PNAS
www.pnas.org

Supplementary Information for

# Autophosphorylation is sufficient to release Mps1 kinase from native kinetochores

Lori B. Koch, Kwaku N. Opoku, Yi Deng, Adrienne Barber, Aimee J. Littleton, Nitobe London, Sue Biggins, Charles L. Asbury.

Correspondence: casbury@uw.edu; sbiggins@fredhutch.org

**This PDF file includes:**

Supplementary Methods
Supplementary Figures S1 to S5
Supplementary Tables S1 to S3
Caption for Supplementary Movie S1
Supplementary References

**Other supplementary materials for this manuscript include the following:**

Supplementary Movie S1



www.pnas.org/cgi/doi/10.1073/pnas.1901653116

## Supplementary Methods

Yeast strain and plasmid construction

*Saccharomyces cerevisiae* strains used in this study are isogenic with the W303 background and described in Supplementary Table S1. Standard genetic techniques were used. *DSN1-6His-3Flag* is described in (1). *MPS1-3V5, PDS1-18Myc,* and *DSN1-3Flag* were made by a PCR-based integration system and confirmed by PCR (6, 7). A strain with *NDC80-3V5-IAA17:KANMX* was made using a standard PCR-based integration system with primers SB1510 and SB1511 and template pSB2067, a gift from Leon Chan, Weis Lab. This strain was subsequently crossed several times and SBY12352 is a derivative. The auxin degron tagging system is described in (8). Strains with *mps1Δ::KanMX:10Myc-MPS1:TRP1* were made by crossing a strain, 2964, provided by Mark Winey (SBY3857). Strains with *MPS1-SNAP* were made by crossing an MTW1-CLIP, DSN1-6His-3FLAG strain from our previous work (SBY10327) with a strain, SBY12459, made using a standard PCR-based integration system with primers SB4170 and SB4171 and template pSB1821 (3, 9). *Dsn1-2D-6His-3Flag* strains were made by crossing to a strain made by transformation of a PCR product from plasmid pSB2439 with primers SB654 and SB2435. All strains and corresponding protocols are available by request.

Spindle assembly checkpoint silencing assays

Spindle assembly checkpoint silencing assays were performed by arresting cells with 10 µg/ml nocodazole for 2 hours, washing cells 3 times in fresh media lacking nocodazole, then resuspending cells in YPDA (YEP + 2% glucose + 0.02% adenine) and taking samples at the indicated times. 1 µg/ml alpha factor was added to the cultures 40-50 minutes after the beginning of the time course to prevent cells from entering a second cell cycle. All experiments were repeated at least 2-3 times.

Purification of native kinetochore particles and Mps1

Kinetochore particles were purified from asynchronously growing yeast by anti-Flag IP of Dsn1-6His-3Flag or Dsn1-3Flag as described in (1) with modifications to the lysis method described in (2). Mps1 was purified from asynchronously growing yeast by anti-V5 IP of Mps1-3V5. Cells

were grown to mid-log in yeast extract peptone dextrose media (YPD) supplemented with 0.2 mM adenine. After harvesting cells, they were washed once in water with 2 mM PMSF and then once in buffer H (25 mM HEPES pH 8.0, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM EGTA, 0.1% NP-40, 15% glycerol with 150 mM KCl for kinetochores or 750 mM KCl for Mps1) with protease inhibitors (at 20 µg/mL final concentration for each of leupeptin, pepstatin A, chymostatin and 200 µM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (0.1 mM Na-orthovanadate, 0.2 µM microcystin, 2 mM β-glycerophosphate, 1 mM Na pyrophosphate, 5 mM NaF). Pelleted cells were resuspended in buffer H (with 150 mM KCl for kinetochores and 750 mM KCl for Mps1) with protease inhibitors and phosphatase inhibitors and then added dropwise to liquid nitrogen to flash freeze. Flash frozen resuspended lysate was processed in a Freezer/Mill (SPEX SamplePrep) submerged in liquid nitrogen and the lysate was clarified by ultracentrifugation at 98,500 g for 90 min at 4 °C. Anti-Flag or anti-V5 conjugated Protein G Dynabeads (Thermo Fisher #100.09D) were incubated with the appropriate lysates for 3 hours with constant rotation at 4 °C, followed by 3 washes with buffer H containing protease inhibitors, phosphatase inhibitors, 2 mM dithiothreitol (DTT) and either 0.15 M KCl (kinetochores) or 1 M KCl (Mps1). Next, beads were washed twice with buffer H containing 150 mM KCl and protease inhibitors. Proteins were eluted from the beads by gentle agitation in elution buffer (0.5 mg/ml 3Flag peptide or 0.5 mg/ml 3V5 peptide in buffer H with 0.15 M KCl and protease inhibitors) for 25 min at room temperature or by boiling in SDS sample buffer. The concentrations of purified kinetochores and Mps1 were determined by comparing the purified material with BSA standards on silver-stained SDS-PAGE gels.

