Fission Yeast Hsk1 (Cdc7) Kinase Is Required After Replication Initiation for Induced Mutagenesis and Proper Response to DNA Alkylation Damage

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ABSTRACT

Genome stability in fission yeast requires the conserved S-phase kinase Hsk1 (Cdc7) and its partner Dfp1 (Dbf4). In addition to their established function in the initiation of DNA replication, we show that these proteins are important in maintaining genome integrity later in S phase and G2. *hsk1* cells suffer increased rates of mitotic recombination and require recombination proteins for survival. Both *hsk1* and *dfp1* mutants are acutely sensitive to alkylation damage yet defective in induced mutagenesis. Hsk1 and Dfp1 are associated with the chromatin even after S phase, and normal response to MMS damage correlates with the maintenance of intact Dfp1 on chromatin. A screen for MMS-sensitive mutants identified a novel truncation allele, *rad35* (*dfp1*-(1–519)), as well as alleles of other damage-associated genes. Although Hsk1–Dfp1 functions with the Swi1–Swi3 fork protection complex, it also acts independently of the FPC to promote DNA repair. We conclude that Hsk1–Dfp1 kinase functions post-initiation to maintain replication fork stability, an activity potentially mediated by the C terminus of Dfp1.

THE Hsk1 protein kinase, the fission yeast ortholog of *Saccharomyces cerevisiae* Cdc7, is a conserved protein essential for the initiation of DNA replication (MASAI *et al.* 1995; BROWN and KELLY 1998; SNAITH *et al.* 2000). Data from many systems suggest that the kinase functions at individual replication origins to activate the prereplication complex (preRC) through phosphorylation of the MCM helicase and other subunits (reviewed in FORSBURG 2004). In fission yeast, Hsk1 kinase activity is limited to S phase by its regulatory subunit Dfp1, which is transcriptionally and post-translationally regulated to restrict its peak of activity to S phase (BROWN and KELLY 1999; TAKEDA *et al.* 1999). The requirement for Dfp1 (in *S. cerevisiae*, Dbf4) is similar to the dependence of CDK kinases on cyclin activity; thus, the Cdc7 kinase family has been dubbed DDK (Dbf4-dependent kinases) (JOHNSTON *et al.* 1999; DUNCKER and BROWN 2003). Hsk1 is a target of the Cds1 checkpoint kinase and undergoes Cds1-dependent phosphorylation during hydroxyurea (HU) treatment *in vivo* and *in vitro* (SNAITH *et al.* 2000). Interestingly, deletion of $\Delta cds1$ partly rescues *hsk1*–1312 temperature sensitivity, which suggests that Hsk1 is negatively regulated by the replication checkpoint. In turn, Cds1 is poorly activated in *hsk1* mutants after HU treatment, indicating that

there may be a feedback loop linking these two kinases (SNAITH *et al.* 2000; TAKEDA *et al.* 2001). *hsk1* mutants are sensitive to HU treatment, with a phenotype suggesting a specific defect in recovery (SNAITH *et al.* 2000).

DDK kinases have substrates outside of the replication initiation pathway. Functional dissection of *Schizosaccharomyces pombe* Dfp1 identifies separate regions that are required for checkpoint response (N-terminal domain; TAKEDA *et al.* 1999; FUNG *et al.* 2002), for centromere cohesion and replication (MIR domain; BAILIS *et al.* 2003; HAYASHI *et al.* 2009) and for proper response to alkylation damage during S phase (C-terminal domain; TAKEDA *et al.* 1999; FUNG *et al.* 2002). Recent studies indicate that the DDK kinase is required for initiation of programmed double-strand breaks in meiosis (SASANUMA *et al.* 2008; WAN *et al.* 2008) and meiotic chromosome orientation (LO *et al.* 2008; MATOS *et al.* 2008). The different domains of Dfp1 are presumed to target the Hsk1 kinase to different substrates. Because kinase activity is limited to S phase, these results suggest that the cell uses the DDK kinase to link various cell-cycle events to S-phase passage.

MMS causes alkylation damage that affects replication forks (WYATT and PITTMAN 2006; KAINA *et al.* 2007). This results in Cds1-dependent slowing of DNA replication forks (LINDSAY *et al.* 1998; MARCHETTI *et al.* 2002). However, $\Delta cds1$ mutants are only modestly sensitive to MMS treatment (LINDSAY *et al.* 1998; MARCHETTI *et al.* 2002), suggesting at least partial independence from the replication checkpoint. In contrast, *hsk1* and *dfp1* C-terminal mutants are extremely MMS sensitive

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(SNAITH *et al.* 2000; TAKEDA *et al.* 2001; FUNG *et al.* 2002; MATSUMOTO *et al.* 2005; SOMMARIVA *et al.* 2005). It has been suggested that this reflects Hsk1 association with the fork protection complex (FPC), which consists of the nonessential proteins Swi1/ScTof1 and Swi3/ScCsm3, which are required for replication fork pausing (NOGUCHI *et al.* 2003, 2004; KRINGS and BASTIA 2004; MATSUMOTO *et al.* 2005; SOMMARIVA *et al.* 2005). In budding yeast, *tof1* mutants treated with HU show uncoupling of replication machinery from the fork (KATOU *et al.* 2003), which underscores the importance of maintaining replication fork stability at sites of pausing or damage. This uncoupling suggests that one function of the FPC, and perhaps Hsk1, is holding together the stalled replisome to facilitate replication fork restart.

However, the FPC may not be the only way Hsk1 contributes to MMS response. Alkylation damage during S phase is repaired by several mechanisms, including homologous recombination, template switching, and translesion synthesis pathways controlled by the Rad6/Rad18 (SpRhp6/SpRhp18) epistasis group (reviewed in VERKADE *et al.* 2001, BARBOUR and XIAO 2003; WYATT and PITTMAN 2006; BRANZEI and FOIANI 2007; ANDERSEN *et al.* 2008). While activation of translesion synthesis may be coupled to a polymerase switching event at the fork, evidence suggests that it occurs behind the replication fork as well [reviewed in BRANZEI and FOIANI (2007); LAMBERT *et al.* (2007)]. Several studies suggest that checkpoint proteins may be intimately involved in the decision between recombination, template switching, and translesion synthesis (PAULOVICH *et al.* 1998; KAI and WANG 2003; LIBERI *et al.* 2005; KAI *et al.* 2007). An intriguing observation links DDK kinases specifically to translesion synthesis. Induced mutagenesis is the result of error-prone bypass of lesions following DNA damage (reviewed in BARBOUR and XIAO 2003; ANDERSEN *et al.* 2008), and budding yeast Cdc7 is one of the few proteins required for induced mutagenesis, outside of the specialized translesion synthesis (TLS) polymerases (NJAGI and KILBEY 1982a,b). Recent data suggest that ScCdc7 participates in TLS (PESSOA-BRANDAO and SCLAFANI 2004), although the mechanism is not clear.

In this study, we investigate the contributions of Hsk1 and Dfp1 to replication recovery mechanisms post-initiation by analyzing its contributions to fork stability and repair. Our data suggest that Hsk1–Dfp1 functions at the replication fork after initiation to promote appropriate modes of recovery independent of the FPC. Mutations that destabilize the replication fork are particularly sensitive to attenuation of Hsk1 activity. *hsk1–1312* phenotypes overlap with, but can be distinguished from, phenotypes associated with FPC components *swi1* and *swi3*, indicating that they perform distinct functions in the response to DNA damage. Hsk1 is likely to perform multiple functions as it has pleiotropic effects: first, in the maintenance of genome integrity, and second, the response to alkylation damage. *hsk1–1312* cells suffer

DNA damage even under permissive conditions and this causes increased rates of mitotic recombination and increased recruitment of Rad22 (ScRad52). We show that both *hsk1*⁺ and *dfp1*⁺ are required for induced mutagenesis in response to alkylation, and epistasis suggests this is through the error-prone TLS pathway. We isolated a novel allele of *dfp1*⁺ in a screen for MMS-sensitive mutations. Our data suggest that the effect is mediated by the C terminus of Dfp1, and we propose that this domain is required to maintain Hsk1 and Dfp1 on the chromatin during alkylation damage to promote appropriate repair and contribute to genome stability after replication initiation.

MATERIALS AND METHODS

Yeast manipulation: *S. pombe* strains were grown in Edinburgh minimal medium (EMM) or Pombe glutamate medium (PMG) and supplemented with adenine, histidine, leucine, and uracil as required (MORENO *et al.* 1991). Crosses were performed as described (MORENO *et al.* 1991). All strains were derived from 972 h. Strain genotypes are shown in [supporting information, Table S1](#). In experiments with temperature-sensitive strains, cultures were grown at 25° and shifted to 36° for 4 hr (approximately one cell cycle). Arrests of temperature-sensitive strains confirmed by flow cytometry were performed as described (DOLAN *et al.* 2004; data not shown). Synthetic lethal mutants were those unable to generate a viable double mutant compared to formation of >20 non-parental wild-type colonies from the same cross.

