Phosphorylation State Defines Discrete Roles for Monopolin in Chromosome Attachment and Spindle Elongation

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Summary

Background: It is unknown how oscillations in Cdk1 activity drive the dramatic changes in chromosome and spindle dynamics that occur at the metaphase/anaphase transition.
Results: We show that the *Schizosaccharomyces pombe* monopolin complex has distinct functions in metaphase and anaphase that are determined by the phosphorylation state of its Mde4 subunit. When Cdk1 activity is high in metaphase, Mde4 is hyperphosphorylated on Cdk1 phosphorylation sites and localizes to kinetochores. A nonphosphorylatable mutant of Mde4 does not localize to kinetochores, appears prematurely on the metaphase spindle, and interferes with spindle dynamics and chromosome segregation, illustrating the importance of Cdk1 phosphorylation in regulating metaphase monopolin activity. When Cdk1 activity drops in anaphase, dephosphorylation of Mde4 triggers monopolin localization to the mitotic spindle, where it promotes spindle elongation and integrity, coupling the late mitotic loss of Cdk1 activity to anaphase spindle dynamics.
Conclusions: Together, these findings illustrate how the sequential phosphorylation and dephosphorylation of monopolin helps ensure the orderly execution of discrete steps in mitosis.

Introduction

For equal segregation of chromosomes to each daughter cell, sister kinetochores must attach to microtubules emanating from opposite poles at metaphase in a bioriented fashion. Most defects in attachment of chromosomes to spindle microtubules trigger the spindle assembly checkpoint, which halts cell-cycle progression and promotes correction of attachment defects [1]. However, merotelic attachments, in which a single kinetochore binds to microtubules from both poles, cannot be sensed by the spindle assembly checkpoint because the requirements for both attachment and tension at kinetochores

are satisfied [2]. Thus, cells with merotelically attached chromosomes proceed into anaphase but develop lagging chromosomes, which often do not segregate properly. Merotelically attached lagging chromosomes are the most common cause of aneuploidy in cultured mammalian cells [3].

Unlike the budding yeast *Saccharomyces cerevisiae* [4], fission yeast and mammalian kinetochores each bind multiple microtubules (2–4 microtubules in the case of fission yeast) [5]. Because fission yeast kinetochores can bind multiple microtubules, mechanisms are required to prevent merotelic attachments. A recent study showed that both centromeric heterochromatin and a protein complex called monopolin are required to prevent merotelic attachments in the fission yeast *Schizosaccharomyces pombe* [6]. The monopolin complex was first identified in *S. cerevisiae*, where it is required during meiosis I to orient duplicated sister kinetochores to the same pole [7–9]. The *S. pombe* monopolin complex consists of the Pcs1 and Mde4 proteins, which localize to the central core of centromeres [6]. Although the fission yeast monopolin complex is not required for mono-orientation of sister chromatids during meiosis I, it is required during mitosis and meiosis II to prevent lagging chromosomes caused by merotelic attachments [6]. It has been proposed that monopolin prevents merotelic attachments by acting as a clamp to align microtubule-binding sites together at kinetochores [6, 7, 10]. How monopolin function is regulated during mitosis in *S. pombe* remains unclear.

The evolutionarily conserved Cdc14 phosphatase is known to dephosphorylate Cdk1 substrates. The *S. pombe* Cdc14-like phosphatase Clp1/Flp1 (hereafter referred to as Clp1) carries out multiple functions in mitosis [11–14]. We have shown previously that Clp1 localizes to kinetochores at metaphase and plays a role in chromosome segregation [15]; however, substrates of Clp1 at kinetochores have not been identified. Here we report that the monopolin subunit Mde4 is a substrate of Clp1. We show that phosphorylation of Mde4 on Cdk1 sites keeps monopolin on kinetochores by preventing its premature localization to the mitotic spindle. Dephosphorylation of Mde4 in anaphase is required for monopolin localization to the mitotic spindle, where it promotes spindle stability and normal spindle elongation. Thus, both phosphorylation and dephosphorylation of Mde4 are important for proper chromosome segregation.

Results

Mde4 Is Phosphorylated during Mitosis and Interacts with the Cdc14-like Phosphatase Clp1

Previous studies have found that *clp1Δ* mutants show chromosome segregation defects, implying that Clp1 substrates regulate chromosome segregation [15]. To identify such substrates, we purified protein complexes containing Clp1 and interacting proteins from mitotic cells with the substrate-trapping mutant of Clp1, Clp1-C286S, which is catalytically inactive but binds more strongly to phosphorylated substrates than wild-type Clp1 does [15, 16]. Tandem affinity purification [17] was used to isolate Clp1-C286S protein complexes from metaphase-arrested cells. Protein complexes were then analyzed by tandem mass spectrometry. Numerous proteins that copurified with Clp1 were identified (data not shown), including the

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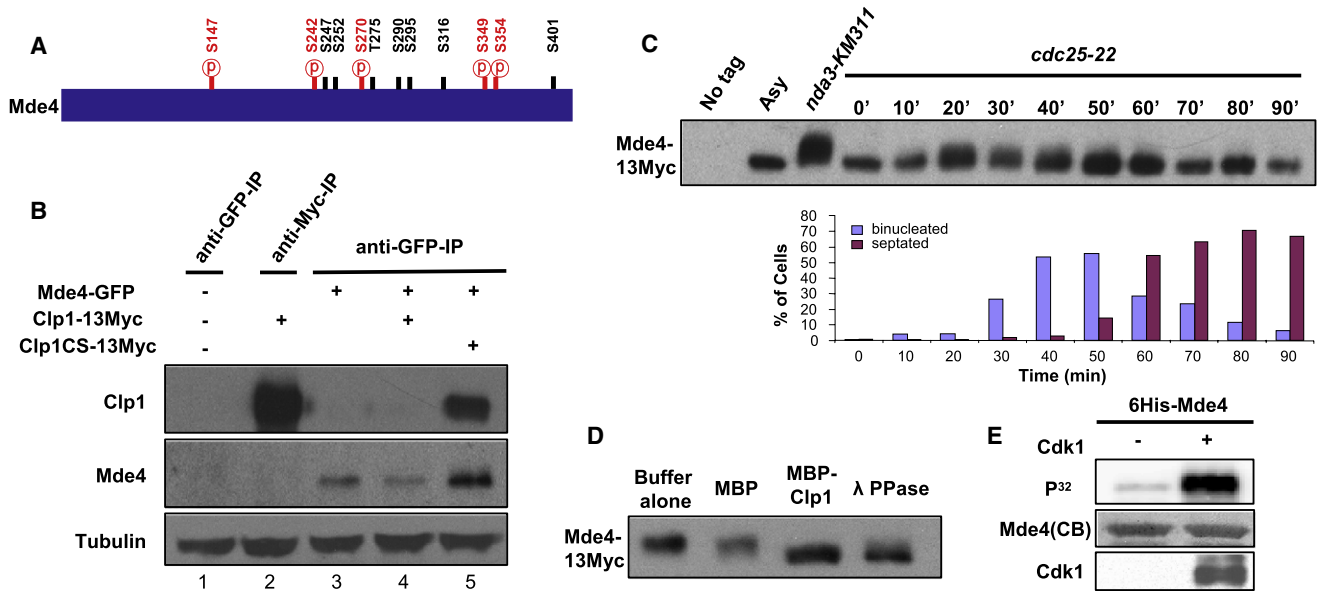
Figure 1. Mde4 Is Phosphorylated during Mitosis and Interacts with Clp1

(A) Schematic representation of Mde4 depicting twelve consensus Cdk1 phosphorylation sites. Five of the twelve predicted phosphorylation sites (indicated with red "p"s) were identified by mass spectrometry analysis of Clp1-C286S-TAP purified from cells arrested at metaphase with the *nda3-KM311* and *mts3-1* mutants and from cells 60 min after release from *cdc25-22* arrest.

