Multiple pathways can bypass the essential role of fission yeast Hsk1 kinase in DNA replication initiation

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Cdc7/Hsk1 is a conserved kinase required for initiation of DNA replication that potentially regulates timing and locations of replication origin firing. Here, we show that viability of fission yeast hsk1Δ cells can be restored by loss of mrc1, which is required for maintenance of replication fork integrity, by cdc7Δ, or by a checkpoint-deficient mutant of mrc1. In these mutants, normally inactive origins are activated in the presence of hydroxyurea and binding of Cdc45 to MCM is stimulated. mrc1Δ bypasses hsk1Δ more efficiently because of its checkpoint-independent inhibitory functions. Unexpectedly, hsk1Δ is viable at 37°C. More DNA is synthesized, and some dormant origins fire in the presence of hydroxyurea at 37°C. Furthermore, hsk1Δ bypass strains grow poorly at 25°C compared with higher temperatures. Our results show that Hsk1 functions for DNA replication can be bypassed by different genetic backgrounds as well as under varied physiological conditions, providing additional evidence for plasticity of the replication program in eukaryotes.

Introduction

Recent studies indicate that the program that regulates eukaryotic DNA replication is very dynamic and shows a great deal of plasticity during development or differentiation (Taylor, 1977; Gilbert, 2007; Hiratani et al., 2008; Hansen et al., 2010; Pope et al., 2010). This “plasticity” of chromosome functions is also evident in other chromosome dynamics such as generation of neocentromere in centromere-less chromosomes (Choo, 1997; Warburton, 2004; Ishii et al., 2008). However, the molecular basis of this plasticity or factors that regulate it is largely unknown. The elucidation of the robustness and plasticity of the replication program would be very important in understanding the molecular basis of the dynamic and plastic nature of chromosome dynamics (Masai et al., 2010).

Cdc7 is a serine-threonine kinase originally identified in budding yeast (Hartwell, 1973, 1974). It forms a heterodimer with the Dbf4 subunit to generate an active kinase (Sclafani, 2000; Masai and Arai, 2002). Initial characterization of budding yeast cdc7-ts mutants revealed that protein synthesis is no longer required for completion of S phase once Cdc7 function is executed (Burke and Church, 1991). Later, it was shown that Cdc7 is required for firing of each origin during S phase (Bousset and Diffley, 1998; Donaldson et al., 1998). Thus, Cdc7 is a key factor for the regulation of replication program by determining when and where DNA replication is initiated on the chromosomes (Patel et al., 2008; Wu and Nurse, 2009; Hayashi et al., 2009; Masai et al., 2010), and understanding how this kinase functions during varied physiological conditions is crucial to unraveling the molecular basis of regulation of replication program.

Cdc7-Dbf4 is conserved and the fission yeast counterpart is Hsk1-Dfp1/Him1 (Masai et al., 1995; Brown and Kelly, 1999; Takeda et al., 1999). hsk1+ is essential for viability, and temperature-sensitive mutants of hsk1, hsk1-89 and hsk1-1329, exhibit a defect in S phase and lose viability at their nonpermissive temperatures (Snaith et al., 2000; Takeda et al., 2001; Matsumoto et al., 2005).

Studies in yeast and Xenopus egg extracts have shown that Cdc7 activity is required for origin binding or chromatin loading of Cdc45, a factor essential for generation of an active replication fork (Jares and Blow, 2000; Zou and Stillman, 2000; Dolan et al., 2004). Furthermore, various studies indicated that MCM is one of the major substrates of Cdc7 kinase for initiation of DNA replication, and that the N-terminal tails of MCM2, -4, and -6 are phosphorylated by Cdc7 in combination with Cdk...
and a checkpoint kinase. This phosphorylation facilitates the interaction of Cdc45 with MCM, contributing to the formation of replication forks (Masai et al., 2006; Sheu and Stillman, 2006; Randell et al., 2010).

Recent reports indicate critical roles for Cdc7 kinase in various chromosome transactions including sister chromatid cohesion, centromeric heterochromatin formation, initiation of meiotic recombination, bypass DNA synthesis, chromatin formation mediated by Caf1, and replication checkpoint regulation (Takeda et al., 2001; Bailis et al., 2003; Pessoa-Brandão and Sclafani, 2004; Gérard et al., 2006; Ogino et al., 2006; Matsumoto et al., 2010; Takayama et al., 2010). These results strongly suggest that Cdc7 has multiple substrates (Takeda et al., 2001; Kim et al., 2008). We and others reported that hsk1+ interacts both genetically and physically with swi1+ and swi3+, which together function as the “replication fork protection complex” (Noguchi et al., 2004). This leads us to propose that Hsk1 plays a role in the stabilization of stalled replication forks (Matsumoto et al., 2005; Sommariva et al., 2005; Shimamoto et al., 2009).

In budding yeast, bypass mutants of cdc7 or dbf4 deletion were previously reported. The first report was bob1 mutant, which is allelic to mcm5. mcm5bob1+ can rescue the growth of both cdc7Δ and dbf4Δ and the mutation was mapped as P38L substitution (Jackson et al., 1993; Hardy et al., 1997). It was later reported that replacement of proline 83 of MCM5 with other “large residues” (lysine or tryptophan) led to recovery of growth of cdc7Δ (Fletcher et al., 2003). More recently, deletions of the MCM4 N-terminal nonconserved segment were found to bypass Cdc7 function for growth. It was proposed that Cdc7 antagonizes the negative effect of the MCM4 N-terminal segment on initiation of DNA replication (Sheu and Stillman, 2010). Indeed, phosphomimetic mutations of Cdc7 target sites of MCM4 and MCM6 were shown to bypass Cdc7 function (Randell et al., 2010). However, similar rescue has not been reported in other organisms and bypass mutations mapped to genes other than mcm have not been identified.

Mrc1 (mediator of replication checkpoint; Claspin in mammalans) is a component of replication fork and is required for replication stress checkpoint (Alcasabas et al., 2001; Tanaka and Russell, 2001; Katou et al., 2003; Osborn and Elledge, 2003). In fission yeast, Mrc1 allows the DNA replication checkpoint sensor kinase Rad3-Rad26 (ATR-ATRIP in mammalans) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003). In fission yeast, Mrc1 allows the DNA replication check point sensor kinase Rad3-Rad26 (ATR-ATRIP in mammals) to activate the effector kinase Cds1 (Chk2 in mammals; Tanaka et al., 2003).

