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The Yeast APC/C Subunit Mnd2 Prevents Premature Sister Chromatid Separation Triggered by the Meiosis-Specific APC/C-Ama1

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Summary

Cohesion established between sister chromatids during pre-meiotic DNA replication mediates two rounds of chromosome segregation. The first division is preceded by an extended prophase wherein homologous chromosomes undergo recombination. The persistence of cohesion during prophase is essential for recombination and both meiotic divisions. Here we show that Mnd2, a subunit of the anaphase-promoting complex (APC/C) from budding yeast, is essential to prevent premature destruction of cohesion in meiosis. During S- and prophase, Mnd2 prevents activation of the APC/C by a meiosis-specific activator called Ama1. In cells lacking Mnd2 the APC/C-Ama1 enzyme triggers degradation of Pds1, which causes premature sister chromatid separation due to unrestrained separase activity. In vitro, Mnd2 inhibits ubiquitination of Pds1 by APC/C-Ama1 but not by other APC/C holo-enzymes. We conclude that chromosome segregation in meiosis depends on the selective inhibition of a meiosis-specific form of the APC/C.

Introduction

The establishment of cohesion between sister chromatids during DNA replication and its persistence until metaphase are both essential for proper chromosome segregation in mitosis (reviewed in Nasmyth, 2001). During metaphase cohesion allows microtubules connecting sister kinetochores with opposite spindle poles to generate tension, which is required to stabilize microtubule-kinetochore interactions. Sister chromatids are linked together by the ring-shaped cohesin complex whose destruction triggers sister chromatid separation and entry into anaphase. This is achieved by separase (Esp1 in budding yeast), a thiol protease that cleaves cohesin's kleisin subunit Scc1 (Uhlmann et al., 2000). For most of the cell cycle separase is kept inactive by binding to an inhibitor called Pds1 or securin (Ciosk et al., 1998; Uhlmann et al., 1999). Pds1 is only removed at the metaphase-to-anaphase transition through proteolysis (Cohen-Fix et al., 1996; Funabiki et al., 1996; Zou et al., 1999), which is triggered by a multisubunit ubiquitin ligase called the anaphase-promoting complex or cyclosome (APC/C) (reviewed in Harper et al., 2002). The APC/C also mediates exit from mitosis by ubiquitinating B type cyclins; the ubiquitination inactivates cyclin-dependent kinase 1 (Cdk1, Cdc28 in budding yeast). Ubiquitin ligation requires association of the APC/C with a member of the Cdc20 family of WD repeat proteins. Cdc20 binds to the APC/C during mitosis and triggers degradation of Pds1 and cyclin B. The related Cdh1 protein activates APC/C during G1.

Inhibitory mechanisms are important to restrict APC/C's activity to anaphase and G1; this restriction facilitates the accumulation of mitotic cyclins and helps to prevent premature destruction of cohesion. Cdh1 is inhibited through phosphorylation by S phase- and M phase-promoting CDKs. In addition, the Emi1/Rca1 protein inhibits Cdh1 from S phase until prophase in animal cells (Grosskortenhaus and Sprenger, 2002; Hsu et al., 2002). The activity of Cdc20 is controlled by the phosphorylation status of the APC/C and a series of different inhibitors. Cdc20 binds to Emi1 prior to mitosis (Reimann et al., 2001a), to RASSF1A during pro-metaphase (Song et al., 2004), and to the mitotic checkpoint complex (MCC) during metaphase until all chromosomes have attached to microtubules in a bipolar manner (Yu, 2002). Restraining APC/C activity to protect cohesion might be an especially demanding task during meiosis, where prophase lasts much longer than in the mitotic cell cycle.

In meiosis, a single round of cohesion establishment during pre-meiotic S phase mediates two rounds of chromosome segregation. This depends on the unique behavior of chromosomes in the first meiotic division (meiosis I) where homologous chromosomes but not sister chromatids segregate (reviewed in Petronczki et al., 2003). During prophase I, homologous chromosomes align and then undergo recombination between non-sister chromatids, which results in the formation of chiasmata connecting maternal and paternal chromosomes. Recombination is initiated by the generation of double-strand breaks (DSBs) through an endonuclease called Spo11 (Keeney et al., 1997). The repair of DSBs is monitored by the pachytene checkpoint, which blocks exit from prophase I as long as unprocessed recombination intermediates are present (Roeder and Bailis, 2000). During meiosis I, sister kinetochores attach to microtubules from the same pole while kinetochore pairs on homologous chromosomes attach to opposite poles. As a result, the tendency of the metaphase I spindle to pull apart homologous chromosomes is resisted by sister chromatid cohesion on chromosomal arms, distal from chiasmata. Destruction of arm cohesion is therefore required for segregation of homologs in meiosis I, while cohesion around centromeres persists until sister chromatids separate in the second division (meiosis II). Protection of centromeric cohesion

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during meiosis I requires the kinetochore protein Sgo1/shugoshin (Watanabe, 2004) and the exchange of cohesins's Scc1 subunit for a meiosis-specific kleisin called Rec8 (Klein et al., 1999; Watanabe and Nurse, 1999). Two waves of separase activity cleave Rec8 first on chromosomal arms to trigger anaphase I and then around centromeres to initiate anaphase II (Buonomo et al., 2000; Kitajima et al., 2003).

APC/C regulation in meiosis differs from that in mitosis. Meiosis I and II are triggered by two rounds of Pds1 degradation, both of which require accumulation of Cdc20 (Lee and Amon, 2003; Salah and Nasmyth, 2000). How APC/C-Cdc20 is activated and inactivated twice in rapid succession is unclear. In several organisms, meiosis-specific APC/C activators have been identified. In *Drosophila* the Cdc20 family members Fzr2 and Cortex are exclusively expressed during spermatogenesis and oogenesis, respectively (Chu et al., 2001; Jacobs et al., 2002). Spore formation, the yeast equivalent of gametogenesis, requires the activators Ama1 in *S. cerevisiae* and Mfr1/Fzr1 in *S. pombe* (Asakawa et al., 2001; Blanco et al., 2001; Cooper et al., 2000). How the activities of different APC/C holoenzymes are regulated and coordinated in meiosis is poorly understood.

To investigate meiosis-specific aspects of APC/C regulation we analyzed *MND2* from budding yeast. *MND2* was discovered as a gene required for meiotic nuclear division but not for proliferation (Rabitsch et al., 2001). Nuclear divisions could be restored in *mnd2Δ* mutants by preventing the formation of DSBs, suggesting that Mnd2 is required for the completion of recombination. However, genetic analysis indicated that Mnd2 has additional functions independent of the recombination process. Later on, the Mnd2 protein was found to be associated with the APC/C in vegetative cells (Yoon et al., 2002). Here we show that Mnd2 prevents APC/C-Ama1 from triggering premature separation of sister chromatids.

Results

Mnd2 Disappears from the APC/C during Anaphase II of Meiosis

To test whether Mnd2 binds to the meiotic APC/C, the complex was immunoprecipitated with anti-Myc antibodies as diploid *APC2myc9* cells of the quickly sporulating SK1 strain progressed through meiosis (Figure 1A). Mnd2 bound to the APC/C during early stages of meiosis but when most cells had completed nuclear division Mnd2 levels dropped and the protein disappeared from the APC/C. In contrast, other subunits remained associated with the complex. Immunofluorescence from a functional Mnd2myc18 revealed that the protein was present in nuclei from G1 until metaphase II but then disappeared during anaphase II (Figure 1B). Increased expression of *MND2* in late meiosis led to constitutive binding to the APC/C, but this did not interfere with the production of viable spores (Figure S1 available with this article online). As cells entered meiosis, Mnd2 was increasingly phosphorylated, as indicated by the appearance of a phosphatase-sensitive shift in gel mobility. In conclusion, Mnd2 is a cell cycle-regulated component of the meiotic APC/C.

Mnd2 Is Required for the Accumulation of Pds1 during Meiotic S- and Prophase I

Next, we induced wild-type (wt) and *mnd2Δ* cells to enter meiosis. The mutant cells replicated DNA normally but then failed to assemble spindles, to divide their nuclei, and to form spores. They also failed to accumulate proteins such as Cdc5 and Cdc20, which appear when wt cells exit from prophase I (Figure 2A). Thus, *mnd2Δ* cells resemble cells arrested in prophase I by the pachytene checkpoint. Consistent with this, the deletion of *SPO11* restores nuclear divisions and sporulation in *mnd2Δ* cells (Rabitsch et al., 2001). *mnd2Δ* cells produced Kip1 and Cin8, whose expression commences around S phase. Interestingly, the mutant cells contained little or no Pds1, although in wt cells Pds1 appears shortly before S phase (Figures 2A and 2B). This was confirmed with strains lacking Ndt80, a transcription factor required for exit from the pachytene stage of prophase I (Chu and Herskowitz, 1998); Pds1myc18 accumulated in *ndt80Δ* cells but not in *ndt80Δ mnd2Δ* double mutants (Figure S2). We conclude that Mnd2 is required for Pds1's accumulation during S- and prophase I. Cells expressing *MND2* solely from the early meiosis *DMC1* promoter accumulated Pds1 normally and produced viable spores with wt kinetics (not shown). Thus, defective meiosis in *mnd2Δ* cells results from the lack of meiosis-specific functions and not from Mnd2's absence during vegetative growth.

To investigate Pds1's fate in *mnd2Δ* cells undergoing nuclear divisions, we analyzed *mnd2Δ spo11Δ* cells. Whereas Pds1myc18 was virtually undetectable after the 2 hr time point in *mnd2Δ* single mutants (Figure 2A), immunoblotting revealed small amounts of Pds1myc18 in *mnd2Δ spo11Δ* cells during nuclear divisions (Figure 2C). Consistent with this, immunofluorescence from Pds1myc18 appeared in *mnd2Δ spo11Δ* cells but only upon entry into metaphase I (Figure 2D). In the *spo11Δ* control cells, Pds1myc18 appeared normally, shortly before S phase. Thus, exit from prophase I allows *mnd2Δ* mutants to produce Pds1. This suggests that Mnd2 is required for Pds1 accumulation during S- and prophase I but not in metaphase I.

Mnd2 Is Required to Prevent Premature Separation of Sister Chromatids

To investigate the behavior of sister chromatids, we analyzed a series of strains, each heterozygous for chromosome V marked with GFP at a different locus: 1.4 kb from the centromere (*CenV*-GFP), 35 kb from the centromere (*URA3*-GFP), and 30 kb from the right telomere (*TelV*-GFP). In wt cells, *TelV*-GFP dots separate in anaphase I whereas *CenV*-GFP and *URA3*-GFP dots separate only in anaphase II (Toth et al., 2000). Up to 60% of *mnd2Δ* cells separated *CenV*-GFP dots, *URA3*-GFP dots, and *TelV*-GFP dots although nuclear division failed in these cells. Dot separation occurred 1.5 to 2 hr earlier (relative to DNA replication) in the mutant cells than in the wt (Figures 2A and S3A–S3C). In fact, the kinetics of sister separation in *mnd2Δ* cells was similar to that in cells lacking the meiotic cohesin subunit Rec8 (Figure S3D). Sisters separated prematurely in *mnd2Δ* cells even if they lacked Ndt80 (Figure S2) or were able