(B) Interaction between Mde4 and Clp1 was determined by immunoprecipitation followed by western blotting with cell lysates from the following asynchronous cultures: wild-type (1), *clp1-13Myc* (2), *mde4-GFP* (3), *mde4-GFP clp1-13Myc* (4), and *mde4-GFP clp1-C286S-13Myc* (5). The bottom row shows the tubulin loading control from whole-cell extracts prior to immunoprecipitation.

(C) Cell-cycle-dependent change in Mde4 phosphorylation was determined by analyzing the gel migration of Mde4-13Myc. *cdc25-22 mde4-13Myc* cells were arrested at the restrictive temperature of 36°C for 4 hr to synchronize them in G2 phase and were then shifted to the permissive temperature of 25°C. Samples were taken at the indicated time points, and the migration shift of Mde4-13Myc (upper panel) and cell-cycle progression (lower panel) were determined by western blot analysis and microscopy, respectively. Lysates from wild-type cells (No tag), asynchronous *mde4-13Myc* cells (Asy), and *mde4-13Myc* cells arrested in mitosis via the *nda3-KM311* mutation (*nda3-KM311*) are also shown.

(D) Immunoprecipitated Mde4-13Myc from *mde4-13Myc clp1Δ* cells arrested in metaphase via the *nda3-KM311* mutation was treated with buffer alone, recombinant maltose binding protein (MBP), MBP-Clp1, or lambda phosphatase (λ PPase) and then analyzed by western blotting.

(E) In vitro kinase assays were performed with Cdk1 immunoprecipitated via Cdc13 (*S. pombe* cyclin B) antibodies from metaphase-arrested *nda3-KM311* cells with bacterially expressed 6His-Mde4 as substrate. γ-^{32}P-labeled protein was detected with a PhosphorImager (Molecular Dynamics), and the gel was stained with Coomassie blue (CB) as a loading control. The level of Cdk1 was determined by western blotting.

S. pombe monopolin proteins, Pcs1 and Mde4. Mde4 has twelve potential Cdk1 phosphorylation sites (SP/TP motifs), five of which were identified as phosphorylated by mass spectrometry (Figure 1A). However, sequence coverage of Mde4 from the mass spectrometry analysis was only 30%, raising the possibility that the other seven Cdk1 sites might be phosphorylated as well. Pcs1 has one potential Cdk1 site, but it was not identified as phosphorylated in vivo (data not shown). To confirm the apparent interaction between monopolin and Clp1 identified via mass spectrometry, we carried out coimmunoprecipitation and western blotting experiments. Mde4-GFP coimmunoprecipitated with the substrate-trapping mutant, Clp1-C286S-13Myc, but not with wild-type Clp1-13Myc (Figure 1B), suggesting that the interaction between Clp1-C286S and Mde4 is mediated by phosphorylation on Mde4. Because Clp1 dephosphorylates sites phosphorylated by Cdk1, whose activity peaks in early mitosis, we investigated whether Mde4 is phosphorylated during mitosis. In synchronous cultures, slower-migrating forms of Mde4-13Myc appeared during early mitosis, decreased during late mitosis, and then disappeared after mitotic exit (Figure 1C). To confirm that the shift was caused by phosphorylation, Mde4-13Myc isolated from metaphase-arrested cells was treated with recombinant Clp1 purified from *E. coli*. Treatment with Clp1 eliminated the migration shift of Mde4-13Myc (Figure 1D),

confirming that Mde4 is phosphorylated in vivo and that Clp1 can dephosphorylate Mde4 in vitro. Moreover, Mde4 purified from *E. coli* could be efficiently phosphorylated by Cdk1 isolated from metaphase-arrested cells, showing that Mde4 can be phosphorylated by Cdk1 in vitro (Figure 1E). To examine the contribution of Clp1 to Mde4 dephosphorylation in vivo, we synchronized wild-type and *clp1Δ* cells in metaphase and then released them from the arrest and monitored Mde4 phosphorylation. Although Mde4 is more highly phosphorylated at the metaphase arrest point in *clp1Δ* cells (see Figure S1A available online), Mde4 still becomes dephosphorylated as these cells exit mitosis (Figure S1B). Thus, although Mde4 specifically purifies in a complex with the substrate-trapping allele of Clp1 and can be dephosphorylated by it in vitro, our data indicate that other phosphatases are able to effect the dephosphorylation in vivo in the absence of Clp1. The identification of these phosphatases will be of considerable interest and will be the subject of future studies. Together, these data show that Mde4 is phosphorylated in early mitosis and then becomes dephosphorylated in anaphase.

Clp1 Promotes Loading of Monopolin onto the Spindle during Anaphase

To test how phosphorylation affects Mde4 function, we examined whether changes in Mde4 phosphorylation state
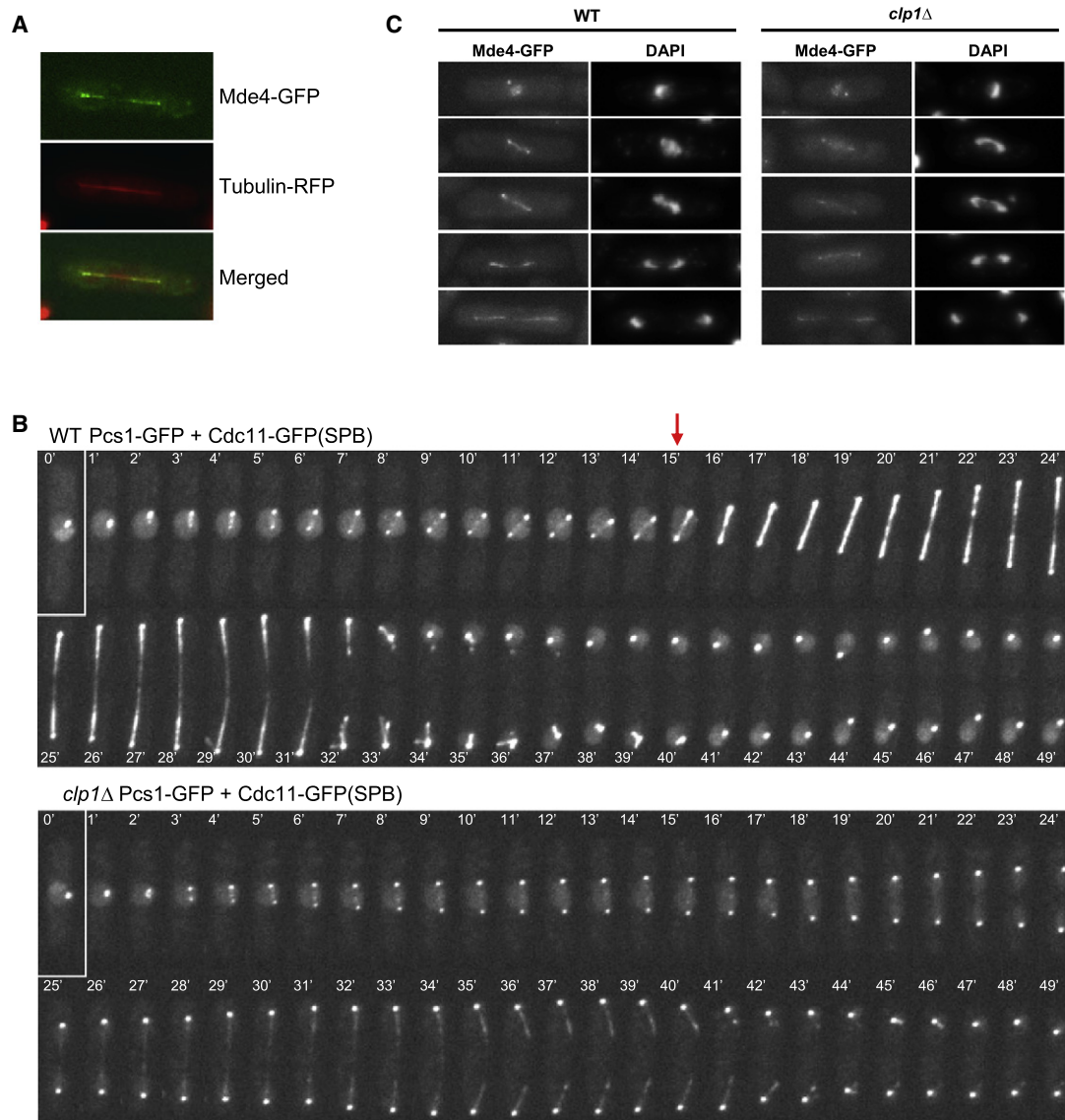
Figure 2. Clp1 Promotes Loading of Monopolin onto the Spindle during Anaphase

(A) *mde4-GFP* cells expressing mRFP-α-tubulin were imaged by fluorescence microscopy.

