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Kinetochore orientation during meiosis is controlled by Aurora B and the monopolin complex.

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Summary

Kinetochores of sister chromatids attach to microtubules emanating from the same pole (co-orientation) during meiosis I and to microtubules emanating from opposite poles (bi-orientation) during meiosis II. We find that the Aurora B kinase Ipl1 regulates kinetochore - microtubule attachment during both meiotic divisions and that a complex known as the monopolin complex ensures that the protein kinase co-orients sister chromatids during meiosis I. Furthermore, the defining of conditions sufficient to induce sister kinetochore co-orientation during mitosis provided insight into monopolin complex function. The monopolin complex joins sister kinetochores independently of cohesins, the proteins that hold sister chromatids together. We propose that this function of the monopolin complex helps Aurora B to co-orient sister chromatids during meiosis I.

Introduction

The mitotic cell division cycle is an alternation of chromosome duplication and segregation. During the meiotic cell division, which generates gametes, DNA replication is followed by two rounds of chromosome segregation. During the first division, meiosis I, homologous chromosomes segregate away from each other. During the second division, meiosis II, sister chromatids separate. Central to accurate chromosome segregation is the correct attachment of chromosomes to the spindle apparatus. During mitosis and meiosis II, sister kinetochores attach to microtubules emanating from opposite spindle poles (bi-orientation). In meiosis I, when homologs segregate away from each other and are hence bi-oriented, sister chromatids segregate to the same spindle pole. Thus, sister kinetochores must attach to microtubules emanating from the same spindle pole, a phenomenon known as monopolar attachment or sister kinetochore co-orientation.

In budding yeast, sister kinetochore co-orientation during meiosis I is brought about by the monopolin complex (reviewed in Marston and Amon, 2004). Cells lacking components of this complex bi-orient sister kinetochores during meiosis I and attempt to separate sister chromatids during the first meiotic division (Rabitsch et al., 2003; Toth et al., 2000; Petronczki et al., 2006). To date four components of the monopolin complex have been identified. Mam1 is a meiosis-specific protein present at kinetochores from pachytene to metaphase I (Toth et al.,

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2000). The monopolin complex components Csm1 and Lrs4 are expressed during both mitosis and meiosis. They reside in the nucleolus until G2 when they are released by the Polo kinase Cdc5 (Clyne et al., 2003; Rabitsch et al., 2003). After their release, Csm1 and Lrs4 form a complex with Mam1 and bind to kinetochores (Rabitsch et al., 2003). In addition Mam1 recruits the ubiquitously expressed casein kinase 1δ/ε Hrr25, which is also required for sister kinetochore co-orientation, to kinetochores during meiosis I (Petronczki et al., 2006). The meiosis-specific protein Spo13 is also necessary for kinetochore co-orientation. In its absence, the monopolin complex initially associates with kinetochores but cannot be maintained there (Katis et al., 2004b; Lee et al., 2004). How the monopolin complex and proteins that regulate its association with kinetochores bring about sister kinetochore co-orientation is not understood.

The protein kinase Aurora B is a key regulator of kinetochore – microtubule attachment. Aurora B (Ipl1 in yeast) forms a complex with INCENP (Sli15 in yeast) and this complex controls many aspects of chromosome segregation including Histone H3 phosphorylation (Hsu et al., 2000), cohesin removal (Resnick et al., 2006; Yu and Koshland, 2005), mitotic and meiotic spindle formation and stability (reviewed in Ducat and Zheng, 2004), chiasmata resolution (Kaitna et al., 2002) and linking of cytokinesis to chromosome segregation (Norden et al., 2006). In budding yeast mitosis, the Ipl1-Sli15 complex was shown to sever kinetochore – microtubule attachments that are not under tension by phosphorylating kinetochore components such as Dam1 (Cheeseman et al., 2002; Dewar et al., 2004; Pinsky et al., 2006; Tanaka et al., 2002). Thereby, Ipl1 generates unattached kinetochores, which activates the spindle checkpoint. The spindle checkpoint inhibits an ubiquitin ligase known as the Anaphase Promoting Complex (APC) or Cyclosome (C; reviewed in Lew and Burke, 2003), whose activity is essential for entry into anaphase through its role in promoting the degradation of Securin (Pds1 in yeast). This degradation leads to activation of a protease known as Separase (Esp1 in yeast). Once active, Separase cleaves a component of cohesin complexes, which hold sister chromatids together. A role for Aurora B in regulating kinetochore – microtubule attachment during meiosis has not been demonstrated.

Here we investigate how Ipl1 and the monopolin complex regulate sister kinetochore orientation during meiosis. We find that Ipl1 is required for homolog bi-orientation during meiosis I as well as sister chromatid bi-orientation during meiosis II. Our data further show that Ipl1 is epistatic to the monopolin complex in the regulation of this process. Importantly, we find that an active monopolin complex is sufficient to promote sister kinetochore co-orientation during mitosis. The ability to induce sister kinetochore co-orientation during mitosis furthermore provided insight into one of the functions of the complex. The monopolin complex links sister kinetochores in a cohesin independent manner.

Results

Aurora B localizes to kinetochores and the spindle during meiosis

To examine the role of Ipl1 in yeast meiosis we analyzed its protein levels and localization. Ipl1 was expressed throughout meiosis, but levels appeared lower as cells entered the meiotic cell cycle (Supplemental Figure 1A; 0 time point). Ipl1 activity, as judged by Histone H3 phosphorylation, mirrored Ipl1 protein levels (Supplemental Figure 1A). The localization of Ipl1 in meiosis resembled that of mitosis (Supplemental Figure 1B; Tanaka et al., 2002; Pereira and Schiebel, 2003). Ipl1 localized to the nucleus in metaphase I and metaphase II. During anaphase I and anaphase II the protein was also found on the meiotic spindle. Analysis of Ipl1 on chromosome spreads revealed that early in meiosis Ipl1 is found on chromosomes but does not localize to kinetochores (data not shown). However, at metaphase I Ipl1 associates with kinetochores as judged by the co-localization with the kinetochore component Ndc10 (Supplemental Figure 1C).

IPL1 is required for the bi-orientation of homologs during meiosis I

To determine Ipl1's function during meiosis we placed the *IPL1* open reading frame under the control of the *SCC1/MCD1* promoter, which is largely repressed during meiosis (Michaelis et al., 1997; *pSCC1-IPL1*). The *pSCC1-IPL1* fusion was expressed during the mitotic cell cycle (Figure 1A) but because Ipl1 is unstable during G1 (Biggins et al., 1999) the protein is rapidly depleted from cells entering the meiotic cell cycle (Figure 1A). Cells carrying the *pSCC1-IPL1* fusion as the sole source of Ipl1 did not exhibit proliferation defects during vegetative growth (data not shown) but progression through the meiotic cell cycle was affected. Cells exhibited a slight delay in entry into S phase (Figure 1B) and a moderate metaphase I and anaphase I delay, with spindles appearing thin and fragile (Figure 1C, data not shown). Despite these delays, 80 % of cells eventually progressed through at least one meiotic division (Figure 1D). Similar results were obtained when Ipl1 was depleted by placing the *IPL1* ORF under the control of the mitosis-specific *CLB2* promoter (Supplemental Figure 2 and data not shown).

To follow the fate of chromosomes during the meiotic divisions in the absence of Ipl1 we integrated a tandem array of tetO sequences near the centromere of chromosome V on both homologs (homozygous CENV GFP dots). These cells also expressed a tetR-GFP fusion, which binds to tetO, to visualize the repeats (Michaelis et al., 1997). The analysis of homozygous GFP dots revealed that 80 % of Ipl1-depleted cells segregated homologs to the same spindle pole rather than, as wild-type, to opposite poles (Figure 1E, F). Similar results were obtained when we analyzed the chromosome segregation behavior of chromosome III or both, chromosome III and V (data not shown). This highly asymmetric chromosome segregation resulted in the two anaphase I DNA masses being of unequal size (data not shown).

During mitosis, cells defective in *IPL1* function preferentially segregate both sister chromatids with the old spindle pole body (SPB) into the bud (Pereira et al., 2001; Tanaka et al., 2002). This is likely due to the fact that the duplication of kinetochore structures and subsequent microtubule capture occur prior to maturation of the newly synthesized SPB. Consequently, both sister chromatids attach to microtubules emanating from the same spindle pole. Owing to cells lacking *IPL1* failing to detach incorrect microtubule attachments, sister chromatids preferentially co-segregate with the old SPB into the bud. Consistent with this idea is the observation that the preferential co-segregation of sister chromatids with the old SPB can be partially rescued by transient microtubule depolymerization (Pereira et al., 2001; Tanaka et al., 2002). Transient treatment with the microtubule depolymerizing drug benomyl during prophase I (4 hours after induction of meiosis) also partially rescued the co-segregation of homologs in Ipl1-depleted meiotic cells. Whereas 80% of homologs co-segregated to the same pole in mock-treated Ipl1-depleted cells, homolog segregation was nearly random (60% co-segregation and 40% separation) when cells were treated with benomyl (Figure 1F; note that the expected ratios for random segregation would be 50%:50%). Our results indicate that *IPL1* is required for accurate homolog segregation during meiosis I. We propose that, as during mitosis, *IPL1* does so by promoting microtubule attachment turnover until all homologs are correctly oriented on the meiosis I spindle.

