Identification of Two Novel Components of the Human NDC80 Kinetochore Complex

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Proper kinetochore function is essential for the accurate segregation of chromosomes during mitosis. Kinetochores provide the attachment sites for spindle microtubules and are required for the alignment of chromosomes at the metaphase plate (chromosome congression). Components of the conserved NDC80 complex are required for chromosome congression, and their disruption results in mitotic arrest accompanied by multiple spindle aberrations. To better understand the function of the NDC80 complex, we have identified two novel subunits of the human NDC80 complex, termed human SPC25 (hSPC25) and human SPC24 (hSPC24), using an immunoaffinity approach. hSPC25 interacted with HEC1 (human homolog of yeast Ndc80) throughout the cell cycle and localized to kinetochores during mitosis. RNA interference-mediated depletion of hSPC25 in HeLa cells caused aberrant mitosis, followed by cell death, a phenotype similar to that of cells depleted of HEC1. Loss of hSPC25 also caused multiple spindle aberrations, including elongated, multinucleated, and fragmented spindles. In the absence of hSPC25, MAD1 and HEC1 failed to localize to kinetochores during mitosis, whereas the kinetochore localization of BUB1 and BUBR1 was largely unaffected. Interestingly, the kinetochore localization of MAD1 in cells with a compromised NDC80 function was restored upon microtubule depolymerization. Thus, hSPC25 is an essential kinetochore component that plays a significant role in proper execution of mitotic events.

The genetic stability of an organism depends on accurate segregation of sister chromatids during mitosis (1, 2). Macronuclear structures termed kinetochores that assemble on the centromeric regions of chromosomes are essential for various aspects of this process (3). Kinetochores form attachment sites for spindle microtubules, recruit microtubule motors and other non-motor proteins that regulate spindle dynamics, and are indispensable for the subsequent alignment of chromosomes at the metaphase plate (3).

Kinetochore also play important roles in the spindle checkpoint (3). Premature separation of sister chromatids prior to their proper attachment to the mitotic spindle results in chromosome missegregation and aneuploidy, contributing to tumorigenesis or birth defects (4, 5). To avoid these dire consequences, cells employ a surveillance mechanism called the spindle checkpoint to monitor the status of microtubule attachment and tension at the kinetochores of sister chromatids (6–11). The spindle checkpoint ensures that chromosome segregation ensues only after all the chromosomes have congressed to the metaphase plate. Unattached kinetochores generate a diffusible signal that inhibits a ubiquitin-protein isopeptide ligase called the anaphase-promoting complex or cyclosome (APC/C) and in turn prevents sister chromatid separation (6–13). It has been postulated that the APC/C inhibitory signal generated by unattached kinetochores consists of a complex of MAD2, CDC20, BUBR1 and BUB3, known as the mitotic checkpoint complex (11). Many checkpoint proteins exhibit dynamic kinetochore localization in mitosis (14, 15). For example, MAD2 localizes only to unattached kinetochores, and the kinetochore-bound pool of MAD2 exchanges rapidly ($t_{1/2} = 24–28 s$) with the cytoplasmic pools (14). MAD2 recruitment to kinetochores depends upon another checkpoint protein, called MAD1, which is constitutively bound to MAD2 and shows the same kinetochore localization pattern as MAD2 (16–18). An attractive model for spindle checkpoint signaling is that MAD2 is recruited to kinetochores by MAD1 (11, 19). Through a poorly understood mechanism, possibly mediated by other checkpoint proteins, MAD2 dissociates from MAD1 and acquires a conformation that is compatible with its incorporation into the mitotic checkpoint complex (11, 19). Little is known about the mechanisms by which the spindle checkpoint proteins are recruited to kinetochores during mitosis. Detailed immunofluorescence analysis revealed that these proteins are recruited to the kinetochores with slightly different timing (20). Moreover, their kinetochore localization patterns vary in response to different spindle-damaging agents (21–23). This suggests that the spindle checkpoint proteins might be recruited to the kinetochores through different mechanisms and via interactions with specific kinetochore proteins. Identification of the kinetochore protein interface to which MAD1-MAD2 associates might provide insights into the mechanism of the generation of the mitotic checkpoint complex.

