Supplemental Data

Phosphorylation State Defines Discrete

Roles for Monopolin in Chromosome

Attachment and Spindle Elongation

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Supplemental Experimental Procedures

Yeast Strains and Plasmids

Strains used in this study are listed in Supplemental Table S1. Genetic crosses and general yeast techniques were performed as described previously [1]. Carboxy-terminal green fluorescent protein (GFP) and 13-Myc epitope tagging of *pcs1*$^+$, *mde4*$^+$, *mde4-12A* and *mde4-12D* was done by PCR-based gene targeting [2]. To construct the *mde4*Δ strains, the entire *mde4*$^+$ coding region was replaced with the *ura4*$^+$ gene cassette by homologous recombination. The *mde4*$^+$ genomic clone (pDM1061), which includes 1kb of 5' UTR and 0.5kb of 3' UTR, was cloned into the pENTR^{TM}/SD/D-Topo plasmid (Invitrogen). The *mde4-12A* (S/T to A) and *mde4-12D* (S/T to D/E) clones were created from the genomic clone pDM1061 using a combination of site-directed mutagenesis to mutate S147, minigene synthesis (Integrated DNA Technologies Inc.) to mutate S242, S247, S252, S270, T275, S290, S295, S316, S349, S354, and primer-directed mutagenesis to change S401. Using LR recombination, the mutated genomic clones were transferred into pIRT2-GW, which is a gateway destination plasmid constructed for this study that is based on the pIRT2 vector. pIRT2-mde4-12A and pIRT2-mde4-12D were transformed into *mde4*Δ::*ura4*$^+$ strain and the resulting transformants were then plated on Edinburgh minimal medium (EMM -Leu) plates including 1mg/ml 5-FOA to select for the *mde4-12A* and *mde4-12D* gene replacement strains. Expression of mRFP-tubulin and Mad2 from the thiamine repressible *nmt1* promoter was either repressed or induced by growing cells in EMM with or without 5 µg/ml of thiamine respectively.

Microscopy

GFP- and mRFP-fusion proteins were observed either after fixation with -20°C methanol or in live cells. DNA was visualized with 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) at 2 µg/ml. Photomicrographs were obtained using a Nikon Eclipse E600 fluorescence microscope coupled to a cooled charge-coupled device camera (ORCA-ER; Hamamatsu, Bridgewater, NJ), and image processing and analysis were carried out using IPLab Spectrum software (Signal Analytics, Vienna, VA). For time-lapse studies, either exponentially growing cells, or cells synchronized by elutriation were concentrated, and 1.8 µl of cell suspension was placed on a microscope slide with a 2% YE agar slab. Time-lapse experiments were made at 24°C by acquiring epi-fluorescence images in Z-planes. 2 x 2 binning was used except for Figure 2B, which did not use binning. Cells were viewed on a spinning disk microscope (Zeiss Axiovert 200 microscope with Argon ion laser system (Mellers Griot)). Images were captured using an IEEE1394 digital CCD camera C4742-80-12AG (Hamamatsu) and UltraVIEW^{TM}RS confocal imaging system software (PerkinElmer).

Immunoprecipitation and TAP Purification

Immunoprecipitation was done as described [3] in the presence of phosphatase inhibitors (1 mM $NaVO_4$ and 5 mM NaF). Mouse monoclonal anti-GFP (A-11120, Molecular Probes) and anti-Myc (sc-40, Santa Cruz Biotechnology) antibodies were used for immunoprecipitation, and mouse monoclonal anti-GFP (sc-9996, Santa Cruz Biotechnology) and rabbit polyclonal anti-Myc (SC-789, Santa Cruz Biotechnology)

antibodies were used for western blotting. Tandem affinity purification (TAP) of Clp1-C286S-TAP was carried out as previously described [4] to isolate Clp1-C286S together with associated proteins from asynchronous cells, cells arrested in metaphase by *nda3-KM311* or *mts3-1* block, and anaphase cells (60 min after released from *cdc25-22* block). Mde4-TAP was also purified from cells arrested in metaphase by *nda3-KM311* block. Purified proteins were separated on SDS-PAGE gels and silver-stained. In parallel, samples were subjected to analysis by tandem mass spectrometry.

