Cell

Monopolar Attachment of Sister Kinetochores at Meiosis I Requires Casein Kinase 1

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DOI 10.1016/j.cell.2006.07.029

SUMMARY

In meiosis, a single round of DNA replication is followed by two consecutive rounds of chromosome segregation, called meiosis I and II. Disjunction of maternal from paternal centromeres during meiosis I depends on the attachment of sister kinetochores to microtubules emanating from the same pole. In budding yeast, monopolar attachment requires recruitment to kinetochores of the monopolin complex. How monopolin promotes monopolar attachment was unclear, as its subunits are poorly conserved and lack similarities to proteins with known functions. We show here that the monopolin subunit Mam1 binds tightly to Hrr25, a highly conserved casein kinase 1 δ/ε (CK1δ/ε), and recruits it to meiosis I centromeres. Hrr25 kinase activity and Mam1 binding are both essential for monopolar attachment. Since CK1δ/ε activity is important for accurate chromosome segregation during meiosis I also in fission yeast, phosphorylation of kinetochore proteins by CK1δ/ε might be an evolutionary conserved process required for monopolar attachment.

INTRODUCTION

Accurate transmission of chromosomes during mitosis and meiosis depends on attachment of microtubules to chromosomal DNA via a proteinaceous interface called the kinetochore. The production of genetically identical daughter cells during mitosis requires the attachment of kinetochores on sister chromatids to microtubules emanating from opposite spindle poles (Hauf and Watanabe, 2004). This bipolar attachment or biorientation relies on sister chromatid cohesion mediated by the cohesin complex, which holds sister DNAs together from DNA replication until anaphase, possibly by trapping DNAs inside a tripartite ring formed between its Smc1, Smc3, and kleisin subunits (Nasmyth, 2005). Sister chromatid cohesion ensures that the tension necessary to stabilize kinetochore-associated microtubules is only generated when sister kinetochores have attached in a bipolar manner. The Ipl1/aurora B kinase eliminates erroneous attachments that fail to generate tension (Dewar et al., 2004). At the onset of anaphase, a protease called separase cleaves cohesin's kleisin subunit Scc1 along the entire length of chromosomes, which causes cohesin's dissociation from chromosomes and segregation of sister chromatids to opposite poles (Uhlmann et al., 2000). Activation of separase depends on degradation of its inhibitor Pds1/securin, which is triggered through ubiquitinylation by the anaphase-promoting complex/cyclosome (APC/C). Alternate rounds of chromosome duplication and segregation ensure that chromosome numbers remain constant during mitotic cell cycles.

In meiosis, a single round of DNA replication is followed by two consecutive rounds of chromosome segregation (called meiosis I and II), which enables diploid germ cells to give rise to haploid gametes. Meiosis I differs from mitosis in four important aspects (Petronczki et al., 2003). First, reciprocal recombination (crossovers) between homologous nonsister chromatids creates the chiasmata that link maternal and paternal chromosomes and produce bivalent chromosomes. Homologs are held together by sister chromatid cohesion along chromosome arms distal to crossovers. Consequently, the tension that stabilizes kinetochore-microtubule interactions can be created by pulling maternal sister centromeres in the opposite direction of their paternal homologs. Second, sister kinetochores attach to microtubules emanating from the

same pole (Hauf and Watanabe, 2004). This monopolar attachment or mono-orientation ensures that maternal and paternal centromeres of bivalents are pulled in opposite directions. Third, chiasmata are resolved by cleavage of cohesin's kleisin subunit Rec8 along chromosome arms but not at centromeres (Buonomo et al., 2000; Kitajima et al., 2003), and this triggers segregation of homologous chromosomes to opposite poles. Cohesin at centromeres is protected from separase at the onset of anaphase I by a complex of the Sgo1/shugoshin protein with protein phosphatase 2A (Kitajima et al., 2006; Riedel et al., 2006; Watanabe, 2005). Lastly, meiosis I is not followed by DNA replication but by a second round of chromosome segregation during which the cohesion between sister centromeres is used to biorient sister kinetochores (Toth et al., 2000; Watanabe and Nurse, 1999). Destruction of this cohesion by a second round of separase activation triggers the disjunction of sister centromeres and the formation of haploid gametes.

Some of these meiosis-specific processes require replacement of cohesin's mitotic Scc1 subunit by the meiosis-specific kleisin Rec8. Cohesin containing Rec8 is essential for the processing of recombinogenic double-strand breaks and for the resistance of centromeric cohesion to cleavage by separase in anaphase I (Petronczki et al., 2003). Furthermore, Rec8-like proteins are required for mono-orientation of sister centromeres during meiosis I in plants and fission yeast (Chelysheva et al., 2005; Watanabe and Nurse, 1999; Yu and Dawe, 2000).

The molecular mechanism responsible for suppressing sister kinetochore biorientation in meiosis I remains poorly understood. Thus far, two classes of proteins have been implicated in promoting mono-orientation: orthologs of Rec8 and meiosis I-specific kinetochore proteins. Mono-orientation in fission yeast depends on Rec8-mediated sister chromatid cohesion at the inner core of centromeres where microtubules attach to chromatin (Yokobayashi and Watanabe, 2005). Cohesin containing the mitotic kleisin Rad21/Scc1 cannot substitute, possibly because it is excluded from this region of the chromosome (Watanabe et al., 2001; Yokobayashi et al., 2003). In contrast, both Rec8- and Scc1-containing cohesin complexes are clearly recruited to the central core of centromeres in budding yeast (Riedel et al., 2006) and can support mono-orientation in meiosis I (Toth et al., 2000).

Centromeric cohesion cannot, however, suffice for mono-orientation because it is required also for biorientation of sister kinetochores in meiosis II (Toth et al., 2000; Watanabe and Nurse, 1999). Indeed, mono-orientation depends on meiosis I-specific functions, which require the Moa1 protein in fission yeast (Yokobayashi and Watanabe, 2005) and the monopolin proteins Mam1, Lrs4, and Csm1 in budding yeast (Rabitsch et al., 2003; Toth et al., 2000). Whereas Mam1 is a meiosis I-specific protein, Csm1 and Lrs4 form a nucleolar protein complex in mitotic cells, which is released from the nucleolus at metaphase I and, together with Mam1, localizes to kinetochores. Mam1, Csm1, and Lrs4 interact in vitro and are recruited to kinetochores in an interdependent manner. Orthologs of these proteins have only been identified in a few fungi, and their sequences do not provide clues to their molecular function.

We show here that Hrr25, the highly conserved casein kinase 1 δ/ε of budding yeast, is a hitherto unknown subunit of monopolin. Hrr25 associates with centromeres at metaphase I, and its catalytic activity as well as its interaction with Mam1 are essential for mono-orientation of sister kinetochores in meiosis I. Our finding of a protein kinase as part of the monopolin complex implies that the molecular mechanism behind sister kinetochore mono-orientation will eventually be revealed by identification of the kinase's substrates at kinetochores.

RESULTS

The Casein Kinase 1 δ/ε Ortholog Hrr25 Is a Subunit of Monopolin

To identify novel proteins associated with monopolin, we used the tandem affinity purification (TAP) strategy (Rigaut et al., 1999) to isolate Mam1 and Lrs4 from extracts of diploid strains that had been arrested in metaphase I due to meiotic depletion of the APC/C activator Cdc20. Purified proteins were detected on gels stained with silver (Figure 1A, left and middle). In parallel, samples were digested in solution with trypsin and subjected to peptide identification by mass spectrometry (M.S.). Proteins associated with Mam1 or Lrs4 but absent from control purifications were ranked by peptide coverage, an approximate measure of protein abundance (Figure 1A, right). Mam1 was associated with high levels of a 60 kDa protein that M.S. identified as Hrr25, the budding yeast ortholog of casein kinase 1 δ/ε (CK1δ/ε). M.S. confirmed that Lrs4 and Csm1 also copurified with Mam1, albeit with much lower stoichiometry. Lrs4 was associated with high levels of its nucleolar companion Csm1 and with smaller amounts of both Mam1 and Hrr25. Interestingly, Lrs4 also bound to the nucleolar anchor protein Net1/Cfi1 and its partner, the Cdc14 phosphatase (Visintin and Amon, 2000).

Hrr25 belongs to the highly conserved δ/ε group of CK1s (Figure S1A) and is the sole budding yeast CK1 lacking a lipid membrane anchor. It has been implicated in DNA repair, stress signaling, and membrane traffic (Knippschild et al., 2005). To identify proteins associated with Hrr25, a TAP-tagged version was purified from metaphase I-arrested cells (Figure 1B). Silver staining and M.S. revealed a multitude of copurifying proteins, including proteins involved in spore formation, cohesin subunits (Smc1, Smc3, and Rec8), the monopolin Mam1, and smaller amounts of Mam1's partners Csm1 and Lrs4. Anti-Myc immunoprecipitations from meiotic *HRR25-myc9 MAM1-ha6* and *MAM1-ha6* cells confirmed that the interaction between Hrr25 and Mam1 is specific (Figure 1C). In summary, our results imply the existence of two high affinity complexes, namely Csm1/Lrs4 and Mam1/Hrr25, which together form with lower efficiency a quaternary complex at metaphase I (Figure 1E).

