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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 Lrs4 and Csm1 (Petronczki et al., 2006; Rabitsch et al., 2003). Shortly before metaphase I, the polo-like kinase (PLK) Cdc5 triggers the release of Lrs4 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 cohesin 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 cohesin 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 monopolar 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 monopolar 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 (Sclafani, 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 monopolin. Thus, activation of DDK both initiates DNA replication and commits meiotic cells to reductional chromosome segregation in meiosis I.

RESULTS

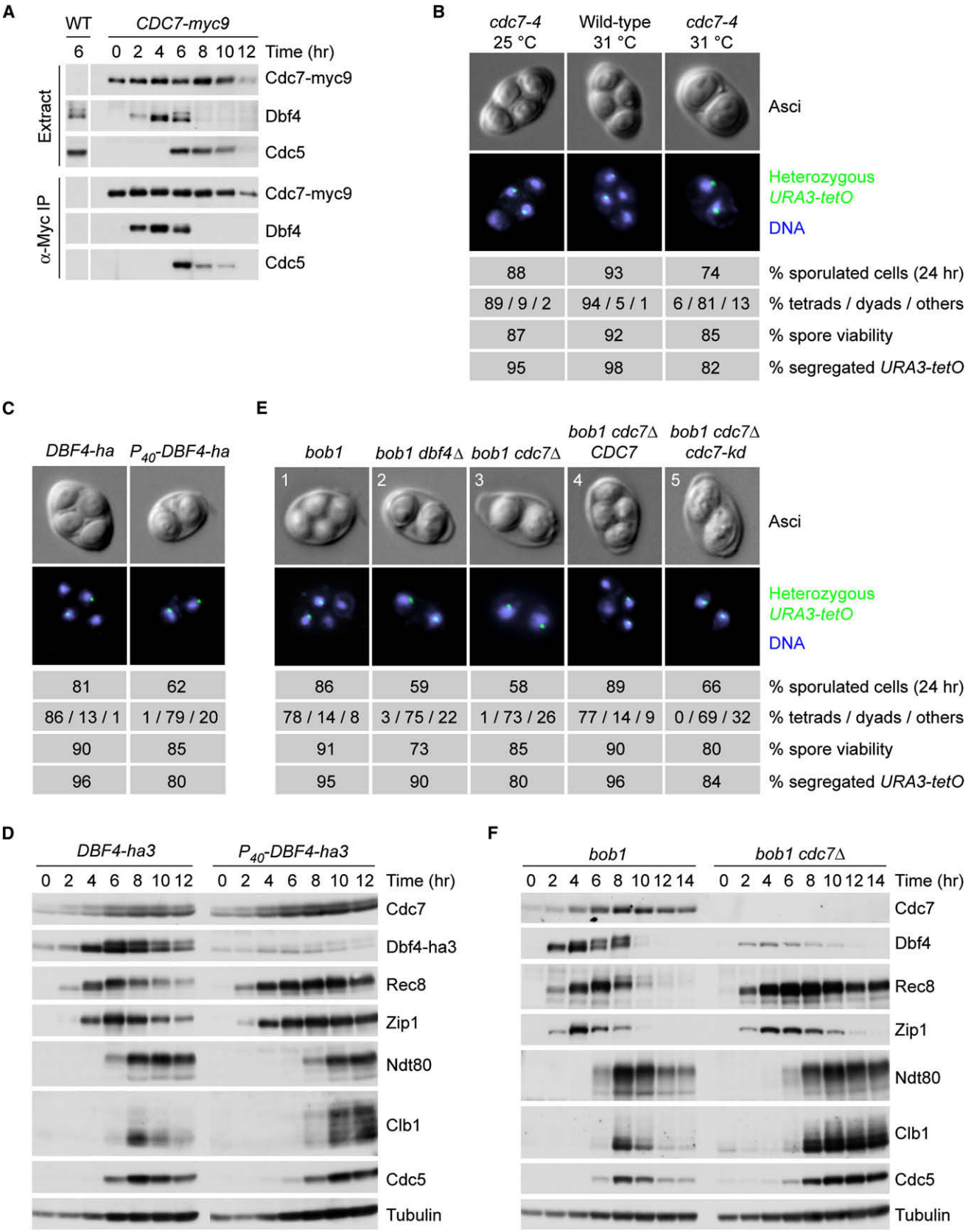
Association of the DDK Cdc7-Dbf4 with the PLK Cdc5 in Meiosis

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 depletion 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 1B). Accordingly, the spores developed into diploid, sporulation-competent cells. Is this phenotype specific to the *cdc7-4* allele or due to reduced DDK activity? To address this, we lowered the expression of *DBF4* by truncating its promoter to 40 bp. These P_{40}-*DBF4-ha3* cells contain only ~15% of normal Dbf4 levels but proliferate and complete premeiotic DNA replication normally. Nevertheless, they produce dyad 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 cdc7Δ* and *bob1 dbf4Δ* 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-kd* allele restored formation of four-spored asci in *bob1 cdc7Δ* cells (Figure 1E, panels 4 and 5). We conclude that DDK activity is essential for reductional chromosome segregation. Time course experiments revealed that *bob1 cdc7Δ* 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, Clb1, 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.


A
WT
CDC7-myc9
6
0 2 4 6 8 10 12 Time (hr)
Extract
Cdc7-myc9
Dbf4
Cdc5
α-Myc IP
Cdc7-myc9
Dbf4
Cdc5
B
cdc7-4 25 °C
Wild-type 31 °C
cdc7-4 31 °C
Asci
Heterozygous URA3-tetO
DNA
88 93 74 % sporulated cells (24 hr)
89 / 9 / 2 94 / 5 / 1 6 / 81 / 13 % tetrads / dyads / others
87 92 85 % spore viability
95 98 82 % segregated URA3-tetO
C
DBF4-ha
P40-DBF4-ha
81 62 % sporulated cells (24 hr)
86 / 13 / 1 1 / 79 / 20 % tetrads / dyads / others
90 85 % spore viability
96 80 % segregated URA3-tetO
E
bob1
bob1 dbf4Δ
bob1 cdc7Δ
bob1 cdc7Δ CDC7
bob1 cdc7Δ cdc7-kd
1 2 3 4 5
Asci
Heterozygous URA3-tetO
DNA
86 59 58 89 66 % sporulated cells (24 hr)
78 / 14 / 8 3 / 75 / 22 1 / 73 / 26 77 / 14 / 9 0 / 69 / 32 % tetrads / dyads / others
91 73 85 90 80 % spore viability
95 90 80 96 84 % segregated URA3-tetO
D
DBF4-ha3
P40-DBF4-ha3
0 2 4 6 8 10 12 0 2 4 6 8 10 12 Time (hr)
Cdc7
Dbf4-ha3
Rec8
Zip1
Ndt80
Clb1
Cdc5
Tubulin
F
bob1
bob1 cdc7Δ
0 2 4 6 8 10 12 14 0 2 4 6 8 10 12 14 Time (hr)
Cdc7
Dbf4
Rec8
Zip1
Ndt80
Clb1
Cdc5
Tubulin