Bulk kinetochore-microtubule binding assay

Alexa 647-labeled microtubules were polymerized by incubating a 1:50 mixture of Alexa 647-labeled bovine tubulin to unlabeled bovine tubulin in polymerization buffer (80 mM PIPES, 1.2 mM $MgCl_2$, 1 mM GTP, 5.7% (v/v) DMSO, 1 mM EGTA pH 6.8). Following polymerization, taxol was added to a final concentration of 10 µM and the microtubules were sheared by passing the solution through a 27½ G needle 10 times. Microtubules were pelleted by centrifugation at 170,000 g for 10 minutes at 23 °C and resuspended in BRB80 with 20 µM taxol. Following the standard purification protocol, kinetochores maintained on beads or mock-treated beads were washed once with 1X BRB80 (80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8) with 20 µM

taxol. To perform the binding assay, the beads were then incubated in 1X BRB80 supplemented with 1 mg/ml κ-casein, 20 μM taxol and either unpolymerized tubulin (0.05 mg/ml final concentration) or taxol-stabilized microtubules (0.009 mg/ml final concentration) with constant rotation for 25 minutes at room temperature. The concentration of microtubules added to the reactions was calculated by comparing the fluorescent intensity of unpolymerized tubulin and the taxol-stabilized microtubule tubulin on an SDS-PAGE gel imaged with a Typhoon Trio (GE Healthcare). Following the binding reaction, the beads were washed once in BRB80 with 20 μM taxol and then the bead-associated proteins eluted by boiling in SDS sample buffer. The kinetochore proteins Dsn1 and Mps1 were visualized by immunoblotting.

Immunoblot Analysis

Immunoblotting and detection using chemiluminescence was performed as described in (2). Commercial antibodies used for immunoblotting were: 9E10 (Covance) at a 1:10,000 dilution for the Myc tag, anti-Flag M2 antibodies (Sigma-Aldrich) at 1:3,000, anti-Pgk1 antibodies (Invitrogen) at 1:10,000, anti-V5 (Invitrogen) at 1:5,000, and rat anti-alpha-tubulin (Accurate Chemical & Scientific) at 1:1,000. Anti-Spc105 antibodies were used at 1:10,000 (1). The anti-Ndc80 antibodies (OD4) were a kind gift from Arshad Desai and were used at a 1:10,000 dilution.

Isolation of fluorescent kinetochore particles

For direct observation of Mps1 levels by fluorescence, we isolated kinetochore particles from yeast strains in which Mps1 and a core kinetochore protein, Mtw1, were tagged with SNAP and CLIP, respectively. These tags allowed the proteins to be efficiently labeled with bright, photostable fluorescent dyes, CLIP-Surface 647 and SNAP-Surface 549 (New England Biolabs), during kinetochore purification. Kinetochores were purified as described above, with the following modifications for fluorescent labeling. After immunoprecipitation of the kinetochores onto magnetic beads, the beads were washed three times in buffer H supplemented with 2 mM dithiothreitol (DTT) and then two times in buffer H/0.15 lacking DTT. The kinetochores were then labeled by suspension in buffer H/0.15 plus 30 μM SNAP-Surface-549 and 30 μM CLIP-Surface-647 for 25 min at room temperature with gentle agitation. Beads were washed an additional two times in buffer H/0.15 and kinetochores were then incubated in elution buffer (0.5 mg/mL 3Flag peptide in buffer H/0.15) for 30 min at room temperature with gentle agitation.

Quantification of the level of fluorescence after SDS-PAGE confirmed that the labeling reaction was specific, and that the amount of labeled protein in the eluate was maximized under these conditions (3). Laser trap assays confirmed that the fluorescent kinetochore particles were fully functional, forming attachments to growing microtubule tips with normal rupture strength.

Preparation of flow channels for fluorescent imaging

Glass coverslips and slides were cleaned in a benchtop plasma cleaner (model PDC-001, Harrick Plasma) for 5 min. Strips of double-sided tape (3M) were then laid onto each slide, perpendicular to the long edge of the slide, and coverslips were pressed onto the tape to create narrow channels, ~10 µL in volume. Before use, each assembly was warmed on a 60°C hot plate for 30 min and pressed again, to create a tighter seal between the double-sided tape, the coverslip, and the slide. Two or three channels were usually created side-by-side on a single slide, and small droplets of nail polish were added in-between the inlets and outlets to prevent fluids pipetted onto one channel from flowing into an adjacent channel.

To block non-specific binding of kinetochore particles, each flow channel was coated with a supported lipid bilayer prepared in the following manner. First, a lipid mixture including a small fraction (~0.1%) of biotinylated lipid was created by dissolving 300 µg of POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; Avanti Polar Lipids, Inc.) and 0.4 µg of biotinyl-cap-PE (1,2-dieoleoyl-*sn*-glycero-3-phosphoethanolamine-N-(cap biotinyl) sodium salt; Avanti) in ~100 µL of chloroform in a glass test tube. The lipid solution was thoroughly mixed, dried by flowing nitrogen over it for 5 min while simultaneously rotating the tube, and then desiccated in a vacuum chamber overnight. Dried lipid 'cakes' were stored for up to several weeks in a vacuum desiccator. A lipid cake was rehydrated by adding 300 µL BRB80 buffer (80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA pH 6.9) and vortexing vigorously. Rehydrated lipids were then sonicated using a clean tip sonicator (model S450A, Branson) submerged directly into the sample for 5 min at 50% duty cycle and a low power setting. The initially cloudy lipid solution was clarified by the direct sonication, confirming that small unilamellar vesicles were created. Clarified lipids were used for up to several hours after the sonication. Each flow channel was pre-wetted with 10 µL of BRB80, coated by adding 10 µL of the clarified lipid mixture and incubating 4 min at room temperature, and then washed with 50 µL of BRB80.

