

# Mps1 Is a Kinetochore-Associated Kinase Essential for the Vertebrate Mitotic Checkpoint

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## Summary

The mitotic checkpoint acts to inhibit entry into anaphase until all chromosomes have successfully attached to spindle microtubules. Unattached kinetochores are believed to release an activated form of Mad2 that inhibits APC/C-dependent ubiquitination and subsequent proteolysis of components needed for anaphase onset. Using *Xenopus* egg extracts, a vertebrate homolog of yeast Mps1p is shown here to be a kinetochore-associated kinase, whose activity is necessary to establish and maintain the checkpoint. Since high levels of Mad2 overcome checkpoint loss in Mps1-depleted extracts, Mps1 acts upstream of Mad2-mediated inhibition of APC/C. Mps1 is essential for the checkpoint because it is required for recruitment and retention of active CENP-E at kinetochores, which in turn is necessary for kinetochore association of Mad1 and Mad2.

## Introduction

The mitotic checkpoint delays anaphase entry until both kinetochores of each duplicated chromosome pair acquire stable attachment to spindle microtubules (Rieder and Salmon, 1998; Zachariae, 1999). In mammals, loss of the checkpoint provokes inaccurate separation of sister chromatids, which generates genomic instability that in turn provokes cell death (Dobles et al., 2000) or tumorigenesis (Michel et al., 2001). Precisely how checkpoint signals are transmitted and received is not yet fully understood. However, it seems clear that unattached kinetochores produce a signal that blocks anaphase onset by inhibiting Cdc20/Fizzy, a protein required for exit from mitosis, which associates with and activates the Anaphase Promoting Complex/Cyclosome (APC/C) (Fang et al., 1998; Kallio et al., 1998; Lorca et

al., 1998; Zachariae, 1999). It is the APC/C which catalyzes the ubiquitination of key regulatory proteins such as securins and cyclins, whose subsequent destruction is required for sister chromatid separation and mitosis exit, respectively (Holloway et al., 1993; Morin et al., 1994; Cohen-Fix et al., 1996; Zou et al., 1999).

The mitotic checkpoint was originally defined in budding yeast, where seven components essential for it have been identified: *MAD1–3* (Li and Murray, 1991), *BUB1–3* (Hoyt et al., 1991; Roberts et al., 1994), and *MPS1* (Winey et al., 1991; Hardwick and Murray, 1995; Weiss and Winey, 1996). *MPS1* was originally identified as a gene essential for spindle pole duplication and mutation in which yielded monopolar spindles (e.g., Winey et al., 1991). Mps1p is a kinase (Lauzé et al., 1995) whose activity seems to be dependent on the presence of *CDC37* (Schutz et al., 1997), a subunit of the Hsp90 chaperone complex in mammals and flies. Overexpression of Mps1p causes mitotic arrest in the absence of spindle disruption, which correlates with hyperphosphorylation of Mad1p (Hardwick et al., 1996). Moreover, Mps1p phosphorylates Mad1p in vitro (Hardwick et al., 1996) and such hyperphosphorylation appears to be required for association of Mad1p with Mad2p (Chen et al., 1999) and with the Bub1p/Bub3p kinase complex (Brady and Hardwick, 2000). On the other hand, Bub1p kinase activity does not depend on the presence of Mps1p (Farr and Hoyt, 1998). Overexpression of Bub1p leads to checkpoint activation in the absence of spindle disruption, but without Mad1p hyperphosphorylation, demonstrating that Mad1p hyperphosphorylation cannot be absolutely required for checkpoint signaling or that it cannot be downstream of Bub1p (Farr and Hoyt, 1998).

The vertebrate homologs of the yeast *MAD1*, *MAD2*, *BUB1*, and *BUB3* genes (Chen et al., 1996; Li and Benzra, 1996; Taylor and McKeon, 1997; Taylor et al., 1998; Chen et al., 1998; Martinez-Exposito et al., 1999) and vertebrate-specific checkpoint components such as MAPK (Minshull et al., 1994; Shapiro et al., 1998; Zecevic et al., 1998), BubR1 (a protein kinase that appears to be a hybrid of yeast Bub1p and Mad3p) (Jablonski et al., 1998; Chan et al., 1999), and CENP-E (a microtubule motor protein of the kinesin family) (Yen et al., 1992; Wood et al., 1997; Yao et al., 2000; Abrieu et al., 2000) have been shown to be kinetochore-associated proteins. As in yeast, formation of complexes seems to be an important step in the checkpoint: Mad2 must form a complex with Mad1 (Chen et al., 1998) and Bub3 with Bub1 and BubR1 (Taylor et al., 1998) in order to be recruited to kinetochores. These complexes associate preferentially with unattached kinetochores. The current model is that an activated form of Mad2 (possibly a tetramer) is assembled on unattached kinetochores, preventing the activation of the APC/C by directly binding to and inhibiting Cdc20/Fizzy (Fang et al., 1998). In agreement with this, it has been recently shown that Mad2 is a transient kinetochore component which rapidly associates and dissociates from unattached kinetochores (Howell et al., 2000). After microtubule attach-

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ment, the amounts of Mad1 (Chen et al., 1998), Mad2 (Chen et al., 1996; Li and Benezra, 1996), Bub1 (Taylor and McKeon, 1997), BubR1 (Jablonski et al., 1998; Chan et al., 1999), MAPK (Shapiro et al., 1998; Zecevic et al., 1998), and Bub3 (Taylor et al., 1998) diminish at kinetochores.

CENP-E probably provides one of the direct links of the vertebrate checkpoint to spindle microtubule capture by the kinetochores. It not only extends from the surface of the kinetochore (Cooke et al., 1997; Yao et al., 1997), but binds directly to BubR1 (Chan et al., 1999) with which it can be found in a stoichiometric complex (Yao et al., 2000). CENP-E is necessary for alignment of chromosomes at metaphase (Wood et al., 1997; Schaar et al., 1997), remaining kinetochore associated through anaphase A (Brown et al., 1996). It is also necessary for activation and maintenance of checkpoint signaling in *Xenopus* egg extracts (Abrieu et al., 2000), with depletion of it eliminating association of the Mad1/Mad2 complex with the kinetochores.

Vertebrate homologs of yeast Mps1p kinase have been identified as *TTK* in humans (Mills et al., 1992; Hogg et al., 1994) and *Esk* in mice (Douville et al., 1992); however, whether these putative kinases have a role in the mitotic checkpoint has not been determined. To test this, we have now isolated the *Xenopus* Mps1 homolog and found it to be a kinetochore-associated kinase that is necessary to establish and maintain the checkpoint at a very early step in vertebrate mitotic checkpoint signaling, including the recruitment to kinetochores of CENP-E.

## Results

### Identifying *Xenopus* Mps1 to Be a 105 kDa Kinase

RT-PCR with degenerate *S. cerevisiae* *MPS1* and mammalian *TTK* primers and total RNA from *Xenopus* metaphase II-arrested eggs was used to generate a probe for screening a  $\lambda$ gt10 cDNA library constructed from the same mRNA source. One positive clone was identified to contain a complete open reading frame of 2649 bp encoding for a protein of 882 amino acids and a predicted molecular weight of about 105 kDa. The predicted polypeptide shares 67% and 59% homology with human *TTK* and *S. cerevisiae* *Mps1p*, respectively (see Supplementary Information at <http://www.cell.com/cgi/content/full/106/1/83/DC1>), including a C-terminal kinase domain in which the homology is greatest.

