

Mps1 phosphorylation of condensin II controls chromosome condensation at the onset of mitosis

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During mitosis, genomic DNA is condensed into chromosomes to promote its equal segregation into daughter cells. Chromosome condensation occurs during cell cycle progression from G2 phase to mitosis. Failure of chromosome compaction at prophase leads to subsequent misregulation of chromosomes. However, the molecular mechanism that controls the early phase of mitotic chromosome condensation is largely unknown. Here, we show that Mps1 regulates initial chromosome condensation during mitosis. We identify condensin II

as a novel Mps1-associated protein. Mps1 phosphorylates one of the condensin II subunits, CAP-H2, at Ser492 during mitosis, and this phosphorylation event is required for the proper loading of condensin II on chromatin. Depletion of Mps1 inhibits chromosomal targeting of condensin II and accurate chromosome condensation during prophase. These findings demonstrate that Mps1 governs chromosomal organization during the early stage of mitosis to facilitate proper chromosome segregation.

Introduction

From yeast to vertebrate cells, Mps1, a dual specificity kinase, plays an essential role in mitotic progression. Mps1 participates in multiple aspects of mitosis: the spindle assembly checkpoint (SAC), chromosome alignment/segregation, and cytokinesis (Liu and Winey, 2012). Previously, several Mps1-associated proteins have been identified. In yeast, the Mps1-mediated phosphorylation of Dam1 contributes to the connection of the kinetochore with microtubules (Shimogawa et al., 2006). Ndc80, a homologue of the mammalian Hec1 protein, is phosphorylated by Mps1 to activate SAC signaling and mitotic arrest (Kemmler et al., 2009). Recently, the kinetochore proteins KNL1/Spc105 and Spc7 were reported as key substrates of Mps1 in SAC signaling (London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012). In mammalian cells, Mps1-dependent phosphorylation of BLM is important for ensuring accurate chromosome segregation (Leng et al., 2006). Moreover, Mps1 phosphorylates Borealin/DasraB, a subunit of the chromosome passenger complex, to control chromosome alignment (Jelluma et al., 2008). These findings collectively demonstrate that Mps1 can control mitotic progression by the phosphorylation of mitotic regulators. However, substrates and

mechanisms by which Mps1 affects mitotic progression remain to be elucidated.

Condensin is a highly conserved complex that contributes to mitotic chromosome condensation and segregation (Hirano, 2012). In vertebrate cells, two types of condensin complexes, condensin I and II, were discovered. The SMC heterodimer, which is composed of SMC2 and SMC4, is a common component of condensin I and II. In contrast, three of the non-SMC subunits are separate, yet similar, proteins. These subunits are CAP-H, CAP-G, and CAP-D2 for condensin I, and CAP-H2, CAP-G2, and CAP-D3 for condensin II. The condensin function is regulated by phosphorylation. A previous study reported that Cdk1 regulates condensin I activity to induce the supercoiling of DNA (Kimura et al., 1998). In several organisms, Aurora B contributes to the association of condensin I to mitotic chromosomes (Giet and Glover, 2001; Lipp et al., 2007; Takemoto et al., 2007; Tada et al., 2011). In budding yeast, the polo kinase Cdc5 phosphorylates condensin and promotes chromosomal condensation (St-Pierre et al., 2009). A recent study has shown that in human cells, Cdk1 and Plk1 regulate activity of condensin II during mitosis (Abe et al., 2011). However, kinases responsible

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Abbreviations used in this paper: 2DICAL, two-dimensional image-converted analysis of liquid chromatography and mass spectrometry; KD, kinase dead; WT, wild type.

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for phosphorylation of condensin to control its functions are largely unknown.

Here, we found that condensin II is a novel Mps1-associated protein and that the phosphorylation of CAP-H2 by Mps1 is required for the mitotic chromosomal localization of condensin II. We concluded that Mps1 plays a crucial role in mitotic chromosome condensation by regulating condensin II.

Results and discussion

Mps1 binds to condensin II in the nucleus

To identify proteins that associate with Mps1 during mitosis, we performed a mass spectrometry analysis. HeLa cells were treated with nocodazole, and the mitotic cell lysates were immunoprecipitated with anti-Mps1 antibodies. By silver staining and by proteome analysis, SMC2 was identified as an Mps1-associated protein (Fig. 1 A). Immunoblot analysis confirmed that Mps1 interacts with SMC2 during mitosis (Fig. 1 B). SMC2 is the core component of condensin complexes, which play essential roles in mitotic chromosome condensation. The condensin function is tightly regulated through the cell cycle. Thus, we next monitored the status of Mps1 binding to SMC2 during the cell cycle. HeLa cells were treated with nocodazole and released from mitosis. Immunoprecipitation and immunoblot analyses revealed that Mps1 is constitutively associated with SMC2 throughout the cell cycle (Fig. 1 C). Furthermore, the association of Mps1 with SMC2 was observed in asynchronous cells (Fig. 1 D).

Condensin I and II display discrete localization during the cell cycle (Hirano, 2012). Condensin I is primarily localized in the cytoplasm during interphase and prophase. By contrast, condensin II is predominantly localized in the nucleus. Given that Mps1 is localized in the nucleus and in the cytoplasm (Stucke et al., 2002; Nihira et al., 2008), we examined the subcellular localization of the association between Mps1 and SMC2. Subcellular fractionation and immunoprecipitation with anti-SMC2 demonstrated that nuclear, but not cytoplasmic, SMC2 specifically interacts with Mps1 (Fig. 1 E and Fig. S1 A). Furthermore, analysis of immunoprecipitates with anti-Mps1 from nuclear lysates revealed that nuclear, and not cytoplasmic, Mps1 interacted with CAP-H2, whereas the binding of Mps1 to CAP-H was not detected in either the nucleus or the cytoplasm (Fig. 1 F), suggesting that Mps1 is associated with condensin II and there is little if any interaction of Mps1 with condensin I. Taken together, these findings demonstrated that Mps1 constitutively interacts with condensin II in the nucleus during the cell cycle progression.

