge ssDNA gaps produced by the removal of UV lesions, and these gaps are reported to drive Mec1 kinase activation (Giannattasio et al. 2010). Exo1-enlarged gaps in these experiments were detected as a “cloud” or smear of chromosomes that migrated only a short distance from the loading well during pulsed-field gel electrophoresis. We did not observe a chromosome cloud in PAA-treated pol3-L612M cells, which may indicate a lower level or shorter ssDNA gaps in our experiments compared to UV-treated cells in the experiments by Giannattasio et al. (2010). Thus, Exo1 degradation as part of MMR in our studies or at sites of UV lesions appears to provide the means to engage the checkpoint response under conditions where checkpoint activation may not occur.

The absence of MMR and the checkpoint response in PAA-treated pol3-L612M cells has serious consequences for the few cells that survive. An unusually high mutation rate for production of PAA-resistant cells is observed for survivors (Figure 6A), which appears to diminish in the presence of HU (Figure 8). PAA resistance is due to the acquisition of a second mutation that encodes an amino acid substitution that suppresses the PAA sensitivity conferred by the L612M substitution (L. Li, A. Oladarin, L. Reha-Krantz, unpublished data). Furthermore, we observed many examples of unusual yeast cell morphology in PAA-treated MMR-deficient pol3-L612M cells (Figure 10). We cannot rule out the possibility that MMR is signaling checkpoint sensor kinases as proposed for mammalian cells, but because ssDNA is required for sensor kinases to bind DNA (Zou and Elledge 2003), we propose that MMR is needed primarily to produce ssDNA gaps. To explain the apparent interaction between MMR proteins and sensor kinases in mammalian cells, we suggest that MMR complexes remain associated with the ssDNA gaps (Figure 9). This is a reasonable proposal because some DNA repair proteins have been observed to remain associated with partially repaired DNA to reduce the chance of

Figure 10 Unusual yeast cell morphology is frequently observed for PAA-treated MMR-deficient pol3-L612M cells. Nuclei are DAPI stained.

DNA repair intermediates becoming genotoxic lesions (Mol et al. 2000).

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Drug-Sensitive DNA Polymerase δ Reveals a Role for Mismatch Repair in Checkpoint Activation in Yeast

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Figure S1  Double mutants that display the PAA-induced synthetically sick/slow growth phenotype. Cells with deletions of the indicated genes and the pol3-L612M allele were plated on gradients from 0 to 0.75 mg/ml PAA.
Figure S3  Double mutants with PAA-induced synthetic lethality. Cells with deletions of the indicated genes plus the pol3-L612M allele were plated on gradients from 0 to 0.75 mg/ml PAA.
Figure S3  Deletion of MEC1 produces synthetic lethality in combination with the pol3-L612M allele and PAA at 1 mg/ml.