Cell, Vol. 106, 95–104, July 13, 2001, Copyright ©2001 by Cell Press

The Mouse Mps1p-like Kinase Regulates Centrosome Duplication

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Summary

The yeast Mps1p protein kinase acts in centrosome duplication and the spindle assembly checkpoint. We demonstrate here that a mouse Mps1p ortholog (esk, which we designate mMps1p) regulates centrosome duplication. Endogenous mMps1p and overexpressed GFP-mMps1p localize to centrosomes and kinetochores in mouse cells. Overexpression of GFP-mMps1p causes reduplication of centrosomes during S phase arrest. In contrast, a kinase-deficient mutant blocks centrosome duplication altogether. Control of centrosome duplication by mMps1p requires a known regulator of the process, Cdk2. Inhibition of Cdk2 prevents centrosome reduplication and destabilizes mMps1p, causing its subsequent loss from centrosomes, suggesting that Cdk2 promotes mMps1p's centrosome duplication function by regulating its stability during S phase. Thus, mMps1p, an in vitro Cdk2 substrate, regulates centrosome duplication jointly with Cdk2.

Introduction

Two precise duplication events, that of the genome and of the centrosome, must be completed as cells progress through the division cycle to ensure faithful transmission of genetic material. Centrosomes are microtubule organizing centers, consisting of a pair of centrioles surrounded by a pericentriolar centromatrix containing the microtubule nucleation capacity of the organelle (Schnackenberg and Palazzo, 1999), that are found at mitotic spindle poles in vertebrate cells. Centrosomes not only contribute to the assembly and function of the mitotic spindle, but are also required for the G1 to S transition (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). Like the genome, centrosomes are segregated to daughter cells by the spindle. In mammalian cells, the centrosome duplication event occurs during S phase, concurrent with DNA replication (reviewed in Hinchcliffe and Sluder, 2001; Stearns, 2001). Centrosome duplication is initiated upon activation of Cdk2 at the G1/S transition, and is regulated by cyclin A- and/or cyclin E- associated Cdk2 (Cdk2/A and/or Cdk2/E) kinase activity (reviewed in Hinchcliffe and Sluder, 2001; Stearns, 2001).

The budding yeast, *Saccharomyces cerevisiae*, has served as a model system for the duplication of centrosomes (Adams and Kilmartin, 2000). Although the vertebrate centrosome and the yeast spindle pole body (SPB, the yeast centrosome equivalent) are morphologically quite different, many SPB components have conserved centrosomal counterparts. The SPB duplication process has been well characterized at an ultrastructural level and many mutations affecting the process have been identified, but the precise molecular mechanisms are not well understood (reviewed in Adams and Kilmartin, 2000). One regulator of SPB duplication is Mps1p (Winey et al., 1991), an essential protein kinase (Lauzé et al., 1995), whose function is required at multiple stages of SPB duplication (Schutz and Winey, 1998), and for the function of the mitotic spindle assembly checkpoint (Hardwick et al., 1996; Weiss and Winey, 1996).

The mitotic spindle assembly checkpoint monitors assembly and function of the mitotic spindle (reviewed in Amon, 1999; Clarke and Gimenez-Abian, 2000) and can prevent the onset of anaphase when chromosomes are not appropriately attached to the mitotic spindle. Upon nuclear envelope breakdown in mammalian cells, checkpoint components bind kinetochores, and generate a signal that inhibits sister chromatid separation and mitotic cyclin degradation. Although it is unclear whether the checkpoint primarily monitors microtubule occupancy at kinetochores, tension between sister kinetochores, or both, a single unattached kinetochore is sufficient to inhibit anaphase onset (see reviews above). The spindle assembly checkpoint thus safeguards against chromosome missegregation and aneuploidy.

Like centrosomal proteins, components of the spindle assembly checkpoint are conserved between yeast and vertebrates (reviewed in Amon, 1999; Clarke and Gimenez-Abian, 2000). These include Bub1p, Bub3p (Hoyt et al., 1991), and Mad1-3p (Li and Murray, 1991), which, like Mps1p, were first identified in yeast. Mps1p, however, is unique among these proteins because it is required for both SPB duplication and the spindle assembly checkpoint. Mps1p-like proteins have been identified in several organisms and include the mouse esk kinase (Douville et al., 1992) and the human PYT/TTK kinase (Hogg et al., 1994; Lindberg et al., 1993; Mills et al., 1992). The observation that Mps1p is conserved among eukaryotes has led to an examination of the functions of vertebrate Mps1p homologs. We have examined the esk protein kinase (Douville et al., 1992), which we designate mMps1p, as the only apparent mouse Mps1p ortholog. Together with an accompanying paper on the *Xenopus* Mps1p ortholog, xMps1p (Abrieu et al., 2001 [this issue of *Cell*]), we provide evidence that, like Mps1p in yeast, the vertebrate Mps1p-like proteins regulate centrosome duplication and the spindle assembly checkpoint. Whereas the control of yeast Mps1p and its localization are unknown, we report that mMps1p localizes to centrosomes and kinetochores in NIH 3T3 cells, and that mMps1p, under the control of Cdk2, regulates centrosome duplication in mouse cells.

Results

The Mps1p Family of Kinases

S. cerevisiae Mps1p is an essential protein kinase required for SPB duplication (the centrosome equivalent

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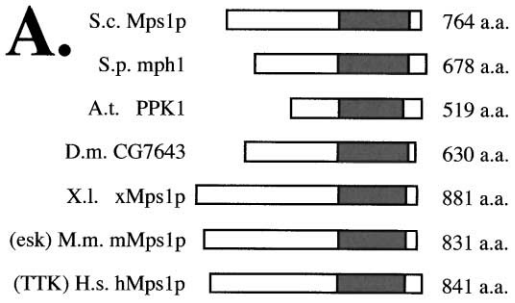

B. **Kinase Domain Similarity**

	to S.c. Mps1p:		to MEKK4:	
	% identity	e value	% identity	e value
S.c. Mps1p	-	-	29%	$5x10^{-12}$
S.p. mph1	52%	$4x10^{-45}$	30%	$2x10^{-14}$
A.t. PPK1	41%	$9x10^{-39}$	27%	$5x10^{-8}$
D.m. CG7643	39%	$1x10^{-25}$	28%	$1x10^{-10}$
X.l. xMps1p	40%	$7x10^{-32}$	29%	$1x10^{-10}$
M.m. mMps1p	41%	$3x10^{-34}$	26%	$6x10^{-11}$
H.s. hMps1p	41%	$2x10^{-33}$	26%	$4x10^{-11}$

Figure 1. The Mps1p Family of Kinases

Mps1p family members were identified by BLASTP or TBLASTN analysis (Altschul et al., 1997) of the nr protein database at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). *Arabidopsis thaliana* (A.t.) PPK1 (GenBank accession AAG51619) and *Drosophila melanogaster* (D.m.) CG7643 (GenBank accession AAF55450) are the results of genome sequencing projects for those organisms; references to *Saccharomyces cerevisiae* (S.c.) Mps1p and its homologs from *Schizosaccharomyces pombe* (S.p.), *Xenopus laevis* (X.l.), *Mus musculus* (M.m.), and *Homo sapiens* (H.s.) are provided elsewhere in the text. (A) Domain structure of Mps1p-like kinases; open boxes represent noncatalytic regions; filled boxes represent kinase domains. (B) Similarity among the kinase domains of the Mps1p-like proteins. Values represent the percentage of identical residues and BLASTP e-values resulting from comparisons restricted to the kinase domains of Mps1p-like proteins and Mps1p or human MEKK4, the next most similar protein to Mps1p.

