Human Mps1 protein kinase is required for centrosome duplication and normal mitotic progression

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The mitotic spindle is essential for the maintenance of genetic stability, and in budding yeast its assembly and function depend on the Mps1 protein kinase. Mps1p is required for centrosome duplication and the spindle checkpoint. Several recent reports demonstrate that vertebrate Mps1 proteins regulate the spindle checkpoint, but reports conflict regarding their role in centrosome duplication. Here we provide multiple lines of evidence that the human Mps1 protein (hMps1) is required for centrosome duplication. A recently described rabbit polyclonal antibody against hMps1 specifically recognizes centrosomes in a variety of human cell types. Overexpression of a dominant-negative version of hMps1 (hMps1KD) can prevent centrosome duplication in a variety of cell types, and active hMps1 accelerates centrosome reduplication in U2OS cells. Finally, we demonstrate that disruption of hMps1 function with pools of hMps1-specific small interfering RNAs causes a pleiotropic phenotype resulting from the combination of severe mitotic abnormalities and failures in centrosome duplication. This approach demonstrates that hMps1 is required for centrosome duplication and for the normal progression of mitosis, and suggests that the threshold level of hMps1 function required for centrosome duplication is lower than that required for hMps1 mitotic functions.

Chromosome segregation is mediated by microtubules emanating from the poles of the mitotic spindle. Proper spindle function requires the regulated duplication of centrosomes, microtubule organizing centers found at mitotic spindle poles, and a quality control mechanism called the spindle checkpoint. Defects in centrosome duplication (1, 2) or in the spindle checkpoint (3) can disrupt normal progression of mitosis leading to chromosome segregation errors and aneuploidy, a hallmark of human tumors. A handful of protein kinase families, including the Cyclin-dependent kinase (cdk), Polo, Aurora, NIMA, Bub (4), and Mps1 (4, 5) families, regulate centrosome duplication and mitotic progression, and thus protect against genetic instability and aneuploidy.

Saccharomyces cerevisiae Mps1p defines a family of protein kinases with apparent orthologues in all vertebrates for which sequencing projects exist (5). Mps1p is a dual-specificity protein kinase (6) primarily required for duplication of the spindle pole body (7), the budding yeast centrosome equivalent organelle (8), and the spindle checkpoint (9, 10). The mouse esk (11) and human TTK/PYT (12, 13) dual specificity kinases have been recognized as Mps1 orthologues and are now referred to as mMps1 and hMps1, respectively (5, 14–17). Studies on the *Xenopus* (18), human (15–17), and zebrafish Mps1 (19) orthologues have now firmly established that vertebrate Mps1 proteins regulate the spindle checkpoint. This checkpoint prevents the onset of anaphase when chromosomes are not properly attached to the spindle and is regulated by several kinetochore proteins. These include six genes first identified in yeast, Bub1p and Bub3p (20), Mad1–3p (21), and Mps1p (9, 10), as well as vertebrate-specific proteins such as the BubR1 protein kinase and the CENP-E kinesin-like protein (3).

Although there is universal agreement that vertebrate Mps1 proteins are required for spindle checkpoint function, reports conflict regarding a role for vertebrate Mps1 proteins in centrosome duplication. In vertebrate systems, centrosome duplication is regulated by cyclin A- and/or cyclin E-associated cdk2 activity, and recent reports have implicated the function of several centrosomally localized cdk2 substrates, including NPM/B23 (22), the centriolar protein CP110 (23), and the mouse orthologue of Mps1 (14, 24). However, another report concluded that the human Mps1 orthologue does not localize to centrosomes and is not required for the ability of human U2OS osteosarcoma cells to undergo centrosome reduplication (15). To clarify this issue and determine whether vertebrate Mps1 proteins universally function in centrosome duplication, we have further explored the relevance of hMps1 to centrosome duplication in human cells.

Materials and Methods

Cells, Cell Culture, and Transient Transfection. HeLa S3 and U2OS cells were grown in DMEM (Sigma) and RPE1 cells were grown in a 1:1 mixture of DMEM and Ham's F12 (Invitrogen). All media were supplemented with 10% FBS (HyClone), 50 units/ml penicillin G (Invitrogen), and 50 μg/ml streptomycin (Invitrogen). Cells were cultured at 37°C in a humidified chamber in the presence of 5% CO_2. For experiments involving overexpression of GFP, GFP-hMps1, GFP-hMps1KD, or GFP-centrin from the SV40 early promoter, cells were transfected 16 h after a 1:10 passage with pHF7 (14), pHF36, pHF56, or pHF80, respectively (see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site, for plasmid construction), using Effectine reagent (Qiagen, Valencia, CA).