(B) *pcs1-GFP cdc11-GFP* (upper panel) and *clp1Δ pcs1-GFP cdc11-GFP* cells (lower panel) were analyzed via time-lapse fluorescence microscopy. Images were collected at 1 min intervals, beginning immediately prior to entry into mitosis. The spindle pole body marker Cdc11-GFP was used to monitor cell-cycle progression. At time 0, both cells are in interphase just prior to mitotic entry. The arrow indicates anaphase onset, when Pcs1 begins to localize to the spindle.

(C) Mde4-GFP localization is shown in wild-type (WT, left panel) and *clp1Δ* cells (right panel). Interphase cells are shown in the top row, and cells from early to late anaphase are shown in the lower panels. Cells were grown asynchronously, fixed, stained with DAPI, and imaged by fluorescence microscopy.

correlated with changes in Mde4 localization. Previous results showed that in interphase, when Mde4 is dephosphorylated, the Mde4-Pcs1 complex localizes to the nucleolus and to the kinetochores, which cluster at the nuclear periphery next to the spindle pole bodies (SPBs) [6, 8]. As cells enter mitosis and Mde4 becomes phosphorylated, Mde4 and Pcs1 leave the nucleolus but remain at the kinetochores [6, 8]. Additionally, we found that in anaphase, when Mde4 becomes dephosphorylated, Mde4 and Pcs1 localize to both ends of the anaphase spindle (Figures 2A and 2B). Because Mde4 is more highly phosphorylated in *clp1Δ* cells just prior to anaphase onset, we examined Pcs1 and Mde4 localization in *clp1Δ* mutants as they progressed through mitosis. Pcs1-

GFP localization was observed by time-lapse microscopy, using cells coexpressing the SPB marker Cdc11-GFP to monitor cell-cycle progression (Figure 2B). In wild-type cells, faint Pcs1-GFP spots, presumably corresponding to the kinetochores, could be observed between the two SPBs in early mitosis. Pcs1-GFP localized to the spindle at anaphase onset (Figure 2B, red arrow) and then to both ends of the spindle at late anaphase. In *clp1Δ* cells, Pcs1-GFP spots at putative kinetochores were observed as in wild-type cells; however, Pcs1-GFP was only observed faintly at both ends of the spindle at late anaphase (Figure 2B). As with Pcs1-GFP, Mde4-GFP localized faintly on the spindle in late, but not early, anaphase in *clp1Δ* cells (Figure 2C). The reduced spindle localization of

Mde4 and Pcs1 in *clp1Δ* cells was not a result of reduced protein levels, because both proteins were present at or above wild-type levels in *clp1Δ* cells (Figure S1A and data not shown). Localization of Mde4 and Pcs1 to the nucleolus and kinetochores in interphase was not affected in *clp1Δ* cells (Figure 2C and data not shown). These results suggest that dephosphorylation of Mde4 may be important for monopolin localization to the spindle. The ability of monopolin to localize to late-anaphase spindles in *clp1Δ* mutants is consistent with our results showing that other phosphatases besides Clp1 can promote Mde4 dephosphorylation.

Loss of Monopolin Causes Defects in Microtubule Attachment to Kinetochores and Activates the Spindle Checkpoint

To test whether spindle localization of monopolin is important for spindle function, we examined spindle dynamics in monopolin mutants. Fission yeast spindle elongation can be divided into three distinct phases [18–20]. During phase I, which begins when cells enter mitosis, the spindle forms and grows to span the nucleus at a length of 2.5–3 μm. The spindle maintains the 2.5–3 μm length in phase II (metaphase/anaphase A) and then elongates during phase III, which corresponds to anaphase B. Mitotic spindles were observed by time-lapse fluorescence microscopy in wild-type, *mde4Δ*, and *pcs1Δ* cells expressing GFP-α-tubulin. A comparison of the kinetics of spindle elongation in *mde4Δ*, *pcs1Δ*, and wild-type cells revealed that cells lacking monopolin spent significantly more time in phase II (Figure 3A). Wild-type cells spent less than 20 min in phase II, but 8 of 14 *mde4Δ* cells and 6 of 8 *pcs1Δ* cells showed a prolonged phase II, suggesting a delay in metaphase. A metaphase delay is often observed in cells when microtubule attachments to kinetochores are defective, which triggers the spindle checkpoint to inhibit anaphase onset. Observation of asynchronous wild-type and *mde4Δ* cells showed that an elevated percent of *mde4Δ* cells displayed spindle checkpoint activation as judged by the presence of Mad2 at kinetochores (Figure 3B). We tested for genetic interactions between the *mde4Δ* mutant and the spindle checkpoint mutants *mad1Δ*, *mad2Δ*, *mad3Δ*, and *bub1Δ*. The *mde4Δ* mutation was synthetic lethal with *bub1Δ*—no double mutants were recovered. Double mutants between *mde4Δ* and *mad1Δ*, *mad2Δ*, or *mad3Δ* showed strong synthetic growth defects (Figure 3C), consistent with the notion that *mde4Δ* cells have defects in the attachment of microtubules to kinetochores that trigger spindle checkpoint-dependent delays.

We observed another difference in spindle morphology in monopolin mutants. Spindle lengths at the phase II/III transition in *mde4Δ* and *pcs1Δ* mutants were longer, 3.52 ± 0.15 μm and 3.47 ± 0.23 μm respectively, than in wild-type cells, 3.07 ± 0.23 μm (Figure 3D). Elongated phase II spindles have also been observed in mutants defective in either microtubule attachment to kinetochores [21] or cohesion between sister chromatids [22], which may suggest that poorly attached kinetochores or reduced cohesion in monopolin mutants causes an imbalance of pushing and pulling forces in the spindle, leading to a somewhat elongated metaphase spindle. Previous results have shown that monopolin is involved in preventing merotelic attachments [6], which are not thought to be monitored by the spindle checkpoint. However, our results suggest an additional role for monopolin in attachment of microtubules to kinetochores or cohesion, which is monitored by the spindle checkpoint.