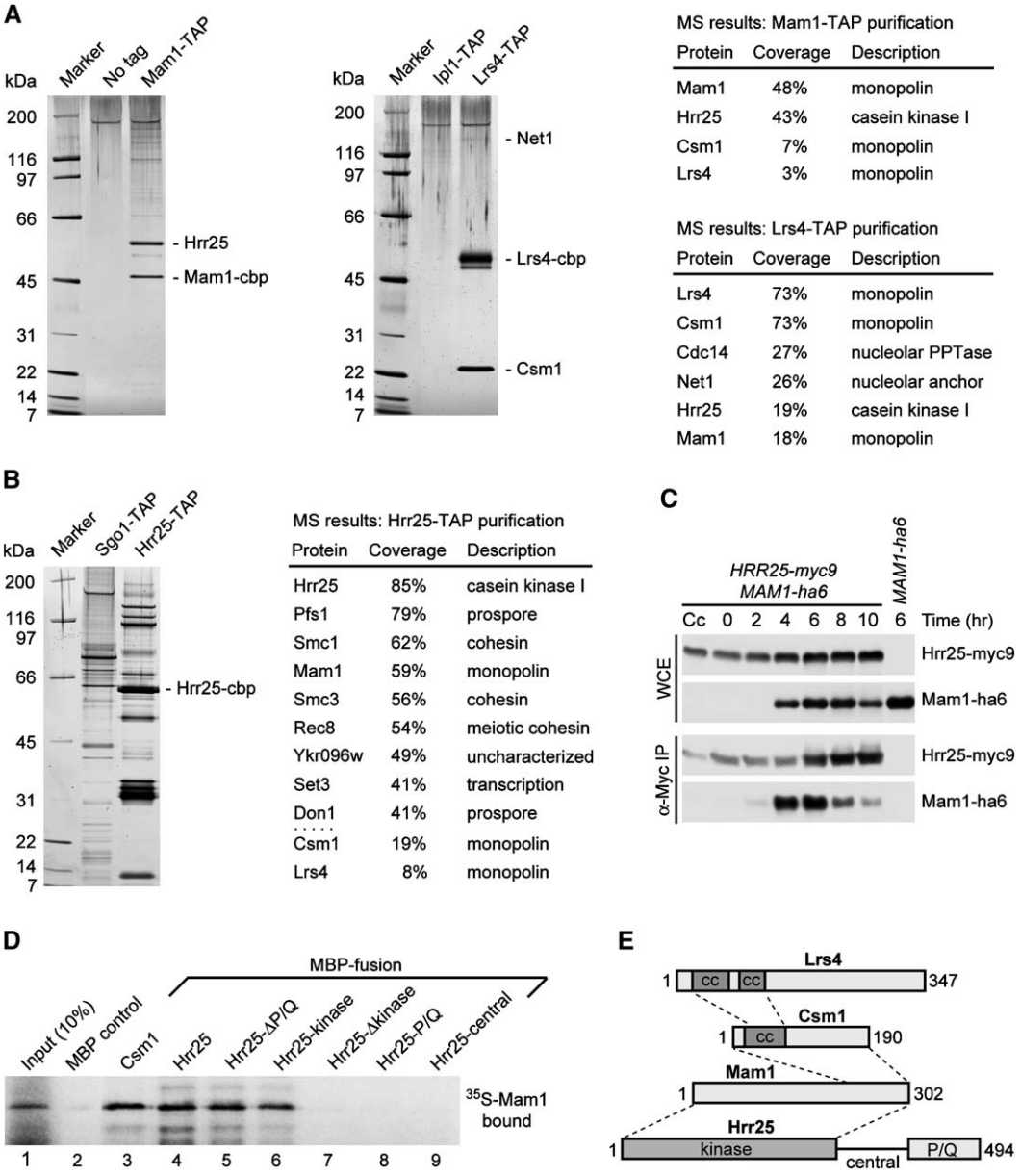

Figure 1. Hrr25 Is a Subunit of the Monopolin Complex

(A) P_{CLB2}-*CDC20* strains containing no tag (K11395, control), *MAM1-TAP* (K11396), *IPL1-TAP* (K11844, control), or *LRS4-TAP* (K11400) were arrested in metaphase I by Cdc20 depletion. Proteins isolated by tandem affinity purification (TAP) were identified by mass spectrometric analysis of tryptic peptides. Left and middle panels show silver-stained protein gels. cbp designates the calmodulin binding peptide remaining on the tagged protein after the TAP procedure. The right panel shows proteins specifically copurifying with Mam1 and Lrs4 ranked by peptide coverage.

(B) Hrr25-associated proteins were identified as in (A) from metaphase I-arrested P_{CLB2}-*CDC20* strains containing *HRR25-TAP* (K12278) or *SGO1-TAP* (control, K12115). Proteins omitted from the list at the dotted line are given in the Supplemental Data.

(C) Immunoblot analysis of whole cell extracts (WCE) and anti-Myc immunoprecipitates from meiotic *HRR25-myc9 MAM1-ha6* (Z6751) and *MAM1-ha6* (Z6750) strains. Cc represents a sample from proliferating cells.

(D) In vitro-translated ^{35}S-labeled Mam1 was incubated with beads carrying maltose binding protein (MBP, lane 2) or MBP fused to Csm1 (lane 3), Hrr25 (lane 4), Hrr25-ΔP/Q (residues 1–394, lane 5), Hrr25-kinase (1–293, lane 6), Hrr25-Δkinase (294–494, lane 7), Hrr25-P/Q (395–494, lane 8), or Hrr25-central (294–394, lane 9). Mam1 added to the beads (input, lane 1) and Mam1 retained on washed beads was gel-separated and detected on a PhosphorImager.

(E) Architecture of the monopolin complex. Dashed lines connect interacting domains. Cc designates coiled coil; P/Q designates P/Q-rich domain.

To test whether Hrr25 and Mam1 interact directly, we incubated in vitro translated Mam1 with beads coupled to recombinant Csm1 and Hrr25 proteins. Mam1 bound, with similar efficiency, to Hrr25- and Csm1-containing beads but not to control beads (Figure 1D, lanes 2–4). Hrr25's N-terminal kinase domain was both necessary and sufficient for the interaction with Mam1 (Figure 1D, lanes 5–9), while its C-terminal P/Q rich domain had no role. These data demonstrate that Mam1 binds directly to Hrr25's kinase domain and indicate that Hrr25 associates with Csm1/Lrs4 indirectly, via Mam1's interaction with Csm1 (Figure 1E).

Essential Roles for Hrr25 in Meiosis Revealed by Kinase Inhibition and Mutation of Two Surface Residues

Though not lethal, deletion of *HRR25* causes cells to grow extremely slowly (Hoekstra et al., 1991), which makes meiotic analysis impossible. To circumvent this problem, we first created a version of the kinase that can be inactivated conditionally. We introduced an I82G mutation that enlarges Hrr25's ATP binding cleft and renders it sensitive to kinase inhibition by the adenine analog 1NM-PP1 (Bishop et al., 2000) (Figure 2A). Cells carrying this analog-sensitive *hrr25-as* allele grew normally in the absence of 1NM-PP1 but very poorly in its presence (Figure 2B). Growth of *hrr25-as* cells in the presence of 1NM-PP1 was restored by wild-type *HRR25* (*HRR25-WT*) but not by the "kinase-dead" *hrr25-KD* allele (Figures 2A and 2B). This suggests that 1NM-PP1 specifically blocks Hrr25-as's kinase activity.

1NM-PP1 blocked formation of spores by *hrr25-as* but not *HRR25-WT* diploid cells (Figure S2A). Staining of the prospore membrane marker Don1 (Knop and Strasser, 2000) revealed that prospore membrane precursors did not concentrate at spindle-pole bodies in metaphase II and therefore failed to engulf chromatin during anaphase II (Figure S2B). To assess the effect of Hrr25 inhibition on meiotic chromosome segregation, we used strains whose two chromosome V homologs were marked at the *URA3* locus by GFP (homozygous *URA3*-GFP). In *HRR25-WT* cells treated with 1NM-PP1, meiosis II spindle elongation produced four equal DNA masses and caused segregation of a single copy of *URA3*-GFP to each of the four spindle poles (Figure 2C). In *hrr25-as* cells, meiosis II spindle elongation failed to produce individualized nuclei due to the absence of prospore membranes. Interestingly, the four spindle poles were associated with very unequal amounts of chromatin, and in 47% of cells, at least one pole lacked a GFP signal (Figure 2C). This suggests that Hrr25's kinase activity is required for correct chromosome segregation during meiosis as well as for spore formation.

Assuming that Hrr25 has a key role in meiosis I monoorientation, we next sought to generate *HRR25* mutants that specifically affect this process without compromising vegetative growth or spore formation. We took advantage of the finding that inactivation of monopolin rescues the lethality of spores produced by *spo11Δ spo12Δ* cells (Rabitsch et al., 2003). A pool of plasmids carrying randomly mutagenized *HRR25* alleles was transformed into *spo11Δ spo12Δ hrr25Δ* cells and selected for alleles that restore spore viability (Figure S1B). Sequencing of the isolated *HRR25* alleles revealed frequent mutation of H25 and E34 (Table S1). These residues are positioned close to each other on the surface of Hrr25's kinase domain near the ATP binding cleft (Figure 2A) but are not involved in enzyme catalysis. As these residues might be crucial for monopolar attachment, we combined the mutations H25R and E34K to generate the allele *hrr25-zo* (Figure 2A).

hrr25-zo supported normal vegetative growth (Figure 2D) and, like deletion of *MAM1*, restored the viability of *spo11Δ spo12Δ* spores (Figure 2E). To assess the effect of *hrr25-zo* on meiotic chromosome segregation in a wild-type background, we analyzed strains homozygous for *URA3*-GFP. *hrr25-zo* cells underwent meiosis and formed spores with normal efficiency. However, while *HRR25-WT* spores contained equal amounts of DNA and a single GFP signal, most *hrr25-zo* spores contained unequal amounts of DNA and more than one or no GFP signal at all (Figures 2F and S1C). Our data imply that *hrr25-zo* causes massive chromosome missegregation resulting in the production of inviable spores. In summary, the analysis of *hrr25-as* and *hrr25-zo* cells shows that kinase activity and two specific surface residues of Hrr25 are essential for correct meiotic chromosome segregation.

Hrr25 Is Required for Meiosis I Nuclear Division

To investigate Hrr25's role in meiotic chromosome segregation, we transferred *HRR25-WT* and *hrr25-as* (both +1NM-PP1) as well as *HRR25-WT* and *hrr25-zo* cells to sporulation medium (SPM). To follow meiotic events, all strains had one chromosome V homolog marked by GFP 35 kb from the centromere at *URA3* (heterozygous *URA3*-GFP) and expressed a Myc18-tagged version of the anaphase inhibitor Pds1. In *HRR25-WT* cells, the metaphase I-to-anaphase I transition is accompanied by the disappearance of Pds1, spindle elongation, division of chromosomal DNA into two equal masses, and cosegregation of sister *URA3* sequences to the same pole (Figures 3B and 3C). These binucleate cells then reaccumulate Pds1 and form a pair of meiosis II spindles. The metaphase II-to-anaphase II transition is accompanied by a second round of Pds1 destruction, segregation of *URA3*-GFP sequences to opposite poles, and formation of four distinct nuclei (Figure 3B). 1NM-PP1 had no effect on the timing of these events in *HRR25-WT* cells (Figure 3A). It also had no effect on DNA replication or meiotic spindle pole body duplication in *hrr25-as* cells (Figure S2C). Furthermore, both *hrr25-as* (+1NM-PP1) and *hrr25-zo* strains formed normal-looking metaphase I cells containing short bipolar spindles and tightly associated sister *URA3*-GFP signals (Figure 3B, middle and lower panel). This suggests that neither Hrr25 inhibition nor the *hrr25-zo* mutation affect sister chromatid cohesion.

