Cdc7-dependent phosphorylation of Mer2 facilitates initiation of yeast meiotic recombination

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Meiosis ensures genetic diversification of gametes and sexual reproduction. For successful meiosis, multiple events such as DNA replication, recombination, and chromosome segregation must occur coordinately in a strict regulated order. We investigated the meiotic roles of Cdc7 kinase in the initiation of meiotic recombination, namely, DNA double-strand breaks (DSBs) mediated by Spo11 and other coactivating proteins. Genetic analysis using *bob1-1 cdc7Δ* reveals that Cdc7 is essential for meiotic DSBs and meiosis I progression. We also demonstrate that the N-terminal region of Mer2, a Spo11 ancillary protein required for DSB formation and phosphorylated by cyclin-dependent kinase (CDK), contains two types of Cdc7-dependent phosphorylation sites near the CDK site (Ser30): One (Ser29) is essential for meiotic DSB formation, and the others exhibit a cumulative effect to facilitate DSB formation. Importantly, mutations on these sites confer severe defects in DSB formation even when the CDK phosphorylation is present at Ser30. Diploids of *cdc7Δ* display defects in the chromatin binding of not only Spo11 but also Rec114 and Mei4, other meiotic coactivators that may assist Spo11 binding to DSB hot spots. We thus propose that Cdc7, in concert with CDK, regulates Spo11 loading to DSB sites via Mer2 phosphorylation.

[**Keywords:** Cdc7; Mer2; meiotic recombination; Spo11; pre-DSB complex]

Supplemental material is available at <http://www.genesdev.org>.

Received October 17, 2007; revised version accepted December 5, 2007.

The meiotic cell division comprises one round of DNA replication and activated homologous recombination followed by two consecutive nuclear divisions, and thereby converts a diploid cell to a haploid gamete with increased genetic diversity. Meiotic recombination is initiated by programmed formation of DNA double-strand breaks (DSBs) catalyzed by the DNA topoisomerase-II-like enzyme Spo11. DSBs are introduced strictly after premeiotic DNA replication, which may accompany reorganization of chromatin structure (Ohta et al. 1994; Wu and Lichten 1994; Borde et al. 2000). In the budding yeast *Saccharomyces cerevisiae*, nine genes (*MEI4*, *MER2/REC107*, *REC102*, *SKI8/REC103*, *REC104*, *REC114*, *MRE11*, *RAD50*, and *XRS2*) in addition to *SPO11* are required for DSB formation (for review, see Keeney 2001). Many genetic and physical interactions among them had been found hitherto, raising the possi-

bility that a pre-DSB complex is established before activating Spo11 at recombination hot spots (Arora et al. 2004; Kee et al. 2004; Sasanuma et al. 2007). Rec102 and Rec104, which can interact with Spo11, may be required for Spo11 activation via the formation of Spo11 multimer (Sasanuma et al. 2007). In addition, it has been reported that Rec114, Mer2, and Mei4 form a complex during meiosis (Li et al. 2006; Maleki et al. 2007). Cytological analysis revealed that the chromosomal association of Rec114 occurs independently from most of the other DSB factors, and Spo11 binding to a DSB hot spot is severely impaired in a *rec114Δ* mutant (Prieler et al. 2005; Maleki et al. 2007). Therefore, Rec114 has been proposed as a factor that recruits Spo11 to chromatin.

Formation of chiasmata, recombination-dependent chromosome connections, is vital for the proper segregation in meiosis I. Thus, for the proper completion of meiosis, it is important to have a strict temporal order of meiotic chromosomal events: DNA replication, DSB formation, recombination, and chromosome segregation. In particular, it has been demonstrated that meiotic DNA replication and recombination are tightly coupled under

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Article is online at <http://www.genesdev.org/cgi/doi/10.1101/gad.1626608>.

spatial and temporal controls. In fission yeast, cells entering into meiosis from the G2 phase of mitosis overleap premeiotic DNA synthesis and cause a fatal impairment in meiotic recombination (Watanabe et al. 2001). In addition, Clb5–Clb6–cyclin-dependent kinase (CDK) activity has crucial roles in triggering both replication initiation and the activation of Spo11 in budding yeast meiosis (Dirick et al. 1998; Stuart and Wittenberg 1998; Smith et al. 2001; Henderson et al. 2006). These studies suggest that cellular regulation coordinates DNA replication and DSB formation. Recently, it was demonstrated that CDK directly regulates DSB formation via the phosphorylation of Mer2 protein (Henderson et al. 2006). The interplay of DNA replication and DSB formation has been also supported by other experiments. In budding yeast, the removal of all ARSs (autonomous replication sequences) from the left arm of chromosome III confers a left-arm-specific delay in DNA replication and meiotic DSB formation, suggesting the coordinated regulation of DNA replication and DSB formation at a chromosomal level. Such control may ensure that DSBs are not formed before the completion of DNA replication (Borde et al. 2000; Murakami et al. 2003).

To uncover such highly integrated regulation, we analyzed meiotic roles of Cdc7, which is required for the initiation of DNA replication and maintenance of replication fork stability. It has been previously reported that reduction of Cdc7 kinase activity in mice confers sterility with a testicular and ovary atrophy, indicating the essential role for Cdc7 in gamete formation (Kim et al. 2003a). Recently, in fission yeast, we demonstrated essential roles for Cdc7 kinase activity in meiotic progression and DSB formation using a temperature-sensitive (*ts*) mutant of *hsk1*⁺, the fission yeast homolog of *CDC7* (Ogino et al. 2006). Similarly, in budding yeast, a *cdc7 ts* mutant was shown to undergo premeiotic DNA replication, but to arrest before meiosis I (Schild and Byers 1978). In addition, it has been recently reported that cells depleting *DBF4* transcripts after the initiation of DNA synthesis arrest before meiosis I (Valentin et al. 2006). Moreover, analysis using chemical genetics of *cdc7-as* mutant suggested a possibility that Cdc7 is required for meiotic DSB formation in budding yeast (Wan et al. 2006).