Mps1 phosphorylates CAP-H2 at Ser492

A previous study showed that the electrophoretic mobility of CAP-H2 is disturbed by phosphorylation during mitosis (Lipp et al., 2007). To examine the mobility of CAP-H2, HeLa cells were treated with nocodazole followed by the release from mitosis. Immunoblot analysis revealed that a band shift of CAP-H2 was observed only in mitotic cells (Fig. 2 A). To investigate whether Mps1 is necessary for the retention of the CAP-H2 band shift during mitosis, HeLa cells were transfected with scrambled siRNA or Mps1 siRNA followed by nocodazole treatment. The results indicated that the mobility retardation of

CAP-H2 was abolished in cells silenced for Mps1, suggesting the possibility that the band shift of CAP-H2 is caused by Mps1-mediated phosphorylation (Fig. 2 B). To identify the phosphorylation sites of CAP-H2 by Mps1, 293 cells were cotransfected with GFP-Mps1 wild-type (WT) or kinase-dead mutant (KD) and Flag-CAP-H2 (Fig. 2 C). Cell lysates were immunopurified with anti-Flag and were then applied for the mass spectrometry analysis. The mass spectral data were evaluated using a two-dimensional image-converted analysis of liquid chromatography and mass spectrometry (2DICAL; Matsubara et al., 2009; Miyamoto et al., 2012). We found that Ser492 is a potential phosphorylation residue of CAP-H2 by Mps1 (Fig. S2 A). To confirm that Mps1 phosphorylates CAP-H2 at Ser492, we raised a specific antibody against phosphorylated Ser492. Immunoblotting with anti-phospho-Ser492 indicated that the expression of GFP-Mps1-WT is associated with the substantial phosphorylation of Ser492 (Fig. 2 D). In contrast, this phosphorylation was completely abrogated by the expression of the GFP-Mps1-KD (Fig. 2 D). Of note, this Ser492 phosphorylation was diminished by treatment with calf intestinal alkaline phosphatase (Fig. S2 B), and the nonphosphorylatable Ser492 mutant, in which Ser492 is substituted to alanine (Flag-CAP-H2-S492A), prevented the reaction with anti-phospho-Ser492 (Fig. 2 D), indicating the specificity of the antibody against Ser492 phosphorylation. We also performed *in vitro* kinase assays using recombinant proteins. The results demonstrated that Mps1 phosphorylates Ser492 *in vitro* (Fig. 2 E). These findings suggest that Mps1 can directly phosphorylate CAP-H2 at Ser492.

Mps1 regulates the mitotic chromosomal localization of condensin II

As shown previously, the mobility shift of CAP-H2 was detected during mitosis (Fig. 2 A). In this context, we speculated that Ser492 phosphorylation was increased during mitosis. HeLa cells were arrested at G1/S using a double thymidine block or at mitosis using nocodazole treatment. Then, the arrest was released into cell cycling, and the phospho-Ser492 status was analyzed. The results indicated that Ser492 of CAP-H2 is phosphorylated during mitosis but not during other cell cycle phases (Fig. 3 A). Notably, the depletion of CAP-H2 from mitotic cells by two different siRNAs abolished the detection of phospho-Ser492, indicating the specificity of the antibody against CAP-H2 phosphorylation (Fig. S3 A). We next examined whether Mps1 phosphorylates CAP-H2 at Ser492 during mitosis. Immunoblot analysis revealed that the depletion of Mps1 abrogated the phosphorylation of Ser492 (Fig. 3 B). To determine if Mps1 regulates the targeting of condensin II to mitotic chromosomes, we performed chromosome fractionation assays (Fig. S1, B and C). The results indicated that the depletion of Mps1 was associated with the dissociation of CAP-H2 from mitotic chromosomes (Fig. 3 C). Furthermore, Ser492-phosphorylated CAP-H2 was enriched in the chromosome fraction of mitotic cells (Fig. 3 C). Chromosome spreading and immunofluorescence analysis also showed that the localization of CAP-H2 on mitotic chromosomes decreased in Mps1-depleted cells (Fig. 3, D and E). These findings demonstrate that Mps1 phosphorylates CAP-H2 and controls the chromosomal recruitment of condensin II during mitosis.

