A Novel Destruction Sequence Targets the Meiotic Regulator Spo13 for Anaphase-promoting Complex-dependent Degradation in Anaphase I*1

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The anaphase-promoting complex (APC) or cyclosome is a multisubunit ubiquitin-protein ligase that ubiquitiniates and thereby promotes the destruction of the mitotic cyclins and the separase inhibitor, securin. The contributions of the APC to progression through the meiotic program are not clear. To clarify the function of the APC in meiosis, we screened several yeast meiotic proteins as APC substrates in vitro. We found that the meiotic regulator Spo13 is an APC substrate that is degraded during anaphase I. Spo13 is expressed only in meiotic cells, where it has multiple functions, including the promotion of monopolar chromosome attachment in the first division. Spo13 ubiquitination by the APC depends on an LxExxN sequence (residues 26–32) that is distinct from previously described destruction sequences of APC substrates. Mutation of one residue, leucine 26, prevented Spo13 ubiquitination by the APC in vitro and stabilized the protein through the meiotic divisions. Analysis of meiotic progression and spore viability of yeast containing the stabilized Spo13 mutant revealed no significant defects, indicating that Spo13 destruction in anaphase I is not essential for meiosis. We propose that Spo13 destruction is one of multiple mechanisms underlying the switch from monopolar to bipolar chromosome attachment between the meiotic divisions.

The anaphase-promoting complex (APC) 2 catalyzes the final step in the ubiquitination of several mitotic proteins, thereby targeting them for destruction in the proteasome (1, 2). The APC has many targets, but its critical substrates are securin, whose destruction triggers sister-chromatid segregation, and the mitotic cyclins, whose destruction leads to the inactivation of cyclin-dependent kinases and the exit from mitosis (3). APC activation in mitosis depends on its sequential association with the activator subunits Cdc20 and Cdh1 (1, 2, 4), which are thought to recruit substrates to the enzyme for ubiquitination. APC activity is first stimulated in early mitosis by association with Cdc20, and the resulting APC-Cdc20 complex targets securin and cyclins for degradation, thereby initiating anaphase. Later in mitosis, the APC associates with Cdh1, resulting in stable APC-Cdh1 activity throughout the following G1.

APC substrates contain amino acid sequence motifs that are required for their ubiquitination and are thought to interact with activator subunits and other sites on the enzyme (5). The most commonly found sequence is the destruction-box (D-box), a highly degenerate motif based on the sequence RxxLxxxN (6, 7). A second common motif is the KEN-box (KENxxxN) (8). In a few rare cases, APC target destruction depends on sequences that are quite distinct from D- and KEN-boxes (9–12). The substrate features that allow recognition by the APC remain poorly understood, and our knowledge of APC-substrate interactions would benefit from a more extensive list of APC substrates.

In addition to its functions in the mitotic cell cycle, the APC is required during meiosis, a specialized form of nuclear division in which a single round of chromosome duplication is followed by two rounds of chromosome segregation (meiosis I and meiosis II). Budding yeast carrying mutations in essential subunits of the APC arrest in metaphase I (13, 14); the APC is also required for spore wall development after meiosis (15). As in mitosis, progression through the two meiotic divisions requires APC-Cdc20-mediated destruction of securin and cyclins (13, 16). In addition, meiotic progression in yeast depends on a meiosis-specific APC activator, Ama1, whose function remains unclear. Cells lacking ama1 arrest after the second meiotic division with elongated spindles and fail to develop spore walls (13, 14, 17). APC-Ama1 also contributes to substrate degradation in anaphase I, as ama1Δ cells do not efficiently degrade securin or the cyclins (13). The APC activator Cdh1 does not seem to contribute significantly to progression through the meiotic divisions.

It is not known if the APC helps govern the meiosis-specific processes that lead to segregation of homologs in the first division and sister chromatids in the second (18). In the first division, both sister chromatids in each homolog must be attached to the same spindle pole (monopolar orientation, in contrast to the bi-orientation of mitosis). Monopolar orientation depends on kinetochore proteins called monopolins, which are released from the kinetochores after the first division, allowing sisters to bi-orient on the second meiotic spindle (19, 20). Another key

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.
2 The abbreviations used are: APC, anaphase-promoting complex; NEM, N-ethylmaleimide; HA, hemagglutinin; DAPI, 4',6-diamidino-2-phenylindole; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein.
feature of the first division is that cohesion between sister chromatids is lost along chromosome arms but protected at the centromeres by the protein Sgo1 (21, 22), so that sister chromatids remain linked for successful segregation in the second division. The mechanisms that trigger the loss of monopolins and the deprotection of centromere cohesion remain unclear, but both occur during anaphase I and one intriguing possibility is that the APC is involved.