Kinase and Phosphatase Assays

Phosphatase assays were performed as described [5]. Immunoprecipitated Mde4-13Myc from *clp1*Δ cells arrested at metaphase using the *nda3-KM311* mutation was incubated in the presence of recombinant MBP-Clp1 or Lambda phosphatase at 30°C for 30 min in phosphatase assay buffer (50 mM Imidazole [pH 6.9], 1 mM EDTA, 1 mM DTT). Reactions were terminated by the addition of sample buffer and boiling, followed by separation on SDS-PAGE and visualization by western blotting.

In vitro kinase assays were performed as described [3]. Recombinant 6His-Mde4, GST-Mde4-137-421, and GST-Mde4-12A-137-421 purified from *E.coli* were incubated at 30°C for 30 min with γ-^{32}P labeled ATP in the presence or absence of Cdc2 immunoprecipitated from metaphase arrested *nda3-KM311* cells using anti-Cdc13 antibodies.

Supplemental References

1. Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol *194*, 795-823.
2. Bahler, J., Wu, J.Q., Longtine, M.S., Shah, N.G., McKenzie, A., 3rd, Steever, A.B., Wach, A., Philippsen, P., and Pringle, J.R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. Yeast *14*, 943-951.
3. Sparks, C.A., Morphew, M., and McCollum, D. (1999). Sid2p, a spindle pole body kinase that regulates the onset of cytokinesis. J Cell Biol *146*, 777-790.
4. Gould, K.L., Ren, L., Feoktistova, A.S., Jennings, J.L., and Link, A.J. (2004). Tandem affinity purification and identification of protein complex components. Methods *33*, 239-244.
5. Wolfe, B.A., McDonald, W.H., Yates, J.R., 3rd, and Gould, K.L. (2006). Phospho-regulation of the Cdc14/Clp1 phosphatase delays late mitotic events in S. pombe. Dev Cell *11*, 423-430.