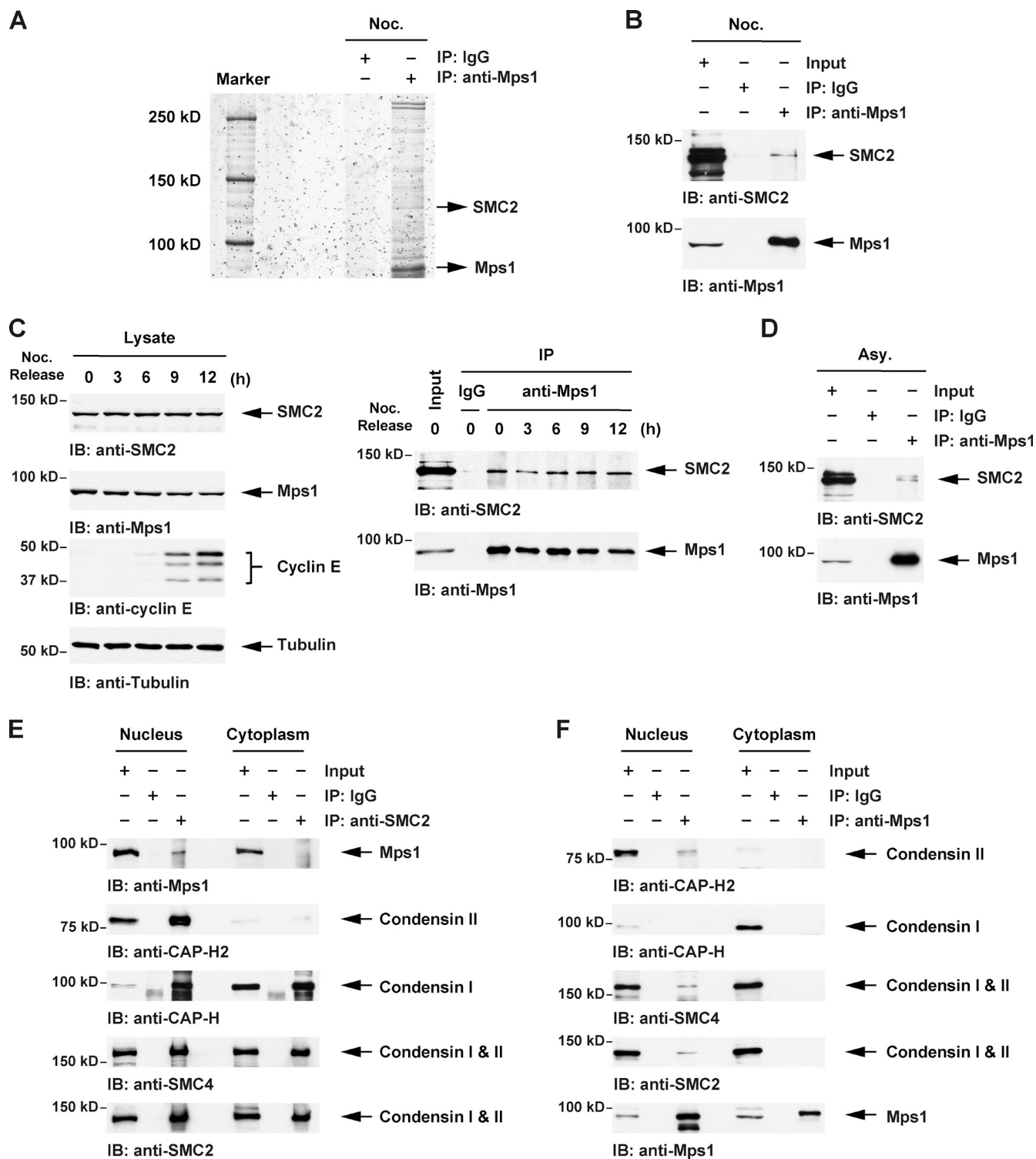


Figure 1. Identification of condensin II as a novel Mps1-associated protein. (A) HeLa cells were treated with nocodazole. The lysates were subjected to immunoprecipitation with IgG or anti-Mps1 followed by silver staining. (B) HeLa cells were treated with nocodazole. The lysates were subjected to immunoprecipitation with IgG or anti-Mps1. The lysate (Input) and the immunoprecipitates (IP) were immunoblotted with anti-SMC2 or anti-Mps1. (C) HeLa cells were treated with nocodazole, and then released for the indicated times. Cell lysates were subjected to immunoprecipitation with IgG or anti-Mps1. Lysates and the immunoprecipitates were immunoblotted with the indicated antibodies. (D) Lysates from asynchronous HeLa cells (Asy.) were subjected to immunoprecipitation with IgG or anti-Mps1. Lysates and the immunoprecipitates were immunoblotted with anti-SMC2 or anti-Mps1. (E and F) Nuclear or cytoplasmic lysates from asynchronous HeLa cells were subjected to immunoprecipitation with IgG, anti-SMC2 (E), or anti-Mps1 (F). These lysates and the immunoprecipitates were analyzed by immunoblotting with the indicated antibodies.

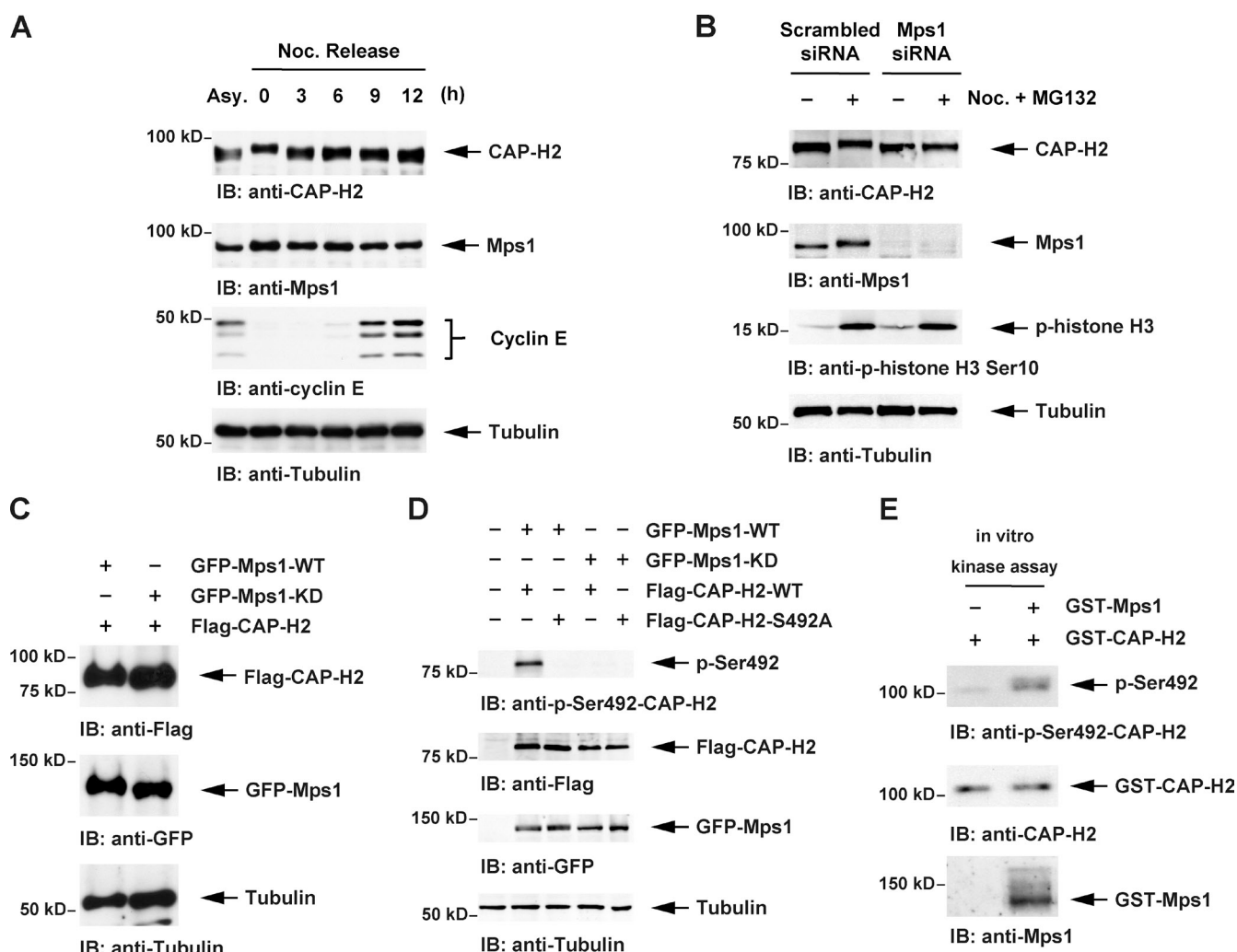


Figure 2. Identification of Ser492 of CAP-H2 as an Mps1 phosphorylation site. (A) HeLa cells were treated with nocodazole and released for the indicated times. Lysates were immunoblotted with the indicated antibodies. (B) HeLa cells were transfected with scrambled siRNA or Mps1 siRNA. At 20 h after transfection, cells were treated with DMSO or nocodazole for 16 h and then treated with MG132 for 1 h. Lysates were immunoblotted with the indicated antibodies. (C) 293 cells were cotransfected with GFP-Mps1-WT or GFP-Mps1-KD and Flag-CAP-H2. Cell lysates were immunoblotted with the indicated antibodies. (D) 293 cells were cotransfected with GFP-Mps1-WT or GFP-Mps1-KD and Flag-CAP-H2-WT or Flag-CAP-H2-S492A. Cell lysates were immunoblotted with the indicated antibodies. (E) Recombinant GST-CAP-H2 was incubated with ATP in the presence or absence of GST-Mps1. The reaction products were analyzed by immunoblotting with the indicated antibodies.