Aurora B regulates meiosis II chromosome segregation

To determine the role of Ipl1 in meiosis II chromosome segregation we examined cells carrying the tetO array on only one of the two homologs (heterozygous CENV GFP dots). Ipl1-depleted cells showed normal segregation of heterozygous CENV GFP dots during the first meiotic division (the GFP signal was present in one of the two nuclei after the first meiotic division; Figure 2A upper panels), indicating that sister chromatids do not separate prematurely during meiosis I. However, 60% of the cells that underwent a second meiotic division mis-segregated chromosomes resulting in the generation of four nuclei of unequal size (Figure 2A lower panels; data not shown). Because Ipl1-depleted cells undergo the second meiotic division with poor

efficiency, we also examined Ipl1-depleted cells deleted for *SPO11*. *SPO11* encodes the topoisomerase-like enzyme responsible for generating recombination-initiating double strand breaks (Bergerat et al., 1997; Keeney et al., 1997), and deletion of *SPO11* allowed Ipl1-depleted cells (as many other meiotic mutants) to progress through the second meiotic division more efficiently (compare Figures 1D and 2B). Mis-segregation of sister chromatids was even more pronounced in Ipl1-depleted cells lacking *SPO11*. 80% of sister chromatids segregated to the same pole during the second meiotic division (Figure 2B). Owing to the meiosis II phenotype of *pSCC1-IPL1 spo11*Δ cells resembling that of *IPL1*-deficient mitotic cells we conclude that *IPL1* is required for sister kinetochore bi-orientation during meiosis II.

Aurora B affects the step-wise loss of sister chromatid cohesion during meiosis

During mitosis, cohesins are lost along the entire length of chromosomes at the onset of anaphase. During meiosis cohesins are lost in a stepwise manner (reviewed in Marston and Amon, 2004). Loss of cohesins from chromosome arms is essential for homologs to segregate during meiosis I. Retention of cohesins around centromeres is necessary for sister chromatids to segregate accurately during meiosis II. To determine whether Ipl1 in addition to kinetochore orientation also regulates the loss of sister chromatid cohesion, we examined the localization of the cohesin subunit Rec8 on chromosome spreads. Cells also carried a tagged version of the kinetochore component Ndc10 to identify centromeric regions of chromosomes. In wild-type binucleate cells Rec8 was found around centromeres (Figure 2C). In contrast, nearly 50 % of Ipl1-depleted binucleate cells lacked centromeric Rec8 (Figure 2C). As a control, we also examined the localization of Rec8 in cells lacking *SGO1*, a gene essential to protect Rec8 from removal around centromeres during meiosis I (Katis et al., 2004a; Kitajima et al., 2004; Marston et al., 2004). In such cells, Rec8 was absent in binucleate cells (Figure 2C). Our results indicate that *IPL1* is required to retain Rec8 at centromeres beyond the first meiotic division, though the gene appears less important than *SGO1*.

Ipl1-depleted cells also exhibited defects in the localization of the cohesion protector Sgo1, which itself associates with centromeric regions from prophase I until metaphase II (Katis et al., 2004a; Marston et al., 2004). Only 50% of mononucleate and binucelate Ipl1-depleted cells exhibited Sgo1 localization (Figure 2D and data not shown). Deletion of *SPO13*, a gene required for the maintenance of Sgo1 at centromeres (Figure 2D; Lee et al., 2004) did not affect Sgo1 localization in mononucleate cells but had more severe effects on Sgo1 localization than Ipl1-depletion in binucleate cells (Figure 2D and data not shown; Katis et al., 2004b, Lee et al., 2004). How Ipl1 affects cohesin loss and why Ipl1 depletion only partially affects Rec8 and Sgo1 localization is at present unclear. The severity of the homolog co-segregation phenotype of Ipl1-depleted cells (80% co-segregation of homologs during meiosis I) argues against incomplete inactivation of Ipl1 being responsible for the partial effects on Rec8 and Sgo1 localization. Parallel pathways could account for the incomplete penetrance of the phenotype. We note that our findings are consistent with observations in *Drosophila*, where the Sgo1 homolog MEI-S332 requires Aurora B and INCENP for its association with pericentric regions (Resnick et al., 2006). Our results indicate that *IPL1* is required for two key aspects of the second meiotic division, sister kinetochore bi-orientation and the correct timing of loss of cohesins from chromosomes.

Depletion of Ipl1 suppresses the co-orientation defect of *mam1*Δ and *spo13*Δ mutants

Having established that Ipl1 regulates kinetochore orientation during meiosis we next examined the relationship between Ipl1 and co-orientation factors. The majority of cells lacking *MAM1* and *SPO11* carrying heterozygous CENV GFP dots segregate sister chromatids during the first observable chromosome segregation phase leading to the formation of binucleate cells with a GFP dot in each of the two nuclei (Toth et al., 2000; Figure 3A). Remarkably, depletion of Ipl1 in such cells led to the co-segregation of sister chromatids to one spindle pole (Figure

3A). Similar results were obtained when Ipl1 was depleted in cells lacking *SPO11* and *SPO13*. *spo13*Δ *spo11*Δ mutants undergo a single meiotic division during which sister chromatids segregate to opposite poles (Klapholz et al., 1985; Figure 3B). Depletion of Ipl1 in these cells led to the co-segregation of sister chromatids (Figure 3B). Our results indicate that bi-orientation of sister kinetochores in *mam1*Δ or *spo13*Δ mutants requires *IPL1* function. Inactivation of *SPO13* or *MAM1* neither changed Ipl1 localization (Supplemental Figures 3A and 3B) nor its ability to phosphorylate Histone H3 (Supplemental Figure 3C) indicating that the two proteins did not affect Ipl1 function. The simplest interpretation of our findings is that Ipl1 performs the same function during meiosis I as during mitosis and meiosis II, that is severing microtubule-kinetochore attachments that are not under tension. The monopolin complex's function is to change sister kinetochores in a way that they are only under tension when homologs are bi-oriented.

An active monopolin complex is sufficient to promote sister kinetochore co-orientation during mitosis

To gain further insights into how the monopolin complex brings about sister kinetochore co-orientation, we wished to define the minimal number of genes necessary for this process to occur during mitosis. The monopolin complex component Mam1 is not expressed during mitosis. Overexpression of *MAM1* alone is, however, not sufficient for sister kinetochore co-orientation to occur during mitosis (Figure 4A; Toth et al., 2000). As Mam1 requires Lrs4 and Csm1 to associate with kinetochores (Rabitsch et al., 2003), the fact that Lrs4 and Csm1 are not released from the nucleolus during mitotic G2 (Toth et al., 2000; Supplemental Figure 4) could be responsible for Mam1's inability to promote sister kinetochore co-orientation during mitosis.

To release Lrs4 and Csm1 from the nucleolus we overexpressed *CDC5* from the galactose inducible *GAL1* promoter. The presence of a single copy of *CDC5* expressed from the *GAL1* promoter did not interfere with cell cycle progression (data not shown) but led to the release of Lrs4 from the nucleolus (Supplemental Figure 4). As Csm1 and Lrs4 localization is interdependent (Rabitsch et al., 2003), Csm1 release is likely to also occur. Lrs4 however, failed to associate with kinetochores in *GAL-CDC5* cells (Figure 4B). Co-overexpression of *MAM1* and *CDC5* from the *GAL1* promoter led to Lrs4 association with kinetochores (Figure 4B) indicating that *CDC5* is required to release the Lrs4-Csm1 complex from the nucleolus and that only when Mam1 is present, are the two proteins efficiently recruited to kinetochores.

Cells overproducing Cdc5 and Mam1 progressed through mitosis with kinetics similar to that of wild type cells (Figure 4C). Degradation of Pds1 was, however, delayed by 15 minutes (Figure 4D) indicating that the spindle checkpoint was transiently activated. The analysis of CENIV-GFP or CENV-GFP dot segregation revealed that 35% of *GAL-CDC5 GAL-MAM1* cells segregated both sister chromatids to the same spindle pole (Figure 4A, data not shown). The co-segregation of sister chromatids depended on the monopolin complex components Lrs4 and Csm1. Deletion of *LRS4* reduced sister chromatid co-segregation to 13%. Inactivation of both *LRS4* and *CSM1* reduced it further to 4% (Figure 4A). Overexpression of *SPO13* did not lead to an increase in *LRS4/CSM1*-dependent sister chromatid co-segregation in *GAL-CDC5 GAL-MAM1* cells (see Supplemental Materials and Supplemental Figure 5) suggesting that high levels of Spo13 do not enhance sister kinetochore co-orientation when Cdc5 and Mam1 are overproduced. We conclude that overexpression of *CDC5* and *MAM1* is sufficient to promote co-orientation of sister kinetochores. This co-segregation of sister chromatids is accompanied by a slight delay in Pds1 degradation suggesting that the lack of tension caused by the co-segregation of sister chromatids leads to Ipl1-dependent microtubule severing which results in a transient activation of the spindle checkpoint.