In this study, using a *cdc7Δ* mutant with the *bob1-1* mutation that only alleviates the replication defect of *cdc7Δ*, we investigated the essential roles of the budding yeast Cdc7 kinase in DSB formation and meiotic progression. We focus here on the molecular mechanism underlying the regulation of DSB formation and show that Cdc7 promotes DSB formation via the phosphorylation of the Mer2 protein. MALDI-TOF mass-spectrometric (TOF-MS) analysis and alanine substitution analysis enabled us to identify *in vivo* bona fide phosphorylation sites in the N-terminal region of Mer2 and led us to conclude that the phosphorylation dependent on Cdc7 is vital for DSB formation via the chromatin loading of Spo11, which may be facilitated by Rec114, a component of the pre-DSB complex. We further demonstrate that Cdc7 is also pivotal for the loading of Rec12 (*S. pombe*

Spo11) to a DSB site in the fission yeast, suggesting a conserved regulatory mechanism for DSB formation by Cdc7.

Results

The bob1-1 cdc7Δ mutation abolishes meiotic DSBs and arrests cells before MI after normal DNA replication

To examine the functions of the Cdc7 kinase in meiosis, we engineered an SK1 strain lacking *CDC7* in the *bob1-1* background, in which the defect of DNA replication in *cdc7Δ* is bypassed. The *bob1-1 cdc7Δ* diploid cells were larger than the wild type, but had a normal cell cycle in mitosis as shown previously (Hardy et al. 1997). Premeiotic DNA replication proceeded with only a slight delay in the *bob1-1 cdc7Δ* mutant (Fig. 1A). Microscopic analysis to monitor the meiotic nuclear division revealed that the *bob1-1 cdc7Δ* mutant arrested with one nucleus before meiosis I, which is consistent with the previously reported phenotype of *cdc7-dbf4* (*S. cerevisiae*) and *hsk1-dfp1/him1* (*S. pombe*) mutants (Fig. 1B; Ogino et al. 2006; Valentin et al. 2006). The proportion of asci with four spores is <7% of the total asci at 24 h in sporulation medium (Supplemental Fig. S1A), while prolonged incubation resulted in the formation of substantial dyads (Supplemental Fig. S1A,B). Importantly, all the tetrad spores were inviable (0/60 viable/total spores in *bob1-1 cdc7Δ*). Meiotic progression, recombination frequency, and spore viability for the *bob1-1* single mutant were indistinguishable from those of the wild type (118/120 viable/total spores in *bob1-1*) (Fig. 1A,C,D).

In general, meiotic recombination-deficient mutants are known to confer very low spore viability. Thus, this result led us to examine the meiotic recombination in the *bob1-1 cdc7Δ* mutant during meiosis. Meiotic recombination at *ARG4*, a well-characterized meiotic recombination hot spot, was severely impaired in *bob1-1 cdc7Δ* (<0.01%; cf. *bob1-1*, 8.1%) (Fig. 1C). We next analyzed meiotic DSB formation at *CYS3* and *YCR048w*, known as strong meiotic recombination hot spots (Wu and Lichten 1994; Vedel and Nicolas 1999). Consistent with the results in Figure 1C, in each locus, meiotic DSBs were completely abolished in *bob1-1 cdc7Δ*, while the frequency of DSB formation in *bob1-1* mutant was similar to that in wild type (Fig. 1D, top and middle panels). Pulse field gel electrophoresis (PFGE) analysis to detect genome-wide meiotic DSBs revealed that no visible DSB is formed in the *bob1-1 cdc7Δ* mutant (Fig. 1D, bottom panel). These results indicate that Cdc7 is essential for DSB formation and meiotic recombination. Because other DSB-deficient mutants such as *spo11Δ* are known to form four inviable spores (80%–90% of total asci contained four spores in *spo11Δ*), the defect in meiotic nuclear division observed in *bob1-1 cdc7Δ* is unlikely to be simply due to its defect in DSB formation. Thus, these results suggest that Cdc7 also participates in progression of meiosis I.

Figure 1. The *bob1-1 cdc7Δ* mutant arrests with one nucleus before meiosis I. (A) Flow cytometry analysis for *bob1-1 cdc7Δ* performed for meiotic cells taken at intervals of 20 min. After synchronization in SPS medium, the cells were transferred to sporulation medium. The strains used were YHS5 (wt), YHS905 (*bob1-1*), and YHS869 (*bob1-1 cdc7Δ*). (B) The meiotic progression in *bob1-1 cdc7Δ*. The number of nuclei (in at least 150 cells) was counted by Hoechst staining. The *spo11Δ* diploid (YHS87) serves as a recombination-deficient control. (C) Return-to-growth analysis of *bob1-1 cdc7Δ*. Recombinants were estimated from colony numbers on SD-ARG and YEPD plates. The two independent analyses gave similar results. Cells were taken from the same culture as in A. (D) The detection of global and local DSB formation. Meiotic DSB formation was detected at the *YCR048w* (top) and *CYS3* (middle) loci. (Top and middle panels) *bob1-1* (YHS754 and YHS893) and *bob1-1 cdc7Δ* (YHS756 and YHS880) are homozygous for the *rad50S::URA3* or *sae2::CgLEU* allele, which permits DSB formation but prevents resection and repair. The DNA samples shown in the bottom panel were prepared in plugs and analyzed by PFGE.

Induction of middle-late meiotic genes is dependent on *Cdc7*

To study the cause of the defect in meiotic progression in *bob1-1 cdc7Δ*, we examined the transcriptional profile of meiotically induced genes by Northern blotting. In the *bob1-1 cdc7Δ* mutant, early meiotic genes including *IME2*, *SPO11*, *REC102*, and *DMC1* were almost normally expressed in the same fashion as in wild type and *bob1-1* (Fig. 2A,B). However, we found that *NDT80* transcripts were not induced until 8 h in the *bob1-1 cdc7Δ* mutant. *Ndt80* is known to play a role as a master transcriptional activator for >150 middle meiotic genes and to be required for meiotic nuclear division (Chu et al. 1998). At 6 h, the amount of *NDT80* transcripts in *bob1-1 cdc7Δ* was reduced to only 8% of that in *bob1-1*

(Fig. 2A,B). Transcriptional induction of *SPS4* and *SPS22*, which are controlled by *Ndt80*, were also abolished (Fig. 2A,B).

