Schizosaccharomyces pombe Cds1^{Chk2} regulates homologous recombination at stalled replication forks through the phosphorylation of recombination protein Rad60

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Summary

The *Schizosaccharomyces pombe rad60* gene is essential for cell growth and is involved in repairing DNA double-strand breaks. Rad60 physically interacts with, and is functionally related to, the structural maintenance of chromosomes 5 and 6 protein complex (Smc5/6). Rad60 is phosphorylated in response to hydroxyurea (HU)-induced DNA replication arrest in a Cds1^{Chk2}-dependent manner. Rad60 localizes in nucleus in unchallenged cells, but becomes diffused throughout the cell in response to HU. To understand the role of Rad60 phosphorylation, we mutated the putative phosphorylation target motifs of Cds1^{Chk2} and have identified two Cds1^{Chk2} target residues responsible for Rad60 dispersal in response to HU. We show that the phosphorylation-defective *rad60* mutation partially suppresses HU sensitivity and the elevated recombination frequency of *smc6-X*. Our data suggest that Rad60 phosphorylation is required to regulate homologous recombination at stalled replication forks, probably by regulating Smc5/6.

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Key words: Checkpoint, DNA repair, SMC5/6

Introduction

Eukaryotic cells need to precisely duplicate their genomes during S phase in every cell cycle. However, exogenous and endogenous sources of DNA damage can block the progression of DNA replication forks, potentially resulting in a range of replication-associated DNA structures including single-strand lesions and double-strand breaks (DSBs) (Lambert and Carr, 2005; Lambert et al., 2007). To cope with replication fork blocks, eukaryotic cells employ a DNA-structure-dependent checkpoint pathway (Carr, 2002; Elledge, 1996). In the fission yeast *Schizosaccharomyces pombe*, Rad3 (a homolog of human ATR) plays a major role in the response to replication fork stalling. Hydroxyurea (HU), which causes deoxyribonucleoside triphosphate starvation by inactivating ribonucleotide reductase, is most widely used to investigate the cellular response to replication fork stalling. In the presence of HU, Rad3^{ATR} is activated and phosphorylates multiple target proteins. This results in activation of checkpoint kinase Cds1, the *S. pombe* homolog of Chk2. Once activated, Cds1^{Chk2} phosphorylates further downstream targets to regulate cell-cycle progression and DNA repair mechanisms (Furuya and Carr, 2003; Kai and Wang, 2003).

S. pombe rad60 was originally identified by screening for genes that are required for homologous recombination (Morishita et al., 2002). The *rad60*$^{+}$ gene is essential for growth and encodes a protein that belongs to the RENi (Rad60, Esc2p, and Nip45) family (Novatchkova et al., 2005). The Rad60 protein is involved in DNA repair through the homologous recombination pathway. Genetic and biochemical analysis demonstrates that it functions in concert with the Smc5/6 complex (Miyabe et al., 2006; Morishita et al., 2002). Smc5/6 is one of the three structural maintenance of chromosome (SMC) complexes. Cohesin, composed of Smc1 and Smc3, maintains the link between two sister chromatids until cells undergo mitosis, whereas condensin, composed of Smc2 and Smc4, is required for condensation of chromosomal DNA during mitosis (Hirano, 2005). *S. pombe smc6* was first identified as a gene that complemented a DNA-damage-sensitive mutant (Fousteri and Lehmann, 2000; Lehmann et al., 1995). Subsequent analysis revealed that the Smc5/6 complex is required for DNA repair by homologous recombination and has an additional essential function (Murray and Carr, 2008). Recent studies suggest that Smc5/6 has multiple functions in homologous recombination (Ampatzidou et al., 2006; Irmisch et al., 2009; Miyabe et al., 2006; Murray and Carr, 2008). *rad60-1*, a temperature-sensitive hypomorph of *rad60*, shows mutual genetic interactions with *smc6*, and the Rad60 protein was shown to physically interact with Smc5/6 complex (Boddy et al., 2003; Morishita et al., 2002). These observations suggested that *rad60* not only shares functions with *smc6* in homologous recombination, but is also required for the essential function of Smc5/6, which is less well characterized.