Establishing sister kinetochore co-orientation during mitosis does not interfere with *IPL1* function

Our *mam1Δ pSCC1-3HA-IPL1* and *spo13Δ pSCC1-3HA-IPL1* double mutant analysis indicated that co-orientation factors either functioned as inhibitors of Ipl1 or were modifying sister kinetochores in a way that Ipl1 was not able to bi-orient them. Several observations, argue against Spo13 and Mam1 inhibiting Ipl1 function. First, overexpression of *CDC5* and *MAM1* during mitosis promotes sister kinetochore co-segregation, which is accompanied by a modest delay in Pds1 degradation (Figure 4D) Second, Ipl1 levels, localization, and overall kinase activity (as judged by Histone H3 phosphorylation) were not affected in *GAL-CDC5 GAL-MAM1* strains (data not shown). Third, we did not detect any genetic interactions between co-orientation factors and *IPL1* gain- and loss-of function alleles. Overexpression of *CDC5* and *MAM1* did not enhance the chromosome segregation defect of temperature sensitive *ipl1-321* mutants (Biggins et al., 1999) at intermediate growth temperatures. At 34°C, *ipl1-321 GAL-CDC5 GAL-MAM1* mutants exhibited the same phenotype as *ipl1-321* mutants (Figure 5A). At 25°C and 30°C the strain showed the same phenotype as the *GAL-CDC5 GAL-MAM1* strain (Figure 5A). Fourth, overexpression of *IPL1* did not affect sister chromatid co-segregation in *GAL-CDC5 GAL-MAM1* cells (data not shown). Finally, the co-segregation of sister chromatids in *GAL-CDC5 GAL-MAM1* cells differed from that observed in *ipl1-321* mutants. Whereas sister chromatids preferentially segregate together with the old SPB into the bud during mitosis in *ipl1-321* mutants (65–70%; Tanaka et al., 2002; Figure 5B), co-segregation of sister chromatids did not show a SPB preference in *GAL-CDC5 GAL-MAM1* cells (47% in the mother, 53% in the daughter; Figure 5B). These observations together with the finding that inactivation of the monopolin complex does not affect Ipl1 localization and kinase activity during meiosis indicates that the monopolin complex does not inhibit Ipl1. Rather, they suggest that the monopolin complex acts on the kinetochore to facilitate co-segregation of sister chromatids.

The monopolin complex joins sister kinetochores independently of cohesins during mitosis

Insights into monopolin complex function came from the analysis of GFP dots in mitotic cells induced to co-segregate sister chromatids. We observed that co-segregating CENIV GFP dots were always tightly paired in *GAL-CDC5 GAL-MAM1* cells (Figure 4A). In contrast, co-segregating telomeric GFP dots were paired only half the time (Figure 6C). The tight association of sister chromatids at centromeres is specific to co-segregation brought about by overproduction of Cdc5 and Mam1 and is not a phenomenon generally occurring when sister chromatids co-segregate to the same spindle pole. We observed two distinct GFP signals during anaphase in wild-type cells carrying GFP dots 1.4 and 2kb away from the centromere of chromosomes IV and V, respectively (Figure 6A). More importantly, in two other mutants that co-segregate sister chromatids, two individual GFP dots were seen in a significant fraction of anaphase cells. In cells lacking cohesins due to the depletion of the cohesin subunit Scc1/Mcd1, approximately 50 % of co-segregating sister chromatids were pulled to the spindle pole individually, as judged by the fact that two distinct GFP dots were visible in one of the two nuclear lobes when sister chromatids segregated to the same pole (Figure 6B). Overexpression of *CDC5* and *MAM1* led to an increase in sister chromatids co-segregation from 29 % to 44 % in such cells and, importantly, sister centromeres remained tightly associated during anaphase under these conditions (Figure 6B). In another mutant that co-segregates sister chromatids, in the *ipl1-321* mutant, two distinct GFP signals were observed in approximately 40 % of cells with co-segregating sister chromatids but GFP dots appeared again as one in most cells when Cdc5 and Mam1 were overproduced in the mutant (Figure 5B).

Could the co-segregation of sister chromatids in *GAL-CDC5 GAL-MAM1* mutants depleted of cohesins be due to only one of the sister kinetochores attaching to a microtubule and the second sister chromatid being dragged along due to cohesin independent linkages? We can exclude

this possibility because in cells lacking cohesins and functional kinetochores (by inactivating *NDC10*) single chromatids are left behind at the metaphase plate during chromosome segregation (data not shown; Tanaka et al., 2002). Together our data indicate that sister chromatids normally segregate independently of each other even under conditions when they co-segregate to the same spindle pole, but overexpression of *CDC5* and *MAM1* induces a tight association between the co-segregating sister chromatids at centromeres that is independent of cohesins.

A *MAM1*-dependent linkage joins sister chromatids in the absence of *REC8*

Next we investigated whether sister kinetochores are also joined by the monopolin complex during meiosis I. If sister kinetochores were linked during meiosis I in a cohesin independent manner sister chromatids should co-segregate to the same spindle pole even in the absence of sister chromatid cohesion. Previous studies indicated that in cells lacking *REC8*, 65% of sister chromatids segregate to the same pole during anaphase I. However, the percentage of cells progressing past prophase I in the absence of *REC8* is exceedingly small (10 – 15%) because of defects in recombination leading to the activation of the recombination checkpoint (Klein et al., 1999). We therefore reinvestigated the segregation behavior of sister chromatids in *rec8*Δ cells in the absence of recombination brought about by the deletion of *SPO11*. Remarkably, more than 80% of sister chromatids segregated to the same spindle pole in *rec8*Δ *spo11*Δ mutants either carrying GFP dots near the centromere (CENV dots) or at chromosome arms (*LYS2* dots; Figure 7A). Furthermore, the majority of CENV GFP dots appeared as one, while chromosome arms (LYS2 dots) were paired only half the time (Figure 7A) indicating that the tight association of sister chromatids is restricted to the centromeric region. Importantly, the co-segregation of sister chromatids was in part dependent on a functional monopolin complex because it was reduced in *rec8*Δ *spo11*Δ *mam1*Δ triple mutants (Figure 7A).

To examine whether the monopolin complex was also affecting the association of sister chromatids prior to meiosis I chromosome segregation we examined the effects of deleting *MAM1* in *rec8*Δ *spo11*Δ cells arrested in prophase I due to the deletion of the transcription factor *NDT80* (Xu et al., 1998). Six hours after the induction of meiosis, CENV GFP dots were paired in 91% of *rec8*Δ *spo11*Δ *ndt80*Δ cells (Figure 7B). In contrast, at chromosome arms GFP dots (LYS2 dots) appeared less frequently paired (60% of cells; Figure 7B). The appearance of only one dot was not due to the lack of DNA replication because most cells had replicated their DNA at the time GFP dots were examined (Figure 7C). Deletion of *MAM1* reduced the pairing of GFP dots in cells carrying CENV GFP dots to 74%. It also reduced pairing of arm sequences from 59% to 37% (Figure 7B), which probably reflects the fact that arm sequences are more likely to interact when centromeres are linked. We conclude that though clearly not the only factor linking sister chromatids at centromeres in the absence of cohesins, the monopolin complex joins sister kinetochores in a cohesin-independent manner during meiosis I.

Discussion

Aurora B kinases affect diverse mitotic events; most prominent among these are chromosome morphogenesis and segregation. We have investigated the protein kinase's role in kinetochore – microtubule attachment during the two meiotic divisions and found that Aurora B is required for homolog bi-orientation during meiosis I as well as sister chromatid bi-orientation during meiosis II. Our data further implicate the meiosis I-specific monopolin complex in allowing Aurora B to bi-orient homologs rather than sister chromatids during meiosis I. Consistent with this central role in determining kinetochore orientation is the observation that the monopolin complex is sufficient to induce co-orientation of sister kinetochores. The ability to establish

sister kinetochore co-orientation during mitosis furthermore provided insights into one of the complex's function: providing a link between sister kinetochores.