Ser492 phosphorylation is required for the targeting of condensin II to mitotic chromosomes

To further examine the significance of the Mps1-dependent phosphorylation of CAP-H2, we generated cell lines that stably express GFP, GFP-tagged CAP-H2 (GFP-CAP-H2-WT), or the S492A mutant (GFP-CAP-H2-S492A). Of note, GFP-CAP-H2 was a resistant form against CAP-H2 siRNA (Fig. 4 A). Both the wild type and mutant CAP-H2 could form condensin complexes in cells (Fig. 4 B), and Ser492 was phosphorylated in wild type, but not S492A mutant during mitosis (Fig. 4 C). To investigate whether Ser492 phosphorylation controls the targeting of condensin II onto mitotic chromosomes, GFP-CAP-H2 stable cell lines were transfected with CAP-H2 siRNA followed by nocodazole treatment. Chromosome fractionation and immunoblot analyses revealed that GFP-CAP-H2-WT largely localized to mitotic chromosomes (Fig. 4 D). In contrast, the

S492A mutant was observed in the soluble fraction (unbound chromosome fraction) and impaired its recruitment to chromosomes (Fig. 4 D). Moreover, a fluorescence microscopic analysis using fixed cells demonstrated that although GFP-CAP-H2-WT primarily accumulates in mitotic chromosomes, the S492A mutant showed a defect in chromosome localization (Fig. 4, E and F). Similar results were obtained with living cells (Fig. S3 B). These findings suggest that the association of condensin II with mitotic chromosomes is dependent on Ser492 phosphorylation of CAP-H2 by Mps1.

Mps1 regulates chromosome condensation in the early phase of mitosis

Previous studies have shown that chromosome condensation during prophase is reduced by the depletion of condensin II (Hirota et al., 2004; Ono et al., 2004). In this regard, we investigated whether Mps1 regulates chromosome condensation at

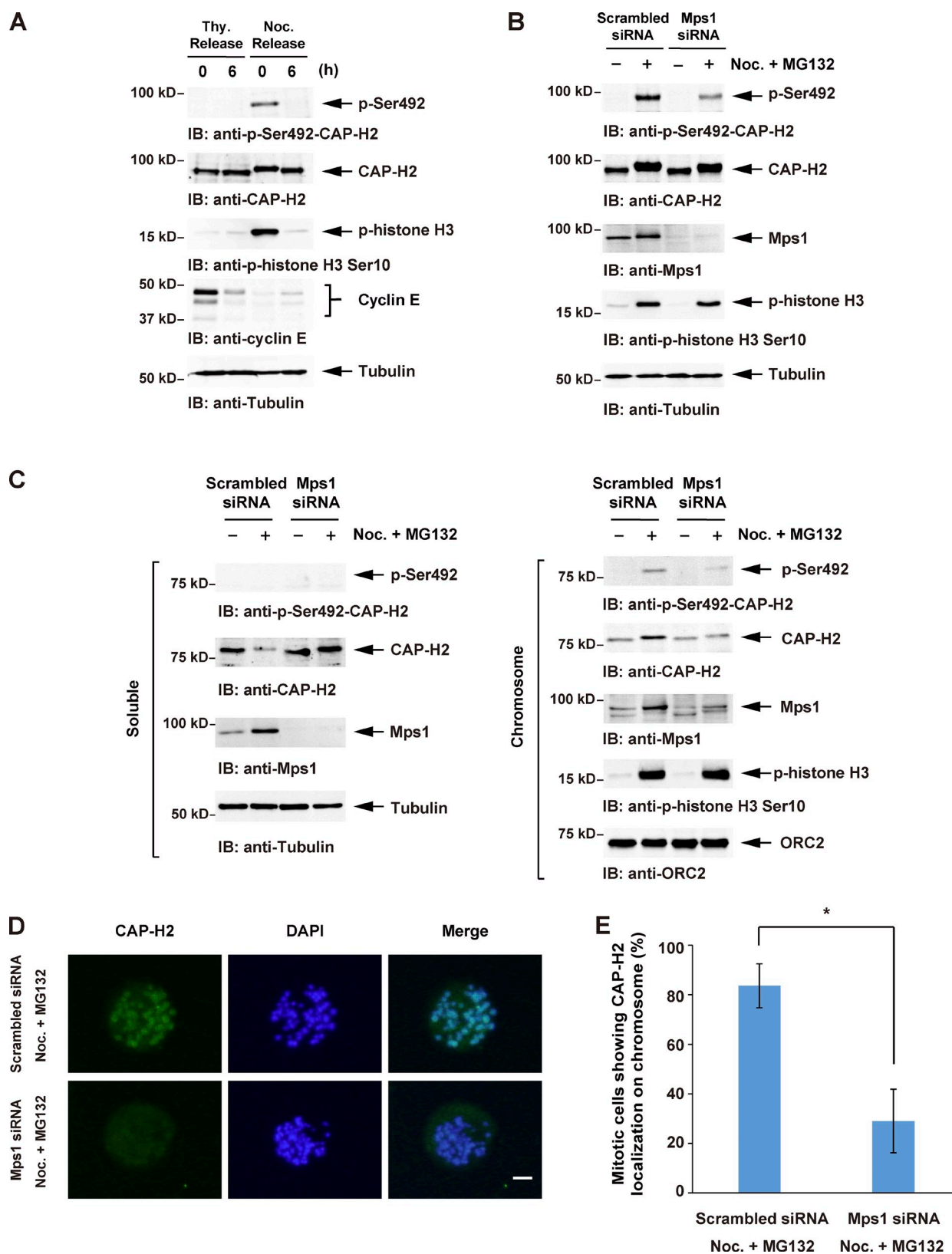


Figure 3. Mps1 regulates the chromosomal localization of condensin II during mitosis. (A) HeLa cells were synchronized by double thymidine block or nocodazole and released for the indicated times. Lysates were immunoblotted with the indicated antibodies. (B and C) HeLa cells were transfected with scrambled siRNA or Mps1 siRNA. These cells were synchronized by nocodazole and then treated with MG132. Cell lysates (B) or soluble or chromosome fractions (C) were immunoblotted with the indicated antibodies. (D) HeLa cells were transfected with scrambled siRNA or Mps1 siRNA. These cells were synchronized by nocodazole and then treated with MG132. The mitotic cells were fixed and the spread chromosomes were stained with anti-CAP-H2 (green). DNA was stained by DAPI (blue). Bar, 20 μ m. (E) The percentage of cells with CAP-H2 localization on mitotic chromosomes was calculated. Data represent the mean \pm SD from three independent experiments (*, $P < 0.05$).

