Dbf4-Dependent Cdc7 Kinase Links DNA Replication to the Segregation of Homologous Chromosomes in Meiosis I

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SUMMARY

Meiosis differs from mitosis in that DNA replication is followed by the segregation of homologous chromosomes but not sister chromatids. This depends on the formation of interhomolog connections through crossover recombination and on the attachment of sister kinetochores to microtubules emanating from the same spindle pole. We show that in yeast, the Dbf4-dependent Cdc7 kinase (DDK) provides a link between premeiotic S phase, recombination, and monopolar attachment. Independently from its established role in initiating DNA replication, DDK promotes double-strand break formation, the first step of recombination, and the recruitment of the monopolin complex to kinetochores, which is essential for monopolar attachment. DDK regulates monopolin localization together with the polo-kinase Cdc5 bound to Spo13, probably through phosphorylation of the monopolin subunit Lrs4. Thus, activation of DDK both initiates DNA replication and commits meiotic cells to reductional chromosome segregation in the first division of meiosis.

INTRODUCTION

Chromosome segregation in mitosis and meiosis depends on the attachment of microtubules to centromeric DNA through large protein complexes called kinetochores. Kinetochore-microtubule interactions are stabilized by tension, which develops when microtubules from opposite spindle poles attach to a pair of physically connected kinetochores. Mitosis generates genetically identical daughter cells because kinetochores on sister chromatids attach to microtubules from opposite spindle poles (bipolar attachment). Sister kinetochores are linked and can develop tension due to ring-shaped cohesin complexes that entrap both sister chromatids during DNA replication. Meiosis generates haploid gametes from diploid germ cells through a single DNA replication phase followed by two rounds of chromosome segregation. Homologous chromosomes (homologs) segregate in meiosis I and sister chromatids disjoin in meiosis II (Petronczki et al., 2003). The meiosis I spindle pulls homologous maternal and paternal centromeres but not sister centromeres in opposite directions because homologs become interconnected after premeiotic DNA replication and sister kinetochores attach to microtubules from the same spindle pole (monopolar attachment). How processes required for the segregation of homologs are coordinated has remained unclear.

Most organisms use reciprocal recombination between maternal and paternal chromatids to create crossovers (visible as chiasmata) that link homologs through sister chromatid cohesion on chromosome arms. Recombination is initiated at DNA double-strand breaks (DSBs) generated by the Spo11 enzyme (Neale and Keeney, 2006) and is facilitated by the synaptonemal complex (SC), a proteinaceous structure that juxtaposes homologs (Page and Hawley, 2004). Processing of DSBs requires recombination enzymes and also cohesin whose Scc1/Rad21 subunit has been replaced by the meiosis-specific Rec8 protein (Klein et al., 1999). Chiasmata enable the meiosis I spindle to pull homologous centromeres in opposite directions but only if sister kinetochores attach to microtubules from the same pole and not from opposite poles as in mitosis or meiosis II (Hauf and Watanabe, 2004). In fission yeast, monopolar attachment depends on sister chromatid cohesion at the inner core of centromeres, which requires meiotic cohesin and Moa1, a meiosis I-specific protein that binds to Rec8 (Watanabe et al., 2001; Yokobayashi and Watanabe, 2005). In budding yeast, both Rec8 and Scc1 can support cohesion and mono-orientation of sister centromeres (Toth et al., 2000). Monopolar attachment depends on the assembly of the monopolin complex from the casein kinase Hrr25, the meiosis I-specific Mam1 protein, and the nucleolar proteins Lsr4 and Csm1 (Petronczki et al., 2006; Rabitsch et al., 2003). Shortly before metaphase I, the polo-like kinase (PLK) Cdc5 triggers the release of Lsr4 and Csm1 from the nucleolus whereupon monopolin localizes to kinetochores (Clyne et al., 2003; Lee and Amon, 2003). Sister centromere cohesion and meiosis I-specific kinetochore modification may represent two aspects of a general mechanism for monopolar attachment. This idea implies, however, the involvement of conserved but hitherto unknown proteins.

As cells enter anaphase I, homologs disjoin because a protease called separase cleaves cohesin’s Rec8 subunit on...
chromosomal arms and thereby resolves chiasmata (Buonomo et al., 2000). Separase is activated upon degradation of its inhibitor Pds1/securin, which results from ubiquitination by the anaphase-promoting complex (APC/C). During anaphase I, centromeric cohesins is protected from separase by a mechanism that requires Rec8 and the kinetochore-associated Sgo1/shugoshin protein (Kitajima et al., 2004). In meiosis II, centromeric cohesins serves to bi-orient sister centromeres (Toth et al., 2000; Watanabe and Nurse, 1999), which are finally disjoined by a second wave of separase activity leading to the formation of haploid gametes.

How premeiotic DNA replication, recombination, and monopolol attachment are initiated in the right order to allow segregation of homologs has remained unclear. Conditions that prevent entry into premeiotic S phase also block DSB formation. One proposal is that DSB formation requires a change in chromatin structure that occurs during the process of DNA replication (Borde et al., 2000). However, basic replication factors are not essential for DSB formation (Hochwagen et al., 2005; Murakami and Nurse, 2001). This led to the view of DNA replication and DSB formation as independent events, which are initiated by a common mechanism. Indeed, the cyclin-dependent kinase (CDK) that promotes S phase also promotes Spo11 activity (Henderson et al., 2006; Smith et al., 2001). Whether regulators of DNA replication and recombination also control monopolol attachment was unclear.