Antibodies. For indirect immunofluorescence (IIF) experiments, the following antigens were visualized with the indicated antibodies and dilutions: γ-tubulin, 1:200 GTU-88 mouse monoclonal antibody (Sigma); α-tubulin, 1:200 DM1A mouse monoclonal antibody (Sigma); kinetochores, 1:500 HCT-100 human centromere positive CREST serum (ImmunoVision, Springdale, AZ); Mad2, 1:200 anti-Mad2 rabbit polyclonal antiserum (Babco, Richmond, CA); CENP-E, 1:200 anti-CENP-E rabbit polyclonal antiserum (25); hMps1, 1:500 hMps1Ag3 (16); BrdUrd, 1:500 anti-BrdUrd rat monoclonal antibody (Accurate Chemicals). Secondary antibodies for IIF were FITC-conjugated donkey anti-human IgG and CY3-conjugated donkey anti-rat (Jackson ImmunoResearch), and Alexa 488- or Alexa 594-conjugated donkey anti-rabbit or anti-mouse IgG (Molecular Probes). Antibodies for immunoblot experiments were as fol-

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Abbreviations: siRNA, small interfering RNA; IIF, indirect immunofluorescence; HU, hydroxyurea.

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lows: α-tubulin, 1:10,000 DM1A; hMps1, 1:1,000 hMps1N1 (15) (Zymed), 1:1,000 hMps1Ag3 (16), or 1:500 SCB540 affinity-purified rabbit anti-hMps1 (Santa Cruz Biotechnology). Secondary antibodies for immunoblot experiments were Alexa 680-conjugated anti-mouse (Molecular Probes) and IRDye800-conjugated anti-rabbit (Rockland, Gilbertsville, PA).

IIF and Cytology. IIF was performed as described (14) with two exceptions; cells were fixed in 4% electron microscopy grade formaldehyde (Ted Pella, Inc., Redding, CA) supplemented with 1 mM $MgCl_2$ and 0.2% Triton X-100 for 10 min at room temperature, and antibody incubations were carried out for 45 min at 37°C in blocking buffer consisting of 0.5% BSA (wt/vol), 0.5% Nonidet P-40 (vol/vol), 1 mM $MgCl_2$, and 1 mM NaN_3 in PBS. Centrosome number was determined by counting γ-tubulin-positive structures. Mitoses were scored as abnormal based on the presence of chromosome alignment or segregation defects; prophase and prometaphase cells, wherein it is too early to detect such defects, were scored as normal. Incorporation of BrdUrd was scored by IIF after incubation of formaldehyde-fixed cells with RNase-free DNaseI (Invitrogen). For all experiments, values represent the mean ± SD of duplicate samples from three independent experiments. At least 100 relevant cells were counted for each duplicate sample.

Immunoblot Analysis. Harvesting and immunoblot analysis of 20–40 μg of cellular protein was performed as described (14). For phosphatase treatment of recombinant proteins, filters were simultaneously incubated with the hMps1N1 and hMps1Ag3 antibodies. For small interfering RNA (siRNA) experiments, filters were simultaneously incubated with the SCB540 anti-hMps1 and DM1A anti-α-tubulin antibodies. After incubation with secondary antibodies, filters were analyzed by using the Odyssey imaging system (Li-Cor, Lincoln NE). hMps1 disruption was estimated by comparing the ratio of hMps1 and α-tubulin fluorescence intensities (each corrected for background) between control and hMps1-siRNA transfections.

hMps1 Fusion Proteins and Phosphatase Treatment. The previously described GST-hMps1400-507 fusion protein (16) and the GST-hMps1-ha and GST-hMps1KD-ha fusion proteins were expressed in *Escherichia coli* (see *Supporting Materials and Methods*). For phosphatase treatment, 1 μg of protein was incubated with 0.4 units/μl λ-phosphatase (New England Biolabs) in 10 μl of phosphatase buffer, or buffer alone for 1 h at 30°C, then analyzed by immunoblot with the hMps1N1 and hMps1Ag3 antibodies.

Cell Synchronization. HeLa and U2OS cells were synchronized in S phase by the addition of hydroxyurea (HU) to 4 mM at the time of transfection. The beginning of S-phase arrest was considered to be 24 h after the addition of HU. Centrosome number was assessed by γ-tubulin staining in HeLa and U2OS cells 24 h after the addition of HU (i.e., at the beginning of S-phase arrest) and in U2OS cells 48 and 72 h after the addition of HU (i.e., after 24 and 48 h of S-phase arrest, respectively). HeLa cells were enriched in G_1 as follows. After a 24-h transfection with 33 nM hMps1- or Lamin-siRNAs, cells were washed twice with serum-free DMEM and incubated for 48 h in the absence of serum. Cells were released from starvation by the addition of FBS to 20%, and centrosome number was assessed by γ-tubulin staining 8 h after serum stimulation. G_1 enrichment was monitored by measuring BrdUrd incorporation over 8 h with or without the addition of serum. For experiments involving HeLa cells expressing GFP-centrin, cells were transfected with pHF80 24 h before siRNA transfection and serum starvation.

Generation and Transfection of siRNA. Pools of siRNAs representing the Lamin A/C cDNA nucleotides 153–719, hMps1 cDNA nucleotides 14–547, and hMps1 cDNA nucleotides 997-1543 were generated *in vitro* by using the Dicer siRNA Generation kit (Gene Therapy Systems, San Diego). PCR products containing these regions flanked by T7 promoter sequences (see *Supporting Materials and Methods* for primer sequences) were transcribed *in vitro*, and the resulting double-stranded RNA was converted into siRNAs with recombinant Dicer enzyme. HeLa cells were transfected with siRNAs in 24-well dishes by using 1 μl of Oligofectamine reagent (Invitrogen), diluted in serum- and antibiotic-free DMEM in a final volume of 0.25 ml and containing the following amounts of siRNA: 2.5 μl of a 20 μM solution per transfection for 21-nt Lamin A/C and rhodamine-labeled nonsilencing siRNAs (Qiagen) (0.74 μg, or 200 nM siRNA) and 2.5 μl of a 50 μg/ml solution per transfection for the siRNA pools (0.125 μg or 33 nM siRNA). In some transfections, half the normal amount of the hMps1 pools (0.0625 μg or 16.5 nM) was used. After 4 h of transfection in serum-free DMEM, a one-third volume of DMEM plus 30% FBS was added to each transfection.