organelle in yeast) and the mitotic spindle assembly checkpoint. Mps1p-like proteins, identified in several eukaryotes, share a similar structural organization consisting of a conserved, C-terminally located kinase domain (Figure 1). However, functional conservation among the Mps1p family has not been explored. In order to extend the functional analysis of this kinase family, we have analyzed the esk protein in cultured mouse cells. The mouse esk protein kinase (Douville et al., 1992) is the only identified mouse ortholog of Mps1p. Douville et al. (1992) identified two splice variants of esk, esk1, and esk2, differing only by the presence of a short predicted transmembrane domain in esk1. Reverse transcriptase PCR experiments indicated that esk2, but not esk1, is expressed in the mouse cell types used in this study (data not shown). We have therefore limited our analysis to esk2, lacking the transmembrane domain, which we refer to hereafter as mMps1p.

mMps1p Localizes to Centrosomes and Kinetochores

In order to determine the localization of endogenously expressed mMps1p in NIH 3T3 cells, we utilized an anti-peptide antibody directed against the C-terminal 20 amino acids of mMps1p (see Experimental Procedures). mMps1p localizes to centrosomes throughout the cell cycle in NIH 3T3 cells, as judged by colocalization with γ-tubulin by indirect immunofluorescence (Figure 2A). mMps1p is also found at centrosomes in primary mouse embryonic fibroblasts (data not shown). The centrosomal staining of the mMps1p antibody was completely blocked by pre-incubation of the antiserum with the peptide against which it was raised (data not shown). We also observed cytoplasmic and nuclear signals with the mMps1p antibody, however neither was significantly blocked by the peptide pre-incubation, suggesting that both are largely nonspecific background.

We also found that the mMps1p antibody stained kinetochores in mitotic cells, as judged by colocalization with a kinetochore-positive human CREST serum (Figure 2A). The kinetochore staining of the mMps1p antibody was also completely blocked by the peptide preincubation (data not shown). The distribution of mMps1p on kinetochores during mitosis is similar to that of characterized vertebrate spindle assembly checkpoint proteins such as Bub1 and Mad2 (reviewed in Amon, 1999; Clarke and Gimenez-Abian, 2000). The function of the Mps1p family of kinases in the spindle assembly checkpoint has been well established by studies of budding and fission yeast Mps1p (Hardwick et al., 1996; He et al., 1997; Weiss and Winey, 1996), and of xMps1p in the accompanying paper (Abrieu et al., 2001). We therefore anticipate that mMps1p also participates in the spindle assembly checkpoint. In support of this idea, mMps1p and GFP-mMps1p (see below) were found on all kinetochores in nocodazole-treated mitotic cells (data not shown).

GFP-mMps1p Localizes to Centrosomes and Kinetochores

We generated stable NIH 3T3-derived cell lines harboring tetracycline-repressible constructs expressing GFP-mMps1p (or GFP alone) to explore the functional relevance of mMps1p centrosomal localization (see Experimental Procedures). Multiple independent clones behaved similarly for each construct. For consistency, we present data generated from a single clone expressing either GFP-mMps1p, or GFP alone. No GFP signal was detected when these cells were grown in the presence of tetracycline, whereas GFP-mMps1p or GFP are expressed at levels detectable by fluorescence microscopy within 6 hr after removal of tetracycline (data not shown). While no specific localization was observed for GFP alone (see Figures 3 and 4), GFP-mMps1p was found at centrosomes throughout the cell cycle, and at kinetochores in mitosis (Figure 2B), similar to the localization of the endogenous, untagged protein as described above. In addition, overexpression of GFP-mMps1p generates a significant cytoplasmic GFP signal (Figure 2B). A similar distribution of GFP-mMps1p was observed when it was transiently expressed from the SV40 promoter (see Figure 4). Comparison of mMps1p and GFP-mMps1p signals on anti-mMps1p immunoblots suggests that GFP-mMps1p was moderately overexpressed (at most 5-fold) from the tetracycline-repressible promoter, whereas it was highly overex-

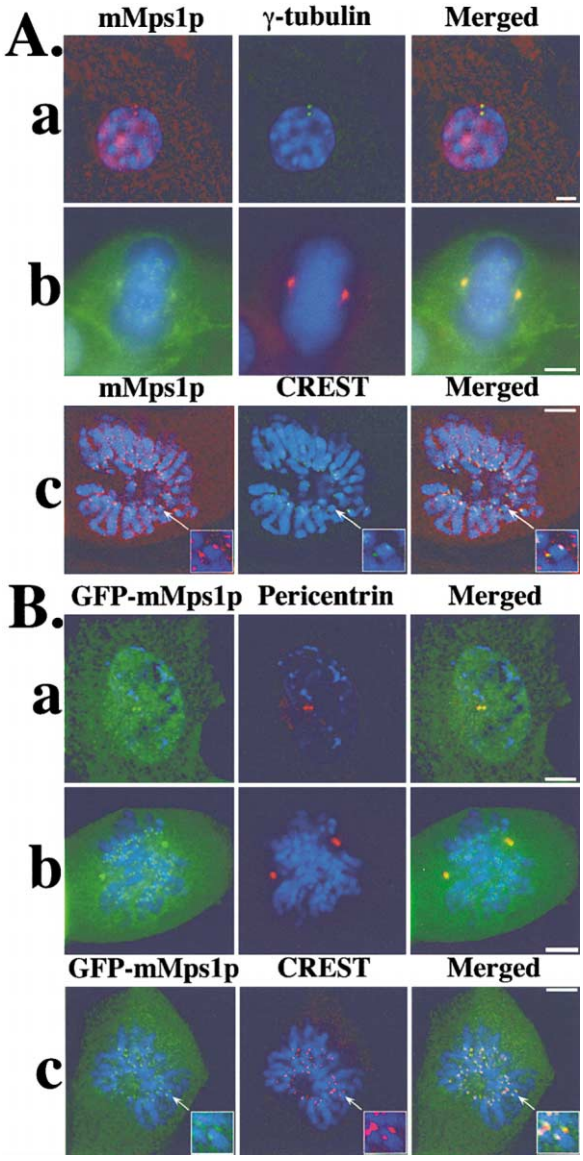

Figure 2. mMps1p and GFP-mMps1p Localize to Centrosomes throughout the Cell Cycle, and to Kinetochores during Mitosis in NIH 3T3 Cells

(A) mMps1p localization in NIH 3T3 cells. NIH 3T3 cells were analyzed by indirect immunofluorescence with antibodies against mMps1p, γ-tubulin, or kinetochores (CREST serum), as indicated. mMps1p centrosomal staining was seen in methanol fixed or Triton X-100 pre-extracted formaldehyde-fixed cells, but kinetochore staining was only seen in formaldehyde-fixed cells (with or without pre-extraction). (a) A methanol fixed interphase cell showing mMps1p (red), γ-tubulin (green), and DNA (blue). (b) A Triton X-100 pre-extracted formaldehyde-fixed mitotic cell showing mMps1p (green), γ-tubulin (red), and DNA (blue). (c) A mitotic cell fixed in formaldehyde (without pre-extraction) showing mMps1p (red), kinetochores (green), and DNA (blue). Bar = 5 μm.