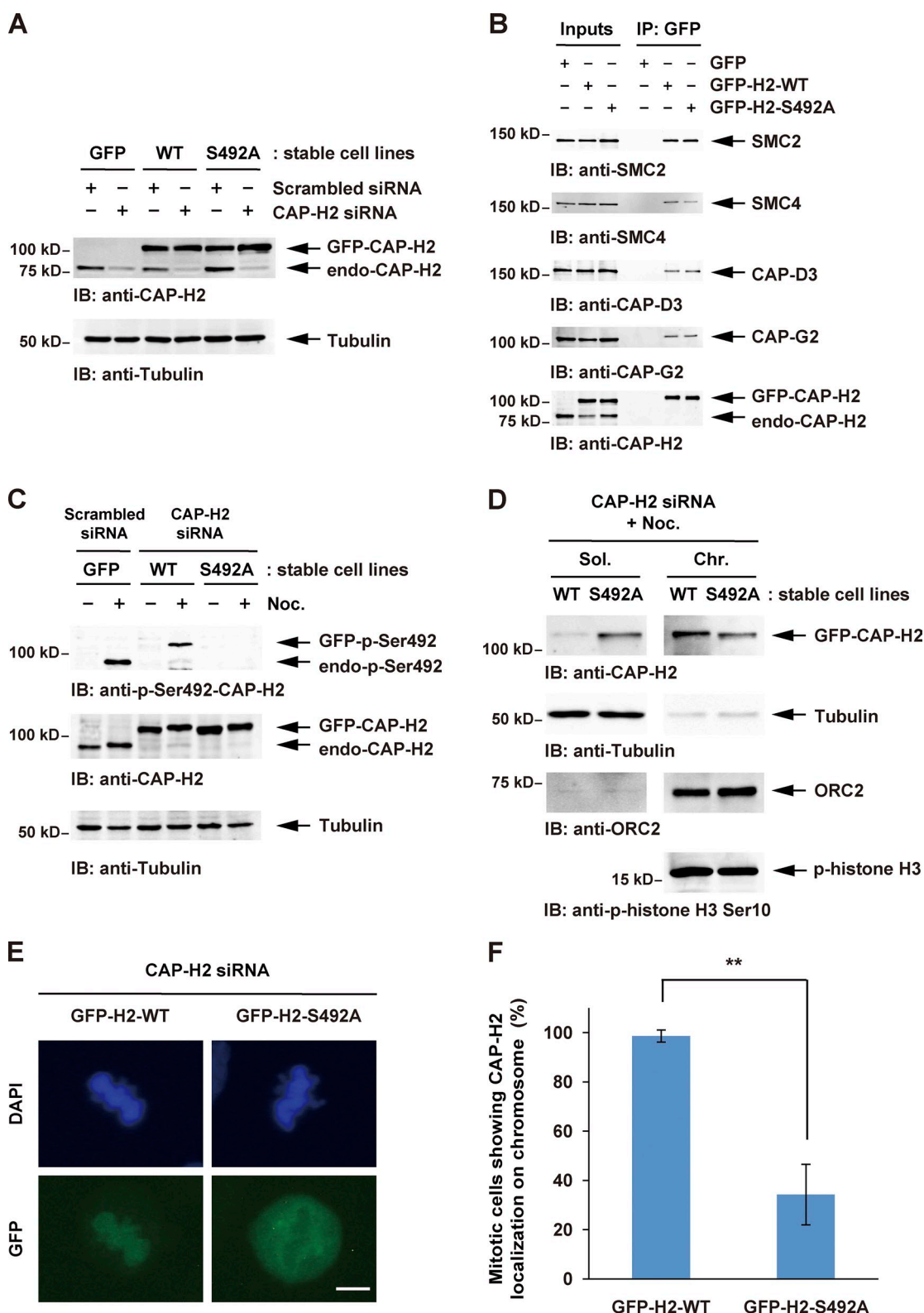


Figure 4. Ser492 phosphorylation is required for the chromosomal localization of condensin II. (A) HeLa cells stably expressing GFP (GFP), GFP-CAP-H2-WT (WT), or GFP-CAP-H2-S492A (S492A) were transfected with scrambled siRNA or CAP-H2 siRNA. Lysates were immunoblotted with the indicated antibodies. (B) Lysates from GFP, WT, or S492A cell lines were immunoprecipitated with anti-GFP and were immunoblotted with the indicated antibodies. (C) GFP, WT, or S492A cell lines were transfected with scrambled siRNA or CAP-H2 siRNA followed by treatment with DMSO or nocodazole. Cell lysates were analyzed by immunoblotting with the indicated antibodies. (D) WT or S492A cell lines were transfected with CAP-H2 siRNA followed by nocodazole treatment. The soluble protein fractions (Sol.) and the chromosome fractions (Chr.) were subjected to immunoblotting with the indicated antibodies. (E) WT or S492A cell lines were transfected with CAP-H2 siRNA and fixed. DNA was stained by DAPI. Bar, 10 μ m. (F) The percentage of cells with GFP-CAP-H2 localization on mitotic chromosomes was calculated. Data represent the mean \pm SD from three independent experiments (**, $P < 0.01$).