The roles of Aurora B during meiosis

Aurora B has been shown to regulate chromosome alignment and segregation, cytokinesis and microtubule dynamics during meiosis in several organisms (Bishop et al., 2005; Kaitna et al., 2002; Ohi et al., 2004; Rogers et al., 2002; Schumacher et al., 1998). Depletion of Aurora B in budding yeast revealed that the protein kinase is required for several aspects of meiotic cell division in this organism too. First, Ipl1-depleted cells were somewhat delayed in entry into pre-meiotic S phase, the basis of which is at present unclear. Second, Ipl1 is required for the coordinated stepwise-loss of cohesion in a fraction of cells, which is consistent with recent results in *Drosophila* (Resnick et al., 2006). The third function of Aurora B that we uncovered during meiosis is that it promotes homolog and sister chromatid bi-orientation during meiosis I and meiosis II, respectively. The mechanisms whereby Ipl1 accomplishes this appear to be the same as during mitosis. The protein kinase severs microtubule-kinetochore attachments that are not under tension. The crucial factor that allows the protein kinase to bi-orient homologs rather than sister chromatids during meiosis I is the monopolin complex.

Establishing sister kinetochore co-orientation during mitosis

By co-overexpressing Cdc5 and Mam1 we were able to induce co-segregation of sister chromatids during mitosis. Does this co-segregation reflect genuine co-orientation of sister kinetochores as it exists during meiosis I or does this regimen lead to non-specific interference with kinetochore function? Abolishing kinetochore function through the inactivation of core kinetochore components such as *NDC10,* leads to spindle elongation in the absence of chromosome segregation, with many chromosomes remaining at the metaphase plate (Goh and Kilmartin, 1993). Interference with kinetochore – microtubule attachment delays/prevents entry into anaphase. These phenotypes are not observed in *GAL-CDC5 GAL-MAM1* cells arguing against a general kinetochore defect in these cells.

Several lines of evidence indicate that the co-segregation of sister chromatids observed in *GAL-CDC5 GAL-MAM1* mutants is also not due to a loss of *IPL1* function. Overproduction of Cdc5 and Mam1 did not enhance the *ipl1-321* phenotype at the semi-permissive temperature, nor did overexpression of *IPL1* affect sister chromatid co-segregation in *GAL-CDC5 GAL-MAM1* cells. Furthermore, the co-segregation phenotype of *GAL-CDC5 GAL-MAM1* mutants differs from that of *ipl1-321* mutants. Finally, the fact that Pds1 degradation was delayed in cells overproducing Cdc5 and Mam1 indicates that Ipl1 is active in these cells. Together, our studies indicate that general kinetochore defects and effects on Ipl1 function are not the reason for the co-segregation of sister chromatids in *GAL-CDC5 GAL-MAM1* cells. The finding that the co-segregation of sister chromatids in cells overproducing Cdc5 and Mam1 depends on the monopolin complex components Csm1 and Lrs4 furthermore leads us to conclude that the co-segregation observed during mitosis reflects genuine co-orientation of sister kinetochores during meiosis I.

Mechanisms of sister kinetochore co-orientation

Aurora B kinases play an essential role in bi-orienting sister kinetochores during mitosis. It was therefore possible that factors promoting the co-orientation of sister kinetochores during meiosis I would be inhibitors of Aurora B function. Our studies indicate that this is not the case. Rather they point towards Ipl1 performing the same function during meiosis I and II as it does during mitosis, that is severing microtubule-kinetochore attachments that are not under tension. The monopolin complex modifies sister kinetochores so that they are only under tension when homologs are bi-oriented. How does the monopolin complex accomplish this? Several lines of evidence indicate that the complex functions as a link between sister

kinetochores that is distinct from cohesins. When overproduced during mitosis Cdc5 and Mam1 induce the co-segregation of sister chromatids, with the two sisters being tightly associated near centromeres but not at arm regions. The tight association of sister centromeres is not observed in other mutants that co-segregate sister chromatids to the same pole during anaphase such as *ipl1-321* mutants or cells depleted for cohesins. Importantly, high levels of Cdc5 and Mam1 are capable of linking co-segregating sister chromatids in cells lacking *IPL1* or cohesin. During meiosis I, we observe that even in the absence of the cohesin subunit *REC8*, 91% of sister chromatids are associated at centromeres during prophase I (*ndt80*Δ block) and preferentially (85%) co-segregate to the same pole during meiosis I. During this co-segregation centromeric sequences appear tightly paired whereas arm sequences were not. Importantly, this association of sister chromatids in *spo11*Δ *rec8*Δ cells is in part dependent on *MAM1* indicating that the protein has sister centromere connecting abilities not only when overproduced during mitosis but also during meiosis I.

How could the joining of sister kinetochores force them to attach to microtubules emanating from the same pole? The fusion of sister kinetochores could put steric constraints onto the two sister kinetochores, hence favoring attachment of both kinetochores to microtubules emanating from the same spindle pole. Ultrastructural analyses of meiosis I spindles in the salamander *Amphiuma tridactylum* and several grasshopper species support this hypothesis (reviewed in Moore and Orr-Weaver, 1998). We favor the idea that at least in yeast, the monopolin complex, in addition to joining sister kinetochores, prevents attachment of microtubules to one of the two sister kinetochores because it is more consistent with ultrastructural analyses of meiosis I spindles in budding yeast. In *S. cerevisiae*, in which kinetochores bind to only one microtubule, the number of microtubules in the meiosis I spindle is more consistent with one microtubule attaching to one homolog (Winey et al., 2005). We note that in other organisms such as *Drosophila* and mouse, sister kinetochores also appear to form a single microtubule binding surface during metaphase I (Goldstein, 1981; Parra et al., 2004). The second observation leading us to favor the model in which the monopolin complex links sister centromeres and prevents one kinetochore from attaching to microtubules is that overexpression of a functional monopolin complex allows 35 % of cells treated with the microtubule depolymerizing drug nocodazole, which causes activation of the spindle checkpoint, to escape the checkpoint arrest (Supplemental Figure 6).

The mechanisms whereby the monopolin complex links sister kinetochores remain to be determined. We propose that after DNA replication sister chromatids are initially topologically linked due to catenation even in the absence of cohesins. Mam1 assembles onto the kinetochores of these sisters joining them at centromeres. Whether this link is able to withstand the pulling forces exerted by microtubules is unclear but we envision that the monopolin complex bridges the sister kinetochores in a way that ensures their concerted movement and conceals one of the two microtubule attachment sites. The monopolin complex could itself bridge sister chromatids or induce changes in kinetochore substructures to induce their interaction with each other. In this regard it is interesting to note that a component of the monopolin complex, Hrr25, forms multimeres only during meiosis I (Petronczki et al., 2006), potentially providing a bridging function. In *S. pombe*, co-orientation factors appear to bring about sister kinetochore co-orientation through cohesin complexes (Yokobayashi and Watanabe, 2005). Our results suggest that in *S. cerevisiae* co-orientation factors themselves have the ability to join sister chromatids. We propose, that this function is important to promote sister kinetochore co-orientation. Whether these linkages simply impose steric constraints or in addition control the attachment of microtubules to kinetochores will be an important question to determine in the future.

Experimental Procedures

Strains and Plasmids—Derivatives of SK1 are described in Supplemental Table 1; derivatives of W303 strains in Supplemental Table 2. To deplete Ipl1 during meiosis, the *IPL1* ORF was placed under the control of the *SCC1* or *CLB2* promoter by the PCR-based method described in Longtine et al., 1998. The *GAL1-10* promoter fusions as well as tagged alleles of various genes are described in the Supplemental Materials.

Sporulation conditions—Cells were grown to saturation in YPD (YEP + 2% glucose) for 24 hours, diluted into YPA (YEP + 2% KAc) at OD_{600} = 0.3 and grown overnight. Cells were then washed with water and resuspended in SPO medium (0.3% KAc [pH = 7.0]) at OD_{600} = 1.9 at 30°C to induce sporulation.

Western blot analysis—Cells were harvested, and incubated in 5% trichloroacetic acid (TCA) and lysed as described in Moll et al., 1991. Immunoblots were performed as described in Cohen-Fix et al., 1996. Antibody concentrations are listed in the Supplemental Materials.

Localization techniques—Indirect *in situ* immunofluorescence was carried out as described in Visintin et al., 1999. Chromosomes were spread as described in Nairz and Klein, 1997. Antibody concentrations are listed in the Supplemental Materials.

CEN GFP dots were analyzed in cells that were fixed in 2.5% formaldehyde for 10 min, washed twice and stored in potassium phosphate buffer pH 7.4. Before the microscopic analysis samples were fixed with 80% EtOH for 10 min, and resuspended in 1 mg/ml 4’,6-diamidino-2-phenylindole (DAPI) solution. 200 cells were counted per time point unless otherwise noted.