Results and Discussion

hMps1 Localizes to Centrosomes in Human Cells. Using a recently described affinity-purified rabbit polyclonal antibody (16) generated against hMps1 amino acids 400–507 (hMps1Ag3), we have detected hMps1 at centrosomes in a variety of human cells, including RPE1 telomerase immortalized human fibroblasts (Fig. 1*A*), U2OS osteosarcoma cells (Fig. 1*B*), normal and tumor-derived human breast cells (data not shown), and HeLa cells (see Fig. 4*C*), as reported by Liu *et al.* (16). Several observations demonstrate that the centrosome staining of the hMps1Ag3 antibody is specific to the hMps1 protein. Preincubation of the antibody with various hMps1 fusion proteins, including full-length GST-hMps1 (data not shown) and GST-hMps1400-507, prevents centrosome staining in U2OS (Fig. 1*C*), HeLa, and RPE1 cells (data not shown). The hMps1Ag3 antibody recognizes a single major band on immunoblots corresponding to hMps1, the only band to disappear after preincubation with hMps1 fusion proteins (Fig. 2*A*). The ability of hMps1Ag3 to recognize centrosomes therefore corresponds to its ability to recognize hMps1. Furthermore, the centrosome staining of the hMps1Ag3 antibody disappears in cells treated with hMps1-siRNAs (see Fig. 4*C*). In contrast, neither preincubation with hMps1 fusion proteins nor hMps1-siRNAs (see Fig. 4*C*) had any affect on the ability of the anti-γ-tubulin antibody GTU-88 to recognize centrosomes (Fig. 1*C*). Together, these data demonstrate that the hMps1Ag3 antibody specifically recognizes hMps1 at centrosomes. As also demonstrated by Liu *et al.* (16), GFP-hMps1 localizes to centrosomes in HeLa cells (Fig. 1*D*), whereas GFP alone does not (Fig. 3*A*). Like Liu *et al.* (16), we also observed kinetochore localization of hMps1 when using both the hMps1Ag3 antibody and GFP-hMps1 (data not shown).

In a recent report, three monoclonal antibodies against hMps1 failed to recognize centrosomes (15). To reconcile that observation with our own, we considered the possibility that protein modification might mask some hMps1 epitopes at centrosomes. We have found that one of those monoclonal antibodies, hMps1N1 (15), is blocked by hMps1 autophosphorylation (Fig. 2*B*, N1). When purified from bacteria, GST-hMps1-ha is heavily autophosphorylated, as evidenced by a mobility shift that disappears on treatment with λ-phosphatase and is not observed for the catalytically inactive GST-hMps1KD-ha (Fig. 2*B*, Ag3). The hMps1Ag3 antibody recognizes both phosphorylated and dephosphorylated GST-hMps1-ha and kinase-deficient GST-hMps1KD-ha (Fig. 2*B*, Ag3). However, the hMps1N1 antibody fails to recognize autophosphorylated, active GST-hMps1-ha (Fig. 2*B*, N1). Furthermore, active phosphorylated GST-