Construction of *dfp1v5::ura4*⁺ strains: A *XhoI*–*NoI* fragment from pmc42X6his–dfp1 (gift of Grant Brown) was cloned into pJAH1172, a *LEU2* vector with a C-terminal 3xv5 epitope tag expressed by *nmt* (J. A. HODSON and S. L. FORSBURG, unpublished data), to create pWPD12. A 2-kb *XhoI*–*SmaI* fragment was excised from WPD12 and cloned into pJK210 (KEENEY and BOEKE 1994) to create pWPD35. pWPD35 was digested with *EcoRI*, and the resultant 6-kb fragment was used to transform strain FY528 by electroporation (KELLY *et al.* 1993). *Ura*⁺ transformants were streaked to yeast extract with supplements and single colonies restreaked to EMM lacking uracil to ensure stable *Ura*⁺ transformants.

UV survival analysis: Strains were grown overnight at 25° to mid-log phase in YES. Cultures were diluted in YES, plated, allowed to dry, and exposed to UV light. Plates were wrapped in aluminum foil and incubated at 25° for 3 to 5 days. Experiments were performed three times with duplicate plates for each experiment.

Mitotic recombination analysis: Single colonies were taken from EMM His[–] plates, inoculated directly into YES, and grown at 25° for 20 to 30 hr. Cell density was counted on a hemacytometer immediately after incubation and before plating 10⁴ cells to two EMM Ade[–] plates. Ade⁺ cells were patched to EMM Ade[–] and EMM Ade[–] His[–] to determine auxotrophies. Mitotic recombination frequency was determined per generation (STEWART *et al.* 1997). Data are the averages of seven or eight independent cultures. Significance was assessed by using Mann–Whitney U-test available online at <http://elegans.swmed.edu/~leon/stats/utest.html>

Rad22YFP microscopy: Logarithmic phase cultures growing at 25° in supplemented EMM were split and HU was added to one culture for a final concentration of 12 mM. Cultures were grown for 3 hr. Cells were collected, washed twice in PBS, and resuspended in PBS. Cells were spotted on slides with poly-L-lysine and air dried. Cells were viewed at 60× on a

DeltaVision Spectris microscope (Applied Precision, Issaquah, WA) and eight 0.5 μ m sections were taken, deconvolved, and projected to one image with softWoRx (Applied Precision, Issaquah, WA). These images were viewed and contrast adjusted in Canvas 8-10 (ACD Systems, Victoria, BC, Canada). For two experiments, 100–500 cells were counted per experiment.

In situ chromatin binding assays: Assays were modified from (DOLAN *et al.* 2004) as follows: *hsk1HA*, *dfp1HA*, and *dfp1-(1-459)HA* strains were viewed with a 1:250 dilution of monoclonal anti-HA 16B12 (BabCO, Berkeley, CA). *dfp1v5* strains were immunostained with a 1:500 dilution of mouse anti-v5 antibody (Invitrogen, Carlsbad, CA). The above samples were incubated with a 1:250 dilution of goat anti-mouse::Alexa-Fluor 546 secondary antibody (Molecular Probes, Eugene, OR). Fixed, stained cells were spotted on microscope slides treated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO). Cells were viewed at 60 $\times$ on a DeltaVision Spectris microscope and images were taken with softWoRx (Applied Precision, Issaquah, WA). *hsk1HA* assays were performed three times; others were performed at least twice. One hundred cells were scored per sample per experiment.

Induced mutagenesis: The fluctuation analysis protocol was adapted from LIU *et al.* (1999). Strains were grown on EMM-uracil at 25°. For each strain, at least 12 independently chosen colonies were inoculated into 5 ml PMG-uracil for overnight culture and incubated at 25°. From each overnight culture, cells cultures at about 0.8 OD₅₉₅ were diluted in YES liquid for 6.5 generations at 25° to mid-logarithmic phase (OD₅₉₅ = 0.8). Half the culture was incubated with 0.0025% MMS for 1 hr while the other half was left untreated. Cells were counted using a hemocytometer and equal numbers were washed twice in 10 ml PMG-uracil, and resuspended in 1 ml PMG-uracil. Twenty-five microliters of a 1:2000 dilution was plated onto YES plates to determine the number of cells surviving after MMS treatment. Cells 2×10^5 were plated onto PMG-FOA plates and incubated at 25° for 10 days. Cells plated onto YES plates were incubated at 25° for 5 days before counting. The uracil reversion rate was calculated according to the following formula: $1 - e^{(1/x) \times \ln((y-z)/(y))}$, where x is the duration of incubation in YES measured in number of generations, y is the total number of cells plated, and z is the number of colonies growing on PMG-FOA plates.

The R software package [Version 2.7.2 (2008-08-25)/The R Foundation for Statistical Computing] was used to determine the statistics and to generate the box plot of the relative mutation rate. The relative mean forward mutation rate was calculated by dividing the mean forward mutation rate for each strain and condition by that of the untreated wild-type strain. Two-sided 95% confidence intervals were calculated from the one sample *t*-test. To test whether there is a difference in the mean reversion rate, the *P*-values were calculated using the Welch two-sample *t*-test to accommodate the differences in sample sizes.

UV mutagenesis: The strain HE686 (*h⁹⁰ smt-0 leu1-32 ura4-D18*) was mutagenized with UV to about 10% viability. Approximately 40,000 treated cells were plated on YES agar (YEA) (about 200 cells per plate) and the resulting colonies were replicated on YEA + 0.01% MMS. Sensitive mutants were picked from the master plates and retested for MMS sensitivity. Ninety-two mutants proved to be MMS sensitive on YEA + 0.01% MMS. Ten of the clearly sensitive strains were analyzed further.

RESULTS

hsk1-1312 sensitivity to replication fork disruption:

To dissect the contributions of Hsk1 to replication fork stability, we compared the damage sensitivity of *hsk1-*

1312 double mutants with mutations in the replication checkpoint (Δ *cds1*, Δ *mrc1*) and the fork protection complex (Δ *swi1*, Δ *swi3*). Since Δ *cds1* partly suppresses *hsk1* temperature sensitivity (SNAITH *et al.* 2000), and Mrc1 contributes to Cds1 activity (TANAKA and RUSSELL 2001; XU *et al.* 2006), we were not surprised to see a similar partial rescue of *hsk1-1312* by Δ *mrc1* (Figure 1). Interestingly, deletion of Δ *cds1* also partly rescues the MMS sensitivity associated with *hsk1-1312*, consistent with previous genetic analysis suggesting that Cds1 negatively regulates Hsk1 (SNAITH *et al.* 2000). Although Δ *cds1* is much less MMS sensitive than *hsk1-1312* (e.g., Figure 1), the most parsimonious explanation for the result is that Cds1 actively restrains some aspect of Hsk1 activity during the MMS response that is required for resistance. But it is also possible that rapid collapse of replication forks after stalling at alkylated bases facilitates survival because it leads more efficiently into recombination-mediated repair or bypass pathways.

Although *hsk1* and *swi1* are reported to be in the same epistasis group for MMS response (MATSUMOTO *et al.* 2005; SOMMARIVA *et al.* 2005), we observed that the double mutants between *swi1* or *swi3* with *hsk1* are more sensitive to UV irradiation damage compared to either single mutant (Figure 1 and data not shown). We also observed that there is a slightly increased sensitivity to low-dose MMS in the *hsk1* Δ *swi3* mutant compared to either single mutation.

We next examined the phenotype of *hsk1-1312* when combined with mutations directly affecting replication fork stability. *hsk1-1312* has negative synthetic interactions (reduced growth rate and reduced permissive temperature) with mutations in the MCM helicase that cause replication fork collapse (SNAITH *et al.* 2000; BAILIS *et al.* 2008). The double mutant *mcm2ts hsk1-1312* has a reduced permissive temperature (SNAITH *et al.* 2000). However, *mcm2ts* is synthetic lethal with Δ *swi1* and synthetic sick with Δ *swi3* (Table 1). This suggests that the fork protection complex is particularly important when MCM helicase activity is abrogated and is consistent with recent work showing replication fork collapse in *mcm2ts* alleles (BAILIS *et al.* 2008). A temperature-sensitive mutant of the GINS subunit *psf2* completes a first round of replication at restrictive temperature (GÓMEZ *et al.* 2005), but is also synthetic lethal with Δ *swi1* and synthetic sick with Δ *swi3* (Table 1). In contrast, *hsk1-1312*, Δ *swi1*, and Δ *swi3* show little synthetic interaction with replication mutants that arrest prior to replication fork activation: *sna41^{goa1}/cdc45ts* (UCHIYAMA *et al.* 2001) and *pol1-1* (D'URSO *et al.* 1995) (Table 1). Together, these interactions suggest that mutants that disrupt early steps of initiation are less sensitive to loss of Swi1 and Swi3 than mutants that carry out substantial DNA synthesis.

