The Role of Dbf4-Dependent Protein Kinase in DNA Polymerase ζ-Dependent Mutagenesis in Saccharomyces cerevisiae

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ABSTRACT The yeast Dbf4-dependent kinase (DDK) (composed of Dbf4 and Cdc7 subunits) is an essential, conserved Ser/Thr protein kinase that regulates multiple processes in the cell, including DNA replication, recombination and induced mutagenesis. Only DDK substrates important for replication and recombination have been identified. Consequently, the mechanism by which DDK regulates mutagenesis is unknown. The yeast mcm5-bob1 mutation that bypasses DDK’s essential role in DNA replication was used here to examine whether loss of DDK affects spontaneous as well as induced mutagenesis. Using the sensitive lys2ΔA746 frameshift reversion assay, we show DDK is required to generate “complex” spontaneous mutations, which are a hallmark of the Polθ translesion synthesis DNA polymerase. DDK co-immunoprecipitated with the Rev7 regulatory, but not with the Rev3 polymerase subunit of Polζ. Conversely, Rev7 bound mainly to the Cdc7 kinase subunit and not to Dbf4. The Rev7 subunit of Polζ may be regulated by DDK phosphorylation as immunoprecipitates of yeast Cdc7 and also recombinant Xenopus DDK phosphorylated GST-Rev7 in vitro. In addition to promoting Polζ-dependent mutagenesis, DDK was also important for generating Polζ-independent large deletions that revert the lys2ΔA746 allele. The decrease in large deletions observed in the absence of DDK likely results from an increase in the rate of replication fork restart after an encounter with spontaneous DNA damage. Finally, nonepistatic, additive/synergistic UV sensitivity was observed in ddc7Δ pol30Δ and ddc7Δ pol30Δ-k127r,k164r double mutants, suggesting that DDK may regulate Rev7 protein during postreplication “gap filling” rather than during “polymerase switching” by ubiquitinated and sumoylated modified Pol30 (PCNA) and Pol32.

Knowledge of how mutations are produced is important for understanding genetic variability in populations and the process of evolution. Cells have evolved sophisticated molecular mechanisms for maintaining the integrity of the genome. Most DNA repair mechanisms have high fidelity and prevent mutations by removing damaged DNA and replacing the resulting gap using the undamaged, complementary strand as a template (Water et al. 2009; Boitex and Jinks-Robertson 2013). If not repaired, some lesions block the replicative DNA polymerases (Polα, δ, and ε) during S phase and require bypass by an alternative error-free or error-prone mechanism. Error-free bypass usually involves a template switch to the undamaged sister chromatid, while error-prone bypass uses translesion synthesis (TLS) DNA polymerases to synthesize new DNA directly across the lesion. There are at least 15 identified TLS polymerases in human cells but only three in the yeast Saccharomyces cerevisiae: Polζ, Polη, and Rev1, which are encoded by the REV3-REV7, RAD30, and REV1 genes, respectively (Boitex and Jinks-Robertson 2013). TLS polymerases have low fidelity and processivity on undamaged DNA templates and lack an associated exonuclease proofreading activity. The misregulation or loss of TLS polymerases is known to result in a number of human diseases, underscoring the importance of this process. Patients suffering from a variant form of xeroderma pigmentosum, for example, lack Polη and have an increased risk for skin cancer (Kennedy and D’Andrea 2006). In Fanconi anemia, another high-risk familial...
cancer syndrome, patients are TLS defective due to a failure to recruit Rev1, a nontemplate directed deoxycytidyl terminal transferase, to the site of DNA damage (Kim et al. 2012).

In early studies by Kilbey (Njagi and Kilbey 1982) and subsequent studies by our laboratory, induced mutagenesis in S. cerevisiae was reduced in hypomorphic cdc7 mutants including an inactive cdc7 kinase-dead (KD) mutant (Hollingsworth et al. 1992; Ostroff and Sclafani 1995; Pessoa-Brandão and Sclafani 2004). Dbf4-dependent kinase (DDK) is conserved from yeast to humans and is composed of the Cdc7 kinase catalytic and Dbf4 regulatory subunit. Dbf4 is low in G1 phase of the cell cycle and required for kinase activity (Sclafani and Holzen 2007). DDK is a Ser/Thr protein kinase that regulates the initiation of DNA replication by phosphorylating one or more members of the Mcm2–7 DNA helicase (Sheu and Stillman 2006; Randell et al. 2010; Sheu and Stillman 2010; Heller et al. 2011; reviewed in Tanaka and Araki 2013). Either mcm5 (mcm5-bob1) (Hardy et al. 1997) or mcm4 (mcm4Δ74-174) (Sheu and Stillman 2010) mutations can bypass the requirement for DDK during replication. However, cdc7Δ mcm5-bob1, dbf4Δ mcm5-bob1, or cdc7Δ dbf4Δ mcm5-bob1 strains are defective in other DDK functions, including the regulation of meiotic recombination (Matos et al. 2008; Wan et al. 2008) and resistance to DNA damaging agents such as UV and MMS (Pessoa-Brandão and Sclafani 2004), suggesting the kinase interacts with and phosphorylates additional substrates that promote mutagenesis and meiotic recombination (Sclafani 2000). In this report, we investigated the role of DDK in mutagenesis and demonstrate DDK is required for spontaneous as well as induced Polβ-dependent mutagenesis. Based on biochemical analyses, we suggest DDK regulates mutagenesis by direct interaction with and phosphorylation of the Rev7 subunit of Polβ. Our results are important, because the conservation of DDK’s role in TLS in human cells (Day et al. 2010; Yamada et al. 2013), and are significant with regard to human disease because overexpression of yeast DDK increases mutagenesis (Sclafani et al. 1988; Sclafani and Jackson 1994), human DDK overexpression is observed in many types of cancer cells (Hess et al. 1998; Nambiar et al. 2007; Bonte et al. 2008; Clarke et al. 2009; Kulkarni et al. 2009), and DDK is a therapeutic target in cancer patients (Rodriguez-Acebes et al. 2010).