To address the possibility of additional APC functions in meiosis, we performed a screen for meiotic substrates of the APC in budding yeast. We identified the meiotic regulator Spo13 as an APC target that is degraded during anaphase I. Intriguingly, Spo13 helps promote monopolar chromosome segregation. The mechanisms that trigger the loss of monopolins and the deprotection of centromere cohesion remain unclear, but both occur during anaphase I and one intriguing possibility is that the APC is involved.

**EXPERIMENTAL PROCEDURES**

**General Yeast Methods**—All strains were based on the W303 background. For galactose-inducible SPO13 expression, the SPO13 coding sequence was inserted in recombination in either a low copy CEN/ARS plasmid (pRS314.1234) or high copy 2-micron plasmid (pAB1234) in frame with a C-terminal TAP tag, under the control of the GAL1–10 promoter. The APC bypass strain (ape2Δ pds1Δ clb5Δ sac1Δ) was a kind gift of David Toczyski (3).

**Measurement of Protein Stability in Vivo**—W303 MAT a yeast, carrying low copy plasmids with galactose-inducible SPO13-TAP or spo13-db-TAP, were arrested in raffinose (2%) media supplemented with either α factor (G1) or nocodazole (M) for 3 h. Once arrested, galactose (2%) was added to the media to induce production of Spo13-TAP or Spo13-db-TAP. After 2 h, dextrose (2%), and cycloheximide (10 μg/ml) were added to simultaneously repress transcription and translation. Samples were withdrawn at the indicated times, protein extracts prepared, and immunoblot analysis performed to assess protein stability.

**APC Reactions**—The desired genes, or fragments of the SPO13 gene (Fig. 2, A–C), were amplified by PCR, using primers that add a T7 promoter upstream of the protein-coding sequence. Substrates were then produced in rabbit reticulocyte lysates (Promega) by coupled transcription and translation in the presence of [35S]methionine, as described previously (25, 26). Alternatively, plasmids containing the SPO13-TAP or the spo13-(L26A)-TAP gene (Figs. 1A and 2D and F) were used as templates for translation in vitro. Following translation, 10 mM N-ethylmaleimide (NEM) was added to translation mixtures to inactivate ubiquitin chain-forming activities that otherwise extend the products of subsequent APC reactions. 10 mM dithiothreitol was then added to inactivate NEM. As described previously (25, 26), substrates (in NEM-inactivated reticulocyte lysates) were mixed with ubiquitin, ATP, E1, E2, APC, and baculovirus-derived Cdh1 and incubated for 45 min at room temperature unless otherwise indicated. In some experiments (Fig. 2F), Cdh1 and Cdc20 were produced by translation in vitro, and the lysate used directly in the APC reactions; NEM was not added in these experiments because it inactivates Cdc20.

**Mitotic Experiments**—To determine the effect of SPO13 overexpression in mitotic cells, W303 MAT a yeast (with plasmids carrying galactose-inducible SPO13-TAP or spo13-db-TAP) were arrested in raffinose (2%) media supplemented with a factor (1 μg ml−1) at 30 °C for 2 h. Galactose was added (2%) for 1 h, and cells were then released into fresh raffinose/galactose media.

**Meiotic Experiments**—All meiotic experiments were performed in the SK1 budding yeast strain. To introduce the spo13-L26A mutation, the SPO13 gene, including promoter, was subcloned into the Yplac211 vector, and site-directed mutagenesis was used to generate the L26A mutant. Digestion with Pst1 was used to integrate at the SPO13 locus, and the vector was selected against on 5-fluorooorotic acid media. PCR and sequencing were used to confirm that the L26A mutation was retained at the SPO13 locus. Diploid yeast were induced to undergo meiosis by the following protocol. Diploids were grown overnight in YPD media (yeast peptone + 2% dextrose) to saturation (A600 = 4–5). The following day, the yeast were diluted (A = 0.2) into YPA media (YP media + 2% acetate) and grown for a further 16 h. The yeast were washed in water and released into SPO media (2% acetate + 0.02% raffinose) at A = 1.8, and samples were processed for immunoblot analysis or immunofluorescence. The antibodies used were α-tubulin clone YOL1/34 (Serotec), α-HA clone 16B12 (Babco), and PAP (Sigma).