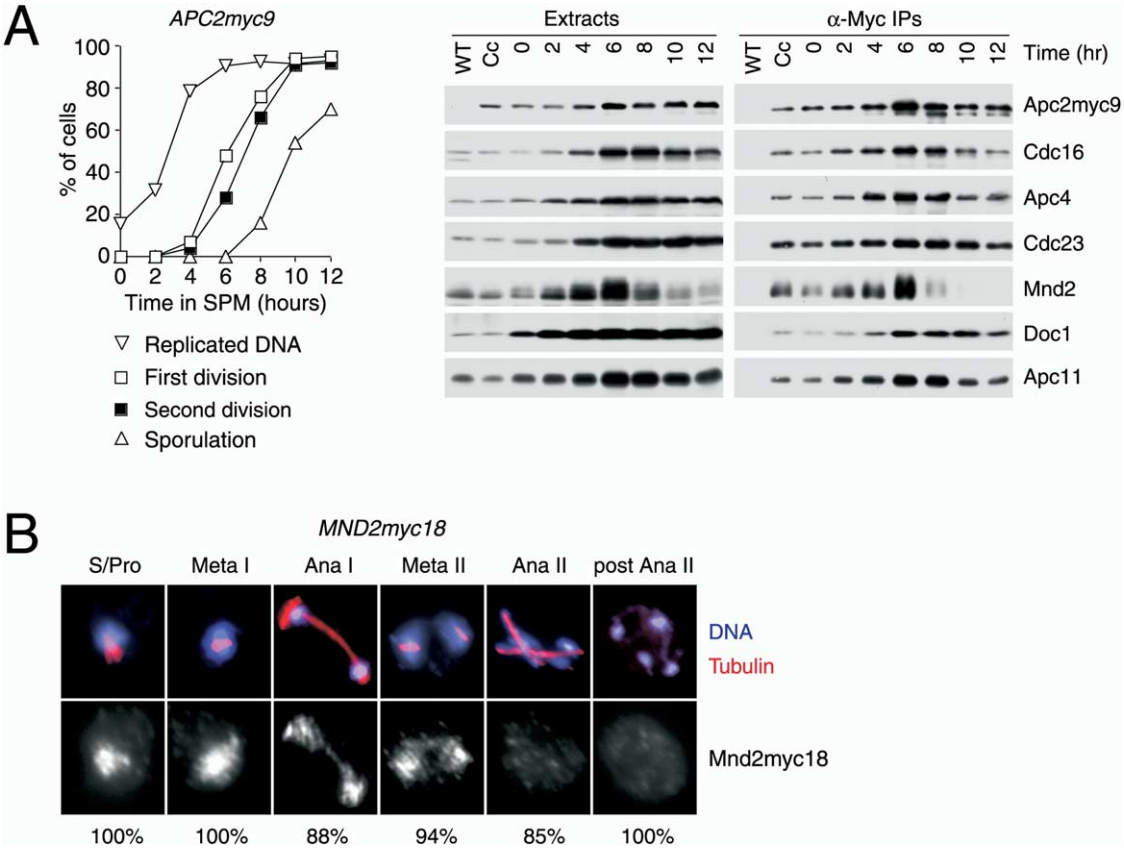


Figure 1. Mnd2 Is Associated with the Meiotic APC/C until Anaphase II

(A) *APC2myc9* cells (Z5187) were transferred to sporulation medium (SPM) and samples were withdrawn every 2 hr. Left: percentages of cells with replicated DNA, first and second nuclear division, and spore formation. Right: immunoblot detection of different APC/C subunits in extracts and anti-Myc immunoprecipitates. Controls were from proliferating wt and *APC2myc9* (Cc) cells.

(B) Staining from DNA (DAPI, blue), tubulin (red), and Mnd2myc18 in meiotic *MND2myc18* cells (Z3023). Percentages of cells represented by the pictures are shown.

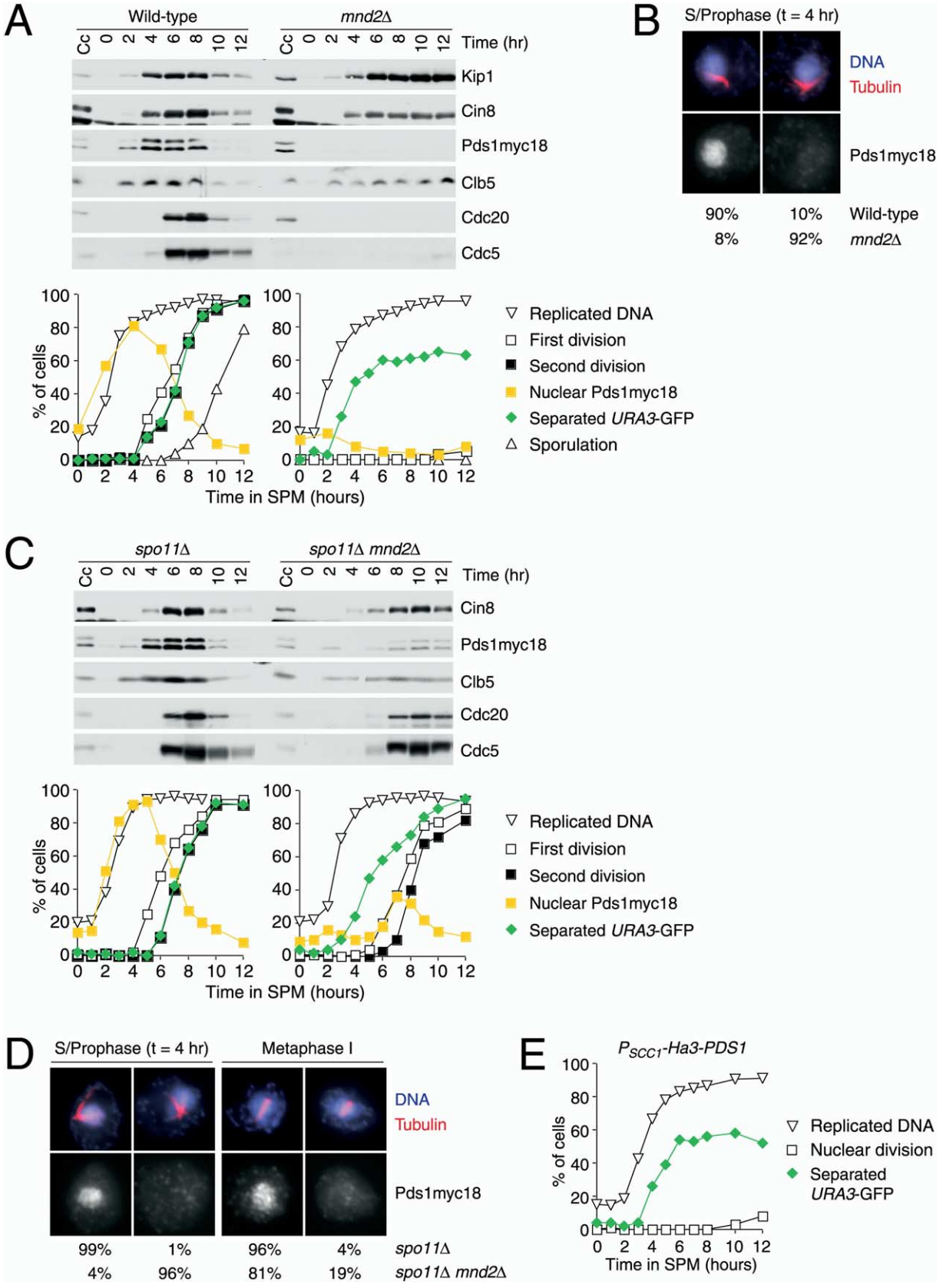
to undergo nuclear division (Figure 2C). We conclude that Mnd2 is required during S- and prophase I to prevent premature separation of sister sequences around centromeres and on chromosomal arms.

Premature sister separation explains why deletion of *SPO13* allows *spo11Δ* cells but not *spo11Δ mnd2Δ* double mutant cells to produce viable spores (Rabitsch et al., 2001). *spo11Δ* cells produce inviable spores because homologs lacking chiasmata segregate randomly. Deletion of *SPO13* causes bi-orientation of sister kinetochores resulting in a single equational division that yields two diploid, viable spores (Klapholz et al., 1985). In *spo11Δ spo13Δ mnd2Δ* cells, Pds1myc18 accumulated only upon entry into metaphase and sister chromatids separated prematurely, prior to spindle formation and nuclear division. These unlinked sister chromatids segregated randomly, which yields inviable spores (Figure S4).

Is Pds1's absence responsible for premature sister separation in *mnd2Δ* cells? Cells lacking Pds1 separate sisters on time in an unperturbed, mitotic cell cycle (Alexandru et al., 1999). To test whether Pds1 is required to prevent premature sister separation in meiosis, we replaced the promoter of *PDS1* with the mitosis-specific *SCC1* promoter. The resulting P_{SCC1}-*Ha3-PDS1* cells proliferated normally but lacked Pds1 in meiosis (not shown). Pds1-depleted cells separated sister chromatids prematurely, with kinetics similar to that of *mnd2Δ* cells and also arrested in a prophase-like state (Figure 2E). Thus, lack of Pds1 is sufficient for premature sister separation in *mnd2Δ* cells.

Mnd2 Is Required to Prevent Premature Cleavage of Rec8 by Separase

To test whether premature sister separation required separase/Esp1, we used the temperature-sensitive (ts) mutation *esp1-2* (Buonomo et al., 2000). Indeed, sister separation was blocked at 34°C in *mnd2Δ esp1-2* cells (Figure 3A). Replacing Rec8 with the noncleavable version Rec8-N (Buonomo et al., 2000) led to a similar result (Figure 3B). These data suggest that premature sister separation in *mnd2Δ* cells depends on the cleavage of Rec8 by separase and argue against a defect in the function of cohesin itself. Next, we compared the behavior of Rec8 (tagged with Ha3) during S- and prophase I in *MND2* and *mnd2Δ* cells. To prevent nuclear division and Rec8 cleavage in *MND2* cells, we used strains lacking Ndt80. Immunoblotting showed that Rec8ha3 per-


A
Wild-type
mnd2Δ
Cc 0 2 4 6 8 10 12
Time (hr)
Kip1
Cin8
Pds1myc18
Clb5
Cdc20
Cdc5
% of cells
Time in SPM (hours)
Replicated DNA
First division
Second division
Nuclear Pds1myc18
Separated URA3-GFP
Sporulation
B
S/Prophase (t = 4 hr)
DNA
Tubulin
Pds1myc18
90% 10% Wild-type
8% 92% mnd2Δ
C
spo11Δ
spo11Δ mnd2Δ
Cin8
Pds1myc18
Clb5
Cdc20
Cdc5
Replicated DNA
First division
Second division
Nuclear Pds1myc18
Separated URA3-GFP
D
S/Prophase (t = 4 hr)
Metaphase I
DNA
Tubulin
Pds1myc18
99% 1% 96% 4% spo11Δ
4% 96% 81% 19% spo11Δ mnd2Δ
E
PSCC1-Ha3-PDS1
% of cells
Time in SPM (hours)
Replicated DNA
Nuclear division
Separated URA3-GFP

sisted in *ndt80Δ* cells but was rapidly cleaved in *ndt80Δ mnd2Δ* cells (Figure 3C). Chromosome spreading revealed that Rec8ha3 stably bound to the chromatin from *ndt80Δ* cells. In contrast, the kleisin initially associated with chromatin in 58% of *ndt80Δ mnd2Δ* cells but then disappeared before cells had completed DNA replication (Figure 3D). Spreads from *ndt80Δ* cells arrested in pachytene (t = 8 hr) showed synapsed bivalents decorated with Rec8ha3 along their axes, whereas *ndt80Δ mnd2Δ* cells yielded diffuse chromatin lacking Rec8ha3. Replacing Rec8ha3 with Rec8-N-ha3 restored cohesin binding to thread-like chromatin structures (Figure 3E). We conclude that Mnd2 is required for the persistence of cohesin on chromosomes during S- and prophase I.

Rec8 is required during prophase I for the assembly of the synaptonemal complex (SC) (Klein et al., 1999), which connects homologs along their entire length in pachytene. To analyze SC formation, we detected the SC component Zip1 (Sym et al., 1993) on chromosome spreads from *ndt80Δ* strains arrested in pachytene. Zip1 decorated chromosomal axes in *ndt80Δ* cells but was absent from the chromatin of *ndt80Δ mnd2Δ* cells. Instead, Zip1 was concentrated in extra-chromosomal aggregates called polycomplexes (Figure 3E). Inactivation of separase by the *esp1-2* mutation or its inhibition by accumulation of Pds1 (see below) restored Zip1's association with chromosomal axes although polycomplexes were more frequent than in *MND2* cells. Interestingly, Rec8-N-ha3 restored cohesion but not efficient SC formation in *ndt80Δ mnd2Δ* cells; 75% of spreads contained little or no chromatin-associated Zip1. These data show that synapsis requires inhibition of separase through Mnd2-dependent accumulation of Pds1. However, blocking only Rec8 cleavage was not sufficient, suggesting that synapsis requires inhibition of additional events triggered by separase.

