Replication checkpoint kinase Cds1 regulates Mus81 to preserve genome integrity during replication stress

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The replication checkpoint kinase Cds1 preserves genome integrity by stabilizing stalled replication forks. Cds1 targets substrates through its FHA domain. The Cds1 FHA domain interacts with Mus81, a subunit of the Mus81–Eme1 structure-specific endonuclease. We report here that Mus81 and Rhp51 are required for generating deletion mutations in fission yeast replication mutants that experience replication stress. A mutation in the Mus81 FHA-binding motif eliminates its Cds1-binding and Cds1-dependent phosphorylation. Furthermore, this mutation exacerbates the deletion mutator phenotype of a replication mutant, and induces a hyper-recombination phenotype in hydroxyurea-treated cells. In unperturbed cells, Mus81 associates with chromatin throughout S phase. In replication mutants grown at semipermissive temperature, Mus81 undergoes minor Cds1-dependent phosphorylation, remains chromatin-associated, generates deletion mutations, and maintains cell growth. Upon S-phase arrest by acute hydroxyurea treatment, Mus81 is not required for cell viability but is essential for recovery from replication fork collapse. Moreover, Mus81 undergoes extensive Cds1-dependent phosphorylation and dissociates from chromatin in hydroxyurea-arrested cells, thereby preventing it from cleaving stalled replication forks that could lead to fork breakage and chromosomal rearrangement. These results provide novel insights into how Cds1 regulates Mus81 accordingly when cells experience different replication stress to preserve genome integrity.

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DNA replication is a process fraught with danger for the integrity of genome (Boddy and Russell 2001; Kolodner et al. 2002; Osborn et al. 2002; Muzi-Falconi et al. 2003). DNA lesions, deoxyribonucleotide (dNTP) depletion, and defective replication proteins are among the many events that can impede replication fork progression. Stalled forks are thought to pose serious threats to genomic integrity because they are barriers to completion of DNA replication and they are unstable structures that can collapse, rearrange, or break (McGlynn and Lloyd 2002). These events can lead to chromosomal rearrangements and deleterious genomic deletions. These genome-destabilizing occurrences are associated with many human diseases, including cancer.

Preservation of genome integrity during replication fork arrest is the responsibility of the replication checkpoint (Elledge 1996; Boddy and Russell 2001; Lopes et al. 2001; Tercero and Diffley 2001; Carr 2002; Osborn et al. 2002; Sogo et al. 2002; Cobb et al. 2003). This genome maintenance system delays the onset of mitosis, thereby providing time to complete replication before cell division. The overall understanding of replication checkpoints is most advanced in studies of budding yeast Saccharomyces cerevisiae and fission yeast Schizosaccharomyces pombe (Lindsay et al. 1998; Boddy and Russell 2001; Lopes et al. 2001; Tercero and Diffley 2001; Osborn et al. 2002; Sogo et al. 2002; Kai and Wang 2003a; Longhese et al. 2003). An important function of the replication checkpoint is to control the stability of stalled forks (Murakami and Okayama 1995; Boddy et al. 1998, 2003; Lindsay et al. 1998; Boddy and Russell 2001; Lopes et al. 2001; Tercero and Diffley 2001; Sogo et al. 2002) and the association of the replisome with the replication fork (Cobb et al. 2003; Lucca et al. 2004; Cotta-Ramusino et al. 2005). A protein kinase called Rad53 in budding yeast and Cds1 in fission yeast is the critical transducer of the replication checkpoint. Mutants defective for the kinase are acutely sensitive to hydroxyurea [HU], a chemical that depletes cellular dNTP pools by inhibiting ribonucleotide reductase (Reichard 1988; Lopes et al. 2001). HU treatment of these mutants leads to formation of pathological DNA structures such as regressed forks and semi-replicated DNA regions, as well as broken forks.
mutations in DNA polymerases such as HU or UV, or by mutations of genes that encode DNA ligase confer a mutator phenotype in fission yeast at their respective semipermissive temperatures. The mutator phenotype is characterized by small sequence alterations (point mutations and single base frame shifts) and deletions of sequences flanked by short direct repeats [Liu et al. 1999; Gutierrez and Wang 2003; Kai and Wang 2003b]. Our previous studies have shown that checkpoint activation in response to stress in a pola mutant up-regulates the translesion DNA polymerase, Polk/DinB. Polk is recruited onto chromatin and physically associates with the checkpoint clamp (Rad9–Rad1–Hus1) in a checkpoint-clamp loader (Rad17–Rfc2-5)-dependent manner. The chromatin-bound Polk then performs mutagenic synthesis, resulting in point mutations and single-base frameshift mutations (Kai and Wang 2003a,b). This chain of events is thought to be a survival strategy to tolerate replication stress and for facilitating replication fork restart. Significantly, a mutation in cds1 enhances the deletion mutation rate in pola mutant cells, further supporting the idea that Cds1 is needed to prevent chromosomal rearrangements associated with fork stalling (Kai and Wang 2003b).

A stalled replication fork, which contains two homologous regions with junctions of double-strand and single-strand DNA, could be an ideal substrate for homologous recombination proteins. The elevated rate of deletion mutations in pola cds1 double-mutant cells (Kai and Wang 2003b) suggests that Cds1 may be required to restrain the activity of recombination enzymes at stalled forks. It is therefore noteworthy that Cds1 associates with Mus81 and Rad60, two proteins that are involved in recombination [Boddy et al. 2000, 2003]. Mus81 associates with Emel to form a DNA endonuclease complex that is required for tolerance of DNA damage that arrests or breaks replication forks [Boddy et al. 2000, 2001]. Mus81–Emel is also essential for crossovers in meiosis [Osman et al. 2003; Smith et al. 2003]. Recombinant Mus81–Emel cleaves synthetic DNA substrates that model a variety of structures, including 3′-flaps, replication forks, D-loops, and nicked Holliday junctions (HJs) [Boddy et al. 2001; Kaliraman et al. 2001; Constantinou et al. 2002, Doe et al. 2002; Abrahm et al. 2003; Bastin-Shanower et al. 2003; Ciccia et al. 2003; Gaillard et al. 2003; Osman et al. 2003; Whitby et al. 2003; Hollingsworth and Brill 2004]. Endogenous Mus81–Emel, but not the recombinant form of the enzyme, also cleaves intact HJs [Gaillard et al. 2003]. These observations are also consistent with the observation that Mus81–Emel activity prevents the accumulation of X-shaped DNA in a thermosensitive pola mutant [Gaillard et al. 2003]. However, the exact range of substrates cleaved by Mus81–Emel in vivo has yet to be established.