**RESULTS**

**Identification of Spo13 as an APC Substrate**—To identify additional APC functions in meiosis, we performed a screen for novel substrates. A list of candidate substrates was compiled from meiosis-specific transcripts (27) that encode proteins containing either of the two well described APC consensus recognition motifs, the D-box or KEN-box. Additional substrates with known involvement in the meiotic divisions were also included. A total of 31 candidates (supplemental Table 1) were analyzed by generating PCR products encoding the meiotic gene downstream of the T7 promoter. Radiolabeled substrates were prepared by coupled transcription and translation in vitro and then incubated with purified APC, Cdh1, ubiquitin, ATP, E1, and E2. The reaction products were analyzed by SDS-PAGE gel electrophoresis for incorporation of ubiquitin (25).

As a positive control, we used the budding yeast securin, Pds1, a known APC substrate in both mitosis and meiosis (16, 28). Two of the 31 candidates, Spol13 and Ndt80, were detectably ubiquitinated. The best substrate was Spol13, which was ubiquitiniated to an extent comparable to that seen with Pds1 (Fig. 1A). Incorporation of ubiquitin in Spol13 depended on both the APC and the activator Cdh1 (data not shown). A second protein, the meiotic transcription factor Ndt80, was ubiquitiniated poorly (data not shown). We therefore focused our efforts on Spol13.
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FIGURE 1. Spo13 is an APC substrate that disappears in anaphase I of the meiotic program. A, Spo13 is a substrate of the APC in vitro. Plasmids encoding Pds1 and Spo13-TAP, and a PCR product encoding Mam1, were transcribed and translated in vitro in the presence of [35S]methionine and mixed with APC, Cdh1, ubiquitin, ATP, E1, and E2 (+), or without APC and Cdh1 (−). Reaction products were analyzed by gel electrophoresis and autoradiography. B, Spo13 is less stable in G1 cells than in M phase cells. Cells were arrested by treatment with α factor (α), or nocodazole (noc), and Spo13-TAP expression was induced by galactose addition to the media. After 2 h, transcription and translation were stopped by addition of dextrose and cycloheximide. Samples were taken at the indicated times and the amount of Spo13 assessed by immunoblot. C, localization of Spo13-HA in meiotic cells. Diploid SK1 yeast cells, and over 100 cells for each stage were analyzed. Representative images are shown.

The remaining 29 candidate proteins displayed no detectable incorporation of ubiquitin by the APC. The monopolin component Mam1 is shown as a representative example in Fig. 1A. This may indicate that these proteins are not APC substrates, but it is also possible that some of these proteins are APC targets whose ubiquitination is difficult to reconstitute in vitro for technical reasons.

In budding yeast, the APC is active in G1 cells but is inactive in cells arrested in M phase (1). Therefore, meiotic APC substrates ectopically expressed in mitotic cells should be unstable in G1 and stable in M phase. Spo13 was expressed from the GAL1–10 promoter in budding yeast cells, which were arrested in either α factor (G1) or nocodazole (M phase). After expression for 2 h, transcription and translation were simultaneously repressed by addition of both dextrose and cycloheximide, and immunoblotting was used to assess protein levels at various times thereafter. Spo13 was more unstable in G1-arrested cells than in M-phase arrested cells, consistent with it being an APC substrate (Fig. 1B).

Disappearance of Spo13 in Anaphase I—We examined the status of Spo13 in meiotic cells (Fig. 1C). Consistent with previous observations, Spo13 was present in metaphase I cells but specifically reduced in anaphase I cells (23), when the APC is active. Unlike securin (16), Spo13 did not re-accumulate in metaphase II cells or thereafter.