Results

Deletion of mrc1 restores the growth of hsk1-null cells

We previously isolated hsk1-89, a temperature-sensitive mutant of hsk1, and have been searching for mutations that genetically interact with this mutant. We have reported that swi1 or swi3 mutant shows synthetic-defective phenotype with hsk1-89 (Matsumoto et al., 2005; Shimamoto et al., 2009). We have then tested for genetic interaction between mrc1Δ and hsk1-89. Unlike in the case of swi1 or swi3, we found that mrc1Δ partially suppresses the growth defect of hsk1-89 under the nonpermissive condition (Fig. 1 A). Although hsk1-89 cannot grow at 30°C, hsk1-89 mrc1Δ can form colonies under this condition. cds1Δ also rescued the growth of hsk1-89 at 30°C, consistent with the previous and recent reports that cds1Δ and mrc1Δ could partially rescue the growth of hsk1Δ (Snaith et al., 2000; Matsumoto et al., 2005; Dolan et al., 2010). However, we always observed better recovery with mrc1Δ compared with cds1Δ (Fig. 1 A).

We then examined whether or not mrc1Δ can rescue the growth defect of hsk1Δ. hsk1Δ is essential for viability at 30°C and germinating spores of hsk1::ura4Δ cease growth after three to four cell divisions (Masai et al., 1995). A cross between hsk1::ura4Δ (pREP41-hsk1c3) and mrc1::kan yielded viable Ura+ Kan- leu- segregants (Fig. 1 B), indicating that hsk1Δ mrc1Δ is viable. The loss of the hsk1Δ coding sequence in these segregants was confirmed by colony-PCR (Fig. S1A). In the same assay, we could recover dpf1/hip1Δ mrc1Δ cells, indicating that mrc1Δ can restore the growth of dpf1/hip1Δ as well. However, cds1Δ or hsk1Δ could not be isolated under the same condition (no leu- segregant in 220 Ura+ segregants analyzed), suggesting that the mechanism of suppression of the hsk1Δ mutation may be different between cds1Δ and mrc1Δ or that the viability through meiosis of cds1Δ hsk1Δ is too low for the survivors to be detected with the number of segregants tested. Therefore, we next examined the viability of double deletions through plasmid loss using 5-fluoroorotic acid (5-FOA) counterselection against ura4Δ.

hsk1::kan cells harboring pREP2hsk1wt (ura4Δ-marker) plasmid were spotted on a plate containing 5-FOA. hsk1Δ cells that lost the plasmid could not grow at 30°C on SD+5-FOA plates, indicating that hsk1Δ is essential for cell viability as reported before (Fig. 1 C). In contrast, mrc1Δ could allow cells to survive in the absence of Hsk1, although the viability of hsk1Δ mrc1Δ was much lower than wild type (Fig. 1 C). mrc1-3Δ (S604AT645AT653A), which is specifically defective in checkpoint function (Zhao et al., 2003; Xu et al., 2006) and cds1Δ also partially restored the growth defect of hsk1Δ. However, both mrc1-3Δ and cds1Δ always exhibited lesser growth recovery than mrc1Δ (Fig. 1 D; see later sections).
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electrophoresis of DNA prepared from the hsk1+/mrc1Δ or dfp1/him1Δ/mrc1Δ cells growing asynchronously showed bubble arc signals indicative of origin firing at ars2004 (Okuno et al., 1997), a well-characterized early-firing origin (Fig. 1 D, filled arrow).

Chromosome integrity is restored in hsk1-89 by mrc1Δ

To gain insight into the mechanism of hsk1Δ bypass by mrc1Δ, we examined hsk1-89 mrc1Δ and hsk1-89 cells at 30°C, the nonpermissive temperature, because the mutant kinase is highly

hsk1Δ mrc1Δ cells grew poorly and frequently exhibited abnormal nuclear morphology (unpublished data), probably due to extensive DNA damage occurring during the course of DNA replication. FACs profiles of the DNA content of growing hsk1Δ mrc1Δ cells also showed DNA degradation (unpublished data), suggesting serious problems in maintaining chromosome integrity. We found that chromosome III containing rDNA repeats was highly rearranged and split into multiple bands (Fig. S2, A–C). Elongation of chromosome I was also detected (Fig. S2, B and C; clone #3). In spite of absence of Hsk1 kinase, which is known to be required for origin firing, two-dimensional gel electrophoresis of DNA prepared from the hsk1Δ mrc1Δ or dfp1/him1Δ mrc1Δ cells growing asynchronously showed bubble arc signals indicative of origin firing at ars2004 (Okuno et al., 1997), a well-characterized early-firing origin (Fig. 1 D, filled arrow).

Figure 1. mrc1Δ can rescue the defect of hsk1Δ function. (A) Fivefold serial dilutions of exponentially growing wild-type, mrc1Δ, hsk1-89, mrc1Δ hsk1-89, cdsΔ hsk1-89, mrc1-3A, and mrc1-3A hsk1-89 cells were spotted on YES agar and incubated at 25 or 30°C for 5 d. (B) MS142 (h* hsk1::ura4+ with pREP41-hsk1c3) was crossed with KT2863 (h+ mrc1::kan). Meiotic products were examined by random spore analyses. Segregants grown on EM+ Leu plates (indicating hsk1::ura4+) were transferred to EM+ Leu and YPAD-containing G418 plates, and incubated at 25°C for 4 d and the numbers of growing clones were counted. (C) Fivefold serial dilutions of indicated cells carrying pREP2hsk1wt plasmid (ura4-marker) were spotted on YES or SD-5-FOA (1 mg/ml) plates and incubated at 30°C for 8 d. (D) hsk1::ura4+ harboring pREP41-hsk1c3 or Δdfp1/him1::ura4+ harboring pREP41-HAHim1(N1) was crossed with mrc1::kan (KT2863) and Ura+ Leu+ segregants (hsk1Δ mrc1Δ or dfp1/him1Δ mrc1Δ, respectively) were selected as in B), and the obtained cells growing asynchronously were analyzed by two-dimensional gel electrophoresis to detect replication intermediates at ars2004, one of the early-firing origins (filled arrow, bubble arc representing initiation; open arrow, Y arc representing passive replication). Bubble arcs can be detected, indicative of the initiation events at this origin in these bypass mutant cells.
Figure 2. The viability and efficiency of S phase progression are partially restored in hsk1-89 by mrc1Δ. (A) Exponentially growing wild-type, mrc1Δ, hsk1-89, and hsk1-89 mrc1Δ cells in YES media at 25°C were shifted to 30°C. At the indicated time, cell numbers were counted, plated at 25°C, and the viability was calculated. (B) Analysis of S phase progression by FACS. Cells indicated were arrested in G1 phase by nitrogen starvation. After release into S phase at 30°C, an aliquot was taken from each culture at the times indicated, and DNA content of collected cells was analyzed by FACS. (C) BrdU incorporation at 30°C in hsk1-89 and hsk1-89 mrc1Δ cells. The cells carrying pREP41-TK plasmid arrested in G1 phase were released into growth in EMM media containing 25 µg/ml BrdU. Genomic DNA was collected at the times indicated, and BrdU was detected by Western blotting with anti-BrdU antibody. The spot indicated as “+HU” represents cells treated with 10 mM HU for 7 h [added at time 0]. The right graph shows quantification of each spot in the Western blot by using ImageJ software. The expression level of TK was identical at both 25 and 30°C between hsk1-89 and hsk1-89 mrc1Δ cells (not depicted).
unstable and enzymatically inactive at this temperature (Shimmoto et al., 2009). Although \textit{hsk1-89} lost its viability at 30°C as previously reported (Matsumoto et al., 2005), the viability at 30°C was significantly improved in \textit{hsk1-89 mrc1\Delta} (Fig. 2 A). Analyses of cell/nuclear morphology and integrity of chromosome DNA indicated that the reduced level of DNA damage and aberrant mitosis may contribute to the improvement of \textit{hsk1-89 mrc1\Delta} viability at 30°C, as shown in Fig. 2 A (Fig. S2, D–G).