(B) GFP-mMps1p localization in NIH 3T3 derived cells. The tetracycline-repressible GFP-mMps1p cell line, GEB4, cultured in the absence of tetracycline for 24 hr was analyzed by indirect immunofluorescence with antibodies against γ-tubulin and kinetochores (CREST serum), as indicated. (a) An interphase cell showing GFP-mMps1p (green), pericentrin (red), and DNA (blue). (b) A mitotic cell showing GFP-mMps1p (green), pericentrin (red) and DNA (blue). (c) A mitotic cell showing GFP-mMps1p (green), kinetochores (red), and DNA (blue). Bar = 5 μm.

pressed (perhaps as much as 50-fold) from the SV40 promoter in transient expression experiments (data not shown).

mMps1p Drives Centrosome Duplication

Centrosome duplication initiates at the G1/S transition in mammalian cells, and is completed during S phase. In at least one cell type, Chinese hamster ovary (CHO) cells, multiple rounds of centrosome duplication are observed under prolonged S phase arrest (termed centrosome reduplication) (Balczon et al., 1995; Kuriyama et al., 1986; Matsumoto et al., 1999). Such extra rounds of centrosome duplication under S phase arrest have not been reported in NIH 3T3 cells. However, the observations that S phase arrest is permissive for centrosome duplication suggested an assay for exploring a role for mMps1p in centrosome duplication. We arrested cells in S phase by double thymidine block, then induced GFP-mMps1p expression under S phase arrest. Not only did GFP-mMps1p localize to centrosomes during this arrest, but it also caused centrosomes to accumulate. After 24 hr, over 50% of cells overexpressing GFP-mMps1p possessed three or more centrosomes (see, for example, Figure 5). More strikingly, after 48 hr of S phase arrest, 84% of cells overexpressing GFP-mMps1p contained three or more centrosomes (Figures 3A and 3B). No such centrosome accumulation was observed when GFP alone was overexpressed (Figures 3A and 3B), or when GFP-mMps1p expression was repressed by tetracycline (Figure 3B). Overexpression of untagged mMps1p also causes centrosome accumulation in S phase arrested cells (data not shown), demonstrating that it is mMps1p overexpression, rather than the presence of the GFP tag, that causes centrosomes to accumulate.

While GFP-mMps1p clearly caused an accumulation of centrosomes, it is difficult to determine whether GFP-mMps1p affected the centrosome duplication process per se, or perhaps promoted cell cycle progression without cell division, allowing cells to enter a second S phase with two centrosomes, which then duplicated normally. However, cells incorporated BrdU within 2 hr if thymidine was removed (greater than 80% at t = 24 hr, data not shown), suggesting that cells had remained arrested in S phase. In addition, we found no evidence of cell cycle progression when we monitored arrested cells expressing GFP-mMps1p every 2 hr over a 24 hr time course, demonstrating that centrosomes accumulated during a prolonged S phase arrest. Although the fraction of cells with three or more centrosomes gradually increased, beginning between 4 and 6 hr, there was no decrease in the percentage of lamin-positive cells (indicating that nuclear envelope breakdown did not occur), and we observed virtually no mitotic figures (Figure 3C). In contrast, mitotic cells were readily observed if thymidine was removed (data not shown). Interestingly, in the arrested population, the number of cells with three centrosomes rose more quickly than the number with four (data not shown), suggesting that centrosomes do not reduplicate synchronously, consistent with earlier observations made in *Xenopus* and sea urchin blastocysts (Gard et al., 1990; Sluder et al., 1990).

To rule out the possibility that GFP-mMps1p causes fragmentation of centrosomes, rather than driving the

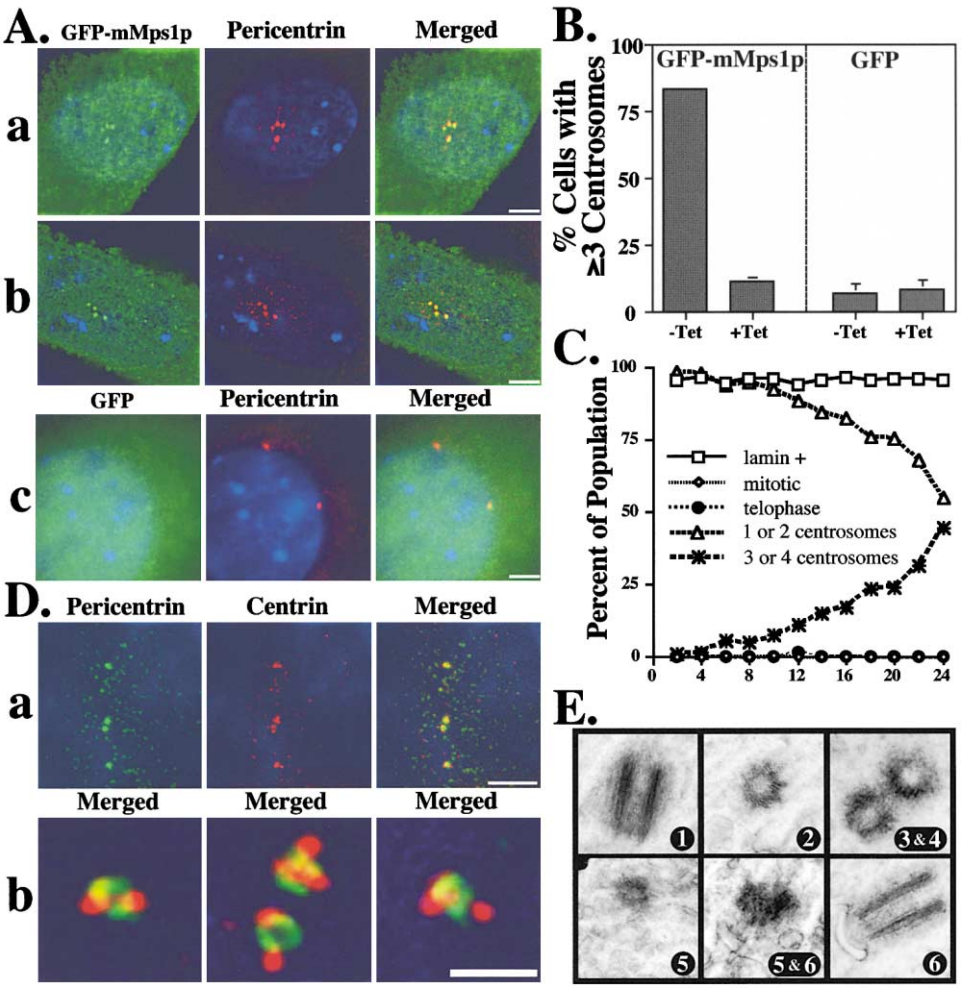

Figure 3. Overexpression of GFP-mMps1p Drives Centrosome Reduplication under S Phase Arrest

(A) GFP-mMps1p causes centrosome reduplication. GEB4 and GC4 cells induced to express GFP-mMps1p or GFP, respectively, while S phase arrested for 48 hr, were fixed in formaldehyde and analyzed by indirect immunofluorescence. (a and b) Two representative GEB4 cells, each having four centrosomes, showing GFP-mMps1p (green), pericentrin (red), and DNA (blue). (c) A representative GC4 cell, with two centrosomes as is normal for S phase arrested NIH 3T3 cells, showing GFP (green), pericentrin (red), and DNA (blue). Bar = 5 μm.