We have analyzed the role of the Dbf4-dependent Cdc7 kinase (DDK) from budding yeast in meiotic chromosome segregation. DDK is known to be essential for the initiation of DNA replication (Scalfani, 2000). The kinase is activated upon binding of the catalytic Cdc7 subunit to the unstable Dbf4 protein and associates with replication origins where it phosphorylates components of the prereplicative complex including the MCM helicase. We show that DDK has postreplicative functions in meiosis: it is essential for DSB formation and kinetochore localization of monopolol. Thus, activation of DDK both initiates DNA replication and commits meiotic cells to reductional chromosome segregation in meiosis I.

RESULTS

Association of the DDK Cdc7-Dbf4 with the PLK Cdc5 in Meiosis

To identify novel regulators of meiotic chromosome segregation, we analyzed proteins that associate with the budding yeast PLK Cdc5. We prepared extracts from diploid cells arrested in metaphase I through meiotic deletion of the APC/C activator Cdc20 and isolated Cdc5 by tandem affinity purification (Rigaut et al., 1999; Figure S1A available with this article online). Mass spectrometric analysis identified several proteins with known functions in meiotic chromosome segregation including APC/C subunits, cohesin components, and Spo13, a regulator of centromeric cohesin (Table S1). In addition, we detected both components of the Dbf4-dependent Cdc7 kinase (DDK), which is essential for the initiation of DNA replication. To confirm this interaction, we purified Cdc7 from Cdc20-depleted cells (Figure S1B). Mass spectrometry identified Dbf4 and Cdc5 among the most abundant proteins that specifically copurify with Cdc7 (Table S2).

Upon induction of meiosis, Cdc7 associates first with Dbf4 (to form DDK) and then with Cdc5 (Figure 1A). It is only during metaphase I that all three proteins are present in the nucleus (Figure S1C). The Cdc7−Cdc5 interaction does not depend on the catalytic activity of either kinase (Figures S2A and S2B). It requires the presence of Dbf4 and the integrity of Cdc5’s polo-box domain (PBD) (Figures S2C and S2D), which can bind to DDK by itself (Figure S2E). This suggests that Cdc5 binds to DDK through an interaction between PBD and Dbf4. Consistent with this, phosphorylation of Dbf4 not only requires Cdc7 (Weinreich and Stillman, 1999) (Figure S2A, arrows) but is further enhanced in a Cdc5-dependent manner (Figure S2F, arrow). These data show that DDK binds to Cdc5 in metaphase I and raise the possibility that DDK has postreplicative functions in meiosis.

Meiosis with Reduced or Absent DDK Activity Generates Diploid Viable Spores

To investigate whether DDK has meiotic functions beyond DNA replication, we analyzed temperature-sensitive cdc7-4 cells, which grow and sporulate normally at 25°C (89% tetrads, 87% viable spores) but arrest in prophase I after induction of meiosis at 34°C (Figure 1B and data not shown). At 31°C, the mutant cells replicated DNA normally but formed only two, mostly viable spores (81% dyads, 85% viable spores) (Figure 1B). The two equal-sized nuclei resulted from the segregation of sister chromatids as revealed by genetic analysis of heterozygous loci on six different chromosomes and by marking one chromosome V homolog with green fluorescent protein (GFP) (Table S3 and Figure S3) at 31°C. The mutant cells produced two diploid spores similar to cdc7-4 cells at 31°C (Figures 1C and 1D), suggesting that reduced levels of DDK activity are sufficient for DNA replication but not for reductional chromosome segregation in meiosis.

To study meiosis in the absence of DDK, we used the bob1 (mcm5-P83L) mutation in the MCM helicase, which bypasses DDK’s essential role in DNA replication (Hardy et al., 1997). bob1 cells sporulated normally and generated four haploid spores (78% tetrads; 91% viable spores) (Figure 1E, panel 1). In contrast, bob1 cdc7-1 and bob1 dbf4-4 cells produced two diploid spores (~74% dyads, ~80% viable spores) with genetic markers similar to those of the “mother” cell (Figure 1E, panels 2 and 3; Table S3). Wild-type Cdc7 but not the kinase-dead cdc7-1 allele restored formation of four-sporated asc in bob1 cdc7-1 cells (Figure 1E, panels 4 and 5). We conclude that DDK activity is essential for reductional chromosome segregation. Time course experiments revealed that bob1 cdc7-1 cells undergo premeiotic DNA replication with normal kinetics (see Figure 2A), express early meiotic proteins (e.g., Spo11, Rec8, and Zip1) on time, and produce proteins important for nuclear division (e.g., Ndt80, Cib1, Cdc5, and Cdc20) (Figure 1F and data not shown). This suggests that the phenotype of DDK mutants does not result from a general defect in meiotic gene expression.
Control of DSB Formation by DDK
cdc7 mutants were shown to lack DSBs (Sasanuma et al., 2008; Wan et al., 2008, 2006). However, the mutants failed to enter metaphase I, probably due to the activation of checkpoint pathways that monitor DNA replication. To test whether DDK is required for recombination also in cells that progress through meiosis, we analyzed DSB-dependent structures on chromosome spreads. Prophase spreads from bob1 cells showed 5–10 foci of the Rad51 recombine and SCs containing the Zip1 protein. In contrast, bob1 cdc7Δ strains lacked Rad51 foci and Zip1 was found in extrachromosomal aggregates (polycomplexes) although Spo11 still associated with chromatin (Figures 2A and S3A). DNA blotting confirmed that bob1:cdc7Δ cells lack DSBs (Figure 2C) suggesting a direct, checkpoint-independent role for DDK in DSB formation. To investigate whether recombination depends on DDK activity during S phase, we constructed bob1 cells expressing CDC7 solely from the prophase I-specific NDT80 promoter (Chu and Herskowitz, 1998). In bob1 PNDT80-CDC7 cells, Cdc7 accumulated together with the Ndt80-dependent Cdc5 protein, which is known to appear in cdc7-4 strains initiated by postreplicative DDK activity. In Cdc7 (Figure 2C). These data suggest that DSB formation can be initiated by postreplicative DDK activity. In Cdc7 (Figure 2C). These data suggest that DSB formation can

After S phase, Tetr-RFP bound to tetO generates one (sisters together) or two (sisters apart) fluorescent dots. Free Tetr-RFP generates a diffuse signal that was used to follow nuclear divisions. In addition, the strains expressed GFP-tagged versions of tubulin and the Cdc14 phosphatase. Cdc14 is released from the nucleolus in early anaphase and recaptured in late anaphase during both meiosis I and II (Marston et al., 2003), which provides a marker for meiotic progression that is independent from spindle morphology.