Since Hsk1 and Swi1/Swi3 (the FPC) function together (MATSUMOTO *et al.* 2005; SOMMARIVA *et al.* 2005), and since Hsk1 is required for proper centromere cohesion and chromosome segregation (BAILIS *et al.*

FIGURE 1.—Synthetic interactions of *hsk1-1312* with replication checkpoint and fork protection complex mutations. (A) *hsk1* temperature sensitivity is suppressed by deletion of $\Delta cds1$ or $\Delta mrc1$ but not $\Delta swi1$. (B) MMS sensitivity of *hsk1* and *dfp1*, $\Delta swi1$, and $\Delta swi3$ single and double mutants. Cells were diluted fivefold on YES under the indicated conditions.

2003), we asked whether the FPC overlaps with Hsk1 in this activity. We compared the interactions between FPC mutants and *hsk1* when combined with mutations in the cohesin subunit Rad21, and the centromeric heterochromatin protein Swi6, which is required for centromere cohesion (BERNARD *et al.* 2001; NONAKA *et al.* 2002; BAILIS *et al.* 2003). We observed that $\Delta swi1$ and $\Delta swi3$ are synthetic lethal with the cohesin mutant *rad21-K1* (Table 1), similar to *hsk1-1312 rad21-K1* (SNAITH *et al.* 2000). In contrast, $\Delta swi1 \Delta swi6$ double mutants were viable although they are more sensitive to thiabendazole treatment than either parent (Table 1).

Previously, we showed that at the permissive temperature, *hsk1-1312* is synthetic lethal with damage checkpoint mutations $\Delta rad3$ and $\Delta chk1$ (SNAITH *et al.* 2000). This contrasts with the suppression of *hsk1-1312* by $\Delta cds1$. Rad3 is the fission yeast ATR homolog and functions as the master kinase for checkpoint activation, and Chk1 is activated by Rad3 in response to DNA damage (reviewed in HARRISON and HABER 2006). We also found that *hsk1-1312* was synthetic lethal with other damage checkpoint response mutants, including $\Delta rad26$, which encodes the fission yeast homolog of ATRIP, which recruits ATR to RPA-coated single-strand DNA (ssDNA) (EDWARDS *et al.* 1999; CORTEZ *et al.* 2001; ZOU and ELLEDGE 2003); $\Delta rad17$, encoding the RFC alternative required for DNA repair (GRIFFITHS *et al.* 1995); and $\Delta rad1$, which deletes a component of the 9-1-1 clamp (KOSTRUB *et al.* 1998; KAUR *et al.* 2001) (Table 1). Double mutants between *hsk1-1312* and $\Delta crb2$ are viable, but extremely slow growing and able to form only microcolonies; Crb2 is a mediator of Chk1 activity (reviewed in HARRISON and HABER 2006).

The dependence on the damage checkpoint is not seen in mutations that affect replication initiation only;

orp1-4, a mutant defective for prereplicative complex formation and replication initiation (GRALLERT and NURSE 1996; DOLAN *et al.* 2004), is viable in combination with $\Delta rad17$ and $\Delta rad1$ (Table 1). The dependency of *hsk1* cells on an intact damage checkpoint suggests that the *hsk1-1312* itself generates DNA damage. The acute sensitivity of *hsk1-1312* to MMS suggests that this damage might result from aberrant repair of replication associated lesions, subsequent to initiation. We investigated these observations in turn.

Increased DNA damage and recombination in *hsk1-1312*: Previously, we showed that *hsk1-1312* has a low, but detectable, level of Chk1 phosphorylation even at the permissive temperature, consistent with a chronic activation of the damage checkpoint (SNAITH *et al.* 2000). To determine whether this reflects active damage, we employed a YFP-tagged Rad22 (ScRad52) to visualize repair foci (LISBY *et al.* 2001, 2003, 2004; DU *et al.* 2003; MEISTER *et al.* 2003, 2005). We observed that 67.3% of *hsk1-1312* cells and 61.6% of $\Delta swi1$ cells growing asynchronously at 25° had at least one Rad22YFP focus, *vs.* only 8.5% of wild-type cells (Figure 2). This is consistent with both *hsk1-1312* and $\Delta swi1$ mutants having some level of constitutive DNA damage that recruits recombination proteins, although in *hsk1* at least, there is no evidence for extensive chromosome breakage by PFGE analysis (SNAITH *et al.* 2000). These results also agree with previously published data showing that both $\Delta swi1$ and $\Delta swi3$ mutants have increased populations of cells with Rad22 foci in asynchronously growing cultures (NOGUCHI *et al.* 2003, 2004) and is consistent with increased damage phenotypes associated with other alleles of *hsk1* (MATSUMOTO *et al.* 2005; SOMMARIVA *et al.* 2005).

TABLE 1
Summary of genetic interactions

Interactions with	Strain	Interaction	Reference
Checkpoint mutants	<i>hsk1-1312 Δrad3</i>	Synthetic lethal	SNAITH <i>et al.</i> (2000)
	<i>hsk1-1312 Δchk1</i>	Synthetic lethal	SNAITH <i>et al.</i> (2000)
	<i>hsk1-1312 Δrad26</i>	Synthetic lethal	This work
	<i>orp1-4 Δrad1</i>	None	This work
	<i>hsk1-1312 Δrad1</i>	Synthetic lethal	This work
	<i>orp1-4 Δrad17</i>	None	This work
	<i>hsk1-1312 Δrad17</i>	Synthetic lethal	This work
	<i>hsk1-1312 Δcrb2</i>	Severely reduced growth (microcolonies)	This work
Replication mutants	<i>hsk1-1312 mcm2ts</i>	Decreased restrictive temperature	SNAITH <i>et al.</i> (2000)
	<i>Δswi1 mcm2ts</i>	Synthetic lethal	This work
	<i>Δswi3 mcm2ts</i>	Slow growth and decreased restrictive temperature	This work
	<i>Δswi1 psf2ts</i>	Synthetic lethal	This work
	<i>Δswi3 psf2ts</i>	Slow growth and decreased restrictive temperature	This work
	<i>hsk1-1312 cdc45ts</i>	Decreased restrictive temperature	DOLAN <i>et al.</i> (2004)
	<i>Δswi1 cdc45ts</i>	None	This work
	<i>Δswi3 cdc45ts</i>	None	This work
	<i>Δswi1 pol1-1</i>	None	This work
Recombination regulators	<i>hsk1-1312 Δrqh1</i>	Synthetic lethal	SNAITH <i>et al.</i> (2000)
	<i>Δswi1 Δrqh1</i>	Slow growth; increased sensitivity to MMS and TBZ	This work
	<i>Δswi3 Δrqh1</i>	Slow growth; increased sensitivity to MMS and TBZ	This work
	<i>hsk1-1312 Δsrs2</i>	No interaction	This work
	<i>hsk1-1312 Δrhp51</i>	Synthetic lethal	
Centromere proteins	<i>hsk1-1312 rad21-K1</i>	Synthetic lethal	SNAITH <i>et al.</i> (2000)
	<i>Δswi1 rad21-K1</i>	Synthetic lethal	This work
	<i>Δswi3 rad21-K1</i>	Synthetic lethal	This work
	<i>hsk1-1312 Δswi6</i>	Viable	BAILIS <i>et al.</i> (2003)
	<i>Δswi1 Δswi6</i>	Increased sensitivity to MMS and TBZ	This work

HU treatment leads to replication fork collapse in mutants lacking the Cds1 checkpoint kinase, which can be visualized by an increase in the Rad22-YFP foci in these cells following addition of HU (Figure 2; BAILIS *et al.* 2008). To determine whether *hsk1-1312* or *Δswi1* mutations result in replication fork collapse in HU, we compared the fraction of cells with Rad22-YFP foci $\pm$ HU under otherwise permissive growth conditions (Figure 2B). We observed no significant increase in Rad22-YFP foci in *hsk1* or *Δswi1* strains, suggesting that the replication fork does not collapse to generate additional breaks in response to HU treatment in these mutants.