Identification of a Novel APC Destruction Sequence in Spo13—Spo13 is one of the proteins we screened that contains no clearly identifiable D-box and KEN-box. To identify the regions of Spo13 important for APC-mediated destruction, we generated a number of fragments of Spo13 by PCR, translated them in vitro, and subjected them to APC reactions. A fragment of the Hsl1 protein was used as a positive control (29). We found that the APC destruction sequence of Spo13 lies in the N terminus (Fig. 2A), as all the N-terminal fragments were efficiently ubiquitinated, while a C-terminal fragment (residues 104–292) was not. The first 15 amino acids of Spo13 contain a sequence (RxxL) that is reminiscent of a D-box. However, fragments lacking this region (residues 15–195 and 1–(Δ6–9)–195) were still ubiquitinated efficiently (Fig. 2B). A fragment lacking the first 30 amino acids of Spo13 (residues 30–195) was resistant to APC-mediated ubiquitination, strongly implicating amino acids 16–30 as the critical residues (Fig. 2B). Analysis of 3-residue truncations through this region revealed that while a fragment of amino acids 24–195 was ubiquitinated by the APC, a fragment of amino acids 27–195 was not, suggesting that residues 25–27 are required for the APC-mediated ubiquitination of Spo13 (Fig. 2B).

The amino acid sequence of this region of Spo13 does not closely resemble any previously described APC destruction motif. To identify the destruction sequence of Spo13, we generated single point mutations (to alanine) in amino acids 24–32 in PCR fragments spanning residues 1–195 of the Spo13 protein. Mutation of three residues inhibited APC activity toward Spo13 (Fig. 2C). Two amino acids, glutamate 28 and asparagine 32, were partly required for full activity. The most critical residue was the leucine at position 26, which was required for the appearance of ubiquitinlated products.

To confirm the importance of this residue, we performed a time course in vitro using either full-length Spo13 or Spo13-L26A (hereafter referred to as Spo13-db). While high molecular weight ubiquitination products and substrate depletion were seen rapidly with wild-type Spo13, neither was observed with Spo13-db, even after 1 h of reaction (Fig. 2D). Thus, ubiquitination of Spo13 in vitro can be largely prevented by a single point mutation of leucine 26. Three residues (Leu26, Glu28, and Asn32) are therefore the key determinants of APC ubiquitination of Spo13, suggesting that the destruction sequence is LxExxxN (Fig. 2E).

APCCdc20 Ubiquitination of Spo13—The APC activator used in these experiments, Cdc1, is not thought to contribute significantly to budding yeast meiosis (13, 14), suggesting that Spo13 is targeted in vivo by the APC in combination with one or both of the other activators, Cdc20 and Ama1. We found that cells...
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To further investigate the possibility that Spo13-induced toxicity is due to inhibition of the APC, we analyzed the effects of Spo13 in a yeast strain engineered to survive in the absence of APC function (23). Consistent with this possibility, overexpression of Spo13 in mitotic cells is known to delay the onset of anaphase and reduce cell viability (30, 31). Our evidence that Spo13 is an APC substrate raised the possibility that Spo13 overexpression blocks mitotic progression and causes lethality by competitively inhibiting substrate binding to the APC. However, we found that overexpression of the spo13-db mutant, which presumably interacts poorly with the APC, caused a delay in anaphase onset and mitotic exit that was greater than the delay induced by overexpression of wild-type Spo13 (Fig. 3B). These results argue that Spo13 is not delaying anaphase onset and causing cell death by binding the APC but through some other mechanism.

To further investigate the possibility that Spo13-induced toxicity is due to inhibition of the APC, the products of Spo13 in a yeast strain engineered to survive in the absence of APC function (3). Overexpression of Spo13 in this strain was lacking Ama1 efficiently degrade Spo13 in anaphase I (data not shown), suggesting that Cdc20 is the relevant activator. However, as meiotic cells lacking Cdc20 do not progress into anaphase I, we could not assess the importance of Cdc20 in Spo13 destruction in vivo. We therefore tested Spo13 ubiquitination by APC in vitro. Active Cdc20 cannot be produced in insect cells or bacteria, but small amounts can be prepared by translation in rabbit reticulocyte lysates. Using this approach, we found that APC was an effective catalyst of Spo13 ubiquitination and that this activity was greatly reduced when Spo13-db was used as substrate (Fig. 2F). These results support the possibility that APC promotes Spo13 degradation in anaphase I.