Three affinity-purified polyclonal antibodies (anti-NP, anti-CP, and anti-NTD) against different portions of the putative Mps1 polypeptide identified a protein of ~105 kDa in CSF egg extracts (Figure 1A, lanes E) indistinguishable from the position of the polypeptide produced by in vitro translation of the full-length reading frame encoded by the *Mps1* clone (Figure 1A, lanes R).

To determine if *Xenopus* Mps1 is a kinase, immunoprecipitates with each of the three anti-Mps1 antibodies were used to phosphorylate myelin basic protein (MBP), previously described as an in vitro substrate of *S. cerevisiae* *Mps1p* (Lauzé et al., 1995). Each of the three antibodies (Figure 1B, bottom panel, lanes 3–5), but not control antibodies (Figure 1B, bottom panel, lanes 1 and 2), immunoprecipitated similar amounts of Mps1 protein

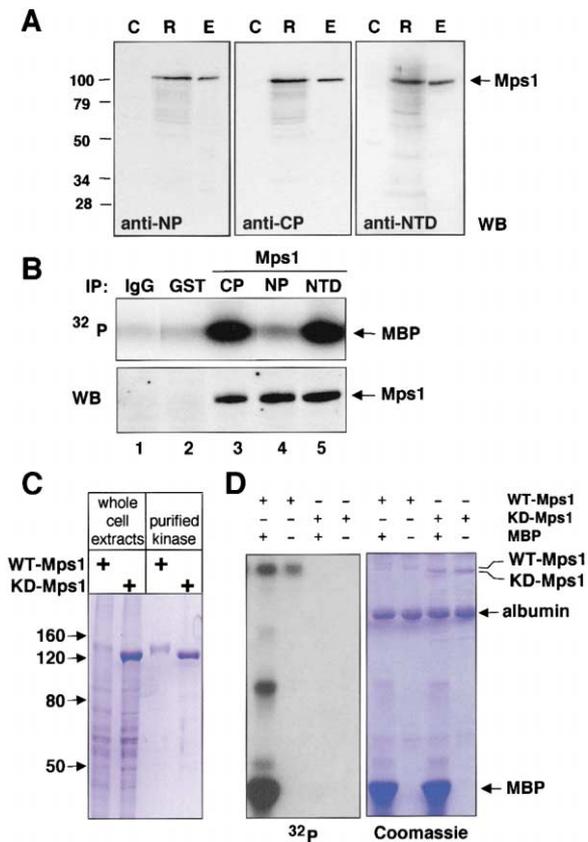


Figure 1. Identification of *Xenopus* Mps1

(A) Characterization of Mps1 antibodies. Reticulocyte lysate without added mRNA (lanes C), in vitro translated Mps1 protein (lanes R), and endogenous Mps1 protein from CSF egg extracts (lanes E) were immunoblotted with three different affinity-purified Mps1 antibodies (anti-NP; anti-CP; and anti-NTD).

(B) Assay for kinase activity of Mps1 in the presence or absence of MBP. Egg extracts were immunoprecipitated with control (lane 1: anti-IgG; lane 2: anti-GST) or Mps1 antibody (lane 3: anti-CP; lane 4: anti-NP; lane 5: anti-NTD). Half of each immunoprecipitate was assayed in the presence of ( $\gamma$ - $^{32}$ P)-ATP for kinase activity toward MBP (top panel) and the other half was used to quantify the level of Mps1 (by immunoblot with anti-NP).

(C) Production of recombinant WT-Mps1-GST or KD-Mps1-GST in insect cells. (Left lanes) whole-cell extracts; (right lanes) after affinity purification over immobilized glutathione.

(D) Recombinant WT-Mps1-GST and KD-Mps1-GST (D685A) were assayed in the presence of ( $\gamma$ - $^{32}$ P)-ATP and added bovine albumin for auto kinase activity and for use of MBP as a substrate. (Left) autoradiograph; (right) Coomassie stain of the same gel.

from CSF egg extracts. Significant kinase activity on the MBP substrate was seen with two of these (Figure 1B, top panel, lanes 3 and 5), although much reduced kinase activity was detected with the anti-NP antibody immunoprecipitates (Figure 1B, compare lanes 4 and 5), suggesting that the anti-NP antibody inhibits Mps1 kinase activity.

Mps1 was also purified as a glutathione S-transferase (GST) fusion protein from insect cells infected with a baculovirus encoding for wild-type *Mps1* (Figure 1C, lanes 1 and 3) or Mps1 containing a D685A point mutation (Figure 1C, lanes 2 and 4) in the putative ATP binding site and that was predicted to inactivate the

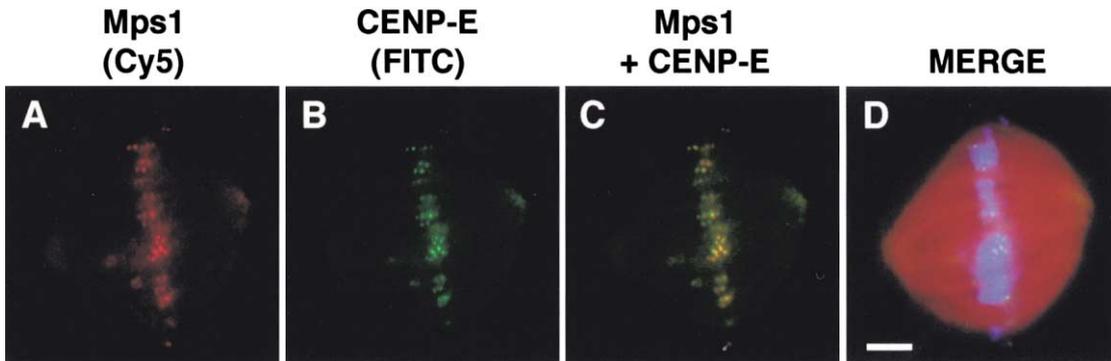


Figure 2. Mps1 Associates with Attached Kinetochores of Aligned Chromosomes

A low concentration of sperm nuclei was added to CSF egg extracts, which were then released from CSF arrest with calcium, cycled for 80 min through S phase to allow chromosome duplication, and arrested again in the subsequent mitosis by addition of CSF extract. This yielded bipolar spindles with aligned chromosomes. (A) Mps1 location detected by anti-DNT (followed by Cy5-labeled anti-rabbit IgG); (B) CENP-E was subsequently localized using biotinylated anti-CENP-E antibody, followed by avidin-FITC. (C) Mps1 and CENP-E co-staining (merged [A] and [B]). (D) Merged chromosomes (DAPI), spindle microtubules (rhodamine tubulin), Mps1 (Cy5), and CENP-E (FITC) staining. Bar, 5  $\mu$ m.

kinase activity (to be referred to as kinase dead KD-Mps1). Using purified recombinant Mps1 protein as a quantitation standard, endogenous Mps1 was found to be  $\sim$ 50 nM in the *Xenopus* extracts (by immunoblotting using  $^{125}$ I-protein A and phosphorimaging). Assay for kinase activity revealed (Figure 1D) that only the wild-type (WT-Mps1, first 2 lanes), but not the kinase-dead (KD-Mps1, last 2 lanes), mutant was able to both autophosphorylate itself and to phosphorylate MBP. On the basis of the sequence homology and kinase activity, we conclude that Mps1 is the *Xenopus* homolog of mammal TTK and *S. cerevisiae* Mps1p.