In order to compare the transcriptional profile of the *bob1-1 cdc7Δ* mutant with that of wild type in the whole genome, we performed transcriptome analysis using a microarray. At 8 h of meiosis in *bob1-1 cdc7Δ*, transcriptional induction of 344 genes, out of a total 404 genes that are clustered as “middle” and “middle-late” meiotic genes (Chu et al. 1998; Primig et al. 2000), was significantly lower than that in the *bob1-1* mutant (Supplemental Fig. S2, top graph). Among the 344 affected genes, meiotic induction of 199 genes was very severely impaired in *bob1-1 cdc7Δ* (>10-fold reduction), and 82 genes could be categorized as genes that are up-regulated by an ectopic expression of *NDT80* in mitosis (Chu et al. 1998).

Figure 2. The reduction of *NDT80* transcripts in the *bob1-1 cdc7Δ* mutant. (A) Northern blotting analysis of RNA in *bob1-1* *cdc7Δ*. Progression of meiosis was confirmed by FACS analysis. YHS5 (wt), YHS905 (*bob1-1*), and YHS869 (*bob1-1 cdc7Δ*) were used. (B) Quantification of each transcript shown in A. Transcripts of each gene in wild type, *bob1-1*, and *bob1-1 cdc7Δ* are indicated as black diamond, black square, and black triangle in the graph, respectively.

Less than 10% of these 82 genes were classified as “metabolic,” “early-middle meiotic,” and “unknown function” genes, although >90% of the 82 genes belonged to “middle” and “middle-late meiotic” genes, including *YSW1*, *SPR28*, and *SPS2* (Supplemental Fig. S2). These results indicate that Cdc7 facilitates meiotic progression via transcriptional induction of middle-late meiotic genes including *NDT80* and some genes of the *Ndt80* regulon.

Mer2 protein is phosphorylated in a Cdc7-dependent manner

We next focused on the role of Cdc7 in meiotic DSB formation. Recently, the Ser30 and Ser271 residues in Mer2 have been shown to be directly phosphorylated by CDK in meiosis (Henderson et al. 2006). Cdc7 has been shown to preferentially phosphorylate a serine residue juxtaposed to the serine residue phosphorylated by CDK in the N-terminal region of Mcm2 and Mcm4 (Cho et al. 2006; Masai et al. 2006). Therefore, we expected Mer2 protein to be a potential target of Cdc7 kinase. To examine the phosphorylation state of Mer2 in *bob1-1 cdc7Δ*, we conjugated Flag-tag sequences to the end of the endogenous *MER2* gene. The Mer2-3Flag protein expressed in diploid cells was specifically pulled down with anti-Flag antibody. After treating the immunoprecipitated fraction with or without calf intestine alkaline phosphatase (CIAP), we examined the mobility shift of Mer2 using SDS-PAGE.

The slower migrating bands of Mer2-3Flag were detected in the *bob1-1* mutant at 4 h of meiosis. Such slower migrating bands were shifted to a faster migrating band by CIAP treatment but not by CIAP with phosphatase inhibitors.

This indicates that the slower migration of Mer2-3Flag is due to phosphorylation (Fig. 3A [lanes 1–3], B [lanes 1–3]). In contrast, Mer2-3Flag extracted from the *bob1-1 cdc7Δ* mutant (4 h of meiosis) migrated faster than that from *bob1-1*, and the former band was shifted to an even lower position after the CIAP treatment (Fig. 3A,B, cf. lanes 1 and 4). These results indicate that Mer2 is phosphorylated depending on Cdc7.

The following observations suggest that CDK can phosphorylate Mer2 in the absence of Cdc7. First, in the *bob1-1 cdc7Δ* mutant, the slightly shifted mobility pattern of Mer2-3Flag could be detected at 4 and 8 h of meiosis, although there was no active Cdc7 (Fig. 3B, lanes 4,5). Second, such a residual band shift in *bob1-1 cdc7Δ* was not detected when we introduced *mer2S30*, *271A-3Flag*, an alanine substitution mutant of the CDK phosphorylation sites (Henderson et al. 2006). Third, the Mer2S30, 271A-3Flag protein exhibited no mobility shift, and the band position was the same as that after the CIAP treatment. These results again suggest that the majority of the Mer2 phosphorylations other than the CDK-mediated ones are dependent on Cdc7 (Fig. 3C).

It has been reported that Mer2 formed a subcomplex with Rec114 and Mei4 in meiosis (Li et al. 2006). Therefore, we also examined the phosphorylation states of Rec114 and Mei4. A faster-migrating band of Rec114-3Flag appeared after the CIAP treatment (Supplemental Fig. S3, arrow). Protein bands derived from some of the degradation products of Rec114-3Flag (Supplemental Fig. S3, indicated by an asterisk) were lost by the CIAP treatment, indicating that Rec114 is also phosphorylated in meiosis (Supplemental Fig. S3, arrowheads). In *bob1-1 cdc7Δ*, the putative phosphorylated, shifted forms decreased in intensity, but did not completely disappear

Figure 3. Identification of the phosphorylation sites of Mer2 using TOF-MS analysis. (A–C) The phosphorylation state of Mer2-3Flag in *bob1-1 cdc7Δ*. After the extracts were prepared from 5×10^9 meiotic cells (4 h, except for lane 5 in B: 8 h). Western blotting was performed for immunoprecipitated Mer2-3Flag. Asterisks indicate degradation products of Mer2-3Flag. Mer2S30, 271A-3Flag was expressed in *bob1-1 cdc7Δ*. The strains used were YHS35, YHS782, YHS775, and YHS1092. (D) The TOF-MS analysis for immunoprecipitated Mer2-3Flag. Immunoprecipitated Mer2-3Flag was eluted with 3XFlag peptide. (Left panel) The silver-stained bands were excised from a gel for TOF-MS analysis. (Right panel) Western blotting with anti-Flag was done to detect Mer2-3Flag in the IP fraction. (E) Results of TOF-MS analysis. Black bold letters in the top amino acid sequence show the peptides identified by TOF-MS, and underlines correspond to the location of detected phosphopeptides. CDK-dependent phosphorylation sites (Ser30, Ser271) are indicated by large letters in the top sequence. One peptide in the C-terminal region (P1) and four peptides in the N-terminal region (P2–P5) were detected. “Modifications” in the right column present the detected number of phosphorylation sites in each peptide.