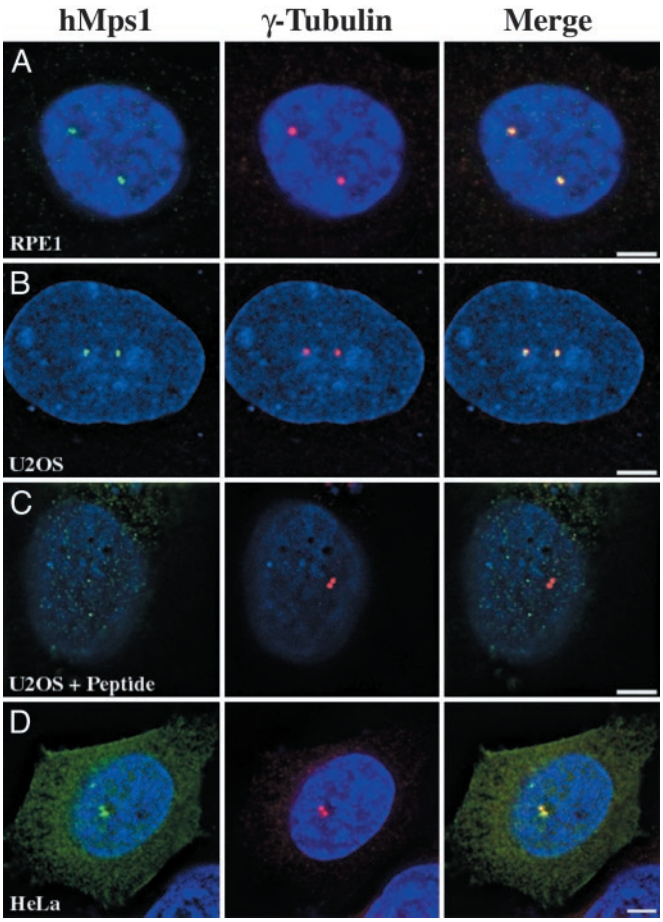

Fig. 1. hMps1 localizes to centrosomes in human cells. (*A–C*) The localization of hMps1 was determined by IIF in asynchronously grown RPE1 (*A*) and U2OS (*B* and *C*) cells with the hMps1Ag3 antibody. (*C*) The specificity of the hMps1Ag3 antibody was verified in U2OS cells by preincubation with the GST-hMps1400-507 fusion protein. hMps1, green; γ-tubulin, red; DNA, blue. (*D*) Asynchronously grown HeLa cells expressing GFP-hMps1were analyzed by IIF. GFP-hMps1 epifluorescence, green; γ-tubulin, red; DNA, blue. (Scale bar, 5 μm.)

hMps1-ha cannot titrate out the hMps1N1 antibody in immunoblot experiments, whereas GST-hMps1KD-ha can (data not shown). Together, these data demonstrate that autophosphorylation of hMps1 reduces the binding of the hMps1N1 antibody

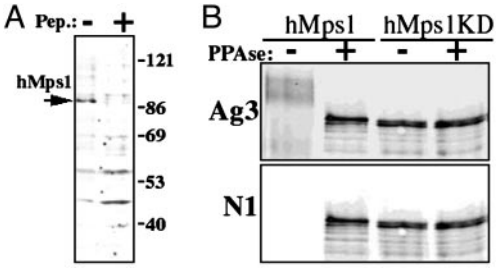

Fig. 2. Specificity of hMps1 antibodies. (*A*) Lysates from HU-arrested HeLa cells were analyzed by immunoblot with the hMps1Ag3 antibody before (−) or after (+) its preincubation with the GST-hMps1400-507 fusion protein. The arrow indicates the position of hMps1, and numbers represent the position of molecular mass markers in kDa. (*B*) Recombinant GST-hMps1-ha and GST-hMps1KD-ha were treated with λ-phosphatase (+) or buffer alone (−), then simultaneously analyzed by immunoblot with the hMps1Ag3 (Ag3) and hMps1N1 (N1) antibodies.

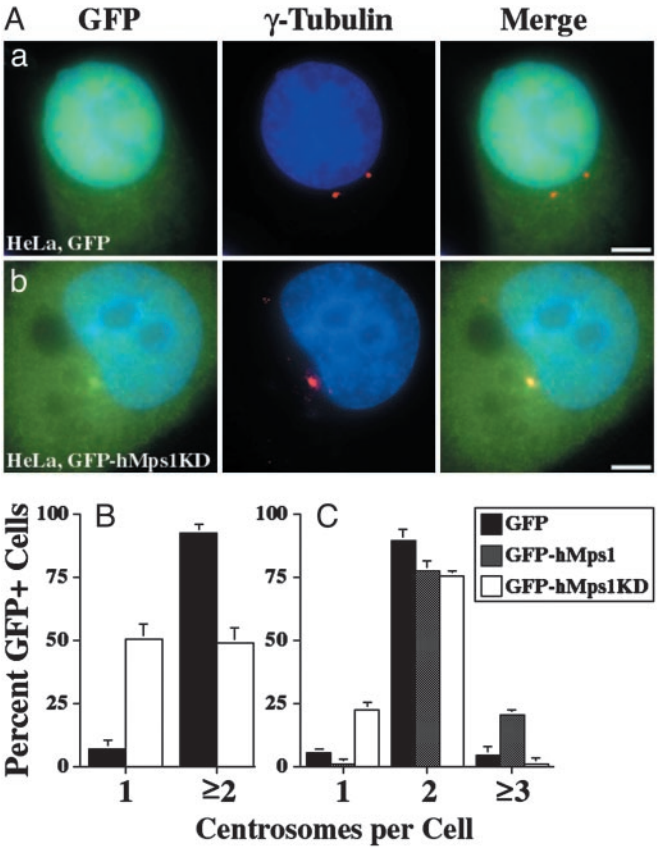

Fig. 3. hMps1 regulates centrosome duplication. (*A* and *B*) HeLa cells expressing GFP or GFP-hMps1KD were arrested in S phase with a 24-h HU treatment and analyzed by IIF. (*A*) Representative HeLa cells expressing GFP alone (*a*) (green) or GFP-hMps1KD (*b*) (green) showing γ-tubulin (red) and DNA (blue). (Scale bar, 5 μm.) (*B*) Centrosome number was determined in HeLa cells expressing GFP or GFP-hMps1KD. (*C*) U2OS cells expressing GFP, GFP-hMps1, or GFP-hMps1KD were arrested in S phase with a 24-h HU treatment. Centrosome number was determined after an additional 24 h of S-phase arrest.

to its epitope. Although the relevance of hMps1 autophosphorylation *in vitro* to hMps1 function *in vivo* is unclear, phosphatase treatment of hMps1 immunoprecipitated from U2OS cells increases its reactivity toward the hMps1N1 antibody (data not shown), suggesting that there are hMps1 species *in vivo* that are not recognized by the hMps1N1 antibody.