#### Mps1 Associates with Kinetochores

To identify the structures with which *Xenopus* Mps1 associates, spindles were assembled in vitro (Figure 2D). Mps1 was found in a punctate pattern in a series of dots at kinetochores of condensed chromosomes (Figure 2A). This pattern coincided with the positions of a known kinetochore component, CENP-E (Figure 2B). Indistinguishable results were obtained with each of the three anti-Mps1 antibodies. Furthermore, after inhibition of microtubule assembly (with addition of nocodazole), Mps1 continued to colocalize with CENP-E (see later, Figures 5 and 6). Thus, Mps1 is a kinetochore-associated kinase both before and after spindle microtubule capture.

#### Mps1 Is an Essential Checkpoint Component

Mature *Xenopus* unfertilized eggs are naturally arrested at metaphase of meiosis II by cytosstatic factor (CSF) (Sagata et al., 1989). Fertilization releases this arrest by transiently increasing cytoplasmic calcium concentration, which activates CaM kinase II (Lorca et al., 1994), allowing the egg to exit mitosis and start embryogenesis. Extracts made from unfertilized eggs (CSF egg extracts) maintain the CSF arrest. Addition of calcium (which mimics fertilization) induces exit into interphase by allowing degradation of cyclin B and cdc2 kinase inactivation. Addition of a high density of sperm nuclei and nocodazole to CSF egg extracts activates the spindle checkpoint (Minshull et al., 1994) that can be assessed by

maintenance of meiotic arrest that is insensitive to CSF destruction, continued stabilization of cyclin B and cdc2 kinase activity (assayed on histone H1), and absence of chromatin decondensation and nuclear reformation.

To assess the role of Mps1 in checkpoint establishment, we first demonstrated that Mps1 is not required for CSF arrest itself. Whether Mps1 was present or depleted from CSF extracts (Figure 3A), radiolabeled cyclin B1 was not degraded (Figure 3B, lanes 1 and 2) and cdc2 H1 kinase activity was maintained (data not shown). In those same extracts, calcium addition led to cyclin B1 degradation (Figure 3B, lane 3) and inactivation of cdc2 kinase activity (data not shown). The same results were obtained with all three Mps1 antibodies (data not shown) and demonstrate that Mps1 depletion has no effect on the CSF-mediated mitotic phase arrest.

To examine the consequence of Mps1 depletion on the establishment of the checkpoint, high levels of sperm nuclei were added to Mps1-depleted or mock-depleted extracts in the presence of nocodazole to disrupt spindle microtubule assembly. After calcium addition, H1 kinase activity was measured and chromatin condensation assessed visually. The mock-depleted extracts activated the checkpoint and remained arrested in mitosis, as revealed by constant H1 kinase activity (Figure 3C, lanes 1 to 3) and continued chromatin condensation (Figure 3C, left panel). Mps1-depleted extracts, on the other hand, rapidly lost H1 kinase activity (Figure 3C, lanes 4 to 6) and reassembled nuclear envelopes around decondensed chromatin (Figure 3C, middle panel).

Binding to and inhibition of the APC/C activator Cdc20/fizzy by an activated form of Mad2 (Fang et al., 1998; Kallio et al., 1998) is thought to be the last signaling step in the checkpoint pathway. Consistent with this, addition of excessive levels of Mad2 can inhibit APC/C activity in *Xenopus* egg extracts, independently of kinetochore signaling (i.e., even without added nuclei) (Chen et al., 1998). To determine if Mps1 depletion has an effect on downstream steps of the kinetochore signaling pathway, an excess of Mad2 (15 times more than the amount normally present) was added to Mps1-depleted extracts.

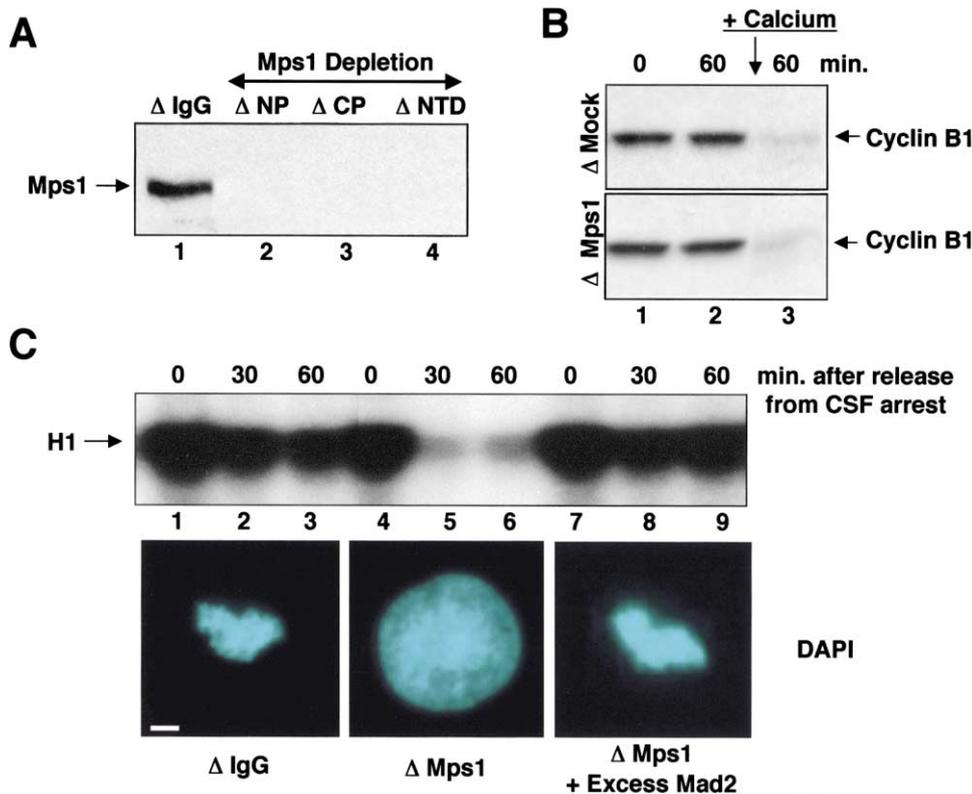


Figure 3. Mps1 Depletion Prevents Activation of the Mitotic Checkpoint

(A) CSF egg extracts were immunodepleted of Mps1 using purified IgG (lane 1) or Mps1 antibodies (anti-NP [lane 2]; anti-CP [lane 3]; anti-NTD [lane 4]). Mps1 content in depleted extracts was assayed by immunoblotting with anti-NP antibody.

(B) Mps1 depletion does not release CSF arrest. Radiolabeled cyclin B1 was visualized by autoradiography in CSF extracts depleted with anti-IgG (top panel) and anti-Mps1 (anti-NP, bottom panel) at 0 (lane 1) and at 60 min (lane 2), and a further 60 min after calcium addition (lane 3).

(C) Depletion of Mps1 prevents activation of the mitotic checkpoint. CSF egg extracts were depleted with anti-IgG (lanes 1–3) or anti-Mps1 antibodies (anti-NP) in the absence (lanes 4–6) or presence of 100 ng/ $\mu$ l recombinant Mad2 (lanes 7–9) and incubated with sperm nuclei and nocodazole for 30 min. Subsequently, histone H1 activity was measured at 0 min (lanes 1, 4, and 7), 30 min (lanes 2, 5, and 8), and 60 min (lanes 3, 6, and 9) after calcium addition. (Bottom panel—DAPI stain) Mps1 (anti-NP) depletion induces chromatin decondensation after release of CSF arrest (middle panel); excess Mad2 (right panel) restores mitotic arrest (assayed 60 min after addition of calcium). Bar, 5  $\mu$ m.