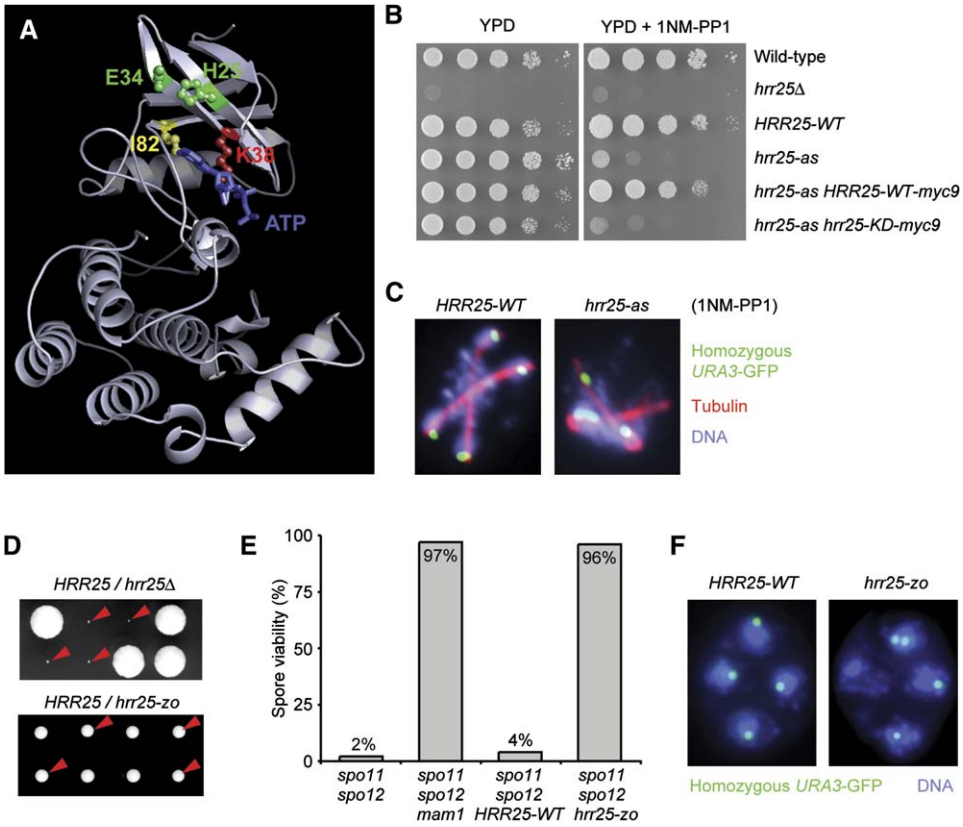

Figure 2. Kinase Inhibition and Mutations of Two Surface Residues Reveal Essential Roles for Hrr25 in Meiosis

(A) Ribbon diagram of Hrr25's kinase domain (residues 1–293) modeled onto the crystal structure of the *S. pombe* Cki1-ATP complex (Xu et al., 1995). Blue sticks represent ATP. Relevant residues are shown in colored ball and stick. H25 and E34 (green) were mutated to R and K, respectively, in *hrr25-zo*; I82 (yellow) was mutated to G in analog-sensitive *hrr25-as*; K38 (red) was mutated to A in kinase-dead *hrr25-KD*.

(B) Serial 10-fold dilutions of wild-type (Z3900), *hrr25Δ* (Z7703), *hrr25Δ::HRR25* (*HRR25-WT*, Z6291), *hrr25Δ::hrr25-as* (*hrr25-as*, Z6292), *hrr25-as HRR25-WT-myc9* (Z6755), and *hrr25-as hrr25-KD-myc9* (Z6757) strains were grown on YPD plates with or without 5 μM 1NM-PP1 at 30°C for 24 hr.

(C) Staining of homozygous *URA3*-GFP, tubulin, and DNA in anaphase II cells of *HRR25-WT* (Z6290) and *hrr25-as* (Z6293) strains treated with 1NM-PP1.

(D) Tetrads produced by heterozygous *HRR25/hrr25Δ* (K12245) and *HRR25/hrr25-zo* (K13550) cells were dissected on YPD and grown at 30°C. Mutant spore clones are labeled.

(E) Dyads produced by *spo11Δ spo12Δ* (K9277), *spo11Δ spo12Δ mam1Δ* (K9278), *spo11Δ spo12Δ HRR25-WT* (K13601), and *spo11Δ spo12Δ hrr25-zo* (K13602) cells were dissected on YPD and grown at 30°C. Spore viability (n = 100) was scored after 72 hr.

(F) Detection of homozygous *URA3*-GFP and DNA in tetrads produced by *hrr25Δ::HRR25* (*HRR25-WT*, K13579) and *hrr25Δ::hrr25-zo* (*hrr25-zo*, K13580) cells.

The first obvious effect of *hrr25* mutations was the abnormal accumulation of mononucleate cells containing a bipolar spindle and low levels of Pds1 (Figures 3B and 3C). These Pds1-negative mutant cells contained stretched but undivided chromatin and, unlike *HRR25-WT* cells, often split *URA3* sequences precociously along their meiosis I spindle axis (*hrr25-as*, 25%; *hrr25-zo*, 28%) (Figure 3B). Despite this massive failure at meiosis I, *hrr25-as* and *hrr25-zo* cells proceeded to form a pair of metaphase II spindles within a single nucleus. Upon Pds1 destruction at the onset of anaphase II, the chromatids within the undivided nucleus segregated simultaneously to four spindle poles (Figure 3B). Sister chromatids were frequently segregated along different spindle axes, presumably because they had been separated precociously. In addition, inhibition of Hrr25-as delayed disassembly of meiosis II spindles, which might be caused by the lack of spore formation (Figure 3A).

hrr25 mutants might fail to perform the meiosis I division due to a defect in the cleavage of Rec8 on chromosome arms, which would prevent the resolution of chiasmata (Buonomo et al., 2000). Detection of Rec8 on chromosome spreads revealed, however, that neither the *hrr25-as* (+1NM-PP1) nor the *hrr25-zo* mutation affected the kinetics of Rec8's chromosomal association, removal from chromosome arms, or retention at centromeres following onset of anaphase I (Figure 3E). Nevertheless, most of the spreads with centromeric Rec8 were mononucleate in *hrr25-as* (+1NM-PP1) and *hrr25-zo* cells but binucleate in *HRR25-WT* cells (Figure S2D). These data suggest that