Statistical analysis—Results are the mean ± standard deviation for n=3 experiments. The statistical significance was evaluated using ANOVA followed by *post hoc* multiple comparison according to the Student-Newman-Keuls method. $p \leq 0.01$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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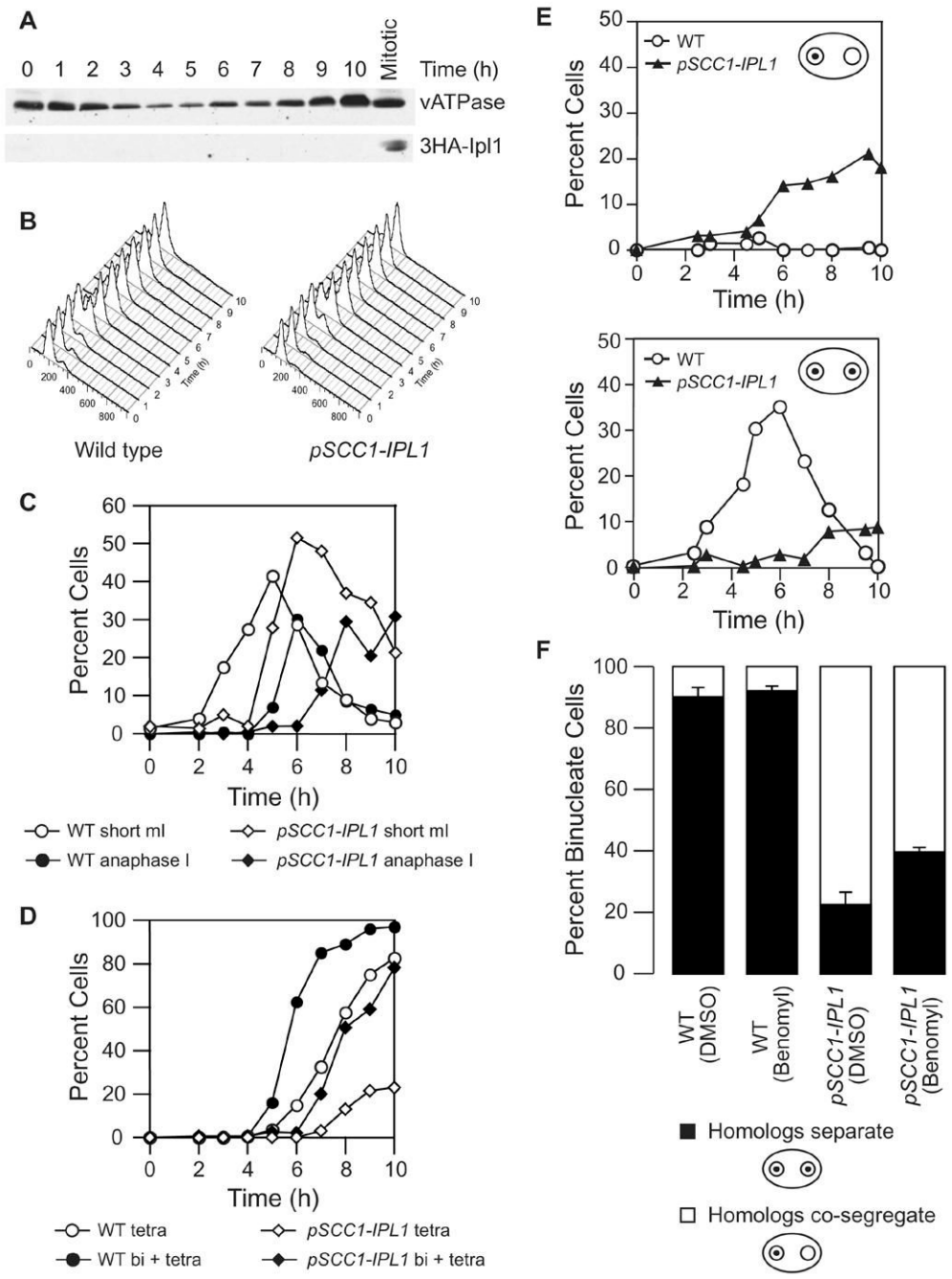

Figure 1. Ipl1 regulates meiosis I chromosome segregation

(A) *pSCC1-3HA-IPL1* cells (A10423) were induced to sporulate to examine 3HA-Ipl1 levels at the indicated times. vATPase was used as a loading control.

(B) Wild type (A5811) and *pSCC1-3HA-IPL1* (A10423) cells were induced to sporulate to determine DNA content by FACS.

(C – E) Wild type (A5715) and *pSCC1-3HA-IPL1* (A14502) cells, both carrying homozygous CENV GFP dots were induced to sporulate to determine spindle morphology (C), nuclear morphology (D) and CENV GFP dot segregation (E).

(F) Strains described in (C) were resuspended in sporulation (SPO) medium containing 120 μg/ml benomyl (benomyl) or DMSO (1%; mock) 4 hours after induction of sporulation. After

30 minutes cells were washed and resuspended in SPO medium. Samples were taken between 3–6 hours thereafter and CENV GFP dot segregation was determined.
Note that we only determined the presence but not the number of GFP dots per nucleus.

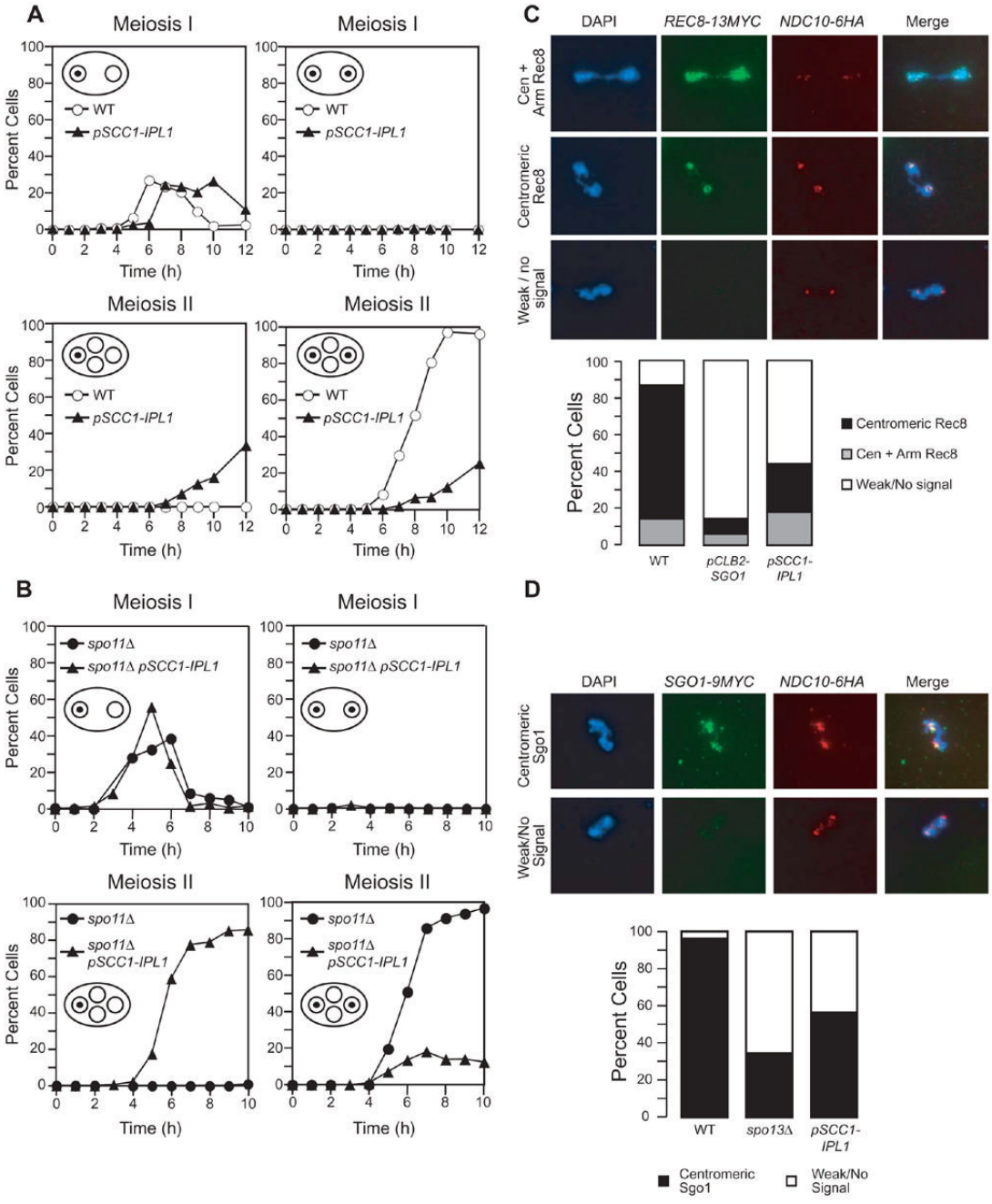

Figure 2. *IPL1* controls multiple meiosis II events

(A) Wild type (A5811) and *pSCC1-3HA-IPL1* (A10423) cells carrying heterozygous CENV GFP dots were induced to sporulate to determine GFP dot segregation at the indicated times.