**\textit{mrc1\Delta} stimulates S phase in \textit{hsk1-89}**

To characterize the effect of \textit{mrc1\Delta} on DNA replication in \textit{hsk1-89}. DNA content was analyzed by flow cytometry of cells arrested at G1 phase by nitrogen starvation and then released into S phase at 30°C. Both wild-type and \textit{mrc1\Delta} cells completed S phase with similar profiles by 5 h after the release (Fig. 2 B, left). On the other hand, DNA content did not significantly change in the first 4 h after the release in \textit{hsk1-89}. After that, a slight rightward shift of the 1C DNA peak was observed in \textit{hsk1-89} by 8 h. In \textit{hsk1-89 mrc1\Delta}, in contrast, DNA replication appeared to be completed in a large population of cells by 8 h (Fig. 2 B, right), suggesting that the bulk rate of DNA synthesis is faster. In \textit{hsk1-89}, a significant number of the cells with less than 1C DNA content appeared by 8 h, whereas these apparently dead cells were not obvious in \textit{hsk1-89 mrc1\Delta} (Fig. 2 B, right; and unpublished data). This result indicates that the S phase progression may be at least partially restored in \textit{hsk1-89} by \textit{mrc1\Delta}. Consistent with this, BrdU incorporation was enhanced in \textit{hsk1-89 mrc1\Delta} by \~1.4 times compared with \textit{hsk1-89} (Fig. 2 C).

**\textit{Mrc1} and \textit{Cds1} negatively regulate the firing of dormant origins**

The results shown above indicate that S phase progression is stimulated by the absence of \textit{Mrc1} in \textit{hsk1-89} and allowed us to assess whether firing of origins is facilitated under the \textit{mrc1\Delta} background. We analyzed initiation sites of DNA replication in \textit{mrc1\Delta} and \textit{cds1\Delta} cells in the presence of hydroxyurea (HU) that depletes dNTPs by the chromatin immunoprecipitation (ChIP)–chip method. In the presence of HU, early-firing origins or efficient origins are fired, but late-firing origins or inefficient origins are not fired in checkpoint-proficient cells (Feng et al., 2006; Heichinger et al., 2006; Patel et al., 2006; Hayashi et al., 2007). Our data indicate that 145 out of 421 silent origins (~34%) were fired in \textit{mrc1\Delta} and a similar result with \textit{cds1\Delta} (Hayashi et al., 2007). Our data indicate that 145 out of 421 silent origins (~34%) were fired in \textit{mrc1\Delta} and a similar result with \textit{cds1\Delta}. These discrepancies may be due to the sensitivity of the detection or stringency of the criteria. They may also be due to the difference in the methods or to the concentration of HU used in the experiments. In any case, the above results suggested that dormant or inefficient origins may be fired in \textit{mrc1\Delta} or \textit{cds1\Delta}, which may contribute to the bypass of \textit{hsk1-89} function. Therefore, we next analyzed initiation sites of DNA replication in \textit{hsk1-89} and \textit{hsk1-89 mrc1\Delta}. In \textit{hsk1-89}, origin firing is wiped out or down-regulated on a genome-wide basis, consistent with the critical role of Hsk1 kinase in firing at preRC. However, in \textit{hsk1-89 mrc1\Delta}, some origins that are not fired in \textit{hsk1-89} are fired (Fig. 3 B), supporting our proposal that increased initiation frequency may be responsible for bypass of \textit{hsk1-89}\textsuperscript{+}.

How are the dormant origins activated in these mutant cells? The conserved target of the replication checkpoint is Cdc45 and its loading at unfired origins is inhibited by replication fork stresses. This checkpoint regulation may be through Cdk or Cdc7, which is required for loading of Cdc45 at origins (Zou and Stillman, 2000; Costanzo et al., 2003; Dolan et al., 2004). We examined interaction of Cdc45 and MCM2 before and after replication stress and did not see any significant difference in cell cycle distribution between wild-type and \textit{mrc1\Delta} cells. A slightly larger amount of MCM2 was coimmunoprecipitated with Cdc45 in \textit{mrc1\Delta} cells in the absence of HU (Fig. 3 C, lanes 3 and 4). After treatment with HU, the interaction was significantly stimulated in \textit{mrc1\Delta} (Fig. 3 C, lanes 7 and 8). The interaction of Cdc45 with MCM2 also increased in \textit{cds1\Delta} (unpublished data). In \textit{hsk1-89}, the interaction of Cdc45 and MCM2 was very weak at 25°C and it was barely detectable after the shift to 30°C, the nonpermissive temperature for this mutant (Fig. 3 D, lanes 7–9), as previously reported (Masai et al., 2006). This interaction increased in \textit{hsk1-89 mrc1\Delta} at both 25 and 30°C (Fig. 3 D, lanes 10–12). These results suggest that the defective checkpoint leads to enhanced initiation at inefficient origins probably through stimulation of Cdc45 loading onto MCM, and that this may at least partly contribute to the rescue of growth of \textit{hsk1\Delta} cells by the DNA replication checkpoint mutations.