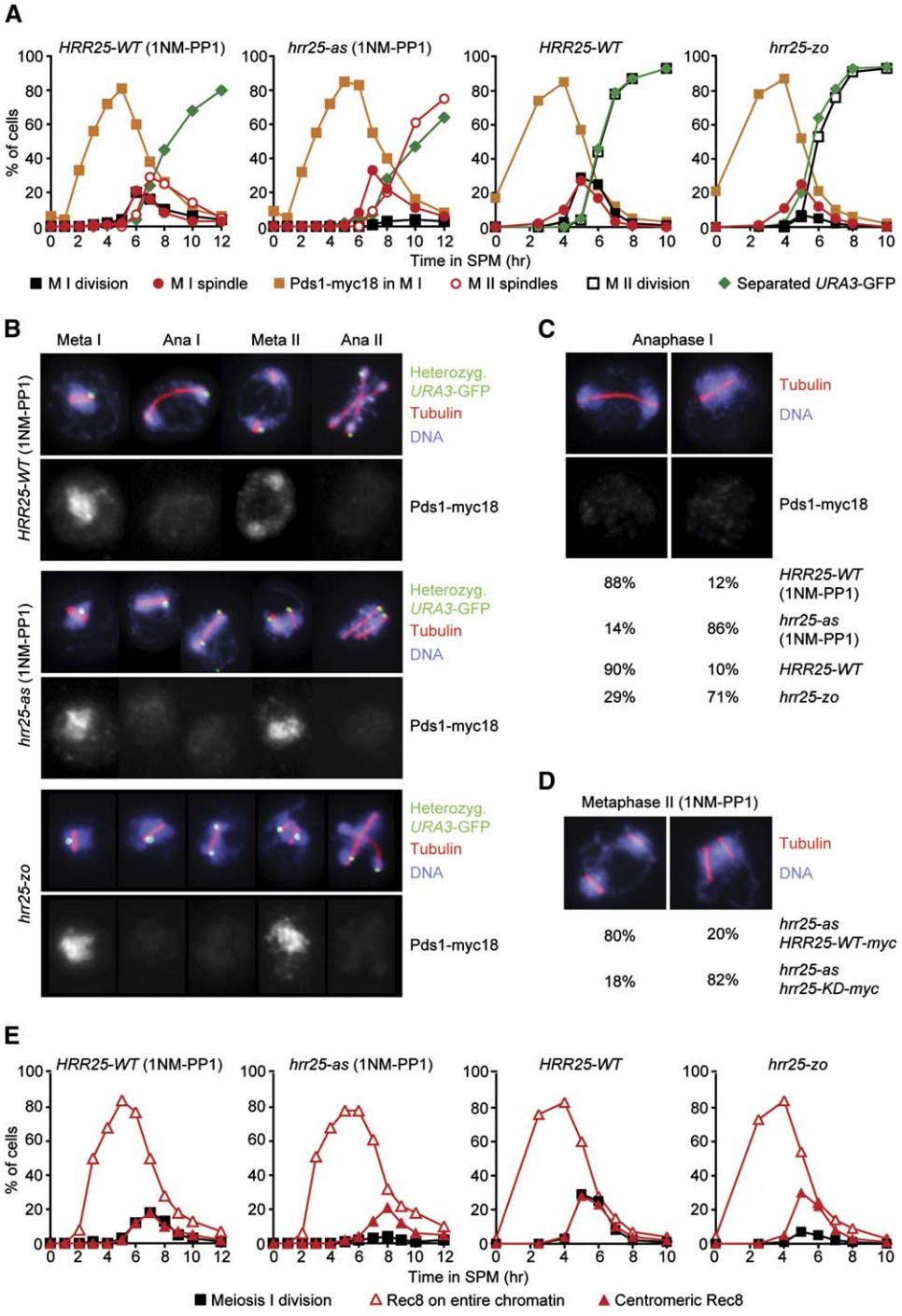

Figure 3. Hrr25 Is Required for Nuclear Division at Meiosis I

(A–C) Immunofluorescence analysis of meiosis in *HRR25-WT* + 1NM-PP1 (Z6291), *hrr25-as* + 1NM-PP1 (Z6292), *HRR25-WT* (K13682), and *hrr25-zo* (K13683) strains containing *PDS1-myc18* and heterozygous *URA3*-GFP. Panel (A) shows percentages of cells with meiosis I division (binucleates), meiosis I spindle, separated *URA3*-GFP, and Pds1-myc18 staining in meiosis I. Meiosis II was quantified by counting cells with meiosis II spindles (*hrr25-as* and *HRR25-WT*) or meiosis II division (tri/tetra-nucleates, *hrr25-zo* and *HRR25-WT*). Panel (B) shows staining of *URA3*-GFP, tubulin, DNA, and Pds1-myc18 at different stages of meiosis. Panel (C) shows percentages of anaphase I cells (Pds1-negative, one bipolar spindle) with divided or undivided nuclei.

(D) Percentages of metaphase II cells (two short bipolar spindles) containing divided or undivided nuclei in *hrr25-as HRR25-WT-myc9* (Z6755) and *hrr25-as hrr25-KD-myc9* (Z6757) strains treated with 1NM-PP1.

(E) Fixed cells and chromosome spreads were prepared from meiotic *HRR25-WT* + 1NM-PP1 (Z6467), *hrr25-as* + 1NM-PP1 (Z6469), *HRR25-WT* (K13682), and *hrr25-zo* (K13683) strains containing *REC8-ha3*. Cells with meiosis I division, spreads with Rec8 on the entire chromatin, and spreads with centromeric Rec8 were quantified.

the failure of *hrr25* mutants to undergo the meiosis I division is not caused by a defect in the removal of Rec8 from chromosome arms.

The phenotype of *hrr25-as* and *hrr25-zo* cells closely resembles that of monopolin mutants (Rabitsch et al., 2003; Toth et al., 2000). These fail to undergo meiosis I because cells attempt to pull sister kinetochores toward opposite poles upon initiation of anaphase but are prevented from disjoining them by the protection from separase of centromeric cohesion. The precocious splitting of some sister *URA3* sequences upon Pds1 degradation is thought to be due to spindle forces sometimes winning the tug of war with pericentric sister-chromatid cohesion.

To confirm that the meiosis I division requires Hrr25's kinase activity, we expressed Hrr25-WT-myc9 or a kinase-dead version in *hrr25-as* cells treated with 1NM-PP1. The wild-type but not the kinase-dead allele rescued their failure to undergo nuclear division and to form spores (Figures 3D and S2E). We conclude that Hrr25's kinase activity as well as residues H25 and E34 are required for the segregation of homologous centromeres to opposite poles in meiosis I but not for destruction of Pds1 or removal of cohesin from chromosome arms.

Hrr25 Is Essential for Mono-Orientation of Sister Kinetochores in Meiosis I

Bipolar attachment of sister kinetochores in monopolin mutants causes the splitting of some sister centromeres during metaphase I (Toth et al., 2000). To test whether this also occurs in *hrr25* mutants, we analyzed Pds1-positive metaphase I cells in which one chromosome V homolog was marked with GFP 1.5 kb from the centromere (heterozygous *CEN5*-GFP). Whereas sister centromeres remained tightly associated in *HRR25-WT* cells, they frequently split along spindle axes in *hrr25-as* (+1NM-PP1) and *hrr25-zo* cells (Figure 4A). We conclude that Hrr25 like Mam1 prevents splitting of sister centromeres at metaphase I.

If meiosis I nuclear division fails in *hrr25* mutants due to erroneous biorientation of sister kinetochores and persistent centromeric cohesion, the following two predictions should hold true. First, abolishing chiasmata formation between homologous chromosomes should not alleviate the division block. Second, elimination of centromeric cohesion at the onset of anaphase I should do so and allow *hrr25* mutants to undergo an equational meiosis I division. To prevent formation of chiasmata we eliminated Spo11, the endonuclease that initiates recombination. We compared *spo11Δ hrr25-as* with *spo11Δ HRR25-WT* cells (+1NM-PP1) and *spo11Δ hrr25-zo* with *spo11Δ HRR25-WT* cells, all of which contained Pds1-myc18 and heterozygous *URA3*-GFP. In the absence of chiasmata homolog segregation no longer depends on Pds1 destruction and removal of arm cohesion. Mono-orientation of sister kinetochores, however, is not affected by deleting *SPO11* in wild-type cells (Toth et al., 2000). Accordingly, most *spo11Δ HRR25-WT* cells underwent meiosis I nuclear division prior to Pds1 destruction, while sister *URA3* sequences invariably cosegregated to the same pole (Figure 4B, left). In contrast, at least 80% of *spo11Δ hrr25-as* and *spo11Δ hrr25-zo* cells failed to undergo the first meiotic division, either before or even after Pds1 destruction. They nevertheless frequently split sister chromatids after Pds1 destruction (Figure 4B, middle and right). Thus, kinase inhibition or the *hrr25-zo* mutation prevents the meiosis I division even in the absence of chiasmata.

To test whether it is centromeric cohesion that blocks nuclear division in *hrr25* mutants, we replaced Rec8 by its mitotic counterpart Scc1 (P_{REC8}-*SCC1*). Scc1 supports cohesion and mono-orientation of sister kinetochores during meiosis I but cannot be protected from cleavage by separase (Toth et al., 2000). Crucially, this enabled *spo11Δ hrr25-as* (+1NM-PP1) and *spo11Δ hrr25-zo* cells to undergo an equational meiosis I. Whereas *spo11Δ* P_{REC8}-*SCC1 HRR25-WT* control cells divided nuclei prior to Pds1 destruction and invariably segregated sister chromatids to the same pole (Figure 4C, left), the corresponding *hrr25-as* and *hrr25-zo* cells divided nuclei only after Pds1 degradation and segregated sister chromatids to opposite poles (*hrr25-as*, 83%; *hrr25-zo*, 100%) (Figure 4C, middle and right).

The persistence of centromeric cohesion during anaphase I not only depends on Rec8 but also on the presence at centromeres of cohesin's protector Sgo1 (Watanabe, 2005). Similar to the results from *spo11Δ* P_{REC8}-*SCC1 hrr25-as* strains, meiotic *spo11Δ hrr25-as* cells depleted of Sgo1 (+1NM-PP1) underwent efficient meiosis I nuclear division in a manner strictly dependent on Pds1 destruction (Pds1-positive, 7% divided; Pds1-negative, 92% divided; not shown). Sgo1-depletion restored meiosis I nuclear division also in recombination-proficient *SPO11 hrr25-as* cells treated with 1NM-PP1 (Figure 4D). The frequency of equational sister segregation was increased in *hrr25-as* cells lacking Sgo1 (Figure 4D) but did not reach the high level observed in *spo11Δ* P_{REC8}-*SCC1 hrr25-as* cells. This might be due to Sgo1's requirement for efficient kinetochore-microtubule attachment. Taken together, our data show that inhibition of Hrr25's kinase activity or mutating residues H25 and E34 causes biorientation of sister kinetochores in metaphase I. Hrr25 is therefore required for monopolar attachment and is an essential component of the monopolin complex.

Two Surface Residues but Not the Kinase Activity of Hrr25 Are Required for Binding to Mam1

To test whether Hrr25's kinase activity is required for the interaction with Mam1, anti-Myc immunoprecipitates were prepared from meiotic *HRR25-WT-myc9 MAM1-ha6* and *hrr25-as-myc9 MAM1-ha6* strains treated with 1NM-PP1. Hrr25's association with Mam1 was not altered by kinase inhibition, even though *hrr25-as-myc9* cells failed to undergo the meiosis I division (Figure 5A). To investigate why mutation of H25 and E34 abolishes mono-orientation, we purified TAP-tagged Hrr25-WT and Hrr25-zo from metaphase I-arrested cells and compared