Materials and Methods

Yeast strains, media, and plasmids

Yeast strains and plasmids used are listed in Table 1 and Table 2, respectively. Yeast strains were grown in yeast extract/peptone/dextrose (YPD) with 2% glucose or in synthetic defined minimal media supplemented with appropriate amino acids and 2% glucose (Pessoa-Brandão and Sclafani 2004). Galactose induction of tagged genes for subsequent immunoprecipitation was as described (Oshiro et al. 1999). Briefly, exponentially growing cells using raffinose as carbon source were induced by the addition galactose to 2% for 2 hr. Because pSF2 has a copper-inducible promoter, pSF2-containing cells were induced by the addition of 0.1 mM CuSO4 for 2 hr.

Genetic methods for yeast strain construction, tetrad analysis, and transformation were as previously described (Pessoa-Brandão and Sclafani 2004). Gene deletions marked with the kanMX cassette were constructed by PCR amplification of an appropriate disruption cassette, transformation, and subsequent selection with G418 (Winzeler et al. 1999). The pol30-K127,K164R allele was introduced at the POL30 locus by two-step allele replacement using XbaI-digested plasmid 721 and verified by sequencing genomic DNA. The mcm5-bob1-2 allele contains a CT-to-TC mutation that converts codon 83 from proline to leucine and creates a Ddel site. This allele was introduced into strain SJR1177 by two-step allele replacement using MluI-digested pRAS490, which contains mcm5-bob1-2 in pRS306 (Pessoa-Brandão and Sclafani 2004; Dohrmann and Sclafani 2006). The cdc7Δ::HIS3 allele was introduced into RSY1183 by one-step allele replacement using a 3-kb MluI–EcoRI fragment from pRS277-cdc7Δ::HIS3 (Jackson et al. 1993). Presence of the cdc7Δ::HIS3 allele was verified by restriction digest and by loss of cdc7 genetic complementation. Strain YSS13 contains Myc-tagged Rev7 and was obtained from Marco Muzi-Falconi (University of Milan, Milan, Italy) (Sabbioneda et al. 2005).

pLPB60 encodes GST-tagged Rev7 and was constructed by insertion of REV7 into the Ncol/BamHI sites of the pYES263 expression vector (Melcher 2000). Plasmid pLPB46, which inserts a FLAG (Sigma-Aldrich) epitope tag immediately after the initiating methionine of Pol30, was constructed by PCR-overlap mutagenesis (Ho et al. 1989). Plasmid pBL211 was used as template for PCR using outside primers M13Fwd (5′-TGTTAAACGACGAGGCAGT-3′) and M13Rev (5′-TCACACAGGAAACAGCTATGAC-3′), complementary to the plasmid backbone, and internal primers Pol30-FlagFwd (5′-ATGATTAGATACGACGAGTATTACCCGA-3′) and Pol30-FlagRev (5′-GAAACCTAGCTCAAATCCGAATCTAATGACAGCAGT-3′); lower case letters indicate the FLAG epitope tag). The resulting PCR fragment, which includes the FLAG-Pol30 construct and the POL30 promoter, was then digested with XhoI and BamHI and cloned into pRS426. The FLAG-Pol30 gene was functional as plasmid pLPB46 complemented a lethal pol30Δ::kanMX4 allele.

All tagged constructs used in this study (Table 1 and Table 2) have been shown to function normally by genetic complementation: Cdc7-myc9 (Oshiro et al. 1999), HA-Dbf4 (Oshiro et al. 1999), HA-Cdc7 (Hardy and Pautz 1996), GST-Mcm2 (Lei et al. 1997), GST-Rev1 and GST-Rev3 (Nelson et al. 1996), and REV7-myc13 (Sabbioneda et al. 2005). GST-Rev7 produced in this study was tested by complementation of a rev7Δ::KanMX4 null mutant.