The ability of Mus81–Emel to cleave DNA structures that model active and stalled replication forks in vitro poses an important question: Does Mus81–Emel cleave replication forks in vivo? Stalled forks can degenerate into broken forks, which can initiate recombination events that can lead to chromosomal rearrangements or deletions. These considerations suggest that there is likely to be a mechanism that prevents Mus81–Emel from actively cleaving stalled or repressed forks, but allows it to cleave the recombination structures that arise during recapture of broken forks. The relevance of such issues has been highlighted by recent studies of Mus81-deficient mice. Homozygous Mus81−/− or heterozygous Mus81+/− mice were shown to be susceptible to spontaneous chromosomal damage and cancer predisposition, indicating that Mus81 is crucial for maintenance of genomic integrity and for tumor suppression [McPherson et al. 2004].

The physical association between Mus81 and Cds1 occurs through the forkhead-associated domain (FHA) of Cds1 [Boddy et al. 2000]. The FHA domain is a phosphopeptide-binding module that selectively associates with the T-X-X-D motif in vitro [Durocher et al. 1999, 2000; Durocher and Jackson 2002; Li et al. 2002]. In budding yeast, FHA-domain interactions mediate Rad53 binding to Rad9, a protein that controls Rad53 activation [Schwartz et al. 2002]. The FHA domains of the Rad53/Cds1 family have been postulated to mediate substrate selection.

A large number of studies have established that Cds1 and Rad53 are required for survival of replication stress, and some have demonstrated physical links between these proteins and others involved in genome maintenance, but there have been few mechanistic insights into exactly how Cds1 and Rad53 function to preserve genome integrity. In this study, we investigate how Cds1 regulates Mus81 when cells experience replication stress induced by a replication mutant or by HU arrest. The results of our study provide novel insights into how the replication checkpoint kinase Cds1 preserves genome integrity when cells experience replication stress.

Results

Deletion of genomic sequences induced by replication stress in replication mutants depends on Mus81–Emel and homologous recombination protein Rhp51

Thermosensitive mutations in several replication genes confer a mutator phenotype at their semipermissive temperature that can be measured in a forward mutation assay that detects inactivation of the ura4+ gene [Liu et al. 1999; Kai and Wang 2003b]. The mutations in ura4− are small sequence alterations (point mutations and single-base frameshifts) or deletions of sequence be-
tween short direct repeats (Liu et al. 1999, Kai and Wang 2003b). The hypomorphic allele cds1.T8A significantly enhances the deletion mutations in a DNA polymerase-α mutant, pola(swi7-H4), at its semipermissive temperature (Kai and Wang 2003b). Double-mutant mus81Δ pola-ts cells grow slowly at permissive temperature and accumulate X-shaped aberrant replication intermediates at the restrictive temperature (Boddy et al. 2000; Gaillard et al. 2003). These genetic interactions prompted us to evaluate whether Mus81 is required for deletion formation in pola(swi7-H4), when cultured at its semipermissive temperature of 30°C. Mutant pola(swi7-H4) exhibited a 35-fold higher mutation rate over the wild-type cells at 30°C. Mutation rates in pola(swi7-H4) were significantly reduced in mus81Δ and eme1Δ backgrounds, both exhibiting about a threefold lower mutation rate than that measured in the pola(swi7-H4) single mutant (Fig. 1A). Analysis of mutation types revealed that the lower mutation rate was mostly due to loss of deletion types (Fig. 1B).

To test whether the decrease of deletion mutation rate was caused by the loss of Mus81–Eme1 endonuclease activity, we examined the effect of the mus81.DD mutation. This allele has two mutations in the nuclease active site of Mus81 (Boddy et al. 2001). The mus81.DD mutation had an effect that was similar to that caused by mus81Δ and eme1Δ mutations (Fig. 1A,B). Thus, deletion mutations in replication mutants arise by a mechanism that is largely dependent on Mus81–Eme1.

To better understand how the deletions are generated, we asked whether the deletion mutations occur through a recombination pathway that depends on the rhp51+ gene. This gene encodes the fission yeast RAD51 homolog (Muris et al. 1998; Jang et al. 1996) required for DNA strand invasion during double-strand break (DSB) repair (Paques and Haber 1999). Deletion of rhp51+ in a pola(swi7-H4) background moderately decreased the overall mutation rate (Fig. 1A) but profoundly changed the mutation spectra (Fig. 1B). Deletion mutations were almost entirely eliminated by rhp51Δ, whereas the rates of point mutations and single-base framshifts were elevated (Fig. 1B). These findings indicated that deletions are generated by a mechanism that involves rhp51+-dependent strand invasion and subsequent processing of the recombination structures by Mus81–Eme1. The elevated rate of point mutations and single-base framshifts found in rhp51Δ cells suggests that cells prefer to use a mutagenic synthesis pathway to tolerate replication stress in the absence of Rhp51 (Kai and Wang 2003b).