Mnd2 Prevents Premature, APC/C-Dependent Proteolysis of Pds1

To test whether APC/C-dependent proteolysis removed Pds1 from *mnd2Δ* cells, we expressed Pds1myc18 from the early meiosis *DMC1* promoter (Figure 4A). Pds1myc18 appeared in wt but not in *mnd2Δ* cells, similar to Pds1 expressed from its own promoter. We then mutated Pds1's destruction box and KEN box, which are required for ubiquitination by the APC/C. The mutant Pds1(mDK)myc18 protein accumulated to high levels and blocked sister separation in both wt and *mnd2Δ* cells (Figure 4A). This suggests that APC/C-dependent proteolysis prevents accumulation of Pds1 in *mnd2Δ* cells and that stabilization of Pds1 is sufficient to restore cohesion through the inhibition of separase. wt cells expressing nondegradable Pds1 accumulated Cdc20 on time, assembled metaphase spindles, and formed dyad spores (thereby fragmenting the chromatin). In contrast, these processes did not occur in the corresponding *mnd2Δ* cells (Figure 4A). Consistent with this, *esp1-2* cells but not *esp1-2 mnd2Δ* double mutants produced Cdc20 and formed spindles at 34°C (not shown). Thus, restoring sister chromatid cohesion is not sufficient to silence the pachytene checkpoint in *mnd2Δ* cells. This suggests a function for Mnd2 in the completion of recombination, which is independent from promoting Pds1's accumulation.

If APC/C was prematurely activated in *mnd2Δ* cells, then reducing APC/C's activity might restore nuclear divisions. Indeed, *mnd2Δ* cells lacking the nonessential APC/C subunit Swm1/Apc13 accumulated Pds1myc18, performed both nuclear divisions, and separated sisters at the onset of anaphase II (Figures 4B–4D). Eighty percent of the double mutant cells produced four-spored asci, albeit with a delay of ~5 hr. Nevertheless, 85% of these spores were viable. Chromosome spreads from *ndt80Δ mnd2Δ swm1Δ* cells arrested in pachytene showed synapsed bivalents decorated with Rec8ha3 and Zip1 along their axes, similar to spreads from *ndt80Δ* cells (Figure 4E). Also the *apc* ts-mutation *cdc27-663* restored progression through meiosis in *mnd2Δ* cells; within 12 hr at 30°C, 60% of *mnd2Δ cdc27-663* cells produced asci containing four spores, 88% of which were viable (Figure S5). Thus, reducing APC/C activity restores nuclear division and sporulation in *mnd2Δ* cells, which supports the idea that Mnd2 restrains APC/C-dependent proteolysis of Pds1 and of proteins required to complete recombination.

Mnd2 Inhibits the Activity of the Meiosis-Specific APC/C-Ama1

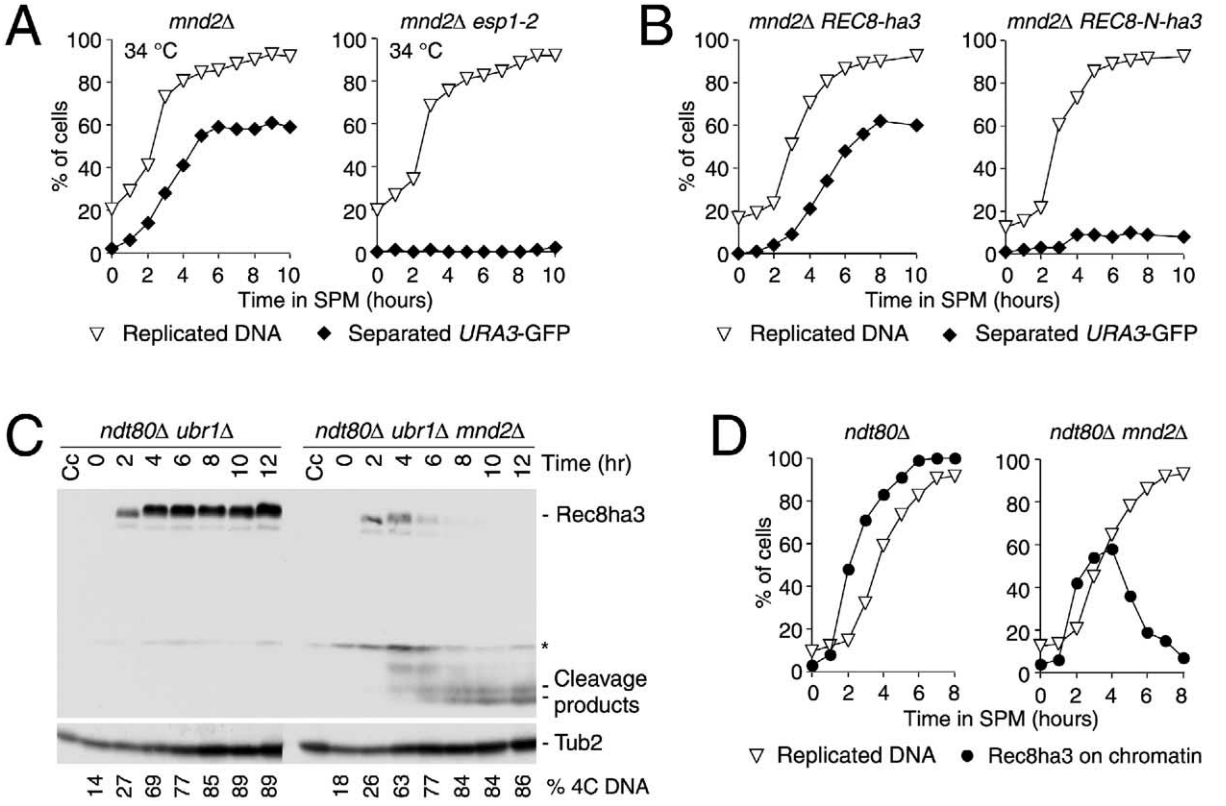
Which APC/C activator is required for Pds1 degradation in *mnd2Δ* cells? First, we removed the meiosis-specific activator Ama1 (Cooper et al., 2000). *mnd2Δ ama1Δ* cells produced Pds1myc18, performed both nuclear divisions, and separated sisters at the onset of anaphase II (Figures 5A–5C). Furthermore, *mnd2Δ ama1Δ* cells lacking Ndt80 stably maintained sister chromatid cohesion as they progressed through prophase I and arrested in pachytene (Figure S2). Spreads from the arrested *ndt80Δ mnd2Δ ama1Δ* cells showed synapsed bivalents decorated with Rec8, similar to

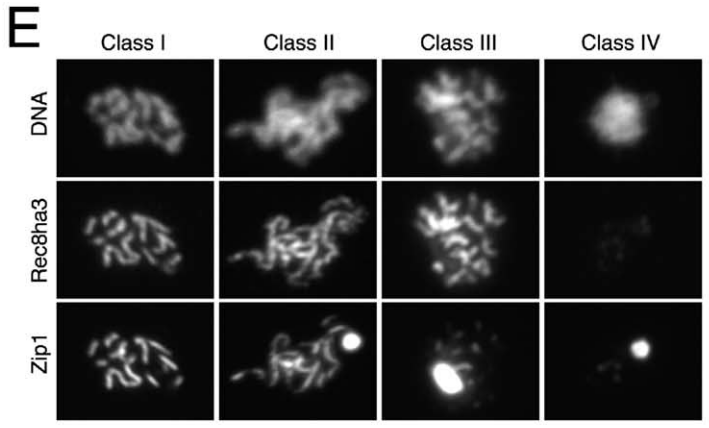
Figure 2. Mnd2 Is Required for Pds1 Accumulation and to Prevent Premature Separation of Sister Chromatids

(A and B) Analysis of *MND2* (wild-type, Z4861) and *mnd2Δ* (Z4860) strains (genotype: *PDS1/PDS1myc18 ura3/URA3-GFP*) in meiosis. (A) Panel, immunoblot analysis of protein levels. Graphs, percentages of cells with replicated DNA, first and second nuclear division, spore formation, Pds1myc18 staining, and separated *URA3*-GFP dots. (B) Staining from DNA (DAPI, blue), tubulin (red), and Pds1myc18 in S/prophase I cells at t = 4 hr. Percentages of cells represented by the pictures are shown.

(C and D) Analysis of *spo11Δ* (Z5799) and *spo11Δ mnd2Δ* (Z4980) strains (genotype: *PDS1/PDS1myc18 ura3/URA3-GFP*) in meiosis. (C) Panel, immunoblot analysis of protein levels. Graphs, percentages of cells with replicated DNA, first and second nuclear division, spore formation, Pds1myc18 staining, and separated *URA3*-GFP dots. (D) Staining from DNA (DAPI, blue), tubulin (red), and Pds1myc18 in cells in S/prophase I and in metaphase I. Percentages of cells represented by the pictures are shown.

(E) Percentages of cells with replicated DNA, separated *URA3*-GFP dots, and divided nuclei in meiotic P_{SCC1}*-Ha3-PDS1 ura3/URA3-GFP* cells (Z4061).





81%	17%	2%	0%	*ndt80Δ REC8-ha3*
74%	24%	2%	0%	*ndt80Δ REC8-N-ha3*
0%	0%	0%	93%	*ndt80Δ mnd2Δ REC8-ha3*
2%	23%	75%	0%	*ndt80Δ mnd2Δ REC8-N-ha3*
21%	76%	3%	0%	*ndt80Δ mnd2Δ* P_{DMC1}*-PDS1(mDK)myc18 REC8-ha3*
26%	70%	4%	0%	*ndt80Δ mnd2Δ esp1-2 REC8-ha3* (34 °C)
84%	14%	2%	0%	*ndt80Δ REC8-ha3* (34 °C)

Figure 3. Mnd2 Is Required to Prevent Premature Cleavage of Rec8 and for Synapsis

(A) DNA replication and separation of *URA3*-GFP dots in *mnd2Δ* (Z4860) and *mnd2Δ esp1-2* (Z3702) strains (genotype: *PDS1/PDS1myc18 ura3/URA3-GFP*) grown at 25°C and transferred to SPM at 34°C.

(B) DNA replication and separation of *URA3*-GFP dots in *mnd2Δ ura3/URA3-GFP* strains containing *REC8-ha3* (Z5578) or *REC8-N-ha3* (Z5472).

(C) Immunoblot detection of Rec8ha3 in meiotic *ndt80Δ ubr1Δ REC8/REC8ha3* strains containing *MND2* (Z4386) or *mnd2Δ* (Z4388). Asterisk, nonspecific bands. % 4C DNA, percentages of cells with replicated DNA. The *UBR1* deletion prevents degradation of Rec8 fragments (Buonomo et al., 2000).

those from *ndt80Δ* single mutants (Figure 5D). These data suggest that during S- and prophase I Mnd2 prevents premature Pds1 degradation and sister separation mediated by APC/C-Ama1. Analysis of *ndt80Δ* strains revealed that Mnd2 inhibits premature, Ama1-dependent degradation of proteins in addition to Pds1; the levels of the cyclin Clb5 and of Sgo1 were low in the *mnd2Δ* mutant but recovered upon deletion of *AMA1* (Figure S2). Ama1 is known to be required in late meiosis for spore formation (Rabitsch et al., 2001). Our data, however, imply that the activator appears much earlier. Indeed, immunofluorescence from a Myc-tagged Ama1 appeared during S phase in the nuclei of wt cells (not shown).