Rad60 interacts with the forkhead-associated (FHA) domain of Cds1^{Chk2} and is phosphorylated in a Cds1-dependent manner (Boddy et al., 2003; Raffa et al., 2006). Rad60 protein disperses throughout the cell when cells are challenged with HU, whereas it normally localizes within the nucleus of unperturbed cells throughout the cell cycle. In *cds1-fha1* mutant cells, Rad60 remains in the nucleus even when cells are challenged with HU. Immunoprecipitated Cds1^{Chk2} phosphorylates the N-terminus of Rad60 in vitro, indicating that

Cds1^{Chk2} directly phosphorylates Rad60 to regulate its localization (Boddy et al., 2003; Raffa et al., 2006).

Recent studies have identified a substrate preference for Cds1^{Chk2} (O'Neill et al., 2002; Seo et al., 2003), showing that an arginine residue at the –3 position is the most important residue for the substrate preference. Peptide phosphorylation is substantially decreased when alanine is substituted for arginine at the –3 position. Thus, Cds1^{Chk2} preferentially phosphorylates serine or threonine residues in an RxxS/T motif. To gain further insight into the regulation and significance of Rad60 phosphorylation by Cds1^{Chk2}, we substituted alanine for serine or threonine in the Cds1^{Chk2} consensus target motifs in Rad60. We identified two residues in Rad60 that are targeted for phosphorylation in a Cds1^{Chk2}-dependent manner and found that these are responsible for the re-localization of Rad60 protein in response to HU treatment. In the absence of this re-localization we observed no quantifiable HU sensitivity or sensitivity to a range of DNA-damaging agents. However, we find that the *rad60* phosphorylation-site mutations suppress specific *smc6* mutations, indicating that Rad60 is required to regulate homologous recombination at stalled replication forks by controlling Smc5/6 activity.

Results

Rad60 residues T72 and S126 are potent targets of Cds1

In vitro, Cds1^{Chk2} kinase exhibits a preference for the substrate motif RxxS/T (O'Neill et al., 2002; Seo et al., 2003). We found three potential phosphorylation sites matching this motif in the Rad60 protein (Fig. 1A). Threonine 72 (T72) and serine 126 (S126) are located in the N-terminal domain and T365 is in the SUMO-like domain 2 at the C-terminus. To examine whether these residues are phosphorylated by Cds1^{Cds1}, we individually or in combination replaced them with alanine and introduced the mutations into the genomic *rad60*$^{+}$ locus. Because the phosphorylated form of Rad60 protein is known to show a significant hypermobility shift, we resolved Rad60 by SDS-PAGE and detected the protein using an anti-Rad60 antibody. As shown in Fig. 1B, four distinct forms of Rad60 were detected, in agreement with previously published results (Raffa et al., 2006), and the level of Rad60 protein was unaffected by the mutations introduced. After treatment with HU, both Rad60-T72A and Rad60-S126A showed an intermediate hypershift (form 1 to form 3 and form 2 to form 3, respectively), whereas almost all of the wild-type Rad60 was converted to form 4. The hypershift essentially disappeared in the *rad60-T72A S126A* double-mutant cells (we will refer to the *rad60-T72A S126A* double mutant as *rad60-2A* and the *rad60-T72A S126A T365A* triple mutant as *rad60-3A*).

Because the Rad60 hypershift is known to be dependent on Cds1^{Chk2}, we next performed the kinase assay in vitro using recombinant Rad60 as substrate for immunoprecipitated Cds1^{Chk2}. Wild-type Rad60 was efficiently phosphorylated in vitro and this phosphorylation was dependent on Cds1^{Chk2} (Fig. 2A). The efficiency of phosphorylation was significantly decreased for Rad60-2A and Rad60-3A proteins, suggesting that T72 and/or S126 are direct targets of Cds1^{Chk2}. Next, we examined the effect of each single mutant on phosphorylation in vitro (Fig. 2A, right panels). T72A decreased the phosphorylation signal to a similar extent as the Rad60-2A mutant protein. S126A also apparently decreased the signal but the effect was less significant. To further clarify the phosphorylation of S126, we expressed truncated proteins to separate S126 from T72. The N-terminal fragment (N1) and middle fragment (M1) encompass T72 or S126, respectively. Both fragments were phosphorylated by Cds1^{Chk2} in vitro, and the corresponding alanine mutants (N1A and M1A) significantly decreased the signal (Fig. 2B). Together with the phosphorylation-dependent hypershift data (Fig. 1) these data led us to conclude that both T72 and S126 are direct targets of Cds1^{Chk2}.