(B) *spo11*Δ(A9498) and *spo11*Δ *pSSC1-3HA-IPL1* (A10425) cells carrying heterozygous CENV GFP dots were induced to sporulate to determine GFP dot segregation at the indicated times.

(C) Wild type (A10483), Ipl1-depleted (A15201) or Sgo1-depleted (A15056) cells carrying a *NDC10-6HA* and a *REC8-13MYC* fusion were induced to sporulate. Chromosome spreads were prepared at 5, 6 and 8 hours after sporulation induction and Rec8 localization was analyzed in binucleate cells (n=50).

(D) Wild type (A10461), *spo13*Δ (A10755) and *pSCC1-3HA-IPL1* (A15169) cells carrying a *NDC10-6HA* and a *SGO1-9MYC* fusion were induced to sporulate to examine Sgo1 localization as described in (C).

Note that we only determined the presence but not the number of GFP dots per nucleus.

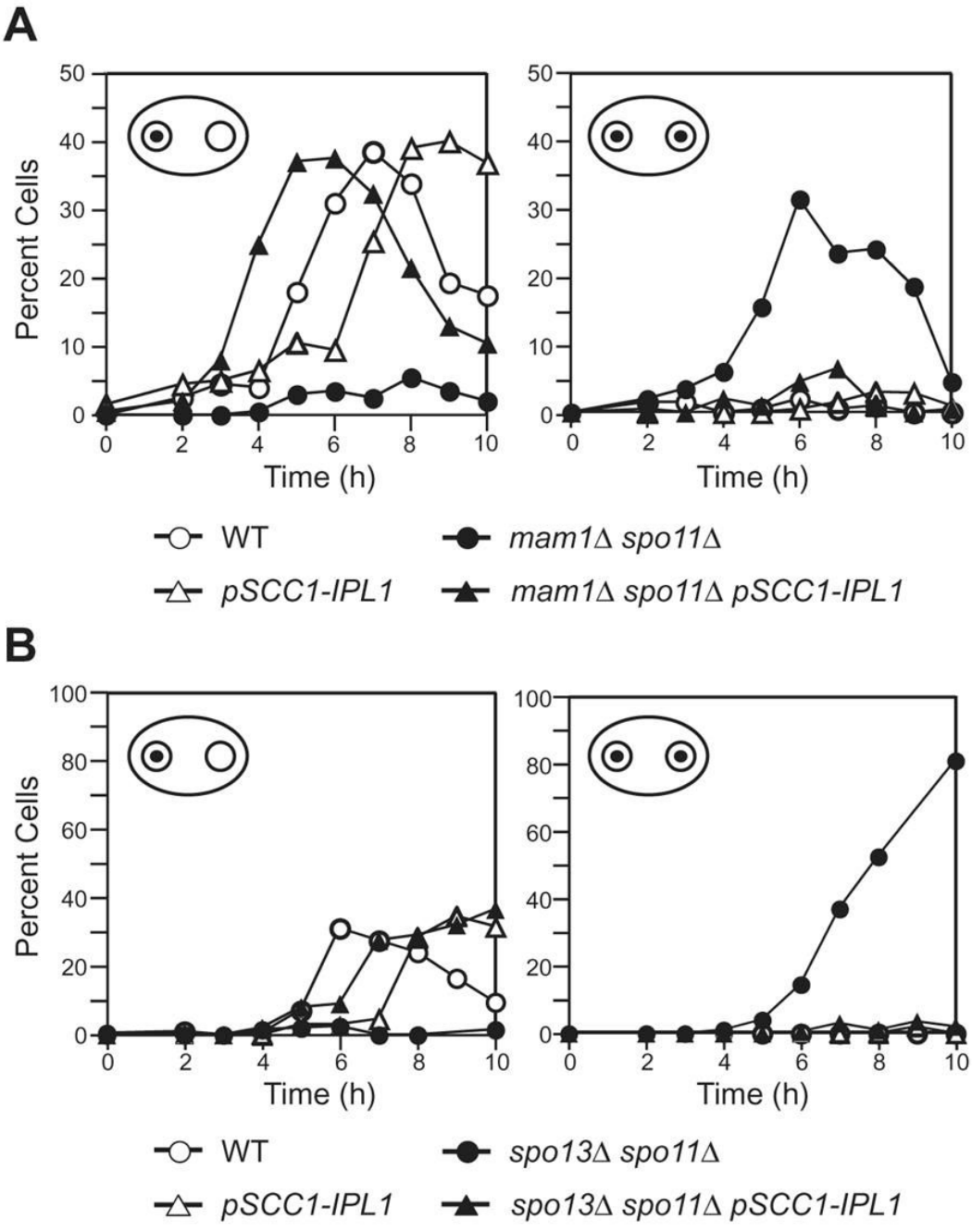

Figure 3. The Ipl1-depletion phenotype is epistatic to that caused by the inactivation of *MAM1* or *SPO13*

Wild type (A5811), *pSCC1-3HA-IPL1* (A10423), *mam1*Δ *spo11*Δ (A8128) and *mam1*Δ *spo11*Δ *pSCC1-3HA-IPL1* (A15164) cells in (A) and wild type (A5811), *pSCC1-3HA-IPL1* (A10423), *spo13*Δ *spo11*Δ (A7170) and *spo13*Δ *spo11*Δ *pSCC1-3HA-IPL1* (A11432) cells in (B), all carrying heterozygous CENV GFP dots, were induced to sporulate to determine GFP dot segregation at the indicated times.

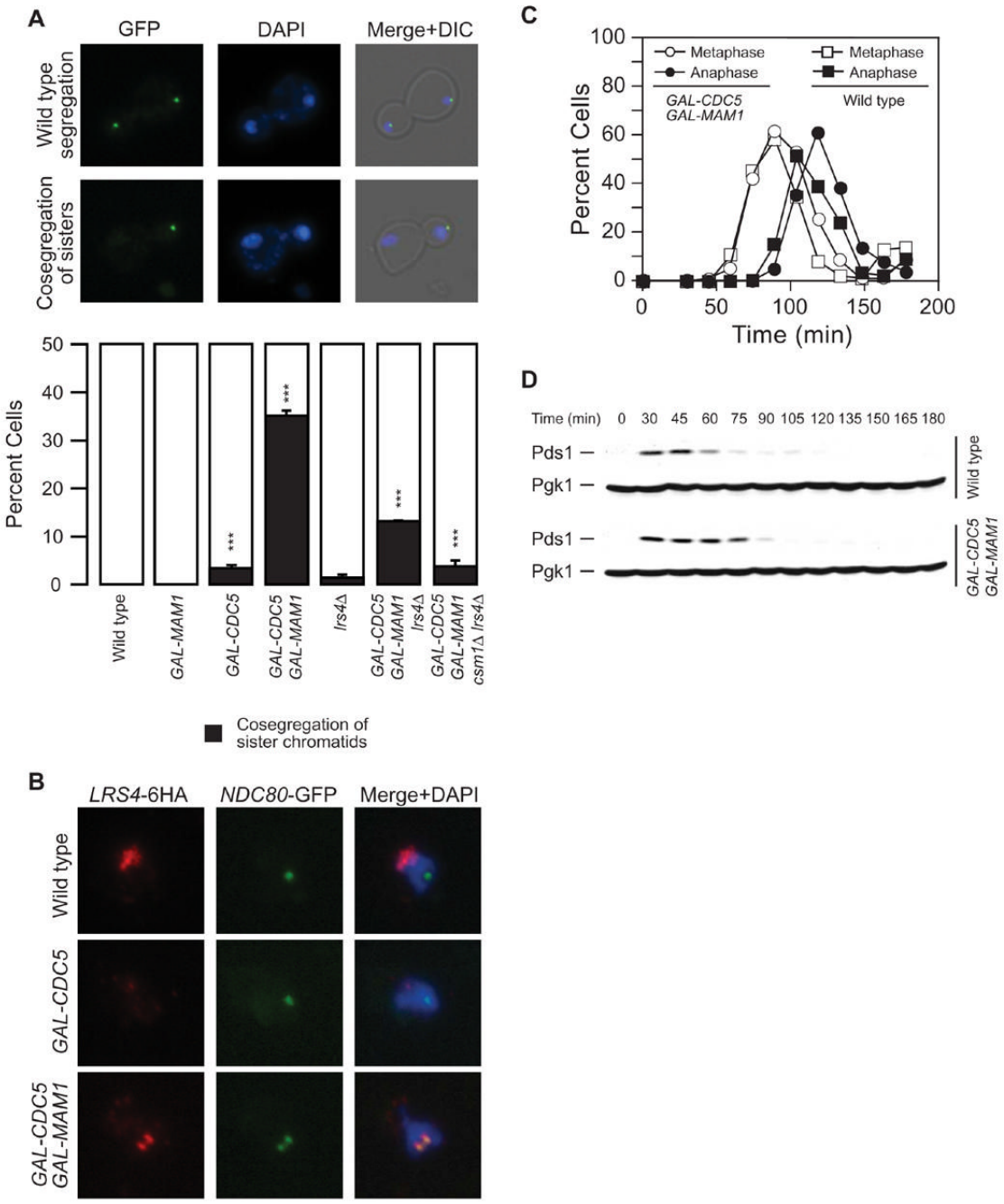

Figure 4. An active monopolin complex is sufficient to promote sister kinetochore co-orientation