Consistent with the increase in recombination centers in *hsk1-1312* under permissive conditions, we observed that *hsk1-1312* is lethal combined with the mutation *Δrhp51* (*RAD51*; Table 1). This suggests that *hsk1-1312* causes intrinsic damage that requires the recombination apparatus for repair, even at permissive temperature. Therefore, we investigated whether *hsk1-1312* shows evidence for increased recombination. Previous work showed that alleles of *hsk1* and *dfp1* mutants

have elevated levels of gene conversion in diploids (SNAITH *et al.* 2000; FUNG *et al.* 2002), consistent with the increase in breaks suggested by the elevated frequency of Rad22-YFP foci. We examined mitotic crossovers frequency in haploids, using a *ade6* tandem heteroallele flanking the *his3⁺* gene (OSMAN *et al.* 2000) to examine the nature of spontaneous recombination in *hsk1-1312*, compared to *swi1-111* or *swi3-146* mutations. The heteroallele can be converted to *ade6⁺* by gene conversion, which retains the intervening *his3⁺* allele and is thought to result from strand exchange and Holliday junction intermediates, or by deletion events, which lose the *his3⁺* marker, and are thought to result from single-strand annealing, replication slippage, or unequal sister chromatid crossing over (OSMAN *et al.* 2000, 2002; CATLETT and FORSBURG 2003). Efficient conversion repair in this system (*Ade⁺ His⁺*) requires homologous recombination proteins including Rhp51 and Rhp54, although mutations in these proteins lead to increased rates of deletion products (OSMAN *et al.* 2000). In contrast, we observed that *hsk1-1312* mutants had a 5.9-fold increase in conversion repair ($P < 0.05$) but an 11.6-fold

FIGURE 2.—Analysis of recombination in *hsk1-1312*. (A) *rad22YFP* (FY2878), *hsk1-1312 rad22YFP* (FY3285), Δ *swi1 rad22YFP* (FY3287), and Δ *cds1 rad22YFP* (FY3286) cultures were grown overnight at 25°. Cultures were split and either no hydroxyurea (async) or 12 mM HU (HU) was added. After 3 hr of growth, cells were washed twice in PBS, and spotted on slides with poly-L-lysine. Images were collected, deconvolved, and counted. Scale bar, 15 μ m. (B) Quantification of cell counts averaged from two experiments. (C) Scheme of *ade6-his3⁺-ade6* cassette used in mitotic recombination assay. (D) Quantification of mitotic recombination frequency per generation relative to wild type. Wild-type (FY2132), *hsk1-1312* (FY3101), *swi1* (FY3098), and *swi3* (FY3100) strains were grown in YES, and two plates of 10^4 cells were plated to EMM Ade⁻. Survivors were patched to Ade⁻ His⁻ to determine histidine prototrophy. Data are the averages of seven or eight independent cultures. Statistical analysis is presented in the text.

decrease in deletion repair ($P < 0.05$) (Figure 2B) relative to wild-type strains.

Unlike *hsk1-1312*, *swi1* mutants had little effect with just a 1.6-fold increase in mitotic recombination ($P < 0.05$; not statistically significant). However, *swi3* mutants showed a 6.3-fold increase of both types of repair ($P < 0.05$) (Figure 2B), consistent with published data showing increased mitotic recombination in these mutants (SOMMARIVA *et al.* 2005).

Hsk1 association with the chromatin in G2 phase cells and after damage: The genetic interactions between *hsk1* and mutations that cause replication fork instability suggested that there may be a role for Hsk1 not just in replication fork activation, but during replication fork progression. If the Hsk1-Dfp1 complex functions after initiation, then these proteins should be associated with the chromatin after replication initiation. To examine the timing of chromatin association, we used an *in situ* chromatin binding assay to analyze the association of Hsk1 and Dfp1 with chromatin (KEARSEY *et al.* 2000). We used a panel of cell-cycle mutants to arrest cells in G1 (*cdc10*), S (*cdc22*), and G2 (*cdc25*) phases and tested the chromatin binding of tagged proteins Hsk1HA and Dfp1V5. We found that Hsk1HA was present in the nucleus throughout the cell cycle, but bound only to chromatin in 2% of G1 cells (Figure 3). Hsk1HA

bound chromatin in 82.7% of S-phase cells and, surprisingly, in 68% of G2 cells (Figure 3). Dfp1V5 was chromatin associated in fewer than 10% of cells arrested by *cdc10* in G1 phase (data not shown). However, Dfp1V5 bound chromatin in 87% of S-phase cells and 67% of G2 cells (Figure 3). These data indicate that the Hsk1-Dfp1 complex is bound to chromatin not only in S phase, but also in G2, which could allow it to act after initiation or even after the conclusion of bulk DNA replication.

We repeated the experiment in Δ *swi1* mutant cells. We found chromatin-bound Hsk1HA in 79.2% of asynchronously growing Δ *swi1* cells and 44.5% of Δ *swi3* cells, compared to 55% of wild-type cells (Figure 3). Thus, Hsk1 recruitment to the chromatin is independent of Swi1. Since Δ *swi1* alone causes DNA damage (Figure 2 and (MATSUMOTO *et al.* 2005; SOMMARIVA *et al.* 2005), we reasoned that the modest enhancement of Hsk1 on the chromatin in Δ *swi1* mutants might mean that Hsk1 is responding to that damage. If this were the case, we predicted that alleles of the Hsk1 kinase that are defective in the damage response might be defective in chromatin binding.

Previously, strains with C-terminal truncations of the Dfp1 protein, *dfp1-(1-376)* and *dfp1-(1-459)*, were shown to be sensitive to MMS but not HU or UV, which led to the identification of the Dfp1 “C-motif” as necessary for

FIGURE 3.—Hsk1 and Dfp1 bind chromatin during S and G2 phases. (A) Cultures of *cdc10 hsk1HA* (FY1000), *cdc22 hsk1HA* (FY1011), and *cdc25 hsk1HA* (FY1006) were grown at 25°, shifted to 36° for 4 hr, and processed for *in situ* chromatin binding. Scale bar, 10.7 μ m. (B) Quantification of data from A; averages of three experiments are presented and error bars show standard deviations. (C) Cultures of *cdc22 dfp1v5* (FY3281) and *cdc25 dfp1v5* (FY3282) were grown at 25°, shifted to 36° for 4 hr, and processed for *in situ* chromatin binding. Scale bar, 10.7 μ m. (D) Quantification of data from (C); averages of two experiments are presented and error bars are standard deviations. (E) Hsk1–Dfp1 and Swi1–Swi3 bind chromatin independently. *hsk1HA* (FY1077), *Δswi1 hsk1HA* (FY3249), and *Δswi3 hsk1HA* (FY3251) cultures were grown at 32° and processed for *in situ* chromatin binding. Scale bar, 10.7 μ m. (F) Quantification of data from E; averages of three experiments are presented and error bars show standard deviations.

response to alkylation damage (FUNG *et al.* 2002). We arrested *dfp1-HA* or *dfp1-(1–459)-HA* strains with HU for 3 hr and released them either to fresh medium or fresh medium containing 0.03% MMS for 1 hr. We found that Dfp1–HA and Dfp1-(1–459)HA were bound to chromatin in HU-arrested cells and cells released into plain medium (Figure 4). However, only 30.5% of cells had chromatin-bound Dfp1-(1–459)HA after release to MMS, compared to 77% of cells with chromatin-bound Dfp1–HA (Figure 4). Thus, reduction in Hsk1–Dfp1 chromatin association correlates with increased damage sensitivity, suggesting that Hsk1–Dfp1 maintenance on the chromatin or at stalled forks may be important for the slowed replication forks and/or repair of the MMS lesions.

Isolation of a new allele of *dfp1*: To identify additional mutations that affect normal MMS response, we performed a genetic screen in an *h⁹⁰ smt-0* background. This strain is not able to make any DSBs at the mating-type locus, which initiates mating-type switching. We used this mutant because the MMS-sensitive mutant *Δrad22* is not viable in a homothallic (*h⁹⁰*) wild-type background (see MATERIALS AND METHODS). The screen yielded mutations in eight genes, of which six were identified by complementation tests (Table 2). We identified

mutations affecting two MRN recombination complex subunits, *nbs1⁺* and *rad32/mre11⁺*. MRN is required for end resection and homologous recombination (reviewed in WILLIAMS *et al.* 2007). We also isolated *snf22* (two alleles), a SNF2-related ATPase characterized for its role in chromatin remodeling in meiosis (YAMADA *et al.* 2004); *swi9/rad16*, the ScRAD1 ortholog required for excision repair and some forms of recombination (CARR *et al.* 1994; FARAH *et al.* 2005); and a mutation in SPBC19G7.10c, encoding an uncharacterized homolog of the topoisomerase-interacting *S. cerevisiae* Pat1 protein required for mRNA decapping (WANG *et al.* 1996; BONNEROT *et al.* 2000). We were unable to identify the genes corresponding to two mutations, *rad37* and *rad39*.

The *rad35-271* mutation proved to be allelic to the Hsk1 regulatory subunit encoded by *dfp1⁺*, and we henceforth call it *dfp1-(1–519)* or *dfp1^{rad35}*. This mutation is a C-terminal truncation that truncates the protein at amino acid 519. Its phenotype is reminiscent of the *dfp1-(1–459)* and *dfp1-(1–376)* mutations analyzed in (FUNG *et al.* 2002), which were shown to be MMS sensitive but competent for replication. C-terminal truncation mutations of Dfp1 function as separation-of-function alleles that allow selective inactivation of just one or two functions of the kinase (TAKEDA *et al.* 1999; FUNG *et al.*

FIGURE 4.—Dfp1ΔC chromatin association with damaged DNA is disrupted. *dfp1HA* (FY1763) (A) or *dfp1ΔCHA* (FY1794) (B) cultures were grown at 32°, arrested with 20 mM HU for 3 hr (HU), and released to plain medium (release) or medium with 0.03% MMS (release + MMS) for 1 hr. Cells were then processed for *in situ* chromatin binding. Scale bar, 10.7 μm. (C) Quantification of data from A and B; averages of two experiments are presented. Error bars show standard deviations.