Next, we compared Ama1's binding to the APC/C as *MND2* and *mnd2Δ* cells progressed through S- and prophase I (Figure 5E). *ndt80Δ* strains were used to prevent exit from prophase I in *MND2* cells. The *MND2* deletion did not affect the expression level of Ama1, but it increased coimmunoprecipitation of the activator with Apc2. In addition, the *MND2* deletion reduced Ama1's modification to a species with decreased gel mobility. This modification was sensitive to phosphatase (not shown) and disappeared after mutating all eight Ser/Thr-Pro sites to Ala-Pro, suggesting that Ama1 is phosphorylated by a kinase with Cdk1-like specificity. The mutant Ama1m8 protein was functional but did not detectably change the behavior of wt and *mnd2Δ* cells. The *MND2* deletion increased also binding of Ama1m8 to the APC/C. This shows that Mnd2 reduces Ama1's association with the APC/C by a mechanism that does not require phosphorylation of the activator on Ser/Thr-Pro sites.

Do Cdc20 and Cdh1 contribute to Pds1 degradation in *mnd2Δ* cells? Cdc20 was undetectable in meiotic *mnd2Δ* cells, suggesting that it was not involved (Figure 2A). This was confirmed with strains expressing *CDC20* solely from the mitosis-specific *CLB2* promoter. P_{CLB2}-*CDC20* cells arrested in metaphase I with high levels of Pds1 whereas *mnd2Δ* P_{CLB2}-*CDC20* cells behaved indistinguishably from *mnd2Δ* single mutants (Figure S6A). However, we detected Pds1 in extracts from *mnd2Δ cdh1Δ* cells induced to enter meiosis. These extracts contained Ama1 but also the mitosis-specific cyclin Clb2, indicating that some cells entered meiosis whereas others continued with aspects of the mitotic cell cycle (Figure S6B). *cdh1Δ* cells were defective in efficiently entering meiosis upon nutrient deprivation. To detect induction of meiosis in individual cells, we analyzed strains containing *REC8-N-ha3*. Six hours after induction of meiosis, >90% of wt and *mnd2Δ* cells but only 70% of the *cdh1Δ* mutants produced Rec8-N-ha3. Analysis of Pds1myc18 in Rec8-N-positive cells revealed that *cdh1Δ* cells behaved similarly to the wt whereas *mnd2Δ cdh1Δ* cells resembled *mnd2Δ* cells (Figure S6C). Analysis of Rec8-N-negative *cdh1Δ* mutants detected Pds1myc18 in most of the cells. We conclude that in the *mnd2Δ cdh1Δ* extracts, Pds1 originated from nonmeiotic cells and that Cdh1 is not required for premature Pds1 degradation in *mnd2Δ* cells.

Destruction of Cdk1 Kinases Activates APC/C-Ama1 during Anaphase I

Next, we analyzed Ama1's function in more detail. Consistent with previous observations *ama1Δ* cells performed DNA replication and nuclear divisions but failed to produce spores (Rabitsch et al., 2001) (Figure S7). The *AMA1* deletion increased the fraction of meiosis I cells containing an elongated spindle in a stretched but not yet divided nucleus. This indicates that spindle elongation was slow. Sixty-seven percent of these *ama1Δ* cells still contained Pds1myc18, which was observed in only 8% of the corresponding wt cells (Figure 6A). Likewise, 89% of *ama1Δ* cells but only 2% of wt cells with a stretched nucleus contained Clb5myc9 (Figure 6B). Thus, Ama1 contributes to rapid spindle elongation and timely degradation of Pds1 and Clb5 during meiosis I. However, these functions are not essential for nuclear division.

Cdc20 is essential for Pds1 degradation (Salah and Nasmyth, 2000), implying that activation of APC/C-Ama1 depends on Cdc20. To test whether APC/C-Ama1 is activated by Cdc20-dependent destruction of Cdk1/Cdc28 kinases, we used P_{CLB2}-*CDC20 cdc28-as1* strains to inhibit Cdk1 in cells depleted of Cdc20. The *cdc28-as1* mutation renders Cdk1 sensitive to the inhibitor 1-NM-PP1 (Bishop et al., 2000). Addition of 1-NM-PP1 to metaphase I-arrested cells triggered rapid spindle disassembly and degradation of Pds1myc18 (Figure 6C). Nuclei did not divide although some cells separated sister chromatids. Spindles also disassembled in the corresponding *ama1Δ* cells but Pds1myc18 remained stable. These data suggest that APC/C-Ama1 is inhibited during metaphase I by Cdk1 and then activated in anaphase I through Cdc20-dependent cyclin degradation. Consistent with this, expression of the M phase cyclin Clb1 from the *DMC1* promoter caused *mnd2Δ* cells to perform nuclear divisions and to form spores, 66% of which were viable (Figure 6D). In contrast, overexpression of the S phase cyclin Clb5 had no detectable effect (not shown), suggesting that inhibition of APC/C-Ama1 is specific to M phase-promoting Cdk1 kinases.

Mnd2 Inhibits APC/C Activity Induced by Ama1 Expression in Mitotic Cells

Our data imply that Mnd2 is required for meiosis but not for proliferation because it specifically inhibits the meiotic APC/C-Ama1. To test this, we expressed a

(D) Quantification of Rec8ha3 binding to chromosome spreads prepared from meiotic *ndt80Δ REC8ha3* (Z4619) and *ndt80Δ mnd2Δ REC8ha3* (Z4621) strains.

(E) Staining from Rec8ha3 and Zip1 on chromosome spreads from *ndt80Δ* strains of the indicated genotypes arrested in pachytene (8 hr in SPM). Spreads were categorized as follows: class I, Rec8 and Zip1 loaded, no polycomplex; class II, Rec8 and Zip1 loaded, polycomplex present; class III, Rec8 loaded, Zip1 loading defective, polycomplex present; class IV, Rec8 and Zip1 not loaded, polycomplex present. Strains from top to bottom: Z4619, Z5402, Z4621, Z4975, Z4976, Z5941, Z4619.

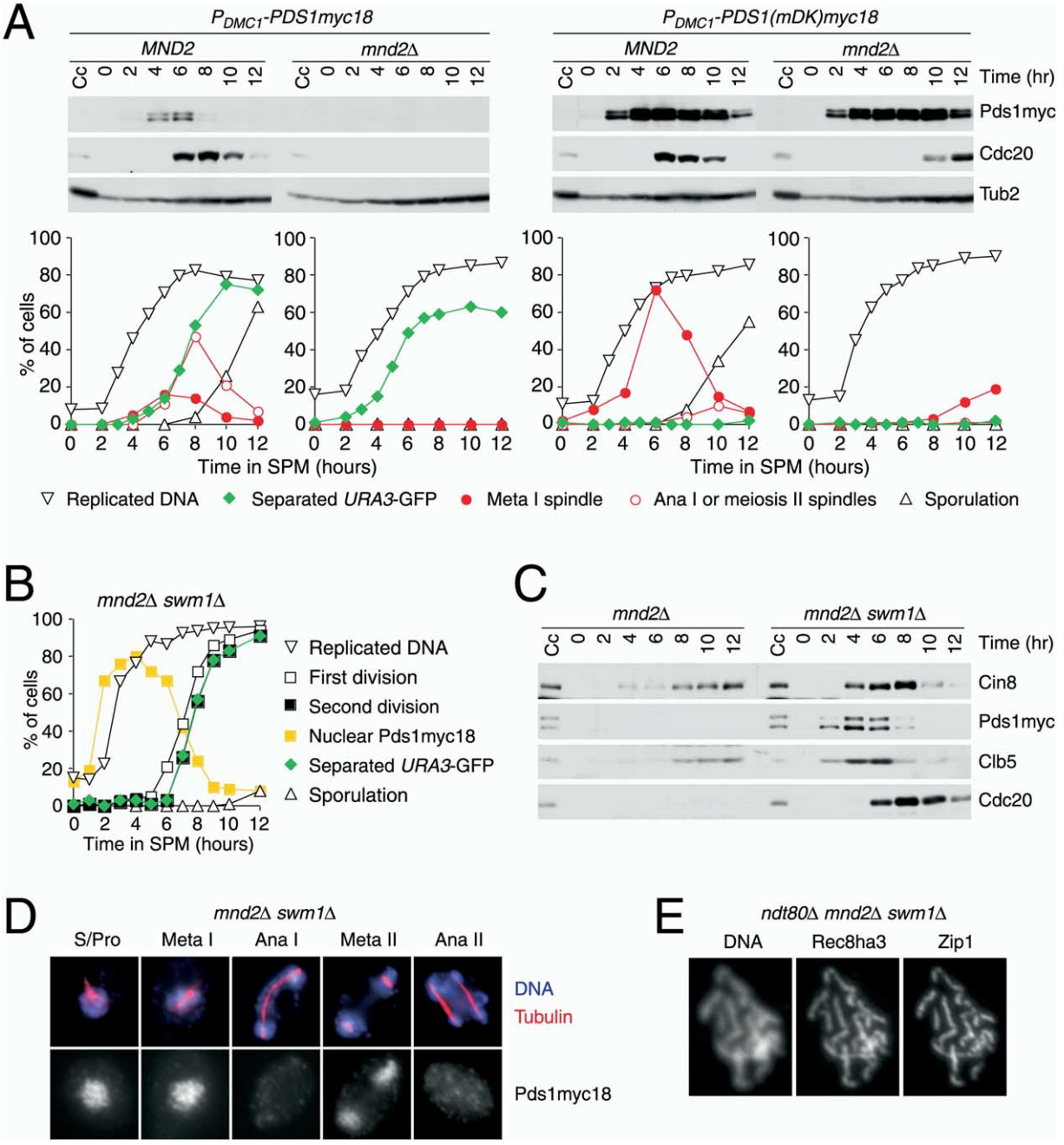


Figure 4. Premature APC/C-Dependent Degradation of Pds1 in *mnd2Δ* Cells

(A) *MND2* and *mnd2Δ* strains (*ura3/URA3-GFP*) containing P_{DMC1}-*PDS1myc18* or P_{DMC1}-*PDS1(mDK)myc18* were induced to enter meiosis. Panels: immunoblot analysis. Graphs: percentages of cells with replicated DNA, separated *URA3*-GFP dots, metaphase I spindles, anaphase I or meiosis II spindles, and spore formation. *MND2* P_{DMC1}-*PDS1myc18* cells produce tetrads while *MND2* P_{DMC1}-*PDS1(mDK)myc18* cells produce dyads. Strains from left to right: Z3464, Z3467, Z3465, Z3493.

(B–D) Analysis of *mnd2Δ swm1Δ* (Z4869) cells (genotype: *PDS1/PDS1myc18 ura3/URA3-GFP*) in meiosis. (B) Percentages of cells with replicated DNA, first and second nuclear division, Pds1myc18 staining, separated *URA3*-GFP dots, and spore formation. (C) Immunoblot analysis of protein levels. Samples from an *mnd2Δ* (Z4860) timecourse were analyzed in parallel. (D) Staining from DNA (DAPI, blue), tubulin (red), and Pds1myc18. Pictures represent ≥90% of cells at the indicated stage. S/prophase cells were from t = 4 hr.