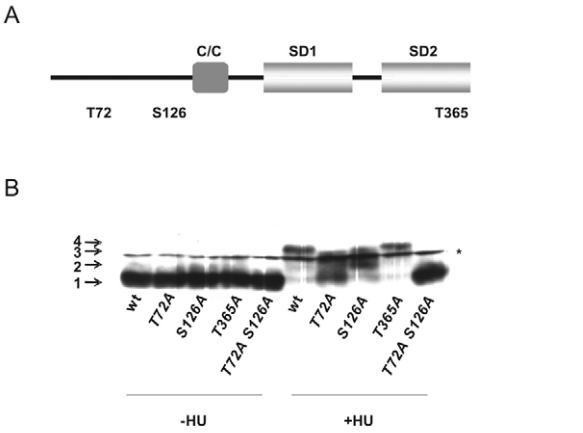

Fig. 1. Phosphorylation of Rad60 in vivo. (A) Schematic representation of Rad60 protein. C/C and SD represent coiled-coil and SUMO-like domains, respectively. There are two SUMO-like domains at the C-terminus of Rad60. (B) Western blot analysis of the indicated strains. Arrows indicate four distinct forms of Rad60 following exposure to 15 mM HU for 4 hours. * indicates a nonspecific band.

Phosphorylation of T72 and S126 are responsible for nuclear de-localization of Rad60 in response to HU

Rad60 is diffused throughout the whole cell in response to HU treatment, whereas it is localized in the nucleus during the normal cell cycle. This HU-dependent re-localization of Rad60 is dependent on Cds1^{Chk2} (Boddy et al., 2003). We thus examined the effects of T72 and S126 phosphorylation-site mutants on the localization of Rad60. Myc-tagged wild-type and mutant Rad60 were expressed from the *rad60* genomic locus and stained with anti-myc antibody. Both the wild-type and mutant versions of Rad60 were localized in the nucleus in the absence of HU (Fig. 3A). Wild-type Rad60 was dispersed throughout the whole cell following treatment with HU and showed only a weak nuclear signal, as previously described. The *rad60-S126A* mutation showed the most striking effect on localization. Rad60-S126A was found only in the nucleus, even after treatment with HU. The Rad60-T365A protein behaved in an identical manner to wild-type Rad60 whereas the Rad60-T72A protein displayed an intermediate pattern of localization. These in vivo results suggest that phosphorylation of S126 is the primary requirement for the re-localization of Rad60 in response to Cds1^{Chk2} activation following HU treatment. Interestingly, we observed that T72 was a better substrate for Cds1^{Chk2} than S126 in vitro. It has been reported that phosphorylation of T72 is required for the interaction of Rad60 with the FHA domain of Cds1 (Raffa et al., 2006). Thus, phosphorylation of T72 might be necessary for efficient phosphorylation of S126 and thus for efficient re-localization of the protein.

Phosphorylation of T72 and S126 are not required for cell viability in response to HU or other DNA-damaging agents

rad60-T72A and *rad60-S126A* mutations both affected the re-localization of Rad60. However, cells expressing either single-mutant proteins or double- and triple-mutant proteins from the *rad60* locus, and at equivalent levels to wild-type protein, are not sensitive to HU, methyl methanesulfonate (MMS) or mitomycin C (MMC) even at concentrations sufficient to reduce the viability of wild-type cells

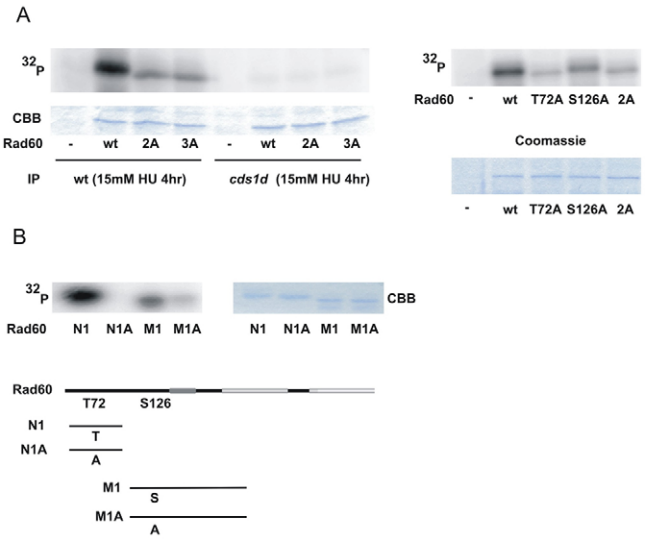

Fig. 2. Phosphorylation of Rad60 in vitro. The Cds1 protein was immunoprecipitated from wild-type (wt) or *cds1Δ* cells treated with 15 mM HU for 4 hours and incubated with recombinant Rad60. Samples were subjected to SDS-PAGE. Gels were stained with Coomassie brilliant blue (CBB) and exposed to a phospho-imager screen after drying (^{32}P). (A) Kinase assay with full-length Rad60. Left: dependence on Cds1. Right: analysis of single amino acid changes. (B) Kinase assay with truncated Rad60. Upper panel shows a gel. Lower panel shows a schematic representation of truncated protein used here.