(Supplemental Fig. S3, arrowheads). In the case of Mei4-3Flag in meiosis, we did not detect a mobility shift in meiosis. Thus, we thereafter focused on the roles of phosphorylation of Mer2 mediated by Cdc7.

Determination of Cdc7-dependent phosphorylation regions in Mer2 protein by TOF-MS analysis

To determine the phosphorylation sites of Mer2 under physiological conditions, we analyzed by a TOF-MS the phosphorylated peptides of Mer2 extracted from meiotic cells (4 h). We detected Mer2-3Flag in immunoprecipitated fraction by silver staining and Western blotting using anti-Flag antibody (Fig. 3D). The total amount of immunoprecipitated Mer2-3Flag protein used for this analysis was ~1.5–2 pmol, sufficient for a TOF-MS analysis. The matched peptide fragments covered 58% of the Mer2 sequence (Fig. 3E, bold letters). As shown in Figure 3E, we identified five species of candidate peptides subjected to phosphorylation, termed P1 to P5. In this analysis, phosphorylation of serine, threonine, and tyrosine residues can be detected. Peptide P1 comprised a tryptic phosphopeptide encompassing residues 269–291, in which Ser271 (a site phosphorylated by CDK) was likely phosphorylated. Peptides P2 to P5 comprised two kinds of peptide with an overlapping region (P2, residues 7–33;

P3–5, residues 5–33) containing another CDK-dependent phosphorylation site (Ser30) (Fig. 3E). These results indicate that at least three sites are phosphorylated in vivo in the peptide P5 among the six serine and three threonine residues (Ser11, Ser15, Ser19, Ser22, Ser29, Ser30, Thr12, Thr26, and Thr28). Therefore, the N-terminal region of Mer2 should be phosphorylated by kinase(s) other than CDK. Lack of phosphorylations in Mer2S30, 271A in *bob1-1 cdc7Δ* indicates that these potential phosphorylation sites at the N-terminal region of Mer2 are phosphorylated in a Cdc7 kinase-dependent manner (Fig. 3C).

The multiple phosphorylation of the N-terminal region of Mer2 contributes to spore viability and DSB formation

To identify the phosphorylation sites at the N-terminal region of Mer2 in detail, we constructed various *mer2* mutants in which potential phosphorylation sites are substituted for alanine residues (Fig. 4A). Since it is known that serine rather than threonine or tyrosine is likely a more preferred phosphorylation target by Cdc7, we focused on the serine residues in the N terminus of Mer2 (Jiang et al. 1999; Cho et al. 2006). Among the *mer2* mutants, the *mer2S11A* mutant conferred almost wild-type levels of normal asci formation and spore viability

sites, and that the spore defect is not due to lack of the phosphorylation by CDK (Fig. 4C). The reduced spore viability in *mer2S11, 15, 19, 22A* mutants is mostly owing to an increase in the frequency of two- and zero-spore-viable tetrads (83.3%), with little or no increase in one- and three-spore-viable tetrads. This suggests that the phosphorylations at those sites are involved in the proper disjunction of homologous chromosomes during meiosis I. Importantly, the *mer2S29A* mutation, an alanine substitution of Ser29 adjacent to the CDK-dependent Ser30 site, conferred a complete loss of spore viability, indicating that Ser29 is essential for viable spore formation (Fig. 4A, top panel). *Mer2S11, 15, 19, 22, 29A* quintuple substitution protein migrated even faster than *Mer2S11, 15, 19, 22A*, at the position identical to that in the *bob1-1 cdc7Δ* mutant (Fig. 4C, lanes 8,5). These results indicate that the Cdc7-dependent phosphorylation sites revealed by electrophoretic mobility shift assay can be fully attributed to phosphorylation at the five serines (Ser11, Ser15, Ser19, Ser22, and Ser29) in the Mer2 N-terminal region.

We next examined whether Mer2 protein ectopically expressed in mitosis could be phosphorylated in a Cdc7-dependent manner. When Mer2 protein was overex-

pressed under the control of *ADH1* promoter in mitotic log-phase cells, we detected the small but significant amount of a slower migrating form of Mer2 (Fig. 4D, lane 2, asterisk). This form was abolished in *bob1-1 cdc7Δ* and in the *mer2S11, 15, 19A* mutant, indicating that the N-terminal region of Mer2 is phosphorylated in mitosis in a Cdc7-dependent manner. Interestingly, in comparison with meiotic Mer2-3Flag, we could not detect the Ser30 phosphorylation of Mer2 in mitosis, although levels of the S-phase cyclin remained high throughout DNA replication and G2 phase (Fig. 4D, bottom panel). This result suggests that the Ser30 phosphorylation by CDK is regulated in a meiosis-specific manner (see Discussion).

Cdc7-dependent phosphorylation of Mre2 is pivotal for meiotic recombination

As shown above, the Cdc7-dependent phosphorylation in the N-terminal region of Mer2 is critical for the spore viability. In addition, Cdc7 is required for the meiotic DSB formation and recombination. Thus, Cdc7-dependent phosphorylation of Mer2 is likely to be crucial for meiotic DSB formation and recombination. To test this notion, we examined the effects of the phosphorylation site mutations on meiotic DSB formation and recombi-

nation. The recombination frequency at the *ARG4* locus (revealed by generation of Arg⁺ prototrophs) was reduced to various extents in the alanine-substituted *mer2* mutants (Fig. 5A). The recombination frequency at *ARG4* was only slightly reduced in *mer2S11A* and *mer2S11, 15A* mutants (*mer2S11A*, 7.9%; *mer2S11, 15A*, 7.7%; wild type, 8.6%) (Fig. 5A). The *mer2S11, 15, 19A* triple mutant showed a partial reduction in recombination frequency (4.4%, nearly half of the wild-type level) (Fig. 5A). The *mer2S11, 15, 19, 22A* quadruple mutant conferred a more severe defect in meiotic recombination frequency (3.3%; i.e., 38% of the wild-type level) (Fig. 5A). Importantly, both the *mer2S29A* single and the *mer2S11, 15, 19, 22, 29A* quintuple mutants showed almost complete loss of meiotic recombination frequency (Fig. 5A,B). Consistent with these results, in the *mer2S29A* single and the *mer2S11, 15, 19, 22, 29A* quintuple mutants, meiotic DSB formation at *YCR048w*, *CYS3*, and *GAT1* hot spots was almost completely abolished (Fig. 5B,C). The *mer2S11, 15, 19, 22A* quadruple mutant exhibited a partial but severe defect (one of four at *YCR048w* and one of 10 at *GAT1* hot spots of the wild-type level) in meiotic DSB formation at *YCR048w* and *GAT1* hot spots (Fig. 5B,C), despite the fact that the high level of phosphorylation at Ser30 by CDK could be still detected in the *mer2S11, 15, 19, 22A* mutant