(B) Centrosome number from the experiment in (A). Values represent the percentage of each population with three or more centrosomes, and are presented as the mean ± standard deviation of three independent experiments. At least 100 cells were counted in each experiment.

(C) Time course of centrosome accumulation. GEB4 cells were arrested in S phase in the absence of tetracycline (see Experimental Procedures), then samples harvested every 2 hr for 24 hr, fixed with formaldehyde, and analyzed with antibodies against lamin and pericentrin to assess nuclear envelopes and centrosome number, and with Hoechst 33342 to identify mitotic figures. ≥ 200 cells were counted for each time point.

(D) GFP-mMps1p drives the complete duplication of centrosomes. GEB4 cells prepared as described in (A) were fixed in methanol and analyzed by indirect immunofluorescence. (a) A representative cell showing pericentrin (green), centrin (red), and DNA (blue). (b) Magnified images of each of the four centrosomes from the cell in (a). Bar = 1 μm.

(E) GEB4 cells prepared as described in (A) were subjected to correlative light and electron microscopy as described in the Experimental Procedures. Shown is a composite of electron micrographs showing six centrioles from one such cell. Serial sections are used to show centrioles 1 and 2, which were separated by a single section, and centrioles 5 and 6, of which only small portions of each were present in the same section. Centrioles 3 and 4 were fortuitously present in the same section.

centrosome duplication process, we performed two additional analyses. First, we used an antibody against centrin, a centriole component (Sanders and Salisbury, 1994), to determine the centriole content in cells with four apparent centrosomes, the vast majority of which possessed eight centrioles (90.6 ± 4.6%; an example is shown in Figure 3D). All such cells had six or more centrioles (n = 101 from three independent experiments). Second, we performed correlative light and electron microscopy on S phase arrested cells induced to overexpress GFP-mMps1p for 48 hr (see Experimental Procedures). Each of three cells examined contained more than four centrioles. Images from one such cell, in which we found three centrosomes, each containing two centrioles, are shown in Figure 3E. This figure shows two centrosomes in serial sections and a third centrosome for which both centrioles were fortuitously in one section. Together, this data suggests that GFP-mMps1p

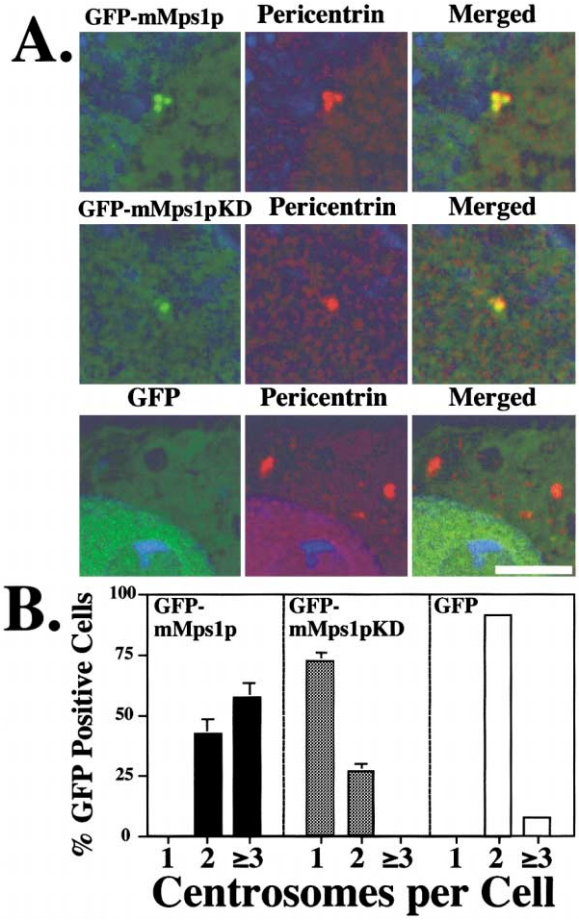

Figure 4. mMps1p Kinase Activity Is Required for Centrosome Duplication

(A) GFP-mMps1pKD blocks centrosome duplication. NIH 3T3 cells transfected with various expression constructs were arrested in S phase for 24 hr (see Experimental Procedures) and analyzed by indirect immunofluorescence. (Top) A representative cell expressing GFP-mMps1p with four centrosomes (three are shown and a fourth is out of the plane of focus) shown by GFP-mMps1p (green) and pericentrin (red); DNA is shown in blue. (Middle) A representative cell expressing GFP-mMps1pKD with a single centrosome shown by GFP-mMps1pKD (green) and pericentrin (red); DNA is shown in blue. (Bottom) A representative cell expressing GFP with two centrosomes shown by pericentrin (red); GFP is shown in green, and DNA in blue. Bar = 5 μm.

(B) Centrosome number from the experiment in (A). Values represent the percentage of GFP-positive cells with a given centrosome number, and are presented as the mean ± standard deviation of three independent experiments.

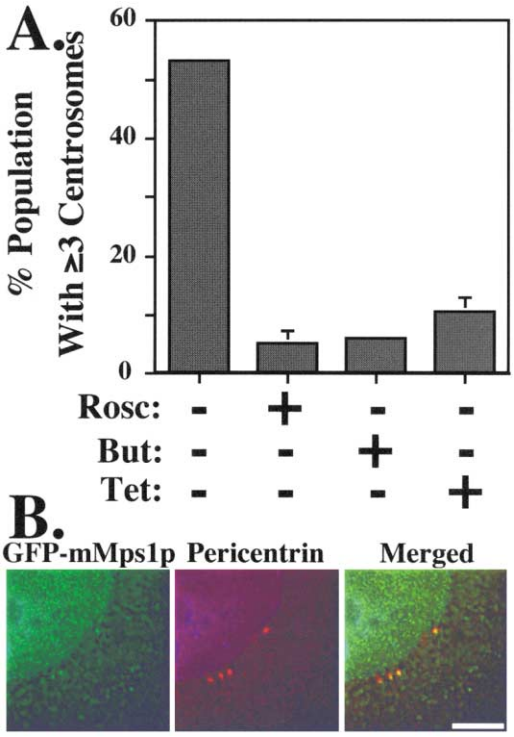

Figure 5. Cdk2 Activity Is Required for mMps1p-Dependent Centrosome Reduplication

(A) Small molecule Cdk2 inhibitors block GFP-mMps1p driven centrosome reduplication. GEB4 cells induced to express GFP-mMps1p were S phase arrested for 24 hr in the presence or absence of 180 μM roscovitine or 180 μM butyrolactone I, as indicated, and centrosome number was determined by indirect immunofluorescence. Values represent the percentage of each population with three or more centrosomes, and were determined as described in Figure 4B.