(Fig. 3B). One explanation for this could be that there are alternative redundant mechanisms that result in the same phenotypic effect as the absence of Rad60 phosphorylation. We therefore examined the effects of deleting various genes (e.g. *rhp51*, *rhp18*, *mus81*, *rqh1*, *srs2*, *brc1*, *slx1*) on the sensitivity of *rad60-2A* to HU. However, loss of Rad60 phosphorylation caused no significant effect in any of these backgrounds (data not shown). There are several independent mechanisms for maintaining or repairing the stalled replication forks, and this could complicate our efforts to detect the effect of phosphorylation of a single DNA repair protein.

rad60-2A suppresses the HU sensitivity of *smc6* mutants

Previous studies have shown that Rad60 interacts with the Smc5/6 protein complex both physically and genetically (Boddy et al., 2003; Morikawa et al., 2004; Morishita et al., 2002). We therefore examined whether *rad60-2A* affected the sensitivity of *smc6* mutants to genotoxic stress. As shown in Fig. 4A, *rad60-2A* suppressed the HU sensitivity of the *smc6-X* mutant. Similar, but less pronounced suppression was observed for the *smc6-74* mutant. *rad60-2A* failed to suppress the UV sensitivity of these *smc6* mutants, consistent with the fact that UV irradiation does not induce Rad60 phosphorylation (data not shown). Smc5/6 has been proposed to function during DNA repair by homologous recombination. However, *rad60-2A* could not suppress the sensitivity of *rhp51Δ* cells to DNA damage (data not shown). Thus, *rad60-2A* does not bypass the requirement for homologous recombination when the Smc6 protein is dysfunctional, but appears to enhance functions of the hypomorphic mutant Smc6.

These results reminded us of the fact that *rad60* has been shown to act as a multicopy suppressor of *smc6-X* (Morishita et al., 2002). However, we observe that multicopy *rad60* suppresses both the HU and UV sensitivity of *smc6* mutants (Fig. 4B). This suppression is less pronounced for *smc6-74* than for *smc6-X*, which is consistent with the suppression by *rad60-2A*. These results suggest that the suppression of *smc6* mutants by *rad60-2A* is due to an excess of Rad60 protein in the nucleus in the presence of HU.

Rad60 phosphorylation modulates proper recombination at ribosomal DNA

To verify the suppression of *smc6* mutants by *rad60-2A* we performed an assay to measure loss of the *ura4*$^+$ gene integrated within one copy of the ribosomal DNA. *smc6-X* cells show a significantly elevated frequency of *ura4*$^+$ loss from the ribosomal DNA (Irmisch et al., 2009). In this assay, loss of the *ura4*$^+$ gene is probably due to ectopic sister chromatid recombination or intrachromosomal recombination. As shown in Fig. 4C, the frequency of *ura4* loss is elevated more than tenfold in *smc6-X* cells and this was partially suppressed by *rad60-2A*. Specifically, the frequency of HU-induced *ura4*$^+$ loss was reduced by a factor of ~50% in the double mutant. Suppression can also be seen in