Mutagenesis assays

UV mutagenesis assays using the CAN1 forward mutation system were as described previously (Hollingsworth et al.
Table 1. *S. cerevisiae* strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant genotype</th>
<th>Background/source or reference</th>
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<tbody>
<tr>
<td>RSY299</td>
<td>MATa his3Δ1 leu2 trp1 can1 cyh2</td>
<td>A364a/Pessoa-Brandão and Sclafani (2004)</td>
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<td>RSY466</td>
<td>MATa leu2 ura3 trp1 his7</td>
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<td>P211</td>
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<td>P235</td>
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<td>SJR1177</td>
<td>MATa ade2-101oc his3Δ200 ura3ΔNco lys2ΔA746 rad1::hisG</td>
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</tr>
<tr>
<td>RSY1183</td>
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<td>S288C/this study</td>
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<tr>
<td>RSY1190</td>
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<td>S288C/this study</td>
</tr>
<tr>
<td>RSY1224</td>
<td>MATa ade2-101oc his3Δ200 ura3ΔNco lys2ΔA746 rad1::hisG mcm5-bob1-2 rev3Δ::kanMX4</td>
<td>S288C/this study</td>
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<tr>
<td>YSS13</td>
<td>MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 rad55 REV7-myc13::kanMX4</td>
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<td>yLPB95</td>
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<td>yLPB224</td>
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<td>YJO235</td>
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</table>

1992). Spontaneous reversion rates of the *lys2ΔA746* frameshift were determined by the method of the median (Lea and Coulson 1949) using data from 12–24 independent cultures. For analysis of reversion spectra, independent *lys*+ revertants were isolated and an appropriate portion of the *lys2* gene was PCR amplified and sequenced (Harfe and Jinks-Robertson 2000).

**DNA damage survival and epistasis analysis**

UV (254 nm) treatment of log-phase cells was as described (Pessoa-Brandão and Sclafani 2004). Briefly, cells were exposed to defined doses of UV, plated on YPD plates, and then surviving colonies were counted after incubation in the dark for 2–3 days at 30°C. The data presented represent the mean and SD of three independent experiments. The following equation describes the predicted survival of a double mutant strain if the interaction between relevant mutant genes is additive, with *S* representing the surviving fraction of cells at a given UV dose (Brendel and Haynes 1973):

\[
-\ln S_{\text{double mutant}} = -\ln S_{\text{mutant 1}} + -\ln S_{\text{mutant 2}} - (-\ln S_{\text{WT}}).
\]

If the observed \(-\ln S_{\text{double mutant}}\) is greater than predicted by the equation, one can conclude that the interaction between the two mutations is synergistic.

**Co-immunoprecipitations and immunoblotting**

Protein extracts were prepared using a glass bead lysis procedure (Pessoa-Brandão and Sclafani 2004) and proteins were separated by SDS–PAGE. Immunoblotting was performed with primary mouse monoclonal anti-HA (12CA5, Roche) or anti-myc (9E10, Babco) antibodies, followed by antimouse HRP secondary antibodies or directly by using antihuman IgG secondary antibodies. HRP conjugates and subsequent visualization by enhanced chemiluminescence (Oshiro et al. 1999; Pessoa-Brandão and Sclafani 2004; Leon et al. 2008). Anti-Pef11 and Anti-ADH antibodies were rabbit polyclonal antibodies obtained from D. Bentley (University of Colorado, Denver) and T. Young (University of Washington, Seattle).

For immunoprecipitation (IP) assays, 1 mg of protein extract was combined with 40 μl of Protein G-Sepharose 4B Fast flow beads (Sigma-Aldrich) and 25 μg of antibody in the presence of protease inhibitors [mini protease inhibitor cocktail tablets (Roche Diagnostics) supplemented with 2 μg/ml pepstatin and 1 mM AEBSF (4-benzenesulfonyl fluoride hydrochloride)] in a total volume of 500 μl. Protein G beads were blocked overnight at 4°C with 3% filtered BSA in lysis buffer (50 mM Tris-HCl pH 7.6, 50 mM NaCl, 0.1% Triton X-100, 0.1% Tween 20, 1 mM EDTA) and then washed three times with 1 ml of lysis buffer + protease inhibitors before use. After overnight incubation at 4°C with end-over-end rotation, the IP reactions were pelleted at 500 × g for 3 min. The supernatant was removed and mixed with 5× SDS sample buffer (1× final) and immediately boiled for 5 min. The IP pellet was washed three times with 1 ml of lysis buffer + protease inhibitors, then resuspended in hot SDS sample buffer and boiled for 5 min. For co-immunoprecipitation (co-IP) assays, strain YSS13 containing an endogenous Myc-tagged REV7 allele was transformed with a high-copy plasmid encoding HA-tagged WT or kinase-dead Cdc7 (pCH766 or pCH777, respectively) or HA-tagged Dbf4 (pYQ118). The signals on immunoblots were quantified using Lab Works (UVP, ver. 4.5) software. In co-immunoprecipitations, we normalize the signal of the second protein by...
dividing by the signal of the protein immunoprecipitated. For example, the Rev7-myc signal was divided by HA-Dbf4 signal in an anti-HA immunoprecipitate to yield the relative amount of Rev7-myc in the HA-Dbf4 immunoprecipitate.

For GST-pulldown assays, 1 mg of protein extract was added to 30 µl of Glutathione Sepharose 4B beads (Amer sham Pharmacia) in the presence of protease inhibitors at a final volume of 200 µl. GST beads were washed two to three times with 500 µl of lysis buffer + protease inhibitors before use, and the GST-pulldown reactions were processed in the same manner as IP reactions.