(A) Wild type (A5244), *GAL-MAM1* (A12315), *GAL-CDC5* (A12325), *GAL-CDC5 GAL-MAM1* (A12312), *lrs4*Δ (A15911), *GAL-CDC5 GAL-MAM1 lrs4*Δ (A15910) and *GAL-CDC5 GAL-MAM1 lrs4*Δ *csm1*Δ (A16882) cells, all carrying CENIV GFP dots, were arrested in G1 using 5 μg/ml α factor, and treated with galactose for 1 hour prior to release. When arrest was complete, cells were released into medium lacking pheromone and containing 2% galactose. Samples were taken to determine GFP dot segregation (data represent the average of 3 experiments; statistically significant changes relative to wild type ($p \leq 0.001$) are indicated by ***).

(B) Wild type (A15127), *GAL-CDC5* (A15926), and *GAL-CDC5 GAL-MAM1* (A15925) cells carrying *LRS4–6HA* and *NDC80-GFP* were grown as described in (A) to determine the localization of Lrs4-HA on chromosome spreads. Lrs4-6HA is shown in red, Ndc80-GFP in green and DNA in blue.

(C, D) Wild type (A15912, squares) and *GAL-CDC5 GAL-MAM1* (A15915, circles) cells, all carrying *PDS1-3HA* fusions, were grown as in (A) except α-factor was re-added (5 μg/ml) 90 minutes after release from the G1 arrest. Samples were taken to determine the percentage of metaphase (closed symbols; C) and anaphase (open circles; C) spindles and Pds1-3HA protein levels (D). Pgk1 was used as a loading control.

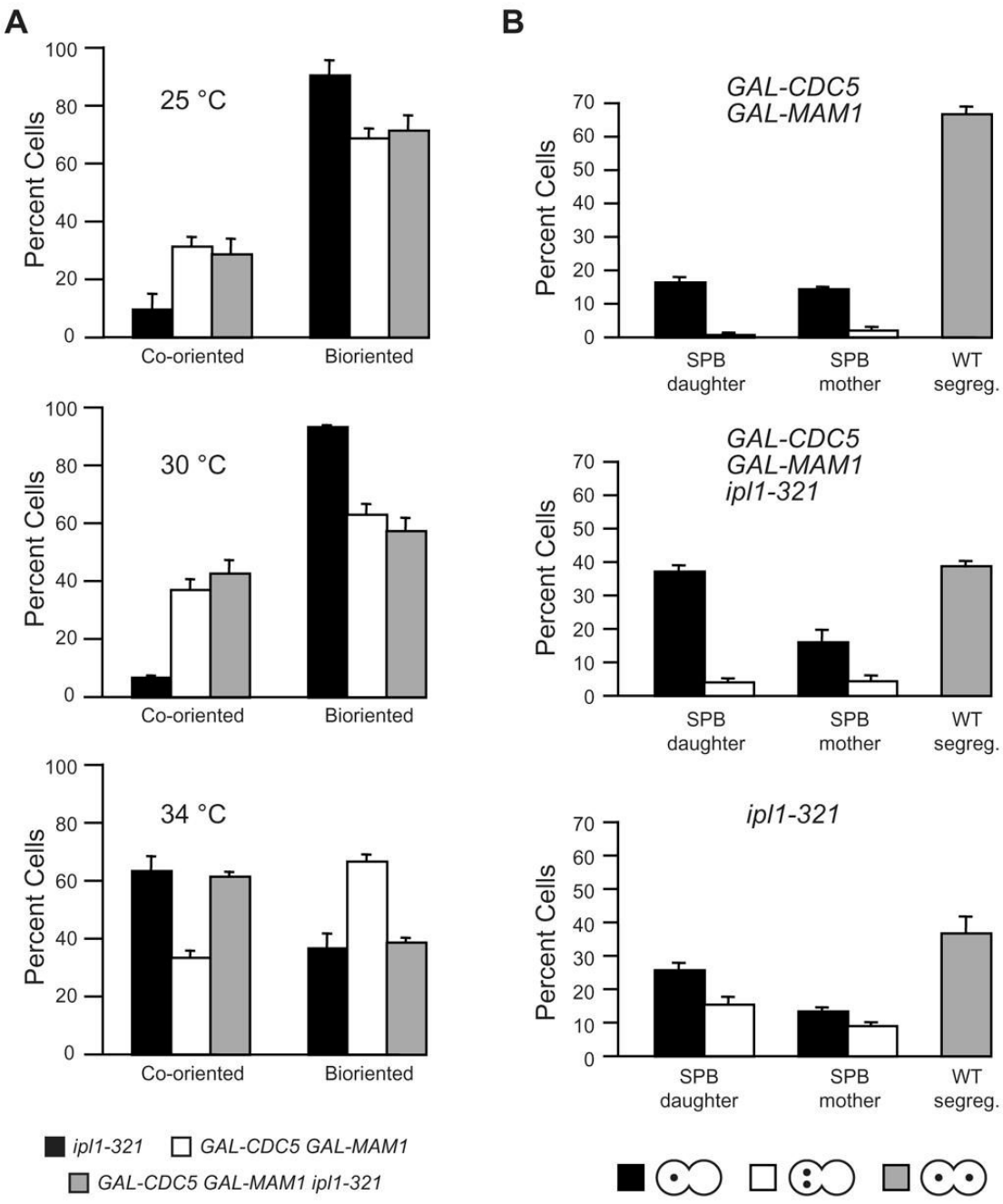

Figure 5. Effects of overproducing Cdc5 and Mam1 on *ipl1-321* mutants

GAL-CDC5 GAL-MAM1 (A12312), *ipl1-321* (A16485), and *GAL-CDC5 GAL-MAM1 ipl1-321* (A15931) cells, all carrying CENIV GFP dots, were arrested in G1 as described in Figure 4A, followed by release into medium lacking pheromone and containing 2% galactose at 25ºC, 30ºC or 34ºC.

(A) The percentage of co-segregating and correctly segregating (bi-oriented) sister chromatids was determined in anaphase cells (data represent the average of 3 experiments).

(B) The percentage of the following three classes of anaphase cells was determined at 34°C: (i) co-segregating sister chromatids that segregated into the bud (SPB daughter), (ii) co-segregating sister chromatids that segregated into the mother (SPB mother) and (iii) correctly

segregating sister chromatids (WT segreg.). Within classes (i) and (ii) the following distinctions were made: co-segregating sister chromatids tightly paired (black bars); co-segregating sister chromatids not paired (white bars).