(E) Detection of Rec8ha3 and Zip1 on chromosome spreads from *ndt80Δ mnd2Δ swm1Δ REC8ha3* cells (Z4977) arrested in pachytene (8 hr in SPM). Pictures represent 73% of spreads (class I). Twenty-two percent were in class II.

cDNA encoding Myc9-Ama1 from the weak, galactose-inducible *GALL* promoter in vegetative, haploid cells containing or lacking Mnd2. *MND2* cells grew normally on galactose medium although some cells produced elongated buds. In contrast, *mnd2Δ* cells failed to divide and arrested with a single, highly elongated bud. This arrest required the APC/C subunit Swm1/Apc13 but not the activator Cdh1 (Figure 7A). Thus, *MND2* is

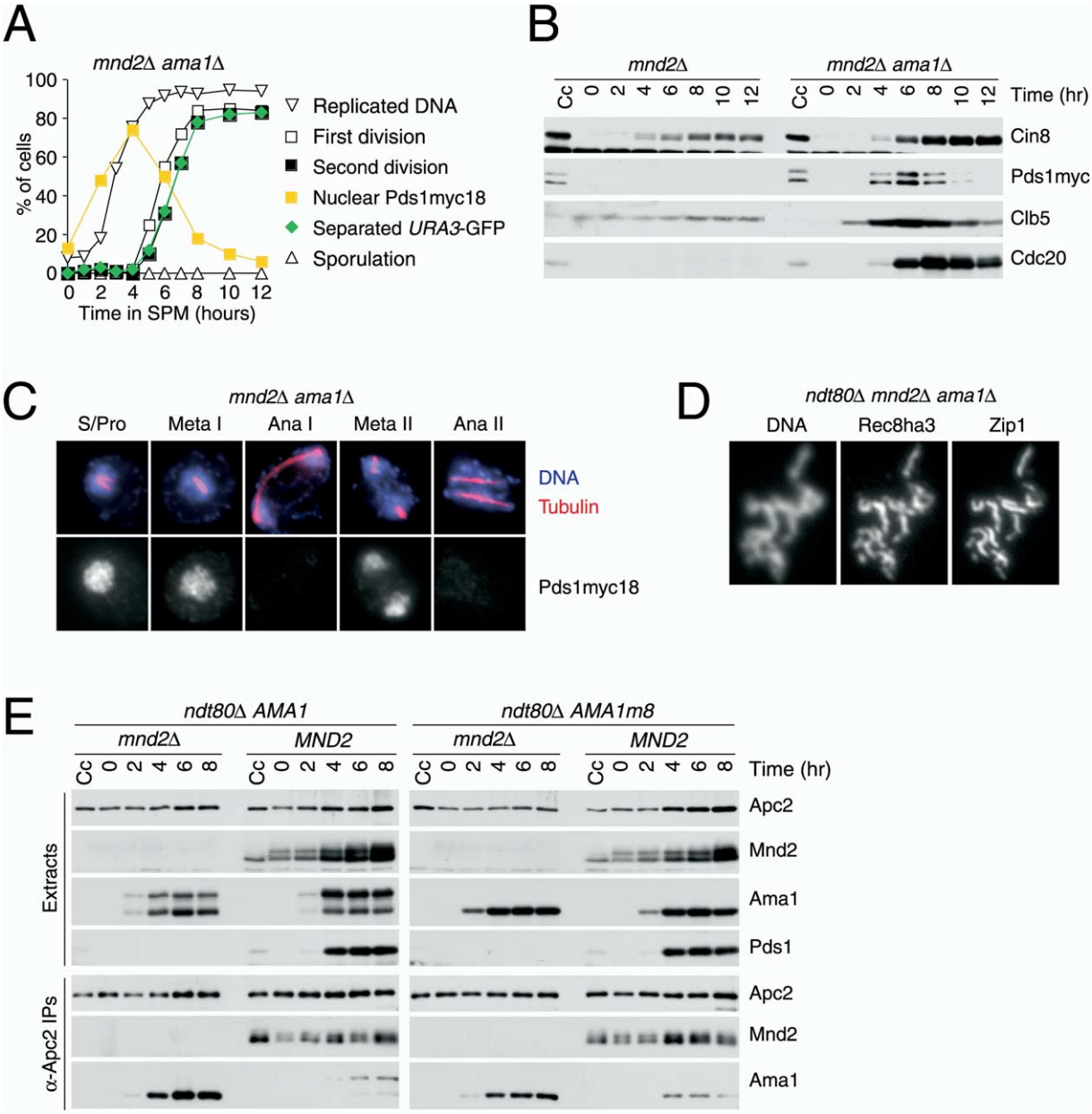


Figure 5. Mnd2 Is Required to Prevent Premature Pds1 Degradation Dependent on APC/C-Ama1

(A–C) Analysis of *mnd2Δ ama1Δ* (Z3813) cells (genotype: *PDS1/PDS1myc18 ura3/URA3-GFP*) in meiosis. (A) Percentages of cells with replicated DNA, first and second nuclear division, Pds1myc18 staining, separated *URA3*-GFP dots, and spore formation. (B) Immunoblot analysis of protein levels. Samples from an *mnd2Δ* (Z4860) timecourse were analyzed in parallel. (C) Staining from DNA (DAPI, blue), tubulin (red), and Pds1myc18. Pictures represent ≥90% of cells at the indicated stage. S/prophase cells were from t = 4 hr.

(D) Detection of Rec8ha3 and Zip1 on chromosome spreads from *ndt80Δ mnd2Δ ama1Δ REC8ha3* cells (Z4620) arrested in pachytene (8 hr in SPM). Pictures represent 81% of spreads (class I). Eighteen percent were in class II.

(E) Immunoblot analysis of extracts and anti-Apc2 immunoprecipitates prepared from meiotic *ndt80Δ AMA1* and *nd80Δ AMA1m8* strains containing *mnd2Δ* or *MND2*. Strains from left to right: Z5588, Z5589, Z5590, and Z5591.

essential for the proliferation of cells that express Ama1.

To analyze the consequences of APC/C-Ama1 activity, G1 cells containing P_{GALL}-*Myc9-cAMA1* and *MND2* or *mnd2Δ* were released into the cell cycle in galactose medium (Figure 7B). Budding and DNA replication occurred normally in both strains. *MND2* cells continued with cell cycle progression whereas *mnd2Δ* cells failed to assemble spindles and arrested in G2 with a single, elongated bud. In *mnd2Δ* cells the APC/C substrates Clb2, Clb5, and Pds1ha6 declined or failed to accumulate whereas Cin8 was stable (Figure 7B). Also overexpression of Cdh1 causes cells to arrest in G2, but Pds1 and Clb5 are stable in this arrest (Schwab et al., 1997; Visintin et al., 1997). We conclude that Mnd2 can inhibit Ama1-dependent degradation of APC/C substrates in

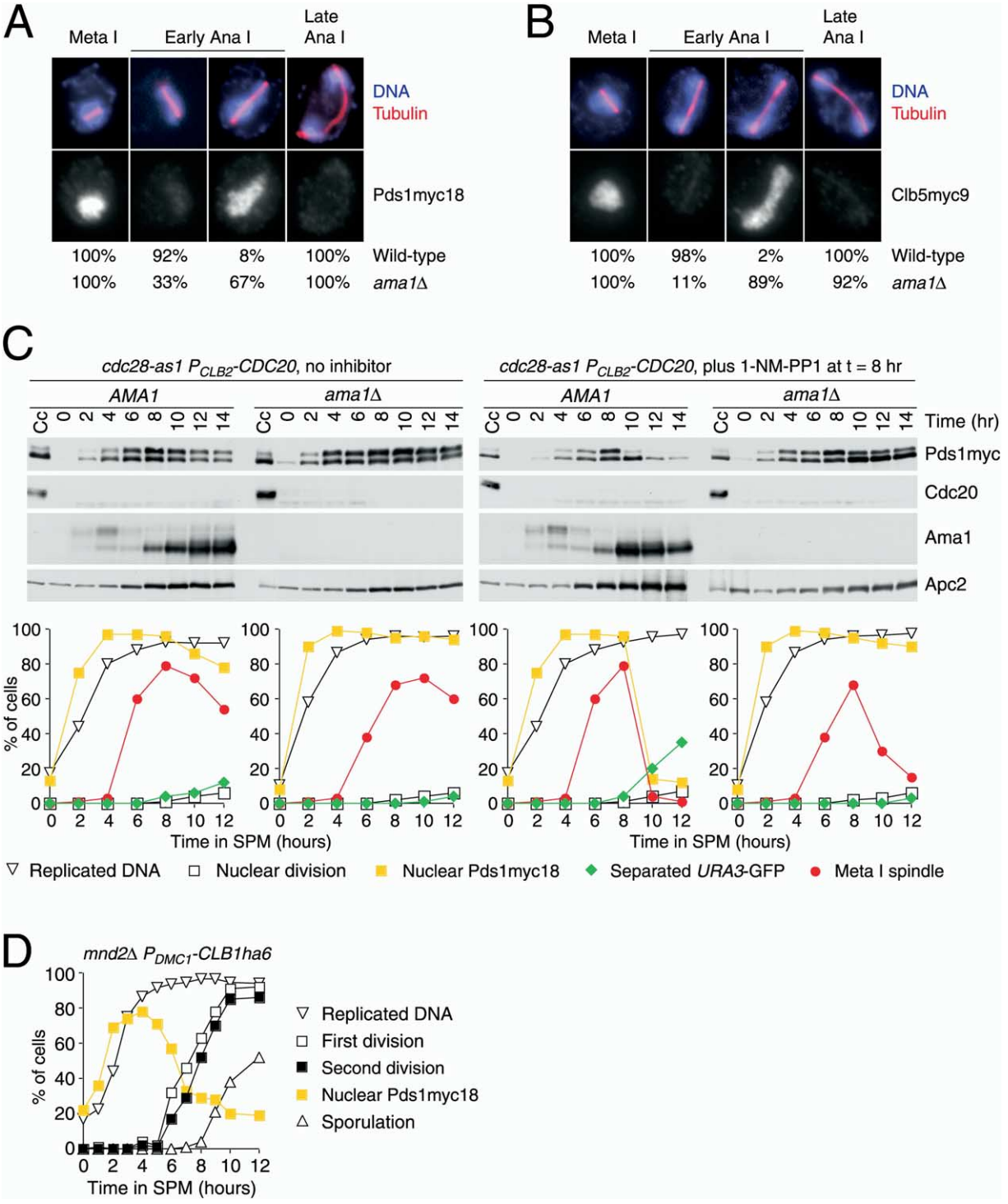


Figure 6. Analysis of Ama1 Function

(A and B) Staining from DNA (DAPI, blue), tubulin (red), and Pds1myc18 (A) or Clb5myc9 (B) in wt and *ama1Δ* cells in meiosis I. Percentages of cells represented by the pictures are shown. (A) *PDS1myc18* (Z2828) and *PDS1myc18 ama1Δ* (Z3264) cells. (B) *CLB5myc9* (Z3289) and *CLB5myc9 ama1Δ* (Z5091) cells.

(C) P_{CLB2}*-CDC20 cdc28-as1 PDS1myc18* strains containing *AMA1* (Z5063) or *ama1Δ* (Z5065) were induced to progress into metaphase I. Cultures were split and 5 μM 1-NM-PP1 was added to one half at t = 8 hr. Panels: immunoblot analysis of protein levels. Graphs: percentages of cells with replicated DNA, divided nuclei, metaphase I spindles, Pds1myc18 staining, and separated *URA3*-GFP dots.

(D) Percentages of cells with replicated DNA, first nuclear division, second nuclear division, Pds1myc18 staining, and spore formation in *mnd2Δ* P_{DMC1}*-CLB1ha6* cells (Z5319) undergoing meiosis.