The T-X-X-D motif of Mus81 is required for its association with Cds1 and Cds1-dependent phosphorylation

It was previously shown that Mus81 undergoes Cds1-dependent phosphorylation in response to HU-induced replication arrest. The phosphorylated form of Mus81 has reduced electrophoretic mobility (Boddy et al. 2000). The cds1.fha1 mutant, which has two point mutations in the FHA domain, abolishes the physical association between Cds1 and Mus81, and eliminates the HU-induced Mus81 phosphorylation. Moreover, Mus81 binds Cds1 kinase dead protein but not with Cds1.fha1 mutant protein (Boddy et al. 2000). These findings indicate that the binding of Mus81 with Cds1 is independent of Cds1 kinase activity, but requires an intact FHA domain. Thus, the FHA domain of Cds1 plays an important role in regulating Mus81 function. However, it is evident that some Mus81–Eme1 functions are not Cds1-dependent because mus81Δ cells display a range of phenotypes that are not observed in cds1Δ cells (Boddy et al. 2000,

Table 1

| Genotype | Mutation rate (× 10−6) | C.I. | Fold increase over wild type | Mutation rate relative to pola(swi7-H4) |
|----------|------------------------|------|-------------------------------|--------------------------------------|
| wild type | 5.70 (5.0-6.1)         | 1    | 1                            |                                      |
| pola(swi7-H4) | 198 (155-231)    | 35   | 1                            | 1.2                                  |
| pola(swi7-H4) | 613 (48.9-73.2) | 11   | 1                            | 13.2                                 |
| pola(swi7-H4) | 59.5 (47.7-79.5) | 10   | 1                            | 13.3                                 |
| mus81Δ    |                        |      | 1                            | 13.3                                 |
| pola(swi7-H4) | 56.4 (45.7-77.7) | 10   | 1                            | 13.3                                 |
| eme1Δ     | 166 (128-217)         | 30   | 1                            | 13.2                                 |

Figure 1. Mutations in pola(swi7-H4) cells with mus81, eme1, and rhp51 mutant backgrounds. A] Mutation rates. Mutation rates are the average of three or more experiments. C.I. is the 95% confidence interval [10−6 per cell division]. Fold increases or decreases in mutation rates relative to the pola(swi7-H4) single mutant are shown as ↑ or ↓, respectively. B] Types of mutations. The rates of each mutation type were calculated by multiplying the rate of 5-FOA survival cells with the percentage of alterations that are shown as deletion or point mutations. "Point mutation" represents both base substitutions and single-base framshift mutations. Bar graphs show the types of mutation rate relative to wild type.
For example, Mus81 is required for formation of viable spores, whereas Cds1 is not. These facts, combined with the evidence that impairment of Cds1 activity significantly increases the deletion mutation rate in a pola(swi7-H4) background (Kai and Wang 2003b), suggest that Cds1 might inhibit a Mus81-promoted event that enhances deletions in pola(swi7-H4) cells.

We tested this hypothesis by identifying a mutation in mus81+ that abolishes its interaction with the FHA domain of Cds1. The FHA domain is a phosphopeptide-binding module with a strong preference for a T-X-X-D motif in which the Thr residue is phosphorylated (Durrocher et al. 1999). Previous studies have shown that the N-terminal 175–314 region of Mus81 protein interacts with the FHA domain of Cds1 (Boddy et al. 2000). Further analyses showed that the FHA-binding site on Mus81 is located between residues 230 and 267 (Fig. 2A). The 230–267 region of Mus81 contains a T-X-X-D motif at position 239. We therefore constructed a strain that replaced the genomic locus of mus81+ with the myc-tagged mus81.T239A allele. We also constructed a strain with myc-tagged mus81+ at its genomic locus. The myc tag does not noticeably affect the activity of Mus81 (our unpublished data). The mus81.T239A mutation abolished the interaction of Mus81 with the FHA domain of Cds1 (Fig. 2B). The mus81.T239A mutation also eliminated the HU-induced hyperphosphorylation of Mus81 (Fig. 2C). These results support the idea that the Thr239 residue in Mus81 is phosphorylated by an as-yet-unidentified kinase and is a prerequisite for Cds1 binding. Subsequent Cds1-dependent phosphorylation of Mus81 results in the slower mobility form of Mus81 protein seen in gel electrophoresis.

In contrast to mus81Δ cells (Boddy et al. 2000), the myc-tagged mus81.T239A cells appeared identical to mus81+ cells in sensitivities to UV and methyl methanesulfonate (MMS), genetic interactions with inactivation of Rqh1 DNA helicase, and spore viability (M.N. Boddy and P. Russell, unpubl.). These observations suggest that Cds1-dependent phosphorylation of Mus81 is not required for Mus81–Eme1 nuclease activity and were consistent with the possibility that this phosphorylation might negatively regulate some cellular functions of Mus81.

The mus81.T239A mutation and cds1.fha1 mutation enhance the deletion mutation rate of a pola mutant

To explore whether Cds1 might negatively regulate Mus81–Eme1, we examined the deletion mutation rate in mus81.T239A pola(swi7-H4) double-mutant cells. Whereas the deletion mutation rate in pola(swi7-H4) cells was elevated 198-fold relative to wild type, the rate in mus81.T239A pola(swi7-H4) cells was elevated 650-fold (Fig. 3A,B). The elevated deletion rate in mus81.T239A pola(swi7-H4) cells was very similar to the 683-fold increase measured in the cds1.fha1 pola(swi7-H4) double mutant, and the 667-fold increase measured in the mus81.T239A cds1.fha1 pola(swi7-H4) triple mutant (Fig. 3A,B). Thus, the mus81.T239A and cds1.fha1 mutations each increased the deletion mutation rate approximately threefold in a pola(swi7-H4) background, suggesting that the mus81.T239A and cds1.fha1 mutations function through a same or overlapping pathway in generating deletion mutations in replication-stressed pola(swi7-H4). These results are consistent with the hypothesis that the interaction of Mus81 with the FHA domain of Cds1 is involved in preventing Mus81 from carrying out a DNA cleavage event that leads to deletion mutations in pola(swi7-H4) cells.