As indicated by a constant H1 kinase activity, this addition overcame the loss of the kinetochore-dependent checkpoint signaling pathway in the absence of Mps1 (Figure 3C, lanes 7–9), compared to Mps1-depleted extracts where no Mad2 was added (Figure 3C, lanes 4–6). The same results were obtained by depletion with each of the three different Mps1 antibodies. Thus, the requirement for Mps1 in establishing the checkpoint occurs upstream of Mad2-mediated inhibition of APC/C.

#### Mps1 Is Necessary for the Maintenance of a Previously Activated Checkpoint

To determine the role of Mps1 in the maintenance of the mitotic checkpoint, nocodazole and a high concentration of sperm nuclei were added to *Xenopus* egg extracts for 30 min in order to first activate the checkpoint. Antibodies were then added (or not) for 15 min, and after addition of calcium in order to release the CSF arrest, the extracts were processed for immunofluorescence or histone H1 kinase assays (see scheme in Figure 4A). Extracts incubated with (Figure 4B, row b) or without (Figure 4B, row a) control antibodies show a constant

H1 kinase activity despite calcium addition. In contrast, within 15 min after addition of Mps1 (anti-NP, anti-CP, or anti-NTD) antibodies (shown in Figure 4B, row d, for the anti-NP antibody), H1 kinase activity was lost and mitotic exit ensued, indicative of loss of the previously established mitotic checkpoint.

Since CENP-E has already been shown to be required for maintenance of a previously activated checkpoint (Abrieu et al., 2000; Figure 4B, row c), we tested whether Mps1 antibody addition may inhibit checkpoint maintenance through a similar mechanism. Mps1 antibodies (anti-NP or anti-CP) were added to CSF egg extracts in which the mitotic checkpoint had been previously activated. This eliminated both the CENP-E and Mad2 (compare Figure 4B, rows a and d) kinetochore association, both of which are normally needed at the kinetochore for maintenance of the checkpoint signaling. While added CENP-E antibody remained kinetochore bound despite eliminating CENP-E function (Figure 4B, row c; Abrieu et al., 2000), Mps1 antibody addition blocked Mps1 binding to kinetochores (direct experiment not shown, but the presence of the added Mps1

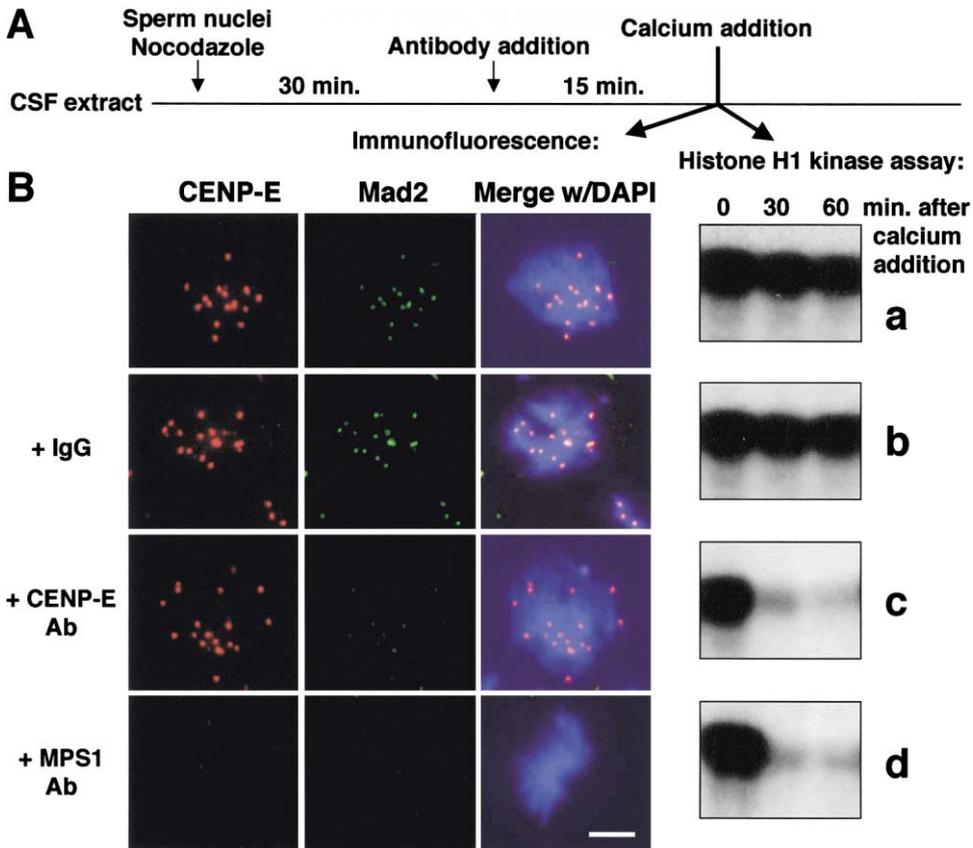


Figure 4. Mps1 Is Required for Maintenance of a Previously Established Mitotic Checkpoint

(A) The checkpoint was initially activated by addition of a high concentration of sperm nuclei and nocodazole to a CSF extract for 30 min. (B) No antibody (a), nonimmune IgG (b), CENP-E (c), or Mps1 (d) antibodies were then added for 15 min and (right) aliquots of each were assayed for histone H1 kinase activity after release from CSF arrest. (Left) Locations of CENP-E and Mad2 were visualized with appropriate secondary antibodies (see Experimental Procedures) and imaged individually or merged with chromatin (DAPI). Bar, 5  $\mu$ m.

antibody at kinetochores would have been detected by the secondary antibody used to visualize CENP-E—Figure 4B, row d). Altogether, these results suggest that Mps1 association with kinetochores is necessary to maintain checkpoint activation in response to spindle damage through its requirement for continued CENP-E and Mad2 binding at kinetochores.