2002; BAILIS *et al.* 2003). However, the construction of the *dfp1(1-459)* and *dfp1(1-376)* alleles was genetically complex, making it comparatively difficult for further genetic analysis with these mutations. Since the MMS phenotypes of *dfp1(1-376)*, *dfp1(1-459)*, and *dfp1(1-519)^{rad35}* are similar (Figure 5; FUNG *et al.* 2002), we continued our analysis using the *dfp1(1-519)* allele as a separation of function mutation and followed it in genetic crosses using MMS sensitivity.

To determine whether *dfp1(1-519)* suffers the same intrinsic damage as *hsk1-1312*, we analyzed the formation of Rad22-YFP foci in exponentially growing cells. We observed a constitutive level of foci in *dfp1(1-519)*, about 70% overall, substantially higher than wild type, but similar to the levels observed in *hsk1-1312*. Follow-

ing 3.5 hr treatment with HU, we observed 27% cells with no foci, 53% with one focus, and 16% with more than one focus, suggesting that like *hsk1-1312*, *dfp1(1-519)* does not affect replication fork stability during arrest (compare with Figure 2). We also observed that *dfp1(1-519)* shows a reduced growth rate with somewhat elongated cells, suggesting an intrinsic level of damage. There is also modest sensitivity to camptothecin, a topoisomerase inhibitor that results in S-phase-specific breaks (Figure S1). However, the *dfp1* alleles show no synthetic phenotype with *Δrhp51*, in contrast to *hsk1*, which is synthetic lethal (Table 1 and Figure 5; FUNG *et al.* 2002), indicating that the foci we observe do not represent sufficient damage to make the cells dependent upon HR repair. Alternatively, the synthetic

TABLE 2
Isolation of MMS sensitive mutants

Mutant	Gene	Function	Reference
226	<i>rad32</i>	Mre11 component of MRN recombination complex	TAVASSOLI <i>et al.</i> (1995)
253	<i>rad32</i>	Mre11 component of MRN recombination complex	TAVASSOLI <i>et al.</i> (1995)
249	<i>swi9/rad16</i>	ScRad1 nuclease ortholog required for excision repair and recombination	CARR <i>et al.</i> (1994)
106	<i>snf22</i>	ATP dependent helicase, Snf2 family	YAMADA <i>et al.</i> (2004)
261	<i>snf22</i>	ATP dependent helicase, Snf2 family	YAMADA <i>et al.</i> (2004)
271	<i>rad35/dfp1</i>	DDK (Hsk1) regulatory subunit	BROWN and KELLY (1998); TAKEDA <i>et al.</i> (1999)
219	<i>rad36/SPBC19G7.10c</i>	ortholog of topoisomerase-associated RNA-degrading factor ScPAT1	Not studied
265	<i>rad37</i>	Not cloned	
278	<i>rad38/nbs1</i>	Nbs1 component of MRN recombination complex	CHAHWAN <i>et al.</i> (2003); UENO <i>et al.</i> (2003)
280	<i>rad39</i>	Not cloned

FIGURE 5.—Hsk1-Dfp1 function in the error-prone postreplication repair pathway. (A) *hsk1* is required for induced mutagenesis. The graph shows the relative mean forward mutation frequency of the *ura4⁺* gene for untreated samples (–) and samples exposed to 0.0025% MMS for 1 hr (+) in wild-type (FY8), *Δswi1::kanMX* (FY4588), *hsk1-1312* (FY1418), and *dfp1^{rad35-271}* (FY3998) strains. The relative forward mutation frequency is the rate compared to that of wild-type untreated cells. The middle line in each box represents the mean, and the upper and lower limits of the box represent 95% confidence interval calculated from the one-sample *t*-test. Each confidence interval was calculated from a sample size of at least 12 independently chosen colonies. (B) Synthetic interactions between *hsk1* and components of the postreplication repair pathway. Cells were plated in 5× dilutions on YES with the indicated amount of MMS. (C) Synthetic interactions of *hsk1-1312*, *Δswi1*, and *Δswi3* with *Δrhps18* in response to UV. Cultures were grown overnight at 25° into log phase. Cells were plated and plates allowed to dry. Plates were exposed to 0, 10, or 25 J/m² UV light. Plates were incubated at 25° for 3–5 days and counted. Values are the average relative viability of three assays; error bars show standard deviations. (D) Synthetic interactions between *dfp1-1-519* and components of the postreplication repair pathway. (E) Synthetic interactions between *hsk1* and deletion of the error-prone polymerases.

lethality in *hsk1-1312* could reflect a combination of initiation and postreplicative events and events at replication initiation that are not defective in the *dfp1* mutant. We considered *dfp1-1-519* as a separation of function mutation, competent for replication but defective in the MMS response.

Hsk1 and Dfp1 in damage repair: Previous genetic analysis of *dfp1* motif C mutations showed that they fall into a separate epistasis group from several known repair pathways (FUNG *et al.* 2002), including those defined by *Δrad13* (nucleotide excision repair XPG/ERCC5; CARR *et al.* 1993), *Δrhps51* (Rad51; homologous

recombination repair; MURIS *et al.* 1993), $\Delta rad2$ (FEN-1 flap endonuclease; MURRAY *et al.* 1994), or $\Delta mag1$ (base excision repair glycosylase; MEMISOGLU and SAMSON 2000), all of which contribute to normal MMS repair (MEMISOGLU and SAMSON 2000). These data suggest that Hsk1–Dfp1 is required for survival of alkylation damage in a pathway that is independent of excision repair and homologous recombination pathways. A likely candidate is the postreplication repair pathway dependent upon Rhp18 (ScRad18) that includes error-free and error-prone branches (reviewed in BARBOUR and XIAO 2003; ANDERSEN *et al.* 2008). This would be consistent with observations in budding yeast suggesting that *ScCdc7* is required for induced mutagenesis in MMS (NJAGI and KILBEY 1982a,b), via the error-prone pathway that responds to alkylation damage.

To investigate this in *S. pombe*, we first examined whether *hsk1-1312* or *dfp1-(1-519)* affect the frequency of induced mutagenesis in fission yeast (Figure 5A). We performed a simple forward mutation assay at the *ura4⁺* locus by calculating the rate of 5-FOA resistance as in (LIU *et al.* 1999), comparing wild type to *hsk1-1312*, *dfp1-(1-519)* and $\Delta swi1$ mutant strains. We plated cells on selective medium in the absence or presence of prior treatment with MMS. First, we observed that there is a slightly higher mutation rate in *hsk1* and *dfp1* compared to wild type in the absence of any exogenous treatment. We repeated the experiment following MMS treatment and observed a dramatic increase in the frequency of mutation in wild type (“induced mutagenesis”) as expected. A similar induction was also apparent in $\Delta swi1$, even though it has a higher basal level of mutation. In contrast, there was no significant elevation of mutation rate in *hsk1-1312* ($P = 0.63$) and only a modest increase in *dfp1-(1-519)* above the levels in untreated cells ($P = 0.06$). This indicates that as in *S. cerevisiae*, *S. pombe* Hsk1 and Dfp1 are required for induced mutagenesis, and importantly, this phenotype is independent of Swi1 and the FPC.

We next examined genetic interactions between *hsk1*, *dfp1*, and the MMS response pathway defined by the ScRad6/SpRhp6 epistasis group. This pathway relies on the ScRad6–Rad18 (*S. pombe* Rhp6–Rhp18) ubiquitin ligase, which ubiquitylates PCNA and has several downstream branches required for translesion synthesis and template switching (reviewed in BARBOUR and XIAO 2003; ANDERSEN *et al.* 2008). Further ubiquitylation of PCNA by the ubiquitin ligase Sc/SpUbc13 and Sc/SpMms2 and the helicase ScRad5/SpRad8 activates an error-free replication bypass system, while the error-prone branch of pathway relies on translesion synthesis by bypass polymerases Pol η (part of a fusion protein encoded by the C terminus of *eso1⁺*), the *dinB* ortholog polk (*mug40/dinB*), and Pol ζ (*rev3*). Studies in budding yeast suggest that ScCdc7 functions in a branch of the translesion synthesis pathway (PESSOA-BRANDAO and SCLAFANI 2004).