**Yeast and Xenopus DDK assays**

Yeast Cdc7 IP kinase assays were performed using Myc-tagged Cdc7 isolated from strain YJO235 and appropriate GST-tagged target proteins as described previously (Oshiro et al. 1999). GST-tagged fusion proteins were purified from strain yLPB132, which contains a cdc7 KD allele (cdc7-D163N) to eliminate phosphorylation of the fusion proteins by endogenous DDK. Anti-Myc immune complexes were washed once with kinase buffer [15 mM MgCl₂, 50 mM Tris-HCl (pH 7.6)] to remove detergents present in the lysis buffer. To these immunoprecipitates, were added 30 µl of kinase buffer plus ATP (50 mM Tris-HCl pH 8.0, 15 mM MgCl₂, 10 µM ATP, 5µCi of γ³²P-ATP (6000 Ci/mmol)) and 1–5 µg of the GST substrate. Reactions were incubated at 30° for 20 min unless otherwise indicated, and reactions were stopped by adding 10 µl of 4X SDS sample buffer and boiling for 5 min. Proteins were resolved by SDS–PAGE and transferred to nitrocellulose, and phosphorylation was analyzed by either autoradiography or PhosphorImager analysis (Molecular Dynamics). Assays were linear with respect to the amount of Cdc7 immunoprecipitated in that more target phosphorylation was observed when increasing amounts of immunoprecipitated Cdc7-Myc9 were used.

Reactions using bacterially produced purified recombinant Xenopus DDK (xCdc7/xDbf4), which was obtained from J. Walter (Harvard University, Cambridge, MA) (Takahashi and Walter 2005), contained 10 µg DDK in 50 mM Na-HEPES buffer pH 7.6, 10 mM MgCl₂, 1 mM DTT, 10 µM ATP, 10 µCi of γ³²P-ATP (6000 Ci/mmol) and 1–5 µg of the GST substrate. Reactions were incubated at 25° for 30 min as described (Takahashi and Walter 2005) and were analyzed similarly to the yeast Cdc7 IP reactions described above.

**Results**

**DDK is required for both UV-induced and spontaneous mutagenesis**

Previous results demonstrated cdc7Δ and rev3Δ mutations are epistatic with regard to survival following UV damage (Pessoa-Brandão and Sclafani 2004), but a requirement of DDK for UV-induced mutagenesis was not assayed. Here we examine UV-induced forward mutations at CAN1 in the presence and absence of Cdc7 protein. Similar to rev3Δ mutants (Lemontt 1971), the cdc7Δ mcm5-bob1 strain had an undetectable level of UV-induced Can⁺ mutants at five different UV doses with >300-fold reduction in mutations at the highest UV dose of 70 J/m² (Figure 1). This is consistent with our previous analyses of hypomorphic cdc7Δ mutants, which have reduced levels of induced mutagenesis under permissive conditions (Ostroff and Sclafani 1995).

The rate of spontaneous mutagenesis at CAN1, measured by the level of mutation in cells without UV in Figure 1, was reduced in the cdc7Δ mcm5-bob1 background, suggesting DDK is required for spontaneous mutagenesis (1.67 for cdc7Δ mcm5-bob1 vs. 9.0 for CDC7 per 10⁶ cells). However, CAN1 is a relatively insensitive assay as even rev3Δ and rev7Δ strains show only small decreases in the rate of spontaneous mutagenesis. A significantly more sensitive test of Polα relevance to spontaneous mutagenesis is achieved by the lys2ΔΔ746 frameshift-reversion assay, where “complex” mutations (CINS-Complex Insertions), in which the selected frameshift is accompanied by one or more base substitution, are a signature of Polα activity (Harfe and Jinks-Robertson 2000). This signature is most evident in the absence of nucleotide excision repair (NER), where the load of endogenous DNA damage is elevated and the need for error-prone bypass is enhanced. We utilized the lys2ΔΔ746 reversion assay to analyze spontaneous mutagenesis in cdc7Δ mcm5-bob1 strains, with RAD1 additionally deleted to eliminate