hMps1 Regulates Centrosome Duplication. To explore a role for hMps1 in centrosome duplication, we initially examined the consequences of high-level expression of hMps1 and hMps1KD (≈50-fold with respect to endogenous; ref. 14 and data not shown) from the SV40 early promoter in cells that do not normally undergo centrosome reduplication. In HU-arrested NIH 3T3 cells, GFP-hMps1 caused centrosome reduplication, and GFP-hMps1KD could prevent normal centrosome duplication (data not shown), as described for mMps1 and Mps1KD (14). Interestingly, GFP-hMps1 did not cause centrosome reduplication in HeLa or RPE1 cells (data not shown). However, GFP-hMps1KD localizes to centrosomes and can prevent normal centrosome duplication in both RPE1 (not shown) and HeLa cells (Fig. 3). Only 5% of HU-arrested HeLa cells expressing GFP alone had one centrosome, indicating they had not yet undergone centrosome duplication, whereas 50% of cells expressing GFP-hMps1KD had a single centrosome (Fig. 3*B*).

Next, we sought to determine whether high-level hMps1

expression could affect centrosome reduplication in U2OS cells. U2OS cells are known to reduplicate centrosomes under HU arrest (15), but after 24 h of S-phase arrest only 5% of U2OS cells expressing GFP alone had done so (Fig. 3*C*). In contrast, roughly 20% of U2OS cells expressing GFP-hMps1 possessed three or more centrosomes. Similar to our observations with mMps1 (14), this increase in centrosome number is due to the increase in hMps1, as opposed to any neomorphic activity of the GFP-tagged version, because similar results were obtained when the untagged kinase was overexpressed. The difference in centrosome number between U2OS cells expressing GFP and GFP-hMps1 is no longer apparent after 48 h of S-phase arrest; between 24 and 48 h the number of cells with three or more centrosomes rose by 4-fold in cells expressing GFP but by only 1.4-fold in cells expressing GFP-hMps1 (data not shown). Therefore, hMps1 can increase the rate of centrosome reduplication in U2OS cells but does not seem to increase its extent. We consider it unlikely that the extra centrosomes arose through any checkpoint influence of hMps1, because a painstaking examination of S phase-arrested cells overexpressing mMps1 revealed no evidence of cell cycle progression (14).

GFP-hMps1KD can also prevent centrosome duplication in U2OS cells, albeit to a lesser degree than observed in HeLa (e.g., Fig. 3*B*) and RPE1 cells; after 24 h of S-phase arrest, only 5% of U2OS cells expressing GFP alone had a single centrosome, whereas ≈23% of U2OS cells expressing GFP-hMps1KD had a single centrosome (Fig. 3*C*). Taken together, our data suggest that both centrosome duplication and centrosome reduplication in U2OS cells are hMps1-dependent. This hypothesis predicts that U2OS cells would be somewhat resistant to hMps1KD compared with nonreduplicating cells, and a modest increase of hMps1 might not increase their capacity for centrosome reduplication. The available data support this model; our data demonstrates that U2OS cells are resistant to hMps1KD as compared with HeLa cells, and Stucke *et al.* (15) have demonstrated that a modest increase in hMps1 levels (6-fold with respect to endogenous) does not increase the extent of reduplication in U2OS cells during a 64-h S-phase arrest.

hMps1 Is Required for Centrosome Duplication. Although overexpression and dominant-negative experiments provide compelling evidence that hMps1 regulates centrosome duplication, it is also important to address the consequences of the loss of hMps1 function. Therefore, we sought to achieve as great a depletion of hMps1 as possible to disrupt its centrosome duplication function(s). To achieve this goal, we generated pools of siRNAs *in vitro* representing ≈500-nt regions of the Lamin A/C and hMps1 cDNAs (see *Materials and Methods*) and transfected them into HeLa cells. At 33 nM, the hMps1-siRNA pools reduced hMps1 levels in HeLa cells by 80% (see *Materials and Methods*) as compared with that in Lamin-siRNA-transfected cells at 72 h posttransfection (Fig. 4*A*). Because the apparent transfection efficiency was 80%, as determined with a rhodamine labeled nonsilencing (RNS) siRNA (data not shown), it is likely that at this late time point close to 100% of hMps1 had been removed from 80% of cells. Compared with mock transfections, no hMps1 depletion was observed in cells transfected with the Lamin-siRNA pool (Fig. 4*A*), a commercial 21-nt Lamin-siRNA, or the RNS-siRNA, (data not shown). Similar levels of hMps1 disruption, and similar overall results, were observed for two different ≈500-nt pools of hMps1-siRNAs (data not shown). Below we present data obtained by using the hMps1 14-547 and Lamin 153-719 siRNA pools.