Figure 5. Recombination frequency and DSB formation in mutants substituted for sites phosphorylated dependent on Cdc7. (A) Measurement of recombination frequency at the *arg4* locus. Cells were plated on media lacking arginine and on complete media and incubated for 4 d at 30°C. (B) Detection of DSB formation around the *YCR048w*, *CYS3*, and *GAT1* regions. All strains (YHS1198, YHS1196, and YHS1204) used here were of the *sae2Δ::CgLEU* background. The experiment was conducted twice and gave the same profile. (C) Quantification of band intensities of a prominent DSB site (indicated by black arrow in B) of *YCR048w* (top graph) and *GAT1* (bottom graph) loci at 6 h of meiosis in the mutants shown in B.

protein (Fig. 4C). These data clearly demonstrate that the Cdc7-dependent phosphorylation of Mer2 plays crucial roles in meiotic DSB formation and recombination. Moreover, it is very likely that meiotic DSB formation and recombination require both the cumulative phosphorylations in the N-terminal region of Mer2 and a critical single phosphorylation at Ser29, which all depend on Cdc7.

As shown in Figure 2, the meiotic induction of *NDT80*-dependent transcription is possibly mediated by the Cdc7-dependent phosphorylation of Mer2. However, the *mer2* mutants with alanine substitutions at the Cdc7-dependent phosphorylation sites could produce asci with four spores. Therefore, there may be alternative Cdc7 targets involved in the activation of the *NDT80*-dependent transcription.

Cdc7-dependent phosphorylation of Mer2 facilitates Spo11 binding to DSB sites

It has been demonstrated that Spo11 binds DSB hot spots when meiotic DSBs are formed and then dissociates from covalent Spo11–DNA intermediates by unknown exonucleases (Neale et al. 2005; Prieler et al. 2005; Sasanauma et al. 2007). We examined whether Cdc7 kinase activity is required for the Spo11 binding to DSB hot spots by conducting chromatin immunoprecipitation (ChIP) analyses. In the *bob1-1* strain, Spo11-3Flag transiently bound the *YCR048w* hot spot, peaking at 3 h of meiosis (Fig. 6A, top right graph). However, little association of Spo11-3Flag at the same DSB site was observed in *bob1-1 cdc7Δ* (Fig. 6A, top middle graph). Moreover, we observed no association of Spo11-3Flag with DSB hot spot in the *mer2S11, 15, 19, 22, 29A* mutant (Fig. 6A, top right graph).

To understand the regulatory mechanism of Spo11 binding to DSB hot spots, we next examined the loading of Rec114-3Flag and Mei4-3Flag to the DSB site in the *bob1-1 cdc7Δ* mutant during meiosis. The Rec114-3Flag and Mei4-3Flag were shown to bind the *YCR048w* hot spot with kinetics similar to that of Spo11 in wild type. Using high-resolution genome tiling arrays, we confirmed the binding of Rec114-3Flag and Mei4-3Flag around the *YCR048w* hot spot at 4 h of meiosis (data not shown). In the *bob1-1 cdc7Δ* mutant, Rec114-3Flag was loaded onto the *YCR048w* hot spot, but the binding was reduced to one-fourth of the wild-type level (Fig. 6B, middle). The similar level of reduction in Rec114-3Flag binding was observed in *mer2Δ* (Fig. 6B, right). These results suggest that the Cdc7-dependent phosphorylation of Mer2 may facilitate the loading of Rec114 to DSB hot spots. Mei4-3Flag efficiently bound the *YCR048w* hot spot in wild type, but not in *bob1-1 cdc7Δ* (Fig. 6C). This result suggests that Mer2 phosphorylation may be required for Mei4 binding to DSB sites. We could detect the meiosis-specific ChIP signal of Mei4-3Flag and Rec114-3Flag to the *YCR048w* hot spot in *spo11Δ*, indicating that hot spot binding is independent of Spo11 protein (Fig. 6D). Therefore, it is likely that the loading of Rec114 and Mei4 to DSB sites precedes the binding of

Spo11 or Spo11–Rec102–Rec104–Ski8 subcomplex to DSB hot spots.

In fission yeast, the accumulation of *Rec12-Flag* (a homolog of Spo11 in fission yeast) at the *mbs1* locus (a natural recombination hot spot) was detected in a *rad50s* background (Fig. 6A, bottom left graph). On the other hand, in the *hsk1-89* mutant (a temperature-sensitive mutant of Cdc7 ortholog in fission yeast), no binding was detected at a restrictive temperature, consistent with the findings in budding yeast (Fig. 6A, bottom right panel; Ogino et al. 2006). These results indicate that Cdc7 kinase has an essential role in Spo11 loading to recombination hot spots in both budding and fission yeasts.

Discussion

In this study, we showed that Cdc7 plays an essential role in the regulation of meiotic DSB formation via phosphorylation of the N-terminal region of Mer2. We also demonstrated that Cdc7 is required for the entry into meiosis I via the proper induction of sporulation genes. Thus, this study provides the evidence that Cdc7 kinase participates in the dual meiotic regulations for the initiation of meiotic recombination and later meiotic progression into meiosis I.