Genetic and biochemical studies in different organisms have led to the identification of a large number of kinetochore pro-
SPC25 (hSPC25) interacted with HEC1 components of the human NDC80 complex. Human bases. We have now purified the human NDC80 complex using the pGEX-4T-1 vector. The resulting plasmids were transformed into Rosetta DE3 bacteria cells. Expression of the GST-HEC1N and GST-HEC1C fusion proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside (250 μM final concentration) to the bacterial culture at A600 = 0.6. The cells were allowed to grow for another 3 h at room temperature. Soluble GST-HEC1 fusion proteins were isolated on glutathione-agarose beads (Amersham Biosciences), eluted with 10 mM glutathione (Sigma), and exchanged into phosphate-buffered saline (PBS) using PD-10 columns (Amersham Biosciences). The two GST-HEC1 fusion proteins were mixed and injected into rabbits for antibody production (Zymed Laboratories Inc.). Crude antibody sera were first precloned with GST-coupled Affi-Gel beads (Bio-Rad) to remove anti-GST antibody and subsequently affinity-purified using either GST-HEC1N or GST-HEC1C fusion protein-coupled Affi-Gel beads. For immunoprecipitation, affinity-purified anti-HEC1 antibodies were used at a final concentration of 1 μg/ml. For immunoprecipitation, affinity-purified anti-HEC1 antibodies were coupled to Affi-Prep-protein A beads (Bio-Rad) at a concentration of 1 mg of antibody/ml of protein A beads. Immunoabsorption of the Human NDC80 Complex—HeLa Tet-On cells (Clontech) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 100 μg/ml each penicillin and streptomycin. At 80% confluence, the cells were treated with nocodazole at a final concentration of 100 ng/ml for 18 h. For each immunoprecipitation experiment, 20 plates (150 mm) of nocodazole-arrested HeLa cells were used. The cell pellet was resuspended in 12 ml of Nonidet P-40 lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM NAD, 10 μg/ml cytochalasin B, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml chymostatin). The suspension was kept on ice for 20 min, followed by brief sonication. The lysate was cleared by centrifugation at top speed in a microcentrifuge for 1.5 h. The supernatant was filtered with 0.45-μm filters and precleared using 200 μl of anti-GST antibody-coupled protein A beads. The NDC80 complex was immunoprecipitated by incubating the lysate with 200 μl of either anti-HEC1N or anti-HEC1C antibody at 4 °C for 2 h. The antibody beads were washed four times with Nonidet P-40 lysis buffer plus 400 mM KCl and once with the lysis buffer. Immunoprecipitated proteins were then eluted with 1 ml of 100 mM glycine HCl (pH 2.5) and concentrated using Microcon centrifugal concentrators (Millipore Corp.). The concentrated proteins were resolved by SDS-PAGE and detected by silver staining. The protein bands were excised and subjected to trypsin digestion, followed by mass spectrometry analysis.

Cloning, Expression, and Purification of Recombinant hSPC25 and Antibody Production—The coding region of hSPC25 was amplified from a human fetal thymus cDNA library (Clontech) by PCR and cloned into the pcDNA3-Myc and pcDNA3-HA vectors. The clones were sequenced to verify the identity of hSPC25. A cDNA fragment of hSPC25 containing nucleotides 1–450 of the hSPC25 coding region (hSPC25N) was cloned into the pGEX-4T-1 vector. The GST-hSPC25N protein was expressed and purified essentially as described above for the purification of GST-HEC1 fusion proteins, except that expression of GST-hSPC25N was induced by the addition of isopropyl-β-D-thiogalactopyranoside (250 μM final concentration) to the bacterial culture at A600 = 0.9. The purified GST-hSPC25N fusion protein was injected into rabbits for antibody production (Zymed Laboratories Inc.). Crude antibody sera were first precloned with GST-coupled Affi-Gel beads to remove anti-GST antibody and subsequently affinity-purified using GST-hSPC25N-coupled Affi-Gel beads.

