Silencing Mitosin Induces Misaligned Chromosomes, Premature Chromosome Decondensation before Anaphase Onset, and Mitotic Cell Death†

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Mitosin (also named CENP-F) is a large human nuclear protein transiently associated with the outer kinetochore plate in M phase. Using RNA interference and fluorescence microscopy, we showed that mitosin depletion attenuated chromosome congression and led to metaphase arrest with misaligned polar chromosomes whose kinetochores showed few cold-stable microtubules. Kinetochores of fully aligned chromosomes often failed to show orientation in the direction of the spindle long axis. Moreover, tension across their sister kinetochores was decreased by 53% on average. These phenotypes collectively imply defects in motor functions in mitosin-depleted cells and are similar to those of CENP-E depletion. Consistently, the intensities of CENP-E and cytoplasmic dynein and dynactin, which are motors controlling microtubule attachment and chromosome movement, were reduced at the kinetochore in a microtubule-dependent manner. In addition, after being arrested in pseudometaphase for approximately 2 h, mitosin-depleted cells died before anaphase initiation through apoptosis. The dying cells exhibited progressive chromosome arm decondensation, while the centromeres were still associated with spindles. Mitosin is therefore essential for full chromosome alignment, possibly by promoting proper kinetochore attachments through modulating CENP-E and dynein functions. Its depletion also prematurely triggers chromosome decondensation, a process that normally occurs from telophase for the nucleus reassembly, thus resulting in apoptosis.

Precise segregation of the chromosome is critical for stable inheritance of genetic material. Such a process is exerted by the spindle and kinetochore during mitosis. The spindle is a specialized dynamic microtubule assembly for chromosome movement in mitosis. Microtubules are nucleated from two spindle poles containing centrosomes in most types of animal cells. The kinetochore is a three-layer organelle located at the primary constriction, or centromere, of the chromosome and serves as the site of spindle microtubule attachment during mitosis (4, 36, 37).

Many proteins exhibit centromere localizations (25, 37). A few of them, for instance, CENP-B and CENP-C, associate with the centromere throughout the cell cycle. The rest, including cytoplasmic dynein, CENP-E, Mad2, BubR1, and mitosin (CENP-F), only exhibit transient associations in mitosis. Both dynein and CENP-E are microtubule-based motors and are important for chromosome movement during congression and segregation (4, 8, 42). Dynein is a protein complex that powers movement towards microtubule minus ends, or spindle poles, with the help of another complex, dynactin. CENP-E, a kinesin motor-like protein, may direct motion in the opposite direction. Nevertheless, inactivation of either motor does not disrupt congression, a process for chromosomes to

achieve alignment at the mitotic equator, possibly due to redundancy of their functions (4).

In *Drosophila melanogaster*, inactivation of dynein genetically or through dynein inhibitors causes multiple mitotic defects, including impaired centrosome positioning, kinetochore alignment, and poleward chromatid movement. Congression, however, still occurs (38, 41). Depleting mammalian CENP-E, on the other hand, results in monooriented chromosomes juxtaposed to spindle poles, decreased numbers of kinetochore microtubules, and markedly attenuated tension across sister kinetochores of fully aligned chromosomes (29, 33, 45, 47, 50). Mad2 and BubR1 are major components of the spindle checkpoint, a mechanism that guarantees anaphase onset only after the bipolar microtubule attachment for every pair of sister kinetochores. Their sensor-like functions are mainly attributed to their rapid depletions or attenuations from kinetochores upon microtubule attachment, so that the checkpoint is subsequently inactivated (4, 31, 40). Motor proteins also contribute to the checkpoint inactivation. Cytoplasmic dynein transports outer kinetochore proteins, including CENP-E, mitosin, and the aforementioned checkpoint proteins, to spindle poles to sequester them from kinetochores upon microtubule attachment (12, 13, 48, 49). CENP-E, on the other hand, activates BubR1 kinase and mediates silencing of BubR1 signaling (26).

Mitosin, also named CENP-F, is a protein of 3,113 amino acid residues located at the outer kinetochore plate in M phase (23, 34, 49, 52, 54). It is expressed in a cell cycle-dependent manner, with the protein levels low in G₁ phase but elevated sharply from the G₁/S boundary as a nuclear protein. It is hyperphosphorylated in M phase, exhibits localizations at kinetochores, spindle, and spindle poles, and is rapidly degraded

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at the end of mitosis (53, 54). Deletion analysis suggests that the "core region" located between residues 2792 and 2887 is responsible for kinetochore targeting, while other regions, including residues 2094 to 2487 and/or 2889 to 3113, stabilize the localization (51). In addition, its kinetochore targeting is also affected by Bub1, RanBP2, CENP-I, and Sgt1 (17, 18, 24, 39, 43). It is farnesylated at the C-terminal CAAX motif, and the modification is important for its kinetochore localization and degradation (1, 15). Moreover, overexpressing certain C-terminal portions of mitotin results in cell cycle delay, mainly in M phase (15, 54).

Although growing lines of evidence suggest a role of mitotin in M-phase progression, this issue has not been explored in details. In addition, a fragment of CENP-F (mitotin) has been shown to interact with CENP-E in a yeast two-hybrid screen (2). Coimmunoprecipitation of both proteins as well as reduction of kinetochore CENP-E in CENP-F-depleted cells further support their interaction (17, 50). Nevertheless, whether mitotin is functionally related to CENP-E remains unexplored. Here, we addressed these questions by studying phenotypes of mitotin depletion using vector-based RNA interference.

MATERIALS AND METHODS

Plasmid construction. The RNA interference vector pBS/U6 (44) was provided by Y. Shi (Department of Pathology, Harvard Medical School, Boston, MA). The following two complementary oligonucleotides were synthesized: 5'-GGAGAATCAAGATTGATGGACTCGAGTCCATCAATCTTTGATTCTCCTTTTTG-3' and 5'-AATTCAAAAAGGGAGATCAAGATTGATGGACTCGAGTCCATCAATCTTTGATTCTCC-3'. After annealing, the oligonucleotide mixture was ligated to pBS/U6 between ApaI (blunted) and EcoRI sites (44). The resultant plasmid, pBS/U6/Mi-1, was identified by restriction digestion and sequencing. It contained a 22-bp inverted repeat (italic in the oligonucleotide sequences) corresponding to nucleotides 200 to 221 of human mitotin cDNA (GenBank accession no. NM-016343) with a 6-bp spacer (XhoI site).