**The checkpoint-independent function of Mrc1 also contributes to the rescue of \textit{hsk1\Delta}**

As shown above, the bypass of Hsk1 function by \textit{mrc1\Delta} is likely to depend on the loss of the DNA replication checkpoint. However, \textit{hsk1-89 mrc1\Delta} or \textit{hsk1\Delta mrc1\Delta} consistently exhibited better growth than \textit{hsk1-89 cds1\Delta} or \textit{hsk1\Delta cds1\Delta}, respectively. This suggests that a checkpoint-independent role of Mrc1 may also be involved in the rescue of \textit{hsk1 mutant}. Checkpoint-independent roles of Mrc1 in replication fork progression or rescue of stalled forks have been suggested (Szyjka et al., 2005; Tourrière et al., 2005). Thus, we examined whether the rescue...
and checkpoint functions were separable in Mrc1. Various deletion derivatives of Mrc1 (Shimmoto et al., 2009) were examined for their ability to convey resistance to HU. Both mrc1(157–781) and mrc1(157–879), containing a DNA-binding domain and a SQ/TQ cluster, restored HU resistance in hsk1-89 mrc1 cells (Fig. 4 C). Indeed, the SQ/TQ cluster (536–674), when tandemly linked (dimer), could restore HU resistance, whereas the monomer SQ/TQ fragment could not (Fig. 4 D). These fragments of Mrc1 were next examined for their ability to inhibit the growth of hsk1-89 mrc1 cells at 30°C, where inhibition of the growth would show that the fragment carries the function involved in the rescue. Mrc1(157–879) inhibited the growth of

Figure 3. Dormant (or inefficient) origins are activated and binding of Cdc45 to MCM is stimulated in checkpoint mutants. (A) Wild-type (WT, KYP103), mrc1Δ, and cds1Δ cells arrested at M phase were released at 30°C, and incubated in the presence of HU and BrdU for 1 h. Mcm4 binding and BrdU incorporating sites were mapped by ChIP-chip assays as described in Materials and methods. Two segments of chromosome I and three segments of chromosome II are shown. (B) hsk1-89 and hsk1-89 mrc1Δ cells were analyzed as in A. The 3,600–3,800 kb segment of chromosome I is shown. (C) Whole-cell extracts were prepared from asynchronous cultures (grown at 30°C) of wild-type (HM545) and mrc1Δ (HM550) cells, each expressing Cdc45-3FLAG–tagged protein from the endogenous locus, after treatment with HU (+HU; 25 mM HU for 3 h) or no treatment (–HU). Left panels show the FACS analyses of DNA content of the wild-type and mrc1Δ cells used for extract preparation. (D) hsk1-89 cdc45-3FLAG (HM316) and hsk1-89 mrc1Δ cdc45-3FLAG (HM319) cells, grown at 25°C to the exponential phase, were harvested at 0, 1, and 3 h after temperature shift to 30°C. In C and D, 10% of the total cell extract used for immunoprecipitation (Input) and immunoprecipitates with anti-FLAG antibody (IP: αFlag) were separated by 7.5% uniform (C) or 4–20% gradient (D) gel, followed by Western blotting analyses using anti-Flag antibody (Cdc45) and anti-Mcm2 antibodies.
Figure 4. **Mrc1 inhibits growth of hsk1-89 at least partially through its checkpoint-independent function.** (A) Schematic representation of the Mrc1 fragments expressed in this experiment. (B) The crude cell extracts from hsk1-89 mrc1Δ cells carrying pREP41-3FLAG, pREP41mrc1-3FLAG, pREP41mrc1[157–781]-3FLAG, pREP41mrc1[157–879]-3FLAG, pREP41mrc1[782–879]-3FLAG, pREP41mrc1[536–673]-3FLAG, or pREP41mrc1[536–678]2-3FLAG grown on EMM without thiamine for 3 d at 25°C were analyzed by Western blotting using anti-Flag monoclonal antibody. The membrane was then probed using anti-α-tubulin without stripping anti-Flag antibody. (C) hsk1-89 mrc1Δ cells carrying either pREP41-3FLAG, pREP41mrc1[157–781]-3FLAG, or pREP41mrc1[782–879]-3FLAG were streaked on EMM media with or without thiamine and incubated at 25 or 30°C for 8 d. To assay HU sensitivity, 5 mM HU was included in the media and the cells were incubated for 10 d at 25°C. (D) Fivefold serial dilutions of exponentially growing cultures of mrc1Δ or hsk1-89 mrc1Δ cells carrying either pREP41-3FLAG, pREP41mrc1[157–781]-3FLAG, or pREP41mrc1[782–879]-3FLAG were spotted on EMM media without thiamine. Top: mrc1Δ cells carrying the indicated plasmid were spotted on EMM with or without 6 mM HU and incubated at 25 or 30°C for 8 d. Bottom: hsk1-89 mrc1Δ cells carrying the same set of the plasmids were spotted on EMM and incubated at 25 or 30°C for 10 d. A Mrc1 fragment [157–781] and a dimer of 536–673 confers HU resistance, but cannot inhibit rescue of hsk1 mutation in mrc1Δ.
Hsk1-89 mrc1Δ at 30°C, whereas Mrc1(157–781) allowed hsk1-89 mrc1Δ to grow at 30°C (Fig. 4 C), indicating that a checkpoint-independent function of Mrc1 involved in the rescue of hsk1-89 lies in the segment 782–879. Furthermore, the dimer of Mrc1(536–673), which confers HU resistance, could not inhibit the growth of hsk1-89 mrc1Δ. These results are consistent with the above conclusion and indicate the separation of checkpoint function and the function required for the rescue of hsk1 mutation by mrc1Δ. However, expression of Mrc1(782–879) alone did not inhibit the growth at 30°C in hsk1-89 mrc1Δ (Fig. 4 C). This could be due to the extremely low expression level of Mrc1(782–879) (Fig. 4 B). Alternatively, combination of Mrc1(782–879) with other segments of Mrc1 may be required.

We examined the initiation efficiency at early-firing origins by two-dimensional gel electrophoresis, and found that the bubble formation is stimulated specifically in hsk1-89 mrc1Δ cells but not in hsk1-89 cds1Δ (Fig. 5), indicating that the firing of early-firing origins may be stimulated by mrc1Δ but not cds1Δ.
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This finding provides further evidence that a checkpoint-independent function of Mrc1 contributes to the rescue process, explaining why more efficient rescue is observed with \( \text{mrc1}^\Delta \) compared with checkpoint mutants.