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 recombinase and SCs containing the Zip1 protein. In contrast, *bob1 cdc7Δ* spreads 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* P_{NDT80}-*CDC7* cells, Cdc7 accumulated together with the Ndt80-dependent Cdc5 protein, which is known to appear in pachytene (Clyne et al., 2003) (Figure 2B). Consistent with DDK activation after S phase, *bob1* P_{NDT80}-*CDC7* cells failed to form Rad51 foci and SCs at early time points but assembled these structures upon accumulation of Cdc7 (Figure 2A, right). We confirmed this result by live-cell imaging of *ZIP1::GFP* strains (Figure S3B). *bob1* cells formed SCs 1 hr after nuclear accumulation of Zip1-GFP whereas *bob1* P_{NDT80}-*CDC7* cells did so only after 3 hr. Parallel analysis of DNA and protein samples showed that in a *bob1* P_{NDT80}-*CDC7* culture, DSBs appear together with Cdc7 (Figure 2C). These data suggest that DSB formation can be initiated by postreplicative DDK activity. In *cdc7-4* and P_{40}-*DBF4-ha3* cells, DDK activity is reduced to levels that support normal DNA replication but not reductional chromosome segregation. These mutants lack DSBs (Figure 2D and data not shown), suggesting that DSB formation requires more DDK activity than DNA replication.

DDK Mutants Fail to Segregate Homologs in Meiosis I but Segregate Sister Chromatids on a Single Spindle in Meiosis II

How can DDK mutants produce viable spores in the absence of chiasmata? One possibility is that DDK mutants are defective in both recombination and segregation of homologs. To observe chromosome segregation in individual cells with high temporal resolution, we performed live-cell imaging. We fused a red fluorescent protein (RFP) to *tet* repressor (TetR), which binds to *tet* operators (*tetO*) integrated into one chromosome V homolog at *URA3*, 35 kb from the centromere (heterozygous *URA3-tetO*). 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*, P_{40}-*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%; P_{40}-*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 frequently overwhelm pericentromeric 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

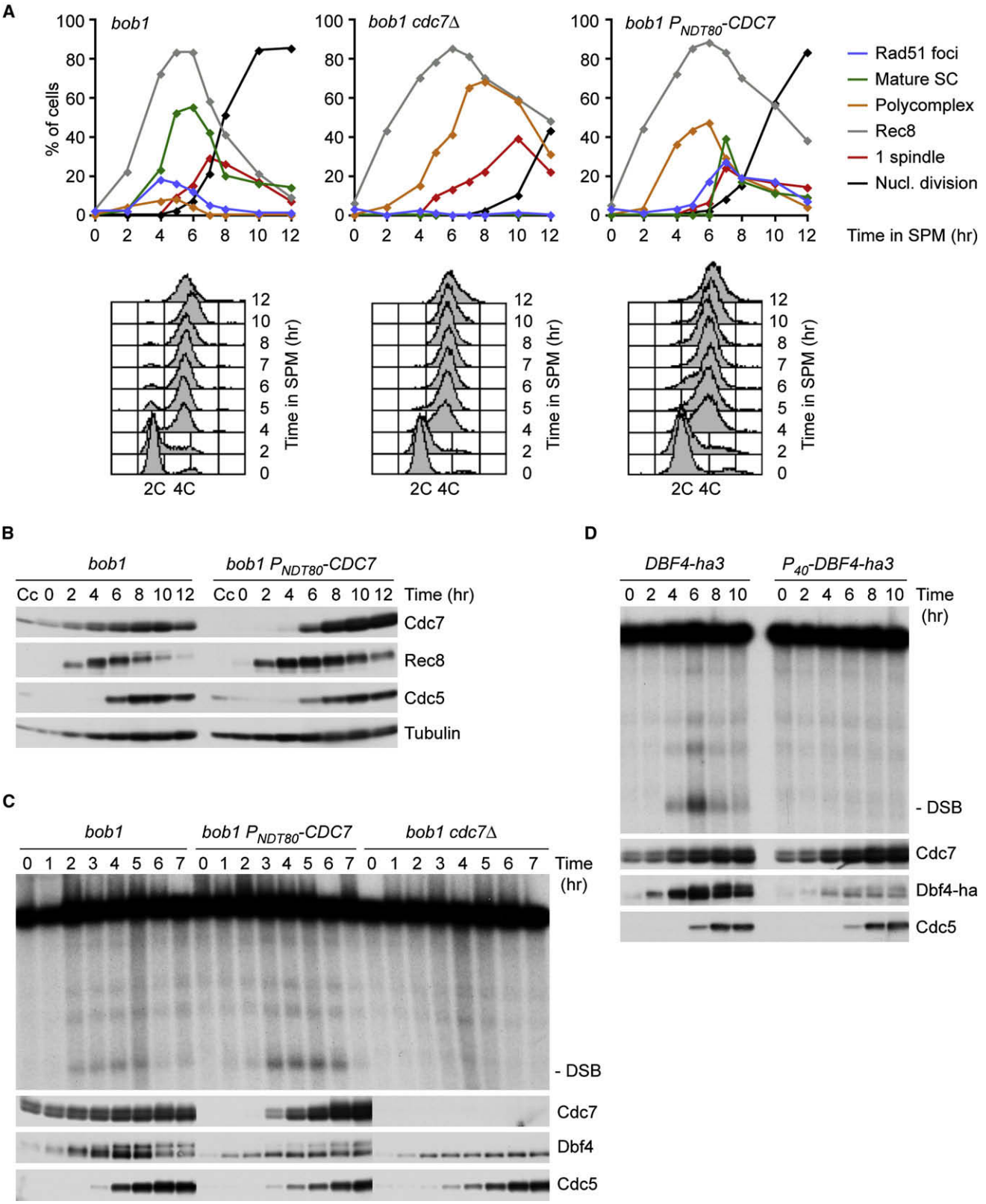
(A) DDK binds to Cdc5. Immunoblots are shown of extracts and anti-Myc immunoprecipitates (IPs) prepared from wild-type (WT, Z2314) and *CDC7-myc9* (Z7103) cells at different times after transfer to sporulation medium (SPM).