Figure 2. A mutation in Mus81 T-X-X-D motif abolishes the Mus81 protein association and phosphorylation by Cds1. [A] Mapping the Cds1-binding site on Mus81. Five overlapping fragments of mus81+ encoding Mus81 from residue 1 to 314 were constructed as a GST-fusion. The GST-fusion Mus81 proteins were expressed from bacteria, purified, and used as affinity matrix for binding of myc-tagged Cds1 from fission yeast cell extracts. [B] Mutation in mus81.T239A abolishes the binding of Mus81 with Cds1. A GST-fusion protein containing the 1–190 region of Cds1, which contains the FHA domain, was overexpressed in strains that expressed 13-myc-tagged forms of wild type or mus81.T239A from the mus81 genomic locus. GST-Cds1-fusion protein was purified with GST-Sepharose and probed for coprecipitating Mus81:13myc. [C] Mutation in mus81.T239A abolishes the HU-induced phosphorylation of Mus81. Strains that expressed 13-myc-tagged forms of wild type or mus81.T239A at endogenous levels were treated with 10 mM HU for 4 h. Mus81 protein was detected by immunoblotting with anti-myc antibody. Wild-type but not mutant Mus81 displayed reduced electrophoretic mobility, indicative of Cds1-dependent phosphorylation (Boddy et al. 2000).
Mus81 is removed from chromatin in a Cds1-dependent manner in response to HU-induced replication stalling

To further understand how Cds1 regulates Mus81–Eme1, we investigated whether Mus81–Eme1 nuclease activity was affected by HU treatment. Cds1 activation by HU treatment had no obvious effect on the in vitro DNA endonuclease activity of immunoprecipitated Mus81–Eme1 complex when assayed with synthetic nicked HJ substrate (P.-H. Gaillard and P. Russell, unpubl.), suggesting that Cds1 does not directly regulate Mus81–Eme1 nuclease activity. We then investigated whether Cds1 might regulate the ability of Mus81 to associate with chromatin. Chromatin association was monitored by preparing an insoluble protein fraction consisting of chromatin and nuclear matrix and then releasing chromatin-bound proteins by DNase I treatment (Kai and Wang 2003b). This analysis revealed that a substantial fraction of Mus81 associated with chromatin in untreated wild-type cells (Fig. 4A). HU treatment led to Mus81 phosphorylation (detected by retarded gel electrophoresis mobility) and a complete dissociation of Mus81 from chromatin (Fig. 4A). In \(\text{pol}(\text{swi7-44})\) cells incubated at 30°C, a mildly phosphorylated form of Mus81 was detected and a substantial amount of Mus81 remained associated with chromatin (Fig. 4A). Incubation of these cells at 34°C led to increased phosphorylation of Mus81 and higher levels of Mus81 dissociating from chromatin (Fig. 4A). These findings indicate that Cds1-dependent phosphorylation of Mus81 regulates the association of Mus81 with chromatin.

To make certain that Cds1 directly regulates the ability of Mus81 to associate with chromatin, we examined chromatin association of Mus81 in wild-type, \(\text{mus81.T239A}\), and \(\text{cds1.fha1}\) cells treated with HU. Consistent with the data shown above, HU treatment of wild-type cells led to Mus81 phosphorylation and its dissociation from chromatin. In both \(\text{mus81.T239A}\) and \(\text{cds1.fha1}\) cells, electrophoretic mobility of Mus81 was unaltered by HU treatment, and a substantial fraction of Mus81 remained associated with chromatin (Fig. 4B). These findings confirmed that the Cds1-dependent phosphorylation of Mus81 negatively regulates Mus81’s association with chromatin.

Mus81 function is not required to survive HU-induced replication arrest but is essential for surviving replication fork collapse

Previous studies have shown that cells with a deletion of \(\text{mus81}^+\) lose viability after chronic HU or camptothecin (CPT) treatment (Boddy et al. 2000; Doe et al. 2002; Doe and Whitby 2004). HU stalls replication forks by depleting dNTPs through inhibition of ribonucleotide reductase (Reichard 1988; Lopes et al. 2001). CPT inhibits the religation step of the topoisomerase I reaction (Porter and Champoux 1989), which leads to accumulation of single-stranded nicks that often result in replication fork collapse. Finding that HU-induced replication arrest promotes the dissociation of Mus81 from chromatin (Fig. 4A,B) raises the question, why are \(\text{mus81}\Delta\) cells sensitive to HU treatment. Studies of the budding yeast response to replication stress induced by acute versus chronic HU treatment have suggested that prolonged HU exposure induces the accumulation of chromosomal...
lesions as evidenced by the recruitment of Mre11 and Rad52 for processing and recombination to restart replication. This results in cells entering mitosis with incompletely replicated chromosomes (Lisby et al. 2004). We hypothesize that chronic exposure of \textit{mus81}/H9004 cells to HU also causes accumulation of DNA lesions in chromosomes, resulting in viability loss of the \textit{mus81}/H9004 cells.

To test this hypothesis, wild-type and \textit{mus81}/H9004 cells were incubated for 8 h in liquid medium supplemented with either 12 mM HU or 30 µM CPT (Fig. 5A). Nearly 70% of the \textit{mus81}/H9004 cells were viable after acute HU exposure for 8 h compared to the wild type, whereas \textit{mus81}/H9004 cells progressively lost viability after 2 h in CPT medium with no detectable viable cells after 8 h. These results indicate that \textit{mus81}Δ cells are not significantly sensitive to acute HU treatment that induces stalled replication forks, but severely sensitive to CPT-induced fork collapse.

We then examined the Cds1 kinase activity in cells exposed to CPT and HU [Fig. 5B] and the Cds1-dependent phosphorylation of Mus81 protein in these cells (Fig. 5C). Cds1 kinase was highly activated in HU-treated cells and marginally activated in CPT-treated cells (Fig. 5A). Consistent with the difference in Cds1 kinase activation by HU and CPT, Mus81 was phosphorylated and detected as a slower-mobility phosphorylated protein in gel electrophoresis in HU-treated cells, whereas Mus81 was only slightly phosphorylated in CPT-treated cells [Fig. 5C]. In contrast to the HU-treated cells in which the phosphorylated Mus81 protein was dissociated from chromatin, the Mus81 protein in CPT-treated cells remained chromatin-associated [Fig. 4C]. These results suggest that replication stalling induced by HU treatment robustly activates Cds1 kinase activity, which, in turn, leads to the phosphorylation of Mus81 and its dissociation from chromatin.