**Effect of temperature on the bypass of hsk1 function: hsk1\^\Delta can grow at a high temperature**

We have noted that hsk1-89 cannot form colonies at 30°C but can do so at 37°C (Fig. 6 A; Matsumoto et al., 2005). hsk1-89 cells pregrown at 37°C exhibited temperature-sensitive phenotype identical to those not exposed to a higher temperature. They could grow at 25 or 37°C, but could not grow at 30°C (Fig. 6 B), excluding the possibility that unknown mutations or rearrangements of the hsk1-89 gene might have occurred during incubation at 37°C and caused the cells to grow at 37°C. This finding prompted us to test if hsk1\^\Delta cells can grow at 37°C. hsk1\^\Delta cells that had grown at 37°C could form colonies at 37°C, but not at 30 or 25°C. Reversible effect of temperature on growth indicates that the growth at 37°C is not due to genetic changes that rescued the growth of hsk1\^\Delta. hsk1\^\Delta mrc1\^\Delta double-mutant cells grew very well at 37 and 30°C, but grew poorly at 25°C. Similarly, hsk1\^\Delta cds1\^\Delta cells grow well at 37°C, but poorly at 30 and 25°C.

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**Figure 6. Temperature-dependent growth of hsk1\^\Delta cells.** (A) Fivefold serial dilutions of exponentially growing cultures of wild-type and hsk1-89 cells were plated on YES agar and incubated at 25, 30, or 37°C for 4 d. (B) Wild-type and hsk1-89 cells were streaked on YES agar and incubated at 37°C for 6 d. From each strain, two growing colonies were picked and restreaked on YES agar and incubated at 25, 30, or 37°C for 4 d. (C) Cells indicated were streaked on SD-supplemented plates with or without 5-FOA and incubated at 25, 30, or 37°C as indicated. Only hsk1\^\Delta ura4\^+ cells carry ura4\^+ on the chromosome. Cells marked with an asterisk are normally maintained in the presence of the plasmid, pREP2hsk1\^\Delta (ura4\^+), because they carry hsk1\^\Delta::kan. (D) Cells harboring either pREP2 or pREP2hsk1\^\Delta as indicated were streaked on SD-supplemented plates containing 5-FOA and incubated at 37°C to induce the loss of plasmids. After loss of hsk1 sequence was confirmed by colony-PCR, those cells growing at 37°C were restreaked on SD-supplemented plates and incubated at 25, 30, and 37°C for 7 d. hsk1\^\Delta cells that had grown at 37°C could form colonies at 37°C, but not at 30 or 25°C. Reversible effect of temperature on growth indicates that the growth at 37°C is not due to genetic changes that rescued the growth of hsk1\^\Delta. hsk1\^\Delta mrc1\^\Delta double-mutant cells grew very well at 37 and 30°C, but grew poorly at 25°C. Similarly, hsk1\^\Delta cds1\^\Delta cells grow well at 37°C, but poorly at 30 and 25°C.
origin firing at six origins by quantitative real-time PCR. The origin at 1546 kb corresponds to ars2004, an early-firing origin, and the other five origins are inefficient or dormant origins that fire in the presence of HU only in \( \text{mrc1}\) or \( \text{cds1}\) background (Fig. 7 B; also see Table S2). BrdU incorporation at ars2004 was not affected by temperature, but that at two dormant origins increased at a higher temperature (Fig. 7 B; ori3152 and ori3161). We then conducted ChIP-chip assays to assess the genome-wide origin-firing pattern at 37°C and compared the result to that at 30°C. We detected a number of loci that were more efficiently fired in the presence of HU at 37°C compared with at 30°C (Fig. S3). These results show that higher temperature permits the firing of some inefficient origins, which may contribute to the rescue of \( \text{hsk1}\) cells.

**Discussion**

Although the conserved Cdc7 kinase is essential for viability in both yeasts and in mammalian cells (Sclafani et al., 1988; Masai et al., 1995; Kim et al., 2003), bypass mutations have been identified in budding yeast. They have been mapped to components of the Mcm complex, consistent with the predicted crucial role of Mcm as a target of Cdc7 in initiation of DNA replication (Jackson et al., 1993; Hardy et al., 1997; Randell et al., 2010; Sheu and Stillman, 2010). In this paper, we have identified the novel pathways for bypass of Hsk1 kinase, the fission yeast homologue of Cdc7 kinase. The results suggest a plastic and dynamic nature of eukaryotic chromosome replication.
Bypass of hsk1Δ by mrc1Δ

We found that mrc1Δ can rescue the growth at a nonpermissive temperature of hsk1-89 as well as the growth of hsk1Δ. The hsk1Δ mrc1Δ grow poorly and accumulate DNA damage as they grow, indicating that rescue of DNA replication is partial. Nevertheless, careful characterization of the rescued double mutant (hsk1-89 mrc1Δ or hsk1Δ mrc1Δ) shows that the rescue is not due to secondary mutations or due to activation of spo4Δ, another Cdc7-like kinase in fission yeast. Tetrad dissection of a diploid, hsk1Δ/*hsk1Δ mrc1Δ/*mrc1Δ, did not generate hsk1Δ mrc1Δ viable colonies (out of 35 tetrads). This is probably due to the requirement of hsk1Δ for the meiotic process that cannot be bypassed by mrc1Δ (Ogino et al., 2006; Matos et al., 2008; Sasanuma et al., 2008; Katis et al., 2010).

hsk1-89 accumulates DNA damage and continues to lose viability at a nonpermissive temperature (Fig. 2 A; Matsumoto et al., 2005). This is probably due to abortive initiation of DNA replication in the absence of sufficiently active Hsk1 kinase. It could also be due to the frequent incidence of stalled replication forks in hsk1-89, which may be caused by the aberrant and infrequent initiation (causing long inter-origin distances). These incidents could eventually lead to the collapse of the replication forks. In contrast, the extent of DNA damage as well as aberrant mitosis is significantly reduced in hsk1-89 mrc1Δ cells (Fig. S2, D–F). This may be due to restored initiation and/or facilitated fork movement in hsk1-89 mrc1Δ. Increased origin firing frequency would reduce inter-origin distances, which may reduce the probability of replication fork stalling. Indeed, DNA synthesis is considerably restored by mrc1Δ in hsk1-89 (Fig. 2, B and C), suggestive of a negative effect of Mrc1 on DNA replication.