Cell culture. HeLa and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% calf serum (Sijiqing Company, Hangzhou, People's Republic of China) at 37°C in an atmosphere containing 5% CO₂. Cells were transfected using the calcium phosphate method and assayed 48 h posttransfection unless otherwise described. After transfection for ~21 h, HeLa cells were initially treated with thymidine (2 mM) for additional 18 h for synchronization at the G₁/S boundary and then cultured for another 9 h to enrich mitotic populations before being processed for immunostaining or live cell imaging. To disassemble microtubules (MTs), cells were released from thymidine block for 6 h and then cultured in the presence of nocodazole (5 µg/ml) for additional 3 h (22).

Antibodies. Antibodies to mitotin, BubR1, p150^{glued} (BD Transduction Laboratories), and α-tubulin (Sigma) were monoclonal. Rabbit antibodies to survivin and activated caspase-3 were from Novus Biologicals and New England Biolabs, respectively. Chicken antimitotin was prepared as described (49). Rabbit antibodies to CENP-E, Mad2, and human Nuf2 were kindly provided by T. J. Yen (Fox Chase Cancer Center, Philadelphia, PA), E. D. Salmon (University of North Carolina at Chapel Hill, Chapel Hill, North Carolina), and J. Kilmartin (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom), respectively. Sheep antibodies to Bub1 and Aurora B were gifts from S. S. Taylor (University of Manchester, Manchester, United Kingdom). Human anticrotomer antibody from patients with CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia) syndrome was a gift from W. R. Brinkley (Baylor College of Medicine, Houston, TX). Secondary antibodies conjugated with Alexa 488, 546, or 633 were purchased from Molecular Probes.

Fluorescence microscopy, measurement, and quantitation. To stain for kinetochore proteins, cells growing on glass coverslips were rinsed twice with PHEM (60 mM piperazine ethanesulfonic acid, 45 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, pH 6.9) followed by permeabilization with 0.5% Triton X-100 in PHEM for 3 min, and then fixed with 3.5% paraformaldehyde in PHEM for 15 min. Cold treatment of cells was performed on ice for 10 min as described (5) before processing on ice for fixation. Indirect immunofluorescence labeling was per-

formed as described previously (49). Fluorescence images were captured by using Leica TCS SP2 laser confocal microscope. Optical sections were scanned at 0.1- to 0.2-µm intervals. Z stack images were then formed by maximal projection. Statistical results were obtained in a blind fashion and presented as mean ± standard error of the mean.

To analyze kinetochore orientations, we first connected both outer edges of each sister kinetochore in an optical section with a straight line based on CREST staining to mark the kinetochore orientation. The angle included between this line and the spindle axis was then measured. Only kinetochores aligned at the spindle midzone were analyzed. Kinetochore pairs located in different optical sections were neglected due to technical difficulties in measuring their angles.

To quantify kinetochore immunofluorescence, an area with relatively homogeneous background was selected in a Z-stack cell image. The mean brightness (B_m) of the area was measured for the CREST antigen and another protein by using Adobe Photoshop. One or several representative regions lacking centromere staining were then chosen within the same area to obtain the averaged background brightness (B_{ab}). The relative kinetochore brightness $B_R = (B_m - B_{ab}) / [B_m(\text{CREST}) - B_{ab}(\text{CREST})]$ to eliminate variations in staining and image acquisition (17). The number of kinetochores included in this area was estimated according to the CREST staining. Several separate areas might be chosen in each cell. The B_R values from multiple measurements were averaged. The relative fluorescence intensity (Fig. 4C and Fig. 5C) was obtained by converting the average B_R from control cells to 1 and then normalizing the value from mitotin-depleted cells accordingly.

Interkinetochore distances were measured as the distance between the outer edges of CREST staining of a sister kinetochore for chromosomes aligned at the spindle midzone (21) using the NIH Image software. Sister kinetochores were identified through their bar-like, dumbbell-like, or symmetric patterns (Fig. 2). Only assured sister kinetochores located in the same optical sections were measured in each cell.

Quantitation results were managed with SigmaPlot and presented as mean ± standard error of the mean.

Flow cytometry. HEK293T cells were seeded in petri dishes containing two glass coverslips. pEGFP-F (Clontech) was cotransfected into the cells with pBS/U6 or pBS/U6/Mi-1 at a ratio of 1:20; 48 h after transfection, cells were fixed in cold 70% ethanol for 1 h and then stained with propidium iodide (20 µg/ml) in the presence of RNase A (200 µg/ml). Samples were analyzed using a fluorescence-activated cell sorter (FACS, Becton Dickinson). The cell cycle distributions of green fluorescent protein (GFP)-positive cells were plotted. Cells growing on coverslips were processed for microscopic examinations.

Live cell imaging. A HeLa cell line expressing histone 2B-GFP (19) was kindly provided by G. M. Wahl (Salk Institute for Biological Studies, La Jolla, California). pDsRed-N1 (Clontech) was cotransfected with pBS/U6 or pBS/U6/Mi-1 at a ratio of 1:10 using Lipofectamine 2000 (Invitrogen). Cells growing on circular coverslips were mounted in Ludin chamber with Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal calf serum (Sijiqing Company, Hangzhou, China) buffered with 20 mM HEPES, pH 7.2. DsRed-positive cells were recorded at 37°C for up to 240 min with exposure intervals of 2 to 3 min using a 60× objective. Cells were not exposed to light between exposure intervals. The Leica AS MDW (multidimensional workstation for live cell imaging) system with a CoolSNAP HQ Monochrome camera (Roper Scientific) (Fig. 6; see supplemental videos 1 to 3 in the supplemental material) and Olympus IX81 microscope with Evolution QEi FAST Monochrome camera (Media Cybernetics) (Fig. 8) gave comparable results with little bleaching.