(B) GFP-mMps1p centrosomal localization and centrosome reduplication are restored upon removal of roscovitine. Roscovitine-treated cells were rinsed and incubated for 24 hr in the absence of roscovitine, fixed in formaldehyde, and analyzed by indirect immunofluorescence. GFP-mMps1p (green), pericentrin (red), and DNA (blue) are shown. Bar = 5 μm.

stimulates the production of new centrosomes (each with two centrioles) during a prolonged S phase arrest.

mMps1p Kinase Activity Is Required for Centrosome Duplication in NIH 3T3 Cells

We created a kinase-deficient point mutation in mMps1p (mMps1pKD) to determine if mMps1p-dependent centrosome reduplication requires mMps1p kinase activity. Analogous mutations abolished kinase activity in yeast (Lauzé et al., 1995) and *Xenopus* (Abrieu et al., 2001) Mps1p, and immunoprecipitated GFP-mMps1pKD lacked kinase activity (data not shown). NIH 3T3 cells were transiently transfected with GFP-mMps1p, GFP-mMps1pKD, or GFP alone (see Experimental Procedures), and S phase arrested for 24 hr. Like GFP-mMps1p, GFP-mMps1pKD localizes to centrosomes during S phase arrest (Figure 4A). However, centrosome reduplication was not observed in cells overexpressing GFP-mMps1pKD. In fact, overexpression of GFP-mMps1pKD blocked even the initial centrosome duplication that occurs in S phase arrested NIH 3T3 cells. Roughly 95% of cells overexpressing GFP alone possess two centrosomes, and 53% of those overexpressing GFP-mMps1p possess three or more centrosomes. However, 73% of S phase arrested NIH 3T3 cells overexpressing GFP-mMps1pKD possess only a single centrosome (Figures 4A and 4B). The observation that overexpression of GFP-mMps1pKD acts as a dominant negative perturbation of centrosome duplication suggests that mMps1p kinase activity is required for centro-

some duplication in NIH 3T3 cells. We also found that GFP-mMps1pKD can block centrosome duplication in both CHO and U2OS cells (data not shown), suggesting that Mps1p activity is a general requirement for centrosome duplication in a variety of cell types.

Cdk2 Kinase Activity Is Required for mMps1p-Dependent Centrosome Duplication

Cdk2 kinase activity is known to be required for centrosome duplication (Hinchcliffe et al., 1999; Lacey et al., 1999; Meraldi et al., 1999; Okuda et al., 2000), and for the centrosome reduplication that occurs in S phase arrested CHO cells (Matsumoto et al., 1999). To address the Cdk2 requirement of mMps1p-dependent centrosome reduplication, we treated S phase arrested cells induced to overexpress GFP-mMps1p with roscovitine for 24 hr as described by Matsumoto et al. (1999). Roscovitine inhibits Cdk1, Cdk2, and Cdk5 (Meijer et al., 1997; Meijer and Kim, 1997), of which Cdk2 is the major activity present in S phase arrested cells (Matsumoto et al., 1999). Because GFP-mMps1p cannot drive centrosome duplication in G1-arrested cells (data not shown), roscovitine was added at the end of the synchronization procedure, after the initial round of centrosome duplication was complete, to ensure cells had entered S phase. Therefore, this experiment specifically examines the Cdk2 requirement of mMps1p-dependent centrosome reduplication. Whereas over 50% of S phase arrested cells expressing GFP-mMps1p possessed three or more centrosomes after 24 hr, only 5% of roscovitine-treated cells possessed more than two centrosomes (Figure 5A). However, the ability of GFP-mMps1p to drive centrosome reduplication was restored upon removal of roscovitine (Figure 5B). Centrosome reduplication was also blocked by butyrolactone I (Figure 5A), another small molecule Cdk2 inhibitor (Meijer and Kim, 1997). Therefore, Cdk2 kinase activity is required for mMps1p-dependent centrosome reduplication.

Centrosomal Localization Is Intrinsic to mMps1p

Surprisingly, roscovitine and butyrolactone I (data not shown) dramatically reduced the overall levels of GFP-mMps1p, which is lost from centrosomes (Figure 6A), but did not affect the expression of GFP alone (as judged by microscopic or immunoblot analysis, data not shown). Roscovitine and butyrolactone I (data not shown) also abolished the centrosomal localization of the endogenous mMps1p in NIH 3T3 cells (Figure 6B). Given the dramatic reduction of GFP-mMps1p, these results might reflect a destabilization of mMps1p, preventing its accumulation at centrosomes (see below). Regardless, the apparent requirement for Cdk2 prompted us to determine if additional requirements for mMps1p centrosomal localization exist. Interestingly, mMps1p kinase activity is not required because GFP-mMps1pKD localizes to centrosomes (see Figure 4). In addition, endogenous mMps1p remained at centrosomes in the absence of microtubules (data not shown), and GFP-mMps1p induced after microtubule depolymerization accumulated at centrosomes (Figure 6C). Therefore, centrosome targeting appears to be intrinsic to mMps1p,

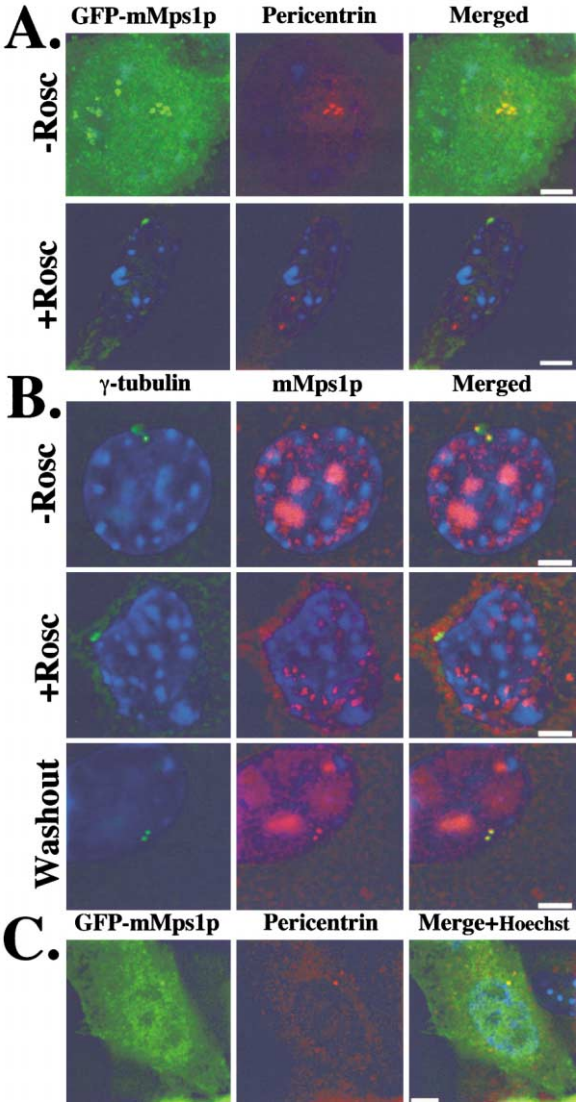

Figure 6. Cdk2 Activity Is Required for the Centrosomal Accumulation of mMps1p and GFP-mMps1p

(A) Loss of GFP-mMps1p in the absence of Cdk2 activity. GEB4 cells were S phase arrested for 24 hr in the presence or absence of 180 μM roscovitine, and analyzed with an antibody against pericentrin. (−Rosc) A representative control cell (which has undergone centrosome reduplication), and (+Rosc) a representative roscovitine-treated cell (with two centrosomes) showing GFP-mMps1p (green), pericentrin (red), and DNA (blue).