At 72 h posttransfection in HeLa cells, centrosome staining by the hMps1Ag3 antibody was absent in hMps1-siRNA cells, but apparent in Lamin-siRNA cells (Fig. 4*C*), further confirming the specificity of this antibody. Roughly 20% of mononucleated hMps1-siRNA cells possessed a single centrosome, suggesting

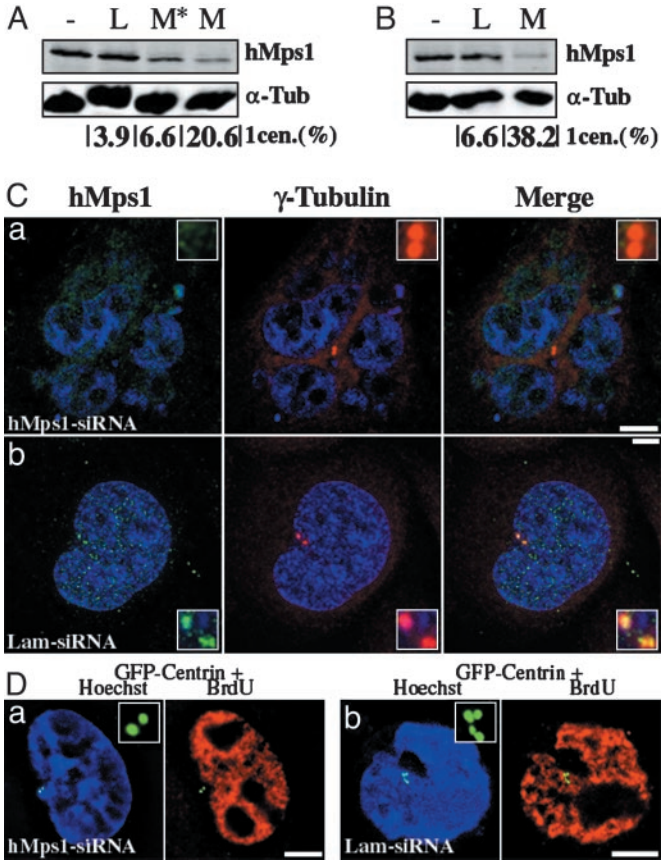

Fig. 4. siRNA-mediated depletion of hMps1 perturbs centrosome duplication. (*A*) At 72 h posttransfection with siRNAs, asynchronously grown HeLa cells were analyzed by immunoblot with antibodies against hMps1 (SCB540) and α-tubulin as indicated. −, Mock transfection; L, 33 nM Lamin-siRNA; M*, 16.5 nM hMps1 siRNA; M, 33 nM hMps1-siRNA. The percentage of cells in each transfection with one centrosome is indicated below the blots [1cen. (%)]. (*B*) HeLa cells were transfected with siRNAs, then enriched in G_1 with a 48-h serum starvation. At 8 h after serum stimulation, cells were analyzed by immunoblot as described in *A*. −, Mock transfection; L, 33 nM Lamin-siRNA; M, 33 nM hMps1-siRNA. The percentage of cells in each transfection with one centrosome is indicated below the blots [1cen. (%)]. (*C*) At 72 h posttransfection with siRNAs, HeLa cells were analyzed by IIF as described in Fig. 1. Representative HeLa cells transfected with 33 nM hMps1-siRNA (*a*) or 33 nM Lamin-siRNA (*b*) showing hMps1 (green), γ-tubulin (red), and DNA (blue). (*D*) HeLa cells expressing GFP-centrin were enriched in G_1 as described in *B*, and BrdUrd was included during the 8-h serum stimulation. Representative HeLa cells transfected with 33 nM hMps1-siRNA (*a*) or Lamin-siRNA (*b*) showing GFP-centrin (green), DNA (blue), and BrdUrd (red). *Insets* in *C* and *D* show 4-fold magnification of centrosomes. (Scale bar, 5 μm.)

that they had failed in centrosome duplication, as compared with 4% of Lamin-siRNA-transfected cells (Fig. 4*A*). Of the hMps1-siRNA cells with a single centrosome, 64% incorporated BrdUrd in a 6-h labeling, demonstrating that they were capable of entering the cell cycle. However, 17% of hMps1-siRNA cells were multinucleate (Figs. 4*Ca* and 5*Bi*), suggesting a high incidence of cytokinesis failures. Cytokinesis failures in hMps1-siRNA-transfected HeLa cells are intriguing because centrosomes control the fidelity of cytokinesis (26), and because a link between Mps1p and cytokinesis is provided by its interaction with Mob1p in yeast (27). If centrosome duplication proceeded normally after cytokinesis failures, multinucleate cells should have four centrosomes. However, 63% of multinucleated hMps1-siRNA-transfected cells have only two centrosomes (Figs. 4*Ca* and 5*Bi*), and the number of cells with a single centrosome is likely an underestimate of centrosome duplication failures.