(B) Sporulation of wild-type (Z4834) and *cdc7-4* (Z8152) cells at 25 and 31°C. Asci 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 (P_{40}-*DBF4-ha3*, Z13218). (C) Asci 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. Asci were analyzed as in (B).

(F) Immunoblot analysis of extracts from meiotic *bob1* (Z9063) and *bob1 cdc7Δ* (Z9064) cells.


A
bob1
bob1 cdc7Δ
bob1 P NDT80-CDC7
% of cells
Rad51 foci
Mature SC
Polycomplex
Rec8
1 spindle
Nucl. division
Time in SPM (hr)
2C 4C
B
bob1
bob1 P NDT80-CDC7
Cc 0 2 4 6 8 10 12
Time (hr)
Cdc7
Rec8
Cdc5
Tubulin
C
bob1
bob1 P NDT80-CDC7
bob1 cdc7Δ
0 1 2 3 4 5 6 7
Time (hr)
- DSB
Cdc7
Dbf4
Cdc5
D
DBF4-ha3
P 40-DBF4-ha3
0 2 4 6 8 10
Time (hr)
- DSB
Cdc7
Dbf4-ha
Cdc5

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 S5A). 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* 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 S5B). 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 cdc7Δ* 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

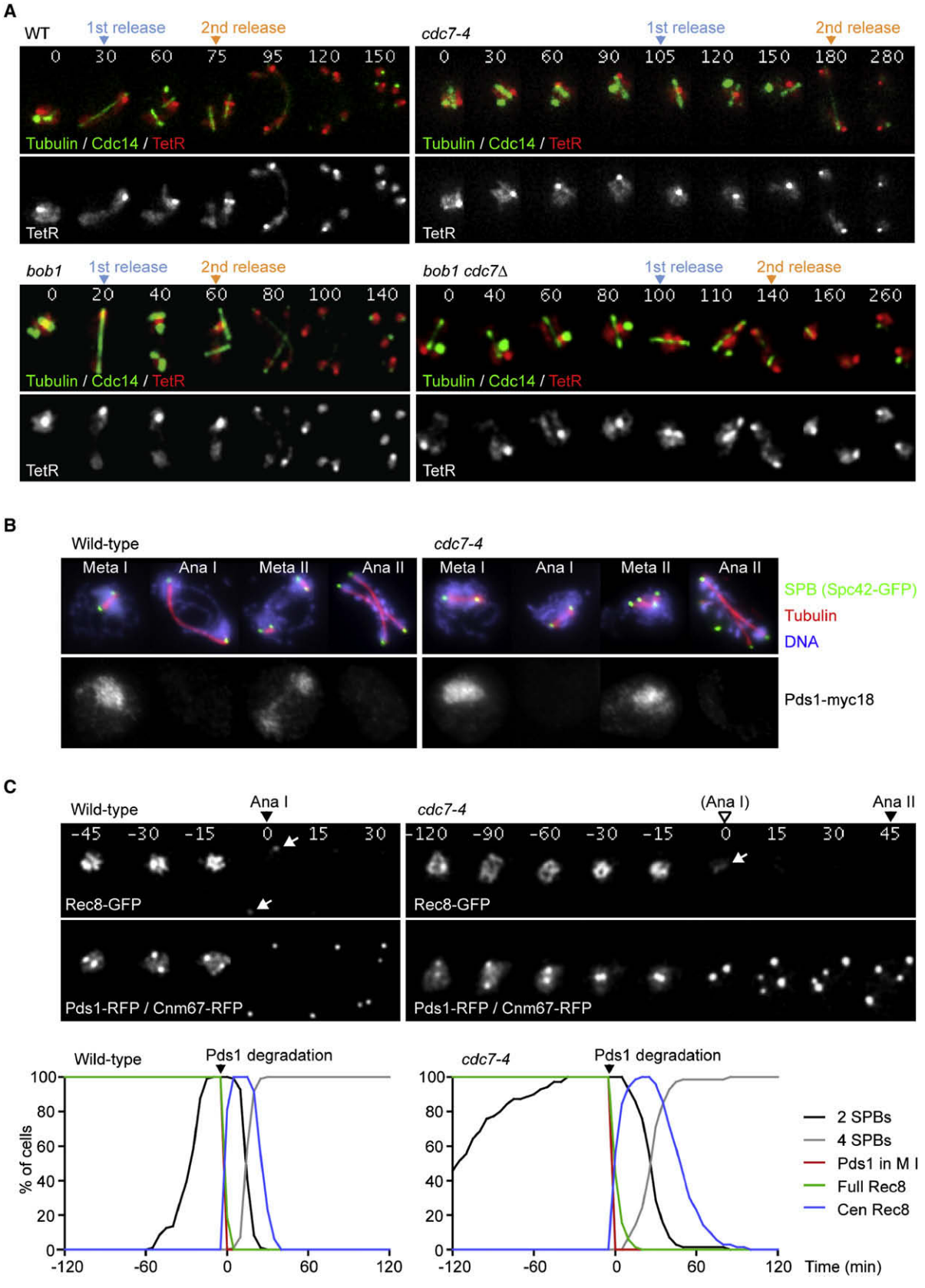
Three criteria have been used to establish that monopolin mutants bi-orient sister kinetochores in meiosis I (Petronczki et al., 2006): (1) Sister kinetochores come under tension in metaphase I. (2) The meiosis I division is blocked even in the absence of chiasmata. (3) "Deprotection" of centromeric 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 Ama1. 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 cdc7Δ* 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 separase access to centromeric cohesion in meiosis I, we used strains that lack Rec8 and instead express the mitotic kleisin Scc1 from the *REC8* promoter (P_{REC8}-*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 cdc7Δ* 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 equational 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* P_{NDT80}-*CDC7* (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 P_{40}-*DBF4-ha3* (Z13218) cells as in (C).