Together, our data support the idea that survival of HU-induced stalled replication forks does not require Mus81. This idea is consistent with a requirement to remove Mus81 from chromatin, thus preventing the counterproductive cleavage of intact stalled replication forks. The acute sensitivity of \textit{mus81}Δ cells to CPT and the chromatin association of Mus81 in CPT-treated cells suggest that Mus81 is required for cells to survive replication fork collapse. This is consistent with the previously proposed role of Mus81 in damage tolerance (Boddy et al. 2000, 2003; Doe et al. 2002; Abraham et al. 2003). The \textit{mus81}T239A mutant displays a hyper-recombination phenotype when replication is arrested by HU

Replication stalling induced by HU treatment activates Cds1 kinase [Boddy et al. 1998; Lindsay et al. 1998; Boddy and Russell 2001]. Activation of Cds1 in fission
yeast and Rad53 in budding yeast is thought to control the stability of stalled replication forks and replisome-fork association in order to prevent fork collapse (Lindsay et al. 1998; Boddy and Russell 2001; Lopes et al. 2001; Sogo et al. 2002). The finding that mus81Δ cells are not significantly sensitive to acute HU treatment (Fig. 5A) and Mus81 protein is dissociated from chromatin upon HU arrest [Fig. 4A] prompted us to analyze the mutator phenotype of acute HU-stressed wild-type cells. Acute HU-treated cells exhibited a mutation frequency comparable to cells without HU treatment (data not shown). It is important to point out here that the mutator phenotype in HU-arrested wild-type cells is entirely different from that seen in replication-stressed pola2(swit7-H4) cells, which induces a 35-fold higher mutation rate and 200-fold higher deletion type of mutations over wild-type cells [Fig. 1].

To further investigate how Cds1 regulates Mus81 in response to HU-induced replication stress, we explored the effect of HU arrest in the mus81.T239A, which contains a mutant Mus81 protein that cannot be regulated by Cds1 [Fig. 2B,C] and remains chromatin-associated after HU treatment [Fig. 4B]. We analyzed the mutation rate of the mus81.T239A mutant on solid media plates containing 2 and 5 mM HU, as well as in liquid culture containing 12 mM HU by the mutator assay. As described above, HU exposure did not affect the mutation rates of wild-type cells. Surprisingly, HU treatment also did not increase the mutation rate of mus81.T239A or cds1.fha1 compared to the cells without HU treatment when measured by the forward mutation analysis [data not shown].

It is possible that the genomic changes caused by HU treatment are undetectable by the mutator assay that we used. For example, recombination events may not be detected by the forward mutation analysis. We thus investigated whether cells with the mus81.T239A mutation would make use of a recombination repair process when replication is stalled by HU treatment. We constructed mus81.T239A into a strain containing a direct repeat of ade6+ heteroalleles flanking a functional ura4+ to measure the recombination frequency [Fig. 6B; Osman et al. 2000; Doe and Whitby 2004; Doe et al. 2004]. The intrachromosomal direct repeats in these strains can recombine with each other and generate a recombinant by multiple pathways (Pagues and Haber 1999; Symington 2002). The deletion types of recombinants in the mouse.T239A strain will exhibit a genotype of ade6+ ura4+, while the conversion types of recombination will exhibit a genotype of ade6+ ura4+. The mus81.T239A mutation did not affect the spontaneous recombination frequency in replication-unperturbed cells when measured by this assay. The recombination frequency in mus81.T239A, however, was enhanced 3.7-fold after acute exposure with HU, whereas the frequency in wild-type cells was enhanced only 1.6-fold [Fig. 6A]. Further analysis showed that the conversion events were increased more than fivefold by HU treatment in mus81.T239A cells as compared to a 1.6-fold increase in wild-type cells treated with HU [Fig. 6C]. In contrast, HU treatment had little effect on the frequency of the deletion-type recombinants in both wild-type and mus81.T239A cells [Fig. 6C]. These results suggest that phosphorylation of Mus81 at Thr239 in HU-treated cells suppresses recombination repair pathways that result in gene conversions.

Mus81 in unperturbed cells

The observation that Mus81 associates with chromatin in wild-type cells without replication stress [see mock-treated or no-HU-treated lanes in Fig. 4A,B], coupled with previous evidence that mus81Δ cells display a checkpoint-dependent cell cycle delay in the absence of exposure to genotoxic agents [Boddy et al. 2000], prompted us to investigate the requirement of Mus81 function in unperturbed cells. We first examined whether Mus81 is chromatin-associated throughout S
phase. Cells were synchronized by cdc10 arrest and release. Samples were removed every 20 min and analyzed for Mus81 chromatin association. A fraction of Mus81 was found chromatin-associated as cells entered and passed through S phase into G2 (Fig. 7A), suggesting a requirement for Mus81 function during normal S-phase progression. We then analyzed the mutation rates in replication-unperturbed cells that had one of following mutations: mus81.T239A, eme1Δ, mus81.DD, mus81.T239A, cds1.fha1, or rhp51Δ. With no replication perturbation, cds1.fha1 mutants exhibited a mutation rate and mutation spectra that were similar to wild type. Cells without Mus81 nuclease activity (mus81Δ, eme1Δ, or mus81.DD) exhibited a mutation rate that was four- to fivefold higher than wild type (Fig. 7B). Further analysis of mutation types in the unperturbed mus81Δ cells indicated that 74% of the mutations were deletions of short genomic sequences (<200 bp, some not flanked by short direct repeats). The remainder had small sequence alterations such as base substitutions or single-nucleotide frameshifts (Fig. 7C; data not shown). A very similar pattern of mutation types was found in unperturbed eme1Δ or mus81.DD mutants. It is notable that the mutation spectra seen in mus81Δ, eme1Δ, and mus81.DD cells without replication stress were different from that observed in the pola(sw17-H4) replication-stressed cells, which exhibited many 50-bp to 1-kb deletions flanked by short direct repeats (Liu et al. 1999; Kai and Wang 2003b). In such a background, Mus81 remains chromatin-associated and generates deletion mutations to tolerate the stress and to promote replication restart for survival (Fig. 8). In cells experiencing replication stalling caused by HU-mediated depletion of dNTPs, Cds1 kinase is robustly activated. In this case, Mus81–Eme1 undergoes Cds1-dependent phosphorylation and dissociates from chromatin (Fig. 8), thus preventing Mus81–Eme1 from cleaving stalled or repressed forks, which could lead to deleterious genomic rearrangements. Furthermore, the Cds1-regulated phosphorylation of Mus81 also prevents a recombination repair process that causes gene conversions in HU-stressed cells. These are several of the mechanisms by which Cds1 preserves replication fork stability when replication is stressed.