The experimental systems were evaluated by recording control cells released for 3 h from thymidine block at 3-min intervals for 6 h. Normal cell divisions were observed repeatedly at end stages of monitoring in either system (data not shown), indicating the correct growth and recording conditions for the time-lapse microscopy. In fact, normal mitoses of neighboring untransfected cells were also found during the experiments (see video S3 in the supplemental material; also, data not shown). In addition, like the vector, a pBS/U6 construct for silencing murine caveolin-1 (provided by K. Liao, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences) (11) also exhibited no effects on either mitotin levels or the M-phase progression of live HeLa cells (data not shown).

Cotransfection efficiency was measured as follows. The HeLa cells transfected with pDsRed-N1 and pBS/U6/Mi-1 were fixed and immunostained for mitotin with Alexa 633. Images of DsRed-positive cells in prometaphase were recorded using a cooled charge-coupled device (SPOT II, Diagnostic Instruments) to allow examination of the mitotin staining because of the invisibility of Alexa 633 to the naked eye; 98.0 ± 1.0% DsRed-positive cells (total $n = 200$, two experiments) were found negative for mitotin. In contrast, mitotin staining was not affected in DsRed-positive mitotic cells cotransfected with pBS/U6 ($n = 60$).

FIG. 1. Silencing mitotin induces M-phase delay and apoptosis. (A) Efficiency of RNA interference. HEK293T cells transfected with pBS/U6 (vector) or pBS/U6/Mi-1 (RNAi) for 24 or 48 h were lysed and subjected to 3 to 12% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting to visualize the indicated proteins. Quantitation results for mitotin from three experiments are shown in the histogram. (B) Cell cycle profiles of GFP-F-positive cells. HEK293T cells were analyzed by FACS 48 h after transfection, with GFP-F as the transfection marker. The mitotic indexes shown in the histogram were obtained from microscopic examinations ($n = 600$, three experiments). (C) Activation of caspase-3 in dying HeLa cells lacking mitotin. HeLa cells cotransfected with pEGFP-F for 72 h were fixed with methanol and stained with an antibody to activated caspase-3. Arrows point to representative apoptotic cells expressing GFP-F. Scale bar, 40 μ m. (D) Statistic results for C. Total $n = 900$, three experiments.

RESULTS

Silencing mitotin by RNA interference results in M-phase accumulation and apoptosis. To explore the role of mitotin, we applied a plasmid-based RNA interference technology (44) to suppress mitotin expression. Neither the vector pBS/U6 nor a construct for RNA interference of murine caveolin-1 (11) affected mitotin expression in HEK293T cells (Fig. 1A; data not shown), while pBS/U6/Mi-1, which was designed to express a small interfering RNA against mitotin mRNA, silenced mitotin (Fig. 1A). In contrast, α -tubulin, CENP-E, and p150^{glued}, a dynactin subunit, were not affected (Fig. 1A). Quantitation of the immunoblots indicated $70.5 \pm 5.5\%$ reduction in mitotin levels at 24 h posttransfection and $96.5 \pm 0.5\%$ reduction at

48 h (Fig. 1A). Indirect immunofluorescence staining indicated that kinetochore mitotin levels were also significantly reduced. The average kinetochore intensity ($n = 499$) in prometaphase was decreased by $98.13 \pm 0.02\%$ compared with the value ($n = 454$) in surrounding control cells. Therefore, pBS/U6/Mi-1 was an appropriate construct for knocking down mitotin expression.

The cell cycle profiles were then assayed using a fluorescence-activated cell sorter (FACS). To identify transfectants, GFP-F, a membrane-associated green fluorescence protein (GFP) for GFP detection after ethanol fixation (16), was used as a transfection marker. After transfection into HEK293T cells for 48 h, the pBS/U6/Mi-1 transfectants showed clear accumulation of G₂/M population with 4n DNA content and apoptotic cells characteristic of DNA content less than 2n (Fig. 1B). Moreover, microscopic examinations of the same samples indicated a 3.3-fold increase in mitotic index of the GFP-F-positive cells (Fig. 1B). Similar effects were seen in samples transfected for 72 h (data not shown). Therefore, the cells were mainly accumulated in M phase, not G₂.

Despite high transfection efficiency, HEK293T cells were not a good choice for morphological analysis due to their small size and poor attachment to coverslips. We therefore switched to HeLa cells. Cell death characteristic of highly condensed mininuclei was also observed in GFP-positive HeLa cells cotransfected with pBS/U6/Mi-1 (Fig. 1C, panels 4 and 6), but not with pBS/U6 (Fig. 1C, panels 1 and 3) for 72 h. To further explore the nature of the cell death, activated caspase-3, which is essential for many types of apoptosis (32), was costained with DNA. The dying HeLa cells transfected with the RNA interference plasmid indeed contained activated caspase-3 (Fig. 1C, panels 4 to 6), while caspase-3-positive cells were rare in populations transfected with the vector (Fig. 1C, panels 1 to 3). There were 6.8-fold more apoptotic cells in mitotin-depleted populations on average than in the mock-transfected populations (Fig. 1D).

Mitotin depletion results in kinetochore malorientation and tension decrease. Since accumulation of mitotic cells suggests defects in mitosis, we examined HeLa cells in detail. Transfectants of pBS/U6/Mi-1 were identified by their strongly declined centromere staining of mitotin compared with surrounding mitotin-positive cells, while the immunostaining by anticentromere antibodies from CREST patients was in either case unaffected (Fig. 2A and B, panels 1 to 5). Quantitation showed that kinetochore mitotin was reduced by $\approx 96\%$ on average in prometaphase cells (see Fig. 4C).

Mitotin-depleted mitotic cells exhibited a high incidence of misaligned chromosomes (Fig. 2B, panels 1 to 5): metaphase-like cells with misaligned chromosomes, or pseudometaphase cells (47) accounted for $41.5 \pm 2.5\%$ ($n = 200$, two experiments) of total mitotic cells, while in surrounding mitotin-positive cells the frequency was only $7.0 \pm 1.0\%$ (Fig. 2E). Many fewer mitotin-depleted cells were in metaphase, anaphase, and telophase (Fig. 2E). On the other hand, the percentages of prophase and prometaphase cells were comparable (Fig. 2E). These results suggest a mitotic delay at metaphase in mitotin-depleted populations, consistent with the flow cytometry results (Fig. 1B).