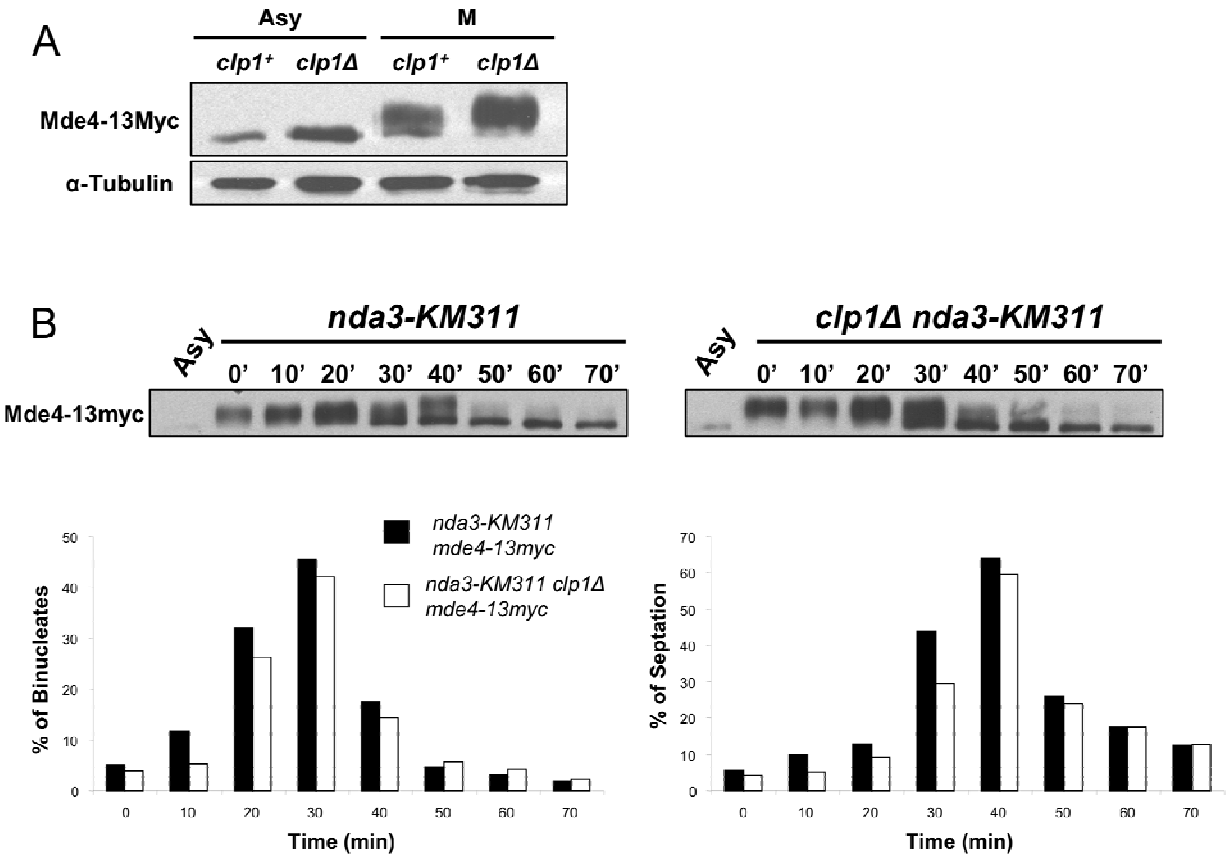

Figure S1. Mde4 Is Hyperphosphorylated at Metaphase in *clp1*Δ Cells but Dephosphorylated after Mitotic Exit

(A) Gel migration of Mde4-13myc was analyzed by immunoprecipitation followed by western blotting of Mde4-13Myc isolated from either asynchronous (Asy) cells or cells arrested in metaphase (M) using the *nda3-KM311* mutation in either a *clp1*$^{+}$ or *clp1*Δ background. Lower panel shows the tubulin loading control.

(B) *mde4-13Myc nda3-KM311* and *mde4-13Myc clp1*Δ *nda3-KM311* cells were arrested at the restrictive temperature of 19°C for 6h to synchronize them in prometaphase, then shifted to the permissive temperature of 30°C. Samples were taken at the indicated time points and the migration shift of Mde4-13Myc (upper panel) and cell cycle progress (lower panel) were determined by western blot analysis and microscopy, respectively.