Expression and Purification of Recombinant HEC1 Proteins and Antibody Production—The coding region of HEC1 was amplified from a human fetal thymus cDNA library (Clontech) using PCR. Two cDNA fragments of HEC1 containing either nucleotides 1–663 (HEC1N) or nucleotides 1285–1929 (HEC1C) of the HEC1 coding region were cloned into the pGEX-4T-1 vector. The resulting plasmids were transfected into Rosetta DE3 bacteria cells. Expression of the GST-HEC1N and GST-HEC1C fusion proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside (250 μM final concentration) to the bacterial culture at A600 = 0.6. The cells were allowed to grow for another 3 h at room temperature. Soluble GST-HEC1 fusion proteins were isolated on glutathione-agarose beads (Amersham Biosciences), eluted with 10 mM glutathione (Sigma), and exchanged into phosphate-buffered saline (PBS) using PD-10 columns (Amersham Biosciences). The two GST-HEC1 fusion proteins were mixed and injected into rabbits for antibody production (Zymed Laboratories Inc.). Crude antibody sera were first precloned with GST-coupled Affi-Gel beads (Bio-Rad) to remove anti-GST antibody and subsequently affinity-purified using either GST-HEC1N or GST-HEC1C fusion protein-coupled Affi-Gel beads. For immunoprecipitation, affinity-purified anti-HEC1 antibodies were used at a final concentration of 1 μg/ml. For immunoprecipitation, affinity-purified anti-HEC1 antibodies were coupled to Affi-Prep-protein A beads (Bio-Rad) at a concentration of 1 mg of antibody/ml of protein A beads. Immunoabsorption of the Human NDC80 Complex—The Ndc80, Nuf2, and Nuf2 complexes, remain to be identified in mammals. Identification of these components will shed light on the structure, assembly, and function of mammalian kinetochores.

The yeast Ndc80 complex consists of four coiled-coil proteins: Ndc80, Nuf2, Spc25, and Spc24 (28). Homologs of Ndc80 and Nuf2 have been identified in various organisms and shown to perform important functions in processes including kinetochore assembly, microtubule attachment, chromosome congression, and the spindle checkpoint (29–32). Nearly all of the spindle checkpoint proteins localize to the outer kinetochore (3). Many central kinetochore proteins, including components of the Ndc80, Mis12/Mtw1, and Ctf19 complexes, remain to be identified in mammals. Identification of these components will shed light on the structure, assembly, and function of mammalian kinetochores.

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Given the importance of the NDC80 complex in both chromosome congression and the spindle checkpoint, we sought to characterize the composition and function of the human NDC80 complex. In particular, despite their likely existence, the mammalian functional homologs of yeast Spc25 and Spc24 have not been identified due to the lack of clear sequence homologs in the data bases. We have now purified the human NDC80 complex using an immunoaffinity approach and identified the Spc25 and Spc24 complexes in the Ndc80 complex of the human NDC80 complex. Human Spc25 (hSpc25) interacted with Hec1 in vitro and in vivo. It localized to the kinetochores during mitosis. RNAi-mediated depletion of hSpc25 in HeLa cells caused a plethora of mitotic defects and the loss of kinetochore localization of Mad1 and Hec1 in the absence of spindle-damaging agents. Therefore, despite the lack of significant sequence similarity, hSpc25 is a functional homolog of yeast Spc25 and a component of the conserved Ndc80 complex. Interestingly, the Ndc80 complex was not required for the kinetochore localization of Mad1 in nocodazole-arrested mitotic cells. This suggests that the Ndc80 complex is not absolutely required for the recruitment of Mad1 to kinetochores upon checkpoint activation.
They contained sequences corresponding to nucleotides 344–366 and 1517–1539 of the coding region of human **HEC1**, nucleotides 161–183 of the coding region of hSPC25, and nucleotides 143–165 of the coding region of MAD2, respectively. The annealing of the siRNAs and subsequent transfection of the RNA duplexes into HeLa cells were performed exactly as described (33). In the case of hSPC25, at 48 h after the first round of RNAi transfection, cells were replated to 30% confluency. At 12 h after replating, the cells were transfected again with either hSPC25 siRNA oligonucleotides alone or with both hSPC25 and MAD2 siRNA duplexes. The time points mentioned in this study for these RNAi experiments refer to the time after the second round of RNAi transfection. Cells transfected with OligofectAMINE alone were used as controls. To determine the cell cycle status, the transfected cells were fixed with 70% ethanol, stained with propidium iodide (PI), and analyzed by flow cytometry (FACS). The phenotypes of these cells were also analyzed by indirect immunofluorescence.