Monopolin May Stabilize Anaphase Spindle Microtubules to Prevent Chromosome Cosegregation

The analysis of spindle elongation in *mde4Δ* cells revealed a function for monopolin in promoting anaphase spindle stability. In 6.4% (3 of 47) of *mde4Δ* cells, the spindles appeared to break and/or collapse (Figure 3E; Figure S2A; Movies S1–S3). Consistent with this, we found that about 8.5% of septated *mde4Δ* cells showed chromosome cosegregation in which one daughter cell inherited all of the chromosomes (Figure 3F). This phenotype likely arises because the nuclear envelope does not break down during mitosis in *S. pombe*. As a consequence of this, when the spindle breaks prematurely, the chromosomes collapse back into a single mass inside the nuclear envelope. Similar results have been observed when the mitotic spindle is cut via laser microsurgery [19, 23]. In those studies, when the spindle was cut near the middle, the mitotic spindle completely collapsed. However, when the mitotic spindle was cut near one SPB, the spindle continued to elongate unidirectionally, with the end of the spindle lacking an SPB pushing out a finger of nuclear envelope as it elongated. Once the broken end of the spindle reached the end of the cell, it pushed against the cell tip, causing the other spindle pole to move to the opposite end of the cell. Of the three cells that showed spindle elongation defects, two showed spindle breakage and collapse (Figure 3E; Movies S1 and S2) and one showed unidirectional spindle elongation, suggesting that the spindle may have broken near one pole (Movie S3 and Figure S2A). Another case of apparent spindle breakage followed by chromosome cosegregation was observed by time-lapse analysis of *mde4Δ* cells expressing histone H3-GFP (Figure S2B). Furthermore, thin protrusions of nuclear envelope possibly caused by broken spindle ends were also observed in some *mde4Δ* cells, but not in wild-type cells (Figure S2C). Taken together, our data suggest that monopolin may have a novel function in stabilization of anaphase spindles.

Characterization of Nonphosphorylatable and Phosphomimetic Mutants of Mde4

To more clearly define the role of phosphorylation of Mde4 in regulating the monopolin complex, we constructed nonphosphorylatable and phosphomimetic mutants. Because incomplete sequence coverage of Mde4 in our mass spectrometry did not allow us to identify all in vivo Cdk1 phosphorylation sites on Mde4, we mutated all twelve of the Cdk1 consensus sites to alanine (S/T to A) to prevent phosphorylation or to acidic residues (S to D and T to E) to create a phosphomimetic mutant. We named them *mde4-12A* and *mde4-12D*, respectively, and constructed strains in which the endogenous *mde4+* locus was replaced by *mde4-12A* or *mde4-12D*. As expected, the Mde4-12A mutant protein was no longer hyperphosphorylated in metaphase-arrested cells (Figure S3A) and Mde4-12A was not phosphorylated by Cdk1 in vitro (Figure S3B), indicating that the major sites of mitotic phosphorylation had been eliminated. Additionally, Mde4-12A did not interact with Clp1-C286S (Figure S3C), showing that phosphorylation is required for interaction with the Clp1-C286S substrate-trapping allele. We do not believe that the 12A and 12D mutations grossly perturbed the Mde4 protein structure, because they did not affect Mde4-GFP localization to kinetochores and the nucleolus in interphase cells (Figure S3D). Additionally, kinetochore and nucleolar localization of Pcs1-GFP was abolished in the absence of Mde4 (Figure S3E) but was retained in *mde4-12A* and *mde4-12D* mutants
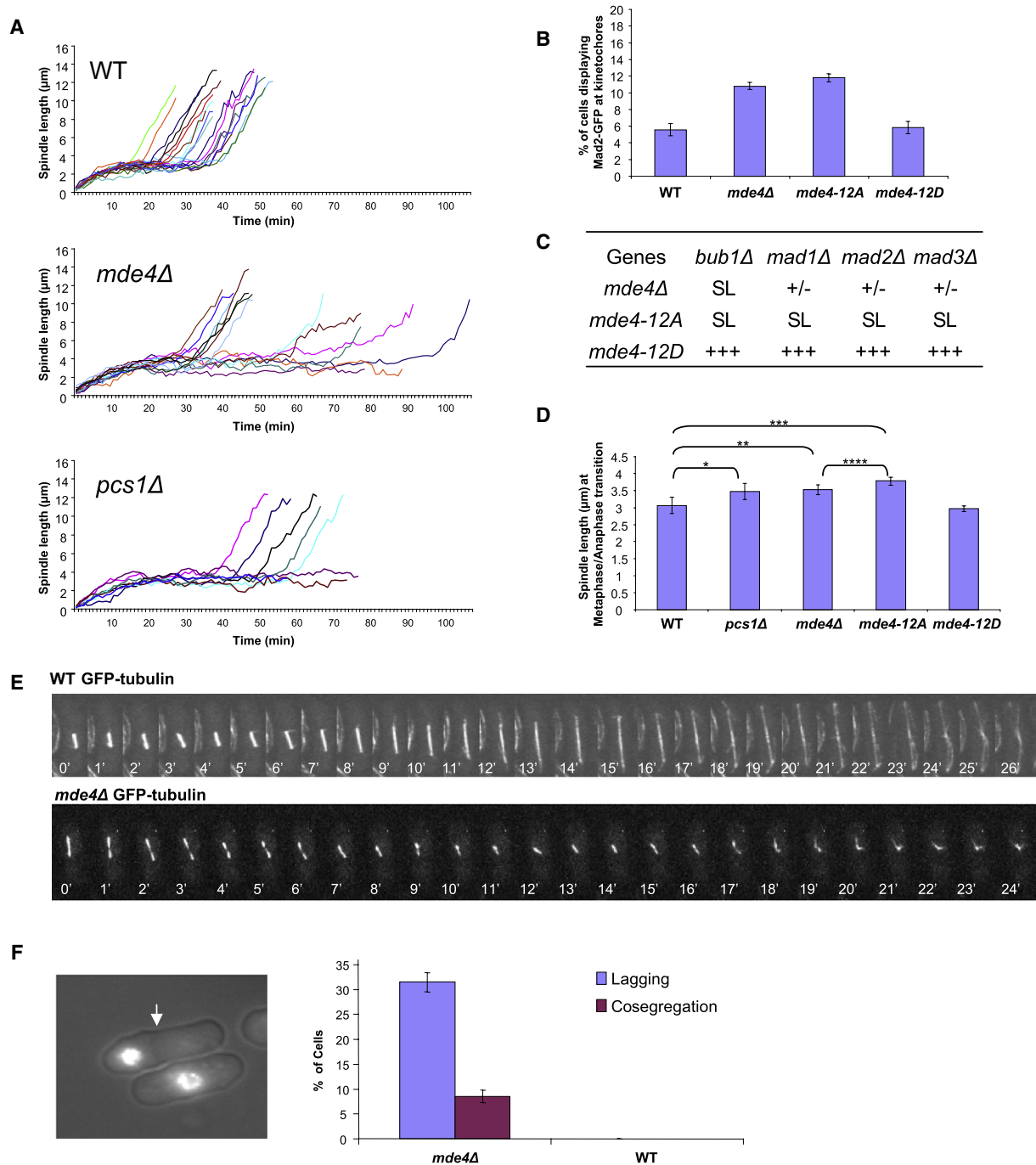
Figure 3. Monopolin Mutants Have Delays in Anaphase Onset and Show Occasional Spindle Collapse

(A) Spindle elongation was monitored in wild-type and monopolin mutant cells. Spindle length was measured every minute from time-lapse sequences of asynchronous *GFP-atb2* (n = 17), *GFP-atb2 mde4Δ* (n = 14), and *GFP-atb2 pcs1Δ* cells (n = 8).

(B) Asynchronous wild-type, *mde4Δ*, *mde4-12A*, and *mde4-12Δ* cells expressing Mad2-GFP were scored for the presence of Mad2-GFP puncta at kinetochores. Error bars represent standard deviation (SD).

(C) Genetic interactions between the *mde4Δ*, *mde4-12A*, and *mde4-12D* mutations and spindle assembly checkpoint mutations. The *mde4Δ*, *mde4-12A*, and *mde4-12D* mutants were crossed with the spindle assembly checkpoint mutants *bub1Δ*, *mad1Δ*, *mad2Δ*, and *mad3Δ*. Genetic interactions are shown as synthetic lethality (SL), strong growth defect (+/−), and normal growth (+++).