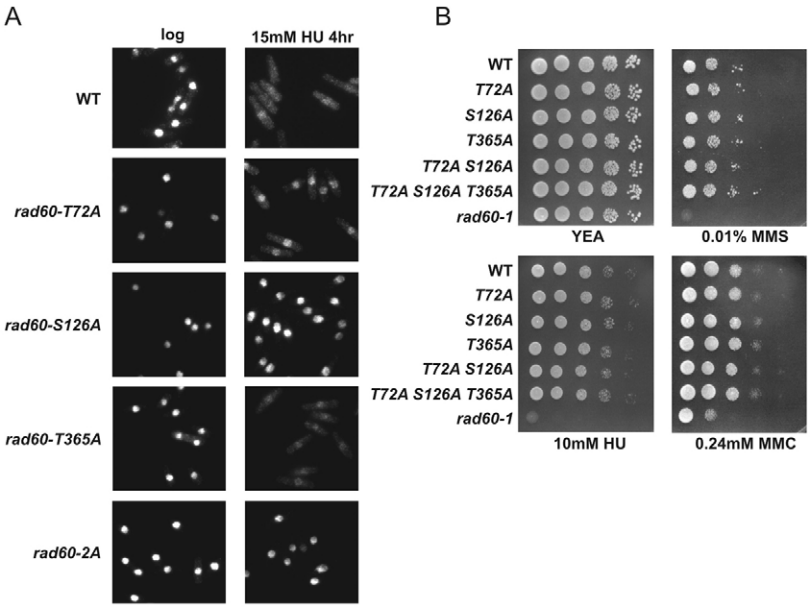

Fig. 3. Localization of mutant Rad60 and genotoxin sensitivity of mutant cells. (A) Rad60 mutant proteins were detected by indirect immunofluorescence of 13× Myc-tagged protein. Cells of indicated strains were treated with or without 15 mM HU for 4 hours and fixed. Rad60-Myc was stained with anti-Myc monoclonal antibody. (B) Sensitivity of cells expressing mutant Rad60 to various DNA damaging agents. Serial dilutions of indicated strains were spotted on YEA with or without HU, MMS or MMC, as indicated.

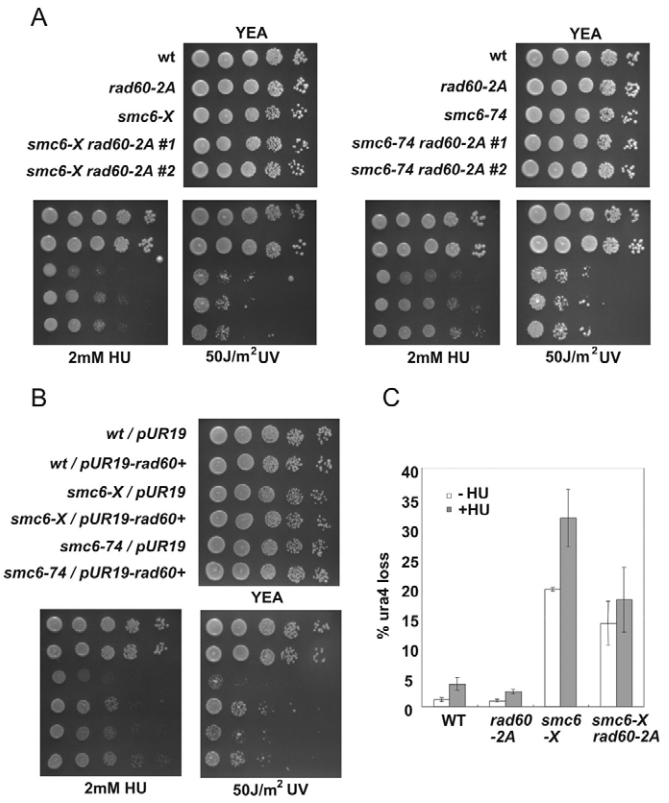

Fig. 4. Genetic interactions of *rad60* mutants with *smc6*. (A) Effect of *rad60-2A* on the sensitivity of *smc6* mutants to HU or UV irradiation. Serial dilutions of indicated strains were spotted on YEA with or without HU. To test sensitivity to UV, cells were irradiated immediately after being spotted on YEA. (B) Effect of multicopy *rad60* on the sensitivity of *smc6* mutants to HU or UV irradiation. Indicated strains carrying pUR19 vector or pUR19 with *rad60*$^{+}$ gene were serially diluted and spotted on YEA with or without HU. To test sensitivity to UV, cells were irradiated after being spotted on YEA. (C) Marker loss from ribosomal DNA. Frequency of the *ura4* loss (%) was calculated and plotted. Error bars show the standard deviation.

untreated cells, though it is less dramatic (Fig. 4C). These results led us to conclude that the suppression of phenotypes of *smc6* mutants by *rad60-2A* is significant. Interestingly, even in the *smc6*$^{+}$ background, the frequency of HU-induced *ura4* loss in *rad60-2A* was significantly lower than that in the wild type (2.4 ±0.3% and 3.7 ±0.9%, respectively). This suggests that the phosphorylation of Rad60 is required for cells to correctly regulate recombination at ribosomal DNA after replication stalls, although phosphorylation of Rad60 does not detectably affect cell viability.