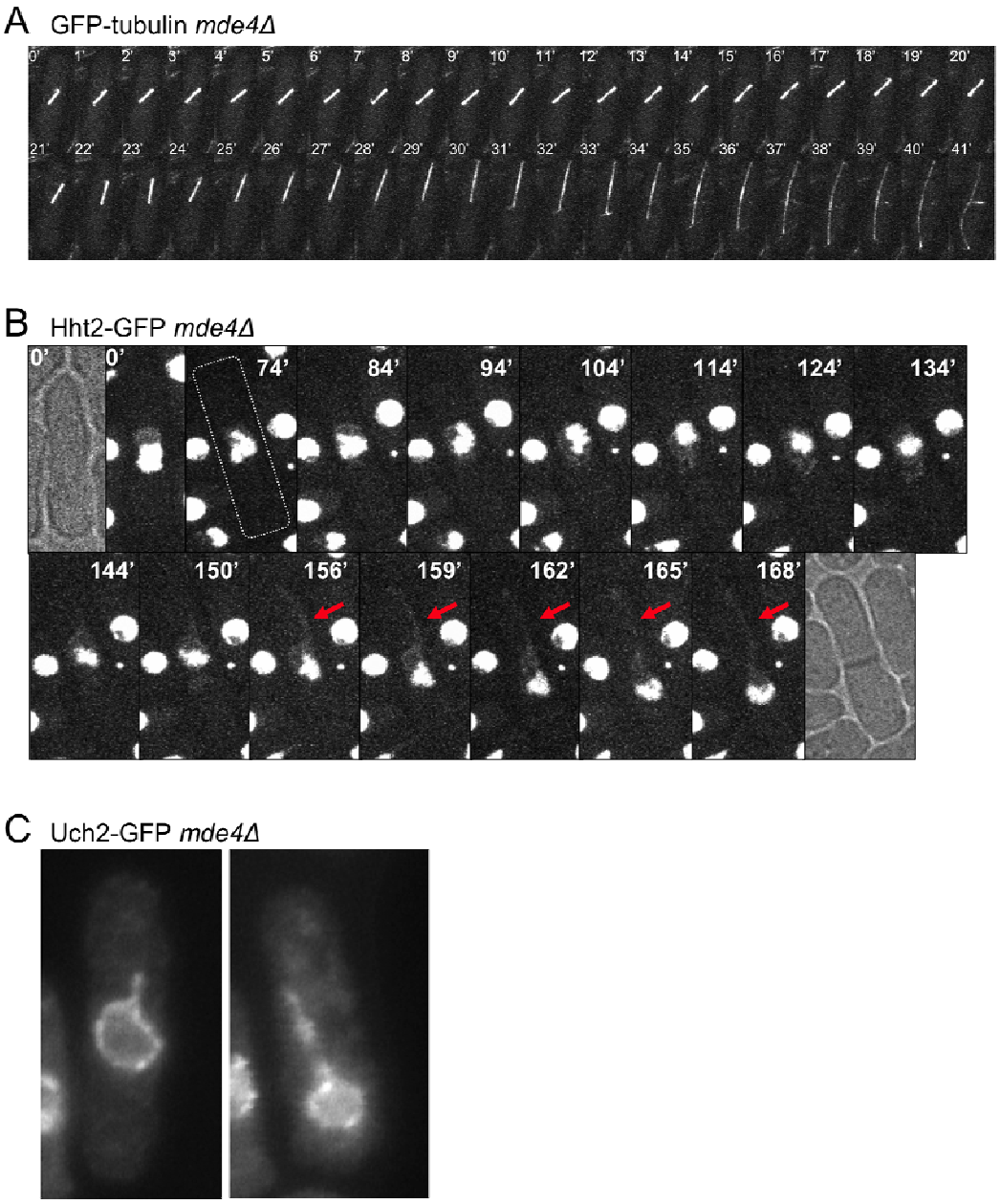

Figure S2. Spindle Breakage near One Pole May Cause Chromosome Cosegregation in *mde4*Δ Cells

(A) Time-lapse analysis of GFP-α-tubulin (GFP-atb2) in an *mde4*Δ cell is shown. The images were collected at one minute intervals.

(B) Time-lapse analysis of histone H3-GFP (hht2-GFP) in an *mde4*Δ cell is shown. The images were collected at one minute intervals. A thin protrusion of nucleoplasmic staining of free Hht2-GFP is indicated (red arrow).

(C) Examples of nuclear envelope protrusion observed in *mde4*Δ cells using the nuclear envelope protein Uch2-GFP, which was visualized by fluorescence microscopy.