Imaging of coverslip-tethered fluorescent kinetochore particles

Isolated kinetochore particles were tethered specifically to lipid-coated coverslip surfaces for viewing by fluorescence. First, 10 µL of 0.25 mg/mL streptavidin (Sigma-Aldrich) in BRB80 was introduced into a lipid-coated flow channel, incubated 4 min, and then washed with 50 µL BRB80. 10 µL of 20 µM biotinylated anti-Penta-His antibody (Qiagen) in BRB80 was then introduced, incubated 5 min, and washed in the same manner. Fluorescent kinetochore particles were diluted in BRB80 to a concentration corresponding to ~140 pM Dsn1, introduced into the channel, incubated 5 min, and then washed with 50 µL of BRB80 supplemented with 0.1 mg/mL κ-casein and an oxygen scavenging system (1 mM DTT, 250 µg/mL glucose oxidase, 30 µg/mL catalase, 25 mM glucose). The inlet and outlet of the channel were sealed, and individual kinetochores on the coverslip surface were viewed in a custom-built multi-color TIRF microscope with a computer-controlled 3-axis piezo specimen stage (4). An automated procedure was developed to rapidly record >200 images for each sample, using LabView software to raster the specimen stage while maintaining image focus. Using Mtw1-CLIP-647 as a fiducial marker, the concentration of kinetochore particles was adjusted to achieve surface densities between 50 and 300 particles per 1,500 $\mu m^2$ field of view. Individual bright spots were detected, and their brightness quantified by integrating over square, 7-by-7-pixel regions (0.28 $\mu m^2$ at the specimen) centered on the maxima. Additional details describing the image analysis are given below.

Imaging of fluorescent kinetochores attached laterally to microtubules

To view kinetochores attached laterally to microtubules, 10 µL of 0.25 mg/mL streptavidin (Sigma-Aldrich) in BRB80 was introduced into a lipid-coated channel, incubated 4 min, and then washed with 50 µL BRB80. 10 µL of 0.05 mg/mL biotinylated anti-α-tubulin antibody (BioLegend) was then introduced, incubated 5 min, and washed with 50 µL BRB80 plus 0.1 mg/mL κ-casein (BBC). 10 µL of Alexa-488-labeled, taxol-stabilized microtubules diluted in BBC were introduced, incubated 5 min, and washed with 50 µL BBC. Fluorescent kinetochore particles were then diluted in BBC to a concentration corresponding to ~140 pM Dsn1, introduced into the channel, incubated 5 min and then washed with BBC plus an oxygen scavenging system (described above). The inlet and outlet of the channel were sealed, and

individual kinetochores and microtubules were viewed in a custom, multi-color TIRF microscope, as described above.

Imaging of fluorescent kinetochores attached to the tips of microtubules

To view tip-attached kinetochores, fluorescent kinetochore particles were first tethered specifically to a lipid-coated coverslip as described above. Short, Alexa-488-labeled microtubules (5 to 10 μm in length) diluted in BBC were then introduced, incubated 5 min, and washed with 50 μL BBC. By thermal diffusion, the microtubules tended to attach to the kinetochores primarily by their ends. The inlet and outlet of the channel were sealed, and individual kinetochores and microtubules were viewed by multi-color TIRF microscopy. End-captured microtubules swiveled freely about their kinetochore-attached ends, occasionally also becoming attached via their sides to additional surface-tethered kinetochores. Surface-tethered kinetochores in the same fields of view that had not captured a microtubule served as internal controls.

Imaging of fluorescent kinetochores tracking with disassembling microtubule tips

To view kinetochores tracking processively with disassembling tips, dynamic microtubule extensions were first grown from coverslip anchored seeds. 10 μL of 0.25 mg/mL streptavidin (Sigma-Aldrich) in BRB80 was introduced into a lipid-coated channel, incubated 4 min, and then washed with 50 μL BRB80. 10 μL of biotinylated, GMPCPP-stabilized microtubule seeds in BRB80 were introduced, and incubated 5 min. 49 μL of microtubule growth buffer was prepared, consisting of 1.5 mg/mL fluorescent tubulin in BBC supplemented with 1 mM GTP and an oxygen scavenging system (described above). 1 μL of undiluted fluorescent kinetochore stock was then added, and the growth buffer was mixed and introduced into the flow channel. 10 min incubation at room temperature allowed fluorescent microtubule extensions to grow from the surface-anchored GMPCPP seeds and kinetochores to bind laterally to the extensions. Disassembly of the microtubules was then triggered by buffer exchange, introducing 15 μL BBC while simultaneously acquiring images by TIRF microscopy. All fluorescence imaging experiments were performed at room temperature.

Analysis of images of fluorescent kinetochores

For each image, we applied the method of (5) to find all local maxima separated by at least 7 pixels. Integrated brightness values were then computed by summing the intensities within square, 7-by-7-pixel regions (0.28 $\mu m^2$ at the specimen) surrounding every local maximum in both color channels. Histograms of these integrated brightness values showed a clear separation between the individual SNAP- and CLIP-dyes and the background fluorescence levels, allowing thresholds to be set for distinguishing dye-labeled particles from background noise. Individual kinetochore particles were identified by the presence of Mtw1-CLIP-647. After registration of the color channels (4), each kinetochore particle was classified as single-color, carrying only Mtw1-CLIP but lacking Mps1-SNAP-549, or dual-color, carrying both Mtw1 and Mps1. Colocalization was then calculated for each image by dividing the number of dual-color particles by the total number of particles. The distributions of integrated brightness values included sub-populations with just one detectable dye molecule, identifiable by their single-step photobleaching behavior, from which the unitary brightness for both Mps1-SNAP-549 and Mtw1-CLIP-647 was estimated (3). Ratios of Mps1 to Mtw1 molecules were then estimated for every particle by dividing their integrated brightness values in the 549-nm and 647-nm color channels by the corresponding unitary brightness and computing the ratio, Mps1-SNAP-549 to Mtw1-CLIP-647.