The DBF4-ha3 and the bob1 control strains formed spindles with similar kinetics as the wild-type (Figures 3A and S4A). Sister URA3-tetO sequences remained tightly associated and cosegregated to the same spindle pole during the first Cdc14 release in anaphase I (>95% reductional segregation). During the second Cdc14 release in anaphase II, sister URA3-tetO sequences segregated to opposite poles on one of the two spindle axes (>95% equational segregation). DDK mutants (cdc7-4, P40 DBF4-ha3, bob1 cdc7Δ, and bob1 dbf4Δ) assembled metaphase I spindles efficiently, albeit with a delay of ~1 hr, and released Cdc14 twice, which indicates progression through meiosis I and II (Figures 3A and S4A). While sister URA3-tetO sequences stayed together in metaphase I, they prematurely split in many of the mutant cells during the first Cdc14 release (cdc7-4, 27%; P40-DBF4-ha3, 34%; and bob1 cdc7Δ, 65%). However, elongation of the metaphase I spindle and nuclear division both failed (>95% mononucleate). The spindle persisted until it finally elongated during the second Cdc14 release, which was accompanied by nuclear division and segregation of sister chromatids to opposite poles (>90% equational segregation). These data suggest that DDK mutants fail to segregate homologs during anaphase I but segregate sister chromatids with high fidelity on a single spindle axis in meiosis II. The meiosis I defect of DDK mutants resembles that of monopolin mutants, which attempt to segregate sister chromatids in anaphase I. The persistence of centromeric cohesion prevents nuclear division but bipolar spindle forces overwhelm pericentric cohesion, which results in premature splitting of URA3 sister sequences (Toth et al., 2000). In meiosis II, however, DDK mutants differ from monopolin mutants, which undergo a tetrapolar division and produce inviable spores.

A defect in the second round of spindle pole body (SPB) duplication could explain why DDK mutants contain only a single spindle axis in meiosis II. To investigate this, we filmed the SPB protein Cnm67-RFP together with GFP-tubulin (Figure S4B). In metaphase I, wild-type and bob1 cells contain two SPBs, which reduplicate in metaphase II, after completion of the meiosis I division. Also cdc7-4 and bob1 cdc7Δ mutants underwent two distinct SPB duplication events. However, in the absence

Figure 1. Analysis of DDK Function in Meiosis
(A) DDK binds to Cdc5. Immunoblots are shown of extracts and anti-Myc immunoprecipitates (IPs) prepared from wild-type (WT, Z2314) and CDC7-myc9 (Z7103) cells at different times after transfer to sporulation medium (SPM).
(B) Sporulation of wild-type (Z4834) and cdc7Δ (Z8152) cells at 25 and 31°C. Ascii were dissected after 24 hr to determine spore viability or fixed to visualize DNA and Tetr-GFP at heterozygous URA3-tetO.
(C and D) Meiosis at 30°C in DBF4-ha3 cells (Z13216) and in cells expressing DBF4-ha3 from a 40 bp-promoter (P40-DBF4-ha3, Z13218). (C) Ascii were analyzed as in (B). (D) Immunoblot detection of proteins. (E) Strains with (1) bob1 (Z10436), (2) bob1 dbf4Δ (Z10508), (3) bob1 cdc7Δ (Z10438), (4) bob1 cdc7Δ::CDC7 (Z10440), or (5) bob1 cdc7Δ::cdc7-kd (kinase-dead, Z11284) were sporulated at 30°C. Ascii were analyzed as in (B).
(F) Immunoblot analysis of extracts from meiotic bob1 (Z9063) and bob1 cdc7Δ (Z9064) cells.
of a meiosis I division, the second round of SPB duplication occurred in mononucleated cells containing a short spindle. Although the SPBs born in meiosis II nucleated short microtubules, they failed to establish stable spindles with their mother SPBs from meiosis I. These data suggest that the persistence of the meiosis I spindle in DDK mutants does not result from a defect in SPB reduplication.

**DDK Mutants Undergo Two Rounds of Pds1 Degradation and Cohesin Cleavage**

To analyze the behavior of Pds1, we detected Pds1-myc18 and spindles in fixed cells by immunofluorescence microscopy (Figure 3B). In addition, we imaged Pds1-RFP and GFP-tubulin during meiosis I in living cells (Figure SSA). In the wild-type, Pds1 degradation results in prompt elongation of the spindle. In cdc7-4 mutants, Pds1 degradation was delayed but once initiated, it occurred at near-normal rates. However, it failed to trigger spindle elongation, leading to the accumulation of Pds1-negative cells with a short spindle and two SPBs. This suggests that the anaphase I-defect of cdc7-4 mutants does not result from a failure to degrade Pds1. cdc7-4 cells re-accumulate Pds1 in metaphase II as indicated by the presence of Pds1 in mononucleate cells with four SPBs. Subsequent degradation of Pds1 triggered the sole nuclear division in these cells. To confirm Pds1's re-accumulation in metaphase II, we detected Mam1, which is degraded in anaphase I (Figure SSB). In the wild-type and the cdc7-4 mutant, we found high levels of Pds1 in ~30% of Mam1-negative cells containing short spindles (metaphase II). These cells were binucleate in the wild-type but mononucleate in the cdc7-4 mutant.