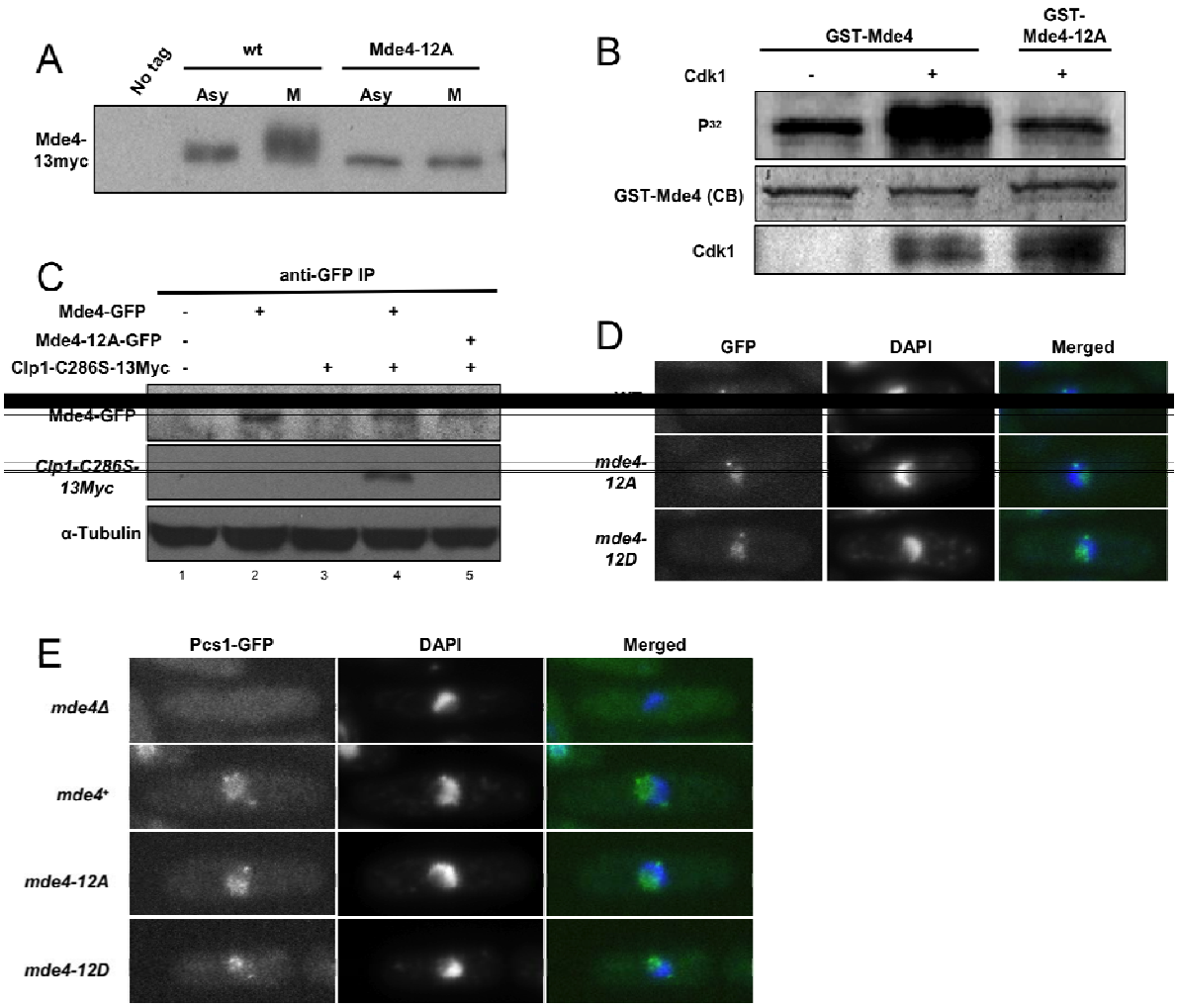

Figure S3. Characterization of *mde4-12A* and *mde4-12D*, Nonphosphorylatable and Phosphomimetic Mutants of Mde4

(A) Mde4 migration shift by phosphorylation was examined in wild-type (No tag), *mde4-13Myc* (wt) and *mde4-12A-13Myc* (12A) cells grown asynchronously (Asy) at 30°C or arrested at metaphase (M) by *nda3-KM311* block at 19°C.

(B) In vitro kinase assays were performed using Cdk1 immunoprecipitated, using Cdc13 (*S. pombe* cyclin B) antibodies, from metaphase arrested *nda3-KM311* cells, and bacterially expressed GST-Mde4-137-421 or GST-Mde4-12A-137-421 were used as substrates. Protein labeled by γ-^{32}P was detected with a Phospho Imager (Molecular Dynamics), and the gel was stained with Coomassie Blue (CB) as a loading control. The level of Cdk1 was determined by western blotting.

(C) Anti-GFP (upper panel) and anti-Myc (middle panel) western blots of anti-GFP immunoprecipitates from cell lysates of the following cultures are shown: wild-type (1), *mde4-GFP* (2), *clp1-C286S-13Myc* (3), *mde4-GFP clp1-C286S-13Myc* (4), *mde4-12A-GFP clp1-C286S-13Myc* (5). Lower panel shows the tubulin loading control from whole cell extracts prior to immunoprecipitation.

(D) Asynchronously growing cells expressing Mde4-GFP, Mde4-12A-GFP, or Mde4-12D-GFP were fixed, stained with DAPI, and DAPI and GFP signals were visualized using fluorescence microscopy. Interphase cells are shown.

(E) Asynchronously growing *mde4*Δ, *mde4*$^+$, *mde4-12A*, and *mde4-12D* cells expressing Pcs1-GFP were fixed, stained with DAPI, and DAPI and GFP signals were visualized using fluorescence microscopy. Interphase cells are shown. Note for (D) and (E) that the nucleolus is the non DAPI staining region of the nucleus, and the spot at the nuclear periphery corresponds to the clustered kinetochores in interphase.