Role of Cdc7 in the progression of meiosis I

Our previous studies have indicated that fission yeast Hsk1 is essential for progression of meiosis, since *hsk1* mutant cells uniformly arrested meiosis with one nucleus (Ogino et al. 2006). We show here that Cdc7 kinase of budding yeast is also critical for the progression of meiosis I. The cells lacking Cdc7 kinase arrested with one nucleus, and the induction of *Ndt80*, an essential regulator for the progression of meiosis I, is severely impaired (Figs. 1, 2). Transcription of *NDT80* is regulated by several pathways. *Ndt80* is activated through the phosphorylation by Ime2, a meiotic kinase required for the initiation of replication (Mitchell et al. 1990; Hepworth et al. 1998). Since replication proceeds normally in cells lacking Cdc7 kinase, Ime2 kinase is supposed to be almost fully activated (Benjamin et al. 2003). Thus, the poor induction of *NDT80* and other defects observed in *bob1-1 cdc7Δ* may not be due to the lack of Ime2 activity. Alternatively, it is likely that some unknown targets of Cdc7 kinase may play crucial roles in meiotic induction of middle and middle-late meiotic genes including *Ndt80*-regulon.

Intriguingly, the *bob1-1 cdc7Δ* diploids formed some asci with dyads after prolonged incubation (>30% asci had dyads) (Supplemental Fig. S1). Microscopic analysis using chromosome tagging by the chromosome tagging system has revealed that the homologous chromosomes segregate equationally in this situation (Valentin et al. 2006; T. Fukuda, H. Sasanauma, and K. Ohta, unpubl.). This result raises the possibility that Cdc7 kinase has other targets to facilitate reductional segregation of homologous chromosomes. Alternatively, the delayed induction of *NDT80* may cause the equational segregation.

Figure 6. ChIP of Spo11, Rec12 (in fission yeast), Rec114, and Mei4 at recombination hot spot. (A) The binding of Spo11/Rec12 with the hot spots in *bob1-1 cdc7Δ*, *mer2Δ* S11, 15, 19, 22, 29A, and *hsk1-89* mutants. The top graphs show the kinetics of Spo11-3FLAG binding to the YCR048w (hot spot) and the SMC1 (cold spot) regions during meiosis. The bottom graphs indicate Rec12-Flag binding to *mbs1* (hot spot) and *fbp1* (cold spot). In fission yeast, the accumulated Rec12-3Flag association is detected by introducing the *rad50S* mutation. G1 arrest and meiotic induction of the *pat1-114* strain were carried out as described previously (Ogino et al. 2006). The strains used were YHS916 (*bob1-1 SPO11-3FLAG*), YHS896 (*bob1-1 cdc7Δ SPO11-3FLAG*), YHS1222 (*mer2Δ* S11, 15, 19, 22, 29A *SPO11-3FLAG*), PKH50 (*rec12-flag*), PKH253 (*hsk1-89 rec12-flag*), YHS866 (*bob1-1 cdc7Δ REC114-3FLAG*), YHS747 (*REC114-3FLAG mer2Δ*), and YHS777 (*bob1-1 cdc7Δ MEI4-3FLAG*). The experiment was conducted at least twice and gave the same profile. (B–D) The association of Rec114 and Mei4 with the YCR048w and SMC1 loci. (B) Rec114-3Flag binding in the wild-type (left), *bob1-1 cdc7Δ* (middle), and *mer2Δ* (right) background. (C) Mei4-3Flag binding in wild-type (left) and *bob1-1 cdc7Δ* (right). (D) Rec114-3Flag (left) and Mei4-3Flag (right) binding in *spo11Δ*. ChIP was performed as for A.

It is intriguing to investigate how Cdc7 regulates modes of chromosome segregation.

Cdc7-dependent phosphorylation of Mer2 is crucial for meiotic DSB formation

We showed that Cdc7 is pivotal for meiotic DSB formation and recombination (Fig. 1). The TOF-MS analysis

revealed that the N-terminal region of Mer2 harbors multiple phosphorylation sites regulated by Cdc7 kinase in vivo (Fig. 3). The alanine substitution of all serine residues [Ser11, Ser15, Ser19, Ser22, Ser29] in this region reduced Mer2 phosphorylation to the *bob1-1 cdc7Δ* level and conferred severe defects in spore viability, meiotic DSB formation, and recombination, indicating that the Mer2 phosphorylation dependent on Cdc7 kinase is vital

for meiotic DSB formation. In addition, we found that the Ser29 residue is a critically important phosphorylation site, since the substitution at this site to alanine exhibited totally impaired DSB formation (cf. the accompanying study by Wan et al. 2008). Moreover, we showed that Ser11, Ser15, Ser19, and Ser22 residues are important for DSB formation as well. These results suggest two modes of phosphorylation in the N-terminal region of Mer2: a critical single phosphorylation site at Ser29 and cumulative phosphorylation sites. In vitro kinase assay revealed that purified Mer2 can be directly phosphorylated by Cdc7–Dbf4 (data not shown).

The N-terminal regions of Mcm2 and Mcm4, which are required for DNA replication, are also known to be directly phosphorylated by Cdc7 (Cho et al. 2006; Masai et al. 2006; Montagnoli et al. 2006). CDK can phosphorylate substrates containing a minimum consensus sequence (Ser/Thr*–Pro) (Ubersax et al. 2003). Cdc7 may preferentially phosphorylate the first serine residue of the “Ser–Ser/Thr–Pro” consensus sequence that has been subjected to prior phosphorylation by CDK. The coordinating sufficient levels of N-terminal phosphorylations of Mcm proteins by Cdc7 kinase and CDK facilitate the assembly of replication complex to initiate DNA replication (Masai et al. 2006; Sheu and Stillman 2006). In the case of Mer2, the “Ser–Ser/Thr–Pro” consensus sequences are located in the N-terminal region (Fig. 3E). Ser29 and Ser30 of Mer2 protein correspond to the first and second serine residues of this consensus sequence, respectively. The latter is known to be phosphorylated by CDK (Henderson et al. 2006). Therefore, these results implicate that Mer2 may be also phosphorylated in a stepwise manner by Cdc7 and CDK, as in the case of Mcm proteins (cf. the accompanying study by Wan et al. 2008).