A
WT
1st release
2nd release
0 30 60 75 95 120 150
Tubulin / Cdc14 / TetR
TetR
cdc7-4
1st release
2nd release
0 30 60 90 105 120 150 180 280
Tubulin / Cdc14 / TetR
TetR
bob1
1st release
2nd release
0 20 40 60 80 100 140
Tubulin / Cdc14 / TetR
TetR
bob1 cdc7Δ
1st release
2nd release
0 40 60 80 100 110 140 160 260
Tubulin / Cdc14 / TetR
TetR
B
Wild-type
Meta I
Ana I
Meta II
Ana II
cdc7-4
Meta I
Ana I
Meta II
Ana II
SPB (Spc42-GFP)
Tubulin
DNA
Pds1-myc18
C
Wild-type
Ana I
-45 -30 -15 0 15 30
Rec8-GFP
Pds1-RFP / Cnm67-RFP
cdc7-4
(Ana I)
Ana II
-120 -90 -60 -30 -15 0 15 30 45
Rec8-GFP
Pds1-RFP / Cnm67-RFP
Wild-type
Pds1 degradation
% of cells
100
80
60
40
20
0
-120 -60 0 60 120
cdc7-4
Pds1 degradation
100
80
60
40
20
0
-120 -60 0 60 120
Time (min)
2 SPBs
4 SPBs
Pds1 in M I
Full Rec8
Cen Rec8

(Figure 4D). Taken together, our data show that DDK is essential for sister kinetochore mono-orientation in meiosis I.

Mono-Orientation of Sister Kinetochores Requires Postreplicative DDK Activity

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 equational 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* P_{NDT80}-*CDC7* cells and control strains. 83% of *bob1* P_{NDT80}-*CDC7* cells underwent two meiotic divisions and produced four equal-sized nuclei, which resembles *bob1* cells (97% tetranucleate) but not *bob1 cdc7Δ* 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* P_{NDT80}-*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.

Deletion of *SPO11* and *MAM1* Recapitulates Chromosome Segregation in DDK Mutants

Are initiation of recombination and monopolar attachment DDK's main postreplicative functions in meiotic chromosome segregation? If so, inactivation of both Spo11 and monopolin should recapitulate the chromosome segregation phenotype of DDK mutants. To test this, we filmed strains expressing GFP-tubulin and histone H2B-RFP (Figure S7). Consistent with previous work (Toth et al., 2000), *mam1Δ* cells produce 3-4 nuclei of unequal size because in anaphase II chromosomes segregate not only along the major spindle axis that persists from meiosis I but also along microtubules nucleated in meiosis II. Accordingly, *mam1Δ* cells produce asci with 3-4 mostly inviable spores (90% triads/tetrads, 10% viable spores). In contrast, *mam1Δ spo11Δ* double mutants resembled *cdc7* mutants in that chromosomes segregated almost exclusively on the main spindle axis (Figure S7). As a result, most asci contained two spores with equal-sized nuclei that developed into viable diploid cells (90% dyads, 75% viable spores). We conclude that blocking DSB formation and monopolin function results in a chromosome segregation pattern similar to that of DDK mutants. This suggests that it is the lack of recombination that prevents DDK mutants from attempting the tetrapolar division characteristic of monopolin mutants. We propose that DDK, after triggering premeiotic DNA replication, promotes the meiosis-specific pattern of chromosome segregation by initiating recombination and monopolar attachment.

DDK Activity Is Required to Recruit Monopolin to Kinetochores

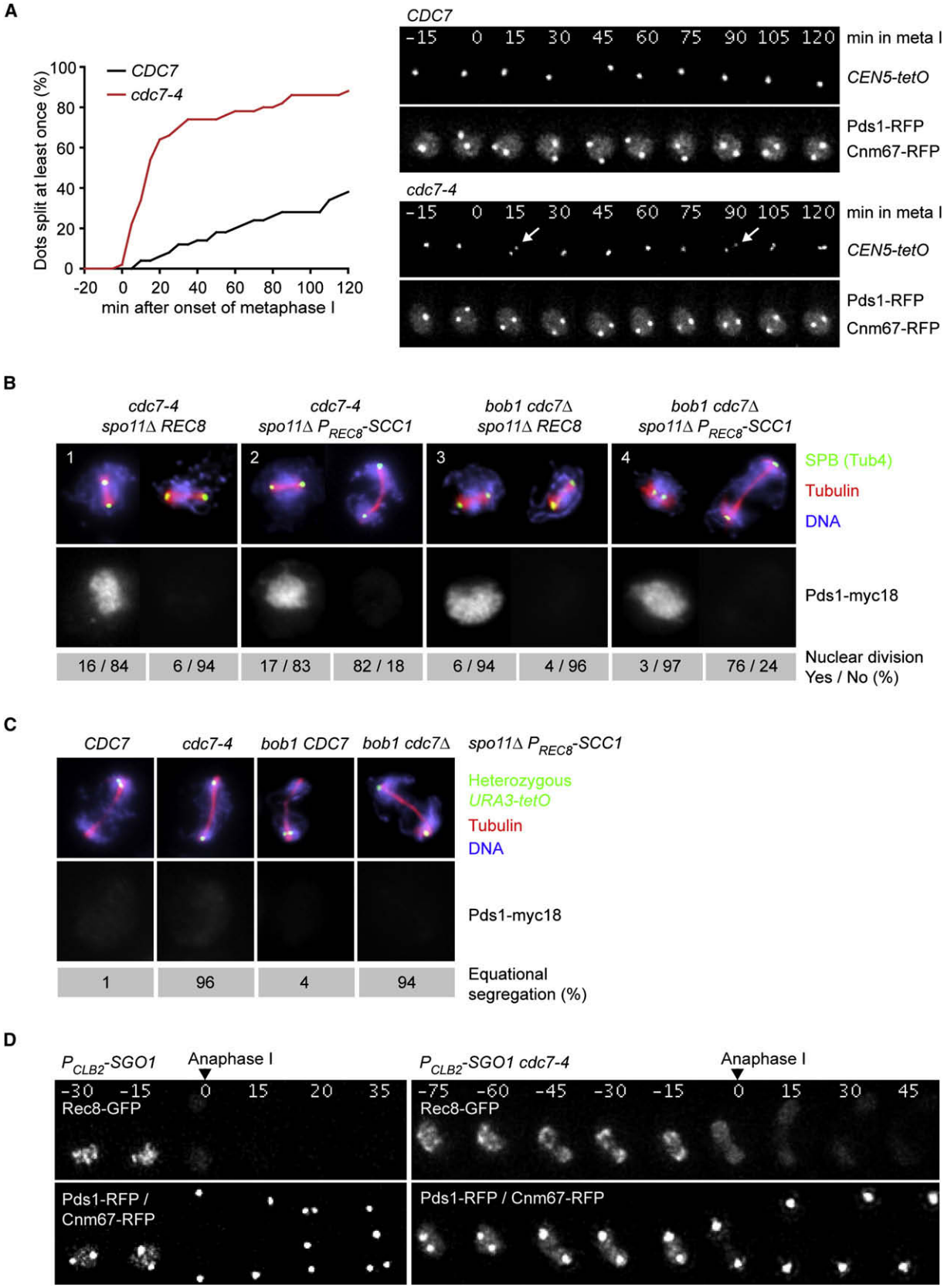
To investigate DDK's function in monopolar attachment, we first asked whether DDK is required to recruit monopolin to kinetochores. We filmed strains expressing Mam1-GFP and the RFP-tagged kinetochore protein Mtw1 (Figures 6A and 6B). During meiosis I, Mam1 colocalized with Mtw1 in *bob1* cells but not in *bob1 cdc7Δ* 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* P_{NDT80}-*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 P_{40}-*DBF4-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