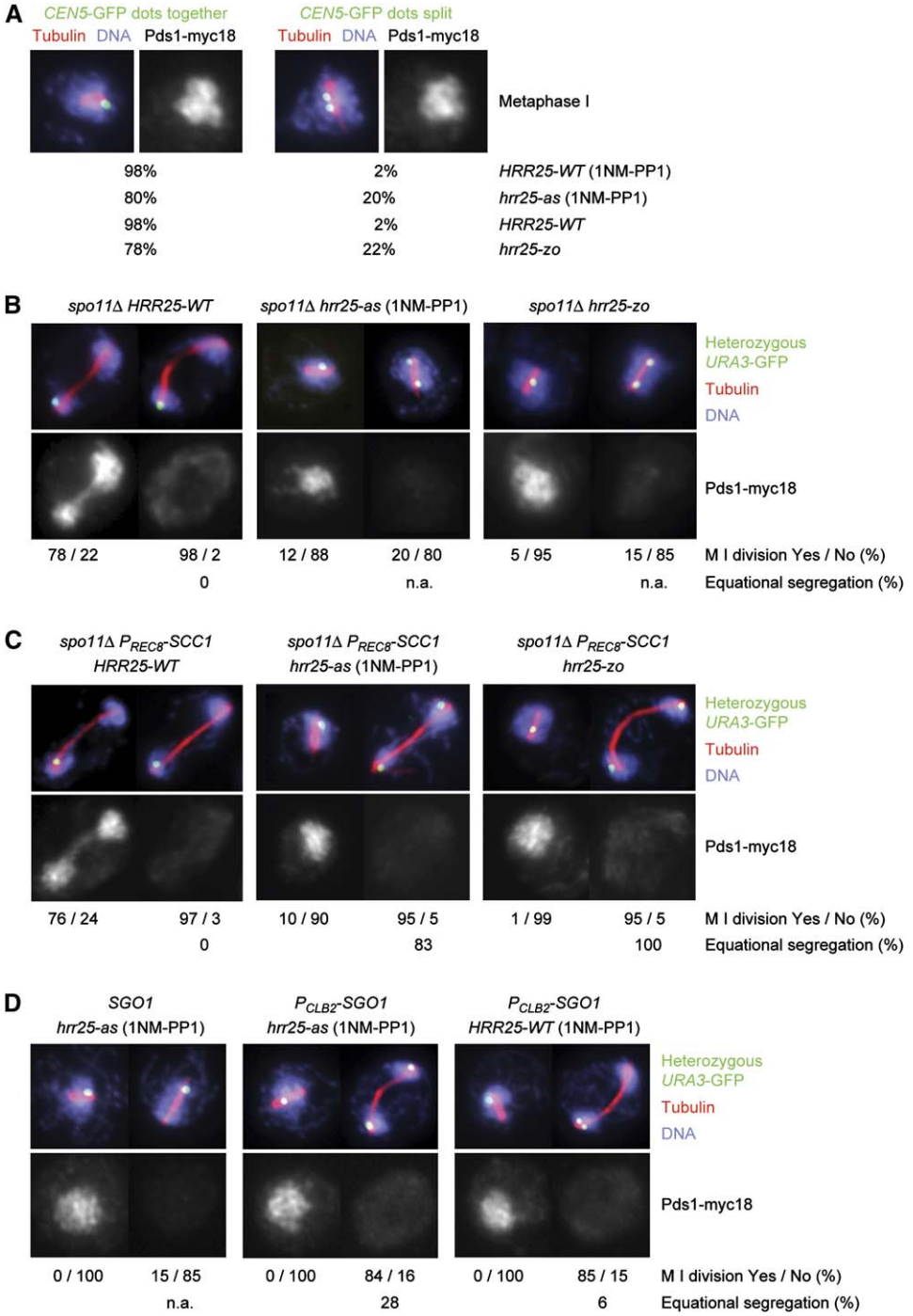

Figure 4. Hrr25 Is Essential for Mono-Orientation of Sister Kinetochores in Meiosis I

(A) Quantification of metaphase I cells (Pds1-myc18 staining, one bipolar spindle) with one or two signals from heterozygous *CEN5*-GFP in *HRR25-WT* + 1NM-PP1 (Z6467), *hrr25-as* + 1NM-PP1 (Z6469), *HRR25-WT* (K13732), and *hrr25-zo* (K13734) strains.

(B and C) Immunofluorescence detection of heterozygous *URA3*-GFP, tubulin, DNA, and Pds1-myc18 in meiosis I cells (one bipolar spindle) of *spo11Δ* strains expressing either Rec8 (B) or Scc1 (C) in meiosis. Nuclear division in Pds1-positive and Pds1-negative cells as well as equational segregation of *URA3*-GFP was quantified. N.a. designates not analyzed. (B) Shown is the analysis of *spo11Δ HRR25-WT* (K13823), *spo11Δ hrr25-as* + 1NM-PP1 (Z6526), and *spo11Δ hrr25-zo* (K13827) strains. (C) Shown is the analysis of *spo11Δ* P_{REC8}*-SCC1 HRR25-WT* (K13824), *spo11Δ* P_{REC8}*-SCC1 hrr25-as* + 1NM-PP1 (Z6528), and *spo11Δ* P_{REC8}*-SCC1 hrr25-zo* (K13828) strains.

(D) *hrr25-as* (Z6626), *hrr25-as* P_{CLB2}*-SGO1* (Z6713), and *HRR25-WT* P_{CLB2}*-SGO1* (Z6712) strains containing *PDS1-myc18* and heterozygous *URA3*-GFP were shifted to SPM/1NM-PP1. Nuclear division in Pds1-positive and Pds1-negative meiosis I cells (one bipolar spindle) as well as equational segregation of *URA3*-GFP was quantified. P_{CLB2}*-SGO1* causes meiotic depletion of Sgo1.

associated proteins by M.S. The yield and peptide coverage of Hrr25-zo were similar to that of Hrr25-WT (Figures 5B and 5C). Remarkably, all but one of the interacting proteins co-purified with comparable efficiency (Figure 5C). M.S. failed to detect any peptide from Mam1 in the Hrr25-zo purification, even though it detected 38% of Mam1 peptides in the Hrr25-WT purification (Figure 5C). To confirm this, Hrr25-WT-TAP and Hrr25-zo-TAP were isolated from *MAM1-myc9* strains. Mam1-myc9 copurified with Hrr25-WT but not with Hrr25-zo (Figure 5D). Taken together, our data show that the mutations H25R and E34K in Hrr25-zo abolish Hrr25's binding to Mam1 but not to other proteins. This explains the highly specific phenotype of the *hrr25-zo* allele and suggests that physical interaction between Hrr25 and Mam1/monopolin is crucial for mono-orientation of sister kinetochores in meiosis I.

Hrr25-Dependent Modification of Mam1 and Rec8

Our data suggest that phosphorylation of kinetochore proteins by Hrr25 may be important for mono-orientation. Mam1 and Rec8 are prime candidates because they bind to Hrr25 and associate with meiosis I kinetochores. To address this, we analyzed by immunoblotting the electrophoretic mobilities of Mam1 and Rec8 as *HRR25-WT* and *hrr25-as* cells treated with 1NM-PP1 progressed into metaphase I. Mam1-myc9 from *HRR25-WT* cells migrated as a double band (Figure 5E), consistent with the finding that Mam1 is a phosphoprotein (Lee and Amon, 2003). In contrast, Mam1-myc9 from *hrr25-as* cells consisted of a single, fast-migrating species suggesting that phosphorylation of Mam1 depends on Hrr25's kinase activity (Figure 5E). Rec8 becomes progressively more phosphorylated as cells approach metaphase I. Inhibition of Hrr25 delayed the onset of Rec8 hyperphosphorylation and limited its extent (Figure 5E).

Our finding that Hrr25 both binds to and phosphorylates Rec8 is of particular interest because Rec8 is necessary for mono-orientation in fission yeast (Watanabe and Nurse, 1999). In budding yeast, Scc1 also supports mono-orientation when expressed instead of Rec8 in meiosis. Thus, if Hrr25's association with cohesin were important for mono-orientation, we might expect Hrr25 to associate also with Scc1. However, we were unable to detect any interaction of Hrr25 with Scc1 in meiotic cells (Figure 5F). We conclude that the association between cohesin and Hrr25 that we currently detect by coimmunoprecipitation is not necessary for mono-orientation. This finding does not exclude the possibility that phosphorylation of centromeric cohesin (whether it contains Scc1 or Rec8) by Hrr25 is nevertheless important for mono-orientation.

Expression, Localization, and Self Association of Hrr25 in Meiosis

Hrr25's localization and expression during meiosis was analyzed in diploid cells homozygous for *HRR25-myc9*. These cells proliferated normally and produced viable spores, suggesting that Hrr25-myc9 is functional. Though Hrr25 is expressed in mitotic cells and at all stages of meiosis, its levels increased markedly as cells approach the meiosis I division (Figure 6B). It was evenly distributed throughout cells during proliferation and early meiosis but accumulated strongly within nuclei from metaphase I onward (Figure 6A). Immunoprecipitations revealed a strong interaction of Hrr25 with itself. Thus, Hrr25-myc9 bound to Hrr25-ha3 in extracts from diploids expressing both versions (Figure 6C). Interestingly, this self association only occurred as cells approach the first meiotic division.

Mam1, in contrast, did not detectably interact with itself and was not required for self association of Hrr25 (Figure S3A). This suggests that a single Mam1 molecule binds to a dimer or multimer of Hrr25. Hrr25's self association explains our unexpected finding that the chromosome segregation defect of *hrr25-zo* mutants is complemented by the kinase-dead *hrr25-KD* allele (Figure S3B). Complexes containing Hrr25-zo and Hrr25-KD presumably possess the two properties necessary for monopolin function, namely CK1δ/ε kinase activity and association with Mam1. We confirmed that Hrr25-KD does indeed bind Hrr25-zo (Figure S3C) and that Hrr25's kinase activity is dispensable for Mam1 binding (Figure 5A). Hrr25's ability to associate with itself allows cells expressing Hrr25-zo and Hrr25-KD to segregate their chromosomes normally, which implies that this property is important for mono-orientation also in wild-type cells.

Hrr25 Associates with Centromeres at Metaphase I

During late prophase and metaphase I, Mam1, Csm1, and Lrs4 associate with kinetochores, which is detectable by chromatin immunoprecipitation (ChIP) (Rabitsch et al., 2003). To determine where Hrr25 might associate with chromatin, we used ChIP followed by hybridization to a high-density oligonucleotide array covering the entire chromosome VI (ChIP/chip) (Katou et al., 2003). First, we analyzed the distribution of Mam1 tagged with FLAG3 in meiosis I cells. As expected, Mam1 strongly associated with sequences around the centromere (Figures 6D and S4A). ChIP/chip detected also Hrr25-myc18 at this location in metaphase I-arrested cells, although the signal was weaker than that of Mam1 (Figures 6E and S4B). Interestingly, Hrr25's accumulation at the core centromere decreased upon deletion of *MAM1*, while association with pericentromeric sequences increased (Figures 6F and S4C). This suggests that recruitment of Hrr25 to core centromeric sequences requires monopolin and that an alternative mechanism mediates Hrr25's binding to adjacent regions. These data imply that Hrr25 promotes mono-orientation during meiosis I when actually associated with kinetochores.

Mam1's Recruitment to Kinetochores Requires Binding to Hrr25 but Not Hrr25 Kinase Activity

To address whether Hrr25's kinase activity is required to recruit other monopolin subunits to kinetochores, we analyzed the expression and localization of Mam1-myc9 in meiotic *HRR25-WT* and *hrr25-as* cells (+1NM-PP1).