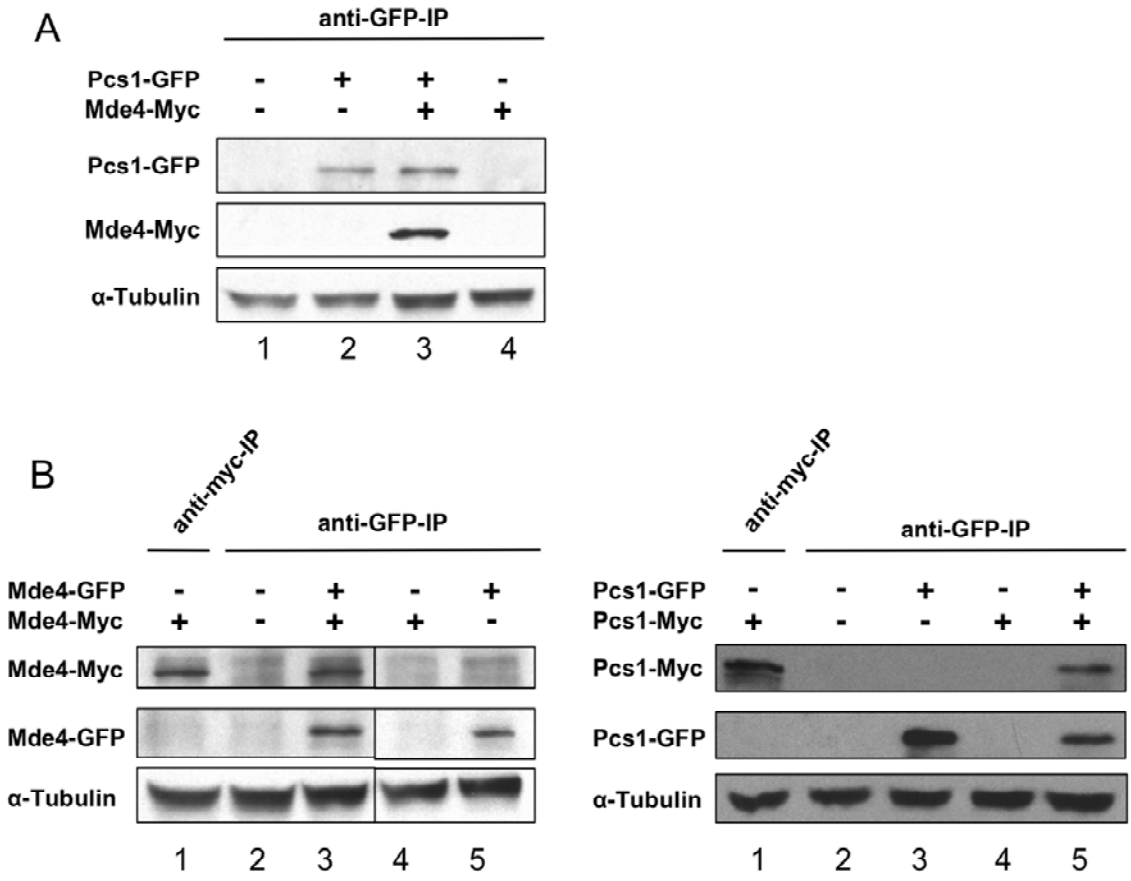

Figure S4. Mde4 and Pcs1 May Form Heterodimers and Possibly Heterotetramers In Vivo

(A) Interaction between Mde4 and Pcs1 was determined by immunoprecipitation followed by western blotting using cell lysates from the following cultures: wild-type (1), *pcs1-GFP* (2), *pcs1-GFP mde4-13Myc* (3), *mde4-13Myc* (4). Lower panel shows the tubulin loading control from whole cell extracts prior to immunoprecipitation.

(B) Multimerization of Mde4 and Pcs1 was determined by immunoprecipitation followed by western blotting using diploid cell lysates from cultures of the following strains: (left panel) *mde4-13Myc* haploid (1), wild-type haploid (2), *mde4-GFP mde4-13Myc* diploid (3), *mde4-13Myc* haploid (4), *mde4-GFP* haploid (5) (left panel), (right panel) *pcs1-13Myc* haploid (1), wild type haploid (2), *pcs1-GFP* haploid (3), *pcs1-13Myc* haploid (4), *pcs1-GFP pcs1-13Myc* diploid (5) (right panel). Samples in left panel were run in the same gel, with nonrelevant lanes excised.