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<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>pRS426</td>
<td>2µ URA3 vector</td>
<td>Sikorski and Hieter (1989)</td>
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<tr>
<td>pLPB46</td>
<td>2µ URA3 FLAG-POL30</td>
<td>This study</td>
</tr>
<tr>
<td>pBL211</td>
<td>ARS CEN URA3 POL30</td>
<td>Ayyagari et al. (1995)</td>
</tr>
<tr>
<td>pYQ118</td>
<td>2µ URA3 GAL1pro-His6-DA-FB4</td>
<td>Oshiro et al. (1999)</td>
</tr>
<tr>
<td>pCH766</td>
<td>2µ HIS3 GAL10pro-4xHA-CDC7</td>
<td>Hardy and Pautz (1996)</td>
</tr>
<tr>
<td>pCH777</td>
<td>2µ HIS3 GAL10pro-3xHA-cdc7(N168A)</td>
<td>Hardy and Pautz (1996)</td>
</tr>
<tr>
<td>pSF2</td>
<td>2µ URA3 Cupro-GST-REV3</td>
<td>Nelson et al. (1996b)</td>
</tr>
<tr>
<td>pEG(KT)</td>
<td>2µ leu2-d URA3 GAL1-10pro-GST</td>
<td>Mitchell et al. (1993)</td>
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<td>2µ URA3 GAL1pro -GST-REV7</td>
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<td>721</td>
<td>Ylp211-P30-pol(31K127/164R)-3' URA3</td>
<td>Steller and Ulrich (2003)</td>
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</table>
NER. The overall $\text{lys2}^{\Delta}A746$ reversion rates were similar in $\text{rad1}^{\Delta}\text{mcm5-bob1}$, $\text{rad1}^{\Delta}\text{cdc7}^{\Delta}\text{mcm5-bob1}$, and $\text{rad1}^{\Delta}\text{rev3}^{\Delta}\text{mcm5-bob1}$ strains ($\sim 3 \times 10^{-9}$ in each strain). The spectrum of revertants from the $\text{rad1}^{\Delta}\text{mcm5-bob1}$ strain (Figure 2A) resembled the spectrum from $\text{rad1}^{\Delta}\text{CDC7 MCM5}$ strains (Harfe and Jinks-Robertson 2000), with $\sim 30\%$ of revertants containing a complex mutation. However, the spectrum was dramatically altered in both the $\text{rad1}^{\Delta}\text{cdc7}^{\Delta}\text{mcm5-bob1}$ and $\text{rad1}^{\Delta}\text{rev3}^{\Delta}\text{mcm5-bob1}$ strains, with an almost complete elimination of complex mutations (Figure 2, B and C, respectively) as reported previously in $\text{rad1}^{\Delta}\text{rev3}^{\Delta}$ strains (Harfe and Jinks-Robertson 2000). These results suggest DDK regulates both induced and spontaneous mutagenesis catalyzed by Pol$\zeta$. In addition to reduction in complex events, the $\text{rad1}^{\Delta}\text{cdc7}^{\Delta}\text{mcm5-bob1}$ strain also exhibited a proportional reduction in the number of large deletions among revertants (from 12/74 in $\text{rad1}^{\Delta}\text{mcm5-bob1}$ to 1/79). These large deletions were not affected...
by the presence or absence of Pol\textsubscript{\textepsilon} and have been attributed to strand misalignment associated with a reduction in the processivity of Pol\textsubscript{\texteta} (Minesinger and Jinks-Robertson 2005).

**DDK interacts with and phosphorylates Rev7**

The epistatic relationship between \textit{cdc7}\textsuperscript{\textDelta} and \textit{rev3}\textsuperscript{\textDelta} with respect to UV survival (Pessoa-Brand\textaod\ and Sclafani 2004) and their similar affects on UV-induced and spontaneous mutagenesis (above) suggest Pol\textsubscript{\textepsilon} might be a target of DDK. We thus investigated if DDK interacts with the Rev3 and Rev7 subunits of Pol\textsubscript{\textepsilon}. We found that a Rev7-Myc13 tagged protein (Sabbioneda et al. 2005) was co-immunoprecipitated with HA-tagged Dbf4, while no co-IP was seen in a mock reaction without the HA-specific antibody (Figure 5A). When Rev7-Myc13 was immunoprecipitated, similar amounts of HA-Cdc7 and inactive HA-Cdc7-N168A proteins (Hardy and Pautz 1996) were co-immunoprecipitated (Figure 3B). When HA-Cdc7 and HA-Cdc7-N168A were immunoprecipitated, about 5–10 times more Rev7-Myc13 co-immunoprecipitated with the inactive HA-Cdc7-N168A protein (Figure 3C), which is consistent with observations that inactive protein kinases bind substrates more efficiently (Wu et al. 1996). Both HA-Cdc7 and HA-Cdc7-N168A proteins appear as doublets on these gels. The doublets have been seen previously without explanation (Hardy and Pautz 1996). It is possible it represents phosphorylation of Cdc7 by another kinase.

No increase was observed in the co-IP of Rev7 with Cdc7 or Dbf4 following treatment of cells with 100 J/m\textsuperscript{2} UV (data not shown). We also failed to detect any interaction of DDK with GST-Rev3 protein using plasmid pSF2 (Table 2) (Pessoa-Brand\textaod\ 2005). Controls with a nonspecific nuclear (Pcf11) or an abundant cytoplasmic protein (Adh1) with either the HA-tagged Cdc7 protein or the Rev7-Myc13 protein were negative (Figure 4).

The co-IP of Rev7-Myc13 with HA-Cdc7 or HA-Dbf4 suggests that Rev7 might be a physiological target of the DDK. We examined whether Cdc7-Myc9 immunoprecipitates, which contain active DDK complex, can phosphorylate purified yeast GST-Rev7 protein \textit{in vitro}. Yeast GST-Mcm2 was used as the positive control for kinase activity and, as expected, Dbf4 was also autophosphorylated in the reaction (Figure 5A). Importantly, purified GST-Rev7 was phosphorylated when incubated with the Cdc7-Myc9 IP, while GST alone was not. However, we estimate that GST-Rev7 is phosphorylated about two to three times less than GST-Mcm2 and may be a less efficient substrate. In support of these findings, similar phosphorylation of GST-Rev7 was observed using purified recombinant \textit{Xenopus} DDK (Takahashi and Walter 2005) (Figure 5B).