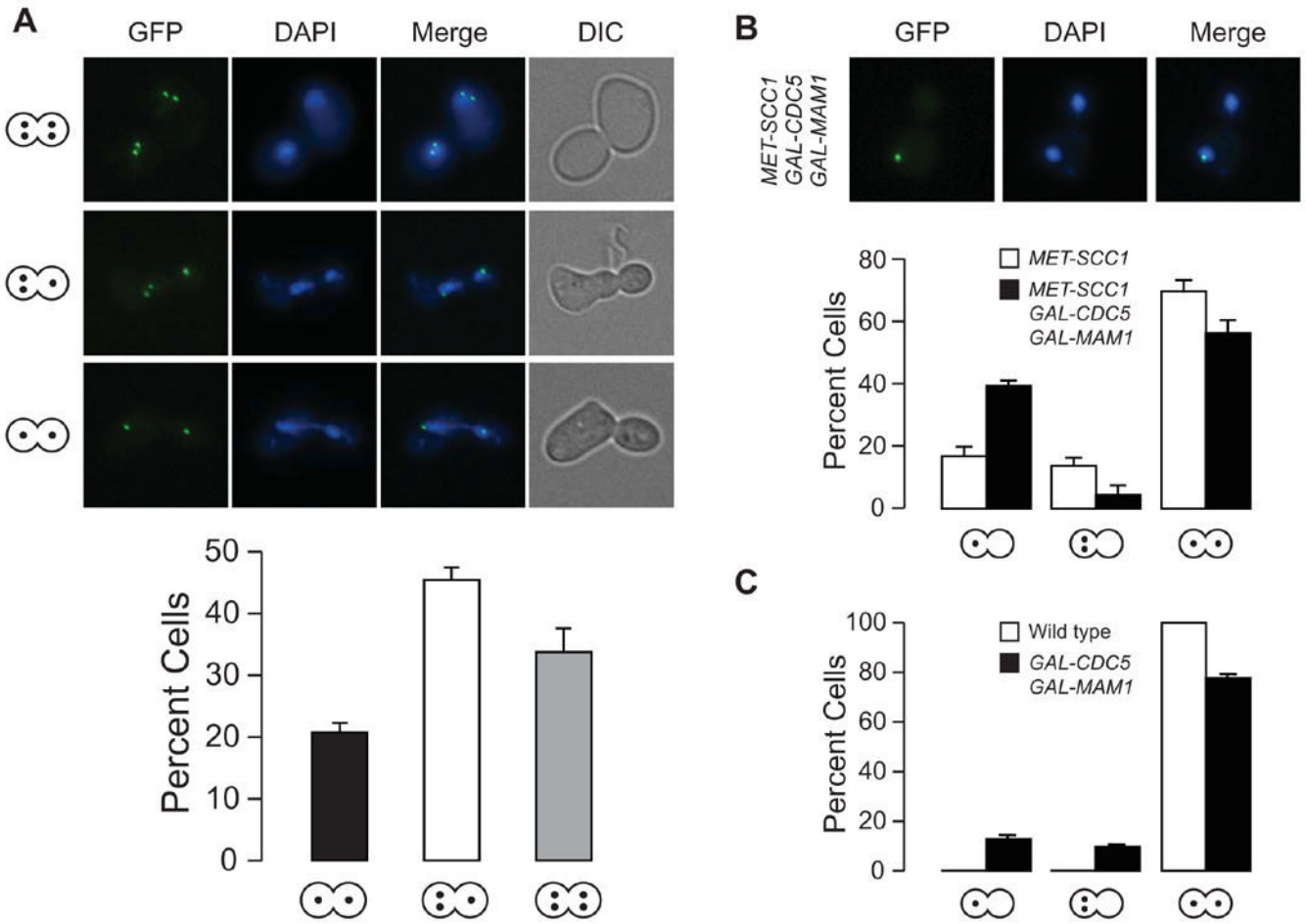

Figure 6. The monopolin complex fuses sister kinetochores together

(A) Haploid wild-type cells carrying GFP dots on both chromosome IV and V (A15978) were arrested in G1 using 5μg/ml α factor. Galactose was added 1 hour prior to release. When arrest was complete cells were released into medium containing galactose but lacking pheromone. The presence of one or two GFP dots in each nuclear lobe was determined in anaphase cells.

(B) Haploid *MET-SCC1* (A16486) and *GAL-CDC5 GAL-MAM1 MET-SCC1* (A16023) cells carrying CENV GFP dots were arrested in G1 in media lacking methionine using 5 μg/ml α factor, preinduced with 2% galactose and 8 mM methionine for 1 h, and released into YEP medium lacking pheromone and containing 2% galactose and 8 mM methionine at 25ºC. Cells were analyzed as in (A).

(C) Wild type (A5237) and *GAL-CDC5 GAL-MAM1* (A16883) cells carrying TELV GFP dots, were grown as described in Figure 4A to determine GFP dot segregation. In this set of strains only 25% of cells co-segregated sister chromatids. The reasons for the lower levels of co-segregation in this strain are unclear.

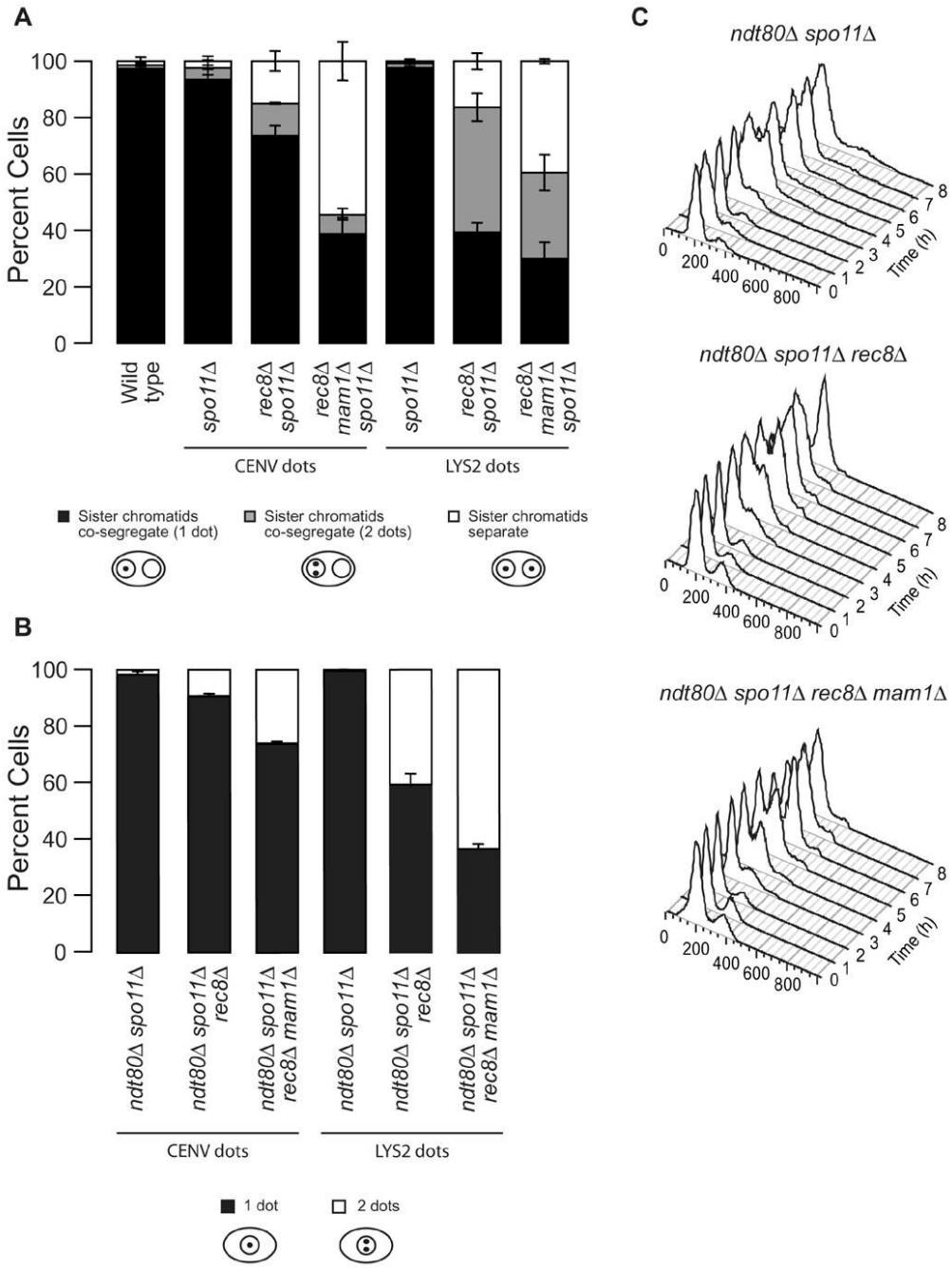

Figure 7. The co-segregation of sister chromatids observed in *REC8*Δ cells depends in part on *MAM1*

(A) Wild type (A5811), *spo11* (A9498), *rec8*Δ *spo11* Δ (A16020) and *rec8* Δ *mam1* Δ *spo11* Δ (A16342) cells carrying heterozygous CENV GFP dots, and *spo11* Δ (A16725) *rec8* Δ *spo11*Δ (A16838) and *rec8*Δ *mam1*Δ *spo11*Δ (A16839) all carrying heterozygous LYS2 GFP dots, were induced to sporulate to examine the distribution of GFP dots in binucleate cells 6 hours after induction. Strains A16838 and A16839 were analyzed after 8 hours. A later time point was chosen for the LYS2 GFP dot strains to allow for a more complete segregation of chromosome arms away from the midzone. (n=100 cells for A16838 and n=150 cells for A16839, n=200 cells for all other strains).

(B, C) *spo11*Δ *ndt80* Δ (A16840) *rec8*Δ *spo11*Δ *ndt80*Δ (A16841) and *rec8* Δ *mam1*Δ *spo11*Δ *ndt80*Δ (A16842) cells all carrying heterozygous CENV GFP dotsΔ and *spo11*Δ *ndt80*Δ (A16835), *rec8*Δ *spo11*Δ *ndt80*Δ (A16836) and *rec8*Δ *mam1*Δ *spo11*Δ *ndt80*Δ (A16837) cells all carrying heterozygous LYS2 GFP dots, were induced to sporulate to examine DNA content (C; CENV dot strains are shown) and the association of GFP dots (B; 6h time point was analyzed).