The ability of checkpoint mutants mrc1-3A and cds1Δ to bypass Hsk1 function for viability suggests that the loss of checkpoint function can reduce the requirement of Hsk1 function for viability and most likely for DNA replication. It is known that the Rad3-Mrc1-Cds1 pathway suppresses firing of new origins and slows down the fork progression in response to fork stall signals (Boddy and Russell, 2001). Indeed, our genome-wide analyses of initiation sites indicate that dormant replication origins are fired in mrc1Δ and cds1Δ cells (Fig. 3 A; also see Table S2), consistent with the results of budding yeast as well as with some reports in fission yeast (Santocanale and Diffley, 1998; Shirahige et al., 1998; Feng et al., 2006; Hayashii et al., 2007; Koren et al., 2010). Increased firing is also observed in hsk1-89 mrc1Δ cells, compared with that in hsk1-89 cells (Fig. 3 B). Thus, the removal of these negative factors somehow deregulates the firing at certain origins and leads to bypass of Hsk1 function.

Checkpoint-dependent bypass of hsk1Δ by mrc1Δ: Chromatin loading of Cdc45 as a critical point of regulation

Previous studies in yeasts, Xenopus egg extracts, and mammalian cells indicate that chromatin loading of Cdc45 is an important step that is regulated by the replication checkpoint (Aparicio et al., 1999; Costanzo et al., 2003; Liu et al., 2006). We observed that binding of Cdc45 to Mcm is stimulated in mrc1Δ (Fig. 3 C) and cds1Δ (unpublished data), which is likely to represent the checkpoint-mediated regulation of Cdc45 loading onto chromatin. This stimulation is observed in the absence of HU (Fig. 3 C), and thus may at least partially explain the bypass of the hsk1Δ function by these mutations. Indeed, interaction between Cdc45 and Mcm2 increased in mrc1Δ in the hsk1-89 background as well (Fig. 3 D).

It has been shown that the replication checkpoint inhibits the loading of Cdc45 through Cdk or Cdc7 kinase (Costanzo et al., 2003). We indeed observed the stimulation of Hsk1 kinase activity in cds1Δ cells by enhanced association between Hsk1 and Dfp1/Him1 (unpublished data). Thus, the checkpoint could inhibit the loading of Cdc45 through Hsk1 in fission yeast as well. However, the ability of checkpoint mutants to rescue hsk1Δ suggests a pathway that can inhibit Cdc45 loading in a manner independent of Cdc7 function. Indeed, Cdk2- and Cdc7-independent checkpoint-induced inhibition of Cdc45 loading was reported in human cells (Liu et al., 2006). We speculate that replication stress induces checkpoint-dependent phosphorylation of multiple target proteins, which inhibits initiation and fork progression in multiple mechanisms. In agreement, recent reports indicate that Sld3 may be a critical target of the replication checkpoint that is inhibited by replication stress (Lopez-Mosqueda et al., 2010; Zegerman and Diffley, 2010).

An additional checkpoint-dependent mechanism for suppression of hsk1-89 may be through abrogation of replication fork stalling. In hsk1-89, “bubbles” start to appear at early-firing origins 80 min after release from M phase arrest and both bubble arc structures (representing the partially replicated segment surrounded by the unreplicated segments) and Y-fork structures accumulate at later time points, suggesting increased fork stalling in hsk1-89 (unpublished data). However, introduction of mrc1Δ, mrc1-3A, or cds1Δ mutations leads to the disappearance of these replication intermediates in hsk1-89 (unpublished data). Thus, reduction of checkpoint-dependent fork arrest may also contribute to the rescue of growth by mrc1Δ, mrc1-3A, or cds1Δ.

Checkpoint-independent function of Mrc1 is involved in the rescue of hsk1Δ

We observed that mrc1Δ bypassed the hsk1 mutations more efficiently than cds1Δ or mrc1-3A. This appears to be due to the checkpoint-independent role of Mrc1. Analyses of deletion derivatives of Mrc1 identified mutants that are capable of restoring the HU resistance but are not able to inhibit the rescue of hsk1-89 in mrc1Δ background. The results indicate that the segment 782–879, which is not required for HU resistance, is involved in the rescue of hsk1 mutation, supporting the role of checkpoint-independent function of Mrc1 in the rescue.

We recently reported that firing at some early-firing origins, detected by two-dimensional gel electrophoresis analyses of replication intermediates, is enhanced in mrc1Δ compared with the wild type (Hayano et al., 2011). This stimulation is observed in mrc1Δ but not in mrc1-3A or cds1Δ background. We show here that the firing of an early-firing origin is enhanced in hsk1-89 by mrc1Δ but not by mrc1-3A or cds1Δ (Fig. 5). These results indicate that a checkpoint-independent function of Mrc1 inhibits the initiation of DNA replication by some unknown mechanism.
Taken together, the operation of both checkpoint-dependent and -independent mechanisms would explain how the suppression of growth of hsk1Δ mutants in mrc1Δ is more efficient than in checkpoint mutants. Potential negative regulation of origin firing by Mrc1 may be conserved in higher eukaryotes because inter-origin distances decreased in the cells depleted for Claspin, a putative mammalian homologue of Mrc1 (Petermann et al., 2008; Scorah and McGowan, 2009), although this could simply be due to slowed fork rate in Claspin-depleted cells.

It is known that bob1Δ mutations (amino acid substitutions at the conserved leucine 83 of Mcm5) bypass requirement for the Cdc7 in budding yeast (Hardy et al., 1997). More recently, a deletion in the MCM4 N-terminal nonconserved segment was shown to bypass Cdc7 (Sheu and Stillman, 2010). Precocious Cdc45 loading was observed in a bob1Δ mutant, and Cdc45 loading was partially restored in mcm4ΔN cdc7Δ mutant (Hardy et al., 1997; Geraghty et al., 2000; Sheu and Stillman, 2010). Thus, various mutations could lead to chromatin loading of Cdc45 in a pathway alternative to the Cdc7-dependent pathway, enabling the bypass of Cdc7 function. This is the first report that mutations in a factor other than Mcm can bypass the Cdc7 function. The results in this manuscript and other reports reinforce the idea that the chromatin loading of Cdc45 is a critical point of regulation for initiation during the normal course of DNA replication as well as for checkpoint responses.