**How and why does Cds1 regulate Mus81 during replication stress?**

Cds1 regulates Mus81 through a physical interaction of the FHA-binding motif, T-X-X-D, of Mus81. FHA-depen-
dent binding of Cds1 and Rad53 to the T-X-X-D motifs is thought to play a critical role in maintaining genome integrity. Furthermore, phosphorylation of the Thr residue in the target’s T-X-X-D motif is a prerequisite for Cds1 binding [Durocher et al. 1999, 2000]. It is not yet known which kinase phosphorylates the Thr239 of Mus81. A previous study showed that an intact FHA domain was required for its interaction with Cds1, but interpretation of this result was complicated by the fact that the cds1.fha1 mutation abolished Cds1 activation [Boddy et al. 2000]. Although a kinase inactive form of Cds1 and the FHA module alone are able to associate with Mus81 in vivo, it was still not clear whether the FHA domain mediated a physiologically significant interaction with Mus81 [Boddy et al. 2000]. The studies described herein show that cells with a mutation of Thr239 in the T-X-X-D motif of Mus81 exhibit a mutator phenotype equivalent to cds1.fha1 (Fig. 3). A robustly activated Cds1 was unable to displace the mutant Mus81 protein from chromatin (Fig. 4B). Thus, the Mus81’s FHA-domain-binding motif, T-X-X-D, indeed has a physiological role for genome maintenance during S phase.

Mus81 is a structure-specific endonuclease [Hollingsworth and Brill 2004]. Structure-specific endonucleases are potentially a double-edged sword: In some situations they are required for maintenance of genomic integrity, but in other circumstances they can create DNA breaks that threaten genomic stability. Replication stalling caused by either exogenous agents or mutations in DNA replication genes could induce regressed forks and create aberrant replication structures. Cleavage of these aberrant replication structures by a structure-specific endonuclease like Mus81–Eme1 could potentially result in DSBs that must be repaired by a fork recapture mechanism of homologous recombination [Seigneur et al. 1998; McGlynn and Lloyd 2002; Krogh and Symington 2004; Symington and Holloman 2004]. These events are potentially error-prone. A safer alternative may be to restore the fork without breaking it.

We show here that Mus81 is not required in cells when replication is arrested by acute HU exposure (Fig. 5A) and is dissociated from chromatin (Fig. 8). In contrast, upon fork collapse induced by CPT treatment, Cds1 is not fully activated; Mus81 remains chromatin-associated (Fig. 4C) and is essential for cell survival (Fig. 5A). These results suggest that Mus81 is required for restart of collapsed replication forks. Cds1 is activated in replication-stressed pola(swi7-H4) cells [Kai and Wang 2003b]; however, cells are continuously growing. In order to maintain active growth of the replication mutant, cells need to tolerate the stress by allowing Mus81 to remain chromatin-associated, thereby generating deletion mutations through a homologous recombination mechanism that requires Rhp51 [Fig. 1]. This hypothesis is consistent with the findings that both Rhp51 and Mus81 are required for tolerance of fork arrest caused by unrepaired UV lesions and for survival of fork collapse caused by CPT [Fig. 5; Boddy et al. 2000; Doe et al. 2002].

An important concept that emerges from our studies is

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### Figure 7.

Mus81 has a role in maintaining genome integrity during the normal cell cycle. (A) Cells were synchronized by cdc10-m17 arrest in G1 at 36.5°C for 4.5 h and released to 25°C. Cell samples were collected at G1 arrest and every 20 min after release from G1 arrest. Cell cycle progression is shown as FACS profile (left panel) and chromatin association of Mus81 protein was shown (right panel). (B) The mutation rate of cds1.fha1, mus81Δ, mus81.DD, mus81.T239A, eme1Δ, and rhp51Δ cells without replication stress. Mutations were calculated as described in Materials and Methods and Figure 1. (C) Types of mutations in unreperturbed mus81Δ and rhp51Δ cells over wild-type cells.
that the activity of a structure-specific endonuclease such as Mus81–Eme1 may not be beneficial for the maintenance of genome integrity in all situations. That is, without strict regulation, Mus81–Eme1 may cleave normal forks that have stalled, which would otherwise be competent to resume replication. Replication fork cleavage may lead to potentially deleterious recombination events. In other situations, cleavage of stalled forks with the inherent risk of genomic deletions may be the best or only option for cell survival.