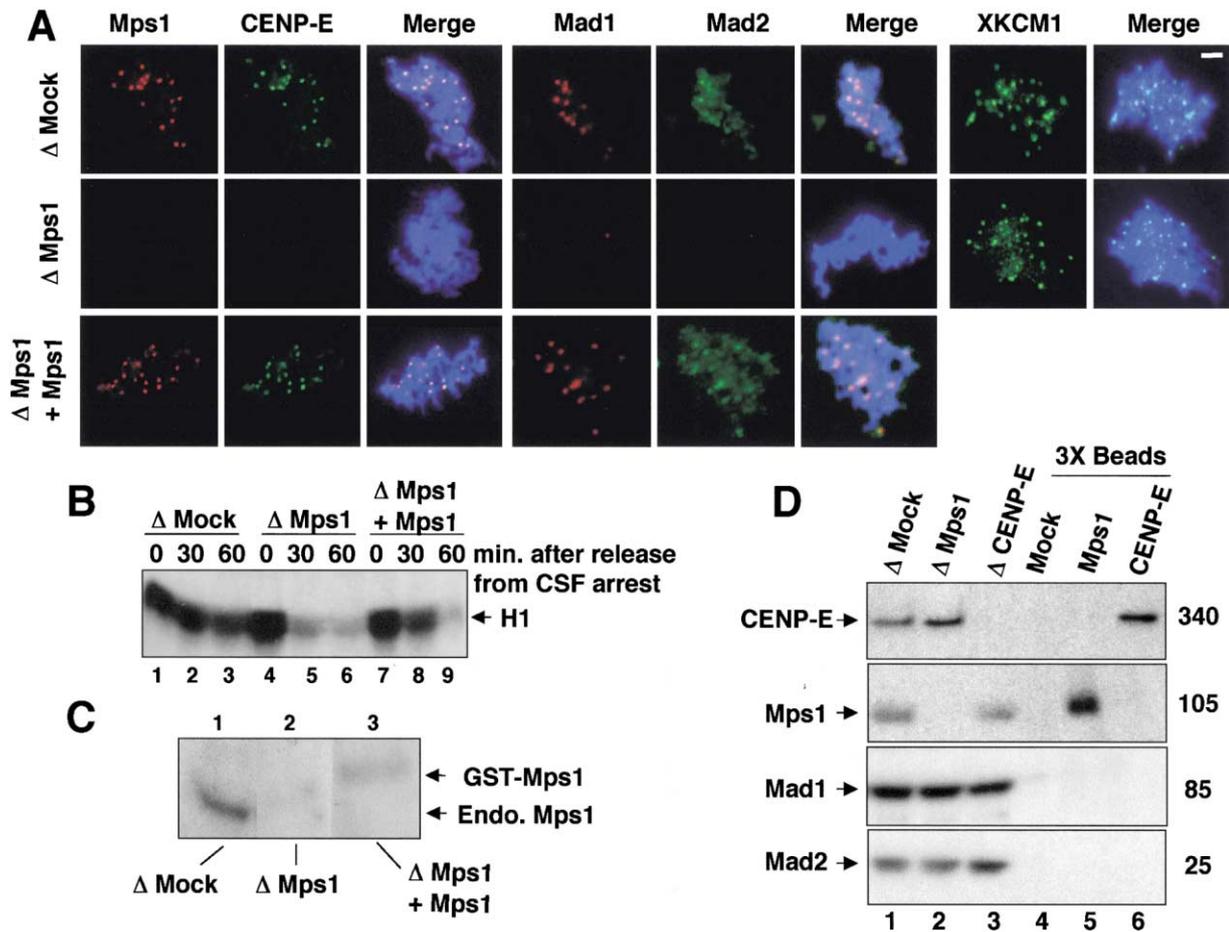
#### Mps1 Activity Recruits CENP-E and Mad1/Mad2 to Kinetochores

To determine the mechanism through which the absence of Mps1 might prevent kinetochore-dependent checkpoint signaling, chromosomes in mock-depleted or Mps1-depleted egg extracts were examined for Mad2 and Mad1 association with kinetochores, which has been shown in *Xenopus* egg extracts to be CENP-E dependent (Abrieu et al., 2000). Mad2 and Mad1 were colocalized at kinetochores in mock-depleted extracts, along with Mps1 and CENP-E (Figure 5A, first row). Mps1 immunodepletion prevented this: CENP-E, Mad2, and Mad1 (compare Figure 5A, first and second rows) all failed to associate with kinetochores, consistent with an inactivated checkpoint.

To rule out the possibility that Mps1 activity was necessary for basic centromere assembly rather than more

specifically in checkpoint signaling, position of XKCM1, another known kinetochore component (Walczak et al., 1996), was determined. As shown in Figure 5A (right panels), whether Mps1 is present or absent from these extracts, XKCM1 localizes to the kinetochores normally, suggesting that the lack of CENP-E, Mad1, and Mad2 recruitment to kinetochores in Mps1-depleted extracts does not occur from a failure of kinetochore assembly. Altogether, these results suggest that Mps1 is necessary for specifically targeting CENP-E (and subsequently Mad1 and Mad2) to the kinetochore.

To determine whether in Mps1-depleted extracts, the checkpoint suppression was a direct consequence solely of the loss of Mps1 function, Mps1 linked to GST was purified from insect cells infected with a baculovirus encoding full-length *Xenopus* Mps1 (see Figure 1C, lane 3). The highest level of recombinant Mps1 we could achieve after readdition to depleted extracts restored Mps1 to approximately half the normal level (Figure 5C, compare lanes 1 and 3). The recombinant Mps1 relocated to kinetochores and restored kinetochore binding of CENP-E, Mad1, and Mad2 (Figure 5A, last row). Furthermore, restoration of this level of Mps1 also partially restored the checkpoint to Mps1-depleted extracts (as assayed by histone H1 kinase activity — Figure 5B, lanes 7–9). On the basis of the apparently complete restoration



**Figure 5. Recombinant Mps1 Restores Association of CENP-E, Mad1, and Mad2 with Kinetochores to Mps1-Depleted Extracts and Induces a Delay in Exit from Mitosis**

(A) CSF egg extracts were mock depleted (anti-IgG, top row) or Mps1 depleted (anti-CP, middle and bottom rows). Recombinant WT-Mps1-GST (bottom row) was added along with sperm nuclei and nocodazole and after 30 min, Mps1, CENP-E, Mad1, Mad2, and XKCM1 locations were visualized by immunofluorescence, as indicated. (Merge) DAPI to visualize the chromatin merged with Mps1/CENP-E or Mad1/Mad2 or XKCM1. Bar, 5  $\mu$ m.

(B) Recombinant Mps1 delays mitotic exit in extracts initially depleted of Mps1. Histone kinase activities at 0, 30, and 60 min following CSF release in (lanes 1–3) mock (IgG) depletion, (lanes 4–6) Mps1 depletion, and (lanes 7–9) Mps1 depletion to which  $\sim$ 20 nM recombinant Mps1 was subsequently added.

(C) Mps1 levels determined by immunoblot (anti-CP) in extracts (lane 1) mock (IgG) depleted of Mps1, (lane 2) Mps1 depleted, and (lane 3) Mps1 depleted and to which  $\sim$ 20 nM WT- Mps1-GST was added.

(D) Depletion of Mps1 does not affect levels of CENP-E, Mad1, and Mad2. CENP-E, Mps1, Mad1, and Mad2 levels (determined by immunoblot) remaining in extracts (lane 1) mock depleted, (lane 2) depleted with Mps1 antibodies, and (lane 3) depleted with CENP-E antibodies. (Lanes 4–6) CENP-E, Mps1, Mad1, and Mad2 bound to the beads used for immunodepletion in mock, Mps1, and CENP-E depletions, respectively, and detected using anti-tail CENP-E, anti-CP Mps1, anti-Mad1, and anti-Mad2 antibodies. (A 3-fold higher proportion of the bead bound fractions relative to the depleted extracts has been analyzed.)

at the immunofluorescence level and the partial one at the level of histone H1 kinase activity, we believe that the absence of checkpoint in Mps1-depleted extracts truly reflects Mps1 depletion and not the depletion of some associated partners. However, to fully resolve this issue, we determined whether Mps1 depletion affected the levels of other checkpoint components. As shown in Figure 5D (lanes 2 and 5), CENP-E, Mad1, and Mad2 are neither co-depleted with Mps1 nor present in the Mps1 immunoprecipitates (Figure 5D, Mps1 row, lane 5). Reciprocally, CENP-E immunoprecipitation does not remove Mps1 from the extract and Mps1, Mad1, or Mad2 are not found in the CENP-E immunoprecipitates (Figure 5D, lanes 3 and 6).

Altogether, these results strongly suggest that Mps1 is required to establish and maintain the mitotic checkpoint by facilitating CENP-E, Mad1, and Mad2 recruitment to the kinetochores.