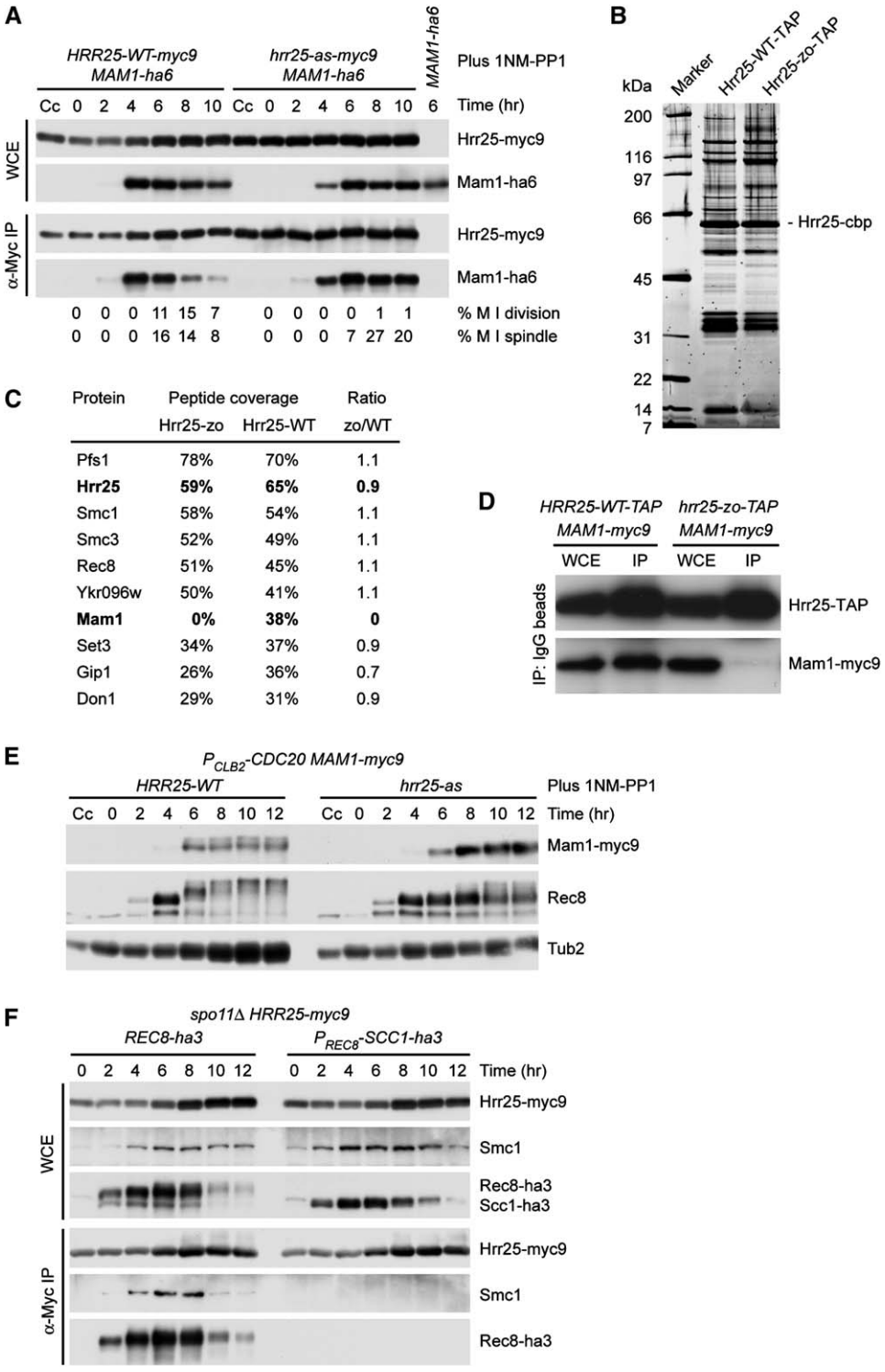

Figure 5. Interactions of Hrr25 with Mam1 and Cohesin

(A) *HRR25-WT-myc9* (Z6751), *hrr25-as-myc9* (Z6752), and *HRR25-WT* (Z6750) strains containing *MAM1-ha6* were shifted to SPM/1NM-PP1. Immunoblot analysis of whole cell extracts and anti-Myc immunoprecipitates is shown together with percentages of cells with meiosis I division and meiosis I spindles. Cc designates a sample from proliferating cells.

(B) Silver-stained gel with TAP purifications from metaphase I-arrested P_{CLB2}-*CDC20* strains containing *HRR25-WT-TAP* (K13786) or *hrr25-zo-TAP* (K13787).

Inhibition of Hrr25 had little or no effect either on the timing of Mam1's nuclear accumulation (Figure 7A) or on its co-localization with the kinetochore protein Ndc10 on metaphase I chromosome spreads (Figure 7B). Next, we analyzed *hrr25-zo MAM1-myc9* cells to test whether the interaction between Mam1 and Hrr25 is required for Mam1's recruitment to kinetochores. Although the timing of Mam1 expression (Figure 7C) and release from the nucleolus of Lrs4 (Figure S5) were both normal, Mam1 failed to associate with kinetochores on metaphase I chromosome spreads (Figure 7D). We conclude that Mam1's association with kinetochores requires Hrr25 to bind Mam1 but not Hrr25's kinase activity. This suggests that Hrr25 hinders biorientation by phosphorylating meiosis I kinetochore proteins, be they monopolin subunits themselves, cohesin subunits, or proteins required for kinetochore function.

Hrr25 Homologs Are Required for Meiosis I in Fission Yeast

To address whether a role for CK1δ/ε in meiotic chromosome segregation might be conserved in evolution, we turned to fission yeast, which possesses two related Hrr25 homologs with overlapping functions (Dhillon and Hoekstra, 1994). Mutants lacking both Hhp1 and Hhp2 grow very slowly, which precluded careful meiotic analysis. We therefore combined an analog-sensitive version of Hhp1 with a deletion of *hhp2*$^{+}$ to create conditional *hhp* mutants. Proliferation of *hhp1-as hhp2Δ* cells was severely impaired in the presence of 1NM-PP1 (Figure 8A). To analyze whether the Hhp kinases have a role in meiosis I, wild-type and *hhp1-as hhp2Δ* cells were sporulated in the presence of 1NM-PP1. Wild-type cells formed two distinct nuclei and segregated sister centromeres to the same pole in anaphase I. In contrast, inhibition of Hhp kinase activity resulted in a high frequency of lagging chromosomes and a slight increase in sister-centromere segregation to opposite poles (Figures 8B and 8C). Elimination in the mutant cells of the centromeric cohesin protector Sgo1 reduced the frequency of lagging chromosomes and dramatically increased equational segregation of sister centromeres (Figures 8B and 8C). This is consistent with the idea that inhibition of Hhp kinase activity causes at least some sister kinetochores to biorient. We cannot rule out, however, that defects in additional processes required for meiosis I nuclear division contribute to the phenotype of fission yeast *hhp* mutants. Taken together, our data show that Hrr25 homologs are essential for meiosis I chromosome segregation in two evolutionarily distant organisms.

DISCUSSION

In budding yeast, suppression of sister kinetochore biorientation in meiosis I depends on the recruitment of the monopolin complex to meiosis I kinetochores. It was unclear, however, how monopolin promotes mono-orientation since biochemical functions have not been proposed for any of its subunits. We first suspected a role for CK1δ/ε activity in mono-orientation due to the copurification of Hrr25 with the monopolin subunit Mam1. To investigate its function, we used two highly specific alleles: *hrr25-zo* selectively blocks the interaction with Mam1 while *hrr25-as* confers sensitivity to a cell-permeable kinase inhibitor. We show that Hrr25's binding to Mam1 but not its kinase activity is required for the recruitment of monopolin to kinetochores. Hrr25's kinase activity is nevertheless essential to suppress biorientation, implying that mono-orientation requires CK1δ/ε activity localized at kinetochores.

CK1s have been implicated in many different processes, including DNA repair, membrane transport, stress signaling, circadian rhythms, and developmental patterning (Knippschild et al., 2005). However, it has never been clear how these kinases achieve sufficient specificity since they are thought to be constitutively active, to function as monomeric enzymes, and to lack regulatory subunits. CK1s have been viewed as rather unsophisticated enzymes with a limited capacity to integrate biochemical and cellular information. Our analysis of Hrr25 during meiosis I paints a very different picture. Hrr25's recruitment to meiosis I kinetochores stems from cell cycle and developmentally regulated assembly of a complex whose subunits are controlled by strikingly diverse mechanisms. These include multimerization of Hrr25 and its accumulation within nuclei shortly before cells embark on the first meiotic division, its direct binding to a subunit that is only expressed during meiosis I (Mam1), and its indirect association with two further subunits (Lrs4/Csm1) that are only released from nucleoli during late prophase. We speculate that a similarly sophisticated repertoire of events, albeit involving distinct regulatory subunits, may be required for other aspects of CK1 function.

Our discovery that CK1δ/ε has an essential role in promoting mono-orientation in budding yeast and possibly an important role also in fission yeast creates a new opportunity to unravel the underlying mechanism. Identification of proteins whose phosphorylation by CK1δ/ε confers mono-orientation should reveal which aspects of kinetochore function are modified during meiosis I to suppress biorientation. We have already identified two candidates,

(C) Comparison of peptide coverage in M.S. protein identifications from the Hrr25-WT and the Hrr25-zo purifications in (B).
(D) Immunoblot analysis of whole-cell extracts and IgG immunoprecipitations from *HRR25-WT-TAP MAM1-myc9* (K14317) and *hrr25-zo-TAP MAM1-myc9* (K14318) strains shifted to SPM for 6 hr.
(E) P_{CLB2}-*CDC20 MAM1-myc9* strains containing *HRR25-WT* (Z7745) or *hrr25-as* (Z7746) were shifted to SPM/1NM-PP1. Protein extracts prepared in trichloroacetic acid were analyzed by immunoblotting. Cc designates a sample from proliferating cells.
(F) Immunoblot analysis of whole-cell extracts and anti-Myc immunoprecipitates from meiotic *spo11Δ HRR25-myc9* strains containing *REC8-ha3* (Z7109) or P_{REC8}-*SCC1-ha3* (Z7181).