Kinase Assays

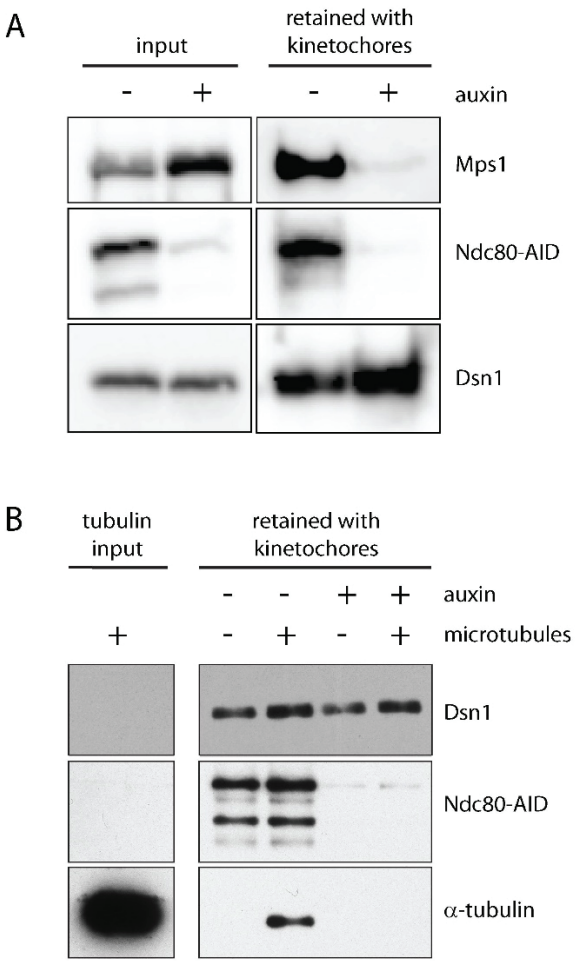
Kinetochores or Mps1 were purified and maintained on beads for kinase assays. After the purification, they were washed once in buffer H/0.15 without inhibitors and then incubated in kinase buffer (28 mM HEPES pH 8.0, 2.8 mM $MgCl_2$, 143 mM KCl, 14% glycerol, 0.1 mM EDTA, 0.5 mM EGTA, 0.1% NP-40, with or without 200 μM ATP) for the indicated period of time in a 30 °C water bath. The kinase buffer was made by diluting 10X kinase buffer (0.5 M HEPES pH 8.0, 0.1 M $MgCl_2$, 0.75 M KCl, 5% glycerol, 2 mM ATP) with buffer H/0.15. To assess release of endogenous Mps1, the supernatants were collected, and the bead-associated proteins were washed once in buffer H/0.15 with protease and phosphatase inhibitors prior to elution. Supernatants and bead-associated proteins were eluted by boiling in SDS sample buffer.

Bulk Binding Assays

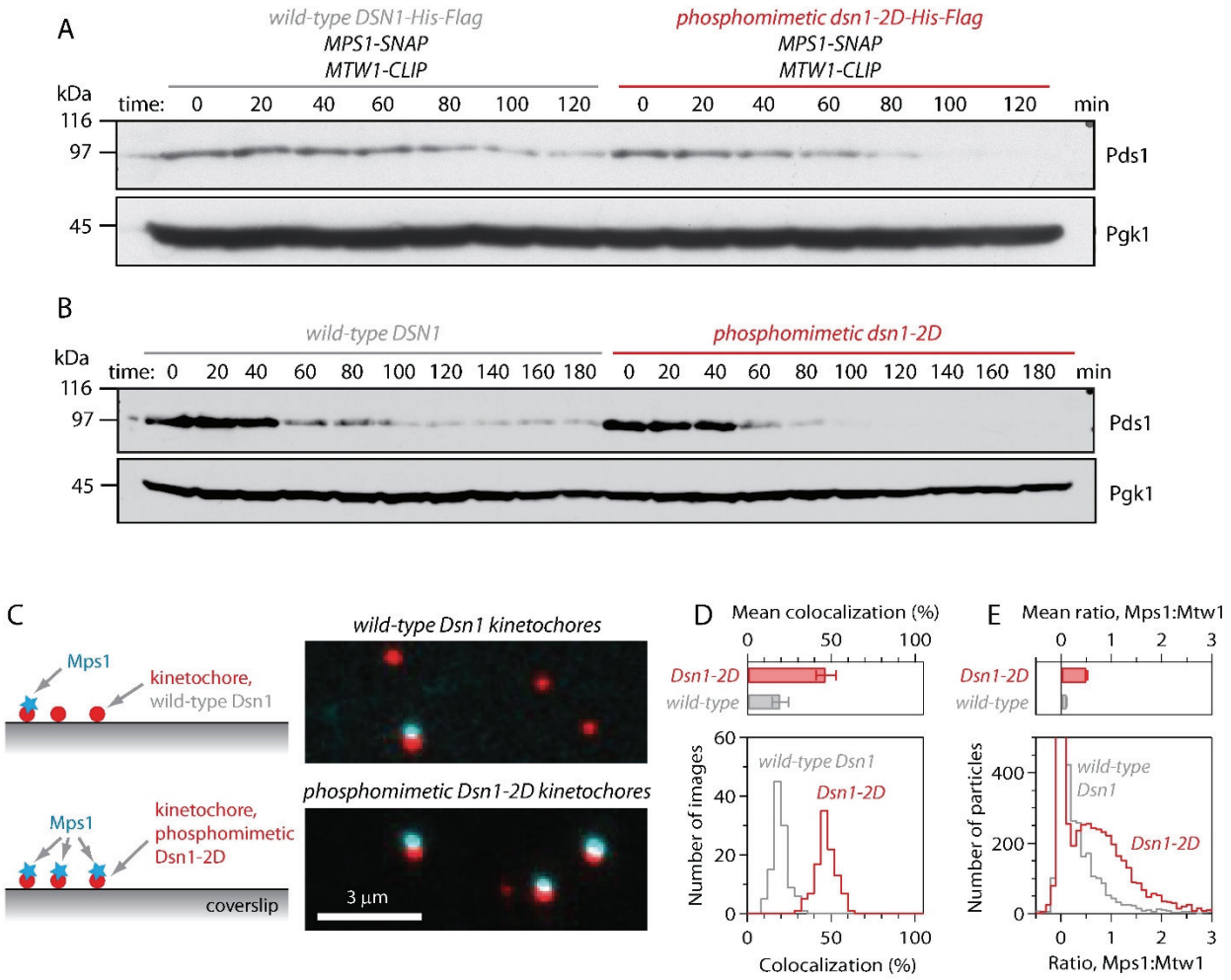
To generate phosphorylated kinetochores lacking Mps1, a kinase reaction was performed with purified kinetochores maintained on beads. Mock reactions were also performed with beads generated from a mock IP with lysate lacking a Flag epitope-tagged protein. Next, the bead-associated proteins were washed twice in buffer H/0.15 without added inhibitors before going through subsequent treatments detailed below.