Generating deletion mutations in replication-stressed pola(swi7-H4) is a tolerance strategy

We have used a mutator assay to quantitatively gauge how cells respond to replication stress. Mutator analysis measures the rate and types of mutations accumulating in cells that survive replication stress. We have shown that fission yeast mutants pola(ts11), pola(ts13), and pola(swi7-H4), as well as budding yeast pol1-1 mutants, all induce a mutator phenotype characterized by high levels of deletion mutations (Liu et al. 1999; Gutierrez and Wang 2003; Kai and Wang 2003b). In fission yeast, the absence of Cds1 exacerbates the deletion mutator phenotype. Mutant pola(swi7-H4) has an intact replication checkpoint that delays the cell cycle transition. Cds1 is moderately activated and autophosphorylated in pola(swi7-H4) at 30°C (Kai and Wang 2003b). If an activated Cds1 exerts negative regulation of Mus81–Eme1 to prevent it from cleaving stalled and/or regressed replication forks in HU-stressed cells, why does pola(swi7-H4) at 30°C with an activated Cds1 kinase exhibit a deletion mutator phenotype? Cds1 was originally identified as a multicopy suppressor of pola(swi7-H4) at 36°C (Murakami and Okayama 1995). Overexpression of cds1+ in pola(swi7-H4) suppresses the pola(swi7-H4) mutation rate 19-fold (Kai and Wang 2003b). In addition, Cds1 kinase activity in the thermosensitive pola(ts13) mutant is 25-fold lower than the Cds1 kinase activity in HU-induced wild-type cells at 25°C (Bhaumik and Wang 1998). A recent study of budding yeast showed that the association of Polα with the replication fork is reduced in a rad53 mutant (Cotta-Ramusino et al. 2005). Together, these findings suggest that Cds1 and Rad53 monitor and control DNA synthesis by Polα. Although Cds1 is activated in pola(swi7-H4) cells, the Cds1 kinase is most likely somewhat compromised in its function in pola(swi7-H4) [Kai and Wang 2003b]. This possibility is consistent with the finding that Mus81 in pola(swi7-H4) cells was nominally phosphorylated and remained chromatin-associated at 30°C [Fig. 4A]. The chromatin-associated Mus81 could cleave stalled or regressed replication forks, resulting in deletion of genomic sequences in pola(swi7-H4) cells at 30°C [Fig. 8].

The pola(swi7-H4) mutant is viable at 30°C. Mus81 is weakly phosphorylated and it remains associated with chromatin. In this situation, Mus81 is available to participate in DNA cleavage events that are required to survive replication stress but run the risk of generating genomic deletions. At 34°C, when pola(swi7-H4) cells experience more severe replication stress and Cds1 is activated to a higher level, Mus81 is phosphorylated to a higher extent, thus resulting in more Mus81 dissociating from chromatin [Fig. 4A]. The low viability of pola(swi7-H4) cells at 34°C prevents us from performing the mutator analysis with this mutant.

Replication stress induced by pola(swi7-H4) at 30°C could also cause lagging-strand discontinuity and lead to spontaneous fork collapse. Fork collapse induced by CPT does not activate Cds1 [Fig. 5B], and Mus81 is not substantially phosphorylated [Fig. 5C] and remains chromatin-associated [Fig. 4C]. Fork collapse is an event that cannot be regulated by Cds1. The deletion mutations
seen in pola mutants at the semipermissive temperature could also be derived from fork collapse.

The results of this study suggest that the deletion mutator phenotype exhibited in replication mutants is a tolerance strategy of replication mutants to survive replication stress and maintain continuous growth.

**Cds1 regulation of Mus81 is a survival strategy for HU-arrested cells**

We show here that replication stress caused by HU treatment does not induce a mutator phenotype in cells. This result is strikingly different from that seen in the replication-stressed pola(swi7-H4) cells. Both HU treatment and pola mutants induce an early S-phase stress. Why do cells respond to these two types of early S-phase stress differentially? The difference in the mutator phenotype seen in these two types of replication-stressed cells suggests that aberrant DNA structures caused by a pola mutant may not be the same as those in HU-treated cells. In HU-arrested budding yeast rad53 mutants, cells exhibit aberrant DNA structures at replication forks (Lopes et al. 2001) and accumulate extensive single-stranded gaps and hemi-replicated intermediates (Sogo et al. 2002; Cottar-Ramusino et al. 2005). Cds1 is required to prevent replication fork collapse in fission yeast cells treated with HU [Noguchi et al. 2003]. These findings clearly demonstrate that Rad53 and Cds1 have a critical role in stabilizing replication forks and preventing fork collapse when replication is stressed by HU treatment. HU treatment of fission yeast cells robustly activates Cds1 [Fig. 5B; Boddy et al. 1998; Lindsay et al. 1998; Boddy and Russell 2001]. We show here that the robustly activated Cds1 in HU-stressed wild-type cells induces extensive Cds1-dependent phosphorylation of Mus81, causing Mus81 to dissociate from chromatin [Fig. 8], thus preventing deleterious cleavage of DNA by Mus81–Eme1. This observation is consistent with the finding that mus81Δ cells are not sensitive to acute HU treatment [Fig. 5A]. Thus, regulation of Mus81 by Cds1-dependent phosphorylation is a survival strategy of HU-treated cells.

We show here that acute HU exposure of wild-type cells does not exhibit any enhancement of either the conversion type or deletion type of recombination events [Fig. 6]. In contrast, mus81.T239A cells that are unable to dissociate Mus81–Eme1 from chromatin during HU treatment [Fig. 4B] exhibit an enhanced frequency of conversion types—but not deletion types—of recombination events [Fig. 6B]. These results suggest that in mus81.T239A cells, DNA products cleaved by the chromatin-associated mutant Mus81–Eme1 nuclease that is unrestrained by Cds1 are processed through double-strand or single-strand break repair pathways that may involve strand exchange and HJ intermediates, and also via conservative one-sided invasion [Osman et al. 2000; Doe and Whitby 2004; Doe et al. 2004], resulting in a gene conversion phenotype.

**Does Mus81–Eme1 have a physiological role in unperturbed cells?**

Replication forks may often encounter abnormal DNA structures or unreppaired DNA damage that can lead to the formation of single-stranded DNA gaps. These gaps can be processed by recombination or gap-filling DNA synthesis. Genetic evidence from budding yeast studies has indicated that Mus81–Mms4 processes potentially toxic recombination intermediates derived from single-stranded gaps [Fabre et al. 2002]. It has been hypothesized that Mus81–Eme1 in fission yeast may process stalled and collapsed replication forks [Boddy et al. 2000; Doe et al. 2002]. That Mus81 associates with chromatin throughout S phase [Fig. 7A] supports these ideas.