To determine whether similar interactions occur in fission yeast, we examined the phenotype of double mutants between *hsk1* or *dfp1* and several components of this pathway (Figure 5 and Figure S2). $\Delta rhp18$ (VERKADE *et al.* 1999) disrupts the E3 ligase that cooperates with the Rhp6/RAD6 E2 ligase for ubiquitylation of PCNA and activation of both error-free and error-prone translesion synthesis (ANDERSEN *et al.* 2008). An allele of PCNA, *pcn1-K164R* is proficient for normal replication but defective in damage-induced ubiquitination, disrupting both branches of the pathway (FRAMPTON *et al.* 2006). $\Delta mms2$ and $\Delta ubc13$ are required for the error-free arm of the pathway (BROWN *et al.* 2002). We also examined disruption alleles of the 40 TLS polymerases, *eso1 Δ C* ($\Delta pol\eta$), and $\Delta rev3$, and a triple deletion *eso1 Δ C* ($\Delta pol\eta$), $\Delta dinB$ $\Delta rev3$.

First, we examined combinations with mutant *hsk1*. All the double mutants were viable, although we observed that *hsk1-1312* $\Delta rhp18$ had a modestly reduced growth rate compared to the single mutants (Figure 5B). This suggests that any endogenous damage in the double mutant caused by *hsk1-1312* does not rely on these damage processing pathways for viability. UV and MMS sensitivity were increased relative to either single parent when *hsk1-1312* was combined with *pcn1-K164R* or $\Delta rhp18$, indicating a combinatorial effect (Figure 5, B and C). When we examined relative viability associated with UV treatment in the double mutants, we observed a modest but distinct reduction in viability in *hsk1*, $\Delta swi1$, or $\Delta swi3$ mutations combined with $\Delta rhp18$. This suggests that Hsk1 and the FPC proteins have some functions in repair independent of the Rhp18 pathway and this would be consistent with a general role in replication fork stability. *hsk1* also showed synthetic phenotypes in combination with $\Delta ubc13$ or $\Delta mms2$. By contrast, no synthetic phenotypes were observed when *hsk1* was combined with mutations in *eso1 Δ C* or $\Delta rev3$, and there was only a slight increase in sensitivity in the quadruple mutant *eso1 Δ C* $\Delta rev3$ $\Delta dinB$ *hsk1* at higher doses (Figure 5E).

Next we examined *dfp1-(1-519)* (Figure 5D). Again, the double mutants with either $\Delta rhp18$ or *pcn1-K164R* were significantly more sensitive to MMS and UV than the parents, and synthetic phenotypes were also observed with $\Delta mms2$. There was only a modest increase in sensitivity with the *eso1* mutant.

These observations suggest that Hsk1–Dfp1 is at least partly independent from ScRad6/SpRhp6 dependent postreplication repair in response to MMS and UV, although these proteins may overlap in function in repair or at other points in cell cycle. Additionally, although *swi1* and *swi3* mutants have little UV sensitivity by themselves (NOGUCHI *et al.* 2003, 2004), we found that $\Delta swi1$ $\Delta rhp18$ and $\Delta swi3$ $\Delta rhp18$ double mutants were also significantly more sensitive to UV or MMS treatment than the parental strains (Figure 5C and Figure S3). $\Delta swi1$ and $\Delta swi3$ showed similar defects in

FIGURE 6.—Model for Hsk1 interaction with TLS pathway. The sliding clamp proteins PCNA (green) and 9-1-1 (pink) mediate the choice between repair mechanisms. Homologous recombination is inhibited by the Srs2 helicase and PCNA sumoylation. Polyubiquitination of PCNA by Rhp6/18 and then Mms2/Ubc13/Rad8 drives error free bypass repair. Error prone translesion synthesis by minor polymerases occurs in response to PCNA monoubiquitination. Hsk1 and Dfp1 appear to operate in this pathway independent of Swi1/3 and Rhp6/18. Modified from KAI *et al.* (2007); ANDERSEN *et al.* (2008); BRANZEI *et al.* (2008).

combination with $\Delta ubc13$ and $\Delta mms2$ (data not shown). This is consistent with a role for the FPC in replication fork stability independent of PRR activation.

When TLS or template switching pathways are inhibited, homologous recombination pathways are used to repair the lesions (Figure 6). In budding yeast, the $\Delta srs2$ mutation suppresses the damage sensitivity of $\Delta rad18$, presumably because $\Delta srs2$ relieves the inhibition of the HR pathway and allows it to substitute for the PRR pathway; however, this is not observed in fission yeast (KAI *et al.* 2007). We observed no genetic interactions between $\Delta srs2$ and $hsk1-1312$ or $dfp1-(1-519)$, and no changes in the damage sensitivity of the double mutants. This suggests that $hsk1-1312$ is epistatic with $\Delta srs2$. In contrast, $hsk1-1312$ is lethal in combination with $\Delta rqh1$ (SNAITH *et al.* 2000), another helicase that antagonizes recombination by a different mechanism (DOE *et al.* 2002; HOPE *et al.* 2006). We observed that $\Delta swi1$ and $\Delta swi3$ are synthetic sick when combined with $\Delta rqh1$ with increased sensitivity to MMS and, curiously, the spindle poison thiabendazole (Table 2). This suggests that unregulated recombination in the $\Delta rqh1$ mutant is deleterious to $hsk1$, $swi1$, or $swi3$ mutants, and particularly so when damage occurs.

DISCUSSION

The Hsk1–Dfp1 (DDK) kinase has a well-studied role in promoting replication initiation. In this study, we

analyzed the phenotypes associated with $hsk1-1312$ and alleles of $dfp1$ to dissect the contributions of the DDK kinase to genome stability after replication initiation. Our work suggests multiple functions for this kinase in promoting replication fork stability and appropriate response to DNA damage caused by alkylating agents during and after S phase.

Hsk1 interacts with Swi1 (ScTof1) and Swi3 (ScCsm3), which constitute the fork protection complex (MATSUMOTO *et al.* 2005; SOMMARIVA *et al.* 2005). The FPC, although not essential for viability, is linked to replication fork pausing and stabilization of the replisome during S-phase arrest (KATOU *et al.* 2003; NOGUCHI *et al.* 2003, 2004; MATSUMOTO *et al.* 2005; SOMMARIVA *et al.* 2005) as well as response to alkylating damage (FOSS 2001; NOGUCHI *et al.* 2003, 2004; MATSUMOTO *et al.* 2005; SOMMARIVA *et al.* 2005). This leads to the model that Hsk1 and the FPC are required for fork stabilization during repair, maintenance of cohesion during repair, and fork recovery required for successful completion of S phase. Consistent with this role at the elongating fork, we observe synthetic phenotypes between $hsk1-1312$, $swi1$, or $swi3$ when combined with mutations that are defective in replication fork stability, such as $mcm2ts$ or $psf2$. In contrast, we see no synthetic phenotypes in combination with mutations that affect the prereplication complex assembly or initiation, such as $orp1$ or $pol1$.

These data suggest that $hsk1-1312$, like FPC, functions at the replication fork after initiation to promote stability, and therefore is not simply a replication initiation factor. Consistent with this model, we observe that Hsk1 associates with chromatin in G2 phase, and this association is enhanced in MMS-treated cells. Importantly, however, while chromatin association requires the C terminus of Dfp1, it does not require the FPC.

We observe that $hsk1-1312$ causes intrinsic damage, which is sufficiently severe to activate the DNA damage checkpoint, and $hsk1$ cells require the checkpoint for viability (SNAITH *et al.* 2000 and this work). There are increased foci corresponding to the recombination protein Rad22–YFP (Rad52) in $hsk1-1312$ even at the permissive temperature; such foci are characteristic of a range of lesions not limited to double-strand breaks (*e.g.*, BAILIS *et al.* 2008). We also observe that $hsk1-1312$ is lethal in the absence of Rhp51 (ScRad51). Thus, we conclude that $hsk1-1312$ generates constitutive damage that renders the cells dependent upon an active recombination system.

Such damage might be expected to increase rates of mitotic recombination, and indeed we observed substantially increased rates of recombination in the form of gene conversion, but reduced levels of deletion repair, measured using an *ade6* heteroallele system (OSMAN *et al.* 2000, 2002). Previous analysis of this system suggests that the conversion repair arises from break-induced strand exchange and Holliday junction intermediates and depends upon HR genes such as $rad22^+$,

while the deletion-type recombination occurs from single-strand annealing, replication slippage, intrachromatid crossing over, or unequal sister chromatid exchange (OSMAN *et al.* 2000, 2002). The *hsk1* phenotype suggests that the homologous recombination pathway is induced. The mechanism could be indirect, reflecting increased levels of damage due to replication fork instability, or could also reflect a role for Hsk1 directly in negatively regulating this pathway, such that its activity is enhanced in *hsk1* mutants. The loss of conversion products could reflect an active downregulation of SSA or other pathways; alternatively, these pathways may be initiated but not resolved, which would result in lethality and thus failure to recover any products. It is interesting to note that $\Delta rhp51$ mutants, which cannot carry out HR and have low levels of conversion products, nevertheless have increased levels of deletion repair (OSMAN *et al.* 2000). Thus, it is possible that the loss of conversion products in *hsk1* implicates the kinase in SSA or other forms of repair.