Discussion

In this study, we have employed site-directed mutagenesis to identify Cds1^{Chk2} target residues in Rad60. Analysis of phosphorylation in vivo and in vitro has identified two target residues, T72 and S126. Phosphorylation of T72 has previously been reported to be required for correct binding between Rad60 and Cds1^{Chk2} (Boddy et al., 2003; Raffa et al., 2006). The FHA domain of Cds1^{Chk2} preferentially binds to TxxD motifs in which threonine is phosphorylated (Durocher et al., 2000). Rad60-T72 is not only located in a putative Cds1^{Chk2} target motif, RxxS/T, but also encompasses the Cds1^{Chk2} FHA binding motif, TxxD. Because we used recombinant protein purified from *E. coli* extract for the kinase assay, it is unlikely that Cds1^{Chk2} binds phosphorylated T72 and phosphorylates another residue in vitro. Therefore, we conclude that T72 is a direct target of Cds1^{Chk2}.

Phosphorylation of S126 is less efficient in vitro than that of T72 although the S126A mutation causes a more striking effect on the re-localization of Rad60 in response to HU treatment (Fig. 2A,B; Fig. 3A). These observations suggest that Cds1^{Chk2} first phosphorylates T72 and stabilize its association with Rad60 and that this stabilization is required for efficient phosphorylation of S126, which is responsible for the re-localization of Rad60. Consistent with this model, S126 is inefficiently phosphorylated in vitro when Rad60-T72A protein is used as a substrate (Fig. 2A). The model is also consistent with the observation that defects in the hypershift of Rad60 are more severe in the *rad60-T72A* mutant than in the *rad60-S126A* mutant (Fig. 1B).

The target consensus recognition of Cds1^{Chk2} is influenced not only by the residue at the –3 position but also, to a lesser extent, by that at position –5. Leucine at –5 increases the phosphorylation of peptide, although the effect is much less dramatic than that of arginine at –3 (O'Neill et al., 2002). There is a leucine at –5 of T72 but not S126. Stable Cds1^{Chk2} binding might be required for S126 to be efficiently phosphorylated. The dispersal of Rad60 is inhibited in the presence of leptomycin B (supplementary material Fig. S1), indicating that Crm1-dependent nuclear export is involved in this dispersal. Although Crm1 requires a nuclear export signal (NES) to export target proteins, S126 is not located in an apparent NES. An NES predictor (NetNES) predicts a putative NES in Rad60 at the C-terminus. However, it has not been determined whether this putative NES is functional for nuclear export. It is also possible that another Rad60-interacting protein containing an NES is required for the nuclear export of Rad60. Further study is thus needed to elucidate the mechanism relating Cds1^{Chk2}-dependent Rad60 phosphorylation to Rad60 re-localization.

Here, we have shown that the hypershift of Rad60 is completely abolished in *rad60-2A* mutant cells and we have failed to detect Cds1^{Chk2}-dependent phosphorylation of the N-terminal portion of Rad60 in vitro when the T72 and S126 residues were changed to alanine. On the other hand, Raffa and collegues identified phosphorylation of S32 and S34 and showed that these residues affect the hypershift of Rad60 in response to HU (Raffa et al., 2006). These observations suggest that Rad60 is tightly regulated by post-translational modifications.

The *rad60-2A* mutation suppressed the HU sensitivity of *smc6* mutants whereas the *rad60-2A* mutant cells were not hypersensitive to DNA-damaging agents (Fig. 3B; Fig. 4A). It has been proposed that Rad60 functions in concert with the Smc5/6 complex because Rad60 physically interacts with Smc5/6 and because hypomorphic mutants of *rad60* show mutual genetic interactions with *smc6*. Our results support a model in which Rad60 assists Smc5/6 in its functions. When the function of Smc5/6 is compromised by hypomorphic mutation, an additional quantity of nuclear Rad60 appears to facilitate the response to replication stress. In addition to the suppression of the frequency of *ura4*$^{+}$ loss from ribosomal DNA in *smc6-X* mutants by the *rad60-2A* mutation, we also observed that the frequency of *ura4*$^{+}$ loss was decreased in *rad60-2A* single mutant cells (Fig. 4C).