prophase. HeLa cells were transfected with scrambled siRNA, Mps1 siRNA, or CAP-H2 siRNA. As reported previously (Ono et al., 2004), prophase cells were monitored by the phosphorylation of histone H3 at Ser10 and by an intact nuclear envelope. The degree of prophase condensation was defined as two categories. In the “weak” category, DAPI and the phospho-histone H3 signal were uniformly distributed in the nucleus (Fig. 5 A, weak). In the “strong” category, a thread-like structure was clearly visible by both DAPI and phospho-histone H3 staining (Fig. 5 A, strong). In contrast to scrambled siRNA-transfected cells, the degree of chromosome condensation during prophase was less in each of the Mps1-depleted cells and CAP-H2-depleted cells (Fig. 5, B and C). To further confirm these results, siRNA-transfected cells were synchronized at G1/S phase and then released. These cells were fixed and prophase condensation was analyzed. The results demonstrated that prophase condensation is reduced by Mps1 and CAP-H2 siRNA (Fig. S3, C and D). Notably, the population of prophase in mitotic cells remained unchanged, indicating that duration of prophase is not affected by Mps1 siRNA or CAP-H2 siRNA (Fig. S3 E). These results thus suggest that the initial phase of mitotic chromosome condensation is induced, at least in part, by Mps1. To investigate the significance of Mps1-mediated phosphorylation of CAP-H2 at Ser492 during early chromosome condensation, we analyzed chromosome condensation during prophase in fixed GFP-CAP-H2 cell lines. As shown previously, a reduction in prophase chromosome condensation was observed in CAP-H2-depleted control cells (Fig. 5, D and E). Importantly, this reduction was rescued by the expression of GFP-CAP-H2-WT and there was little if any effect on the expression of S492A mutant (Fig. 5 D). These results indicate that prophase chromosome condensation by condensin II requires Ser492 phosphorylation. Taken together, these findings support the model in which Mps1 phosphorylates Ser492 of CAP-H2 to control the chromosomal targeting of condensin II for proper chromosome condensation during the initial phase of mitosis (Fig. 5 F).

Mps1 controls chromosome condensation to maintain genome stability

Our results clearly show that Mps1 can phosphorylate CAP-H2 at Ser492 and regulates the localization of condensin II to the mitotic chromosome (Figs. 3 D and 4 E). This is one of the phosphorylations on CAP-H2 because the depletion of Mps1 did not completely abolish the mitotic mobility shift of CAP-H2 (Figs. 3 B and 4 C). In this context, a previous study reported that Cdk1 phosphorylates CAP-D3 at Thr1415 and that this phosphorylation promotes the further phosphorylation of the condensin II complex by Plk1 (Abe et al., 2011). Notably, nonphosphorylatable CAP-D3 at Thr1415 impairs condensin II activity but not chromosomal recruitment during mitosis (Abe et al., 2011). In contrast, the chromosomal localization of condensin I was largely dependent on the phosphorylation of CAP-H at Ser70 by Aurora B (Tada et al., 2011). CAP-H2 and CAP-H belong to the “kleisin” protein superfamily, both of which conserve the structural similarities. These findings thus suggest the possibility that the chromosomal recruitment of condensins depends on the phosphorylations of kleisin family proteins. Importantly,

there is no evidence that Aurora B has any role for the regulation of condensin II (Lipp et al., 2007). In this regard, we show for the first time that Mps1 kinase controls the chromosomal localization of condensin II.

Accumulating lines of evidence have revealed the molecular mechanisms by which Mps1 controls chromosome dynamics, including chromosome alignment and segregation (Liu and Winey, 2012). The current study uncovers a novel Mps1 function in the maintenance of the chromosome architecture, including condensation. Ser492 of CAP-H2 is phosphorylated by Mps1, and this phosphorylation is required for prophase chromosome condensation (Fig. 5 F). Defects in the chromosome structure cause chromosome segregation errors during anaphase and genome instability. In this regard, the processes that regulate mitotic chromosome condensation are also crucial for genome stability. Taken together, we conclude that Mps1 functions in not only the regulation of chromosome dynamics but also chromosome structures through the process of mitosis to maintain genome stability.

Materials and methods

Cell culture and cell synchronization

HeLa cells and 293 cells were cultured in DMEM containing 10% heat-inactivated FBS, penicillin, and streptomycin. Cells were maintained at 37°C in 5% CO₂. To synchronize at mitosis, cells were treated with 30 ng/ml nocodazole (Sigma-Aldrich). In some experiments, cells were treated with 200 ng/ml nocodazole and 10 μM MG132. Mitotic cells were collected in suspension by gently shaking cells off the culture dishes (mitotic shake-off method). To synchronize at the G1/S phase, HeLa cells were synchronized using a double thymidine block method. Cells were treated with 2 mM thymidine (Wako Pure Chemical Industries, Ltd.) for 14 h, washed twice with fresh medium, and released for 10 h. Thymidine was added again to a final concentration of 2 mM to synchronize cells at the G1/S phase.