Table S1. Yeast Strains

Strain Number	Relevant Genotype
DM105	*leu1-32 ura4-D18 ade6-210 h-*
DM107	*leu1-32 ura4-D18 ade6-216 h-*
DM108	*leu1-32 ura4-D18 ade6-216 h+*
DM774	*clp1-13Myc::KanR leu1-32, ura4-D18, ade6-216 h-*
DM916	*nda3-KM311 leu1-32 ura4-D18 ade6-21X h-*
DM1093	*mad2Δ::ura4+ ura4-D18 leu1-32 h-*
DM1799	*bub1Δ::ura4+ leu1 ura4 ade6 h+*
DM1801	*mad1Δ::ura4+ leu1 h-*
DM1802	*mad3Δ::ura4+ leu1 ura4 ade6 h-*
DM2272	*mad2-GFP::KanR leu1-32 ura4-D18 h-*
DM2488	*clp1-C286S-13Myc::KanR ura4-D18 leu1-32 ade6-21X h-*
DM2962	*pcs1-GFP::leu2+ ura4 leu1-32 ade6-210 h-*
DM3301	*leu1-32::pSV40-GFP-atb2-Leu1+ ura4-D18 leu1-32 ade6-21X h+*
DM3719	*mde4-13Myc::KanR leu1-32 ura4-D18 ade6-210 h-*
DM3728	*mde4-13Myc::KanR leu1-32 ura4-D18 ade6-210 h+*
DM3729	*mde4-GFP::KanR leu1-32 ura4-D18 ade6-210 h-*
DM3745	*mde4-13myc::KanR clp1D::Ura4+ leu1-32 ura4-D18 ade6-21X h-*
DM3751	*mde4-13Myc::KanR nda3-KM311 ura4-D18 leu1-32 ade6-21X h-*
DM3754	*mde4-GFP::KanR clp1-C286S-13Myc::KanR leu1-32 ura4-D18 ade6-21X h-*
DM3796	*mde4-GFP::KanR clp1-13Myc::KanR leu1-32, ura4-D18, ade6-21X h+*
DM3804	*mde4-GFP::KanR clp1Δ:ura4+ ura4-D18 leu1-32 ade6? h-*
DM3818	*mde4-13Myc::KanR clp1Δ::ura4+ nda3-KM311 ura4-D18 leu1-32 ade6-21x h-*
DM3831	*pcs1-GFP::leu2+ Mde4-13Myc::KanR ura4-D18 leu1-32 ade6-210 h-*
DM3835	*mde4-13Myc::KanR cdc25-22 leu1-32? ura4-D18 ade6? h-*
DM3839	*mde4-13Myc::KanR clp1-C286S-GFP::ura4+ cdc25-22 leu1-32? ura4-D18 ade6? h-*
DM3911	*mde4-GFP::KanR leu1-32 ura4-D18 ade6-216 h-*
DM3912	*mde4Δ::KanR ura4-D18 leu1-32 ade6-21X h+*
DM3917	*mde4Δ::ura4+ leu1-32 ura4-D18 ade6-210 h-*
DM3962	*mde4Δ::ura leu1-32::pSV40-GFP-atb2-leu1+ ura4-D18 leu1-32 ade6? h-*
DM3989	*pcs1Δ::ura4+ leu1-32::pSV40-GFP-atb2-leu1+ ura4-D18 leu1-32 ade6? h-*
DM4111	*pcs1-GFP::leu2+ leu1-32 ura4-D18 ade6-210 h-*
DM4117	*pcs1-13Myc::KanR leu1-32 ura4-D18 ade6-216 h+*
DM4172	*mad2-GFP::KanR mde4Δ::Ura4+ ura4-D18 leu1-32 h+*
DM4218	*pcs1-GFP::KanR leu1-32 ura4-D18 ade6-210 h-*
DM4243	*pcs1-GFP::KanR mde4Δ::KanR leu1-32 ura4-D18 ade6-21X h?*
DM4331	*pcs1-GFP::KanR cdc11-GFP::KanR ura4-D18 leu1-32 ade6-21x h+*
DM4333	*pcs1-GFP::KanR cdc11-GFP::KanR clp1Δ::ura4+ ura4-D18 leu1-32 ade6? h-*
DM4336	*mde4-12A leu1-32 ura4-D18 ade6-210 h-*
DM4338	*pcs1-GFP::KanR mde4-12A leu1-32 ura4-D18 ade6-210 h+*
DM4362	*mde4-12A-13Myc::kanR leu1-32 ura4-D18 ade6-210 h-*
DM4363	*mde4-12D leu1-32 ura4-D18 ade6-210 h-*
DM4365	*pcs1-GFP::KanR mde4Δ::mde4_wt leu1-32 ura4-D18 ade6-210 h+*
DM4372	*pcs1-GFP::KanR mde4-12D leu1-32 ura4-D18 ade6-210 h+*
DM4408	*mde4Δ::mde4_wt-GFP::KanR leu1-32 ura4-D18 ade6-210 h-*
DM4410	*mde4-12A-GFP::kanR leu1-32 ura4-D18 ade6-210 h-*