(B) mMps1p centrosomal accumulation requires Cdk2 activity. NIH 3T3 cells were S phase arrested for 24 hr in the presence or absence of 180 μM roscovitine, then fixed in methanol and analyzed by indirect immunofluorescence. γ-Tubulin (green), mMps1p (red), and DNA (blue) are shown for (−Rosc) an untreated cell, (+Rosc) a roscovitine-treated cell, and (Washout) a roscovitine-treated cell that was S phase arrested for an additional 24 hr following the removal of roscovitine. Bar = 5 μm.

(C) Recruitment of GFP-mMps1p to centrosomes does not require microtubules. Microtubules were depolymerized in GEB4 cells in the presence of tetracycline (see Experimental Procedures). GFP-mMps1p was then induced for 6 hr in the presence of 400 ng/ml nocodazole, and formaldehyde-fixed cells were analyzed by indirect immunofluorescence. Presented is a representative cell showing GFP-mMps1p (green), pericentrin (red), and DNA (blue). Bar = 5 μm.

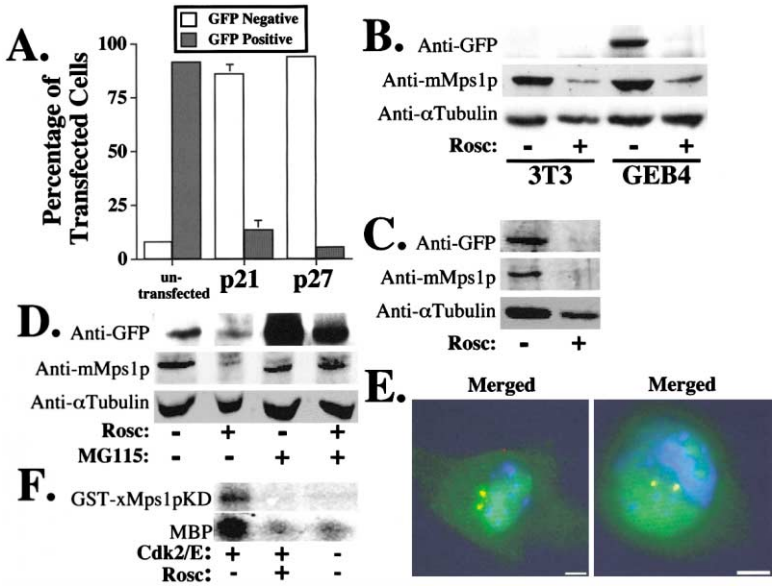

Figure 7. Cdk2 Activity Stabilizes mMps1p and GFP-mMps1p during S Phase

(A) GFP-mMps1p is not detected in $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ transfected cells. GEB4 cells transfected with $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ expression plasmids were S phase arrested for 24 hr in the absence of tetracycline (see Experimental Procedures), and analyzed with antibodies against $p21^{CIP1/WAF1}$ or $p27^{KIP1}$. Cells positive for $p21^{CIP1/WAF1}$ or $p27^{KIP1}$ were scored for expression of GFP-mMps1p and compared to nontransfected controls.

(B) mMps1p and GFP-mMps1p protein levels are reduced in roscovitine-treated cells. NIH 3T3 and GEB4 cells were S phase arrested for 24 hr in the presence or absence of 180 μM roscovitine as indicated, then harvested and subjected to immunoblot analysis (see Experimental Procedures) with antibodies against mMps1p, GFP, and α-tubulin.

(C) GFP-mMps1pKD protein levels are reduced by roscovitine. NIH 3T3 cells transiently expressing GFP-mMps1pKD were S phase arrested for 24 hr in the presence or absence of 180 μM roscovitine as indicated, then harvested and subjected to immunoblot analysis with antibodies against mMps1p, GFP, and α-tubulin.

(D) Loss of mMps1p and GFP-mMps1p in response to roscovitine is proteosome dependent. GEB4 cells were S phase arrested for 24 hr in the presence or absence of 180 μM roscovitine and/or 5 μM MG115 as indicated, then harvested and subjected to immunoblot analysis with antibodies against mMps1p, GFP, and α-tubulin.

(E) GFP-mMps1p localizes to centrosomes in cells treated with both roscovitine and MG115. Presented are two representative cells treated as in (D) and analyzed by indirect immunofluorescence showing GFP-mMps1p (green), pericentrin (red), and DNA (blue). Bar = 5 μm.

(F) Mps1pKD proteins are in vitro Cdk2 substrates. *Xenopus* Cdk2/E (150 ng) was combined with 4 μg myelin basic protein (MBP) and 400 ng GST-xMps1pKD or approximately 500 ng immunoprecipitated GFP-mMps1pKD (data not shown), in the presence of 180 μM roscovitine or the equivalent volume of DMSO. Controls lacking Cdk2 were identical to the roscovitine-treated samples.

and does not require its kinase activity, microtubules, or Cdk2 activity (see below).

Cdk2 Activity Is Required for the Stability of mMps1p

In addition to the dramatic decrease in GFP-mMps1p levels caused by roscovitine (Figure 6A), GFP-mMps1p was undetectable in cells overexpressing the cyclin-dependent kinase inhibitors $p21^{CIP1/WAF1}$ or $p27^{KIP1}$ (Figure 7A), and both $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ prevented centrosome reduplication (data not shown). Immunoblot analysis further demonstrates that levels of the mMps1p, GFP-mMps1p (Figure 7B), and GFP-mMps1pKD (Figure 7C) proteins are reduced by roscovitine treatment. To determine if Cdk2 activity affects mMps1p protein stability per se (as opposed to its transcription or translation), we examined mMps1p in the presence of the proteosome inhibitor MG115 (Rock et al., 1994), which prevented the loss of mMps1p and GFP-mMps1p in response to roscovitine (Figure 7D). MG115 also restored the centrosomal localization of GFP-mMps1p in roscovitine-treated cells (Figure 7E), suggesting mMps1p centrosomal localization does not require Cdk2 activity. Therefore, the major contribution of Cdk2 to the regulation of mMps1p is to promote its stability.

Our data demonstrate that mMps1p is required for centrosome duplication. Overexpression of GFP-mMps1p drives the duplication process under permissive conditions, whereas GFP-mMps1pKD prevents the duplication process altogether. Roscovitine did not affect the in vitro kinase activity of immunoprecipitated GFP-mMps1p (data not shown), yet prevented mMps1p-dependent centrosome reduplication, as did three additional Cdk2 inhibitors. Cdk2 activity is required for mMps1p stability, and mMps1p is an in vitro substrate of Cdk2 (Figure 7F), offering the possibility of direct regulation of mMps1p by Cdk2. We propose that Cdk2-dependent stabilization of mMps1p is part of a mechanism restricting centrosome duplication to S phase.

Discussion

We have shown that mMps1p localizes to centrosomes throughout the cell cycle, and to kinetochores during mitosis. The dynamics of mMps1p's distribution to kinetochores is consistent with a role in the spindle assembly checkpoint, as shown in the accompanying paper on xMps1p (Abrieu et al., 2001).

In this report, we have concentrated on the requirement for mMps1p in centrosome duplication, and have shown that mMps1p regulates centrosome duplication in cultured mouse cells. mMps1p localizes to centrosomes throughout the cell cycle, and this localization does not require Cdk2 activity, microtubules, or mMps1p kinase activity. Furthermore, overexpression of mMps1p drives the complete and faithful reduplication of the centrosome pair normally present in S phase arrested NIH 3T3 cells. Finally, a kinase-deficient version of mMps1p blocks centrosome duplication upon S phase entry in NIH 3T3 cells. This is consistent with observations that a kinase-deficient version of Mps1p prevents SPB duplication in yeast (Huneycutt and M.W., unpublished observations). We suggest that mMps1pKD inhibits centrosome duplication by displacing the endogenous

active mMps1p from the centrosome and/or by titrating out a key regulator or substrate into nonfunctional complexes.