Temperature affects the viability of hsk1Δ cells

It was quite unexpected that hsk1Δ can grow at 37°C (Fig. 6). Furthermore, the growth of hsk1Δ mrc1Δ or hsk1Δ cds1Δ showed temperature dependency; better growth at 37°C and very little growth at 25°C. This suggests a possibility that DNA replication is stimulated at a higher temperature. Indeed, overall incorporation of nucleotide increases at 37°C compared with lower temperatures (Fig. 7 A). ChIP-chip analyses showed increased BrdU incorporation at 37°C at some dormant origins, which are normally not fired at 30°C in the presence of HU (Fig. S3 and Table S2). Among 61 dormant origins activated at 37°C, while 42 are also activated in either mrc1Δ or cds1Δ, 19 origins are not activated in either mrc1Δ or cds1Δ suggesting that another mechanism is involved in the origin activation at 37°C. These results suggest that the bypass of hsk1 at a high temperature is due to enhanced DNA synthesis.

How does high temperature facilitate DNA replication? First the checkpoint pathway may be shut off or partially impaired when the temperature is increased. Second, limiting factors for origin firing may be overproduced at a high temperature. Sld3 and Cdc45 are essential for firing, but may not be present in sufficient amount for activation of late origins. It was shown in budding yeast that overproduction of Cdc45, Sld3, and Sld7 leads to the firing of late origins in the presence of HU (Tanaka and Araki, personal communication). Third, high temperature may bypass a negative conformation of MCM, which was suggested to be awaked by Cdc7 kinase in budding yeast (Sheu and Stillman, 2010). Fourth, strand opening that may precede the synthesis that may be facilitated. In in vitro initiation at oriC, the chromosomal origin of DNA replication of the E. coli chromosome, a temperature above 38°C is required for the initial strand opening (Bramhill and Kornberg, 1988). Finally, chromatin structure may change with temperature. It has been reported that high temperature can generate relaxed chromatin, which may be favored for initiation (Weintraub et al., 1982; Pfaffle and Jackson, 1990). The first possibility is unlikely because the replication does depend on temperature even in hsk1Δ mrc1Δ or hsk1Δ cds1Δ lacking the checkpoint pathway. We did not see significant change in the level of Cdc45 or Sld3 at different temperatures, excluding the second possibility as well (unpublished data). Other possibilities are now being examined.

Plastic DNA replication program revealed by the differential requirement for Cdc7 kinase

Viability of hsk1Δ mrc1Δ as well as that of hsk1Δ at 37°C indicates that DNA replication can be initiated in the absence of Hsk1. The loading of Cdc45 at early-firing origins, although inefficient and delayed, was observed in hsk1Δ cells at 30°C (unpublished data). We speculate that Cdc45 could be loaded onto a fork by virtue of other kinases such as Cdc2 in the absence of Hsk1, albeit with reduced efficiency. We have found that, although Cdc7−/− blastocysts do not grow in vitro and Cdc7−/− embryos die between E3.5 and E6.5, Cdc7−/− p27−/− blastocysts grew to a significant extent and the embryos with the latter genotype could survive up to E7.5–9.5 (unpublished data), suggesting that increased Cdk activity could partially suppress deficiency of Cdc7 function in the growth of mammalian cells.

These results indicate that conditions that facilitate Cdc45 loading onto origins could bypass the requirement of Cdc7/Hsk1 for initiation. This may reflect the robustness and plasticity of the replication program that controls site selection and timing of initiation to a substantial degree, in a manner dependent on the differentiation and developmental stages or cell types (Hiratani et al., 2008; Hiratani and Gilbert, 2009; Hansen et al., 2010). Our findings present additional examples of plastic regulation of the eukaryotic DNA replication program and provide a novel system by which one can study the basis of robustness and plasticity of eukaryotic DNA replication program that enables cells to respond to varied internal and external signals to permit their survival. More extensive studies on the genetic and environmental conditions that might affect the requirement of Hsk1/Cdc7 for DNA replication may provide important clues as to how the replication program is regulated under a variety of cellular and metabolic conditions.

Materials and methods

Strains and general methods

All the strains used in this study are listed in Table S1. All epitope-tagged strains were made by integration of a 3Flag- or 13Myc-tagged gene fragment into the original chromosome loci. All the tags were at the C terminus. Cells were cultured in YES media containing 0.5% yeast extract, 2% glucose, and 0.1 mg/ml each of adenine, uracil, and leucine (Sigma-Aldrich). For FACS analyses, cells were fixed with 70% ethanol. After treatment with RNaseA at 37°C for 2 h, DNA was stained with 4 mg/ml propidium iodide.
Disruption of the hsk1+ or dfp1+ him1 gene
First, wild-type cells (YM71) were transformed with pREP41-hsk1c3 carrying the wild-type hsk1+ gene (Masa et al., 1995), and then the entire open reading frame (ORF) of the genomic hsk1+ gene of a resultant transformant was replaced with ura4+ gene generating a hsk1-l-disrupted strain (MS142). Second, the ura4+ marker of MS142 was replaced with Kan+ gene generating another hsk1-l strain (MS170). MS170 was then transformed with pREP2his16, and from the resultant ura- leu transformants, leu- cells were selected that had lost pREP41-hsk1c3. A resultant strain (MS143) could not grow on a 5-FOA plate at 30°C and was used for further experiments. A dfp1+/him1-l-disrupted strain (1019) in which the 0.44 kb HindIII fragment containing the essential Dbf4-motif-M located in the middle of the dfp1+/him1+ ORF was replaced with ura4+ gene was described previously (Fakuda et al., 1999).

Two-dimensional gel analysis
Genomic DNA was prepared as described previously (Arcangioli, 1998). In brief, 1.5 × 10^6 cells, treated with 1% vol/vol NaN3, were suspended in SP1 buffer at pH 5.6 (1 M sorbitol, 50 mM citrate phosphate, and 40 mM EDTA) containing 0.7 mg/ml lyticase. After incubation at 37°C for 1 h, the spheroplasts were suspended in 0.5% low-melting agarose and poured into CHEF plug molds (Bio-Rad Laboratories) to make agarose plugs. The plugs were treated with DB buffer (1% laurel sarcosine, 0.25 mg/ml proteinase K, and 25 mM EDTA) and an appropriate restriction enzyme (40 U of EcoT22I or BglII per plug) followed by incubation at 37°C for 1 h. The plugs were then melted by incubation at 70°C for 10 min with gentle mixing. The restriction enzyme (40 U/plug) was added again and the solid samples were incubated again at 37°C for 1 h. Subsequently, the solid samples were treated with 4U β-agarase I (NEB) and 40 µg/ml RNaseA (Sigma-Aldrich) at 37°C for 1 h. DNA was precipitated by addition of 0.7 vol of isopropanol and 0.1 vol of 3 M NaOAc. The DNA samples were analyzed on 0.32% agarose in the first dimension and on 0.9% agarose in the second dimension in TBE buffer at 4°C. The DNA was transferred to N+ membrane (GE Healthcare) and cross-linked with UV. The DNA probes were prepared using Megaprime DNA Labeling System (GE Healthcare). The specific signals were detected by imaging plates (BAS2000; FujiFilm). The primers used for the probe to detect initiation at ars2004 are as follows: sense, 5'-AAAGTGCGTGCACTGGCATTAG-3'; antisense, 5'-TGAGAGTAAGCTCAACGCGTACTAG-3' (Hayano et al., 2011).