Although *swi1* and *swi3* mutants also have increased Rad22YFP foci, we do not see the same spectrum of recombination events in the *ade6* heteroallele as we see with *hsk1* (Figure 2 and SOMMARIVA *et al.* 2005). The increase in deletion events in *swi3* cells is consistent with a specific function in replication fork instability, and the absence of these events in *hsk1* suggests that its defects may be functionally separated from the FPC. Similar phenotypes to those observed with FPC mutants are also reported for other repair mutants including MMS-sensitive repair mutants $\Delta rad16/swi9$ (ScRad1) and $\Delta swi10$ (ScRad10) (DOE *et al.* 2000; OSMAN *et al.* 2000). These genes encode a nuclease that is involved in excision repair but also associated with recombination (CARR *et al.* 1994; FARAH *et al.* 2005, 2009). The differences in mutation spectra between *hsk1-1312* and *swi1* and *swi3* suggest that these proteins make nonidentical contributions to genome stability and recombination.

Inappropriate activation of the recombination pathway is antagonized by several helicases, including the Rqh1 (Bloom syndrome) helicase, which blocks formation of recombinogenic structures and is required for replication fork stability, and Srs2, which antagonizes formation of Rad51 filaments (reviewed in BARBOUR and XIAO 2003; BRANZEI and FOIANI 2007; LAMBERT *et al.* 2007). *hsk1-1312* is lethal in combination with $\Delta rqh1$ (SNAITH *et al.* 2000), but has no additional phenotype combined with $\Delta srs2$ (this work). This is consistent with a general defect in *hsk1* in replication fork stability that is additive with the defects in $\Delta rqh1$ and suggests that Hsk1 may function in a common genetic pathway with Srs2.

We performed a screen for additional mutations that cause MMS sensitivity. Mutations were identified in the MRN complex subunits *nbs1*⁺ and *rad32*⁺ (*MRE11*), in an ortholog to the RNA decapping enzyme *S. cerevisiae* *PAT1*, in *rad16*⁺ encoding a nuclease required for re-

combination and excision repair (CARR *et al.* 1994; FARAH *et al.* 2005), and in the *snf22*⁺ helicase (YAMADA *et al.* 2004). We also isolated *rad35*, a novel allele of *dff1* that truncates the extreme C terminus of the protein at residue 519. Additional *dff1-(1-519)* serves as a separation of function mutation that specifically affects the MMS response of cells.

Blocked replication forks can be recovered without repair using either homologous recombination or the lesion bypass system. Data from *S. cerevisiae* suggest that *cde7* mutants disrupt induced mutagenesis and function in the Rad6–Rad18 pathway of bypass repair via translesion synthesis (NJAGI and KILBEY 1982a,b). This would be a function consistent with a requirement for DDK in replication fork stability, since lesion bypass requires assembly of nonreplicative polymerases at the fork (BRANZEI and FOIANI 2005; ANDERSEN *et al.* 2008). Induced mutagenesis following MMS exposure depends largely on the error-prone translesion synthesis pathway (BARBOUR and XIAO 2003; ANDERSEN *et al.* 2008). Both *hsk1* and *dff1* cells have a slightly higher basal mutation rate than wild-type cells. However, treatment with MMS did not further increase the rate of mutation in *hsk1-1312* and only modestly increased the mutation frequency in *dff1-(1-519)*. In contrast, both $\Delta swi1$ and wild-type cells had a robust induction of mutagenesis following treatment with MMS. This indicates first that Hsk1 and Dfp1 are required for induced mutagenesis in response to alkylation damage and, second, that the FPC is not required for induced mutagenesis. Thus, Hsk1–Dfp1 have a separate role from the FPC in promoting this response.

We observed a modest synthetic interaction between *hsk1* or *dff1* with $\Delta rhp18$ and other components of the PRR pathway including *pcn1-K164R*, $\Delta mms2$, and $\Delta ubc13$, and the error-prone polymerases $\Delta eso1$, $\Delta dinB$ (*mug40*), or $\Delta rev3$ (there is no *dinB* ortholog in budding yeast). These data suggest that the Hsk1–Dfp1 kinase complex affect the error-prone repair pathway independent of the Rhp18 PCNA-ubiquitylation pathway that has been identified previously. This is consistent with observations in budding yeast, which suggest that Cdc7 functions in a distinct epistasis group in the error-prone repair pathway (PESSOA-BRANDAO and SCLAFANI 2004).

We propose that the function of Hsk1–Dfp1 in the proper response to MMS depends upon association of the kinase with the chromatin during the MMS response, via the C terminus of Dfp1. In fact, Hsk1 may be recruited by specific damage recognition or repair proteins. For example, in a recent proteomics study, budding yeast Cdc7 was isolated in an affinity purification using MGMT (*O*₆-methylguanine–DNA methyltransferase (NITURE *et al.* 2005). This enzyme is responsible for directly removing methyl groups from DNA damaged by alkylating agents such as N-methyl-N-nitrosourea (MNU) (KAINA *et al.* 2001, 2007; WYATT and PITTMAN 2006);

fission yeast uses a different enzyme to deal with these lesions (PEARSON *et al.* 2006). Therefore, we propose that Hsk1 may contribute to the choice of repair mechanism by direct regulation of repair proteins at the replication fork.

There are other examples of Rhp18-independent inputs into the PRR pathway. A recent study suggested that phosphorylation of the Rad9 checkpoint protein on T225 by Rad3/ATR specifically activates the error-free translesion synthesis pathway (KAI *et al.* 2007). Interestingly, mutation of *rad9-T225C* combined with mutations of Δ *rhp18* leads to a dramatic increase of gene conversion, but not deletion recombination (KAI *et al.* 2007). The authors suggest that loss of the *rhp18*⁺-mediated lesion bypass system is synergistic with mutations that inhibit inappropriate recombination, leading to a hyper-recombinant phenotype. Thus, the recombination response is intimately linked to the PRR response. Our data suggest that Hsk1 is required for error-prone repair; at least in genetic terms, this may antagonize the effects of Rad9-phosphoT225. Interestingly, the hyperrecombinant phenotype of *hsk1*, and the lack of genetic interaction with Δ *srs2*, could suggest that Hsk1 also inhibits the recombination response to alkylating damage.

Our data suggest several roles for the Hsk1-Dfp1 that contribute to genome stability after the initiation of DNA synthesis. We agree with previous studies that Hsk1 functions in concert with the FPC to promote fork stability during fork pausing. This is consistent with evidence showing that reduction of Cdc7 in murine ES cells causes slowing of replication, and its complete depletion causes p53-dependent apoptosis (KIM *et al.* 2002, 2003). However, our data show that Hsk1 also fulfills an FPC-independent function that promotes error-prone repair. We suggest that this is dependent on the C terminus of Dfp1, which may directly associate with repair proteins. Further studies will be necessary to identify the target for Dfp1 association and likely substrates for Hsk1 activity in the repair process.

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GENETICS

Supporting Information

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Fission Yeast Hsk1 (Cdc7) Kinase Is Required After Replication Initiation for Induced Mutagenesis and Proper Response to DNA Alkylation Damage