Mitotin-depleted pseudometaphase cells were most frequently seen to contain two to five misaligned chromosomes

FIG. 2. Mitosin depletion causes chromosome misalignment. HeLa cells transfected with pBS/U6/Mi-1 were fixed and labeled as shown. Mitosin-positive cells in the same sample served as controls. Scale bar, 10 μ m. (A) A typical control metaphase cell. Images are from a typical optical section to show kinetochore pairs decorated by the CREST antigen. (B) A typical pseudometaphase cell lacking mitosin. Arrowheads point to representative misaligned kinetochores, their corresponding chromosomes, and associated polar microtubules. Panels 1 to 5 are the Z stack images. Panels 6 and 7, 9 and 10, and 12 and 13 are amplified (2 $\times$) locals from appropriate optical sections to show microtubule-kinetochore relationships of misaligned chromosomes. Cartoons delineating the image sets are shown in panels 8, 11, and 14. The chromosome at the upright corner of panel 14 was purposely drawn away from its original position to avoid overlap. (C) Number of misaligned chromosomes per cell. Only mitosin-depleted pseudometaphase cells were counted. Total $n = 200$, two experiments. Inset, BubR1 staining (Z stack image) of a mitosin-depleted cell showing kinetochore pairs of polar chromosomes (arrowheads). The corresponding images for CREST antigen and DNA were not shown. (D) Statistics for orientations of kinetochores in the spindle midzone. The angle included between a kinetochore pair and the spindle long axis (parallel to the white line in the inset) was measured as described in Materials and Methods. Total $n = 134$ for kinetochores in mitosin-depleted cells or $n = 157$ for control cells. Inset, an amplified local (2 $\times$) from the cell in B showing misoriented kinetochores (arrows) at the metaphase plate. (E) Graph showing the percentages of cells in different stages of mitosis. Total $n = 200$, two experiments.

(Fig. 2C), the majority of which lay juxtaposed to one or both spindle poles (Fig. 2B, panels 1 to 5, arrowheads). The polar chromosomes were not derived from premature sister chromatid segregation because the characteristic double-dot patterns of BubR1 were visible at their centromere regions (Fig. 2C, inset; data not shown). Moreover, their centromeres were frequently (17 of 26) seen to associate with aster microtubule bundles when the corresponding Z sections of confocal image sets were examined for 6 pseudometaphase cells (Fig. 2B, panels 6 to 14; data not shown), suggestive of monooriented attachments (Fig. 2B, panels 8, 11, and 14). Bipolar attach-

ments were occasionally seen (2 of 26) (Fig. 2B, panels 12 to 14). The rest (7 of 26) was difficult to judge, mostly due to locations in areas with high microtubule density. Interestingly, many of the monooriented kinetochores (6 of 17) exhibited clear syntelic attachments, i.e., both sister kinetochores attached by microtubules from the same pole (Fig. 2B, panels 6 to 11). The actual incidence might be higher, considering difficulties in estimating microtubule attachment at other kinetochore pairs.

The kinetochore orientations of aligned chromosomes also exhibited abnormalities. In control metaphase cells, kine-

chores were oriented roughly in the direction of the spindle long axis (Fig. 2A). However, kinetochores positioned in other directions were frequently noticed in mitosis-depleted cells (Fig. 2B, panels 1 to 5; Fig. 2D, inset). When the angles included between sister kinetochore orientations and the spindle long axis were measured (see Materials and Methods), a clear difference was seen: kinetochores oriented between 15 to 30 degrees and more than 30 degrees away from the long axis were, respectively, 2.5-fold and 8.3-fold more frequent in mitosis-depleted cells (Fig. 2D). Since kinetochore pairs located in different optical sections were not analyzed due to technical difficulties, these data might have underestimated the actual situations of kinetochore malorientation in mitosis-depleted cells. Despite this, the difference was already very significant.

We also compared tension across the kinetochore by measuring the distances between sister centromeres based on CREST serum staining. The longer the distance, the more tension across the kinetochore (29, 37, 50). In control metaphase cells, the average sister kinetochore separation was $1.59 \pm 0.01 \mu\text{m}$ ($n = 102$). In mitosis-depleted pseudometaphase cells, the value was $1.26 \pm 0.02 \mu\text{m}$ ($n = 106$) for chromosomes aligned at the spindle midzone. The difference was very significant between the two populations ($P < 10^{-5}$). In contrast, the value was $0.97 \pm 0.01 \mu\text{m}$ ($n = 100$) in nocodazole-treated cells. Given that the kinetochore distance in nocodazole-treated cells represented the neutral state, silencing mitosis decreased the net replacement of a kinetochore from its neutral position by 53% on average in fully aligned chromosomes, significantly attenuating tension across the sister centromere.

Mitosis depletion induces spindle abnormalities without impairing the stability of bipolar K-fibers. The microtubule-kinetochore association is a result of complicated yet concerted coordination between dynamic microtubules and kinetochore activities (4, 8, 42). Proper spindle organization and microtubule-kinetochore interactions also contribute to normal mitosis. In mitosis-depleted HeLa cells, $16.0 \pm 4.4\%$ ($n = 300$) exhibited multipolar spindles (Fig. 3A, panels 1 to 4), while the incidence was $6.3 \pm 3.1\%$ ($n = 300$) in surrounding control cells. γ -Tubulin was found at each pole of multipolar spindles in mitosis-depleted cells (data not shown), suggesting that such spindles may result from extra centrosomes in polyploid cells due to failed or abnormal cytokinesis (27). In addition, $39.4 \pm 5.9\%$ ($n = 300$) of mitosis-depleted cells in late prometaphase or metaphase showed abnormal bipolar spindles that were distorted and/or contained microtubule bundles overgrown across the spindle equators (Fig. 3A, panels 5 to 8, arrows, and data not shown). Some of these long bundles were probably not kinetochore fibers, because they extended from one pole to the other (Fig. 3A, panels 5 to 8, arrows). Similar spindle abnormalities were found in only $8.5 \pm 0.5\%$ of control cells. Furthermore, the spindles were generally much longer in mitosis-depleted cells at pseudometaphase. The average spindle length was $13.5 \pm 0.4 \mu\text{m}$ ($n = 25$), in contrast to $9.1 \pm 0.2 \mu\text{m}$ ($n = 15$) in control cells from late prometaphase to metaphase.