Furthermore, the present results lead us to speculate a role of cumulative phosphorylations of the remaining N-terminal Ser11, Ser15, Ser19, and Ser22. After the stepwise phosphorylation at Ser29 and Ser30, the N-terminal region may be converted to more preferred targets for Cdc7 kinase due to an increase in negative charge, leading to further phosphorylation of this N-terminal segment by Cdc7. Alternatively, the Ser11, Ser15, Ser19, Ser22, and Ser29 residues may be phosphorylated independently of CDK. However, the latter possibility is unlikely, since the phosphorylations at Ser11, Ser15, and Ser19 on Mer2 were substantially reduced in mitotic cells, in which the phosphorylation of Ser30 is absent (Fig. 4D). Therefore, it is most likely that the Cdc7-dependent phosphorylation in the Mer2 N-terminal region progressively occurs after the priming phosphorylation by CDK. It is intriguing to investigate the coordinated fashion of Mer2 phosphorylation by Cdc7 and CDK kinases (cf. the accompanying study by Wan et al. 2008).

It is possible that other targets of Cdc7-dependent phosphorylation may be involved in DSB formation. For example, Rec114 protein is also phosphorylated depending on Cdc7 in meiosis (Supplemental Fig. S3). Moreover, it has been reported that Rec104, a factor interacting with Spo11, is also phosphorylated in meiosis (Kee et al.

2004). These results raise the possibility that the establishment and activation of the pre-DSB complex at DSB hot spots is under integrated regulation via phosphorylations on various factors. This notion is very similar to the regulation of the prereplicative complex (pre-RC) at replication origins that involves multiple layers of phosphorylation. Our results demonstrate that two sets of kinases, CDK and Cdc7–Dbf4 (DDK), are commonly involved in both meiotic DSB formation and replication. It is an interesting future study to investigate the common molecular basis of the activation of pre-DSB and pre-RC complex by combined actions of CDK and Cdc7.

The loading mechanism of Spo11 on meiotic recombination hot spot

Given that Mer2 protein is regulated by Cdc7 and CDK via phosphorylation, the next question is how Mer2 regulates DSB formation. We demonstrated that the *mer2S11, 15, 19, 22, 29A* mutant or the cells lacking Cdc7 kinase fail to load Spo11 to meiotic DSB sites, indicating that the defect of DSB formation is caused by the lack of the Spo11 binding to DSB hot spots (Fig. 6). Previous studies had revealed that the binding of Spo11 to DSB hot spots requires at least three proteins: Rec102, Rec104, and Rec114 (Prieler et al. 2005). Rec102 and Rec104 can form a subcomplex with Spo11 (Kee et al. 2004). Recently, it has been reported that Rec114 interacts with Mei4 and Mer2 to form another type of subcomplex in meiosis (Li et al. 2006). In this study, we demonstrated that Rec114 and Mei4 bind to DSB hot spots with kinetics similar to that of meiotic DSB formation. In addition, we found that Rec114 and Mei4 bound to DSB hot spots in the *spo11Δ* mutant (Fig. 6D). Moreover, the chromatin binding of Rec114 occurs independently of Spo11 and most of the other DSB factors (Maleki et al. 2007). All these results suggest that Rec114 (or Rec114–Mer2–Mei4 complex) acts as a regulatory subunit for the Spo11 loading to DSB sites (Fig. 7). Importantly, the chromatin binding of both Rec114 and Mei4 was impaired in *bob1-1 cdc7Δ*. Therefore, Cdc7-dependent phosphorylation of Mer2 may contribute to stabilize or enhance the assembly of the Rec114–Mer2–Mei4 complex on chromatin around DSB sites (Fig. 7).

Temporal regulation of DNA replication and DSB formation by CDK and DDK

We demonstrated the essential role of Cdc7 in meiotic DSB formation. Taken together with previous studies, it is most likely that the activities of both CDK and Cdc7 are needed for the phosphorylation of Mer2 during meiosis, and thereby facilitate meiotic DSB formation. Given such dual regulation by distinct entities of kinases, we speculate about the physiological significance of the regulation. The activity of CDK is required for various meiotic events such as meiotic chromosome segregation, premeiotic DNA replication, or DSB formation in concert with other kinases (Benjamin et al. 2003; Brar et

Figure 7. A schematic model of establishment of pre-DSB complex at meiotic DSB hot spot. Following the progression of the replication fork and opening of the chromatin structure, the Rec114–Mer2–Mei4 complex is assembled at the DSB hot spot region (Step I), and undergoes CDK- and Cdc7-dependent phosphorylation of Mer2, which may stabilize the complex on the chromatin. Then the Spo11 complex, including Rec102, Rec104, and Ski8, is loaded onto hot spot (Step II). The Rec114–Mer2–Mei4 complex possibly serves as a scaffold that retains the Spo11 complex at the DSB hot spot. Finally, an activated complex would stimulate Spo11-catalyzed DSB formation.

al. 2006; Henderson et al. 2006). In order to regulate DSB formation, it is known that the activity of CDK with Clb5, 6 is required for the Mer2 phosphorylation besides its function in meiotic DNA replication. Therefore, it is possible that the activity of CDK serves to coordinate DSB formation and premeiotic DNA replication via Mer2 phosphorylation. Since Mer2 expressed in mitotic cells was not phosphorylated at Ser30, Mer2 phosphorylation by CDK might be regulated so that it occurs strictly during meiosis.

On the other hand, Borde et al. (2000) suggested that DSB formation is also regulated to occur only in the regions where the DNA replication forks have passed through. Thus, some unknown mechanisms modulate DSB formation at the chromosomal level. DDK has important roles in S-phase checkpoint response in addition to replication initiation (Kim et al. 2003b; Kim and Masai 2004; Matsumoto et al. 2005; Sommariva et al. 2005). These results suggest that DDK monitors the progression of the replication forks. Thus, we speculate that Cdc7, through its association with replication forks, phosphorylates Mer2 in a manner coupled to the progression of replication forks, permitting DSB formation only within the replicated regions. This notion seems to be consistent with our chromosome-wide DNA microarray

ChIP analysis indicating that the distribution of Spo11 along the chromosomes gradually spreads from early-replicating to late-replicating regions (K. Kugou, T. Fukuda, H. Sasanuma, S. Mori, Y. Katou, T. Itoh, K. Matsumoto, T. Shibata, K. Shirahige, and K. Ohta, unpubl.). Therefore, we propose that Cdc7 functions as a coordinator for the interplay between replication and regional control of meiotic chromosomal events, which may ensure the strict temporal order of meiotic events.