To determine whether Pds1 degradation triggers cohesin removal from chromosomal arms in DDK mutants, we filmed strains expressing Rec8-GFP together with Pds1-RFP and Cnm67-RFP (Figure 3C). Wild-type cells containing Pds1 showed strong Rec8-GFP signals in the form of dots and threads, which represent cohesin bound to chromatin. Degradation of Pds1 was accompanied by a dramatic loss in Rec8-GFP signal intensity and movement of SPBs to opposite poles of the dividing nucleus. However, two Rec8-GFP foci persisted in the vicinity of SPBs until they finally disappeared shortly after SPB reduplication in meiosis II (Figure 3C, arrows). These foci represent centromeric cohesin because they were eliminated upon meiotic depletion of the centromeric cohesin protector Sgo1 (see Figure 4D). In cdc7-4 cells, Pds1 degradation occurred with a delay and failed to trigger spindle elongation but was inevitably accompanied by a sharp drop in Rec8-GFP signal intensity. One or two foci of Rec8-GFP were protected from destruction by an Sgo1-dependent process until the second round of SPB duplication (Figure 3C). Thus, cohesin is destroyed in a stepwise manner in the cdc7-4 mutant as in the wild-type. We confirmed this result by detecting Rec8-ha3 on chromosome spreads (Figure S5C). Spreads with centromeric Rec8 were mostly bilobed in control strains but undivided in cdc7-4 and bob1 cdc7-4 mutants. Bilobed spreads only appeared upon complete removal of Rec8. Our data suggest that in cdc7 mutants, removal of cohesin from chromosomal arms fails to trigger spindle elongation and nuclear division, which only occur upon loss of centromeric cohesion in meiosis II. These results support the idea that bi-orientation of sister kinetochores blocks the meiosis I division in DDK mutants.

**DDK Is Essential for Mono-Orientation of Sister Kinetochores in Meiosis I**

Three criteria have been used to establish that monopolin mutants bi-orient sister kinetochores in meiosis I (Petronczki et al., 2006): (1) Sister kinetochores come under tension in metaphase I. (2) The meiosis I division is blocked even in the absence of chiasmata. (3) “Deprotection” of centromeric cohesion restores the meiosis I division. To determine whether sister kinetochores experience tension in DDK mutants, we looked for the transient splitting of sister centromeres that results from bipolar microtubule forces. We filmed strains in which one chromosome V homolog was marked with GFP at the centromere while Pds1 and Cnm67 were tagged with RFP (Figure 4A). Exit from metaphase I was blocked by eliminating the APC/C activators Cdc20 and Am1. Sister centromeres split only rarely during the metaphase I arrest in control cells. In contrast, sister centromere splitting increased abruptly when cdc7-4 cells entered metaphase I, which satisfies the first criterion. DDK mutants lack chiasmata because they cannot form DSBs. Accordingly, spo11Δ cdc7-4 and spo11Δ bob1 cdc7-4 strains behaved similar to their SPO11 counterparts. They failed to undergo nuclear division in the presence of two SPBs (meiosis I), even after degradation of Pds1, which satisfies the second criterion (Figure 4B, panels 1 and 3). To grant separate access to centromeric cohesion in meiosis I, we used strains that lack Rec8 and instead express the mitotic slecin Scc1 from the REC8 promoter (PREC8-SCC1). Scc1 supports sister chromatid cohesion and mono-orientation of sister kinetochores in meiosis I but cannot be protected from separase (Toth et al., 2000). We used spo11Δ strains because Scc1 does not support meiotic DSB repair. Exchange of Scc1 for Rec8 caused spo11Δ cdc7-4 and also spo11Δ bob1 cdc7-4 cells to divide their nuclei upon Pds1 degradation in the presence of two SPBs (Figure 4B, panels 2 and 4) and to segregate sister chromatids to opposite poles (Figure 4C). Thus, cleavage of both arm and centromeric cohesion restores an equal meiosis I division satisfying the third criterion. The persistence of centromeric cohesion during anaphase I requires both Rec8 and its protector Sgo1. Accordingly, depletion of Sgo1 from meiotic cdc7-4 cells caused nuclear division in the presence of two SPBs, as Pds1 disappeared at the onset of anaphase I.

**Figure 2. DDK is Required for Recombination**

(A and B) Meiosis in bob1 (Z10436), bob1 cdc7Δ (Z10438), and bob1 P40-DBF4-ha3 (Z11821) cells. (A) Top: percentages of chromosome spreads with Rad51 foci (blue), SCs (green), polycomplexes (orange), or Rec8 on chromatin (gray), and of cells with one spindle (red) or a divided nucleus (black). Bottom: cellular DNA content. (B) Immunoblot detection of proteins.

(C) DNA blot analysis of the YCR048w hotspot and immunoblot analysis of protein levels in cultures of strains described in (A).