The microtubule-kinetochore interactions, however, were not disrupted in mitosis-depleted cells because clear kinetochore microtubules, or the K-fibers (37), were visible (Fig. 2B, panels 1 to 5; Fig. 3A, panels 1 to 4, arrowheads). This obser-

FIG. 3. Silencing mitosis affects spindle morphology but not kinetochore fiber stability. Scale bar, 10 μm . (A) Representative abnormal spindles in mitotic HeLa cells. Panels 1 to 4, a mitosis-depleted cell with multiple spindle poles. Arrowheads indicate typical kinetochores, their kinetochore fibers, and corresponding chromosomes. Panels 5 to 8, two mitosis-depleted pseudometaphase cells (mitosis staining not shown) with long microtubule bundles across the half spindles (arrows). (B) Cold stability of kinetochore microtubules. Cells were treated on ice for 10 min before fixation. Arrowheads indicate kinetochores of misaligned chromosomes.

vation was further corroborated by cold treatment, which disassembles most spindle microtubules but keeps K-fibers (35). Cold-stable microtubules diminish when the microtubule-kinetochore attachment is impaired (5, 18). In mitosis-depleted cells, K-fibers still existed after cold treatment and were virtually indistinguishable from control cells (Fig. 3B). The relative amounts of cold-stable microtubules (arbitrary units) were 1.00 ± 0.06 in control mitotic cells and 0.87 ± 0.07 in mitosis-depleted ones ($n = 25$). The difference was not statistically significant ($P = 0.14$). Interestingly, the misaligned chromosomes ($n = 60$) failed to exhibit clear cold-stable K-fibers (Fig. 3B, arrowheads), indicating that their microtubule attachments (Fig. 2B) are not resistant to the cold treatment.

Mitosis contributes to dynamics of kinetochore CENP-E and dynein/dynactin. The malorientation and reduced tension for kinetochores of aligned chromosomes (Fig. 2) implied certain defects of the force-generating machinery, most likely kinetochore motors (4, 8, 42). CENP-E is a kinesin motor-like protein whose inactivation also leads to misaligned polar chromosomes and tension reduction (29, 33, 47, 50). Furthermore, its potential interaction with and partial dependency on kinetochore localization on CENP-F (mitosis) (2, 17, 50) imply a functional relationship between them. Consistently, we also observed reduction of kinetochore CENP-E in mitosis-de-

FIG. 4. Mitosin depletion attenuates kinetochore CENP-E in a microtubule-dependent manner. Scale bar, 10 μ m. (A) Reduction of kinetochore CENP-E after silencing mitosin. Panels 1 to 4, a typical surrounding control cell. Panels 5 to 8, a typical mitosin-depleted cell. (B) Kinetochore BubR1 is not affected after silencing mitosin. (C) Quantitation results for kinetochore intensities of the indicated proteins. The number over each bar indicates the minimal number of kinetochores that were quantified. Noc, nocodazole. (D) Kinetochore localization of CENP-E in the absence of microtubules. HeLa cells were treated with nocodazole for 3 h to disassemble microtubules before fixation.

pleted cells (Fig. 4A). In prometaphase cells, the average kinetochore intensity of CENP-E was reduced to $\approx$ 28% of that in control cells (Fig. 4C). A similar value (30%) was obtained from metaphase and pseudometaphase cells. In contrast, kinetochore BubR1 was not affected after silencing mitosin (Fig. 4B and C). Neither was kinetochore Mad2, Bub1, hNuf2, Aurora B, or survivin significantly altered (data not shown).

The partial influence appeared to preclude mitosin as the protein responsible for kinetochore targeting of CENP-E. Rather, it might stabilize the CENP-E-kinetochore association. In fact, many outer kinetochore proteins bind kine-

chores dynamically in M phase. Their steady-state levels are thus the net results of dynamic association versus dissociation (12, 14). Moreover, their kinetochore levels often decline in accordance with the extent of microtubule-kinetochore attachment (10, 20).

To further understand how the kinetochore CENP-E was influenced by mitosin, we examined whether its reduction in mitosin-depleted cells was related to microtubule attachments. Indeed, after 3 h treatment with nocodazole, the kinetochore CENP-E levels increased to 75% of control values on average (Fig. 4C and D).

FIG. 5. Mitosin depletion reduces kinetochore dynactin in a microtubule-dependent manner. Scale bar, 10 μ m. (A) Reduction of kinetochore p150^{glued} after silencing mitosin in prometaphase. Panels 1 to 4, a control cell. Panels 5 to 8, a typical mitosin-depleted cell. Arrowhead points to the isolated chromosome with stronger kinetochore p150^{glued}. (B) Kinetochore localization of p150^{glued} in the presence of nocodazole for 3 h. (C) Relative intensity of kinetochore p150^{glued}. The number over each bar indicates the minimal number of kinetochores that were quantified. Noc, nocodazole.

Due to the importance of dynein in spindle organization and chromosome movement, we also examined dynactin, which is essential for dynein's kinetochore targeting and functions in mitosis (4, 6, 9, 46). Dynactin was analyzed because its subunit p150^{glued} showed stronger kinetochore staining than dynein (data not shown). We found that p150^{glued} also displayed weaker kinetochore staining in mitosin-depleted prometaphase cells than in mitotic phase-matched control cells (Fig. 5A and data not shown). Its average centromere intensity was $\approx 22\%$ of the control value (Fig. 5C). Phase match was important because kinetochore dynactin is sensitive to and is markedly reduced upon microtubule attachment (6). We also noticed that, in early prometaphase, when tensions across the kinetochore were not apparent according to the CREST staining, less p150^{glued} was accumulated at spindle poles in control cells than in mitosin-depleted cells (Fig. 5A). Moreover, in

contrast to the general low levels of kinetochore dynactin, bright staining was often seen at kinetochores away from the spindle in cells lacking mitosin (Fig. 5A, arrowhead; and data not shown), suggesting microtubule-dependent reduction of kinetochore dynactin. Indeed, in the presence of nocodazole, the centromere localization of p150^{glued} in mitosin-depleted cells was completely restored to the levels in control cells (Fig. 5B and C). Therefore, mitosin depletion attenuated both kinetochore CENP-E (Fig. 4) and dynactin and dynein (Fig. 5) in the presence of microtubules.