*Assay to analyze the effect of kinetochore phosphorylation on Mps1 binding*: Bead-associated kinetochores or mock-treated beads were incubated in buffer H/0.15 supplemented with 1mM $MnCl_2$ and 200 units of λ-phosphatase (NEB#PO753L) in a 30 °C water bath for 20 minutes. Mock phosphatase reactions also included phosphatase inhibitors (0.1 mM Na-orthovanadate, 0.2 μM microcystin, 2 mM β-glycerophosphate, 1 mM Na pyrophosphate, 5 mM NaF). Next, the beads were washed 3 times in buffer H/0.15 with protease and phosphatase inhibitors. Each reaction was then incubated with 1 ng of purified native Mps1-V5 in buffer H/0.15 with protease inhibitors and phosphatase inhibitors with gentle agitation at room temperature for 25 minutes. Following the binding reaction, bead-associated proteins were washed once in buffer H/0.15 with protease and phosphatase inhibitors and the proteins were eluted by boiling in SDS sample buffer.

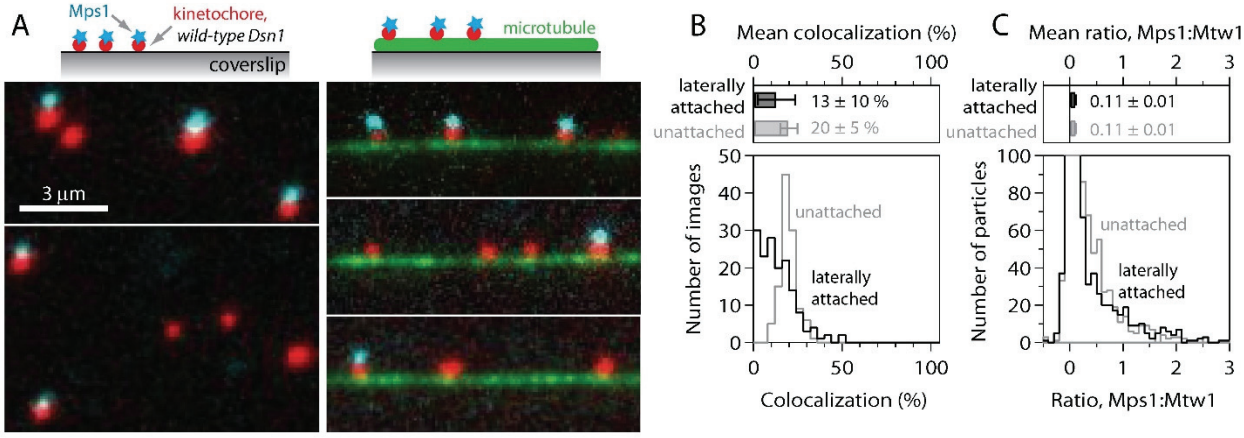
*Assay to analyze the effect of Mps1 autophosphorylation on kinetochore binding*: All kinetochores or mock-treated beads were dephosphorylated by incubation in buffer H/0.15 supplemented with 1 mM $MnCl_2$ and 200 units of λ-phosphatase (NEB#PO753L) in a 30 °C water bath for 20 minutes. Next, bead-associated proteins were washed twice in buffer H/0.15 with protease and phosphatase inhibitors. Each reaction of bead-bound kinetochores or mock treated beads was then incubated with 5 ng native Mps1-V5 or 5 ng autophosphorylated P-Mps1-V5 in buffer H/0.15. After the binding reactions, the bead-associated proteins were washed once in buffer H/0.15 with protease and phosphatase inhibitors and the proteins were eluted by boiling in SDS sample buffer.