Analysis of the spo13-db Mutant in Mitotic Cells—To demonstrate that the L26A mutation stabilizes the protein by preventing its degradation in vivo, we expressed either wild-type SPO13 or spo13-db from the GAL1-10 promoter in cells arrested in G1 phase. After induction for 2 h, transcription and translation were simultaneously repressed with cycloheximide and dextrose, and protein stability was monitored over the next hour. While Spo13 was degraded over the time course of the assay, the Spo13-db protein was fully stabilized (Fig. 3A). Thus, the Spo13-db mutant is resistant to APC-mediated ubiquitination both in vitro and in vivo.

Previous work suggests that Spo13 may act as an APC inhibitor (23). Consistent with this possibility, overexpression of Spo13 in mitotic cells is known to delay the onset of anaphase and reduce cell viability (30, 31). Our evidence that Spo13 is an APC substrate raised the possibility that Spo13 overexpression blocks mitotic progression and causes lethality by competitively inhibiting substrate binding to the APC. However, we found that overexpression of the Spo13-db mutant, which presumably interacts poorly with the APC, caused a delay in anaphase onset and mitotic exit that was greater than the delay induced by overexpression of wild-type Spo13 (Fig. 3B). These results argue that Spo13 is not delaying anaphase onset and causing cell death by binding the APC but through some other mechanism.

To further investigate the possibility that Spo13-induced toxicity is due to inhibition of the APC, the products of Spo13 in a yeast strain engineered to survive in the absence of APC function (3). Overexpression of Spo13 in this strain was

and Asn contribute to ubiquitination of Spo13 by the APC. PCR products encoding fragments of Spo13-(1-195) and containing single point mutations (to alanine) were analyzed as in A, D, Spo13-db (L26A) is resistant to ubiquitination by the APC. Plasmids encoding either Spo13-TAP or Spo13-db (L26A)-TAP were transcribed and translated in vitro in the presence of [35S]methionine. In separate reactions, plasmids encoding either Cdh1 or Cdc20 were transcribed and translated in vitro in the presence of [35S]methionine and mixed with APC, Cdh1, ubiquitin, ATP, E1, and E2. Samples were withdrawn at the indicated time points and analyzed by gel electrophoresis and autoradiography. E, the APC recognition sequence of Spo13. F, Spo13 is ubiquitinated by Cdc20-dependent APC activity. Plasmids encoding either Pds1, Spo13-TAP, or Spo13-db-TAP were transcribed and translated in vitro in the presence of [35S]methionine. In separate reactions, plasmids encoding either Cdh1 or Cdc20 were transcribed and translated in vitro in the absence of radioactive label. Each radiolabeled substrate was then incubated alone (control reactions, c) or incubated with APC, ubiquitin, ATP, E1, and E2. As described under "Experimental Procedures," the products of these reactions are longer than those in other experiments because NEM was added in other experiments to inactivate non-specific ubiquitin chain-extending activities in translation mixtures.
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A Spo13-TAP Spo13-db-TAP time (min) 0 5 10 20 40 60

B % budded cells % bi-nucleate cells Time (min) 0 120 240 60

C Dextrase Galactose

FIGURE 3. Analysis of Spo13-db (L26A) in mitosis. A, Spo13-db (L26A) is stable in G1 phase. Cells were arrested in a factor (G1), and Spo13-TAP or Spo13-db-TAP production was induced by galactose addition to the media. After 2h, transcription and translation were stopped by addition of dextrose and cycloheximide. Samples were taken at the indicated times and the amount of Spo13 assessed by immunoblot. B, overexpression of Spo13-db interferes with mitotic progression. Cells carrying low copy plasmids with Spo13-TAP, spo13-db-TAP, or no insert (control) under the control of the GAL1–10 promoter were arrested in G1 with a factor, and galactose was added to the media. After 1h, cells were released from the arrest into fresh raffinose/galactose media, and a factor was added back after 100 min to arrest cells in the subsequent G2 phase. Cells were sampled at the indicated times for analysis of budding index (left) and chromosome segregation by DNA staining with DAPI (right). 100 cells were analyzed for each time point. C, Spo13 overexpression is toxic in the absence of APC activity. High copy plasmids with no insert or Spo13-TAP under the control of the GAL1–10 promoter were transformed into wild-type cells (wt) or cells engineered to be viable despite the deletion of the essential APC subunit Apc2 (apcΔ). Cells were plated on dextrose (left) or galactose media (right) and grown at 25°C for 3 days.