Time-lapse microscopy reveals defective chromosome congression followed by cell death in mitosin-depleted cells. For global views of mitotic cell behaviors, we performed time-lapse microscopy using a HeLa cell line stably expressing histone 2B-GFP as a chromosome marker (19). When 15 live cells cotransfected with pBS/U6 and pDsRed-N1, a plasmid expressing the

FIG. 6. Mitosin depletion induces M-phase block followed by cell death. HeLa cells stably expressing histone 2B (H₂B)-GFP, which labels chromatin, were transfected for ≈ 48 h. DsRed was used as a transfection marker. Transfectants in prophase or early prometaphase were randomly picked for time-lapse microscopy. Images were recorded every 2 to 3 min for up to 240 min. DIC, differential interference contrast. Scale bar, 10 μ m. (A) Representative images showing the M-phase progression of a typical DsRed expressor cotransfected with pBS/U6. Also see supplemental video 1. (B and C) Representative images for two DsRed-positive cells cotransfected with pBS/U6/Mi-1. Also see supplemental videos 2 and 3. (D) Consecutive frames for the cell in C to show chromosome movement. Arrows point in the direction of movement.

red fluorescence protein DsRed (Clontech) as a transfection marker, were monitored from prophase or early prometaphase, all of them started anaphase within 45 ± 2 min and completed mitosis within 58 ± 2 min. Congression was rapid; after nuclear envelope breakdown, all chromosomes moved quickly towards the metaphase plate to achieve full alignment in 18 ± 1 min. All these cells divided normally with no visible lagging chromosomes

(Fig. 6A; see video S1 in the supplemental material; also data not shown).

The situation was quite different in DsRed expressors cotransfected with pBS/U6/Mi-1; 17 out of 18 cells that had been monitored for 240 min contained various numbers of chromosomes unable to migrate to the metaphase plate but remaining in the vicinity of spindle poles during congression. The remain-

ing cell stayed in prometaphase. Three of the 17 cells exhibited only one unaligned chromosome at late stages of recording (Fig. 6C; see video S3 in the supplemental material; also data not shown). Nevertheless, anaphase onset was not seen in any of the 18 cells. From prophase or early prometaphase, the average time required for most chromosomes to achieve alignments was 32 ± 3 min ($n = 17$) (Fig. 6B and C; see videos S2 and S3 in the supplemental material), which was significantly longer than the time required for full chromosome alignment in control cells (18 ± 1 min). As time went on, the chromosomes became gradually decondensed in all cells and then, in 15 cells, aggregated to form mininuclei at 151 ± 7 min, a hallmark of cell death (Fig. 6B and C; see videos S2 and S3 in the supplemental material). Among the dying ones, four cells displayed plasma membrane blebbing reminiscent of apoptosis following chromosome deformation (30). These aberrations were not due to the experimental conditions, since normal mitoses of surrounding untransfected cells were seen in our raw image sets throughout the experiments (see video S3 in the supplemental material; also data not shown). Collectively, these results are in agreement with those from fixed cells (Fig. 1 and 2), indicating critical roles of mitotin in chromosome congression and mitotic cell survival. Moreover, they clarified that the cell death seen in fixed cells (Fig. 1) occurred in mitosis.

Abnormal chromosome movement was observed in mitotin-depleted cells. In vector-transfected cells, all chromosomes, even those close to poles, moved rapidly towards the metaphase plate (Fig. 6A; see video S1 in the supplemental material). In DsRed expressors cotransfected with pBS/U6/Mi-1, chromosomes near the metaphase equator congressed relatively rapidly, while those away from it often failed to move likewise and then tended to become misaligned (Fig. 6B to D; see videos S2 and S3 in the supplemental material). Occasionally, some unaligned chromosomes were seen to move away from the equator, possibly towards spindle poles (Fig. 6D). Some were also seen to move abruptly plateward after certain time points (Fig. 6D), indicating that congression of the polar chromosomes can occur gradually when their kinetochores are attached by proper microtubules.

Mitotin depletion-induced cell death coincides with premature chromosome decondensation. In an attempt to explore the cause of cell death, we carefully examined HeLa cells fixed after 13 h of release from thymidine block. About $75.5 \pm 2.5\%$ of dying or dead cells ($n = 400$, two experiments) contained highly condensed mininuclei of various numbers and sizes typical of apoptosis, some of which were accompanied by bright microtubule bundles reminiscent of spindle remains (Fig. 7, panels 21 to 25). Surprisingly, the remaining $24.5 \pm 3.5\%$ still exhibited brightly stained spindles and thus should be in earlier stages of cell death (Fig. 7, panels 1 to 20). Moreover, some cells were seen with several chromosomes decondensed into amorphous chunks of chromatin, while the remaining chromosomes still maintained typical morphologies (Fig. 7, panels 1 to 5). Such changes appeared to reflect initial stages of cell death, since cells with increasing extents of chromosome decondensation were also observed (Fig. 7, panels 6 to 15, arrows; data not shown) and the live cell recording showed progressive chromosome decondensation prior to chromatin condensation

and membrane deformation (Fig. 6B and C; see videos S2 and S3 in the supplemental material; also data not shown).