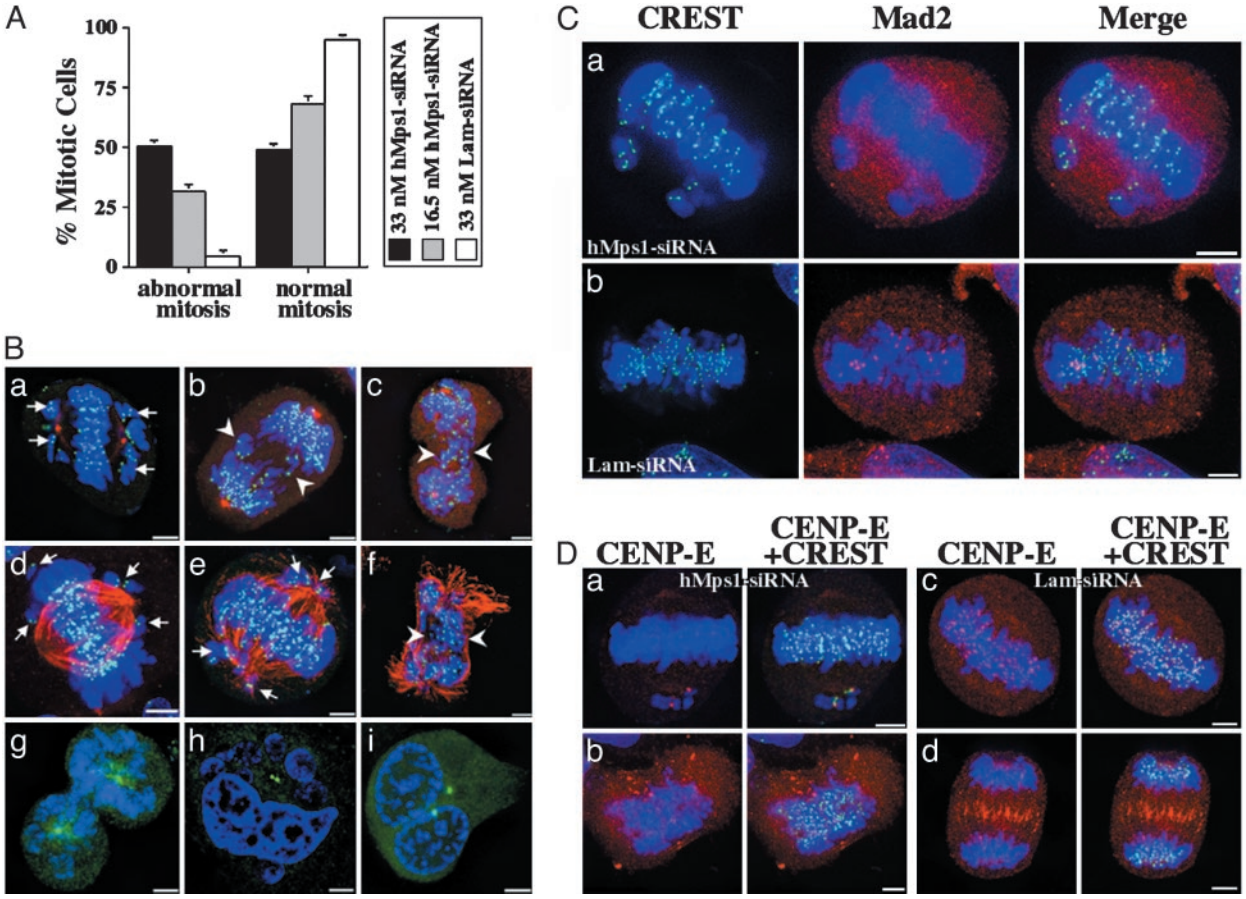

Fig. 5. siRNA-mediated depletion of hMps1 perturbs normal mitotic progression. (*A* and *B*) HeLa cells were analyzed for mitotic abnormalities by IIF 72 h after transfection with hMps1- or Lamin-siRNAs. (*A*) Cells were scored for the presence (abnormal) or absence (normal) of chromosome alignment and segregation defects. (*B*) Representative chromosome alignment and segregation defects in hMps1-siRNA-transfected HeLa cells. (*a–c*) Kinetochores (green) and γ-tubulin (red). (*d–f*) Kinetochores (green) and α-tubulin (red). (*g–i*) γ-tubulin (green). (*a*, *d*, and *e*) Representative chromosome alignment defects; arrows indicate unaligned and/or unattached chromosomes. (*b*, *c*, *f*, and *g*) Representative chromosome segregation defects; carets indicate unsegregated chromosomes. (*g*) A representative cell attempting to divide through unsegregated chromosomes. (*h*) A representative micronucleated cell with elongated nuclear morphology. (*i*) A binucleated prophase cell with two centrosomes. (*a–i*) DNA is shown in blue. (Scale bar, 5 μm.) (*C*) HeLa cells were analyzed by IIF to determine the distribution of Mad2 72 h after transfection with siRNAs. Representative HeLa cells transfected with 33 nM hMps1-siRNAs (*a*) or Lamin-siRNAs (*b*) showing kinetochores (green), Mad2 (red), and DNA (blue). (Scale bar, 5 μm.) (*D*) HeLa cells were analyzed by IIF to determine the distribution of CENP-E 72 h after transfection with siRNAs. Representative HeLa cells transfected with 33 nM hMps1-siRNAs (*a* and *b*) or Lamin-siRNAs (*c* and *d*) showing kinetochores (green), CENP-E (red), and DNA (blue). (*a*) Decreased binding of CENP-E to kinetochores at metaphase. (*b*) Failure of CENP-E to redistribute to the spindle midzone during anaphase. (*c*) Normal kinetochore binding of CENP-E at metaphase. (*d*) Normal redistribution of CENP-E to the spindle midzone during anaphase. (Scale bar, 5 μm.)