(D) A comparison of spindle lengths of wild-type and monopolin mutant cells at the metaphase/anaphase transition. Mean spindle lengths were compared between wild-type and *pcs1Δ* (*p < 0.01), *mde4Δ* (**p < 0.0001), or *mde4-12A* (***p < 0.0001) as well as between *mde4Δ* and *mde4-12A* (****p < 0.0001) by t test. Error bars represent SD.

(E) Time-lapse analysis of GFP-tubulin-expressing wild-type cells (upper panel) and *mde4Δ* mutant cells (lower panel) is shown. Images were collected at 1 min intervals.

(F) Left: an example of the chromosome cosegregation phenotype observed in *mde4Δ* cells (arrow indicates septum position). Right: the frequency of anaphase cells with lagging chromosomes and septated cells with cosegregated chromosomes for *mde4Δ* and wild-type cells. Error bars represent SD.
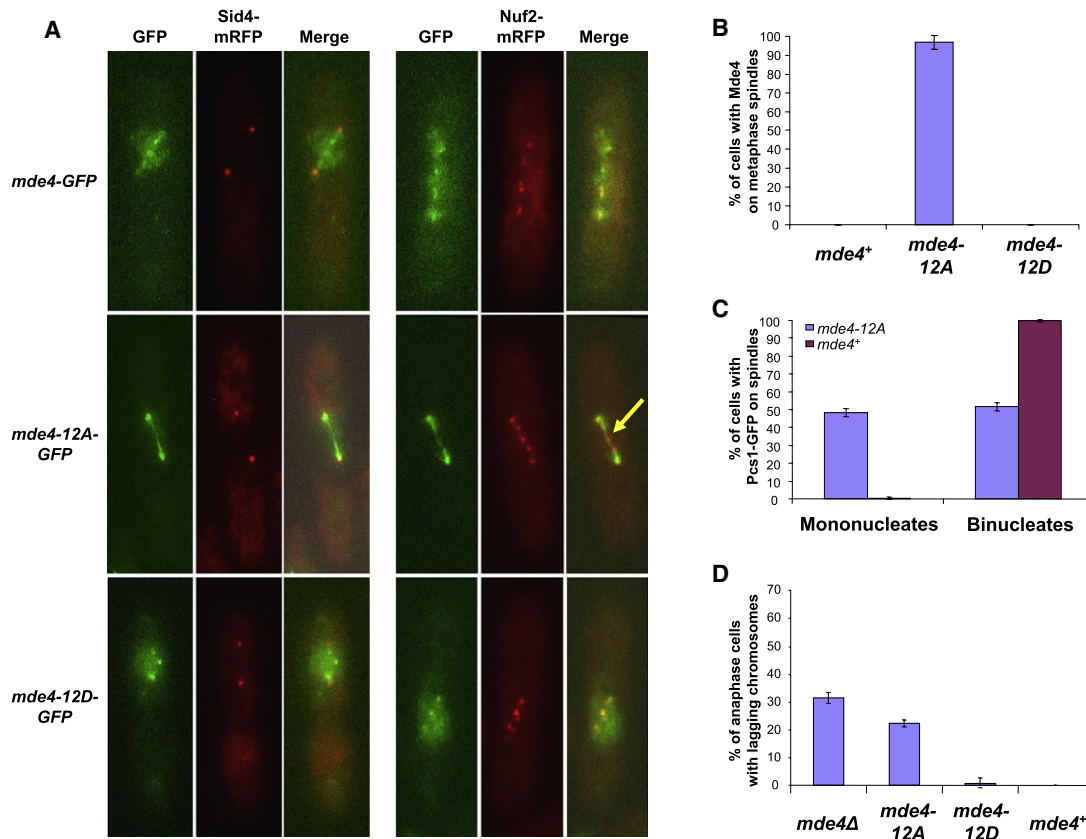
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Figure 4. *Mde4-12A* Prematurely Localizes to the Spindle Instead of Kinetochores before Anaphase Onset and Displays Lagging Chromosomes in Anaphase

(A) Localization of Mde4, Mde4-12A, and Mde4-12D at metaphase was examined in the strains *mde4-GFP sid4-mRFP*, *mde4-12A-GFP sid4-mRFP*, and *mde4-12D-GFP sid4mRFP* (left panel) and *mde4-GFP nuf2-mRFP*, *mde4-12A-GFP nuf2-mRFP*, and *mde4-12D-GFP nuf2-mRFP* (right panel). Cells were arrested in metaphase by overexpression of Mad2 via the pREP3X-Mad2 plasmid for 19 hr in the absence of thiamine. Cells were fixed and imaged by fluorescence microscopy. The yellow arrow indicates a kinetochore that does not appear to label with Mde4-12A-GFP.

(B) Frequency of Mde4 localization to the metaphase spindle. *mde4-GFP sid4-mRFP*, *mde4-12A-GFP sid4-mRFP*, and *mde4-12D-GFP sid4-mRFP* cells were arrested at metaphase by Mad2 overexpression for 19 hr. Cells showing spindle localization were counted among metaphase-arrested cells exhibiting unseparated condensed chromosomes and separated spindle pole bodies. Error bars represent SD.

(C) Quantification of Pcs1 spindle localization in preanaphase and postanaphase *mde4+* and *mde4-12A* cells. *pcs1-GFP* and *pcs1-GFP mde4-12A* cells were grown asynchronously at 30°C and then fixed and stained with DAPI. Of cells that showed Pcs1 spindle localization, the percentage that were mononucleate or binucleate is shown. Error bars represent SD.

(D) Frequency of lagging chromosomes in *mde4* mutants was determined by counting the percent of anaphase cells with lagging or unevenly segregated chromosomes from asynchronously growing *mde4Δ*, *mde4-12A*, *mde4-12D*, and wild-type cells. Error bars represent SD.

(Figure S3E), suggesting that the Cdk1 site mutations do not disrupt formation of the monopolin complex.

Phosphorylation of Mde4 Is Required to Prevent Monopolin Localization to the Prometaphase/Metaphase Spindle

Unlike wild-type Mde4, Mde4-12A-GFP was often observed on the spindle in preanaphase cells (data not shown), suggesting that it may load onto the spindle prematurely. To investigate this in greater detail, we arrested cells at metaphase by overexpressing the spindle assembly checkpoint protein Mad2, which prevents cyclin B and securin destruction by suppressing the activity of the APC/C-Cdc20 complex [24]. Under these conditions, Mde4-GFP and Mde4-12D-GFP were seen as several faint dots between the two spindle pole bodies, which were labeled with Sid4-mRFP (Figure 4A). These dots colocalized with a kinetochore protein, Nuf2-mRFP (Figure 4A). In contrast to Mde4-GFP, Mde4-12A-GFP localized to the metaphase spindle and could not be detected at kinetochores (Figure 4A, yellow arrow). Mde4-12A localized to the metaphase spindle

in 97% (n = 115) of metaphase cells, in contrast to Mde4 (n = 126) and Mde4-12D (n = 103), which did not localize to the spindle in metaphase cells (Figure 4B). To examine how the *mde4-12A* mutation affects Pcs1 localization, we counted cells exhibiting spindle localization of Pcs1-GFP in asynchronous *mde4+* and *mde4-12A* cells and classified them into mononucleate (early mitosis) and binucleate cells (anaphase). In wild-type cells, Pcs1-GFP localization to the spindle was observed almost exclusively in anaphase cells (307 of 308). In *mde4-12A* cells, Pcs1-GFP localized normally to anaphase cells but was also observed in preanaphase cells; specifically, 48% (n = 350) of cells showing Pcs1-GFP localization to the spindle were mononucleate (Figure 4C). Thus, in each assay, the non-phosphorylatable Mde4-12A mutant localizes the monopolin complex inappropriately to the prometaphase/metaphase spindle, implying that Cdk1 phosphorylation acts to prevent premature localization of Mde4 to the mitotic spindle.