In some of the dying cells, although chromosomes were extensively decondensed, their centromeres were still labeled brightly for the CREST antigen and associated with the spindle. Accordingly, DNA speckles of various sizes and intensities were seen to colocalize with the centromere staining, indicating that they actually contained centromeric chromatins. Nevertheless, these chromatins were excluded from but very likely still connected to the large mass of decondensed chromatins (Fig. 7, panels 6 to 15, arrowheads). In contrast, some cells exhibited heavily distorted bipolar spindles and sphere-like mininuclei of various sizes (Fig. 7, panels 16 to 20, arrows), suggesting that they were in later stages of cell death. Even so, centromere staining by the CREST serum still associated with the spindle and some was seen with chromatin speckles (Fig. 7, panels 16 to 20, arrowheads). Collectively, we concluded that these mitotin-depleted cells were experiencing a rare situation in which their chromosome arms decondensed prematurely in pseudometaphase when their centromeres were still associated with spindle microtubules so that the centromeric chromatins were precluded and away from the amorphous chromatin chunks. Such cells died with their decondensed chromosomes collapsing further into mininuclei (Fig. 7, panels 16 to 25).

Proteasome activity is dispensable in mitotin depletion-induced cell death. To confirm whether the mitotin-depleted cells died before anaphase onset, cells were treated with MG132, an inhibitor of the proteasome. Since proteasome activation is essential for chromatin segregation (4, 31, 40), its inhibition blocks cells in metaphase (3, 10). We found that $25 \mu\text{M}$ of MG132 (Sigma) was sufficient to arrest HeLa cells in metaphase for at least 4 h with few side effects regardless of cotransfection with pDsRed-N1 and pBS/U6 (Fig. 8A and data not shown). Of 12 DsRed-positive prometaphase/metaphase cells cotransfected with pBS/U6/Mi-1, however, eight still showed chromosome decondensation and aggregation characteristic of cell death during 4 h of recording (Fig. 8B and data not shown). Membrane deformation was also observed in many (5 of 8) of the dying cells (Fig. 8B and data not shown). Therefore, mitotin-depleted cells die before chromosome segregation or anaphase onset.

DISCUSSION

Our data suggest that mitotin is a multifunctional protein essential for proper M-phase progression in HeLa and HEK293T cells. Mitotin depletion by RNA interference resulted in multiple abnormalities in M phase, including attenuated congression rates, juxtapolar chromosomes, premature chromosome decondensation, and cell death.

Mitotin is required for full chromosome alignment, probably through interaction with CENP-E. The formation of pseudometaphase cells in the absence of mitotin (Fig. 2) closely resembles the phenotype of CENP-E depletion (27, 29, 33, 47, 50). First, 80 to 90% of living mitotic HeLa cells exhibits misaligned polar chromosomes upon repression of mitotin (Fig. 6 and data not shown) or CENP-E by RNA interference (45). Second, depletion of either protein results in increased incidence of spindle distortion and decreased tension across the sister kinetochore (Fig. 2 and 3) (29, 50). Third, kinetochores

FIG. 7. Mitosin-depleted cells die in M phase coincident with premature chromosome decondensation. Representative mitotic cells with various extents of decondensed chromosomes were shown, hopefully to trace the progress of cell death. Arrowheads point to typical centromeres, and arrows indicate decondensed chromosomes or mininuclei. Panels 1 to 5, a cell containing both compacted chromosomes (arrowheads) and decondensed ones (arrows). Panels 6 to 15, two dying mitotic cells with chromosomes decondensed into amorphous chunks. Note that centromeres and their centromeric chromatins were still associated with the spindles and were excluded from the chromatin chunks. Panels 16 to 20, a cell with bended spindle and mininuclei, probably representing a later stage of cell death. Panels 21 to 25, a dead cell with leaking cytoplasmic contents. Scale bar, 10 μ m.

of the polar chromosomes in either type of cell lack stable microtubules, as demonstrated by electron microscopy studies (29, 33) or cold treatment (Fig. 3B). These similarities, together with the significant reduction of kinetochore CENP-E in mitotin-depleted cells (Fig. 4) and the potential interaction between the two proteins (2, 50), suggest that both proteins function in the same pathway for full chromosome alignment. Since neither protein is essential for congression of most chromosomes, they may be involved in initial processes of the kinetochore-microtubule interaction (29, 33).

Mitosin, however, is not functionally identical to CENP-E. For instance, mitotin-depleted cells hardly divided, even in the presence of only one misaligned chromosome (Fig. 6C; supplementary video 3; data not shown). Instead, they tended to undergo cell death after entering mitosis for ≈ 3 to 4 h. In contrast, the majority of CENP-E-depleted HeLa cells enter

anaphase after a prolonged mitotic delay, the length of which, usually 2.5 to 8 h, correlates positively with the number of misaligned chromosomes (45). Only about 10% cells die during or after mitosis (45). Furthermore, CENP-E is an activator of BubR1 kinase, and its depletion suppresses the autophosphorylation of BubR1 (26, 45). In contrast, mitotin depletion did not affect BubR1 autophosphorylation (data not shown).

Mitosin facilitates proper microtubule-kinetochore attachments. Our current data suggest that mitotin helps to establish proper microtubule-kinetochore interactions. The high incidence of polar chromosome formation and transient microtubule association of polar chromosomes (Fig. 2 and 3) strongly suggest defects in efficient microtubule-kinetochore interactions and/or stabilization of such interactions. The slowed congression (Fig. 6) provided another support. In fact, CENP-E is involved in stabilization of microtubule attachment (29, 33).

FIG. 8. Mitotin-depleted cells die before anaphase onset. HeLa cells stably expressing histone 2B (H₂B)-GFP were transfected for $\approx$ 48 h. DsRed was used as a transfection marker. Transfectants in prometaphase or metaphase were randomly picked for time-lapse microscopy in the presence of 25 μ M MG132. Images were recorded every 3 min for up to 240 min. DIC, differential interference contrast. Scale bar, 10 μ m. (A) Representative images of a DsRed-positive cell cotransfected with pBS/U6. (B) A typical cell cotransfected with pBS/U6/Mi-1.

Mitotin may serve as a partner in this process. On the other hand, during early mitosis, errors such as syntelic and merotelic attachments can occur (3, 21). They must be corrected to allow normal congression and chromatid segregation, guaranteeing genome stability (3, 21). We did find syntelic attachment in at least 23% of polar chromosomes (Fig. 2B), which, if not destabilized, may hinder proper attachment. Mitotin depletion may thus lead to defects in a mechanism for the correction of such errors. Apparently, more work needs to be done to clarify this issue, since syntelic attachment may also be a result of polar localization of chromosomes where microtubules from the same pole exist in high density.