The effects of mMps1p on centrosome duplication allowed us to further investigate the role of a known regulator of centrosome duplication, Cdk2, in this process. Inhibition of Cdk2, either with small molecule inhibitors or by overexpression of cyclin-dependent kinase inhibitor proteins, blocks the ability of mMps1p to drive centrosome duplication in S phase arrested cells. We have further demonstrated that Cdk2 activity stabilizes mMps1p, suggesting that one function of Cdk2 in centrosome duplication is to promote the stabilization and subsequent centrosomal accumulation of mMps1p. Our finding that Cdk2 activity stabilizes mMps1p during S phase is consistent with the observation that the level of the TTK protein kinase, the human Mps1p ortholog, rises at the G1/S transition and peaks during S phase (Hogg et al., 1994).

We believe that the primary contribution of Cdk2 to mMps1p function is to promote the stability of mMps1p under conditions that are permissive for centrosome duplication. Given that mMps1p is an in vitro Cdk2 substrate, such regulation might reflect a direct phosphorylation of mMps1p by Cdk2, wherein the phosphorylated form becomes protected from proteosome-mediated degradation. The suggestion that Cdk2 promotes the stability of mMps1p during S phase does not preclude other mechanisms to stabilize (or destabilize) mMps1p at other points in the cell cycle. Detailed structure/function studies will be required to identify the determinants of mMps1p localization, and to determine how Cdk2 acts to control mMps1p stability.

Recently, Okuda et al. (2000) identified Nucleophosmin/B23 (NPM/B23) as a Cdk2 substrate required for the initiation of centrosome duplication. NPM/B23 is present at unduplicated centrosomes where it prevents the initiation of centrosome duplication until it becomes phosphorylated by Cdk2. NPM/B23 then dissociates from centrosomes, and is not reacquired until mitosis, upon nuclear envelope breakdown. The stability of mMps1p and mMps1p-dependent centrosome reduplication also require Cdk2 activity, thereby demonstrating the existence of an additional function for Cdk2 in centrosome duplication. We assume that NPM/B23 must be released from centrosomes before mMps1p can function in centrosome duplication, and suggest that while Cdk2 acts through NPM/B23 to initiate centrosome duplication, it also acts through mMps1p to drive the duplication process.

One model suggested by our data orders Cdk2 and mMps1p in a linear pathway, with mMps1p, like NPM/B23, downstream of Cdk2. One obvious prediction of this model is that mMps1p is a direct substrate for Cdk2 in vivo as well as in vitro. While it is possible that Cdk2 and mMps1p act in different regulatory steps of centrosome duplication, or that mMps1p is upstream of Cdk2, we consider these possibilities unlikely because of the dependence of mMps1p stability on Cdk2 activity. Regardless of how the interaction between Cdk2 and mMps1p operates, it is clear that both of these protein kinases are critical to centrosome duplication.

The duplication of centrosomes and subsequent spindle assembly are as critical to the equal segregation of genetic material at mitosis as is the precise duplication of the genome. The study of yeast Mps1p provides insight into the possible consequences of defects in vertebrate Mps1p proteins. Cells lacking Mps1p function cannot duplicate the SPB or activate the spindle assembly checkpoint, and execute mitosis with a monopolar spindle generating aneuploid progeny. Our results demonstrate that mMps1p regulates centrosome duplication in mouse cells, and the results of Abrieu et al. (2001) establish that vertebrate Mps1p proteins function in the spindle assembly checkpoint. Defects in these processes can lead to aneuploidy (Cahill et al., 1998; Lingle and Salisbury, 2000), and defects in vertebrate Mps1p proteins may therefore lead to cancer. Although a screen of human colorectal cancers found no tumor-associated mutations in the human Mps1p ortholog, TTK (Cahill et al., 1999), it seems likely that TTK mutations may be present in tumors which display high levels of centrosome abnormalities, such as breast tumors (Lingle et al., 1998). Previous work on yeast Mps1p (Lauzé et al., 1995; Weiss and Winey, 1996), the accompanying report on the xMps1p (Abrieu et al., 2001), and our analysis of mMps1p indicate that Mps1p function contributes to maintenance of genomic integrity in a unique combination of roles, regulating both centrosome duplication and the spindle assembly checkpoint.

Experimental Procedures

Cells and Culture

NIH 3T3 cells were grown in DME (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; GIBCO, Rockville, MD). Chinese hamster ovary cells were grown in Ham's F12 (GIBCO) supplemented with 10% FBS. The human U2OS osteosarcoma cell line was grown in McCoy's 5A (GIBCO) supplemented with 10% FBS. MEF Tet-OFF (Clontech, Palo Alto, CA) were grown in DME supplemented with 10% FBS and 100 μg/ml G418 (US Biological, Swampscott, MA). All culture media were supplemented with 50 U/ml Penicillin G (GIBCO), 50 μg/ml Streptomycin sulfate (GIBCO), and cultures were maintained in a humidified 37°C incubator equilibrated with 5% CO_2.

Plasmids, Mutagenesis, and GFP Tagging

The mMps1p expression plasmid, pECE-esk, was a kind gift from John Bell (Douville et al., 1992). Reverse transcriptase PCR was performed as described by Yucel et al. (2000). To create mMps1pKD, the D637A mutation was created in the ATP binding pocket of the esk kinase domain by a two-step PCR "stitching" procedure (Fisk and Yaffe, 1999). The final PCR product was digested with NcoI and BstBI, and swapped into pECE-esk to create pHF13, which was sequenced to verify that the D637A mutation was the only nucleotide change.

mMps1p was tagged with GFP at the N terminus in pECE by PCR to create pHF11. The PCR product used to tag mMps1p with GFP was cloned into pECE to create pHF7. Tetracycline-repressible GFP-mMps1p was created by cloning the 3602 bp SalI-XbaI fragment of pHF11 into the SalI and XbaI sites of the pTRE2 vector (Clontech) to create pHF21. Tetracycline-repressible GFP was created by cloning the 747 bp SalI- XbaI fragment from pHF7 into the SalI and XbaI sites of pTRE2 to create pHF23. To create GFP-mMps1pKD, a 1324 bp D637A-containing PmlI-EcoRV fragment was swapped with the PmlI-EcoRV fragment of pHF11 to create pHF15. The $p21^{Cip1}$ and $p27^{Kip1}$ expression constructs were the kind gift of Erik Knudsen. The pβActproHyg vector, which expresses the Hygromycin B resistance gene driven by the β-actin promoter was the kind gift of Stephen Langer.

Transfections

For transient expression of GFP, GFP-mMps1p, and GFP-mMps1pKD, $p21^{Cip1}$ or $p27^{Kip1}$, cells were transfected with the respec-

tive expression plasmids using Effectine Reagent (Qiagen, Valencia, CA) during the recovery period of the double thymidine block procedure (see below). NIH 3T3-derived cells harboring tetracycline-repressible GFP and GFP-mMps1p were created by cotransfecting MEF-TetOff cells with pβActproHyg and pHF23 or pHF21, respectively, using Superfect reagent (Qiagen), and selecting for clones resistant to 400 μg/ml G418 and 200 μg/ml Hygromycin B (CalBiochem, San Diego, CA). Drug-resistant clones were screened visually for GFP expression. Once established, tetracycline-repressible cell lines were maintained in DME supplemented with 10% FBS, 50 μg/ml G418, and 50 μg/ml Hygromycin B.