**Immunoprecipitation, Immunoblotting, and Protein Binding Assays**—Lysates of HeLa Tet-On cells transfected with HEC1 and hSPC25 plasmids were prepared and immunoprecipitated as described for the immunopurification of the NDC80 complex. Myc-HEC1 or Myc-hSPC25 was immunoprecipitated from lysates with mouse monoclonal anti-Myc antibody (Roche Applied Science) coupled to protein A beads. After washing, samples were resolved by SDS-PAGE and analyzed by immunoblotting with anti-hemagglutinin (HA) antibody at a final concentration of 1 μg/ml. For analysis of the interactions between endogenous HEC1 and hSPC25 proteins, the anti-HEC1 immunoprecipitates were blotted with anti-hSPC25 antibody at a 1 μg/ml final concentration. Horseradish peroxidase-conjugated goat anti-rabbit or goat antimouse IgG (Amersham Biosciences) were used as a secondary antibody, and the immunoblots were developed using ECL reagent (Amersham Biosciences) following the manufacturer’s protocols.

Plasmids encoding Myc-HEC1, human NUF2 (NUF2), and hSPC25 were cotranslated in rabbit reticulocyte lysate in the presence of [35S]methionine. The lysate was immunoprecipitated using either anti-Myc or anti-GST (as a negative control) antibody. The beads were washed four times with Nonidet P-40 lysis buffer. The immunoprecipitated proteins were dissolved in SDS sample buffer, separated by SDS-PAGE, and analyzed by autoradiography.