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

(A) Time-lapse series of meiosis in wild-type (Z9316, 31°C), *cdc7-4* (Z9318, 31°C), *bob1* (Z10712), and *bob1 cdc7Δ* (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 wild-type (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.


A
CDC7
cdc7-4
Dots split at least once (%)
min after onset of metaphase I
CDC7
-15 0 15 30 45 60 75 90 105 120
min in meta I
CEN5-tetO
Pds1-RFP
Cnm67-RFP
cdc7-4
-15 0 15 30 45 60 75 90 105 120
min in meta I
CEN5-tetO
Pds1-RFP
Cnm67-RFP
B
cdc7-4 spo11Δ REC8
cdc7-4 spo11Δ P REC8 -SCC1
bob1 cdc7Δ spo11Δ REC8
bob1 cdc7Δ spo11Δ P REC8 -SCC1
SPB (Tub4)
Tubulin
DNA
Pds1-myc18
16 / 84
6 / 94
17 / 83
82 / 18
6 / 94
4 / 96
3 / 97
76 / 24
Nuclear division
Yes / No (%)
C
CDC7
cdc7-4
bob1 CDC7
bob1 cdc7Δ
spo11Δ P REC8 -SCC1
Heterozygous
URA3-tetO
Tubulin
DNA
Pds1-myc18
1
96
4
94
Equational
segregation (%)
D
P CLB2 -SGO1
Anaphase I
-30 -15 0 15 20 35
Rec8-GFP
Pds1-RFP /
Cnm67-RFP
P CLB2 -SGO1 cdc7-4
Anaphase I
-75 -60 -45 -30 -15 0 15 30 45
Rec8-GFP
Pds1-RFP / Cnm67-RFP

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 (*rec8Δ*) and DSBs (*spo11Δ*) (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 cdc7Δ* 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). Lrs4 from cells lacking Cdc5 or Spo13 shows a similar increase in gel mobility as the protein from *bob1 cdc7Δ* 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 *spo13Δ* 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 *spo13Δ* 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 TTTTP 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 premeiotic 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 P_{40}-*DBF4*

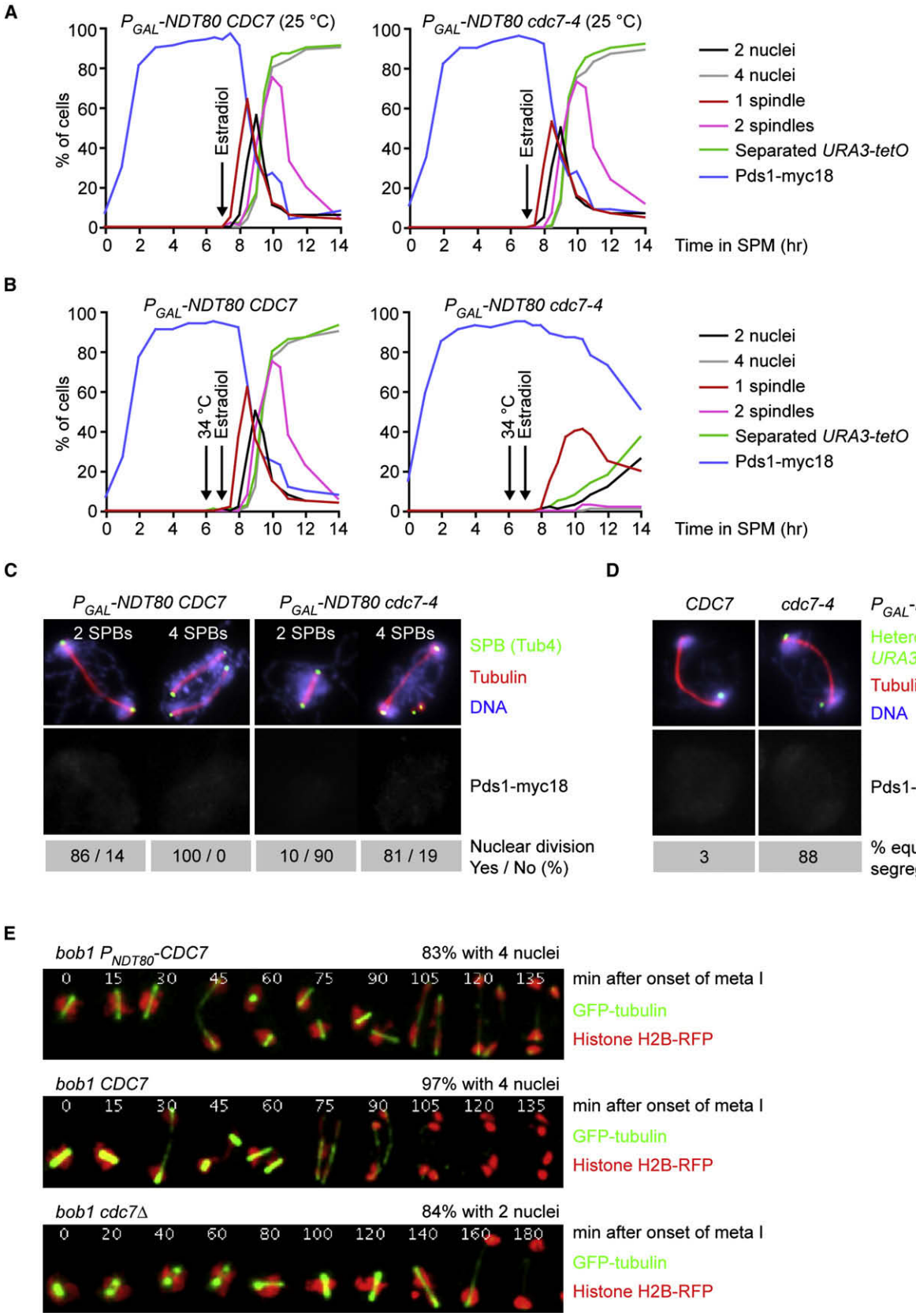
Figure 4. Mono-Orientation of Sister Kinetochores in Meiosis I Requires DDK