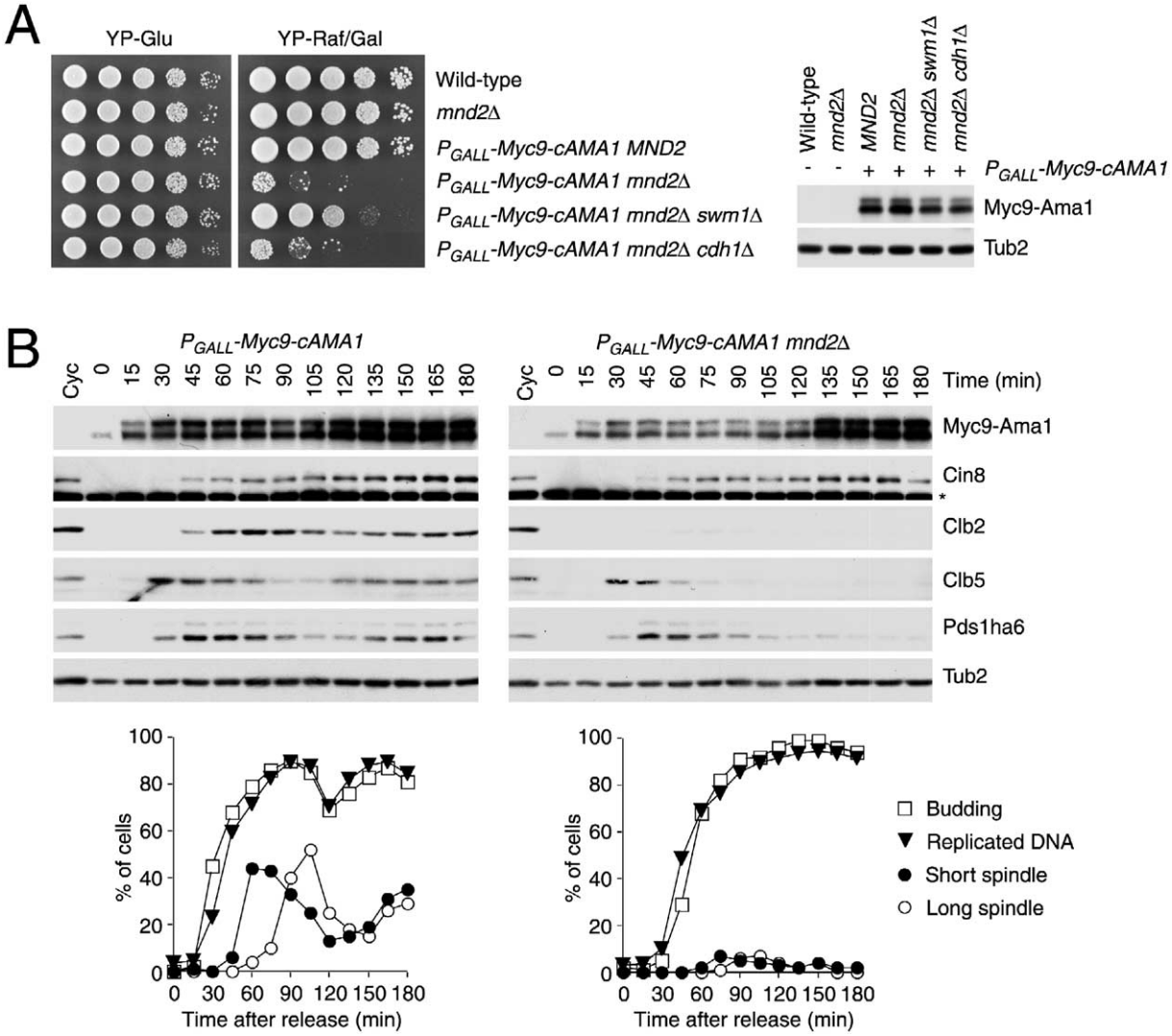


Figure 7. Mnd2 Is Essential for the Proliferation of Cells that Express Ama1

(A) Left: serial ten-fold dilutions of haploid W303 strains with the indicated genotypes were spotted on YP-glucose (P_{GALL} off) and YP-raffinose/galactose (P_{GALL} on) plates and grown for 2 days at 30°C. Strains from top to bottom: Z651, Z2483, Z3653, Z3750, Z4312, Z4313. Right: Myc9-Ama1 expression was confirmed by immunoblotting.

(B) Haploid W303 P_{GALL}-*Myc9-cAMA1 PDS1ha6* strains containing *MND2* (Z4113) or *mnd2Δ* (Z4118) were grown in YP-raffinose medium (Cyc, P_{GALL} off) at 25°C and arrested in G1 with α factor. Galactose was added for 30 min and cells were released at t = 0 into YP-raffinose/galatose medium (P_{GALL} on) lacking α factor at 30°C. Panels: immunoblot analysis of protein levels. Asterisk, nonspecific bands. Graphs: percentages of cells with a bud, replicated DNA, a short spindle, and a long spindle.

vegetative cells, i.e., in the absence of other meiosis-specific proteins.

Mnd2 Inhibits Ubiquitination of Pds1 by APC/C-Ama1 In Vitro

To analyze Mnd2's function in vitro, large-scale cultures of *ndt80Δ ama1Δ CDC16-TAP* strains containing or lacking *MND2* were induced to enter meiosis. After completion of DNA replication, APC/C was isolated by tandem affinity purification (TAP). Complexes from *mnd2Δ* and control cells had similar subunit composition, except for Mnd2 (Figure 8A). To determine the fraction of APC/C that contains Mnd2 the protein was immunodepleted from a solution of wt APC/C. This resulted in the near-complete removal of the other subunits, suggesting that during prophase I the vast majority of complexes contain Mnd2 (Figure 8B). Consistent with this, in vitro-translated Mnd2 bound to APC/C from *mnd2Δ* cells but not to wt APC/C (not shown).

Next, we added purified APC/C to ubiquitination reactions containing ^{35}S-Pds1 as a substrate and different activators produced by in vitro translation. Activators unable to bind the APC/C due to a mutation of the terminal arginine served as negative controls. Cdh1 and Cdc20 activated ubiquitination by both the wt and the mutant APC/C (Figure 8C). Ama1, in contrast, could only activate ubiquitination of Pds1 by the APC/C lacking Mnd2. This reaction required Ama1's terminal arginine (Figure 8C) and the degradation motifs in Pds1 (not shown). These data suggest that Mnd2 specifically pre-

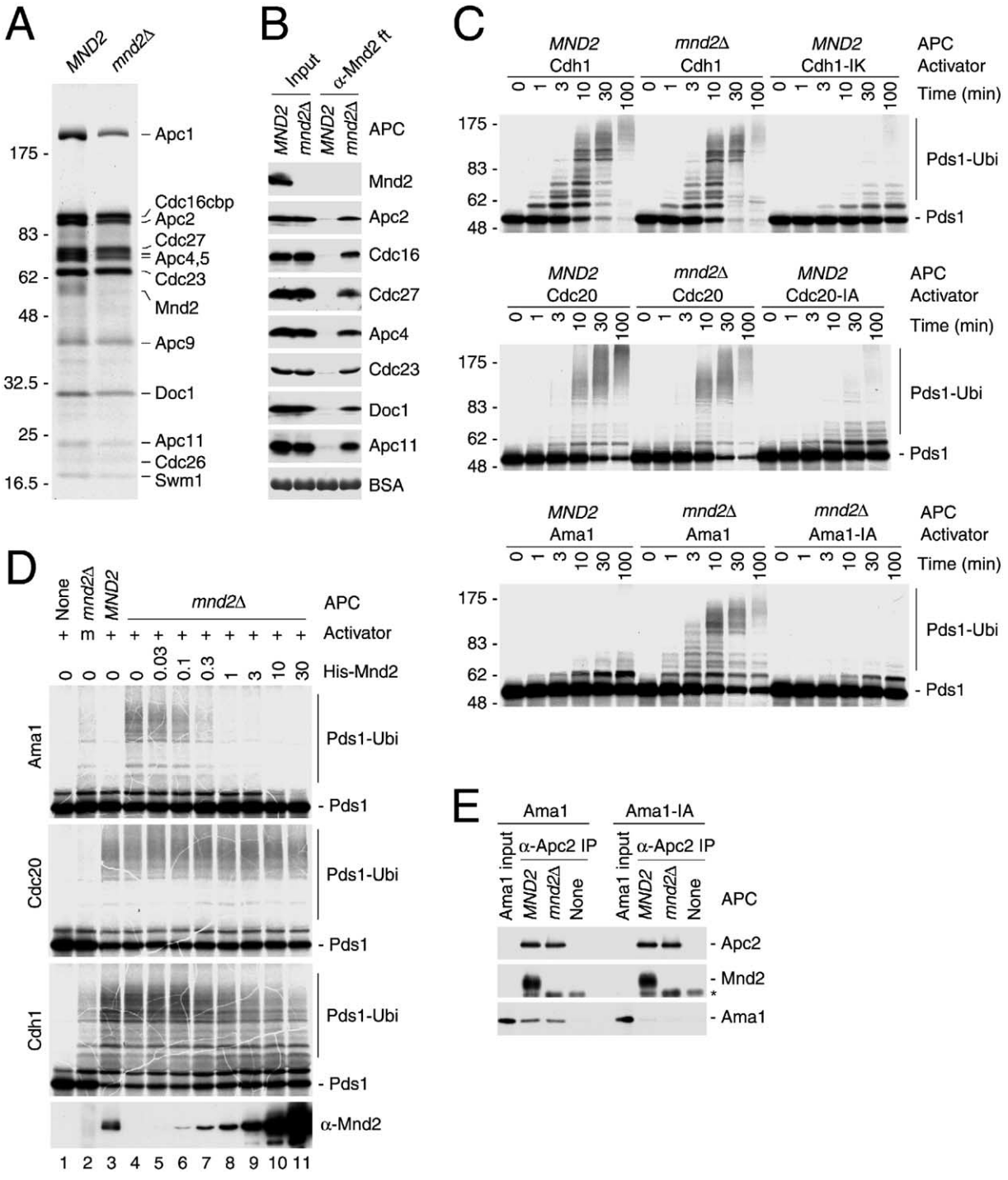


Figure 8. Mnd2 Specifically Inhibits Ubiquitination of Pds1 by APC/C-Ama1 In Vitro

(A) *MND2* (Z4355) and *mnd2⊿* (Z4356) strains containing *CDC16-TAP ndt80⊿ ama1⊿* were induced to enter meiosis for 8 hr in parallel large-scale cultures and APC/C was purified with the TAP method. Gel-separated APC/C stained with Coomassie blue is shown. Cdc16cbp, Cdc16 with the calmodulin binding peptide from the TAP tag.

(B) APC/C containing or lacking Mnd2 from (A) in a buffer with BSA was passed through an α-Mnd2 antibody column. Equal amounts of input and flowthrough (ft) were analyzed by immunoblotting. BSA was stained with Coomassie blue.

(C) APC/C containing or lacking Mnd2 from (A) was added to ubiquitination cocktails containing ^{35}S-labeled Pds1 and the activators Cdh1 (top), Cdc20 (middle), or Ama1 (bottom). The mutant activators Cdh1-IK, Cdc20-IA, and Ama1-IA served as negative controls. Reactions were incubated at 23°C for the indicated times, gel-separated, and detected by fluorography.

(D) APC/C from *mnd2⊿* cells was incubated with different amounts of recombinant His6-Mnd2 and added to ubiquitination cocktails containing the activators indicated on the left (lanes 4–11). Control reactions lacked APC/C (lane 1), contained APC/C from *mnd2⊿* cells plus mutant

vents Ama1 but not Cdh1 or Cdc20 from activating ubiquitination of Pds1. Similar results were obtained with APC/C purified from proliferating *CDC16-TAP* and *CDC16-TAP mnd2Δ* strains (not shown), which is consistent with Mnd2-dependent inhibition of Ama1 expressed in vegetative cells.

To test whether Mnd2 itself was sufficient for inhibition, APC/C from *mnd2Δ* cells was incubated with increasing amounts of bacterially expressed His6-Mnd2 and then added to the ubiquitination cocktail. Recombinant His6-Mnd2 inhibited Ama1-dependent ubiquitination at a concentration similar to that of endogenous Mnd2 in the reaction with wt APC/C (Figure 8D). Ubiquitination activated by Cdc20 was not affected and the Cdh1-dependent reaction was inhibited slightly only at high concentrations of His6-Mnd2. These data show that recombinant Mnd2 lacking yeast-specific modifications is sufficient to inhibit the activity of APC/C-Ama1 toward Pds1. Finally, we compared binding of in vitro-translated, unmodified Ama1 to APC/C containing or lacking Mnd2 (Figure 8E). Ama1 associated with both complexes with similar efficiency. At least in vitro, binding to the APC/C of Mnd2 and Ama1 is not mutually exclusive.