Cell Synchronization and Drug Treatments

For double thymidine block, cells were passaged at a 1:5 dilution into DME containing 10% FBS and 4 mM thymidine (Sigma), then synchronized as described (Stein et al., 1994), with the exception that we used 4 mM thymidine. The end of the double thymidine block was considered the beginning of an S phase arrest (t = 0 hr). At this point, cells were then transferred into 6 well dishes at 2×10^5 cells per well in 2.0 ml of DME containing 10% FBS and 4 mM thymidine.

For Cdk2 and proteosome inhibition experiments, cells were arrested as described above, and released into medium containing 4 mM thymidine together with Roscovitine (CalBiochem), Butyrolactone I (ICN, Costa Mesa, CA), or MG115 (CalBiochem), as described in the text. Roscovitine and Butyrolactone I were resuspended at 50 mM DMSO and used at a final concentration of 180 μM. MG115 was resuspended at 10 mM in DMSO and used at a final concentation of 5 μM. The amount of DMSO was identical in all samples.

To depolymerize microtubules, cells were incubated for 30 min at 37°C in the presence of 400 ng/ml Nocodazole (US Biological), then placed on ice for 2 hr. Cells were then returned to 37°C in the presence of 400 ng/ml Nocodazole for varying times, fixed, and analyzed by indirect immunofluorescence as described below. For experiments involving induction of GFP-mMps1p, cells were washed four times with ice cold medium containing 400 ng/ml nocodazole before being returned to 37°C in the presence of 400 ng/ml Nocodazole. An antibody to α-tubulin was used to verify that microtubules had been depolymerized prior to the induction of GFP-mMps1p, and remained depolymerized throughout the experiment.

Antibodies and Indirect Immunofluorescence

Affinity-purified antibodies against mMps1p (esk; SC541 and the SC541-P peptide immunogen representing the C-terminal 20 amino acids of esk), $p21^{Cip1}$ (F-5), and $p27^{Kip1}$ (C-19) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit antibody against pericentrin was obtained from BAbCO (Richmond, CA). The DM1A and GTU-88 monoclonal antibodies against α-tubulin and γ-tubulin, respectively, were obtained from Sigma. Human kinetochore-positive CREST serum was obtained from Immunovision (Springdale, AR). The anti-centrin monoclonal antibody 20H5 (Sanders and Salisbury, 1994) was the kind gift of Jeffrey Salisbury. The XB10 antibody against Lamin A and C was obtained from BAbCO (Richmond, CA). A rabbit polyclonal antibody against GFP was the kind gift of Charles Zuker. Secondary antibodies were obtained from Amersham (Piscataway, NJ) or Jackson Immuno Research Laboratories (West Grove, PA). All fluorescently labeled secondary antibodies were minimally species cross reactive.

For indirect immunofluorescence, cells were grown on 13 mm round glass coverslips coated with 1 μg/ml Pronectin F (Stratagene, La Jolla, CA). Cells were fixed in PBS containing 4% formaldehyde (Ted Pella, Redding, CA) for 10 min at room temperature, or in −20°C methanol for 10 min at −20°C, then washed four times with 0.5 ml PBS. In some experiments, cells were extracted for 1 min at room temperature with 0.1% Triton X-100 in PBS, then fixed with formaldehyde. Fixed cells were incubated for 1 hr at room temperature in blocking solution (PBS, 0.1% Triton X-100, 5% FBS, 0.2 M Glycine). All antibody incubations were 1 hr at room temperature in a dark humidified chamber, followed by four PBS washes. Primary antibodies were diluted 1:200 in blocking buffer, with the exception of the anti-mMps1p antibody which was diluted 1:10, and were used consecutively. Secondary antibodies were diluted 1:1000 and were combined in one incubation with 1 μg/ml Hoechst 33342 (Sigma). Coverslips were mounted on glass microscope slides using Citifluor (Ted Pella). Standard fluorescence microscopy was carried out using a Leica DMRXA/RF4/V automated microscope with a Cooke Sensi-Cam digital camera. Images were collected and subjected to no neighbors or nearest neighbors deconvolution algorithms using the Slidebook software package (Intelligent Imaging Innovations, Denver, CO).

Electron Microscopy

For correlative light and electron microscopy, cells induced to express GFP-mMps1p plated onto Pronectin F coated Cellocates coverslips (Eppendorf, Hamburg, Germany) were S phase arrested for 48 hr as described above. Cells that appeared to have four centrosomes were identified by live cell fluorescence microscopy. Coverslips were then fixed for 30 min at room temperature in a buffer containing 0.15 M Sodium Cocodylate (Ted Pella) (pH 7.0) and 0.2% glutaraldehyde (Ted Pella), and processed for electron microscopy as described previously (McDonald, 1984).

Immunoblotting and Kinase Assays

NIH 3T3 cells or cells induced to express GFP-mMps1p were S phase arrested for 24 hr in the presence or absence of 180 μM roscovitine and/or 5μM MG115, removed from plates using trypsin/EDTA, harvested by centrifugation, rinsed in PBS, and lysed at $3–5 \times 10^6$ cells/ml in RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.2% SDS) containing Sigma's mammalian protease inhibitor cocktail (P8340.) Cells were lysed on ice for 30 min, and DNA sheared by ten passages through a 25G needle. Protein concentration was determined by UV absorbance. Samples were resuspended at approximately 2 mg/ml in sample buffer [25 mM Tris-HCl (pH 6.8), 2.5% SDS (w/v), 0.25% bromophenol blue (w/v), 12.5% glycerol (v/v), 2.5% β-mercaptoethanol (v/v)], and approximately 40 μg protein analyzed by immunoblot analysis as described previously (Yucel et al., 2000). Cdk2 kinase assays were performed essentially as described (Hartley et al., 1997).

Acknowledgments

We thank J.C. Bell, J.L. Salisbury, S. Langer, N.G. Ahn, E.S. Knudsen, C. Zuker, K.E. Knudsen, T. Stearns, and W.E. Theurkauf for advice and reagents used in this study. We are particularly grateful for the kind gifts of *Xenopus* Cdk2/E (J.L. Maller, UCHSC) and GST-xMps1pKD (A. Abrieu, J.A. Kahana, and D. Cleveland, UCSD), and to the Cleveland and Labbe laboratories for sharing data prior to publication. Deconvolution microscopy was made possible in part by a gift from Virginia and Mel Clark. We are indebted to Mary Morphew of the Boulder Laboratory for 3D Fine Structure (RR0592, J.R. McIntosh, P.I.) for her work on the correlative light and electron microscopy study. We would also like to thank J.K. Yucel, M.H. Jones, S.L. Jaspersen, A. Stemm-Wolf, S. McBratney, and C.P. Mattison for critical reading, and Jennifer Yucel for daily support. This investigation has been aided by a grant from The Jane Coffin Childs Memorial Fund for Medical Research to H.F., and supported by the National Institutes of Health (GM51312 to M.W.).

Received January 9, 2001; revised June 1, 2001.

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