(D) Analysis of DNA and protein samples from DBF4-ha3 (Z13216) and P40-DBF4-ha3 (Z13218) cells as in (C).
and release at 25°C to undergo two normal meiotic divisions (Figure 5A). Thus, arrest system. Control and (2008) have independently developed a similar synchronisation

Does DDK promote mono-orientation directly or indirectly via DNA replication? To address this question, we sought to inactivate DDK after S phase but prior to the onset of metaphase I. This should result in sister kinetochore biorientation in the direct but not in the indirect scenario. To reversibly arrest cells in pachytene, we used an estradiol-inducible promoter to control expression of the Ndt80 transcription factor, which is essential for exit from pachytene (Chu and Herskowitz, 1998). Carlile and Amon (2008) have independently developed a similar synchronisation system. Control and cdc7-4 cells transferred to sporulation medium (no estradiol) at 25°C replicated DNA with similar kinetics and arrested in pachytene with normal-looking SCs (Figures S6A and S6B). Induction of NDT80 (plus estradiol) at 25°C caused cdc7-4 cells to synchronously enter metaphase I and to undergo two normal meiotic divisions (Figure 5A). Thus, arrest and release at 25°C has no detectable effect on chromosome segregation in cdc7-4 cells. NDT80 induction after shifting cultures to 34°C prompted normal meiotic divisions in control cells (Figure 5B, left), cdc7-4 cells entered metaphase I (Figure 5B, right) but Pds1 degradation failed to trigger anaphase I leading to the accumulation of Pds1-negative cells with a short spindle and two SPBs (Figure 5C). After SPB reduplication in meiosis II, nuclear division occurred with equal segregation of sister chromatids (Figures 5C and 5D). We conclude that meiosis I nuclear division requires postreplicative DDK activity.

To test whether postreplicative activity is sufficient for monopolar attachment, we filmed GFP-tubulin and histone H2B-RFP in bob1 PNDT80-CDC7 cells and control strains. 83% of bob1 PNDT80-CDC7 cells underwent two meiotic divisions and produced four equal-sized nuclei, which resembles bob1 cells (97% tetranucleate) but not bob1cdc7 Δ mutants (84% binucleate) (Figures 5E and S6C). This suggests that postreplicative DDK activity is sufficient for both recombination and monopolar attachment. Consistent with this, bob1 PNDT80-CDC7 tetrads contained mostly viable spores (88%). Thus, our data suggest that activation of DDK in late prophase I is sufficient for correct segregation of replicated chromosomes.

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DDK Activity Is Required to Recruit Monopolin to Kinetochores

To test whether postreplicative activity is sufficient for monopolar attachment, we filmed strains expressing Mam1-GFP and the RFP-tagged kinetochore protein Mtw1 (Figures 6A and 6B). During meiosis I, Mam1 localized to the main spindle axis, which is consistent with the idea that postreplicative DDK activity is sufficient for normal segregation of replicated chromosomes. Mam1 failed to colocalize with Mtw1 in cdc7-4 cells but not in bob1cdc7 Δ cells. This localization defect likely extends to other monopolin subunits because the kinetochore localization of monopolin components is interdependent (Rabitsch et al., 2003). Thus, our data suggest that DDK is essential to recruit monopolin to kinetochores in metaphase I. Mam1 localized to kinetochores in bob1 PNDT80-CDC7 cells, which is consistent with the idea that postreplicative DDK activity is sufficient for normal segregation of replicated chromosomes. Mam1 failed to colocalize with Mtw1 in cdc7-4 and PND-CBP4-ha3 mutants whose DDK activity is reduced but still sufficient for DNA replication. This implies that recruitment of monopolin to kinetochores requires more DDK activity than DNA replication. Detection of Mam1 and the kinetochore protein Ndc10 by chromatin immunoprecipitation as well as chromosome spreading confirmed that monopolin recruitment depends on both components of DDK and on Cdc7’s catalytic activity (Figures S8A and S8B). Does monopolin’s kinetochore localization depend on preceding DDK-dependent processes? Mam1 colocalized with Ndc10 in cells

Deletion of SP011 and MAM1 Recapitulates Chromosome Segregation in DDK Mutants

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Figure 3. The Single Division of DDK Mutants Corresponds to Meiosis II

(A) Time-lapse series of meiosis in wild-type (Z3916, 31°C), cdc7-4 (Z9318, 31°C), bob1 (Z10712), and bob1 cdc7 Δ (Z10716) cells with GFP-tubulin, Cdc14-GFP, TetR-RFP, and heterozygous URA3-tetO. TetR-RFP labels URA3-tetO (dots) and the nucleoplasm (diffuse signal). Arrows mark Cdc14’s first and second release from the nucleolus. Minutes after onset of metaphase I are indicated.

(B) Staining of Spc42-GFP at SPBs, tubulin, DNA, and Pds1-myc18 in wild-type (Z8510) and cdc7-4 (Z8511) cells fixed at different stages of meiosis at 31°C. (C) Meiosis at 31°C in cdc7-4 (Z9737) and cdc7-4 (Z9738) cells with Rec8-GFP, Pds1-RFP, and Cnm67-RFP at SPBs. Top: time-lapse series starting with SPB separation. Numbers indicate minutes before and after Pds1 degradation (t = 0). Arrows mark centromeric Rec8. Bottom: the presence of two SPBs (black), four SPBs (gray), Rec8 on the entire chromatin (green), centromeric Rec8 (blue), and Pds1 in meiosis I (red) was quantified every 5 min in 100 individual cells, in which Pds1 degradation was set to t = 0.
undergoing meiosis without DNA replication due to depletion of the replication initiation factor Cdc6 (Hochwagen et al., 2005) (Figure S8C). It also bound to kinetochores in cells lacking sister chromatid cohesion (rec8J) and DSBs (spo11J) (Figure S8C). Thus, monopolin’s kinetochore localization does not require processes associated with DNA replication or recombination.