Cells harboring mus81Δ, eme1Δ, and mus81-DD without replication stress all exhibited a four- to fivefold higher mutation rate over the wild-type cells [Fig. 7B]. Furthermore, these cells induce a mutation spectrum that is quite different from that exhibited in the replication-stressed pola(swi7-H4) cells. These findings suggest that in addition to Mus81–Eme1, other factors are involved in maintaining genomic integrity by generating these types of deletion mutations to tolerate occasional genomic obstacles [Boddy et al. 2000]. A recent study of mammalian Mus81 has shown that Mus81-deficient mice and cells are hypersensitive to a DNA cross-linking agent. Mus81-deficient mice exhibit susceptibility to spontaneous chromosomal damage and predisposition to lymphoma and other types of cancer [McPherson et al. 2004]. The results shown in this study suggest a role of Mus81–Eme1 in processing DNA structures that often arise during unperturbed DNA replication, and this role may be generally conserved among eukaryotes.

**Conclusion**

Cds1 is thought to play a critical role in controlling replication fork stability and replisome–replication fork association when cells experience replication arrest by HU. HU treatment of cells has often been used and is generally accepted as an approach to induce replication block for studying cellular responses to S-phase perturbation. Results of our studies suggest that Cds1 regulates Mus81 appropriately according to type or extent of replication stress. When S phase is blocked by HU treatment, the robustly activated Cds1 kinase negatively regulates Mus81, causing it to dissociate from chromatin. This prevents Mus81–Eme1 nuclease from cleaving otherwise normal replication forks that can resume replication when dNTP levels are restored. This negative regulation of Mus81 by Cds1-dependent phosphorylation in HU-arrested cells can also prevent the recombination repair process that causes gene conversion. In cells with mutations in replication genes, such as in pola mutants at its semipermissive growth condition, cells need to maintain continuous growth. Thus, Mus81 undergoes a limited Cds1-dependent phosphorylation and remains chromatin-associated to generate some deletions of genomic sequence flanked by short direct repeats to toler-
ate the replication stress in order to maintain cell growth. In Figure 8, we present a model that accounts for our data on how Cds1 regulates Mus81 in cells experiencing different types of replication stress to maintain genome integrity and cell survival.

Materials and methods

General methods

All yeast media, YEp5S, minimal medium (MM), and 5-fluoroorotic acid (5-FOA) medium were prepared as described in Moreno et al. [1991]. Except where stated, all yeast strains were propagated at 25°C. All genetic operations were performed as described in Gutz et al. [1974]. Epitope tagging of mus81+, mus81.T239A, or cds1+ genes with either the myc-tag or the HA-tag at the chromosomal loci were performed as described (Bahl er et al. 1998). The epitope-tagged mus81+ does not affect the mutator phenotype of the cells, cell growth, or cells’ damage sensitivity.

Mutator analysis

Strains for the mutator analysis were first grown on minimal medium without uracil to ensure each strain having ura4+ at its genomic locus. The ura4− cells were then plated on minimal medium with complete supplements and incubated at the semi-permissive temperature of 30°C. Eleven or 15 individual colonies were picked, suspended in sterile water, and plated on minimal medium agar containing 5-FOA at 1 mg/mL. Colonies resistant to 5-FOA were scored after incubating at 25°C for 3–4 d. Mutation rates on ura4− were calculated by fluctuation analysis using the method of the median as described in Lea and Coulson [1949]. Experiments of mutation rate analysis were performed as previously described [Kai and Wang 2003b], and all comparisons had a “significant” p level of p < 0.005. For mutation spectra analysis, genomic DNA was isolated from 5-FOA-resistant colonies, and the mutant ura4+ gene was PCR-amplified and analyzed by 1.5% agarose gel electrophoresis as described in Kai and Wang [2003b]. The ura4− PCR products were then gel-purified and sequenced to determine the mutation types generated. At least 100 5-FOA− colonies from each strain were analyzed for the size of ura4+ PCR product by agarose gel electrophoresis, and 20 ura4− PCR products were sequenced.

Recombination assay

Mitotic recombination frequencies were determined using strains containing an intrachromosomal recombination sub-state consisting of a nontandem direct repeat of ade6+ heteroalleles flanking a functional ura4+ gene [Fig. 6B]. Frequencies of spontaneous recombinants were determined by fluctuation analysis as described by Osman et al. [2000]. For each strain, seven independent colonies were used for each assay. Each assay was repeated at least three times. To determine HU-induced recombination frequencies, single colonies were picked and cultured in YES media. Cells were cultured in YES media with or without 12 mM HU for 5 h at 30°C and plated on media selective for ade6+. The ade6− recombinants were replicated onto minimal media lacking both adenine and uracil.

Median recombination frequencies of each strain were determined from three independent assays. The average recombination frequencies and percentage of conversion-types were determined from these medians, according to Lea and Coulson [1949]. All comparisons had a “significant” p level of p < 0.005.

Construction of mus81 mutant strains

Mutant strains mus81.DD and mus81∆ used in this study have been described [Boddy et al. 2000; Boddy and Russell 2001]. Mutant strain mus81.T239A was constructed by PCR copying primers that contain the mutation of Thr359 to Ala [Mus81 FHA Forward, CTGATGGTGTGAGCGCCGATATAGTTGAT CAGG; Mus81 FHA Reverse, CCTGTACACATCTTATGC GGCTTACACGTCATCA]. Two primers [Forward, GCAT TGGCAGTGAAGGAGATTTGC; Reverse, GACCTTGGCG GAAAGTGCTGGCCC] outside the Thr359 region were used to amplify the genomic Mus81:myc:kanMX locus [Boddy et al. 2000]. The resulting construct was used to replace the genomic copy of mus81+ by kanMX selection. The rhp5−A strain was a generous gift from Masaru Ueno of Shizuoka University, Japan.

Chromatin fractionation assay

The chromatin fractionation assays were performed as described in Kai et al. [2001] and Kai and Wang [2003b].

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Replication checkpoint kinase Cds1 regulates Mus81 to preserve genome integrity during replication stress

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