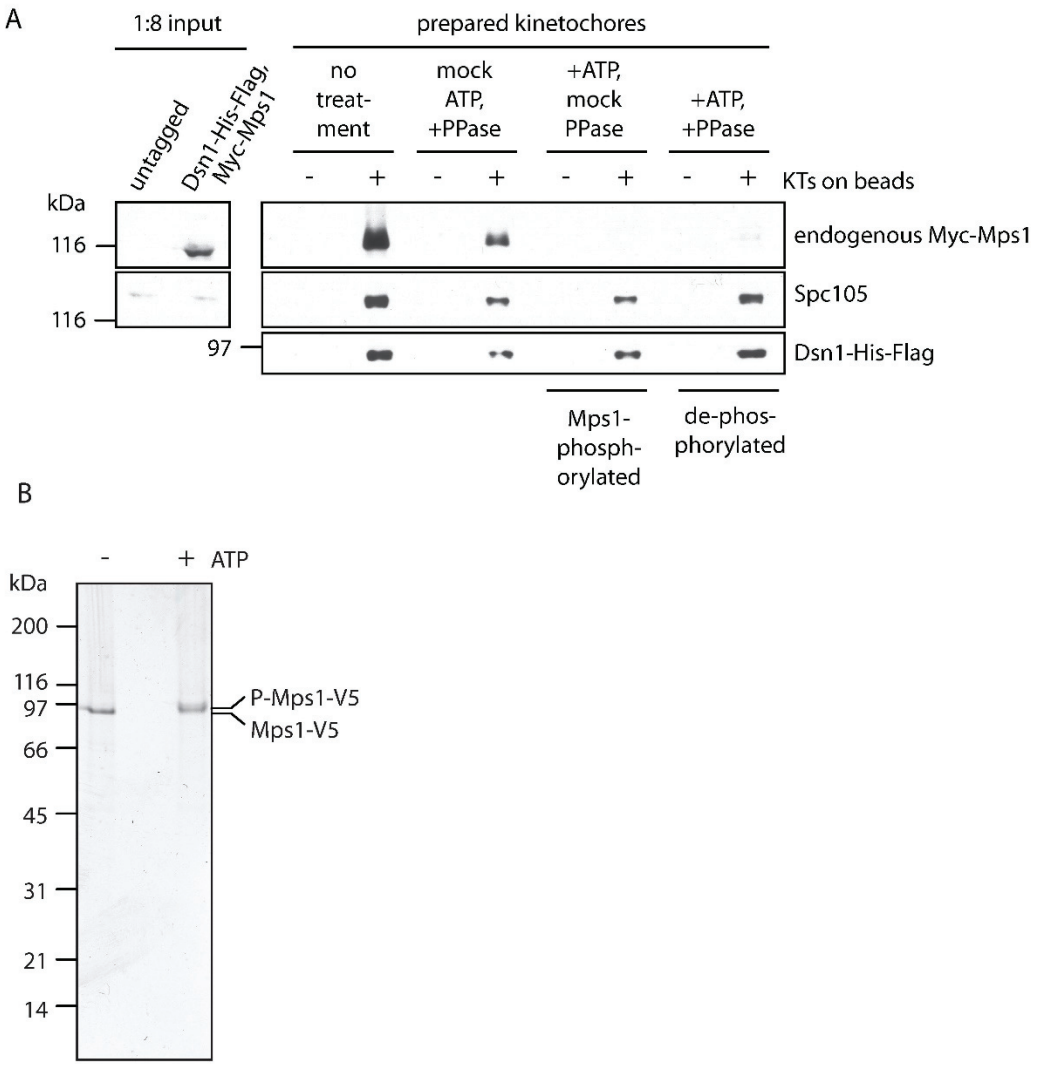

**Supplementary Figure S1. Ndc80 is required for retention of Mps1 on purified kinetochores and for microtubule attachment.** Yeast expressing Ndc80-AID (SBY12352) were grown to mid-log phase and then 500 μM of auxin (3-indoleacetic acid, IAA) or vehicle alone (DMSO) was added. Cells were harvested two hours later. (A) Kinetochores were bound to beads, washed, and then the levels of Mps1 and kinetochore proteins were assessed by SDS-PAGE followed by immunoblotting. (B) Kinetochores were bound to beads and either mock-treated or incubated with taxol-stabilized microtubules. The levels of kinetochore proteins and the amount of α-tubulin retained after washing the beads were then assessed by SDS-PAGE followed by immunoblotting.