Localization of monopolin at kinetochores has been proposed to prevent merotelic attachments, which cause
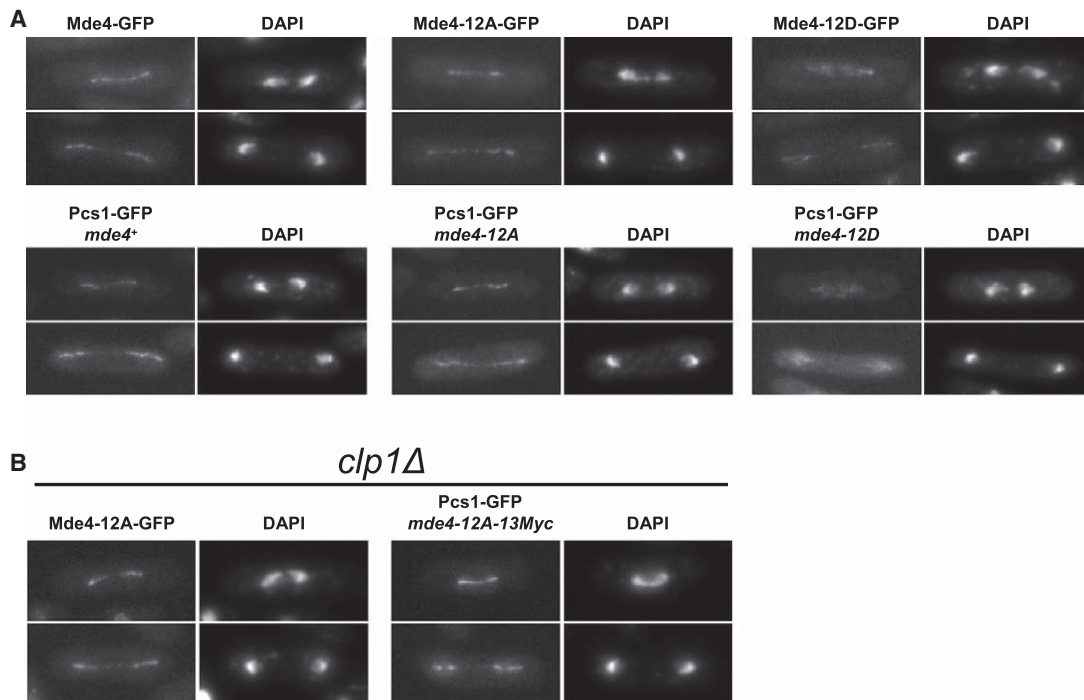
Figure 5. Mde4-12D Localizes Poorly to Anaphase Spindles

Cells were grown asynchronously, fixed, and imaged by fluorescence microscopy.

(A) Early- and late-anaphase localization of wild-type Mde4-GFP, Mde4-12A-GFP, and Mde4-12D-GFP (upper panel) and Pcs1-GFP in *mde4+*, *mde4-12A*, and *mde4-12D* cells (lower panel).

(B) Early- (top row) and late-anaphase localization (bottom row) of Mde4-12A-GFP in *clp1Δ* and Pcs1-GFP in *mde4-12A clp1Δ* cells.

lagging chromosomes [6, 8]. We observed that 22% of *mde4-12A* cells (n = 304) exhibited lagging chromosomes in anaphase, comparable to the 31% observed in *mde4Δ* cells (n = 327) (Figure 4D). In contrast, less than 1% of *mde4-12D* cells (n = 311) in anaphase showed lagging chromosomes (Figure 4D). Our data show that Cdk1 phosphorylation on Mde4 is important for its role in preventing merotelic attachment of kinetochores to spindle microtubules. One explanation for this could be that Cdk1 phosphorylation promotes kinetochore localization of Mde4 by inhibiting premature localization of Mde4 to microtubules in prometaphase/metaphase.

Mde4 Dephosphorylation Promotes Localization of Monopolin to Anaphase Spindles

We used the *mde4-12A* and *mde4-12D* mutants to explore the role of Mde4 dephosphorylation in monopolin localization to the anaphase spindle (Figure 5A). Mde4-GFP and Mde4-12A-GFP localized similarly to the spindle in early and late anaphase. In contrast, Mde4-12D-GFP showed a diffuse nuclear signal in anaphase, with less than 10% of cells showing faint spindle localization. Pcs1-GFP localized in *mde4+*, *mde4-12A*, and *mde4-12D* cells the same as each Mde4 mutant protein (Figure 5A, lower panel), consistent with the notion that the phosphorylation status of Mde4 regulates localization of the monopolin complex to the spindle. The poor localization of Mde4-12D to the spindle was similar to that observed for wild-type Mde4 in early-anaphase *clp1Δ* cells, presumably because Mde4 is not dephosphorylated as rapidly in *clp1Δ* cells. If this is the case, then disruption of Mde4 phosphorylation should rescue the Mde4 spindle localization defect in *clp1Δ* cells. This proved to be the case: Mde4-12A localized strongly to anaphase spindles in *clp1Δ* cells (Figure 5B). Additionally, expression of Mde4-12A was sufficient to restore Pcs1 association with the mitotic spindle (Figure 5B). Together, these results show that Cdk1 phosphorylation on Mde4 must be removed to allow the monopolin complex to localize to anaphase spindles.

Both *mde4-12A* and *mde4-12D* Cells Display Distinct Defects in Spindle Elongation