(A) Live-imaging at 31°C of *CDC7* (Z10367) and *cdc7-4* (Z10268) cells arrested in metaphase I due to the lack of APC/C activators (P_{CLB2}-*CDC20 ama1Δ*). Cells contain TetR-GFP at heterozygous *CEN5-tetO*, Pds1-RFP, and Cnm67-RFP at SPBs. Left: accumulation of metaphase I-cells that split *CEN5-tetO* once or more (n = 50). Right: time-lapse series. Arrows mark split *CEN5-tetO*.

(B) Percentage of nuclear division upon Pds1 degradation in meiosis I (two SPBs) in *spo11Δ* cells with (1) *cdc7-4* (Z8224, 31°C), (2) *cdc7-4* P_{REC8}-*SCC1* (Z8445, 31°C), (3) *bob1 cdc7Δ* (Z10747), or (4) *bob1 cdc7Δ* P_{REC8}-*SCC1* (Z10379).

(C) Percentage of equational *URA3-tetO* sister segregation in binucleated, Pds1-negative *spo11Δ* P_{REC8}-*SCC1* cells with *CDC7* (Z8444, 31°C), *cdc7-4* (Z8445, 31°C), *bob1 CDC7* (Z10366), or *bob1 cdc7Δ* (Z10379).

(D) Meiosis at 31°C in *CDC7* (Z10122) and *cdc7-4* (Z10123) cells depleted of Sgo1 (P_{CLB2}-*SGO1*). Cells contain Rec8-GFP, Pds1-RFP, and Cnm67-RFP at SPBs. Numbers indicate minutes before and after Pds1 degradation (t = 0). Time-lapse series start at SPB separation.


A
P_{GAL}-NDT80 CDC7 (25 °C)
P_{GAL}-NDT80 cdc7-4 (25 °C)
% of cells
Estradiol
2 nuclei
4 nuclei
1 spindle
2 spindles
Separated URA3-tetO
Pds1-myc18
Time in SPM (hr)
B
P_{GAL}-NDT80 CDC7
P_{GAL}-NDT80 cdc7-4
34 °C
Estradiol
C
2 SPBs
4 SPBs
SPB (Tub4)
Tubulin
DNA
Pds1-myc18
86 / 14
100 / 0
10 / 90
81 / 19
Nuclear division
Yes / No (%)
D
CDC7
cdc7-4
Heterozygous
URA3-tetO
3
88
% equational
segregation
E
bob1 P_{NDT80}-CDC7
83% with 4 nuclei
bob1 CDC7
97% with 4 nuclei
bob1 cdc7Δ
84% with 2 nuclei
min after onset of meta I
GFP-tubulin
Histone H2B-RFP

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 spore 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 was recently found to phosphorylate Mer2 on residues that are essential for DSB formation (Sasanuma et al., 2008; Wan et al., 2008). How DSB formation is delayed until after DNA replication is less clear. We show that DSB formation requires more Dbf4 protein than replication. This supports a model in which increasing DDK activity first reaches the threshold for replication and only later that for DSB formation (Murakami and Keeney, 2008). DDK is also regulated by DNA damage and replication checkpoints, which might keep DDK activity low until replication has been completed (Duncker and Brown, 2003). Coregulation of replication and recombination is probably an evolutionary conserved function of DDK because defects in both processes have also been observed in a DDK mutant of fission yeast (Ogino et al., 2006).

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, spore formation is clearly delayed in DDK mutants, which might result from the delay in the onset of Pds1 degradation in meiosis I. This process requires Cdc5 (Clyne 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.

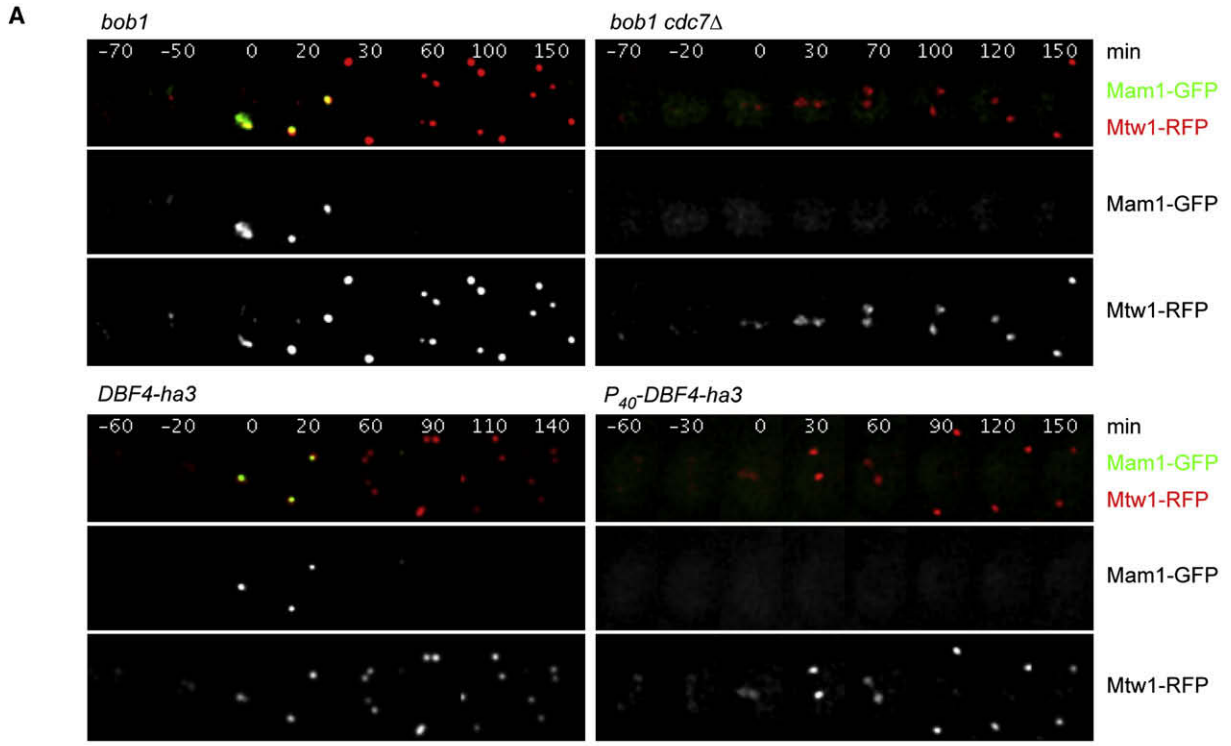
DDK mutants produce viable spores because they segregate sister chromatids in meiosis II on a single spindle axis that originates from metaphase I. Meiosis II spindle formation requires anaphase I and thus depends only indirectly on DDK. In DDK mutants, deprotection of centromeric cohesion restores both 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 *mam1Δ 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.