We also showed that Hsk1 kinase is vital for the recruitment of Spo11 to a DSB site in the distantly related fission yeast, suggesting that this regulatory mechanism may be conserved in other eukaryotes. In fission yeast, the obvious counterpart of Mer2 has not been found yet. Thus, we speculate that other meiotic regulator(s) or functional homologs of Mer2 may be involved in Hsk1-mediated regulation of meiotic recombination in fission yeast. Further elucidation of the regulatory mechanism of DSB formation by Cdc7 kinase would shed light on the conserved system for regulation of meiotic recombination initiation.

Materials and methods

Yeast strains and plasmid construction

The *MCM5* gene was cloned in pRS306 carrying a *URA3* marker (Sikorski and Hieter 1989), and the mutations (cytosine to thymine at base 248 and adenine to guanine at base 246 from the start codon, called the *bob1-1* mutation) were introduced to generate the *bob1* mutation (P83L) and a *SacI* cutting site. The resultant plasmid pRS306-*bob1-1* was linearized by *SnaBI* treatment and transformed in wild-type haploid cells. The transformant carrying two copies of the *MCM5* gene and *URA3* marker were streaked on the plate containing 5-FOA to pop out a wild-type copy of the *MCM5* gene, and the *bob1-1* mutant was obtained. The *CDC7* gene was deleted with a *kanMX4* marker in the *bob1-1* background. To construct Flag-tagged strains, we used the standard PCR-mediated integration method (De Antoni and Gallwitz 2000). The resulting *REC114-3FLAG* and *MEI4-3FLAG* diploid strains showed normal spore formation and spore viability, but the *MER2-3FLAG* diploid strain showed a slight reduction in spore viability compared with wild type. The *MER2* gene containing the promoter region (upstream 500 base pairs [bp]) was cloned in pRS306, and the various point mutations were introduced. To construct tagged *mer2* mutants, the 3xFlag sequence was conjugated at the end of the *mer2* ORF by PCR. The resultant plasmid pURS306-U500*mer2-3FLAG* was linearized by *EcoRI* treatment, integrated into a haploid cell, and further popped out by counterselection on the plate containing 5-FOA. The *cMER2* gene was amplified by PCR from a cDNA library generated from an RNA sample of 4 h in meiosis and cloned in the *KpnI/SacI* site of pAUR123 (TaKaRa-Bio Co. Ltd.). The *BamHI* fragment containing the *ADH1* promoter, *MER2*cDNA, and a terminator cassette was swapped to the YCp50 vector to express in yeast.

Immunoprecipitation (IP), Western blotting, and antibodies

IP was performed as described previously (Sasanuma et al. 2007), with a slight modification. IP was conducted using anti-Flag M2 monoclonal antibody-conjugated Sepharose beads (Sigma). To treat the IP fraction with CIAP (TaKaRa-Bio Co. Ltd.), the immunoprecipitates were washed well with NEB buffer #3 (50 mM Tris-Cl at pH 7.9, 10 mM MgCl₂, 100 mM NaCl, 1 mM DTT) to

remove inhibitors against CIAP in lysis buffer completely. After eluting with 150 μ g/mL 3xFlag peptide (Sigma), the IP fraction was divided into three aliquots. The first aliquot was added to buffer (untreated), the second was treated with 36 U of CIAP, and the third was treated with the phosphatase plus inhibitors (20 mM NaVO₃, 100 mM NaF, 50 mM EDTA). Each aliquot was incubated for 2 h at 37°C. SDS-PAGE was performed by low electrical current to detect the subtle mobility shift of phosphorylated Mer2. Anti-phosphorylated Mer2 antibody (anti-S30^P; Abcam) was used at 1/5000 dilution.

TOF-MS analysis

For TOF-MS analysis to identify the phosphorylation site of Mer2, meiotic cell lysate expressing Mer2-3Flag was prepared from 6 L of SPM culture (YHS35; the diploid *MER2-3FLAG* strain) ($\sim 1.2 \times 10^{11}$ cells) and incubated with anti-Flag agarose beads for 5 h at 4°C. After washing several times with lysis buffer, Mer2-3Flag was eluted with 3xFlag peptide. After the IP fraction was separated by a 4%–20% gradient polyacrylamide gel (Dai-ichikagaku), the gel was stained with a silver staining KIT (Wako). The band corresponding to Mer2-3Flag protein was excised from several lanes, and TOF-MS analysis was performed by Aprosience Co. Ltd..

Northern blotting

Total RNA was prepared from $\sim 2 \times 10^8$ cells according to the method described in Elder et al. (1983). For Northern analysis, 10 μ g of total RNA was denatured with formamide, separated on 1% agarose gels containing formaldehyde, and blotted on a charged nylon membrane (BioDyne B membrane; PALL, EA). Probes were described previously for *IME2*, *SPS4*, *SPS22/YCL048w*, *DMC1*, and *CMD1* (Kugou et al. 2007). The probes for *SPO11* and *REC102* were prepared from DNA fragments using a random-priming kit (GE Healthcare). The DNA fragment of full-length *SPO11* ORF was used to detect the *SPO11* transcript. The 406-bp EcoRV fragment from a cloned *REC102* gene in pCR2.1 (Invitrogen) was gel-purified and used to detect the *REC102* transcript.

Acknowledgments

We are grateful to W. Lin and other laboratory members for constructive comments on the manuscript. We also thank R. Nakazawa for DNA sequencing and A. Nakamura for cDNA cloning of the *MER2* gene. This work was supported by grants from the following sources: basic research from the Bio-oriented Technology Research Advancement Institution (to T.S. and K.O.), and grants-in-aid for scientific research on priority areas from the Ministry of Education, Science, Culture, and Sports, Japan (to K.O.). We dedicate this manuscript to the memory of Y.K.

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Cdc7-dependent phosphorylation of Mer2 facilitates initiation of yeast meiotic recombination

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Genes Dev. 2008, **22**:

Access the most recent version at doi:[10.1101/gad.1626608](https://doi.org/10.1101/gad.1626608)

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