#### Mps1 Kinase Activity Is Required to Activate the Checkpoint

Using a recombinant kinase-dead version of Mps1 (KD-Mps1), the requirement of Mps1 kinase activity in checkpoint signaling was examined. Kinase activity was not needed for Mps1 localization to kinetochores (Figure 6A, compare Mps1 levels in rows a and b) even in the absence of endogenous Mps1 (Figure 6A, compare Mps1 levels in rows a and c). However, kinetochore

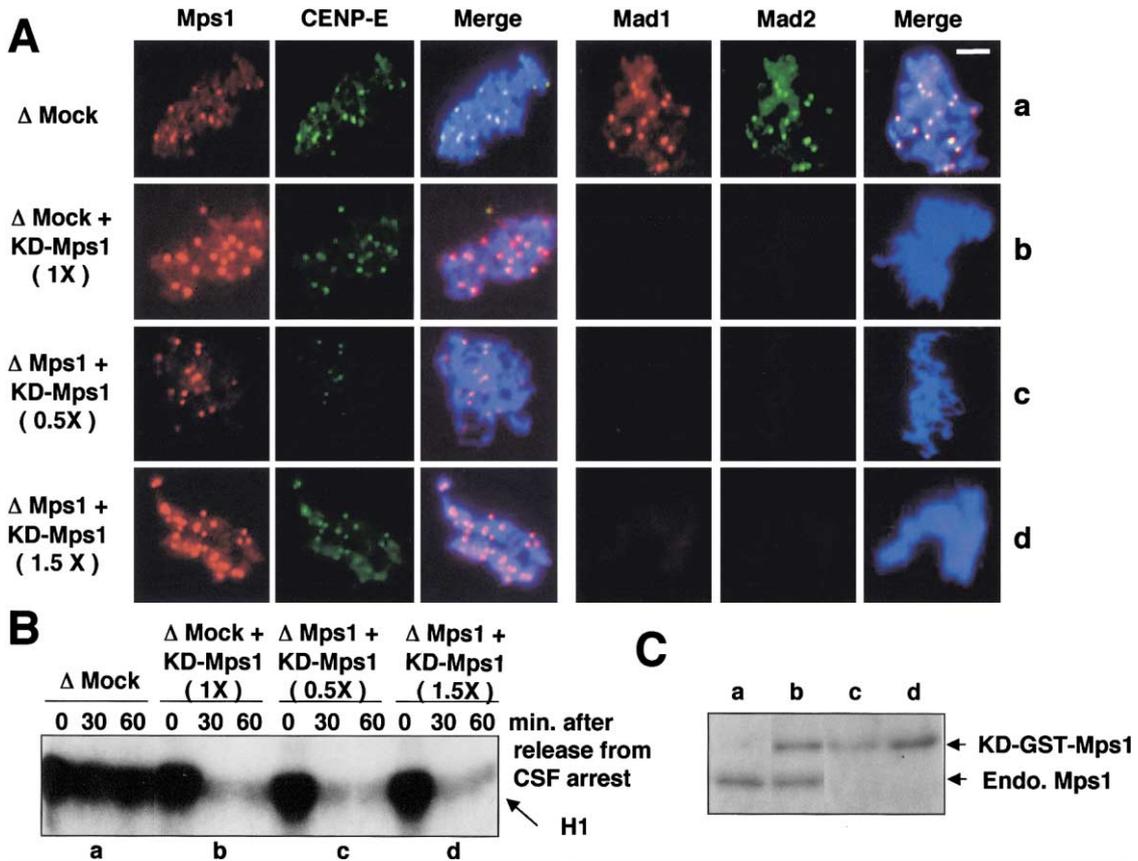


Figure 6. Mps1 Kinase Activity Is Required for Mad1/Mad2 Recruitment to Kinetochores and Checkpoint Activation

(A) CSF egg extracts were depleted with mock (IgG, rows a and b) or Mps1 (anti-CP, rows c and d) in the absence (row a) or presence of added recombinant KD-Mps1 [row b: 50 nM (1X); row c: 25 nM (0.5X); row d: 75 nM (1.5X)]. Thirty minutes after activating the checkpoint with sperm nuclei and nocodazole, the extracts were assayed for immunofluorescence either for Mps1/CENP-E or Mad1/Mad2. Chromatin, visualized with DAPI, and Mps1/CENP-E or Mad1/Mad2 co-staining were merged. Bar, 5  $\mu$ m.

(B) The same extracts were assayed for histone H1 kinase activity at 0, 30, and 60 min after calcium addition.

(C) Levels of endogenous and recombinant KD-Mps1 from the same experiment were detected by immunoblot (anti-CP).

bound KD-Mps1 did not allow recruitment of Mad1 and Mad2 to kinetochores (Figure 6A, rows b and c) nor did it restore checkpoint signaling as measured by histone H1 kinase activity (Figure 6B, compare a–c). Moreover, KD-Mps1 dominantly disrupted checkpoint signaling even when added to levels similar to that of the endogenous Mps1 (see Figure 6C, b): under these conditions, neither Mad1 nor Mad2 were recruited to kinetochores (Figure 6A, row b) and histone H1 kinase activity was not maintained after release from CSF (Figure 6B, b).

Despite disruption of kinetochore signaling, the dominant KD-Mps1 did stimulate CENP-E association with kinetochores in a dosage-dependent manner: addition to Mps1-depleted extracts of the KD-Mps1 mutant to half the normal level of Mps1 (Figure 6C, row c) resulted in a reduced level of CENP-E at kinetochores (Figure 6A, row c), while close to normal levels of CENP-E were kinetochore bound when KD-Mps1 levels were increased above normal levels (Figures 6A and 6C, rows d). In contrast, subnormal levels of WT-Mps1 yielded full recruitment of CENP-E to the kinetochores (Figures 5A, last row, and 5C). Thus, while not strictly required to recruit CENP-E to the kinetochores, the kinase activity of Mps1 facilitates more efficient CENP-E binding.

## Discussion

We have shown here that a vertebrate Mps1 is a kinetochore-associated kinase that is essential for activation and maintenance of kinetochore-dependent mitotic checkpoint. Mechanistically, its action must be upstream of the diffusible Mad2 inhibitory complex since the loss of checkpoint signaling can be overcome in a kinetochore-independent manner by high levels of added Mad2. Furthermore, we have shown that kinase activity of Mps1 is required to activate the checkpoint, probably by recruiting Mad1 and Mad2 and a normal amount of CENP-E to kinetochores. Previously, kinetochore bound CENP-E had itself been shown to be required for efficient association of the Mad1/Mad2 complex with unattached kinetochores (Abrieu et al., 2000), so the simplest view is that Mps1 activity may be required to control CENP-E recruitment and activation at the kinetochores. This implies that Mps1 acts upstream of CENP-E (modeled in Figure 7). At a minimum, it is clear that since CENP-E, Mad1, and Mad2 remain in Mps1-depleted extracts but do not target to unattached kinetochores, none of them can be activated without some Mps1 kinase-dependent event, albeit we do not