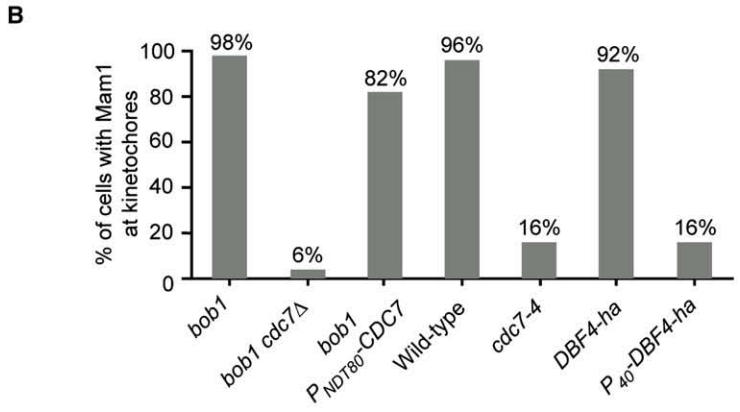
Our data identify DDK as a key regulator of meiotic chromosome segregation in yeast. After induction of meiosis, accumulation of Dbf4 activates DDK, which initiates DNA replication

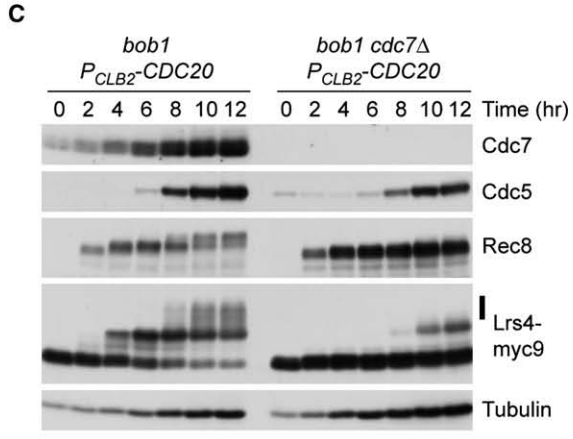
Figure 5. Monopolar Attachment Requires Postreplicative DDK Activity

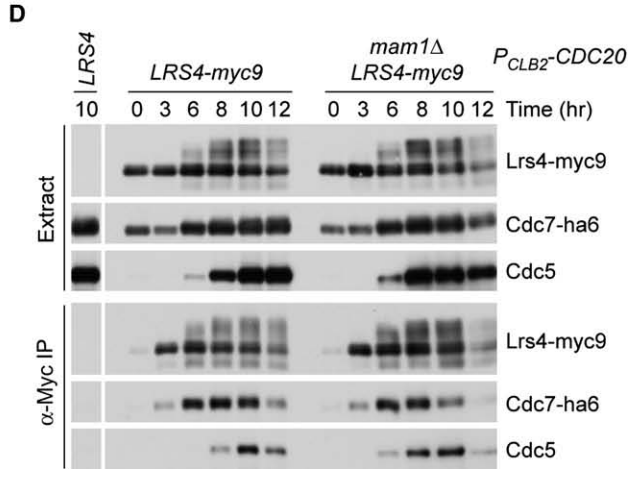
(A–D) Cdc7 inactivation after S phase. *CDC7* (Z8291) and *cdc7-4* (Z8292) cells containing Pds1-myc18, Rec8-ha3, TetR-GFP, heterozygous *URA3-tetO*, and an estradiol-inducible *NDT80* gene (P_{GAL}-*NDT80*) were transferred to SPM (no estradiol) at 25°C causing cells to arrest in pachytene. In (A), cultures were kept at 25°C and estradiol was added at t = 7 hr. In (B), cells were shifted to 34°C at t = 6 hr and estradiol was added at t = 7 hr. Percentages of cells with two nuclei (black), four nuclei (gray), one spindle (red), two spindles (pink), separated *URA3-tetO* dots (green), and Pds1-myc18 staining (blue) were determined by immunofluorescence analysis of fixed cells. (C) Nuclear division at 34°C was quantified in Pds1-negative cells with two (anaphase I) or four SPBs (anaphase II). (D) Percentage of equational *URA3-tetO* sister segregation in binucleated Pds1-negative cells at 34°C.

(E) *CDC7* induction after S phase. Time-lapse series are shown of GFP-tubulin and histone H2B/Htb1-RFP in *bob1* cells with P_{NDT80}-*CDC7* (Z11683), *CDC7* (Z11680), or *cdc7Δ* (Z12220).