**Rev7 protein binds to Cdc7 protein in the absence of Dbf4**

Because an inactive kinase-dead Cdc7 kinase can still bind Rev7 protein (Figure 3), we tested whether Cdc7 kinase is able to bind to Rev7 in the absence of Dbf4 protein (Figure 6A). Because \textit{DBF4} and \textit{CDC7} are essential genes, we used strains containing the \textit{mcm5-bob1} mutation that bypasses the lethality of both \textit{dbf4}\textsuperscript{\Delta} and \textit{cdc7}\textsuperscript{\Delta} mutations (Hardy et al. 1997). Strain RSY1445 (Table 1), which also contains an integrated \textit{REV7-MYC13} gene, was transformed with the HA-tagged \textit{CDC7} plasmid pCH766 (B and C, HA-Cdc7), or pCH777 (HA-Cdc7-KD). In B and C, extracts were from cells containing a tagged wild-type (WT) or kinase-dead (KD) Cdc7, as indicated. Lane 1, input extract only; lane 2, supernatant of IP; lane 3, supernatant of mock IP with no antibody; lane 4, pellet of mock IP; lane 5, pellet of IP. Twenty percent of the IP was used for SDS–PAGE as compared to 5% of the supernatants or the input extract.

![Figure 3](Image)

**Figure 3** Co-IP of Rev7 with Dbf4 or Cdc7. Cell extracts were incubated with anti-HA (A and C) or anti-Myc (B) antibody, and precipitated proteins were separated by SDS–PAGE. Western blots were probed individually with an anti-HA or anti-Myc antibody to detect tagged proteins in the immunoprecipitate. Strain YSS13 (Rev7-Myc) with plasmid pYQ118 (A, HA-Dbf4), pCH766 (B and C, HA-Cdc7), or pCH777 (HA-Cdc7-KD). In B and C, extracts were from cells containing a tagged wild-type (WT) or kinase-dead (KD) Cdc7, as indicated. Lane 1, input extract only; lane 2, supernatant of IP; lane 3, supernatant of mock IP with no antibody; lane 4, pellet of mock IP; lane 5, pellet of IP. Twenty percent of the IP was used for SDS–PAGE as compared to 5% of the supernatants or the input extract.
immunoprecipitated with HA-Dbf4 protein is at least 25- to 50-fold reduced when Cdc7 protein is absent. In contrast, the amount of Rev7-myc13 protein co-immunoprecipitated with HA-Cdc7 protein is only reduced ~1.3-fold when Dbf4 protein is absent. We conclude Rev7 protein binding of DDK is mediated primarily by the Cdc7 kinase subunit and does not require the Dbf4 subunit, although Dbf4 protein likely contributes to stabilize the binding.

Loss of DDK is not epistatic with the loss of PCNA post-translational modifications and Pol32 in survival following UV irradiation

We investigated whether DDK involvement in TLS was regulated by interacting with subunits of Polô that are important in Polô-dependent mutagenesis using genetic epistasis analysis (Figure 7) (Boiteux and Jinks-Robertson 2013). Polô also interacts with Pol32, a nonessential subunit of Polô that is required for Pol ô-dependent mutagenesis (Gerik et al. 1998; Huang et al. 2000). In biochemical analyses, Polô holoenzyme is a four subunit complex called Polô4 that contains Polô2 and Polô1 in addition to the core Rev3 and Rev7 proteins (Makarova et al. 2012). Polô also interacts with PCNA (encoded by POL30), a homotrimeric sliding clamp that promotes DNA polymerase processivity (Ayyagari et al. 1995). PCNA is modified at K127 and K164 by sumoylation and ubiquitination, respectively, to modulate postreplication repair (PRR) pathways (reviewed in Boiteux and Jinks-Robertson 2013). Monoubiquitination of PCNA by Rad6-Rad18 promotes TLS by Polô and Polô1, the pol30-K164R and pol30-K127R, K164R alleles are epistatic with rev3Δ for survival following UV irradiation (Stelter and Ulrich 2003). Polyubiquitination of monoubiquitinated PCNA by Ubc13-Mms2-Rad5 promotes error-free damage avoidance (Hoeger et al. 2002).

Given the epistasis between cdc7Δ and rev3Δ with respect to survival following UV treatment, we examined whether the pol30-K127R, K164R or pol32Δ allele is similarly epistatic to a cdc7 null mutation. Instead of epistasis, we observed at least an additive and possible synergistic interaction between pol30-K127R, K164R and cdc7Δ mcm5-bob1 at three different UV doses (Figure 7A, Table 3). Furthermore, pol32Δ also showed a nonepistatic relationship with cdc7Δ mcm5-bob1 (Figure 7B, Table 3). We also examined possible physical interactions of Cdc7 and Dbf4 with GST-Rev1, Pol32-HA, or FLAG-Pol30 proteins using co-IP and GST pulldown experiments but failed to detect any interactions (Pessoa-Brandão 2005).