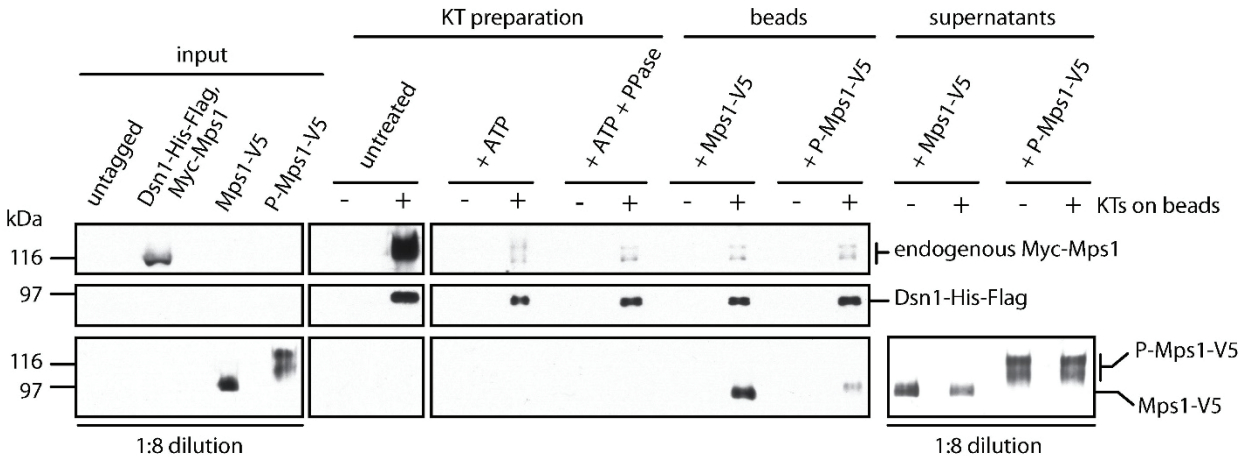

**Supplementary Figure S2. Phosphomimetic Dsn1-2D improves retention of Mps1 on isolated kinetochores without disrupting checkpoint silencing in vivo.** (A and B) *dsn1-2D* cells silence the spindle assembly checkpoint with wild-type-like kinetics. Yeast strains with wild-type Dsn1 or with phosphomimetic Dsn1-2D were arrested in prometaphase by exposure for 3 hours to the microtubule-depolymerizing drug, nocodazole. After nocodazole was washed out of the media, samples were collected at indicated timepoints and analyzed for levels of the anaphase inhibitor, Pds1 (securin), as well as a loading control, Pgk1 (phosphoglycerate kinase 1), by SDS-PAGE and immunoblotting. Sustained levels of Pds1 during nocodazole arrest indicate that the cells can generate robust checkpoint 'wait' signals. The drop in Pds1 levels 80 to 100 min after release from nocodazole indicates that the cells entered anaphase and therefore silenced the 'wait' signals. (A) Wild-type strain with *DSN1-His-Flag*, *MPS1-SNAP*, and *MTW1-CLIP* (SBY16381), versus phosphomimetic strain with *dsn1-2D-His-Flag*, *MPS1-SNAP*, and *MTW1-CLIP* (SBY16417). (B) Wild-type strain with *DSN1* (SBY4880), versus phosphomimetic strain with *dsn1-2D* (SBY8066). (C) Schematic diagram (left) and fluorescence images (right) of kinetochore particles carrying wild-type Dsn1 (top, from SBY12571) or phosphomimetic Dsn1-2D (bottom, from SBY15285). Both were labeled with Mps1-SNAP-549 (cyan) and Mtw1-CLIP-647 (red) and tethered to coverslips. Colors are offset vertically; cyan-red pairs are colocalized particles. (D) Percentages of kinetochore particles retaining Mps1. Bars show means (± SD) from N = 111 and 108 images of wild-type and Dsn1-2D particles, respectively. Histograms show corresponding distributions. (E) Approximate ratios of Mps1 to Mtw1 molecules, estimated from particle brightness relative to the brightness of single Mps1-SNAP-549 and Mtw1-CLIP-647 molecules. Bars show mean ratios (± SEM) from N = 8,863 and 6,891 wild-type and Dsn1-2D particles, respectively. Histograms show corresponding distributions.




**Supplementary Figure S3. Isolated wild-type kinetochores retain Mps1 when attached laterally to microtubules.** (A) Fluorescence images of wild-type kinetochore particles (from SBY16381) carrying Mps1-SNAP-549 (cyan) and Mtw1-CLIP-647 (red), tethered to coverslips (left) or attached laterally to microtubules (green, right). Colors are offset vertically; cyan-red pairs are colocalized, dual-color particles. Fields containing multiple colocalized particles, as shown in the topmost images, were rare. (B) Percentages of wild-type kinetochore particles retaining Mps1. Bars show means (± SD) from N = 157 and 111 images of microtubule-attached and coverslip-tethered particles, respectively. Histograms show corresponding distributions. (C) Approximate ratios of Mps1 to Mtw1 molecules, estimated from particle brightness relative to the brightness of single Mps1-SNAP-549 and Mtw1-CLIP-647 molecules. Bars show mean ratios (± SEM) from N = 2,540 and 2,500 laterally attached and coverslip-tethered particles, respectively. Histograms show corresponding distributions.




**Supplementary Figure S4. Preparation of purified kinetochores and Mps1 for binding experiments.** (A) Preparation of kinetochores phosphorylated by Mps1 or de-phosphorylated and lacking endogenous Mps1, for use in the experiment of Figure 4D. Kinetochores were purified (from SBY9190) via immunoprecipitation of Dsn1-His-Flag and maintained on beads. Control beads lacking kinetochores were also generated by immunoprecipitation from a wild-type strain lacking any tagged proteins (SBY3). Beads with or without kinetochores were incubated in kinase buffer with or without ATP. The ATP-treated samples were then divided and treated either with λ-phosphatase or with λ-phosphatase plus phosphatase inhibitors. All samples were analyzed by SDS-PAGE and immunoblotting. (B) Full-length Mps1 purified from budding yeast and induced to autophosphorylate by treatment with ATP. Mps1-V5 was purified (from SBY12412) via immunoprecipitation under stringent conditions to remove co-purifying proteins, incubated with or without ATP, and analyzed by silver-stained SDS-PAGE.




**Supplementary Figure S5. Purified native Mps1, but not autophosphorylated Mps1, binds dephosphorylated kinetochores lacking endogenous Mps1.** Kinetochores (KTs) were purified (from SBY9190) via immunoprecipitation of Dsn1-His-Flag and maintained on beads. Immunoprecipitation from a wild-type strain lacking any tagged proteins (SBY3) generated control beads lacking kinetochores. Immobilized kinetochores and control beads were incubated in kinase buffer with ATP to release endogenous Myc-Mps1 (+ATP), washed, and then dephosphorylated with λ-phosphatase (+ATP +PPase). Following phosphatase treatment, kinetochores or control beads were washed and incubated with either purified Mps1-V5 or autophosphorylated P-Mps1-V5 (prepared as in Figure 4B). All samples were analyzed by SDS-PAGE and immunoblotting. This figure shows results from the same experiment as in Figure 4E. Additional inputs and preparative steps are included here to illustrate the ATP-dependent release of endogenous Mps1 from kinetochores.