To better characterize spindle dynamics in *mde4-12A* and *mde4-12D* mutants, spindle elongation was observed by time-lapse microscopy in these cells. Spindle elongation in *mde4-12A* cells resembled spindle elongation in *mde4Δ* cells (compare Figure 6A with Figure 3A) in that 50% (10 of 20) of *mde4-12A* cells remained longer in phase II than *mde4+* cells. Unlike in *mde4Δ* cells, however, we did not observe spindle collapse or breakage in *mde4-12A* cells. The extended phase II in *mde4-12A* cells suggests that, like *mde4Δ* cells, they have microtubule attachment defects that trigger a spindle assembly checkpoint-dependent delay. To test this possibility, we crossed *mde4-12A* to various spindle checkpoint mutants. The *mde4-12A* mutant was synthetically lethal with the *mad1Δ*, *mad2Δ*, *mad3Δ*, and *bub1Δ* checkpoint mutants (Figure 3C), suggesting that the extended duration at phase II in *mde4-12A* cells is caused by activation of the spindle checkpoint. Notably, this interaction was even stronger than that observed for *mde4Δ*, because *mde4Δ* was only lethal with *bub1Δ*. Additionally, *mde4-12A* cells displayed significantly longer spindle lengths at phase II/III transition (3.78 ± 0.12 μm) than both wild-type and *mde4Δ* cells (Figure 3D). These results suggest that *mde4-12A* mutants may have
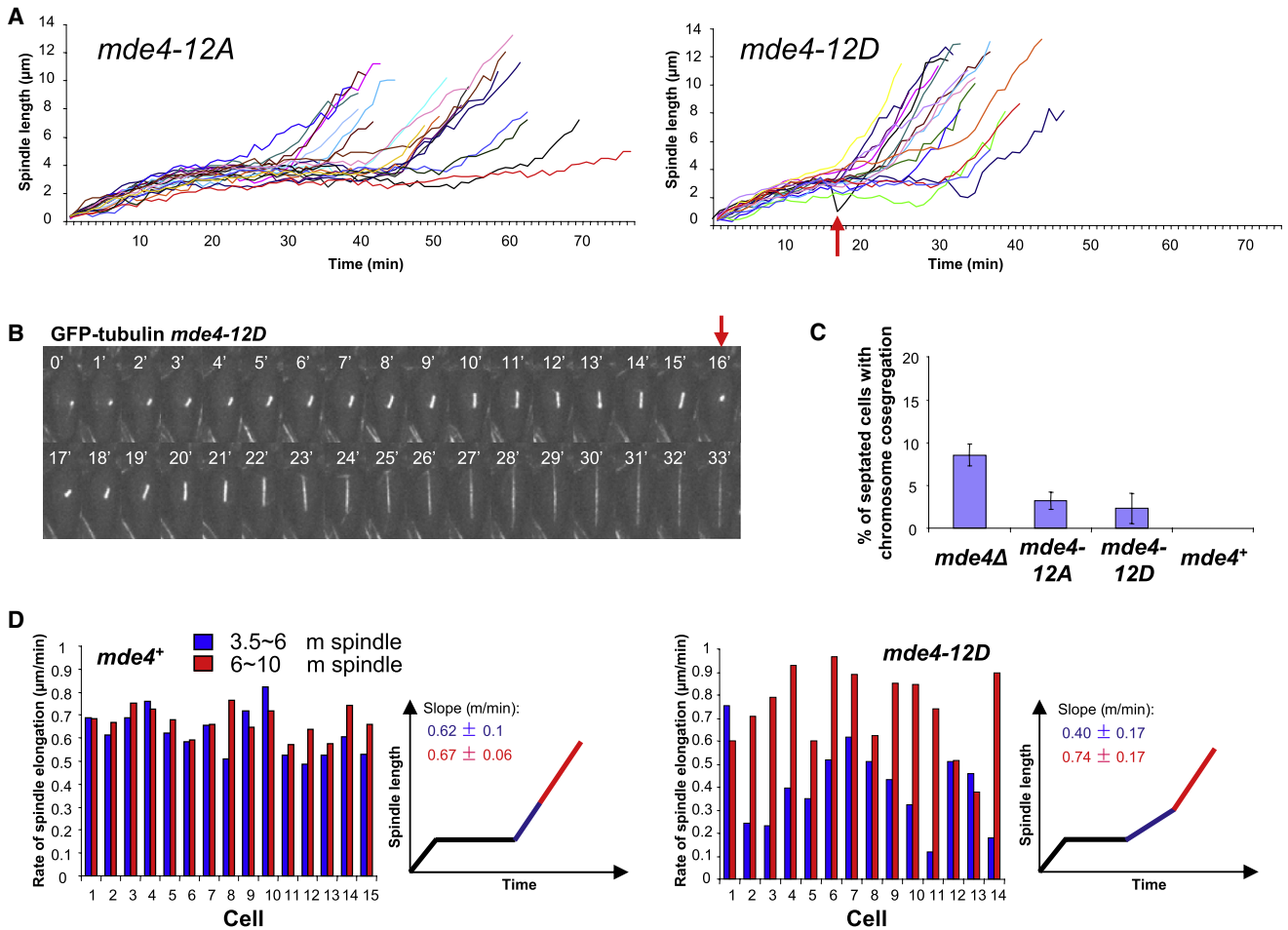
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Figure 6. Both *mde4-12A* and *mde4-12D* Cells Display Distinct Defects in Spindle Elongation

(A) Spindle elongation was monitored in *mde4-12A* and *mde4-12D* cells. Spindle length was measured every 1 min from time-lapse sequences of asynchronous *GFP-atb2 mde4-12A* (n = 20) and *GFP-atb2 mde4-12D* cells (n = 17). The red arrow indicates spindle collapse in the cell shown in (B).

(B) Spindle collapse and re-elongation was observed in 1 of the 17 *GFP-atb2 mde-12D* cells shown in (A). Images were collected every 1 min, and spindle collapse was reconfirmed by examining a 3D view of the spindle from stacked images (data not shown) at the time of spindle shortening (red arrow).

(C) Frequency of chromosome cosegregation in *mde4* mutants was determined by counting the percent of septated cells with a single DNA mass in only one daughter cell. Error bars represent SD.

(D) Comparison of spindle elongation rate between *mde4*[+] (n = 15) and *mde4-12D* cells (n = 14). Spindle elongation rates were obtained by measuring the slope of plots of spindle length over time via linear regression in individual *mde4*[+] and *mde4-12D* anaphase cells. Slopes were calculated for early (spindle lengths of 3.5–6 μm, shown as blue bars) and late anaphase (spindle lengths of 6–10 μm, shown as red bars). Each pair of red and blue bars represents an individual cell.

greater defects in microtubule attachment to kinetochores than *mde4Δ* cells. This could be explained if the defects in *mde4-12A* cells are due not only to loss of Mde4 from the kinetochores in metaphase but also to premature localization of Mde4 to the spindle.

Overall, *mde4-12D* cells showed spindle behavior similar to wild-type cells, but most cells (14 of 16) displayed a slightly shorter phase II (Figure 6A; Figure 3A). Spindle lengths at the phase II/III transition in *mde4-12D* cells were 2.97 ± 0.09 μm, similar to wild-type cells (Figure 3D), and *mde4-12D* cells did not show negative genetic interactions with the spindle checkpoint mutants *mad1Δ*, *mad2Δ*, *mad3Δ*, and *bub1Δ* (Figure 3C), suggesting that microtubule attachment in *mde4-12D* cells is normal. A small fraction of *mde4-12D* cells exhibited spindle collapse followed by recovery (Figure 6B). Furthermore, 2.3% (13 of 561) of *mde4-12D* cells showed chromosome cosegregation (Figure 6C). Although this represents a minority of *mde4-12D* cells, this was not observed in an equivalent number of wild-type cells (Figure 6C). These data are consistent with the view that reduced levels of Mde4 on the spindle may compromise its stability. Unexpectedly, we also found that *mde4-12D* cells displayed a reduced rate of spindle elongation in early anaphase B (Figure 6D). We measured the spindle elongation rate during early (spindle length 3.5–6 μm) and late (spindle length 6–10 μm) phase III. Although the rate of spindle elongation in wild-type cells was the same in early and late phase III (~0.65 μm/min), the rate of spindle elongation in *mde4-12D* cells was slower during early phase III (~0.40 μm/min) but was similar to wild-type cells in late phase III (~0.74 μm/min). Taken together, our data suggest that dephosphorylation of Mde4 at Cdk1 sites triggers spindle localization of monopolin, which promotes proper spindle elongation in early anaphase and enhances spindle stability.
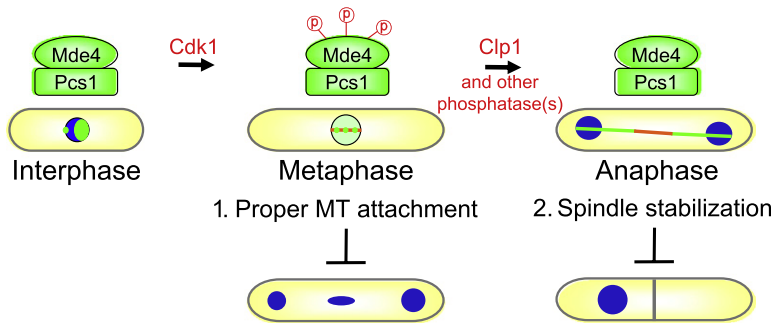
Figure 7. Model of Phosphorylation-Dependent Regulation of Chromosome Segregation by the Monopolin Complex

Left: Mde4 and Pcs1 (green) localize to the nucleolus (large green spot in nucleus) and the clustered kinetochores (smaller green spot at periphery of the nucleus) in interphase. Middle: in early mitosis, Mde4 becomes phosphorylated (probably by Cdk1), which is required to maintain Pcs1-Mde4 at kinetochores (three small green spots) to promote proper attachment of microtubules (MTs) to kinetochores to prevent lagging chromosomes in anaphase. Right: in anaphase, Clp1 and other phosphatases dephosphorylate Mde4 to promote loading of monopolin onto the spindle (shown in orange) to stabilize it and allow for proper elongation to prevent chromosome cosegregation.