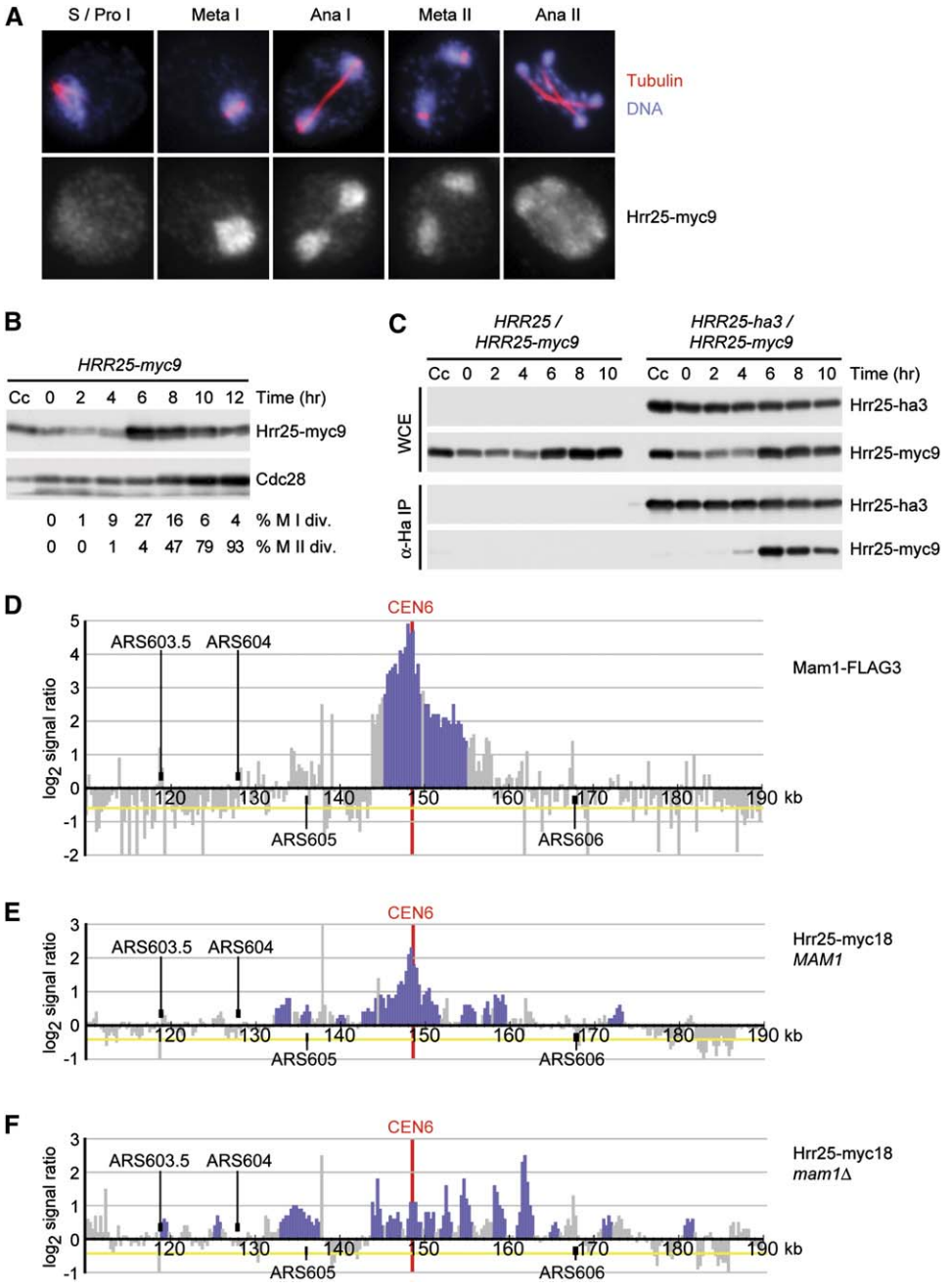

Figure 6. Expression and Localization of Hrr25 in Meiosis

(A and B) Detection of Hrr25-myc9 by immunofluorescence, in panel (A), and immunoblotting of whole cell extracts, in panel (B), in *HRR25-myc9* cells (Z4093), at different stages of meiosis is shown together with percentages of cells with meiosis I and meiosis II division. Cdc28 served as loading control. Cc designates a sample from proliferating cells.

(C) Immunoblot analysis of whole cell extracts and anti-Ha immunoprecipitates from meiotic cells heterozygous for *HRR25*/*HRR25-myc9* (Z6884) or *HRR25-ha3*/*HRR25-myc9* (Z6885). Cc designates a sample from proliferating cells.

(D) *MAM1-FLAG3* cells (SKY10106) were processed for ChIP/chip (Katou et al., 2003) with antibodies to FLAG after 6 hr in SPM + benomyl (80 μg/ml). The ratio between signals from immunoprecipitated DNA fragments and signals from whole genomic DNA is plotted in $\log_2$ scale along an 80 kbp region around the centromere of chromosome VI. Blue bars, significant enrichment of precipitated material. Gray bars, statistically not significant signals. Yellow line, average signal ratio of loci not enriched in the precipitated fraction.

(E and F) *HRR25-myc18* (K14179), shown in panel (E), and *HRR25-myc18 mam1Δ* (K14433), shown in panel (F), strains containing P_{CLB2}-*CDC20* were arrested in metaphase I (6 hr in SPM) and processed for ChIP/chip with antibodies to Myc as in (D).

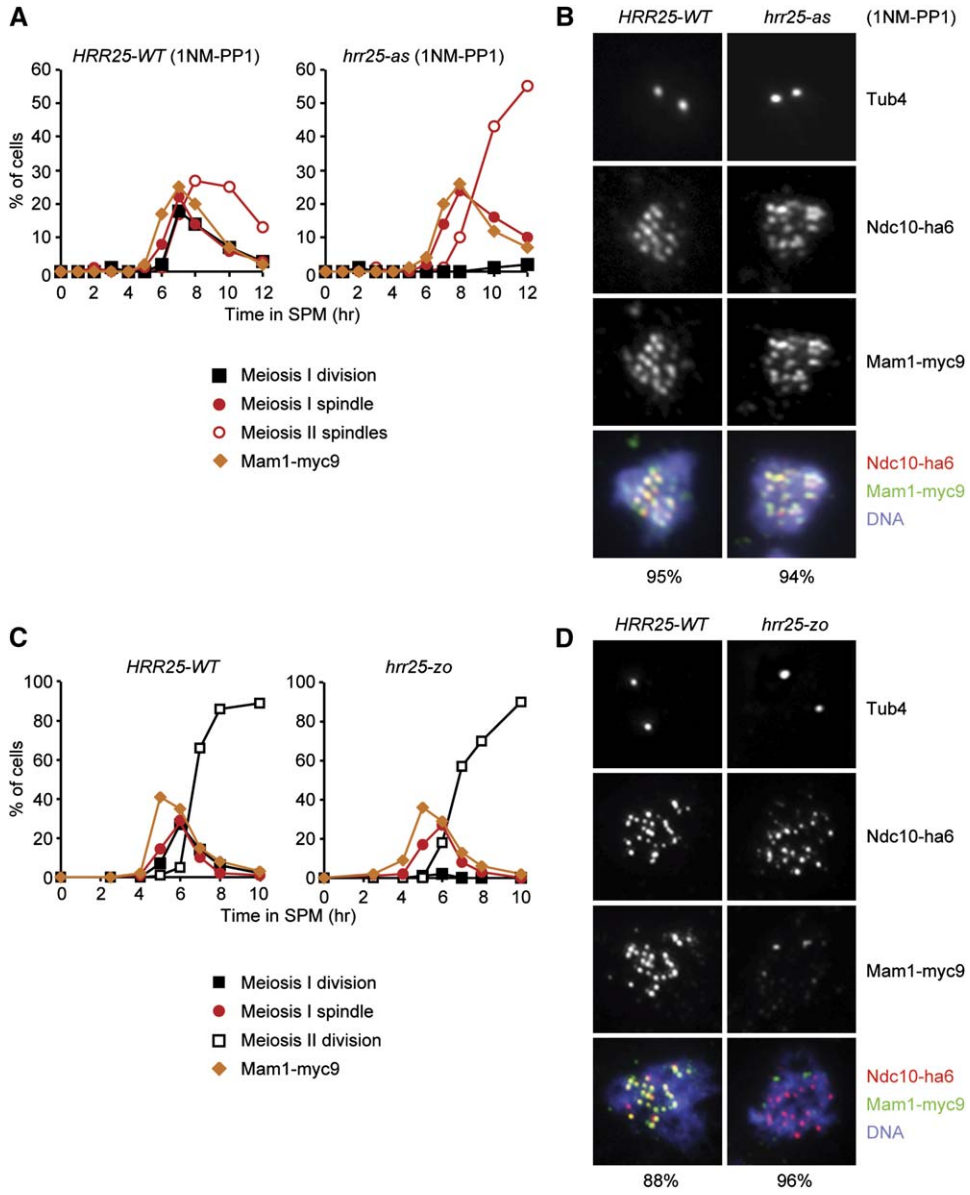

Figure 7. Mam1 Localization to Kinetochores Requires Interaction with Hrr25 but Not Hrr25's Kinase Activity

(A and B) *MAM1-myc9 NDC10-ha6* strains containing *HRR25-WT* (Z6882) or *hrr25-as* (Z6883) were shifted to SPM/1NM-PP1. Fixed cells and chromosome spreads were analyzed. Shown in panel (A) are percentages of cells with meiosis I division, meiosis I spindle, meiosis II spindles, and Mam1-myc9 staining. Shown in panel (B) is the colocalization of Mam1-myc9 with Ndc10-ha6, analyzed on spreads from metaphase I cells (t = 7 hr) showing two spindle pole bodies (which contain Tub4) and an undivided cluster of Ndc10 signals. Images represent the indicated percentages of metaphase I spreads (n = 60).