Regardless, the hMps1-siRNA phenotype in cycling HeLa cells is too complex to permit a straightforward analysis of centrosome duplication.

We also analyzed centrosome duplication in HeLa cells that had been enriched in G_1 by serum starvation. At 8 h after serum stimulation, 38% of hMps1-siRNA cells possessed a single centrosome, as compared with 6.6% of Lamin-siRNA cells (Fig. 4*B*). We verified that these cells had failed in centrosome duplication by using HeLa cells expressing GFP-centrin. Roughly 85% of hMps1-siRNA cells with two centrioles (representing 32% of the entire population) were BrdUrd-positive, indicating that they had entered S phase but failed to undergo centrosome duplication (Fig. 4*D*). In contrast, control cells quickly duplicated centrosomes on entering S phase, and only 13% of Lamin-siRNA cells with two centrioles (representing only 0.8% of the entire population) had incorporated BrdUrd. Therefore, hMps1-siRNA caused a 40-fold increase in the number of S-phase cells with a single centrosome. Given that this experiment achieved only a 65% reduction in hMps1 levels (Fig. 4*B*), and that serum starvation of HeLa cells allows only G_1 enrichment and not a true synchronization, this level of failed centrosome duplication is quite striking.

hMps1 Is Required for Normal Mitotic Progression. The hMps1-siRNA phenotype in cycling HeLa cells is consistent with failures in both centrosome duplication and normal mitotic progression. At 72 h after hMps1-siRNA transfection, 50% of mitotic cells displayed visible chromosome segregation defects (Fig. 5*A*). These defects included unattached and unaligned chromosomes at metaphase, lagging chromosomes during anaphase, and anaphase in the presence of unattached chromosomes (Fig. 5*B*). The more severe anaphase defects (Fig. 5*B c*, *f*, and *g*) may lead directly to cytokinesis failures. Anecdotal evidence for this suggestion is provided by cells attempting to cleave through unsegregated DNA (Fig. 5*B c* and *g*), and by micronucleated cells with an elongated nuclear morphology (Fig. 5*Bh*) reminiscent of the distribution of unsegregated DNA in anaphase and at cytokinesis (Fig. 5*B b*, *c*, *f*, and *g*). Micronuclei in such cells are kinetochore positive (data not shown), suggesting they arose from missegregated or unattached chromosomes rather than through chromosome breakage (28).

We observed that the recruitment of Mad2 to kinetochores requires hMps1 in the absence of microtubule perturbations (Fig. 5*C*), providing further evidence that hMps1 is required for normal mitotic progression. It is therefore not surprising that hMps1-siRNA-treated HeLa cells fail to arrest in response to nocodazole (data not shown). Similar requirements for hMps1 in the activation of the spindle checkpoint (15), normal mitotic progression (16), and Mad2 recruitment (17) have recently been reported. However, we have observed much more severe mitotic defects than observed in these previous studies. Furthermore, unlike these previous hMps1-siRNA studies, which found no requirement for hMps1 in the distribution of CENP-E, we observed pleiotropic CENP-E defects, including reduced kinetochore binding and failure of CENP-E to redistribute to the spindle midzone during anaphase (Fig. 5*D*). The variability made the CENP-E defects difficult to quantify, but no such defects were observed in control cells. These results are consistent with the requirement for xMps1 in the localization of CENP-E to kinetochores revealed by immunodepletion of xMps1 from *Xenopus* extracts (18).

Different hMps1 Thresholds Exist for Centrosome Duplication and Mitosis. When HeLa cells are transfected with 16.5 nM hMps1-siRNAs, we achieved half the hMps1 depletion (Fig. 4*A*) and half the number of mitotic defects as observed at 33 nM hMps1-siRNAs (Fig. 5*A*). However, there was almost no detectable defect in centrosome duplication (Fig. 4*A*). Therefore, we conclude that centrosome duplication requires lower levels of hMps1 than that required for hMps1 mitotic functions, and levels of hMps1 depletion sufficient to disrupt mitotic progression are not necessarily sufficient to disrupt centrosome duplication.

Conclusions

In this report, we provide several lines of evidence supporting a role for hMps1 in centrosome duplication. We have shown that hMps1 localizes to centrosomes in a variety of human cell types using a recently described antibody (16) and demonstrate that this antibody is specific for hMps1. Furthermore, overexpression of hMps1KD can prevent centrosome duplication in NIH 3T3, HeLa, RPE1, and U2OS cells, and overexpression of hMps1 can accelerate centrosome reduplication in U2OS cells. Finally, siRNA-mediated depletion of hMps1 in HeLa cells causes a pleiotropic phenotype representing failures in both centrosome duplication and in the normal progression of mitosis. Although a previous study concluded that hMps1 is not required for centrosome duplication (15), we suspect that the level of hMps1 depletion achieved was insufficient to disrupt centrosome duplication, because it failed to reveal the requirement of hMps1 for normal mitotic progression (ref. 16 and this work). We have shown that relatively low levels of hMps1 are required for centrosome duplication, levels that are not sufficient to support normal mitotic progression. Taken together, our data demonstrate that hMps1 is required for centrosome duplication, and that the dual nature of Mps1 function in centrosome duplication and spindle checkpoint function is conserved among the vertebrate Mps1 proteins.

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