Discussion

For faithful segregation of genetic material, chromosomes must be attached to microtubules in a bioriented manner at metaphase and segregated to each daughter cell through spindle elongation at anaphase. Our studies show that regulated phosphorylation of the monopolin complex allows it to carry out discrete functions in each of these steps (Figure 7). When cells enter mitosis, Mde4-Pcs1 is released from the nucleolus, and Mde4 becomes phosphorylated on Cdk1 sites. Mde4 phosphorylation does not appear to cause release of Mde4 from the nucleolus, because Mde4-12A is still released from the nucleolus in early mitosis. Instead, phosphorylation of Mde4 is required to prevent localization of the Mde4-Pcs1 complex to the metaphase spindle. Furthermore, not only does the Mde4-12A protein localize prematurely to the spindle, it also appears to localize poorly to the kinetochores. This could either be because monopolin localization to kinetochores requires Mde4 phosphorylation or because the Mde4-12A protein has higher affinity for the spindle than for the kinetochores. We prefer the latter model, because the Mde4-12A protein localizes normally to kinetochores in interphase when there is no spindle to compete with the kinetochores for binding to Mde4. Our data indicate that Mde4 phosphorylation on Cdk1 sites serves the dual function of (1) allowing monopolin to localize to kinetochores, where it can carry out its function in promoting proper chromosome attachment, and (2) blocking premature localization of monopolin to the spindle, which might interfere with metaphase spindle dynamics. After anaphase onset, Clp1 along with other phosphatases dephosphorylates Mde4 to allow the monopolin complex to load onto the anaphase spindle, where it is required for both spindle stability and for proper spindle elongation.

Monopolin Function at Kinetochores
Previous studies in budding and fission yeast led to a model for monopolin function that proposed that the monopolin complex acts to clamp microtubule-binding sites together at the kinetochore to ensure that they attach to microtubules from the same spindle pole [6–8, 10]. Further work in budding yeast showed that monopolin may also function to clamp the rDNA repeats together to keep them in register and prevent unequal crossing-over [25]. Budding yeast monopolin requires separate adapters to target it to the meiosis I kinetochores and the rDNA repeats. These results suggest that monopolin can act as a crosslinker for different structures through the use of unique adaptor molecules. Many crosslinking molecules such as the microtubule crosslinker Ase1 (PRC1 in humans) form multimeric complexes [26, 27]. Consistent with this, we found that Mde4 and Pcs1 not only form heterodimers

(Figure S4A) but also may form either heterotetramers or higher-order multimers, because Mde4-GFP pulled down Mde4-13Myc from cell lysates from diploid cells expressing both tagged proteins (Figure S4B). Similar results were observed for cells expressing two different tagged versions of Pcs1 (Figure S4B). Thus, it is possible that in *S. pombe*, the Mde4-Pcs1 complex acts to crosslink microtubule-binding sites, spindle microtubules, and possibly rDNA repeats. However, candidate adaptor molecules for these locations have not been identified. Tandem affinity purification of Mde4 and Pcs1 did not identify other kinetochore or microtubule-binding proteins that could serve as adapters for Mde4-Pcs1 at these locations (unpublished data; see also [6]).

In addition, our finding that monopolin mutants have extended metaphase delays and depend on the spindle checkpoint for normal growth suggests that monopolin may have an additional function of promoting attachment of microtubules to kinetochores, because merotelic attachments are not thought to trigger the spindle checkpoint [2]. One way to reconcile these data with the earlier model would be to suppose that monopolin acts to clamp together and stabilize parallel microtubule ends at the kinetochore. Thus, monopolin could both stabilize attachments and favor kinetochore binding to microtubules from the same pole. Alternatively, monopolin could carry out separate functions in preventing merotelic attachments and promoting or stabilizing microtubule attachment to kinetochores.

Role of Monopolin Function in Spindle Elongation
Our work has uncovered a novel function for monopolin in promoting elongation and stability of the mitotic spindle. The spindle breakage phenotype that we observed in *mde4Δ* cells indicates that Mde4 may have a structural role in maintaining spindle integrity. The *mde4-12D* mutant, which localizes poorly to the mitotic spindle, also has spindle integrity defects as judged by the chromosome cosegregation phenotype observed in fixed cells and the occasional spindle collapse observed in time-lapse analysis. Both the exclusion of monopolin from antiparallel microtubules in the spindle midzone and the spindle breakage phenotype could be explained if monopolin prefers to bind to and crosslink only parallel microtubules. By crosslinking spindle microtubules, monopolin could promote spindle stability much like the spindle midzone crosslinker Ase1 [26, 27]. In an attempt to study the microtubule-binding properties of monopolin in vitro, we were able to produce small amounts of Mde4-Pcs1 complex in bacteria; however, the bacterially produced protein complex did not bind to microtubules in vitro (unpublished data), suggesting that monopolin either does not bind to microtubules directly or requires other proteins or secondary modifications that

are only present in vivo. In addition to the spindle collapse defect, we also found that *mde4-12D* mutant cells display slowed spindle elongation in early anaphase. We also observed a slowed rate of spindle elongation in *mde4Δ* cells (data not shown); however, the phenotype in these cells may be complex because, unlike *mde4-12D* mutant cells, *mde4Δ* cells have a high incidence of lagging chromosomes, which also slows spindle elongation [28]. The cause of slowed spindle elongation in *mde4-12D* cells is not known but could be explained if monopolin has a role in loading and/or activation of microtubule motors on the spindle in early anaphase.

Reversal of Cdk1 Phosphorylation and Anaphase Spindle Function

Upon entry into mitosis, Cdk1 phosphorylation, either directly or indirectly, drives disassembly of the interphase microtubule cytoskeleton and assembly of the mitotic spindle. However, cells are able to maintain a mitotic spindle even when Cdk1 activity drops in anaphase. This and other studies have shown that anaphase spindle stability is maintained at least in part by the loading of certain proteins onto the spindle specifically in anaphase [29–31]. Tightly coupling the localization and/or activity of these proteins to the level of Cdk1 activity seems to be a common strategy, because a number of spindle-associated proteins are inhibited in early mitosis by Cdk1 activity and then dephosphorylated in anaphase. Examples include in budding yeast Sli15 (human INCENP), Ase1, and Fin1 [29–31]. Dephosphorylation by Cdc14 promotes spindle localization of Sli15 and Fin1 [30, 31] and promotes the activity of Ase1 [29]. Similar to our findings with nonphosphorylatable Mde4, nonphosphorylatable variants of Sli15 and Fin1 localize prematurely to the mitotic spindle, which results in chromosome segregation defects [30, 31]. Premature localization of monopolin to the metaphase spindle also seems to cause additional defects in spindle dynamics or microtubule attachment to kinetochores, because double mutants between *mde4-12A* and spindle checkpoint mutants such as *mad2Δ* were lethal, in contrast to *mde4Δ mad2Δ* mutants, which were sick but viable.

Conservation of Monopolin Function?

The large number of microtubule-binding sites at each mammalian kinetochore suggests that they should be particularly vulnerable to merotelic attachments. Consistent with this, time-lapse analysis of cultured mammalian cells has shown that merotelic attachments are the major cause of chromosome missegregation [3]. Therefore, we expect that mammalian cells have mechanisms to clamp together microtubule-binding sites on each kinetochore to ensure that they become attached to microtubules from the same spindle pole. It will be interesting in future studies to identify structural or functional homologs of monopolin that act to prevent merotelic attachments in mammalian cells.

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