It has been reported that cohesin, another SMC complex that is required for sister chromatid cohesion, regulates the length of ribosomal DNA repeats in *S. cerevisiae* (Kobayashi and Ganley, 2005). On the one hand, Smc5/6 also localizes on ribosomal DNA in *S. cerevisiae* and is required for proper separation of this region during mitosis (Torres-Rosell et al., 2005). On the other hand, Smc5/6 is shown to be required for efficient sister chromatid

Table 1. *S. pombe* strains used in this study

Strain	Genotype	Source
MP10	*h⁻ leu1-32 ura4-D18*	(Morishita et al., 2002)
MP11	*h⁺ leu1-32 ura4-D18*	(Morishita et al., 2002)
501	*h⁻ leu1-32 ura4-D18 ade6-704*	Wild-type strain
AMC231	*h⁻ leu1-32 ura4-D18 ade6-704 cds1::ura4⁺*	(Lindsay et al., 1998)
rad60 T72A	*h⁺ leu1-32 ura4-D18 rad60.T72A*	This study
rad60 S126A	*h⁺ leu1-32 ura4-D18 rad60.S126A*	This study
rad60 T365A	*h⁺ leu1-32 ura4-D18 rad60.T365A*	This study
rad60-2A	*h⁺ leu1-32 ura4-D18 rad60-2A*	This study
rad60-3A	*h⁺ leu1-32 ura4-D18 rad60-3A*	This study
MPR111	*h⁺ leu1-32 ura4-D18 rad60-1*	(Morishita et al., 2002)
rad60-13Myc	*h⁺ leu1-32 ura4-D18 rad60-13Myc::kanMX*	This study
rad60 T72A-13Myc	*h⁺ leu1-32 ura4-D18 rad60.T72A-13Myc::kanMX*	This study
rad60 S126A-13Myc	*h⁺ leu1-32 ura4-D18 rad60.S126A-13Myc::kanMX*	This study
rad60 T365A-13Myc	*h⁺ leu1-32 ura4-D18 rad60.T365A-13Myc::kanMX*	This study
rad60-2A-13Myc	*h⁺ leu1-32 ura4-D18 rad60-2A-13Myc::kanMX*	This study
smc6-X	*h⁺ leu1-32 ura4-D18 smc6-X*	(Lehmann et al., 1995)
smc6-74	*h⁺ leu1-32 ura4-D18 smc6-74*	(Verkade et al., 1999)
smc6-X rad60-2A	*h⁺ leu1-32 ura4-D18 smc6-X rad60-2A*	This study
smc6-74 rad60-2A	*h⁺ leu1-32 ura4-D18 smc6-74 rad60-2A*	This study
J1597	*h⁺ ura4-DS/E leu1/YIp2.4 pUCura4⁺-7*	(Thon and Verhein-Hansen, 2000)
J1600	*h⁺ ura4-DS/E leu1/YIp2.4 pUCura4⁺-7 smc6-X*	This study
J1597 rad60-2A	*h⁺ ura4-DS/E leu1/YIp2.4 pUCura4⁺-7 rad60-2A*	This study
J1600 smc6-X rad60-2A	*h⁻ ura4-DS/E leu1/YIp2.4 pUCura4⁺-7 smc6-X rad60-2A*	This study

recombination: in *smc6* mutants, ectopic recombination is elevated whereas sister chromatid recombination is decreased (De Piccoli et al., 2006). Elevated ectopic recombination in the ribosomal DNA at the expense of sister chromatid recombination is consistent with the increased *ura4⁺* loss we observed in *S. pombe*. Thus, to ensure ectopic recombination in specific situations, such as the maintenance of ribosomal DNA repeats, cells might need to regulate Smc5/6 by reducing the concentration of Rad60 in the nucleus.

In *S. pombe*, a recombination protein Mus81 is also phosphorylated in Cds1^{Chk2}-dependent manner (Boddy et al., 2000). The *mus81-T239A* mutation abolishes the interaction of Mus81 with Cds1 in a similar manner to the abolition of the Rad60-Cds1^{Chk2} interaction in the *rad60-T72A* mutant. Interestingly, the regulation of Mus81 in response to replication stress closely resembles that of Rad60. Mus81 dissociates from chromatin in the presence of HU whereas Mus81-T239A protein remains chromatin-associated. However, *mus81-T239A* mutation enhances recombination frequency of cells after HU treatment (Kai et al., 2005). This is exactly the opposite of what we have observed in *rad60-2A* cells. However, *mus81* is essential for growth of *rad60* mutants (Boddy et al., 2003; Morishita et al., 2002), suggesting that these genes have overlapping functions. Slx1/4, another structure-specific endonuclease, has also been shown to be involved in recombination at ribosomal DNA repeats in *S. pombe* (Coulon et al., 2004; Coulon et al., 2006). In *S. cerevisiae*, the non-catalytic subunit Slx4 is phosphorylated in an Mec1^{ATR}-dependent manner and is required for phosphorylation of ESC4 protein (Flott and Rouse, 2005; Roberts et al., 2006), which is required for restart of stalled replication forks (Rouse, 2004). *ESC4* is a homolog of *S. pombe brc1*, a multicopy suppressor of *smc6-74* (Lee et al., 2007; Sheedy et al., 2005; Verkade et al., 1999). Multicopy *brc1* suppresses the sensitivity of *smc6-74* but not *smc6-X* to DNA damage, but multicopy *rad60* suppresses *smc6-X* more dramatically than it does *smc6-74* (Fig. 4B). Taken together, these data clearly indicate that checkpoint responses regulate multiple pathways to overcome the difficulties induced by replication stress. Understanding the intricacies of this regulation is a complex but important job.