Fluorescence microscopy
 Yeast cells incubated at 30°C were fixed in 70% ethanol at 4°C and stained in PBS containing 0.2 µM DAPI and 0.8 µg/ml Fluorescent Brightener 28 (Sigma-Aldrich), which visualize nuclear DNA and septum, respectively. Yeast cells with rad22YFP were harvested and suspended in PBS, and were analyzed without fixation. Samples were analyzed on a microscope (Axiohot; Carl Zeiss) with a Plan-NEOFLUAR 40x/0.75 objective lens (Carl Zeiss). Images were taken using a camera (ORCA-ER; Hamamatsu Photonics) and were exported as TIFFs. Images were contrast adjusted with Photoshop (Adobe). No manipulations other than contrast and brightness adjustments were conducted.

ChiP-microarray analyses (ChiP-chip)
 Chromatin-immunoprecipitation (ChiP) was performed as described previously (Katou et al., 2003; Yabuuchi et al., 2006) with some modifications. Collected cells (2.5 × 10^6 cells) were disrupted by Multi-beads Shocker (Yasu-kikai Co.) and DNA was sheared to 600 bp by sonication. Immuno-precipitation was performed using monoclonal anti-c-Myc antibody (Nacalai Tesque) or monoclonal anti-Flag M2 antibody (Sigma-Aldrich). After incubation of the lysate with antibody and protein G beads (Invitrogen) for 3 h, beads were washed three times with the lysis buffer (Yabuuchi et al., 2006). Immunoprecipitated DNA was purified by MiniElute Reaction Cleanup kit (QIAGEN). The chromatin-immunoprecipitated DNA samples were amplified by in vitro transcription as described previously (Liu et al., 2003), labeled with biotin, and hybridized to S. pombe Tiling 1.0R Array (Affymetrix). ChiP signals were validated using the criteria as described previously (Hayashi et al., 2007). In brief, we compared ChiP fraction with the total genomic fraction at M phase and judged the ChiP signal validity using the following three criteria. First, the reliability of strength of signal was judged by detection P-value of each locus (p ≤ 0.001). Second, reliability of the binding ratio was judged by change P-value (p ≤ 0.001). Third, clusters consisting of at least three contiguous loci that filled the above two criteria were selected because it is known that protein-DNA interaction at a single site will result in immunoprecipitation of DNA fragments that hybridized not only to the locus of the actual binding site, but also to its neighbors. The raw and processed microarray data are accessible through GEO Databases under accession numbers GSE31650 and GSE28182.

BrDU incorporation and Birp
 The cells carrying AUR1::1-Adh1-1-TK-Adh1::scENT1 (a plasmid expressing the mammalian TK gene) were grown in 100 ml of YES medium to 5.0 × 10^6 cells/ml, arrested at M phase by incubation for 5 h at 20°C, and then released for 60 min at 30°C in the presence of 200 µM BrDU and 25 mM HU. Cells (8.0 × 10^6 cells) were treated with 10 mM Tris-HCl and 100 mM EDTA containing 0.1% sodium azide, and total genomic DNA was purified with Genomic-tip 100/G and Genomic DNA Buffer set (QIAGEN). The genomic DNA was sheared by sonication and BrdU-incorporated DNA was immunoprecipitated as described previously (Katou et al., 2003). The amount of immunoprecipitated DNA was quantified with real-time PCR using SYBR Premix Ex Taq (Takara Bio Inc.) in the LightCycler 480 (Roche). The following primers were used for amplifying the DNA: ara2004, Fwd-5'TGGTGAATTCTGAGATCC-3' and Rev-5'TAATGAGATGCTGAGATCC-3'; Chr2, 5280kb, Fwd-5'TGATAAAGGTGGTACAG-3' and Rev-5'TTAAAGACCCGACTAGC-3'; Chr1, 3152kb, Fwd-5'TCAGGTTGTGAGCATT-3' and Rev-5'CGGTTGTTGTGTTGCTTTC-3'; Chr2, 3161kb, Fwd-5'GGATTGTGTTGTTGCTTTC-3' and Rev-5'ATTACCTCAAGTTGGC-3'; Rev-5'ATGAGTACAGTCAACGCGTACTAG-3' and Rev-5'SCATTCCACTAGCTAATT-3'.

Incorporation of [3H]-thymidine into yeast cells
 The wild-type or hsk1+ cells harboring a plasmid expressing TK and scENT1 pregrown in EMM at 37°C to 5 × 10^6 cells/ml were split into three parts, and [3H]-thymidine was added to each culture (5 µCi per ml culture) at time 0. The three cultures were kept incubated at 30, 35, and 37°C. At the times indicated, 100 µl of each culture was withdrawn, and added 1 ml of 1% trichloroacetic acid solution. After incubation on ice for 10 min, the solution was passed through glass-fiber filters (FC/C; GE Healthcare). The filters were washed three times with solution containing 1 N HCl and 20 mM pyrophosphate, followed by wash with 10% ethanol. The filters were dried and then exposed to X-ray film (Kodak). The radioactivity trapped on the filters was counted by scintillation counter.

Online supplemental material
Fig. S1 presents genetic evidence showing that loss of mrc1 rescue hsk1+ cells, and is related to Fig. 1. Fig. S2 presents data on chromosome integrity, DNA damages, and checkpoint responses in hsk1+ cells, and is related to Fig. 2. On the basis of the data presented, we discuss the roles of DNA damage checkpoint and DBS repair in suppression of hsk1+ mutation by mrc1+. Fig. S3 shows ChIP-chip analyses of replication initiation sites at 30 and 37°C and is related to Fig. 7. Table S1 shows fission yeast strains used in this study. Table S2 lists a list of preRC and BrdU-incorporation sites in the wild-type, mrc1Δ, and cds1Δ cells that were identified in Chip-chip assays and is related to Fig. 4. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201107025/DC1.

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