**William P. Dolan, Anh-Huy Le, Henning Schmidt, Ji-Ping Yuan, Marc Green
and Susan L. Forsburg**

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TABLE S1**Strains used**

Strain number	Genotype	Reference
FY8	<i>h⁺</i> (975)	Lab stock
FY261	<i>h⁺ can1-1 leu1-32 ade6-M216 ura4-D18</i>	Lab stock
FY528	<i>h⁺ his3-D1 ade6-M210 ura4-D18 leu1-32</i>	Lab stock
FY865	<i>h⁻ Δ<i>cds1</i>::<i>ura4 ura4-D18 leu1-32</i></i>	T. Wang
FY945	<i>h⁻ hsk1-1312 ura4-D18 leu1-32 ade6-M210</i>	(SNAITH <i>et al.</i> 2000)
FY986	<i>h⁺ hsk1-1312 ura4-D18 leu1-32 ade6-M216</i>	(SNAITH <i>et al.</i> 2000)
FY999	<i>h⁺ hsk1-1312 Δ<i>cds1</i>::<i>ura4⁺ ura4-D18 leu1-32</i></i>	(SNAITH <i>et al.</i> 2000)
FY1000	<i>h⁻ cdc10-V50 hsk1HA::ura4⁺ ura4-D18 leu1-32 ade6-M210</i>	(SNAITH <i>et al.</i> 2000)
FY1006	<i>h⁻ cdc25-22 hsk1HA::ura4⁺ ura4-D18 leu-32 ade6-M210</i>	(SNAITH <i>et al.</i> 2000)
FY1011	<i>h⁻ cdc22-M45 hsk1HA::ura4⁺ ura4-D18 leu-32 ade6-M210</i>	(SNAITH <i>et al.</i> 2000)
FY1077	<i>h⁻ hsk1HA::ura4⁺ ura4-D18 leu1-32 ade6-M216</i>	(SNAITH <i>et al.</i> 2000)
FY1193	<i>h⁺ Δ<i>srs2</i>::<i>kanMX leu-32 ura4-D18 ade6-M210</i></i>	G. Freyer
FY1418	<i>h⁺ hsk1-1312</i>	This work
FY1763	<i>h⁺ leu1::(<i>dff1</i>⁺<i>6his3HA leu1</i>⁺) dff1-D1 ura4-D18 ade6-M216</i>	(FUNG <i>et al.</i> 2002)
FY1764	<i>h⁺ leu1::(<i>dff1</i>(1-376)-<i>6his3HA leu1</i>⁺) dff1-D1 ura4-D18 ade6-M216</i>	(FUNG <i>et al.</i> 2002)
FY1794	<i>h⁺ leu1::(<i>dff1</i>(1-459)-<i>6his3HA leu1</i>⁺) dff1-D1 ura4-D18 ade6-M216</i>	(FUNG <i>et al.</i> 2002)
FY2132	<i>h⁺ ade6-L469/pUC8/<i>his3⁺/ade6-M375 leu1-32 his3-D1</i></i>	(CATLETT and FORSBURG 2003)
FY2878	<i>h⁻ rad22::YFP:<i>kanMX4 ade6-M210 ura4-D18 leu1-32</i></i>	G. Freyer
FY2879	<i>h⁻ Δ<i>mrc1</i>::<i>ura4 ade6-704 leu1-32 ura4-D18</i></i>	T. Carr
FY2983	<i>h⁻ Δ<i>mrc1</i>::<i>ura4⁺ hsk1-1312 ura4-D18 leu1-32 ade6</i></i>	This work
FY3084	<i>h⁺ orp1-4 Δ<i>rad1</i>::<i>ura4⁺ ura4-D18 ade6-M210</i></i>	This work
FY3089	<i>h⁺ orp1-4 Δ<i>rad17</i>::<i>ura4⁺ ura4-D18 ade6-M210</i></i>	This work
FY3098	<i>h⁺ swi1-111 ade6-L469/pUC8/<i>his3⁺/ade6-M375 his3-D1</i></i>	This work
FY3100	<i>h⁺ swi3-146 ade6-L469/pUC8/<i>his3⁺/ade6-M375 his3-D1</i></i>	This work
FY3101	<i>h⁺ hsk1-1312 ade6-L469/pUC8/<i>his3⁺/ade6-M375 his3-D1 leu1-32</i></i>	This work
FY3121	<i>h⁻ leu1-32 ura4-D18 swi1::KanMX</i>	(NOGUCHI <i>et al.</i> 2003)
FY3122	<i>h⁻ leu1-32 ura4-D18 swi3::KanMX</i>	(NOGUCHI <i>et al.</i> 2004)
FY3123	<i>h⁻ Δ<i>rhpl8</i>::<i>ura4⁺ ura4-D18 leu1-32 ade6-704</i></i>	(VERKADE <i>et al.</i> 2001)
FY3124	<i>h⁺ Δ<i>rhpl8</i>::<i>ura4⁺ ura4-D18 leu1-32 ade6-704</i></i>	(VERKADE <i>et al.</i> 2001)
FY3126	<i>h⁺ Δ<i>ubc13</i>::<i>ura4⁺ ade6-M210 ura4-D18 leu1-32 his3-D1</i></i>	(VERKADE <i>et al.</i> 2001)
FY3180	<i>h⁺ hsk1-1312 Δ<i>rhpl8</i>::<i>ura4⁺ ura4-D18 leu1-32 ade6-M216</i></i>	This work
FY3182	<i>h⁺ Δ<i>swi1</i>::<i>kanMX Δrhpl8::ura4⁺ ura4-D18 leu1-32 ade6-704</i></i>	This work
FY3184	<i>h⁺ Δ<i>swi3</i>::<i>kanMX Δrhpl8::ura4⁺ ura4-D18 leu1-32 ade6-704</i></i>	This work
FY3224	<i>h⁺ Δ<i>swi1</i>::<i>kanMX6 ura4-D18 leu1-32 ade6-M210</i></i>	This work
FY3249	<i>h⁻ Δ<i>swi1</i>::<i>kanMX hsk1HA::ura4⁺ ura4-D18 leu1-32 ade6-M216</i></i>	This work
FY3251	<i>h⁻ Δ<i>swi3</i>::<i>kanMX hsk1HA::ura4⁺ ura4-D18 leu1-32 ade6-M216</i></i>	This work
FY3253	<i>h⁺ Δ<i>swi3</i>::<i>KanMX psf2-209 ura4-D18 leu1-32</i></i>	This work

FY3254	<i>h² Δswi3::KanMX cdc19-P1 ura4-D18 leu1-32</i>	This work
FY3255	<i>h² Δswi3::KanMX pol1-1 ura4-D18 leu1-32 ade6-M210</i>	This work
FY3257	<i>h⁻ Δswi3::KanMX Δrqh1::ura4⁺ ura4-D18 leu1-32</i>	This work
FY3260	<i>h⁺ Δswi3::KanMX cdc45ts ura4-D18 leu1-32 ade6-M210</i>	This work
FY3262	<i>h⁺ Δswi3::KanMX Δrad3::ura4⁺ ura4-D18 leu1-32</i>	This work
FY3264	<i>h⁺ Δswi1::KanMX cdc45ts ura4-D18 leu1-32</i>	This work
FY3266	<i>h⁺ Δswi1::KanMX pol1-1 ura4-D18 leu1-32 ade6-M210</i>	This work
FY3267	<i>h⁻ Δswi1::KanMX Δswi6::ura4⁺ ura4-D18 leu1-32 ade6-M210</i>	This work
FY3268	<i>h² Δswi1::KanMX Δrqh1::ura4⁺ ura4-D18 leu1-32</i>	This work
FY3271	<i>h⁺ dff1v5::ura4⁺ ura4-D18 leu1-32 ade6-M210 his3-D1</i>	This work
FY3272	<i>h⁺ hsk1-1312 swi3-13myc::KanMX ura4-D18 leu1-32 ade6-M210</i>	This work
FY3280	<i>h⁺ cdc10-V50 dff1v5::ura4⁺ ura4-D18 leu1-32 ade6</i>	This work
FY3281	<i>h⁺ cdc22-M45 dff1v5::ura4⁺ ura4-D18 leu1-32 ade6 his3-D1</i>	This work
FY3282	<i>h⁺ cdc25-22 dff1v5::ura4⁺ ura4-D18 leu1-32 ade6 his3-D1</i>	This work
FY3285	<i>h⁺ hsk1-1312 rad22YFP::KanMX6 ura4-D18 leu1-32 ade6</i>	This work
FY3286	<i>h⁻ Δcds1::ura4⁺ rad22YFP::KanMX6 ura4-D18 leu1-32 ade6?</i>	This work
FY3287	<i>h⁻ Δswi1::KanMX6 rad22YFP::KanMX6 ura4-D18 leu1-32 ade6-M210</i>	This work
FY3509	<i>h⁻ eso1ΔC::kanMX6 ura4-D18 leu1-32 ade-704?</i>	M. O'Connell
FY3511	<i>h⁻ rev3::hphMX6 ura4-D18 leu1-32 ade-704?</i>	M. O'Connell
FY3513	<i>h⁻ pcn1-K164R::ura4 leu1-32 ade6-704 ura4-D18</i>	M. O'Connell
FY3532	<i>h⁻ hsk1-1312 swi1::kanR ura4-D18 leu1-32 ade6-M210</i>	This work
FY3543	<i>h⁻ hsk1-1312 Δsrs2::kanR ura4-D18 leu1-32 ade-M210</i>	This work
FY3545	<i>h⁻ hsk1-1312 Δubc13::ura4⁺ ura4-D18 leu1-32 ade-M210 his3-D1</i>	This work
FY3582	<i>h² eso1ΔC::kanMX6 hsk1-1312 ura4-D18 leu1-32 his3-D1 ade6-704</i>	This work
FY3584	<i>h² pcn1-K164R::ura4⁺ hsk1-1312 ura4-D18 leu1-32 his3-D1 ade6-M210</i>	This work
FY3611	<i>h⁺ rev3::hphMX6 hsk1-1312 ura4-D18 leu1-32 ade6-M210 his3-D1</i>	This work
FY3999	<i>h⁺ rad35-271</i>	This work
FY4588	<i>h⁺ Δswi1::kanMX</i>	This work
HE686	<i>h⁹⁰ smt-0 leu1-32 ura4-D18</i>	This work

FIGURE S1.—Synthetic interactions between *dfp1*-(1-519) and components of the post replication repair pathway. Cells were plated in 5X dilutions on YES with the indicated amount of drug.

FIGURE S2.—Synthetic interactions between *hsk1* and components of the post replication repair pathway on different DNA damaging compounds. Cells were plated in 5X dilutions on YES with the indicated amount of drug.

FIGURE S3.—Synthetic interactions between *hsk1* or FPC components *swi1* or *swi3*, and *rhp18*. Cells were plated in 5X dilutions on YES with the indicated amount of drug.