Next, we asked whether DDK is required for the assembly of monopolin. This involves Cdc5-dependent release of a Csm1-Lrs4 complex from the nucleolus and its association with another complex consisting of Mam1 and two or more Hrr25 molecules. In the absence of Cdc7, monopolin subunits accumulated to normal levels, escaped from the nucleolus on time, and efficiently associated with their binding partners (Figures S9A–S9E). These data suggest that DDK is dispensable for the assembly of monopolin but required for localizing the assembled complex to kinetochores. DDK might regulate the localization of monopolin by phosphorylating its subunits. As cells approach metaphase I, Lrs4 shows a mobility shift (Katis et al., 2004), which results from phosphorylation (Figure S10A). To analyze whether this phosphorylation requires DDK, we detected by immunoblotting Lrs4-myc9 from Cdc20-depleted bob1 and bob1 cdc7J cells. The absence of Cdc7 strongly reduced Lrs4’s mobility shift demonstrating that DDK contributes to the hyperphosphorylation of Lrs4 in metaphase I (Figure 6C). We analyzed the other monopolin subunits on gels containing Phos-tag, which retards the mobility of phosphoproteins (Kinoshita-Kikuta et al., 2007). While Csm1 appeared unmodified, Mam1 and Hrr25 were phosphorylated in a DDK-independent manner (Figures S10B and S10C). Thus, hyperphosphorylation of Lrs4 is the sole DDK-dependent modification we can detect in the monopolin complex.

DDK Collaborates with PLK/Cdc5 and Spo13 in the Phosphorylation of Lrs4

Modification of Lrs4 in metaphase I requires not only DDK but also Cdc5 and Spo13 (Katis et al., 2004). Processor Cdc5 or Spo13 shows a similar increase in gel mobility as the protein from bob1 cdc7J cells (Figure S11A). The common requirement of DDK, Cdc5, and Spo13 for the hyperphosphorylation of Lrs4 is reflected in protein-protein interactions: Cdc5 binds to DDK (Figure 1A) and both kinases copurify with Lrs4, even in the absence of Mam1 (Figure 6D). Furthermore, Cdc5 binds to Spo13 (Table S1) and the phosphorylation of Spo13 requires both Cdc5 and DDK (Figures S12A–S12C). In spo13J mutants, the monopolin complex fails to localize to kinetochores although its subunits are released from the nucleolus (Katis et al., 2004; Lee et al., 2004) and interact with each other normally (Figures S13A–S13D). Thus, in both spo13J and DDK mutants, defective kinetochore localization of monopolin correlates with reduced phosphorylation of Lrs4. We speculate that kinetochore localization actually depends on the hyperphosphorylation of Lrs4.

To investigate how Spo13 (which is not a kinase) promotes the phosphorylation of Lrs4, we analyzed its interaction with Cdc5, which is mediated by Cdc5’s PBD (Figure S12D). Spo13 contains a STSTP motif, which might represent a binding site for the PBD (Elia et al., 2003). Mutation of this motif to TTTPP generated Spo13-m2, which appears and disappears like the wild-type protein but hardly binds to Cdc5 (Figures 7A and 7B). The spo13-m2 mutation reduced Lrs4’s hyperphosphorylation in metaphase I (Figure 7C) and prevented monopolin’s localization to kinetochores as revealed by chromosome spreading (Figure 7D) and live-imaging (Figure S13E). Consistent with sister kinetochore biorientation, spo13-m2 cells frequently split centromeric sister sequences in metaphase I (Figure 7E). These data suggest that Spo13 promotes hyperphosphorylation of Lrs4 and thereby kinetochore recruitment of monopolin through its binding to Cdc5. Unlike Spo13 and DDK, Cdc5 is essential for the nucleolar release of Lrs4-Csm1. This implies that Cdc5 first triggers the nucleolar release of monopolin subunits in a Spo13- and DDK-independent reaction and then collaborates with Spo13 and DDK to recruit the monopolin complex to kinetochores.

To address how DDK and Cdc5 collaborate in the hyperphosphorylation of Lrs4, we considered the following possibilities: (1) DDK and Cdc5 might independently phosphorylate Lrs4 at different sites. This appears unlikely since the gel mobility of Lrs4 from cells lacking either DDK or Cdc5 is similar to that of Lrs4 from cells lacking both kinases (Figure S11B). (2) Phosphorylation of Lrs4 by DDK could generate a binding site for Cdc5’s PBD. However, Cdc5 binds normally to Lrs4 in the absence of Cdc7, arguing against a role for DDK as Cdc5’s priming kinase (Figure S11C). (3) Finally, we found that DDK is dispensable for the Cdc5-Spo13 interaction (data not shown). We speculate that DDK and Cdc5 collaborate by a mechanism that involves their physical association.

DISCUSSION

DDK is required for the initiation of both premitotic and promeiotic DNA replication (Ogino et al., 2006; Sclafani, 2000; Valentin et al., 2006). Our finding that DDK binds to the PLK Cdc5 in metaphase I suggested that DDK has additional, postreplicative functions in meiosis. We used two strategies to inactivate DDK without invoking checkpoint mechanisms that block progression of meiosis in response to replication defects. (1) The P40-DBF4
and the cdc7-4 mutations reduce DDK activity to levels permissive for normal DNA replication but insufficient for DDK’s postreplicative functions. (2) The bob1 mutation enabled us to analyze cells that undergo DNA replication and spindle formation in the complete absence of Cdc7 or Dbf4. We show that DDK is required for DSB formation in prophase I and for mono-orientation of sister kinetochores in metaphase I. In the absence of these events, meiosis is converted into a division that resembles mitosis in that it generates two genetically identical diploid spores.