Discussion

The persistence of sister chromatid cohesion from S phase until metaphase is a fundamental aspect of the eukaryotic cell cycle because it allows chromosomes to segregate long after their duplication. This is especially obvious in meiosis where the first nuclear division is preceded by an extended prophase, which can last for several decades in the case of human oocytes. Furthermore, cohesion around centromeres is not destroyed until the second division. Since separase is present throughout the cell cycle, its ability to destroy cohesin is controlled by several, partially redundant mechanisms, which include association with the inhibitor Pds1 and phosphorylation of Scc1 by polo kinase (Alexandru et al., 2001; Ciosk et al., 1998). For this reason Pds1 is not required to prevent premature cohesin destruction in a normal, mitotic cell cycle in budding yeast (Alexandru et al., 1999). In contrast, we find that in meiosis Pds1 is essential for the persistence of cohesion. Accumulation of Pds1 depends on the inhibition of APC/C-dependent proteolysis during S- and prophase I.

Mnd2 Preferentially Inhibits the Meiosis-Specific APC/C-Ama1

Our conclusion that Mnd2 is required to prevent premature recognition of Pds1 by APC/C-Ama1 is based on the finding that Pds1's absence in *mnd2Δ* cells depends on the presence of Ama1, the integrity of the APC/C, and the degradation motifs in Pds1. Consistent with this, Mnd2 inhibits in vitro Pds1's ubiquitination by APC/C-Ama1 but not by other APC/C holo-enzymes. Ama1 appears in the nucleus during pre-meiotic S phase, i.e., at the time when cells establish cohesion between sister chromatids. In contrast, Cdc20 does not appear until metaphase I in wild-type cells (Salah and Nasmyth, 2000), and it was absent in *mnd2Δ* cells, which is consistent with Ndt80-dependent expression of *CDC20*. Cdh1 is present in *mnd2Δ* cells but it was not required to prevent accumulation of Pds1. Consistent with this, Cdh1 dissociated normally from the APC/C as *mnd2Δ* cells entered pre-meiotic S phase (our unpublished data). We conclude that Mnd2 preferentially, if not exclusively, inhibits APC/C-Ama1. This explains why Mnd2 is essential for meiosis but not for a normal, mitotic cell cycle (Rabitsch et al., 2001). Transcription of *AMA1* and the splicing of its mRNA are both restricted to meiosis (Cooper et al., 2000). Accordingly, *MND2* is essential for the proliferation of cells that express an *AMA1* cDNA.

Mnd2 is not only required for the appearance of Pds1 but for the normal accumulation of a specific set of APC/C substrates. APC/C-Ama1 activity in *mnd2Δ* cells did not affect accumulation of Kip1 and Cin8 but strongly reduced the levels of the cyclin Clb5, which is consistent with delayed Clb5 degradation during anaphase I in *ama1Δ* cells. Normal Clb5 levels might be important for efficient DNA replication while rapid degradation might facilitate timely elongation and/or disassembly of the anaphase I spindle. APC/C-Ama1 activity reduced also the levels of Sgo1 in *mnd2Δ* cells. Low Sgo1 levels seem to result from APC/C-dependent ubiquitination rather than cleavage by separase because cells lacking Pds1 contained normal amounts of Sgo1 (our unpublished data). Similar conclusions have been drawn from Sgo1's behavior in mitotic cells (Indjeian et al., 2005). Sgo1 is required to protect centromeric cohesion in meiosis I and disappears from centromeres at the onset of anaphase II (Katis et al., 2004; Kitajima et al., 2004; Marston et al., 2004). Thus, APC/C-dependent degradation of Sgo1 might facilitate sister chromatid separation in meiosis II. It is unknown, however, how Sgo1 is spared from degradation in meiosis I. Inactivation of separase restored sister chromatid cohesion and synapsis in *mnd2Δ* cells but was not sufficient to silence the pachytene checkpoint. We propose that in *mnd2Δ* cells APC/C-Ama1 triggers premature degradation of a protein required to complete recombination.

Mnd2, an Inhibitory Subunit of the APC/C Particle

Several inhibitors of the APC/C have been identified including Emi1/Rca1, Mad2L2/Mad2β, RASSF1A, and the MCC. These inhibitors associate with the APC/C through an activator protein and were not identified as stoichiometric APC/C components. Emi1 is thought to

activators (lane 2) or APC/C from *MND2* cells plus activators (lane 3). Reactions were started with ^{35}S-Pds1, incubated for 35 min at 23°C, gel-separated, and analyzed by fluorography. Bottom panel: Mnd2 detected by immunoblotting.
(E) APC/C containing or lacking Mnd2 from (A) was incubated with ^{35}S-Ama1 or ^{35}S-Ama1-IA and immunoprecipitated with antibodies to Apc2. Immunoprecipitates (100%) and the Ama1 input (30%) were gel-separated and analyzed by immunoblotting (for Apc2 and Mnd2) and fluorography (for Ama1). Asterisk, antibody bands.

prevent binding of substrates to the activator (Reimann et al., 2001b), whereas Mad2 and Mad2L2 might block release of substrates from the activator (Pfleger et al., 2001). Mnd2 differs from these inhibitors in that it is a stoichiometric APC/C subunit in proliferating cells (our unpublished data) and in meiotic prophase I. How Mnd2 inhibits APC/C-Ama1 is unknown. In vivo, Mnd2 reduced Ama1's association with the APC/C during prophase I. In vitro, however, similar amounts of Ama1 associated with APC/C purified from wt cells and *mnd2Δ* mutants. This argues against a simple model in which binding to the APC/C of Ama1 and Mnd2 is mutually exclusive. One possibility is that Mnd2 does not prevent binding of Ama1 alone but prevents binding of an Ama1-substrate complex. Alternatively, Mnd2 might inhibit both the assembly of APC/C-Ama1 and its ligase activity. Our in vitro assay might have reproduced only the latter mechanism.

Regulation of APC/C-Ama1 by Mnd2 and Cdk1/Cdc28

Several pieces of evidence suggest that APC/C-Ama1 is regulated by both Mnd2 and Cdk1: (1) Pds1's accumulation during metaphase in *mnd2Δ spo11Δ* cells implies that APC/C-Ama1 is inhibited by an Mnd2-independent mechanism. (2) Ectopic expression of Clb1 during S- and prophase I in *mnd2Δ* cells inhibits APC/C-Ama1 and restores Pds1 accumulation. (3) Cdk1 kinase activity prevents Ama1-dependent degradation of Pds1 in metaphase I-arrested cells. (4) APC/C-Ama1 becomes active during anaphase I when Cdk1 activity drops. Taken together, these findings suggest that APC/C-Ama1 is inhibited by Mnd2 during S- and prophase I and by Cdk1 in metaphase I. Cdc20-dependent cyclin degradation then activates APC/C-Ama1 in anaphase I. Thus, activation of APC/C-Ama1 depends on that of APC/C-Cdc20, which might be important for the ability of the spindle checkpoint to block degradation of Pds1 and cyclins. Ama1 might be important for APC/C activity in anaphase I because Cdc20 is degraded at this stage (Salah and Nasmyth, 2000).

Activation of APC/C-Ama1 during anaphase I occurs in the presence of Mnd2, implying that Mnd2's inhibitory function has been inactivated. We speculate that Mnd2 is inactivated during metaphase I by an Ndt80-dependent mechanism, which might involve a change in Mnd2's phosphorylation status. The phosphorylation of Mnd2 strongly increases as cells exit from prophase I and induce Ndt80-dependent genes. The kinase(s) responsible for this event remain to be identified. We do not know whether Mnd2 is reactivated and then inactivated again as cells progress through meiosis II. Phosphorylation-dependent inactivation of Mnd2 could explain why degradation of Mnd2 during anaphase II is not essential for sporulation, which requires APC/C-Ama1 activity. Phosphorylation and degradation might have redundant functions in Mnd2's inactivation in late meiosis.

Implications for the Control of Meiotic Chromosome Behavior

Cleavage of Rec8 in meiosis I depends on the polo kinase Cdc5 (Clyne et al., 2003; Lee and Amon, 2003). Cdc5 is required for efficient degradation of Pds1 and for the hyperphosphorylation of Rec8, which was proposed to target the kleisin for cleavage. Cells lacking Pds1, however, separate sisters although they contain little if any Cdc5 activity. Phosphorylation by Cdc5 might facilitate but is probably not essential for Rec8's cleavage, as is the case for Scc1 in mitosis (Alexandru et al., 2001). We note, however, that Rec8 is phosphorylated by an unknown kinase as cells enter S phase; this might be sufficient to promote cleavage.

Loading of Rec8 onto chromosomes is required for SC formation (Klein et al., 1999). Accordingly, we find that this process also depends on Mnd2. Inhibition of separase was sufficient to restore SC formation in *mnd2Δ* cells, whereas blocking only the cleavage of Rec8 was not sufficient. This raises the possibility that synapsis depends on Pds1's ability to prevent cleavage of Rec8 and that of another separase substrate. A potential candidate for this substrate is the mitotic kleisin Scc1. Although Scc1 levels decline as cells enter meiosis, a fraction of Scc1 remains bound to chromatin until the first division (Klein et al., 1999).

Maintaining cohesion throughout meiotic S- and prophase I is a task that all sexually reproducing organisms have to accomplish. So far, database searches did not reveal obvious orthologs of Mnd2 in animals. Nevertheless, the homology between Mnd2 and Apc15 from fission yeast shows that Mnd2-related proteins exist in evolutionary distant organisms (Yoon et al., 2002). Meiosis-specific APC/C activators are present in yeasts and in *Drosophila*, implying that meiosis-specific regulation of the APC/C is generally important for the control of gametogenesis.

Experimental Procedures

Yeast Strains

Diploid SK1 strains were used for all meiotic experiments. Mutations are homozygous, unless stated otherwise (e.g., *REC8ha3* is homozygous, *REC8/REC8ha3* is heterozygous). Haploid W303 strains were used in Figure 7. SK1 strains containing *esp1-2*, *REC8ha3*, *REC8-N-ha3* (Buonomo et al., 2000), and *cdc28-as1* (Benjamin et al., 2003) have been described. SK1 strains containing *CenV*, *ura3*, and *TelV* marked with GFP have been published previously (Klein et al., 1999; Toth et al., 2000). Strain constructions and genotypes of all strains are described in Supplemental Data.

Meiotic Timecourse Experiments

Unless specified, induction of meiosis was performed at 30°C. Single colonies grown on YP-glycerol plates were spread on YPD plates and grown for 24 hr. Cells were inoculated into YPA (YP plus 2% K-acetate) to OD_{600} ~0.3 and grown for 12 hr (OD_{600} ~1.7). When ≥90% of cells were unbudded, strains were inoculated into sporulation medium (SPM, 2% K-acetate) to OD_{600} ~3.4 (t = 0). For large-scale cultures, cells from 20 YPD plates were inoculated into 8 liters of YPA, grown for 12 hr, and transferred to 6 liters of SPM. Cultures were aerated with pressurized air in a single 10 liter beaker. Cellular DNA content was measured on a Becton Dickinson FACScan flow cytometer with CellQuest software. Cells were stained with DAPI to score the first (two or more nuclei) and the second division (three or four nuclei). Indirect immunofluorescence (Salah and Nasmyth, 2000), observation of GFP-marked sister chromatids (Michaelis et al., 1997), and chromosome spreading (Loidl et al., 1998) were performed as described. 1-NM-PP1 (Bishop et al., 2000) was from Cellular Genomics (Branford, Connecticut).

Analysis of Proteins

Protein levels were analyzed by immunoblotting after breaking cells with glass beads in 10% trichloroacetic acid. For immunoprecipi-

tations, 0.2 M PMSF in DMSO was diluted 1:100 into the culture. Cells were washed with cold water plus PMSF/DMSO and processed for immunoprecipitations as described (Camasses et al., 2003), except that the breakage buffer contained the protease inhibitors 5 mM Pefabloc, 2 mM PMSF, 2 mM benzamidine, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and Complete (Roche). Rabbit antisera were raised against His6-Mnd2, His6-Ama1, and GST-Pds1 expressed in *E. coli*. Antibodies to APC/C subunits, Cdc20, and Cdh1 have been described (Camasses et al., 2003; Schwickart et al., 2004). Clb5 (sc-6704, Santa Cruz), Cdc5 (sc-6732, Santa Cruz), Ha (12CA5), and Myc9 (9E10) were detected with the indicated antibodies. Antisera to other proteins were provided by P. Hieter (Cdc16), A. Hyman (Cin8 and Kip1), D. Drechsel (Clb2), W. Seufert (Tub2), and K. Schmekel (Zip1).