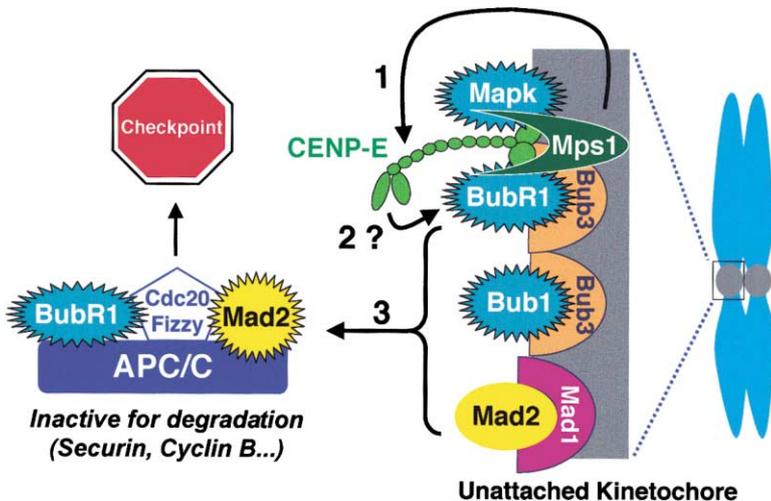


Figure 7. Mps1 Is Required for Recruitment of Mitotic Checkpoint Components to Kinetochores

Mps1 is essential for efficient recruitment to kinetochores of CENP-E, a microtubule motor previously demonstrated to be required for checkpoint activation in *Xenopus* egg extracts. CENP-E recruitment does not require the kinase activity of Mps1, but activation of the checkpoint (reflected in the recruitment of Mad1/Mad2 to kinetochores) does. Substrates for Mps1 may include other checkpoint components (such as Bub1, BubR1, or Map kinase, all of which are kinases themselves, or CENP-E). Mps1-dependent recruitment of CENP-E and Mad1/Mad2 are essential features of production and release from kinetochores of a diffusible inhibitor of APC/C.

know which, if any, are direct substrates for Mps1 kinase activity.

#### Possible Mechanisms of Action of Mps1 in the Kinetochores-Dependent Checkpoint

How does Mps1 govern CENP-E and thus Mad1/Mad2 kinetochore association? Since Mps1 is a kinase, a plausible way by which Mps1 could modulate CENP-E kinetochore association would be by phosphorylation. Indeed, we have shown that Mps1 kinase activity is fully required for Mad1 and Mad2 recruitment to the kinetochores, and partially required for CENP-E to be kinetochore localized, since some CENP-E (but not normal levels) binds to kinetochores in the absence of Mps1 kinase activity (but *not* in the absence of Mps1 protein). However, we do not know if the CENP-E recruited to the kinetochore by the KD-Mps1 is active in microtubule capture or whatever role CENP-E plays in initiating checkpoint signaling. Indeed, we have already shown that when CENP-E antibody is added to extracts, CENP-E can be present at kinetochores in an "inhibited" conformation (e.g., Abrieu et al., 2000; Figure 4B, c), unable to promote recruitment of Mad2. Thus, the most plausible possibility is that some CENP-E is recruited to a large kinetochore complex that includes Mps1, with Mps1 kinase activity required to stabilize this association of CENP-E at the kinetochores (Figure 7). Further action of the Mps1 kinase acting on CENP-E and/or other kinetochore substrates is necessary for facilitating Mad1 and Mad2 recruitment at the kinetochore.

Resolving further this issue will necessitate identifying direct substrates of Mps1 and/or identifying the direct target(s) of CENP-E action (including its likely influence on activity of the checkpoint kinase BubR1). We have no evidence that Mps1 and CENP-E do associate directly in a complex either before (Figure 5D) or after checkpoint activation (not shown), albeit it remains a possibility that such an interaction could exist and that we have failed to detect it because it reflects only kinetochore bound Mps1 and CENP-E, which might be a small fraction of each that is present in the extracts. Consistent with this, recombinant CENP-E can be weakly phosphorylated by recombinant WT-Mps1, but not KD-Mps1. Another possibility is that Mps1 and CENP-E do not interact directly,

but that some other unidentified component bridges between them, allowing Mps1 to recruit CENP-E indirectly.

In yeast, checkpoint arrest has been correlated to Mad1p hyperphosphorylation (i.e., retarded mobility on gels), and Mps1p seems to be, at least *in vitro*, the kinase responsible for this modification (Hardwick et al., 1996). However, the significance of Mad1p hyperphosphorylation remains unknown and in the *Xenopus* system Mad1 hyperphosphorylation has not been observed under checkpoint conditions (Chen et al., 1998). Additionally, we are not able to detect any *Xenopus* Mad1 hyperphosphorylation by Mps1 *in vitro* (not shown).

Like CENP-E, Mps1 is found here not only to be associated with unattached kinetochores, but also remains on already aligned chromosomes. Thus, Mps1 kinetochore association cannot be dependent on attachment status. From this, one could speculate that Mps1 may play at least two roles. One is to insure the presence of transducer proteins (e.g., CENP-E) on unattached kinetochores (where the checkpoint would normally be on), thus delaying anaphase. The other could be to insure the presence of CENP-E, itself necessary for chromosome congression, on attached chromosomes (checkpoint off), thus facilitating congression and possibly anaphase movement as well. Indeed, in *in vitro* assembled spindles, CENP-E does not localize to the kinetochores in Mps1-depleted extracts (not shown). (The degree to which deficits in Mps1 result in congression and in anaphase defects remains to be determined.)

Finally, the requirement of Mps1 to recruit CENP-E at the kinetochore must be fairly specific since removal of Mps1 does not prevent XKCM1, another member of the kinesin family, from kinetochore localization (Figure 5A; data not shown in noncheckpoint conditions). This strongly diminishes the possibility that Mps1 compromises the checkpoint simply by preventing kinetochore assembly, thereby leaving the most plausible explanation that Mps1 is directly or indirectly regulating CENP-E recruitment at the kinetochores.

#### Mps1 and Multiple Steps in the Checkpoint

An appreciation has emerged from a flurry of efforts in the past two years that the mitotic checkpoint pathway,

at least in *S. cerevisiae*, can be separated into two branches. One involves the *MAD1-3*, *BUB1*, and *BUB3* genes and controls anaphase onset and sister chromatid cohesion in a kinetochore-dependent way. The other branch involves *BUB2* and seems to regulate later events in cell cycle progression, including anaphase spindle positioning (Bloecher et al., 2000; Pereira et al., 2000). Where (or whether) yeast Mps1p fits into either branch (or both) has not been firmly established. In one vertebrate, we have shown here that Mps1 contributes directly to the kinetochore-dependent pathway. But there are hints that unlike the other known checkpoint components, Mps1 may participate in both branches. Mps1p overexpression in yeast inhibits Dbf2p in a Bub2p-dependent manner (Fesquet et al., 1999). While homologs of *BUB2* have not yet been identified in other species and the existence of such a bifurcated pathway remains to be verified in metazoans, the role of Mps1 at mammalian centrosomes and in their duplication (Fisk and Winey, 2001 [this issue of *Cell*]) would make it a very plausible candidate for a mitotic role in this pathway as well.

#### Experimental Procedures

##### Isolation of Mps1 cDNA and DNA Constructs

*Xenopus* Mps1 cDNA was amplified from total mRNA of *Xenopus* metaphase II-arrested eggs using the following degenerated primers: 5' TAYATGGTIATGGARTGYGG 3' and 5' TTNGCDATICCRAAR TCDAT 3' (N = A, C, G or T; Y = C or T; R = A or G; D = A, G, or T; I = Inosin) derived from *S. cerevisiae* *MPS1* and mammal *TTK* sequences. The amplified PCR product (300 bp) was used to probe  $5 \times 10^5$  plaques from a  $\lambda$ gt10 cDNA library constructed from mRNA from metaphase II-arrested eggs (Clontech). Three phage positive were isolated, the cDNAs subcloned into Bluescript, and sequenced. One clone had a complete open reading frame of 2649 bp, encoding a putative 882 amino acids protein. This clone was named Mps1.