**DDK Links DNA Replication, Recombination, and Monopolar Attachment**

Our data extend previous work suggesting that regulators of DNA replication have a key role in linking recombination to premeiotic S phase. DSB formation requires the S phase-promoting CDK Cdc28-Clb5, which phosphorylates Mer2/Rec107, a protein essential for Spo11 activity (Henderson et al., 2006; Smith et al., 2001). The finding that cdc7 mutants lack DSBs indicated that recombination is initiated, like replication, by a collaboration between Cdc28-Clb5 and DDK (Wan et al., 2006). However, these mutants arrested in prophase I, probably due to checkpoint activation in response to replication defects. We show that DDK is essential for DSB formation in sporulating cells, which implies a direct, checkpoint-independent role for DDK in DSB formation. Consistent with a direct role, also DDK is recently found to phosphorylate Mer2 on residues that are essential for DSB formation in sporulating cells, which implies that it generates two genetically identical diploid spores.

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It has been proposed that DDK is required for the induction of Ndt80-dependent genes and thus for exit from prophase (Lo et al., 2008; Sasanuma et al., 2008). In our hands, however, cells lacking Cdc7 or Dbf4 readily enter metaphase I. Nevertheless, DDK activity in meiosis I. This process requires Cdc5 (Cluye et al., 2003; Lee and Amon, 2003), which might be assisted in this task by DDK. Consistent with this idea, Cdc5 associates with the APC/C in metaphase I.

We show that DDK is also essential for monopolar attachment of sister kinetochores in meiosis I. Bi-orientation of sister kinetochores allows DDK mutants to generate viable spores because it prevents the segregation of unlinked homologs. Previous work in fission yeast has linked monopolar attachment to premeiotic DNA replication through the establishment of Rec8-dependent sister chromatid cohesion at centromeres (Watanabe and Nurse, 1999; Watanabe et al., 2001). Two lines of evidence suggest, however, that DDK promotes monopolar attachment directly rather than through its role in replication. First, DDK inactivation after the completion of DNA replication and cohesion establishment causes bi-orientation of sister kinetochores suggesting that monopolar attachment depends on postreplicative DDK activity. Furthermore, bob1 cells that activate DDK after the completion of replication undergo two normal-looking divisions. This implies that postreplicative DDK activity even suffices for monopolar attachment. Second, reducing DDK activity with a kinase inhibitor (Lo et al., 2008) or by genetic manipulation (this work) blocks the recruitment of monopolin to kinetochores. However, the complex still binds to kinetochores in cells lacking Rec8 or in the absence of premeiotic DNA replication. We propose that DDK controls monopolin localization and thereby monopolar attachment by promoting the hyperphosphorylation of Lrs4 in metaphase I. This modification is dispensable for the nucleolar release or the association of monopolin subunits but correlates with the localization of the complex to kinetochores. Proving this idea will require the identification and mutation of the relevant phosphorylation sites.

DDK mutants produce viable spores because they segregate sister chromatids in meiosis II on a single spindle axis that originates from metaphase I. Meiosis II spindle formation requires anaphase I and thus depends only indirectly on DDK. In DDK mutants, deprotection of centromeric cohesion restores both anaphase I and assembly of a pair of new meiosis II spindles. However, these spindles fail to segregate chromatids. In anaphase II, cells contain only two nuclei from each of which a spindle extends “into the void.” We suspect that after erroneous bi-orientation in meiosis I, kinetochores remain attached to microtubules from meiosis I SPBs and then fail to attach to those from meiosis II SPBs. In monopolin mutants, most sister kinetochores bi-orient in meiosis I and remain attached as cells enter metaphase II. However, a few, previously mono-oriented, kinetochores now attach to microtubules from the new SPBs, which causes a “tetrapolar” meiosis II division (Toth et al., 2000). The lack of chiasmata in mom1Δ spo11Δ and DDK mutants further augments bi-orientation because monopolar attachments can no longer generate tension. Thus, sister chromatids segregate almost exclusively on the meiosis I spindle axis.

Our data identify DDK as a key regulator of meiotic chromosome segregation in yeast. After induction of meiosis, accumulation of Dbf4 activates DDK, which initiates DNA replication...
and establishes a period of high DDK activity that persists until Dbf4’s degradation in anaphase I. High DDK activity initiates recombination and monopolar attachment, which commits cells to segregating homologous chromosomes in the subsequent meiosis I division. Degradation of Dbf4 in anaphase I is probably triggered by the APC/C, which is known to recognize Dbf4 in mitosis (Ferreira et al., 2000). Unlike other APC/C substrates, Dbf4 does not reappear in meiosis II. This might help to suppress a second round of DNA replication after meiosis I and to prepare for equational chromosome segregation in meiosis II. Other mechanisms, such as degradation of Mam1, also contribute to inactivating the machinery for homolog segregation (Toth et al., 2000). It remains to be investigated whether DDK’s role in promoting reductive chromosome segregation in meiosis I has been conserved in evolution similar to its function in DNA replication. Conversion of meiosis into an equational division has implications for animal cloning and for efforts to transfer apomixis (asexual seed production) to crop plants.