APC/C Purification and Ubiquitination Assay

APC/C purified with the TAP method from large-scale meiotic cultures was used for ubiquitination assays as described (Schwickart et al., 2004). Yeast His6-Ubc4 and His6-Mnd2 were expressed in *E. coli* and purified on Ni-NTA agarose (Qiagen) followed by anion exchange chromatography on Poros HQ20 (Applied Biosystems). ^{35}S-Pds1, activators, and the mutants Cdc20-IA (R610A), Cdh1-IK (R566K), and Ama1-IA (R593A) were generated by in vitro transcription/translation (TNT, Promega).

Supplemental Data

Supplemental Data include text, seven figures, and one table and can be found with this article online at http://www.cell.com/cgi/content/full/120/6/773/DC1/.

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References

Alexandru, G., Zachariae, W., Schleiffer, A., and Nasmyth, K. (1999). Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage. EMBO J. *18*, 2707–2721.

Alexandru, G., Uhlmann, F., Mechtler, K., Poupart, M.A., and Nasmyth, K. (2001). Phosphorylation of the cohesin subunit Scc1 by Polo/Cdc5 kinase regulates sister chromatid separation in yeast. Cell *105*, 459–472.

Asakawa, H., Kitamura, K., and Shimoda, C. (2001). A novel Cdc20-related WD-repeat protein, Fzr1, is required for spore formation in Schizosaccharomyces pombe. Mol. Genet. Genomics *265*, 424–435.

Benjamin, K.R., Zhang, C., Shokat, K.M., and Herskowitz, I. (2003). Control of landmark events in meiosis by the CDK Cdc28 and the meiosis-specific kinase Ime2. Genes Dev. *17*, 1524–1539.

Bishop, A.C., Ubersax, J.A., Petsch, D.T., Matheos, D.P., Gray, N.S., Blethrow, J., Shimizu, E., Tsien, J.Z., Schultz, P.G., Rose, M.D., et al. (2000). A chemical switch for inhibitor-sensitive alleles of any protein kinase. Nature *407*, 395–401.

Blanco, M.A., Pelloquin, L., and Moreno, S. (2001). Fission yeast mfr1 activates APC and coordinates meiotic nuclear division with sporulation. J. Cell Sci. *114*, 2135–2143.

Buonomo, S.B., Clyne, R.K., Fuchs, J., Loidl, J., Uhlmann, F., and Nasmyth, K. (2000). Disjunction of homologous chromosomes in meiosis I depends on proteolytic cleavage of the meiotic cohesin Rec8 by separin. Cell *103*, 387–398.

Camasses, A., Bogdanova, A., Shevchenko, A., and Zachariae, W. (2003). The CCT chaperonin promotes activation of the anaphase-promoting complex through the generation of functional Cdc20. Mol. Cell *12*, 87–100.

Chu, S., and Herskowitz, I. (1998). Gametogenesis in yeast is regulated by a transcriptional cascade dependent on Ndt80. Mol. Cell *1*, 685–696.

Chu, T., Henrion, G., Haegeli, V., and Strickland, S. (2001). Cortex, a Drosophila gene required to complete oocyte meiosis, is a member of the Cdc20/fizzy protein family. Genesis *29*, 141–152.

Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M., and Nasmyth, K. (1998). An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. Cell *93*, 1067–1076.

Clyne, R.K., Katis, V.L., Jessop, L., Benjamin, K.R., Herskowitz, I., Lichten, M., and Nasmyth, K. (2003). Polo-like kinase Cdc5 promotes chiasmata formation and cosegregation of sister centromeres at meiosis I. Nat. Cell Biol. *5*, 480–485.

Cohen-Fix, O., Peters, J.M., Kirschner, M.W., and Koshland, D. (1996). Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. Genes Dev. *10*, 3081–3093.

Cooper, K.F., Mallory, M.J., Egeland, D.B., Jarnik, M., and Strich, R. (2000). Ama1p is a meiosis-specific regulator of the anaphase promoting complex/cyclosome in yeast. Proc. Natl. Acad. Sci. USA *97*, 14548–14553.

Funabiki, H., Yamano, H., Kumada, K., Nagao, K., Hunt, T., and Yanagida, M. (1996). Cut2 proteolysis required for sister-chromatid separation in fission yeast. Nature *381*, 438–441.

Grosskortenhaus, R., and Sprenger, F. (2002). Rca1 inhibits APC-Cdh1(Fzr) and is required to prevent cyclin degradation in G2. Dev. Cell *2*, 29–40.

Harper, J.W., Burton, J.L., and Solomon, M.J. (2002). The anaphase-promoting complex: it's not just for mitosis any more. Genes Dev. *16*, 2179–2206.

Hsu, J.Y., Reimann, J.D., Sorensen, C.S., Lukas, J., and Jackson, P.K. (2002). E2F-dependent accumulation of hEmi1 regulates S phase entry by inhibiting APC(Cdh1). Nat. Cell Biol. *4*, 358–366.

Indjeian, V.B., Stern, B.M., and Murray, A.W. (2005). The centromeric protein Sgo1 is required to sense lack of tension on mitotic chromosomes. Science *307*, 130–133.

Jacobs, H.W., Richter, D.O., Venkatesh, T.R., and Lehner, C.F. (2002). Completion of mitosis requires neither fzr/rap nor fzr2, a male germline-specific *Drosophila* Cdh1 homolog. Curr. Biol. *12*, 1435–1441.

Katis, V.L., Galova, M., Rabitsch, K.P., Gregan, J., and Nasmyth, K. (2004). Maintenance of cohesin at centromeres after meiosis I in budding yeast requires a kinetochore-associated protein related to MEI-S332. Curr. Biol. *14*, 560–572.

Keeney, S., Giroux, C.N., and Kleckner, N. (1997). Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. Cell *88*, 375–384.

Kitajima, T.S., Miyazaki, Y., Yamamoto, M., and Watanabe, Y. (2003). Rec8 cleavage by separase is required for meiotic nuclear divisions in fission yeast. EMBO J. *22*, 5643–5653.

Kitajima, T.S., Kawashima, S.A., and Watanabe, Y. (2004). The conserved kinetochore protein shugoshin protects centromeric cohesion during meiosis. Nature *427*, 510–517.

Klapholz, S., Waddell, C.S., and Esposito, R.E. (1985). The role of the SPO11 gene in meiotic recombination in yeast. Genetics *110*, 187–216.

Klein, F., Mahr, P., Galova, M., Buonomo, S.B., Michaelis, C., Nairz, K., and Nasmyth, K. (1999). A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis. Cell *98*, 91–103.

Lee, B.H., and Amon, A. (2003). Role of Polo-like kinase CDC5 in programming meiosis I chromosome segregation. Science *300*, 482–486.

Loidl, J., Klein, F., and Engebrecht, J. (1998). Genetic and morphological approaches for the analysis of meiotic chromosomes in yeast. Methods Cell Biol. *53*, 257–285.

Marston, A.L., Tham, W.H., Shah, H., and Amon, A. (2004). A genome-wide screen identifies genes required for centromeric cohesion. Science *303*, 1367–1370.

Michaelis, C., Ciosk, R., and Nasmyth, K. (1997). Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell *91*, 35–45.

Nasmyth, K. (2001). Disseminating the genome: joining, resolving, and separating sister chromatids during mitosis and meiosis. Annu. Rev. Genet. *35*, 673–745.

Petronczki, M., Siomos, M.F., and Nasmyth, K. (2003). Un menage a quatre: The molecular biology of chromosome segregation in meiosis. Cell *112*, 423–440.

Pfleger, C.M., Salic, A., Lee, E., and Kirschner, M.W. (2001). Inhibition of Cdh1-APC by the MAD2-related protein MAD2L2: a novel mechanism for regulating Cdh1. Genes Dev. *15*, 1759–1764.

Rabitsch, K.P., Toth, A., Galova, M., Schleiffer, A., Schaffner, G., Aigner, E., Rupp, C., Penkner, A.M., Moreno-Borchart, A.C., Primig, M., et al. (2001). A screen for genes required for meiosis and spore formation based on whole-genome expression. Curr. Biol. *11*, 1001–1009.

Reimann, J.D., Freed, E., Hsu, J.Y., Kramer, E.R., Peters, J.M., and Jackson, P.K. (2001a). Emi1 is a mitotic regulator that interacts with Cdc20 and inhibits the anaphase promoting complex. Cell *105*, 645–655.

Reimann, J.D., Gardner, B.E., Margottin-Goguet, F., and Jackson, P.K. (2001b). Emi1 regulates the anaphase-promoting complex by a different mechanism than Mad2 proteins. Genes Dev. *15*, 3278–3285.

Roeder, G.S., and Bailis, J.M. (2000). The pachytene checkpoint. Trends Genet. *16*, 395–403.

Salah, S.M., and Nasmyth, K. (2000). Destruction of the securin Pds1p occurs at the onset of anaphase during both meiotic divisions in yeast. Chromosoma *109*, 27–34.

Schwab, M., Lutum, A.S., and Seufert, W. (1997). Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. Cell *90*, 683–693.

Schwickart, M., Havlis, J., Habermann, B., Bogdanova, A., Camasses, A., Oelschlaegel, T., Shevchenko, A., and Zachariae, W. (2004). Swm1/Apc13 is an evolutionarily conserved subunit of the anaphase-promoting complex stabilising the association of Cdc16 and Cdc27. Mol. Cell. Biol. *24*, 3562–3576.

Song, M.S., Song, S.J., Ayad, N.G., Chang, J.S., Lee, J.H., Hong, H.K., Lee, H., Choi, N., Kim, J., Kim, H., et al. (2004). The tumour suppressor RASSF1A regulates mitosis by inhibiting the APC-Cdc20 complex. Nat. Cell Biol. *6*, 129–137.

Sym, M., Engebrecht, J.A., and Roeder, G.S. (1993). ZIP1 is a synaptonemal complex protein required for meiotic chromosome synapsis. Cell *72*, 365–378.

Toth, A., Rabitsch, K.P., Galova, M., Schleiffer, A., Buonomo, S.B., and Nasmyth, K. (2000). Functional genomics identifies monopolin: a kinetochore protein required for segregation of homologs during meiosis I. Cell *103*, 1155–1168.

Uhlmann, F., Lottspeich, F., and Nasmyth, K. (1999). Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. Nature *400*, 37–42.

Uhlmann, F., Wernic, D., Poupart, M.A., Koonin, E.V., and Nasmyth, K. (2000). Cleavage of cohesin by the CD clan protease separin triggers anaphase in yeast. Cell *103*, 375–386.

Visintin, R., Prinz, S., and Amon, A. (1997). CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. Science *278*, 460–463.

Watanabe, Y. (2004). Modifying sister chromatid cohesion for meiosis. J. Cell Sci. *117*, 4017–4023.

Watanabe, Y., and Nurse, P. (1999). Cohesin Rec8 is required for reductional chromosome segregation at meiosis. Nature *400*, 461–464.

Yoon, H.J., Feoktistova, A., Wolfe, B.A., Jennings, J.L., Link, A.J., and Gould, K.L. (2002). Proteomics analysis identifies new components of the fission and budding yeast anaphase-promoting complexes. Curr. Biol. *12*, 2048–2054.

Yu, H. (2002). Regulation of APC-Cdc20 by the spindle checkpoint. Curr. Opin. Cell Biol. *14*, 706–714.

Zou, H., McGarry, T.J., Bernal, T., and Kirschner, M.W. (1999). Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. Science *285*, 418–422.