Discussion

In this report, we have investigated the role of DDK in Polô-dependent TLS and mutagenesis. Previous studies showed DDK regulates mutagenesis induced by various DNA damaging agents (Figure 1) (Njagi and Kilbey 1982; Hollingsworth et al. 1992; Ostroff and Sclafani 1995; Pessoa-Brandão and Sclafani 2004), but DDK had not been similarly implicated in spontaneous mutagenesis (Sclafani 2000). Because DDK and Polô are in the same DNA-
The role of DDK in the DNA damage checkpoint is equivocal in that it is not clear whether DDK activity itself or some downstream event is inhibited during DNA damage (Sclafani and Holzen 2007). Yeast DDK function is inhibited and late origin firing is blocked by Rad53 phosphorylation of the DDK regulatory subunit Dbf4 (Chen et al. 2013); mutation of the Dbf4 phosphorylation sites to alamines removes the inhibition (Zegerman and Diffley 2009; Lopez-Mosqueda et al. 2010). However, even though MCM proteins are known DDK substrates during replication (Sclafani and Holzen 2007), DDK phosphorylation of Mcm2 protein in vitro is not reduced (Oshiro et al. 1999) and phosphorylation of the Mcm7 protein is only modestly reduced during checkpoint activation (Weinreich and Stillman 1999). Furthermore, yeast Mcm4 is still phosphorylated during DNA replication-checkpoint arrest in vivo (Sheu and Stillman 2006). Recently, it was shown that DDK phosphorylation of yeast Eco1, which is required to establish chromosome cohesion, is inhibited by Rad53 phosphorylation of Dbf4 (Chen et al. 2013). Similarly, DDK phosphorylation of Mer2 during meiosis is inhibited by Rad53 phosphorylation of Dbf4 (Blitzblau and Hochwagen 2013). Yet, in human cells, DDK phosphorylates both Claspin (Kim et al. 2008) and hMcm2 (Tenca et al. 2007) during DNA damage both in vivo and in vitro. Therefore, DDK is active during checkpoint responses depending on the system, substrate, and assay used. Based on the current report, we infer that DDK must be active during DNA damage checkpoint activation to allow for TLS. Similarly, human DDK on chromatin remains active during checkpoint activation to facilitate TLS by Polη (Yamada et al. 2013). It was proposed there are two distinct pools of DDK with the soluble pool inhibited to block late origin firing, while the chromatin-bound pool remains active to facilitate TLS (Yamada et al. 2013). It is also possible the effect of checkpoint activation on DDK function is either substrate specific (Blitzblau and Hochwagen 2013; Lyons et al. 2013) or affects different downstream effectors.

Figure 6 Analysis of the co-IP of Rev7 by each subunit of DDK. Cell extracts were incubated with anti-HA antibody, and precipitated proteins were separated by SDS-PAGE. Western blots were probed individually with an anti-HA or anti-Myc antibody to detect tagged proteins in the immunoprecipitate. (A) WT strain YSS13 (lanes 1–5) or dbf4Δ strain RSY1445 (lanes 6–10) with HA-Cdc7 plasmid pCH766 was used. (B) WT strain YSS13 (lanes 1–5) or cdc7Δ strain yLPB163 (lanes 6–10) with HA-Dbf4 plasmid pYQ118 was used. Lanes are labeled the same in A and B. Lane 1, input extract only; lane 2, supernatant of mock IP with no antibody; lane 3, supernatant of IP; lane 4, pellet of mock IP; lane 5, pellet of IP; lane 6, input extract only; lane 7, supernatant of mock IP with no antibody; lane 8, supernatant of IP; lane 9, pellet of mock IP; lane 10, pellet of IP; 20% of the IP was used for SDS–PAGE as compared to 5% of the supernatants or the input extract.

In addition to a reduction in CINS, the cdc7Δ mcm5-bob1 strain also showed a reduction in the number of large deletions that revert the lys2Δ746 allele (Figure 2). These large deletions have been attributed to a decrease in Polβ processivity, as they increase in pol30Δ-46 and pol32Δ mutants (Minesinger and Jinks-Robertson 2005). The large deletions are thought to be produced when lesion-stalled replication restarts downstream (3’) during lagging-strand synthesis. It has been suggested that DDK may be required to facilitate replication restart after checkpoint activation (Duncker and Brown 2003), and this, in principle, could explain the loss of large deletions in the cdc7Δ mcm5-bob1 background. From our recent data, we have ruled out this hypothesis because replication restart not only occurs in cdc7Δ mcm5-bob1 strains, but the restarted replication forks proceed at a faster rate than in wild-type or mcm5-bob1 cells (Zhong et al. 2013). Presumably, the increased fork rate reflects an excess of replication factors and reduced Rad53 checkpoint signaling due to reduced origin firing. Based on the previously discussed processivity hypothesis (Minesinger and Jinks-Robertson 2005), we propose the reduction in large deletions in the cdc7Δ mcm5-bob1 mutant may result from increased processivity when a replication fork restarts after encountering DNA damage.

damage response pathway (Pessoa-Brandão and Sclafani 2004) and Polζ is required for spontaneous mutagenesis, the role of DDK in spontaneous mutagenesis was investigated using the sensitive lys2ΔA746 reversion assay (Harfe and Jinks-Robertson 2000). Significantly, we found DDK is required for the production of complex frameshift events (CINS), which are a distinctive mutational signature of Polζ (Harfe and Jinks-Robertson 2000; Sabbioneda et al. 2005) (Figure 2). We conclude DDK is required for Polζ-dependent spontaneous, as well as induced, mutagenesis.