**Regulation of DDK-Dependent Events in Meiosis**

DNA replication, DSB formation, and monopolar attachment differ in their requirements for DDK activity. We propose that these differences help in properly coordinating these events. DSB formation and monopolar attachment require more DDK activity than replication so that their initiation can only occur after that of premeiotic S phase. Monopolar is recruited to kinetochores after recombination, as cells approach metaphase I, because this requires Cdc5 in addition to DDK. Another level of regulation is added by Spo13, which we have identified as a Cdc5-associated protein. DDK collaborates with Cdc5 on the hyperphosphorylation of Rec8 but requires both Cdc5 and Spo13 for the hyperphosphorylation of Lrs4. Consistent with this idea, Spo13 itself is phosphorylated in a Cdc5- and DDK-dependent manner. A Spo13 mutant with reduced affinity for Cdc5 shows phenotypes as a Spo13 deletion including defects in monopolar recruitment to kinetochores, protection of centromeric cohesion, and entry into a second meiotic division. This suggests that Spo13 functions mainly through binding to Cdc5. We envision that Spo13 activates Cdc5 toward a specific set of substrates in meiosis. How DDK and Cdc5 collaborate is currently unclear. Since DDK and Cdc5 can bind independently to Lrs4, it appears unlikely that one kinase serves as a substrate-priming kinase for the other one. We speculate that the interaction between DDK and Cdc5 is relevant for their ability to collaborate, for instance through mutual phosphorylation. DDK has been viewed as the classical S phase kinase whose function is largely confined to the initiation of DNA replication. However, DDK’s ability to collaborate with the M phase kinase PLK implies a potential to control a wide range of postreplicative processes in both meiosis and mitosis.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**

All experiments were performed with the diploid SK1 strains listed in Table S4. Strain constructions are detailed in Supplemental Data. CDC7 and DBF4 were deleted in bob1 strains (Hardy et al., 1997). cdc7-kd (K76A) and spo13-m2 (S132T,S134T) contain the indicated mutations. A Gal4-estrogen receptor fusion was used for estradiol-inducible expression from the GAL promoter (Benjamin et al., 2003). For live-imaging, proteins were tagged with eGFP and the RFPs mCherry or tdTomato (Shaner et al., 2004). URA3-tetO and CENS-tetO loci (Toth et al., 2000) were visualized with TetR-GFP or TetR-tdTomato.

**Induction and Analysis of Meiosis**

Meiosis was induced at 30°C as described (Petronczki et al., 2006). cdc7-4 and control cells were grown in YP-acetate medium at 25°C, transferred to sporulation medium (t = 0), and shifted to 31°C after 1 hr. The DSB assay, immunoprecipitation, immunofluorescence microscopy, chromosome spreading, and ChiP were performed as described (Klein et al., 1999; Petronczki et al., 2006; Rabitsch et al., 2003). Proteins isolated by tandem affinity purification (Rigaut et al., 1999) were analyzed by mass spectrometry as detailed in Supplemental Data.

**Live-Cell Imaging**

Cells were induced to enter meiosis for 4 hr, immobilized on glass slides with Concanavalin A, and imaged on an Applied Precision DeltaVision RT system with an Olympus IX70 microscope, an UPPlanSApo 100x/1.4NA/olil objective, an eGFP/mCherry dual band filter set (F89-021, AHF Analysetechnik,Tuebingen, Germany), and a Photometrics CoolSnap HQ CCD camera. The microscope was placed in a temperature-controlled chamber and equipped with an objective heater. Z-stacks of 8 sections (1 μm apart) were acquired every 5 or 10 min for 12 hr using a 10% neutral density filter and exposure times of 50–150 ms. Z-stacks were deconvolved and combined into a single maximum intensity projection with SoftWoRx (Applied Precision).

**SUPPLEMENTAL DATA**

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, Thirteen Figures, and Four Tables and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01363-9.

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**Figure 6. Regulation of the Monopolin Complex by DDK**

(A and B) Live-imaging of Mam1-GFP and Mtw1-RFP during meiosis in bob1 (Z12450), bob1 cdc7-J1 (Z12459), bob1 P<sup>APC/C</sup>CDC7 (Z12479), wild-type (S11, C, Z11252), cdc7-4 (S11, C, Z11362), DBF4-ha3 (Z13368), and P<sup>APC/C</sup>DBF4 ha3 (Z13449) cells. (A) Time-lapse series. (B) Quantification of cells with Mam1 at kinetochores in 50 cells per strain.

(C) Immunoblot analysis of extracts from P<sup>CLEF</sup>CDC20 LRS4-myc9 cells with bob1 (Z3447) or bob1 cdc7-J1 (Z10850). The bar marks DDK-dependent hyperphosphorylation of Lrs4.

(D) Analysis of Lrs4’s interaction with Cdc7 and Cdc5 in anti-Myc IPs from P<sup>CLEF</sup>CDC20 CDC7-ha6 cells with LRS4 (Z11529), LRS4-myc9 (Z11528), or LRS4-myc9 mam1-J (Z13559).
Figure 7. Monopolar Attachment Requires Binding of Spo13 to Cdc5

(A) Immunoblot detection of proteins from MAM1-myc9 NDC10-ha6 cells with SPO13-WT (Z12183) or spo13-m2 (Z12184).

(B) Analysis of the Spo13-Cdc5 interaction in anti-Myc IPs from PCLB2-CDC20 cells with Myc9-SPO13 (Z11436) or Myc9-spo13-m2 (Z11437). The asterisk denotes antibody bands.

(C) Immunoblot analysis of Lrs4 hyperphosphorylation (marked with a bar) during metaphase I in extracts from PCLB2-CDC20 LRS4-myc9 cells with Ha3-spo13-m2 (Z11942) or Ha3-SPO13-WT (Z11941).
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(D) Analysis of Mam1-mdc9 localization to kinetochores (Ndc10-ha6) on chromosome spreads from SPO13-WT (Z12183), spo13-md2 (Z12184), and spo13D (Z10726) cells in meiosis I (two SPBs).

(E) Live-imaging of Pcl2–CDC20 spo13–WT (Z12558) and Pcl2–CDC20 spo13–md2 (Z12559) cells arrested in metaphase I. Cells contain TetR–GFP at heterozygous CENS–tetO, Pds1–RFP, and Cnm67–RFP at SPBs. Left: accumulation of metaphase I-cells that split CENS–tetO once or more (n = 50). Right: time-lapse series. Arrows mark split CENS–tetO.

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