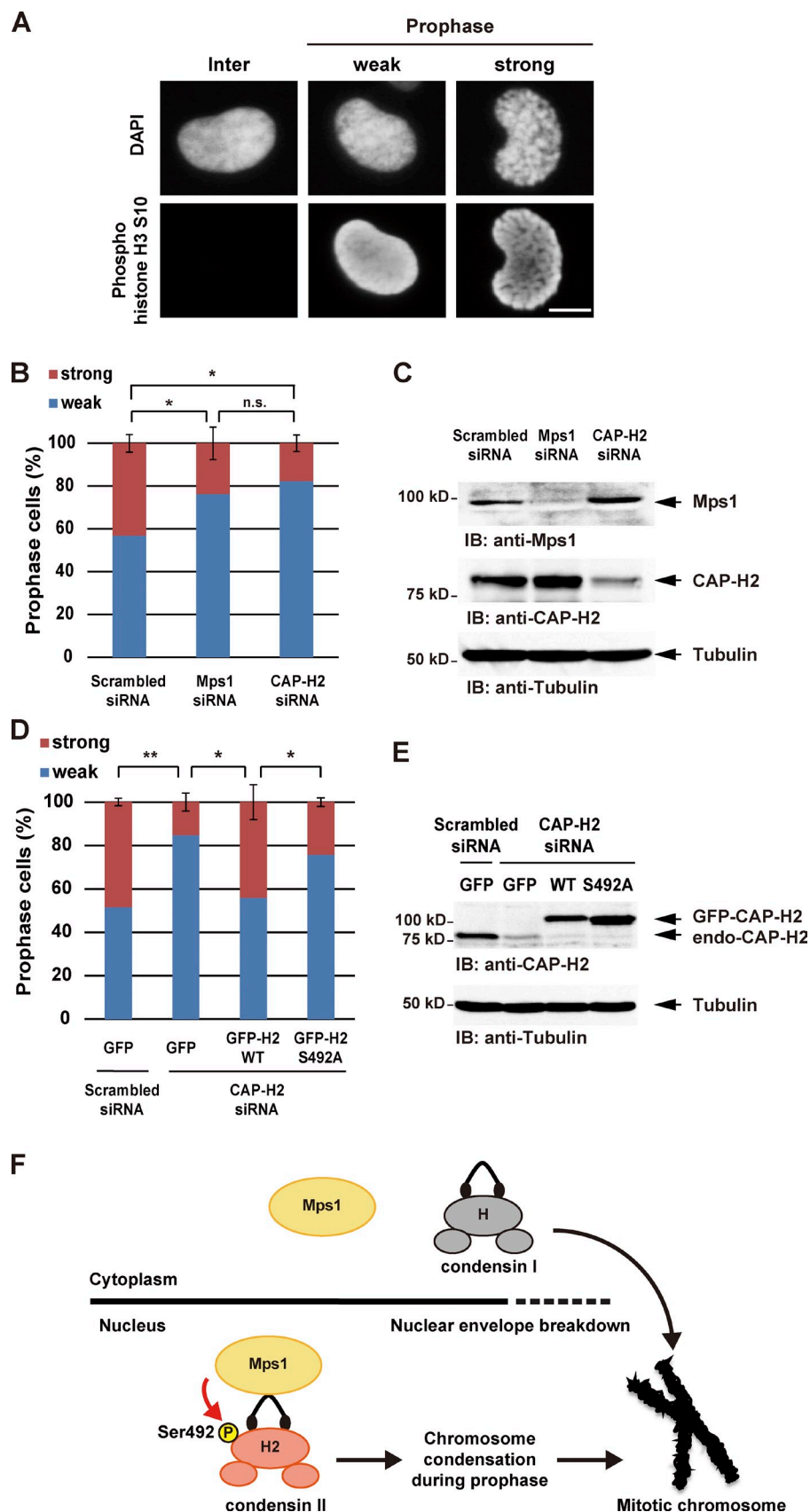
Preparation of a phospho-specific antibody

To generate a phospho-specific antibody against Ser492-phosphorylated CAP-H2, phosphopeptides (QETEL(pS)QRIRD) were used to immunize rabbits (Operon). The anti-serum was affinity purified against the phosphorylated peptides.

Immunoblot and immunoprecipitation

Cells were harvested, washed in PBS, and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM PMSF, 1 mM DTT, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1% NP-40) with phosphatase inhibitor (10 mM NaF and 1 mM Na₃VO₄). After centrifugation, the supernatants were isolated and used as cell lysates. To obtain whole-cell lysate, cells were suspended in SDS-lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM PMSF, 1 mM DTT, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1% SDS, and 1% NP-40) and sonicated. Lysates were incubated with normal rabbit IgG (Santa Cruz Biotechnology, Inc.), rabbit anti-Mps1 (Santa Cruz Biotechnology, Inc.), or rabbit anti-SMC2 (Bethyl Laboratories, Inc.) for 1 h at 4°C. Then, the solutions were incubated with protein A-Sepharose CL-4B (GE Healthcare) for 2 h at 4°C. For the immunoprecipitation of GFP-tagged proteins, lysates were incubated with anti-GFP agarose (Medical & Biological Laboratories Co.). For the immunoprecipitation of Flag-tagged proteins, lysates were incubated with anti-Flag agarose (Sigma-Aldrich). The beads were washed three times in lysis buffer and resuspended in lysis buffer. Cell lysates and immunoprecipitated proteins were boiled for 5 min, separated by SDS-PAGE, and transferred onto nitrocellulose membranes. The membranes were blocked with Blocking One (Nacal Tesque), washed three times in PBS with 0.05% Tween 20, incubated with rabbit anti-SMC2 (Bethyl Laboratories, Inc.), rabbit anti-Mps1 (Santa Cruz Biotechnology, Inc.), mouse anti-cyclin E (Santa Cruz Biotechnology, Inc.), mouse anti-tubulin (Sigma-Aldrich), rabbit anti-SMC4 (Bethyl Laboratories, Inc.), rabbit anti-CAP-H (Bethyl Laboratories, Inc.), rabbit anti-CAP-H2 (Bethyl Laboratories, Inc.), rabbit anti-CAP-G2 (Bethyl Laboratories, Inc.), rabbit anti-CAP-D3 (Bethyl

Figure 5. Mps1 regulates chromosome condensation during prophase. (A) Fixed HeLa cells were stained with anti-phospho-histone H3 Ser10. DNA was stained by DAPI. Degrees of prophase condensation were defined in the two categories and the representative cells are shown. Bar, 10 μ m. (B) HeLa cells were transfected with the indicated siRNAs and fixed. The percentage of each category, as defined in A, was calculated. Data represent the mean \pm SD from three independent experiments (*, $P < 0.05$; n.s., not significant). (C) The efficiency of protein depletion by siRNAs in B was analyzed by immunoblotting. (D) GFP, GFP-CAP-H2-WT, or GFP-CAP-H2-S492A cell lines were transfected with scrambled siRNA or CAP-H2 siRNA and fixed. The percentage of each category, as defined in A, was calculated. Data represent the mean \pm SD from three independent experiments (*, $P < 0.05$; **, $P < 0.01$). (E) The efficiency of protein depletion by siRNA in D was analyzed by immunoblotting. (F) A model of condensin II regulation by Mps1. Mps1 interacts with condensin II in the nucleus during the cell cycle. In the early phase of mitosis, Mps1 phosphorylates CAP-H2 at Ser492 and recruits condensin II on chromatin to induce mitotic chromosome condensation.



Laboratories, Inc.), rabbit anti-phospho-histone H3 Ser10 (EMD Millipore), mouse anti-GFP (Nacalai Tesque), rabbit anti-Flag (Cell Signaling Technology), rabbit anti-lamin B1 (Santa Cruz Biotechnology, Inc.), mouse anti-ORC2 (Medical & Biological Laboratories Co.), or rabbit anti-phospho-CAP-H2-Ser492. Membranes were then washed three times in PBS with 0.05% Tween 20, incubated with peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Inc.) or peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Inc.), and washed three times in PBS with 0.05% Tween 20. Immune complexes were visualized by using a Western Light-ning Plus-ECL substrate (PerkinElmer).