In this report, we have shown that checkpoint kinase Cds1^{Chk2} regulates homologous recombination at the ribosomal DNA repeats through the phosphorylation of Rad60 at T72 and S126. The phosphorylated form of Rad60 disperses from the nucleus and this dispersal appears to promote ectopic recombination, possibly by influencing the function of Smc5/6. In *S. cerevisiae* and *S. pombe*, Smc5/6 has been shown to localize at centromeric and repeated sequences, including the ribosomal DNA repeats (Pebernard et al., 2008; Torres-Rosell et al., 2005). It has also been reported that Smc5/6 accumulates at the sites of DNA double-strand breaks or collapsed replication forks (Lindroos et al., 2006). *S. cerevisiae ESC2* is a putative homolog of *rad60*, and has been shown to be involved in chromatin silencing and sister chromatid cohesion (Dhillon and Kamakaka, 2000; Ohya et al., 2008). However, there is no evidence that *S. pombe* Smc5/6 has similar activity, and a correlative interaction between ESC2p and Smc5/6 has not been reported. One possibility is that Rad60 plays a role in regulating the localization of Smc5/6 on chromatin. Further studies are required to elucidate the function of Rad60, and how this relates to Smc5/6 functions.

Materials and Methods

S. pombe strains, media and methods

The *S. pombe* strains used in this study are listed in Table 1. *S. pombe* cells were grown in medium supplemented with yeast extract (YE) or in Edinburgh minimal medium (EMM). Standard genetic and molecular procedures were employed as described previously (Moreno et al., 1991). To examine the sensitivity to drugs, serial dilutions of cells were spotted on YE plates (YEA) containing each drug, and incubated at 30°C for 3-4 days. NLS sequences were identified using NetNES predictor (la Cour et al., 2004).

Western blot

Total protein was extracted in buffer G (50 mM Tris-HCl, 100 mM NaH_2PO_4, 6 M guanidine hydrochloride, pH 8.0). For SDS-PAGE, proteins were precipitated with

trichloroacetic acid and resuspended in 1× SDS-PAGE sample buffer. Subsequently, proteins were transferred to PVDF membranes and probed with affinity-purified anti-Rad60 (BioAcademia, Osaka, Japan). Detection was performed with HRP-conjugated secondary antibody and ECL Advance Western Blot Detection Kit (GE Healthcare).

Cds1^{Chk2} kinase assay

GST-Rad60 was expressed in *E. coli*, and purified on glutathione Sepharose (GE healthcare). Purified protein was incubated with immunoprecipitated Cds1^{Chk2} as described previously (Lindsay et al., 1998). Samples were subjected to 10% SDS-PAGE and gels were dried and exposed to phospho-image screens after staining with Coomassie brilliant blue.

Indirect immunofluorescence

Strains expressing Myc-tagged Rad60 from the native locus were used for indirect immunofluorescence. Cells were fixed with 3.7% formaldehyde and processed as described previously (Caspari et al., 2000). Processed cells were stained with anti-Myc monoclonal antibody (9E10) and Alexa-Fluor-488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR). Stained cells were observed under an epifluorescence microscope and photographed.

Assay of *ura4* loss at ribosomal DNA

In three separate experiments, 11 independent single colonies for each strain were inoculated into 10 ml YE (2% YE, 6% sucrose, pH 5.5) medium and grown to stationary phase. 1×10^3 cells were plated on YEA, grown for ~5 days at 30°C and then the colonies were replica plated to medium without uracil. To assay for *ura4* loss after treatment with HU, colonies were inoculated as above, grown to mid-log phase and treated with 10 mM HU for 4 hours. Cells were then washed, resuspended in YE and grown to stationary phase. The *ura4* loss assay measures marker loss from a single *ura4+* gene integrated into one ribosomal DNA repeat.

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