DM4412	*mde4-12D-GFP::kanR leu1-32 ura4-D18 ade6-210 h-*
DM4435	*sid4-mRFP::ura4+ mde4-GFP::KanR ura4-D18 leu1-32 ade6? h+*
DM4437	*sid4-mRFP::ura4+ mde4-12D-GFP::KanR ura4-D18 leu1-32 ade6? h-*
DM4438	*sid4-mRFP::ura4+ mde4-12A-GFP::KanR ura4-D18 leu1-32 ade6? h+*
DM4443	*hht2-GFP::ura4+ mde4Δ::ura4+ ura4-D18 leu1-32 ade6? h+*
DM4450	*nuf2-mRFP::Ura4+ mde4-wt-GFP::KanR his2? leu1-32 ura4-D18 h+*
DM4452	*nuf2-mRFP::Ura4+ mde4-12A-GFP::KanR his2? leu1-32 ura4-D18 ade6? h+*
DM4453	*nuf2-mRFP::Ura4+ mde4-12D-GFP::KanR his2? leu1-32 ura4-D18 ade6? h+*
DM4474	*hht2-GFP::ura4+ ura4-D18 leu1-32 h+*
DM4491	*uch2-GFP::ura4+ ura4-D18 leu1-32 h+*
DM4509	*leu1-32::pSV40-GFP-atb2-Leu1+ mde4-12A ura4-D18 leu1-32 ade6? h-*
DM4510	*leu1-32::pSV40-GFP-atb2-Leu1+ mde4-12D ura4-D18 leu1-32 ade6? h-*
DM4547	*mde4-12A-GFP::KanR clp1-C286S-13Myc::KanR leu1-32 ura4-D18 ade6-21X h+*
DM4551	*mde4-12A-13Myc::KanR nda3-KM311 leu1-32 ura4-D18 ade6? h+*
DM4604	*mde4-12A-GFP::KanR leu1-32 ura4-D18 ade6-210 h+*
DM4646	*mad2Δ::URA4 mde4-12D-GFP::KanR leu1-32 ura4-D18 ade6? h-*
DM4648	*bub1Δ::URA4 mde4-12D-GFP::KanR leu1-32 ura4-D18 ade6-21X h+*
DM4650	*mad1Δ::URA4 mde4-12D-GFP::KanR leu1-32 ura4-D18 ade6? h+*
DM4652	*mad3Δ::URA4 mde4-12D-GFP::KanR leu1-32 ura4-D18 ade6-21X h+*
DM4737	*mad2-GFP::KanR mde4-12D ura4-D18 leu1-32 h-*
DM4793	*mde4Δ::KanR uch1-GFP::ura4+ ura4-D18 leu1-32 ade6? h+*
DM4959	*mde4-12A-GFP::KanR clp1Δ::ura4+ ura4-D18 leu1-32 h+*
DM4960	*pcs1-GFP::leu+ mde4-12A-13myc::KanR clp1Δ::ura4+ ura4-D18 leu1-32 h-*
DM5005	*Mad2-GFP::KanR mde4-12A ura4-D18 leu1-32 h+*