**Supplementary Table S1. Yeast strains used in this study.**

| Strain | Genotype |
|---|---|
| SBY3 | *Mat* a *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 LYS2 can1-100 bar1*Δ |
| SBY3857 | *Mat* a *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 LYS2 can1-100 bar1-1 mps1::KAN::10Myc-MPS1:TRP1* |
| SBY4880 | *Mat* a *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 LYS2 can1-100 bar1-1 PDS1-18myc:LEU2* |
| SBY8066 | *Mat* a *ura3-1 leu2,3-112 his3-11::DSN1-2D:HIS3 trp1-1 ade2-1 lys2*Δ *can1-100 bar1-1 PDS1-18myc:LEU2 dsn1::KANMX* |
| SBY9190 | *Mat* a *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 LYS2 can1-100 bar1-1 DSN1-6His-3Flag:URA3 mps1::KAN::10Myc-MPS1:TRP1* |
| SBY10327 | *Mat* a *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 LYS2 can1-100 bar1-1 DSN1-6His-3Flag:URA3 MTW1-CLIP:KanMX* |
| SBY12412 | *Mat* a *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 LYS2 can1-100 bar1-1 MPS1-3V5:KanMX* |
| SBY12352 | *Mat* a *ura3-1 leu2,3-112 his3-11::pADH1-OsTIR1-myc:HIS3 trp1-1 DSN1-6His-3Flag:KanMx ade2-1 LYS2 can1-100 bar1-1 mps1::KanMx::10xmyc-MPS1::TRP1 Ndc80-3V5-IAA17:KanMX* |
| SBY12459 | *Mat* alpha *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 LYS2 can1-100 bar1-1 DSN1-6His-3Flag:URA3 MPS1-SNAP:HPH* |
| SBY12571 | *Mat* a *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 LYS2 can1-100 bar1-1 DSN1-6His-3Flag:URA3 MTW1-CLIP:KanMX MPS1-SNAP:HPH* |
| SBY15285 | *Mat* a *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 LYS2 can1-100 bar1-1 dsn1-2D-6His-3Flag:URA3 MTW1-CLIP:KanMX MPS1-SNAP:HPH* |
| SBY16381 | *Mat* a *ura3-1, leu2,3-112 his3-11 trp1-1 ade2-1 LYS2 can1-100 bar1-1 DSN1-6His-3Flag:URA3 MTW1-CLIP:KanMX MPS1-SNAP:HPH PDS1-18myc:LEU2* |
| SBY16417 | *Mat* a *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 LYS2 can1-100 bar1-1 dsn1-2D-6His-3Flag:URA3 MTW1-CLIP:KanMX MPS1-SNAP:HPH PDS1-18myc:LEU2* |

**Supplementary Table S2. Plasmids used in this study.**

| Plasmid | Description | Source |
|---|---|---|
| pSB1821 | *SNAP:HPH* | Aaron Hoskins, Moore Lab, U. Mass. Med. |
| pSB2067 | *pFA6-3V5-IAA17:KANMX* | Leon Chan, Karsten Weis Lab, U.C. Berkeley |
| pSB2439 | *dsn1-2D-6His-3Flag:URA3* | *this study* |

**Supplementary Table S3. Primers used in this study.**

| Primer | Description | Sequence (5' to 3') |
|---|---|---|
| SB654 | *DSN1* forward primer at 200bp | GTGTCGAATTTGAGCCCCG |
| SB1510 | *NDC80* C-terminal tagging forward | GGAAACGTCATTGAAGAGTTACGAAATTTGGAGTTTGAAACTGAACAT AACGTAACAAATcggatccccgggttaattaa |
| SB1511 | *NDC80* C-terminal tagging reverse | CGGAAAGGTGGGGCTGAGCTTTGCTGTAGATTGCTCGGGTATTATAT ATCATTTATTTTAgaattcgagctcgtttaaac |
| SB2435 | Reverse primer to amplify *DSN1-6His-3FLAG:URA3* from pSB2439 | GTATATATAAATGTATGACTGTGTAATGTTACATATGCAGAAGTATCCG ATTTTTTTTTGATTTTTTCTTTTATTGATTCGGTAATCTCCGAACAG |
| SB4170 | SNAP tagging C-terminus of *MPS1* forward | CAATGATGTGGTAGACACTGTTTTAAGGAAATTTGCAGATTACAAAATT GGT TCT GGT GGT TCT GGT atg gac aaa gac tgc gaa atg aag cgc ac |
| SB4171 | SNAP tagging C-terminus of *MPS1* reverse | G TAT TTA TGT TCA TAA CTG GCA CAT GCT TTT CTT CCT TAT GCG GCT CTT gagctcgttttcgacactggatggc |

**Caption for Supplementary Movie S1. Kinetochores retain Mps1 when tracking with disassembling microtubule tips.** A kinetochore particle (from SBY15285) tracks with the disassembling tip of a microtubule (green) while carrying Mps1-SNAP-549 (cyan) and Mtw1-CLIP-647 (red). Colors are deliberately offset; the cyan-red pair is a colocalized, dual-color kinetochore particle.

## Supplementary References


1. Akiyoshi B*, et al.* (2010) Tension directly stabilizes reconstituted kinetochore-microtubule attachments. *Nature* 468(7323):576-579.

2. Miller MP, Asbury CL, & Biggins S (2016) A TOG Protein Confers Tension Sensitivity to Kinetochore-Microtubule Attachments. *Cell* 165(6):1428-1439.

3. Sarangapani KK*, et al.* (2014) Sister kinetochores are mechanically fused during meiosis I in yeast. *Science* 346(6206):248-251.

4. Deng Y & Asbury CL (2017) Simultaneous Manipulation and Super-Resolution Fluorescence Imaging of Individual Kinetochores Coupled to Microtubule Tips. *Methods Mol Biol* 1486:437-467.

5. Crocker JC & Grier DG (1996) Methods of digital video microscopy for colloidal studies. *J Colloid Interf Sci* 179(1):298-310.

6. Knop M*, et al.* (1999) Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast* 15(10B):963-972.

7. Longtine MS*, et al.* (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. *Yeast* 14(10):953-961.

8. Nishimura K, Fukagawa T, Takisawa H, Kakimoto T, & Kanemaki M (2009) An auxin-based degron system for the rapid depletion of proteins in nonplant cells. *Nat Meth* 6(12):917-922.

9. Shcherbakova I*, et al.* (2013) Alternative spliceosome assembly pathways revealed by single-molecule fluorescence microscopy. *Cell reports* 5(1):151-165.