The L26A mutation did not lead to a loss of Spo13 function in meiosis. 95% of spo13-db yeast formed tetrads after sporulation, compared with 97% of Spo13 cells and only 7% of cells carrying a deletion in the Spo13 gene.

To assess the phenotype of the L26A mutation in more detail, we compared meiotic progression in Spo13 and spo13-db cells. Progression through the two chromosome divisions was very similar in the two strains, as similar accumulation of 2n and 4n DNA species was seen (Fig. 4C). Thus, the continued presence of Spo13 during and after anaphase I did not have a profound effect on meiotic progression. We also dissected tetrads to assess viability. Wild-type yeast were 98% viable and spo13-db yeast were 97% viable, indicating no loss of viability from the continued presence of Spo13 in late stages of meiosis.

DISCUSSION

The APC is necessary for meiotic progression, as yeast with reduced APC function arrest in meiotic metaphase I with unsegregated chromosomes (13, 14). Other than this observation, and the characterization of yeast lacking the AMA1 gene (17), little is known about how the APC contributes to the unique regulatory features of the meiotic program. Our obser-
vation that Spo13 is an APC substrate in anaphase I demonstrates that the APC does have functions in the control of meiosis-specific processes.

The primary sequence of Spo13 contains no close matches to either of the well described APC recognition sequences, the D- or KEN-boxes (6, 8), and we identified a novel destruction sequence, LxExxxN, that is required for its ubiquitinization by the APC in vitro and its degradation in vivo. The amino acid requirement of the Spo13 destruction box sequence is distinct from any other previously described APC substrate destruction box sequences, although it resembles slightly the end of the D-box sequence (RxRxLxExxN). Spo13 could not have been predicted to be an APC substrate solely on the basis of its sequence. We suspect that there are a number of degenerate sequences that can act as destruction motifs, and the Spo13 sequence is one such variant. Clearly, more analysis of the sequence requirements of D- and KEN-boxes is needed before we can identify novel APC substrates based on their primary sequence alone.

Sister-chromatid pairs make monopolar attachments in meiosis I and switch to bipolar attachments in meiosis II. A failure to switch attachment modes from monopolar to bipolar would be catastrophic for high fidelity chromosome segregation. The regulatory molecules that mediate the switch from monopolar to bipolar attachment are currently unknown, but the APC is an excellent candidate because it is activated just before the monopolin complex is lost from chromosomes in anaphase I (16). Spo13 is a key regulator of monopolar chromosome attachment, as spo13Δ cells fail to load adequate amounts of the monopolin complex and make mostly bipolar attachments at metaphase I (23, 24). How Spo13 specifically promotes monopolin loading is unknown, but our observations that Spo13 is an APC substrate suggested that degradation of Spo13 allows the unloading of the monopolin complex in anaphase I. However, spores from cells containing the stabilized spo13-db mutant were fully viable, demonstrating that degradation of Spo13 alone is not the only mechanism to ensure unloading of monopolin in anaphase I. How else might the APC contribute to the chromosome attachment switch? We could find no evidence from either our in vivo or in vitro assays that the known subunits of the monopolin complex (Mam1, Lrs4, and Csm1) (19, 20) are APC substrates (data not shown). At least two other regulators of monopolar chromosome attachment, the protein kinase Cdc5 (32, 33) and the Cdc7 kinase-associated protein Dbf4 (34), are known APC substrates in mitosis (35, 36). We introduced stabilized mutant versions of these proteins into spo13-db cells to test if their degradation contributes to loss of monopolin. However, the triple mutant (spo13-db, cdc5-db, dbf4-db) displayed normal meiotic progression and spore viability (data not shown). Furthermore, in anaphase I, both the Mam1 and Lrs4 subunits of the monopolin complex efficiently disappeared from chromosomes in the triple mutant, similar to wild-type cells (data not shown). Thus, additional mechanisms must exist to drive the down-regulation of monopolin chromosome attachment between the meiotic divisions.

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