Mitotin stabilizes kinetochore CENP-E and cytoplasmic dynein and dynactin against microtubule-dependent stripping.

Mitotin is apparently not required for kinetochore targeting of dynactin and dynein or CENP-E. Instead, it appears to facilitate their retentions on kinetochores. Kinetochore dynein and dynactin are sensitive to microtubule attachment and mainly dissociate in a microtubule-dependent manner (6, 10, 20). Microtubule disassembly even increases kinetochore dynein by 20- to 100-fold (10). Kinetochore CENP-E also exhibits similar but smaller microtubule-dependent oscillations (10). In mitotin-depleted cells, the steady-state levels of the kinetochore CENP-E and dynactin were further reduced (Fig. 4 and 5). Since their levels were largely rescued after microtubule disassembly (Fig. 4 and 5), the reduced intensities in the presence

of microtubules are attributed to overstripping upon microtubule attachment. Such alterations might affect the normal functions of CENP-E and dynein and dynactin in the microtubule-kinetochore interaction and tension generation (4, 8, 42), contributing to decreased congression rates, the formation of polar chromosomes, and malorientation of aligned kinetochores.

Although kinetochore CENP-E can be transported to spindle poles by dynein/dynactin (13), its reduction in mitotin-depleted cells is unlikely due to increased dissociations of dynein/dynactin. Instead, these two events appear to be independent, since inactivation of dynein/dynactin does not influence kinetochore CENP-E and vice versa (6, 50). Furthermore, the kinetochores BubR1 and Mad2, which are also transported by dynein/dynactin (12, 13), were not affected in mitotin-depleted cells (Fig. 2C and data not shown). Mitotin may stabilize kinetochore CENP-E through direct interaction (2, 50). Transport of kinetochore mitotin by dynein/dynactin (49) suggests a certain association between them, either directly or indirectly. Mitotin is independently found to bind NudE1 and NudE, two homologous regulators of dynein, in yeast two-hybrid screens (data not shown) (7, 22), which may provide clues to link mitotin to dynein/dynactin.

In addition to mitotin, Spc24 and Spc25, two components of the Ndc80/hNuf2 complex essential for stable microtubule-kinetochore attachment (5, 27), also affect retentions of kinetochore dynein/dynactin and CENP-E (28). Silencing either Spc24 or Spc25 in HeLa cells attenuates kinetochore dynactin and CENP-E only in the presence of microtubules without affecting CENP-F (mitotin) (28). Distinct from CENP-E and mitotin, which are not critical for maintenance of the kinetochore-microtubule attachment (Fig. 2 and 3) (29, 33), Spc24 and Spc25 are required for both establishment and maintenance of the attachment (28). Moreover, mitotic cells lacking any constituent of the Ndc80/hNuf2 complex manifested long microtubule bundles across the spindle and longer spindles (5, 27, 28). Similar phenotypes were also seen in mitotin-depleted cells (Fig. 3A), further supporting a role of mitotin in facilitating microtubule-kinetochore interactions. Moreover, these results provide interesting insights into possible relationships among the proteins involved in microtubule-kinetochore interactions.

Mitotin depletion induces mitotic cell death correlated with premature chromosome decondensation. The way in which mitotin-depleted cells die is also intriguing. First, the cells usually died before anaphase onset, according to live cell recordings in the absence and presence of MG132 and spindle morphologies found in dying cells (Fig. 6 to 8; supplemental videos 2 and 3). The dying cells contained activated caspase-3, indicating apoptosis (32). The incidence of cell death is high: the majority of mitotic cells showed signs of cell death within 4 h of monitoring (Fig. 6; Fig. 8; data not shown). Possibly, a portion of the cells may survive by entering G₁ abnormally (27) and exhibit multipolar spindles in the next round of mitosis (Fig. 3). Second, cells in early stages of death showed several unique features. They contained various extent of decondensed chromosomes. The spindle was not disassembled in these cells because they were seen even in dying cells with mininuclei (Fig. 7). Moreover, centromeres, which were labeled by the CREST antigen together with spot-like chromatin

DNA, were excluded from the decondensed chromatin (Fig. 7), suggesting that the decondensation occurs mainly at chromosome arms. These phenotypes, which have not been previously reported to our knowledge, may be unique to mitosis depletion. Alternatively, they might be common for mitotic cell death, a poorly studied type of cell death.

The aberrant chromosome decondensation appears to be the major cause of cell death upon mitosis depletion. The majority of CENP-E-depleted HeLa cells by RNA interference initiated anaphase after the pseudometaphase block for 2.5 to 8 h (45), while treatment with antisense RNA arrested cells with condensed chromosomes for more than 20 h (50). The relatively early onset of mitotic cell death (2 to 4 h) (Fig. 6) thus strongly suggests that death is a direct result of mitosis deficiency instead of pseudometaphase arrest. This is further corroborated by cell death in the presence of MG132, since control cells showed only metaphase arrest (Fig. 8). Moreover, death always occurred following progressive chromosome decondensation (Fig. 6 to 8; see videos S2 and S3 in the supplemental material). The relatively normal spindle morphologies and the spindle associations of centromeres (Fig. 7) suggest that the mitotic apparatus is still functional even in dying cells. Anaphase onset was not observed prior to cell death in live cells, suggesting that the spindle checkpoint is not inactivated. The MG132 block (Fig. 8) further confirmed that chromosome decondensation and cell death were not due to aberrant anaphase initiation. It is therefore possible that such premature chromosome decondensation accidentally interrupted mitotic cell homeostasis, leading to cell death by activating a checkpoint that monitors the orchestration of mitotic events.

Our results thus suggest a role of mitosis in chromosome decondensation, a process that is normally initiated from telophase for reassembly of nuclei in daughter cells (36). Silencing mitosis seems to decouple chromosome decondensation from M-phase progression and trigger this event oddly before anaphase (Fig. 6 to 8). In fact, in intact cells, the majority of mitosis is rapidly degraded at the end of mitosis (23, 52), coincident with the timing of nucleus reassembly. Whether mitosis degradation controls the timing of chromosome decondensation is also an interesting issue deserving further investigation.

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