In addition to a reduction in CINS, the cdc7Δ mcm5-bob1 strain also showed a reduction in the number of large deletions that revert the lys2ΔA746 allele (Figure 2). These large deletions have been attributed to a decrease in Polβ processivity, as they increase in pol30Δ-46 and pol32Δ mutants (Minesinger and Jinks-Robertson 2005). The large deletions are thought to be produced when...
As an example of DDK being active during checkpoint activation, human Rad18 is phosphorylated by DDK to produce a binding site for Polh during TLS at sites of replication fork stalling (Day et al. 2010; Yamada et al. 2013). However, the “S box” region of human Rad18 that is phosphorylated by human DDK is not conserved in either budding or fission yeast and it was suggested DDK in yeast may phosphorylate other TLS proteins (Day et al. 2010). In support of this idea, we show in this report that Rev7 subunit of Polz may be the target as DDK binds to (Figure 3) and phosphorylates Rev7 protein (Figure 5). Therefore, there is conservation of DDK function as DDK regulates TLS polymerases in both humans and yeast.

Furthermore, we show Rev7 binds largely to the Cdc7 kinase subunit and not to the Dbf4 regulatory subunit (Figure 6). Although Dbf4 protein is required for Cdc7 kinase to phosphorylate substrates (Kitamura et al. 2011), and may bind substrates in the Zn++ binding domain of motif C (Hughes et al. 2012), it has not been determined whether Cdc7 or Dbf4 protein alone can bind substrate proteins. In contrast, the N-terminal domain of Dbf4 is required for DDK to bind to MCM2–7 complex in the pre-replication complex in vivo (Duncker et al. 2002; Varrin et al. 2005) and in vitro (Sheu and Stillman 2006; Francis et al. 2009). In cyclin-dependent kinases (CDKs), the main recognition site for the substrate is in the CDK subunit, while the cyclin hydrophobic patch stabilizes the interaction and increases potential

Figure 7 Survival of single- and double-mutant strains following UV irradiation. (A) UV sensitivity of cdc7Δ mcm5-bob1 with pol30-K127,K164R. (B) UV sensitivity of cdc7Δ mcm5-bob1 with pol32Δ. Viability of relevant mutant strains was determined following exposure of log-phase cultures to UV. Error bars represent the standard deviation of the mean value of three independent experiments.
for substrate phosphorylation (reviewed in Morgan 2007). By analogy, we propose Cdc7 protein can bind Rev7 without Dbf4 unlike binding to the Mcm4 substrate, which requires Dbf4.

Although there are a number of possible interpretations of additive and synergistic interactions with DNA repair pathways (Brendel and Haynes 1973), one interpretation of the interaction seen between cdc7Δ and pol32Δ or pol30-K127R,K164R mutations (Figure 7, Table 3) is Pol32 protein and the Pol30 (PCNA) modifications regulate multiple TLS and also replicative polymerases (Boiteux and Jinks-Robertson 2013), while DDK regulates TLS differently by regulating Pol3 through regulation of Rev7. DDK may regulate TLS lesion bypass during “gap-filling” rather than during “polymerase switching” (Waters et al. 2009). In the gap-filling model, repriming of replication occurs downstream of the lesion leaving a gap, which is subsequently filled in by TLS. This is consistent with the pol30 mutant being more sensitive to UV than the cdc7 mutant (Figure 7).

Previously, we have shown Cdc7 kinase activity to be required for all three DDK functions: DNA replication, meiosis, and mutagenesis as a Cdc7 KD mutant is null in all three cellular systems (reviewed in Sclafani and Jackson 1994). Similar to DDK’s role in DNA replication in which phosphorylation of the MCM helicase loads Cdc45 and GINS proteins to initiate replication (Tanaka and Araki 2013), and in meiosis, where DDK phosphorylation loads Mer2 onto chromatin to initiate recombination (Matos et al. 2008; Wan et al. 2008), we propose DDK is likely to regulate Pol3 by phosphorylating Rev7 and loading it onto chromatin. Once on chromatin, Rev7 would then bind to Rev3 polymerase subunit to produce an active Pol3 complex. This may explain why we were unable to coIP Rev3 with DDK as Rev3-Rev7-DDK complexes may not exist in the cell. Consistent with this idea, human DDK phosphorylates Rad18 on chromatin to load Pol3 for TLS (Day et al. 2010; Yamada et al. 2013). Thus, we speculate DDK has evolved to regulate TLS and mutagenesis (Yamada et al. 2013) and to act as a multipurpose regulator for loading of proteins onto substrate DNA and chromatin.

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**Table 3 Mathematical analysis of UV survival data**

<table>
<thead>
<tr>
<th>Strain–genotype</th>
<th>UV dose (J/m²)</th>
<th>Expected −ln S&lt;sub&gt;am&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; (for additive interaction)</th>
<th>Observed −ln S&lt;sub&gt;am&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>yLPB226 cdc7Δ pol30-K127, K164R</td>
<td>20</td>
<td>2.29</td>
<td>3.16 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4.42</td>
<td>5.52 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>6.43</td>
<td>6.91 ± 0.11</td>
</tr>
<tr>
<td>yLPB95 cdc7Δ pol32Δ</td>
<td>20</td>
<td>1.46</td>
<td>1.63 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.27</td>
<td>3.27 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>3.91</td>
<td>4.96 ± 0.13</td>
</tr>
</tbody>
</table>

<sup>a</sup> S<sub>am</sub>, surviving fraction for double mutant. If the −ln S<sub>am</sub> observed is greater than expected, then the interaction is synergistic (Brendel and Haynes 1973).

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