Silver staining and mass spectrometric analysis

Silver staining was performed using Silver Stain Plus (Bio-Rad Laboratories). The gels were analyzed for mass spectrometry, which was performed by the Cellular and Proteome Research Laboratory, Medical Research Institute, Tokyo Medical and Dental University (Tokyo, Japan). To identify phosphorylation sites on CAP-H2, a 2DICAL shotgun proteomics analysis was performed as described previously (Matsubara et al., 2009; Ono et al., 2009, 2012; Miyamoto et al., 2012). In brief, 2DICAL analyzed the data of mass-to-charge ratio (m/z), peak intensity, retention time (RT), and each sample generated by liquid chromatography and mass spectrometry as the elemental data; it displayed various two-dimensional images with different combinations of axes using these four elements. From the m/z -RT image, peaks derived from the same peptide in the direction of acquiring time were integrated. By adding algorithms to ensure reproducibility of m/z and RT, the same peak was compared precisely across different samples, and a statistical comparison of identical peaks in different samples led to the discovery of specific differentially expressed peptide peaks.

Subcellular fractionations

For nuclear and cytoplasmic fractionations, cells were harvested, washed in PBS, and resuspended in A buffer (10 mM Hepes, pH 7.6, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 10 μ g/ml leupeptin). After centrifugation, the cell pellets were resuspended in B buffer (10 mM Hepes, pH 7.6, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.2% NP-40, 1 mM DTT, 0.5 mM PMSF, and 10 μ g/ml leupeptin). After centrifugation, the supernatants were isolated and used as cytoplasmic lysates. The cell pellets were resuspended in S buffer (0.25 M sucrose, 10 mM Hepes, pH 7.6, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 10 μ g/ml leupeptin). After centrifugation, the cell pellets were resuspended in D buffer (50 mM Hepes, 400 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, and 10 μ g/ml leupeptin). After centrifugation, the supernatants were isolated and used as nuclear lysates.

Chromosomal fractionation was performed as described previously (Méndez and Stillman, 2000). In brief, cells were harvested, washed in PBS, and resuspended in chromatin-A buffer (10 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 0.1% Triton X-100, 1 mM DTT, 0.5 mM PMSF, and 10 μ g/ml leupeptin). The supernatants were isolated and used as S2 (soluble cytoplasmic fraction). The pellets were then washed in chromatin-A buffer and incubated in the absence or presence of micrococcal nucleases (New England Biolabs, Inc.) at 37°C for 2 min. After centrifugation, the pellets were resuspended in chromatin-B buffer (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 10 μ g/ml leupeptin) and incubated on ice for 30 min. The supernatants were isolated and used as S3 (soluble nuclear fraction). The pellets were washed in chromatin-B buffer, added with Laemmli buffer, sonicated, boiled for 5 min, and centrifuged. The supernatants were isolated and used as P3 (chromosome fraction). P3 was used as the chromatin fraction, and the mixture of S2 and S3 was used as the soluble fraction.

In vitro kinase assay

Recombinant GST-CAP-H2 (Abnova) was incubated in kinase buffer (20 mM Hepes, 10 mM $MgCl_2$, 0.1 mM Na_3VO_4 , and 2 mM DTT) with GST-Mps1 (Invitrogen) and ATP for 15 min. The reaction products were boiled for 5 min and subjected to immunoblot analysis.

siRNA transfections

The transfection of siRNA was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. The sequences of siRNAs were as follows: Mps1 siRNA, 5'-ugaacaaagugagagacauTT-3' and 5'-augucucucacuuuguucaTT-3'; CAP-H2 siRNA1, 5'-gcugcaggacucacacagTT-3' and 5'-cugguggaaguccugcagcTT-3'; and CAP-H2 siRNA2, 5'-ggauuucaggaacacagTT-3' and 5'-cguguuacucugaaauccTT-3'. Of note, the lowercase letters represent complementary RNA sequences for the target regions.

Plasmid construction and stable cell lines

GFP-Mps1 was constructed as described previously (Nihira et al., 2008). Mps1 cDNA was cloned into pEGFP-C1 vector. Kinase-dead mutant of Mps1 (D664A) was generated by site-directed mutagenesis. CAP-H2 cDNA was cloned into the pEGFP-C1 vector or the pcDNA3-Flag vector. Various mutations were introduced by site-directed mutagenesis. siRNA-resistant forms of CAP-H2 were generated by introducing silent mutations in the targeting regions for CAP-H2 siRNA1. To generate stable cell lines that express GFP-CAP-H2, HeLa cells were transfected with a plasmid encoding GFP-CAP-H2 using the X-tremeGENE 9 DNA transfection reagent (Roche). Stably expressing cell clones were selected by culture with medium containing G418.

Chromosome spreading

Cells were harvested, washed in PBS, resuspended in 75 mM KCl, and incubated for 15 min. Carnoy's fixative (methanol/acetic acid = 3:1) was added to the suspension. After washing twice with Carnoy's fixative, the cell pellets were resuspended in Carnoy's fixative. Fixed cells were dropped onto glass slides. An immunofluorescence analysis of spreading chromosomes was performed using rabbit anti-CAP-H2 (Bethyl Laboratories, Inc.).

Fluorescence microscopy

Cells cultured in chamber slides were fixed with 3% paraformaldehyde. Fixed cells were permeabilized with 1% Triton X-100 in PBS and incubated with 10% goat serum in PBS for 1 h. Cells were incubated with rabbit anti-phospho-histone H3 Ser10 (EMD Millipore) followed by a reaction with fluorescein isothiocyanate- or tetramethyl rhodamine isothiocyanate-conjugated secondary antibodies. DNA was stained with DAPI. Fixed cells were imaged at room temperature using an all-in-one type fluorescence microscope (Bio-Zero BZ-8000; Keyence) equipped with a Plan Apochromat 20 \times /0.75 NA objective lens (Nikon). Images were acquired with BZ Analyzer software (Keyence). Live cells were cultured in DMEM containing 10% FBS at 37°C in 5% CO₂ and were imaged using a DeltaVision Core system equipped with a microscope (IX71; Olympus), UPlan SApo 20 \times /0.75 NA objective lens (Olympus), and CoolSNAP HQ² camera (Photometrics). DNA was stained with 0.2 μ g/ml Hoechst 33342. The living cell images were acquired with SoftWoRx software (Applied Precision). Imaging data were processed using Photoshop (Adobe).

Online supplemental material

Fig. S1 shows the purity of subcellular fractionations. Fig. S2 shows that Mps1 phosphorylates Ser492 of CAP-H2. Fig. S3 shows Ser492 phosphorylation during mitosis. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201308172/DC1>.

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