A
bob1
bob1 cdc7Δ
-70 -50 0 20 30 60 100 150
-70 -20 0 30 70 100 120 150
min
Mam1-GFP
Mtw1-RFP
Mam1-GFP
Mtw1-RFP
DBF4-ha3
P40-DBF4-ha3
-60 -20 0 20 60 90 110 140
-60 -30 0 30 60 90 120 150
min
Mam1-GFP
Mtw1-RFP
Mam1-GFP
Mtw1-RFP


B
% of cells with Mam1 at kinetochores
100
80
60
40
20
0
98%
6%
82%
96%
16%
92%
16%
bob1
bob1 cdc7Δ
bob1 PNDT80-CDC7
Wild-type
cdc7-4
DBF4-ha
P40-DBF4-ha


C
bob1 PCLB2-CDC20
bob1 cdc7Δ PCLB2-CDC20
0 2 4 6 8 10 12
0 2 4 6 8 10 12
Time (hr)
Cdc7
Cdc5
Rec8
Lrs4-myc9
Tubulin


D
LRS4
LRS4-myc9
mam1Δ LRS4-myc9
PCLB2-CDC20
10
0 3 6 8 10 12
0 3 6 8 10 12
Time (hr)
Extract
Lrs4-myc9
Cdc7-ha6
Cdc5
α-Myc IP
Lrs4-myc9
Cdc7-ha6
Cdc5

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 reductional 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. Monopolin 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 similar phenotypes as a *SPO13* deletion including defects in monopolin 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 *CEN5-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 UPlanSApo 100x/1.4NA/oil objective, an eGFP/mCherry dual band filter set (F89-021, AHF Analysentechnik, 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 cdc7Δ* (Z12459), *bob1* P_{NDT80}-*CDC7* (Z12479), wild-type (31°C, Z11252), *cdc7-4* (31°C, Z11362), *DBF4-ha3* (Z13368), and P_{40}-*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_{CLB2}-*CDC20 LRS4-myc9* cells with *bob1* (Z9447) or *bob1 cdc7Δ* (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_{CLB2}-*CDC20 CDC7-ha6* cells with *LRS4* (Z11529), *LRS4-myc9* (Z11528), or *LRS4-myc9 mam1Δ* (Z13505).

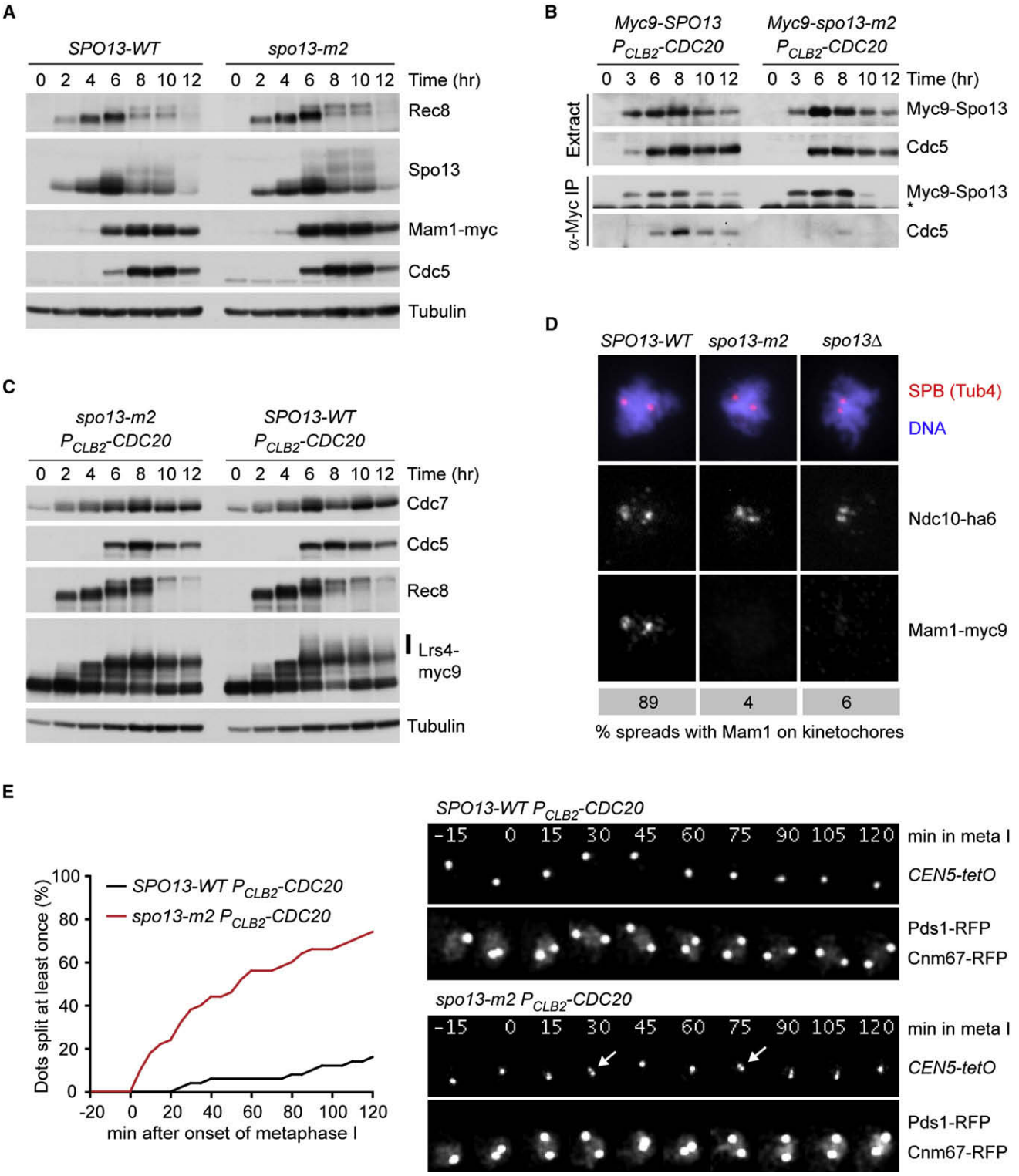

Figure 7. Monopolar Attachment Requires Binding of Spo13 to Cdc5

(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 P_{CLB2}-*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 P_{CLB2}-*CDC20 LRS4-myc9* cells with *Ha3-spo13-m2* (Z11942) or *Ha3-SPO13-WT* (Z11941).

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(D) Analysis of Mam1-myc9 localization to kinetochores (Ndc10-ha6) on chromosome spreads from *SPO13-WT* (Z12183), *spo13-m2* (Z12184), and *spo13Δ* (Z10726) cells in meiosis I (two SPBs).

(E) Live-imaging of P_{CLB2}-*CDC20 SPO13-WT* (Z12558) and P_{CLB2}-*CDC20 spo13-m2* (Z12559) cells arrested in metaphase I. Cells contain TetR-GFP at heterozygous *CEN5-tetO*, Pds1-RFP, and Cnm67-RFP at SPBs. Left: accumulation of metaphase I-cells that split *CEN5-tetO* once or more (n = 50). Right: time-lapse series. Arrows mark split *CEN5-tetO*.

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