##### Immunization Procedures, Antibody Production, Immunoprecipitation, and Immunoblotting

Rabbit polyclonal antibody anti-CP was generated against a peptide (C-GTTVSQNRTRTK) (the cysteine was used for coupling) corresponding to the C-terminal sequence of the Mps1 protein (residues 870 to 882). Antibody anti-NP was produced to the peptide (C-MDD EDISERKLIKIA) corresponding to the N-terminal sequence (residues 1 to 14). Peptides were coupled to thyroglobulin using m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (Pierce) for immunization and to immobilized bovine serum albumin for affinity purification.

Antibody anti-NTD was generated to a GST fusion protein corresponding to the N-terminal domain of the Mps1 protein (residues 1 to 626). Purified fusion protein was used to immunize rabbits and sera were depleted of reactivity against GST by passage over a GST column, and then affinity-purified on immobilized Mps1 fusion protein.

For immunoprecipitation (Figure 1B), CSF egg extracts were diluted with RIPA buffer (10 mM  $\text{Na}_2\text{H}_2\text{PO}_4$ , 300 mM NaCl, 50 mM NaF, 80 mM Na  $\beta$ -glycerophosphate, 5 mM EDTA, 0.5% Na deoxycholate, 1% Triton X-100) and incubated for 1 hr at 4°C with affinity-purified antibodies previously bound to protein A-Sepharose beads. Immunoprecipitates were washed three times with RIPA and twice with 50 mM Tris (pH 7.5).

Immunoblots were blocked with TBS (20 mM Tris [pH 8], 150 mM NaCl) containing 5% nonfat dried milk and probed with affinity-purified primary antibodies for 1 hr at room temperature in TBS 0.05% Tween. Primary antibodies were visualized using the ECL kit (Amersham, Figures 1 and 3) or  $^{125}\text{I}$  protein A (Amersham, Figures 5 and 6).

##### Recombinant Protein Production

Mps1 and human cyclin B1 were in vitro transcribed and translated in reticulocyte lysate, using the TNT system (Promega), either in the presence or absence of ( $^{35}\text{S}$ )-methionine as indicated.

*Xenopus* Mad2 protein was made in bacteria as described (Abrieu et al., 2000).

A baculovirus encoding full-length Mps1 fused to GST was constructed by standard methods (Bac-to-Bac, GIBCO). A kinase-dead version (KD-Mps1) was constructed by introducing a point mutation (D685A) in subdomain VII of the catalytic domain (by analogy with Mps1p—Lauzé et al., 1995), prior to insertion into baculovirus. Extracts of SF9 cells were collected after infection with either virus, and the corresponding fusion proteins purified over glutathione-Sepharose beads (Pharmacia) by standard methods.

##### Kinase Assays

For MBP (myelin basic protein) kinase assays, Mps1 immunoprecipitates were incubated at room temperature for 20 min with: MBP (Sigma) 5  $\mu\text{g}$ , 25 mM Hepes (pH 7.5), 10 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  ATP, and 1  $\mu\text{Ci}$  ( $\gamma$ - $^{32}\text{P}$ )-ATP. Recombinant WT-Mps1 and KD-Mps1 were assayed in the presence or absence of 15  $\mu\text{g}$  MBP as described, and in the presence of 0.1 mg/ml BSA.

For histone H1 kinase assays, 1  $\mu\text{l}$  of extract was frozen in liquid nitrogen at indicated time points. Extract samples were thawed by the addition of 9  $\mu\text{l}$  of H1 mix including ( $\gamma$ - $^{32}\text{P}$ )-ATP (Chen and Murray, 1997) and incubated for 10 min at room temperature. Reactions were stopped by adding Laemmli gel sample buffer, loaded onto a denaturing gel, and analyzed by autoradiography.

##### *Xenopus* Egg Extracts, Spindle Assembly In Vitro, Immunodepletion, and Antibody Addition

Fresh CSF extracts were prepared from unfertilized *Xenopus* laid eggs as described by Murray (1991). To visualize microtubules, rhodamine-labeled bovine brain tubulin (Wood et al., 1997) was added to CSF egg extracts at 50  $\mu\text{g}/\text{ml}$ . The spindle assembly checkpoint was activated at room temperature by the addition of a high concentration (9,000/ $\mu\text{l}$ ) of demembrated sperm nuclei (Murray, 1991) and nocodazole (10  $\mu\text{g}/\text{ml}$ ). Exit from metaphase arrest was induced by addition of  $\text{CaCl}_2$  to 0.4 mM. Progress through mitosis was monitored by fluorescence microscopic examination of 1  $\mu\text{l}$  aliquots squashed under a coverslip, after fixation of the sample in formaldehyde containing DAPI (1  $\mu\text{g}/\text{ml}$ ).

For immunodepletion, 100  $\mu\text{g}$  of affinity-purified antibodies or nonimmune rabbit IgG were bound for 30 min to 100  $\mu\text{l}$  Dynal beads protein A, and then added to 100  $\mu\text{l}$  of CSF egg extracts for 1 hr at 4°C. For Figure 5D, beads were gently washed three times with PBS and then analyzed by immunoblot. For antibody addition experiments, affinity-purified antibodies or nonimmune rabbit IgG were added to CSF egg extracts at 100  $\mu\text{g}/\text{ml}$ .

##### In Vitro Cyclin B1 Degradation

1  $\mu\text{l}$  of ( $^{35}\text{S}$ )-methionine-labeled human cyclin B1 from in vitro translation was added to 20  $\mu\text{l}$  of CSF egg extracts. At the indicated time points, 2  $\mu\text{l}$  reactions were stopped by adding Laemmli buffer, loaded into a denaturing gel, and analyzed by autoradiography.

##### Immunofluorescence

Immunofluorescence with *Xenopus* extracts was performed as previously described (Wood et al., 1997) except that spindles were prefixed for 10 min with 2% formaldehyde before centrifugation onto coverslips (Figure 2). For Mps1/CENP-E and Mad1/Mad2 costaining experiments (Figures 5 and 6), the structures were prefixed for 10 min with 1% formaldehyde. Anti-X-Mad2 antibody was raised and purified as described (Abrieu et al., 2000). Anti-XKCM1 was a gift from C. Walczak. CENP-E was detected using anti-tail CENP-E polyclonal affinity-purified antibodies (Wood et al., 1997). Initial experiments for Mad1 were done with antibody to *Xenopus* Mad1 provided as a generous gift of R.H. Chen. Subsequent efforts were performed with an affinity-purified rabbit polyclonal antibody raised against a bacterially produced GST fusion with *Xenopus* Mad1.

Anti-CENP-E and anti-Mad2 antibodies were labeled with biotin (long arm)-NHS (Vector Laboratories) and detected using avidin-FITC. In co-staining experiments, a nonbiotinylated antibody was used first, followed by Texas red or Cy5-conjugated anti-rabbit antibody, and then the biotinylated primary antibody was used, followed by avidin-FITC. Chromatin was visualized by staining with DAPI (1  $\mu\text{g}/\text{ml}$ ). Spindles were visualized using rhodamine-labeled bovine brain tubulin as described (Wood et al., 1997). The exposure times

were identical in all conditions within each independent figure, in order to compare fluorescence intensities.

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#### Accession Numbers

The *Xenopus Mps1* sequence reported in this paper was deposited in GenBank under accession number AF250290.