(C and D) Analysis of fixed cells and chromosome spreads from meiotic *MAM1-myc9 NDC10-ha6* strains containing *HRR25-WT* (K14250) or *hrr25-zo* (K14251). Shown in panel (C) are percentages of cells with meiosis I division, meiosis I spindle, meiosis II division, and Mam1-myc9 staining. Shown in panel (D) is the colocalization of Mam1-myc9 with Ndc10-ha6 on metaphase I spreads (n = 25) at t = 5 hr, analyzed as in (B).

Mam1 and Rec8, both of which bind tightly to Hrr25 and localize to centromeres in meiosis I. However, the physiological significance of their phosphorylation remains to be analyzed in detail. While Hrr25's kinase activity is not needed for recruiting monopolin to kinetochores, phosphorylation of Mam1 might nevertheless change the properties of monopolin once bound to kinetochores.

The finding that Rec8 is an Hrr25 target is particularly intriguing, as kleisins have been implicated in the mono-orientation process in fission yeast and plants (Chelysheva et al., 2005; Watanabe and Nurse, 1999; Yu and Dawe, 2000). Might recruitment of Hrr25 to kinetochores increase phosphorylation of Rec8 at centromeres and hence modulate cohesin's activity in a manner that facilitates

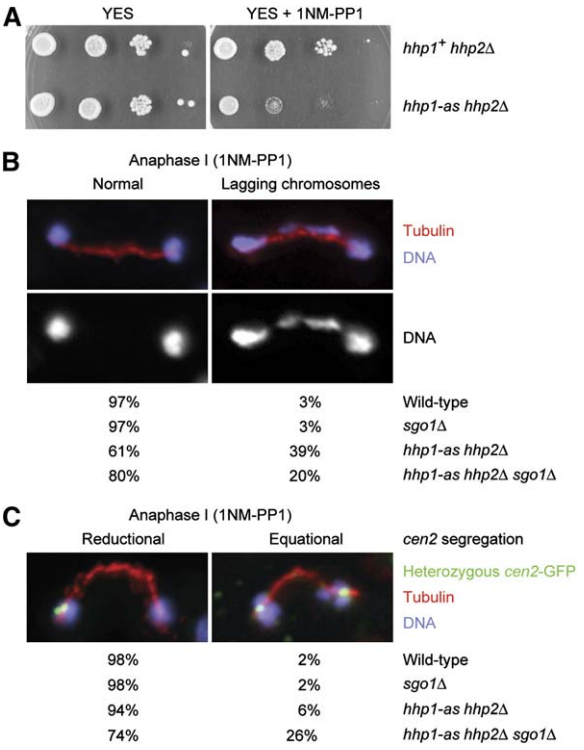

Figure 8. Hrr25 Homologs Are Required for Meiosis I in Fission Yeast

(A) Serial dilutions of haploid *hhp1*$^{+}$ *hhp2Δ* (K13619) and *hhp1-as hhp2Δ* (K14637) strains were grown on YES plates with or without 25 μM 1NM-PP1 for 72 hr at 32°C.

(B and C) Wild-type (K12225/K11318), *sgo1Δ* (K12269/K11793), *hhp1-as hhp2Δ* (K14637/K14809), and *hhp1-as hhp2Δ sgo1Δ* (K14791/K14788) strains were mated and then sporulated in the presence of 1NM-PP1. Shown in panel (B) is the staining of tubulin and DNA in anaphase I cells together with percentages of lagging chromosomes. Shown in panel (C) is the quantification of reductional and equational segregation of heterozygous *cen2*-GFP in late anaphase I cells.

mono-orientation? This hypothesis has the attractions both of simplicity and of involving most of the known players. However, several observations are difficult to reconcile with this model, at least at this stage. A large fraction of the entire cellular pool of Rec8 is associated with and phosphorylated by Hrr25 in a process that does not require Mam1. We suggest, therefore, that the observed association between Hrr25 and meiotic cohesin is not directly involved in the mono-orientation process. This is consistent with our finding that Hrr25 associates with Rec8- but not with Scc1-containing cohesin complexes, and yet both types of cohesin support mono-orientation mediated by Hrr25. The actual function of Rec8 phosphorylation by Hrr25 throughout chromosomes is unclear, as kinase inhibition has no drastic effect on cohesin's association with or removal from chromosomes.

Our ChIP/chip data indicate that Hrr25 has two distinct modes of chromatin binding, namely one that requires Mam1 and another that does not. In the absence of Mam1, Hrr25 associates with several loci in pericentromeric regions and on chromosomal arms. Since the pattern of these loci resembles Rec8's chromosomal distribution (Riedel et al., 2006), we suggest that Hrr25 binds to these sites as part of the Mam1-independent Hrr25-Rec8 complex that we detect in whole cell extracts. Mam1 is essential for the hyperaccumulation of Hrr25 in a limited region around the core meiotic centromere, a region that coincides with that occupied by Mam1 itself. We suggest that it is this subpopulation of Hrr25 that is responsible for mono-orientation. It is conceivable that also this pool of Hrr25 phosphorylates cohesin. The presence of Mam1 or other kinetochore proteins might alter the phosphorylation reaction in a manner that is crucial to the mono-orientation process. In summary, our data are compatible with the notion that Hrr25's phosphorylation of cohesin at kinetochores is important for mono-orientation, but they do not exclude the possibility that mono-orientation is mediated by the phosphorylation of a different set of proteins.

Our discovery of a highly conserved CK1 in the budding yeast monopolin complex raises the possibility that the mono-orientation mechanism has been conserved during evolution. Indeed, inhibition of Hrr25 orthologs in fission yeast also causes severe chromosome segregation defects during meiosis I and a high frequency of equational segregation of sister kinetochores when centromeric cohesion is not protected by Sgo1. Our findings should therefore encourage an investigation of CK1's role in meiosis in animals. Defects in the mono-orientation process might contribute to the missegregation of chromosomes during meiosis I in oocytes, which is the leading cause of pregnancy loss and developmental disabilities in humans (Hassold and Hunt, 2001). Analyzing CK1 function in animals will not be trivial due to the multitude of functions performed even by individual members of the CK1 family and the ensuing pleiotropy caused by simple gene deletions. However, we describe here, possibly in unique detail, the sorts of methods that permit the dissection of different functions for this multifunctional kinase, namely alterations that enable its inhibition at defined stages of a life cycle and, more novel still, mutations that alter its association with specific regulatory subunits.

EXPERIMENTAL PROCEDURES

S. cerevisiae Strains and Induction of Meiosis

Diploid SK1 strains were used for all experiments. Full genotypes are listed in Table S2. Details of strain construction are given in the Supplemental Data. To construct *hrr25* mutants and control strains, plasmids with different *HRR25* alleles were integrated into the promoter of *hrr25Δ*. The *hrr25-as* allele was generated according to Bishop et al. (2000). To identify *hrr25* mutations defective in mono-orientation, *HRR25* amplified by mutagenic polymerase chain reaction was introduced together with a gap-repair plasmid into sporulation-competent haploid *spo11Δ spo12Δ hrr25Δ* cells. Alleles that restored spore viability and caused chromosome missegregation in meiotic *hrr25Δ* cells were sequenced, and the mutations H25R and E34K combined to create *hrr25-zo*. Meiosis was induced at 30°C as described (Buonomo

et al., 2000). 1NM-PP1 (Bishop et al., 2000) from Cellular Genomics (Branford, CT) was used at 5 μM.

Analysis of Meiotic Cells

Immunofluorescence microscopy of cells and chromosome spreads was performed as described (Rabitsch et al., 2003). ChIP followed by hybridization to the Affimetrix high-density oligonucleotide array of chromosome VI was performed as described (Katou et al., 2003). Data have been deposited in the GEO database at the NCBI (accession number GSE4792).

Purification and Analysis of Proteins

P_{CLB2}-*CDC20* strains expressing the spindle pole body marker Spc42-GFP and a TAP-tagged protein were transferred to 10 l of aerated SPM (Oelschlaegel et al., 2005). When >60% of cells showed separated Spc42-GFP signals (~7 hr), a solution of 0.2 M PMSF in DMSO was diluted 1:100 into the culture, and cells were harvested. Proteins isolated by a modified TAP procedure (Riedel et al., 2006) were identified by mass spectrometric analysis of tryptic peptides as detailed in the Supplemental Data. Immunoprecipitations and the in vitro binding assay were performed as described (Oelschlaegel et al., 2005; Rabitsch et al., 2003).

S. pombe Experiments

Genotypes of all strains are listed in Table S3. Strain construction and immunofluorescence were performed as described (Rabitsch et al., 2004). To introduce *hhp1* alleles into cells, plasmids with *hhp1*$^+$ or *hhp1-as* (M84G) were integrated into the promoter of the *hhp1Δ* locus. Sister centromeres were observed using *cen2*-GFP (Yamamoto and Hiraoka, 2003). To analyze meiosis, h^+ and h^- strains were mated on PMG-N plates for 13 hr at 25°C and transferred to PMG-N agar plus 25 μM 1NM-PP1.

Supplemental Data

Supplemental Data include five figures, three tables, experimental procedures, and references and can be found with this article online at http://www.cell.com/cgi/content/full/126/6/1049/DC1/.

ACKNOWLEDGMENTS

This paper is dedicated to our sadly deceased friend and colleague, Peter Kirsten Rabitsch, who pioneered the discovery and characterization of monopolin proteins. We thank A. Amon, K. Gull, B. Habermann, Y. Hiraoka, S. Kearsey, J. Kilmartin, M. Knop, A. Schleiffer, W. Seufert, K. Shokat, A. Strunnikov, P. Uluocak, Y. Watanabe, A. Yamamoto, C. Zhang, and M. Zerial for help, reagents, and advice. Research in the Mechtler lab is supported by the Austrian Proteomics Platform (APP) and the 6th EU framework MitoCheck. Work in the Zachariae lab is supported by the Max Planck Society. Work in the Nasmyth lab was supported by Boehringer Ingelheim and the Austrian Industrial Research Promotion Fund (FFF).

Received: January 16, 2006
Revised: May 16, 2006
Accepted: July 17, 2006
Published: September 21, 2006

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Accession Numbers

The accession number in the GEO database at the NCBI for the ChIP/chip data reported in this paper is GSE4792.