**Immunofluorescence**—For the immunostaining of endogenous HEC1, hSPC25, MAD1, BUB1, and BUBR1, HeLa cells were grown to 40% confluency and transfected with different plasmids or siRNA duplexes on chambered cover slides. Cells were washed with PBS and extracted with 0.5% Triton X-100 in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 4 mM MgSO4) for 5 min at 37 °C. Cells were then fixed with 4% paraformaldehyde in PHEM buffer for 20 min at 37 °C, washed three times with 0.2% Triton X-100 in PBS, and incubated with blocking solution containing 3% bovine serum albumin in PHEM buffer for 1 h. Cells were incubated with primary antibodies in 3% bovine serum albumin in PHEM buffer for 1 h, washed three times with PBS plus 0.05% Tween 20, and further incubated with fluorescein secondary antibodies (Molecular Probes, Inc.) at 1:500 dilution. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were acquired with a Zeiss Axiovert 200M fluorescence microscope. Images were acquired with a CCD camera using the Intelligent Imaging software and further processed with Adobe Photoshop. The staining protocol for cyclin A1, cyclin B, securin, and Myc-hSPC25 was essentially the same as described above, except that cells were not detergent-extracted prior to fixation. The immunostaining of tubulin and poly(ADP-ribose) polymerase-1 (PARP1) in the hSPC25/HEC1 RNAi cells was performed as described above, except that the cells were fixed with methanol at −20 °C. Antibodies used were as follows: mouse monoclonal anti-Myc antibody at 1 μg/ml, CREST serum at 1:1000 dilution (Immuno-Vision), rabbit anti-cyclin A antibody at 1 μg/ml (Santa Cruz Biotechnology), rabbit anti-cyclin B1 antibody at 1 μg/ml (Santa Cruz Biotechnology), rabbit anti-securin antibody at 1 μg/ml (a gift from Hui Zou), mouse monoclonal anti-PARP1 antibody at 1:1000 dilution (Cell Signaling), rabbit anti-MAD1 antibody at 4 μg/ml, rabbit anti-HEC1 antibody at 2 μg/ml, rabbit anti-BUB1 antibody at 1 μg/ml, rabbit anti-BUBR1 antibody at 1 μg/ml, and mouse anti-tubulin antibody at 1:1000 dilution. Annexin V staining was performed on HEC1 RNAi cells that were released from thymidine arrest for 16 h using a fluorescein isothiocyanate-conjugated annexin V apoptosis detection kit (PharMingen) according to the manufacturer’s protocol. Briefly, cells were washed twice with annexin V binding buffer and incubated with fluorescein isothiocyanate-conjugated annexin V and PI for 15 min. Apoptotic cells were scored by positive annexin V staining and negative PI staining.

**Cold- or Calcium-sensitive Microtubule Staining**—For cold-sensitive microtubule staining, at 48 h after hSPC25 siRNA treatment, cells were incubated with ice-cold medium for 10 min, followed by extraction with 0.5% Triton X-100 in PHEM buffer for 5 min. For calcium treatment, samples were permeabilized with 100 mM PIPES, 1 mM MgCl2, 0.1 mM CaCl2, and 0.1% Triton X-100 (pH 6.9). Cells were subsequently fixed with methanol and immunostained with anti-tubulin antibody and CREST, the cells were scored as described above.

**Cell Synchronization Experiments**—At 24 h after transfection of HeLa cells with HEC1 siRNA oligonucleotides, the mitotic cells were removed by mitotic shake off, and thymidine (2 μM final concentration) was added to the remaining cells. Another 18 h later, the mitotic and dead cells were again washed off, and the remaining cells were released into fresh medium. Samples were taken at 2-h intervals for 16 h and stained with annexin V or anti-PARP1 antibody to determine the percentage of apoptotic cells. To determine the timing of the appearance of spindle defects, samples were taken at 1-h intervals starting at 9 h after the release from thymidine arrest. These cells were fixed and stained with anti-tubulin antibody and DAPI. The effect of microtubule depolymerization on MAD1 staining and apoptosis in HEC1 RNAi cells was examined by adding nocodazole to these cells at 7 h after thymidine release. Immunostaining was performed at 7 h after nocodazole addition.

**RESULTS**

**Identification of hSPC25 by Immunopurification of the NDC80 Complex**—To characterize the human NDC80 complex, we raised antibodies against human HEC1. Upon immunoblotting, the affinity-purified anti-HEC1 antibody detected a 70-kDa band in HeLa cell lysates that was identical in size to the untagged HEC1 protein translated in vitro in rabbit reticulocyte lysate (Fig. 1A). The intensity of this band decreased significantly in lysates of HeLa cells transfected with HEC1-specific siRNA, indicating that this band belonged to HEC1 (see Fig. 4A). The affinity-purified anti-HEC1 antibody was coupled to protein A beads and used to immunopurify the human NDC80 complex from HeLa cell lysates (Fig. 1B). There were four proteins present in stoichiometric amounts in the anti-HEC1 immunoprecipitates. These proteins were absent in immunoprecipitates of anti-GST antibody (Fig. 1A). To facilitate discussion, they were named p70, p50, p25, and p24 on the basis of their molecular masses.

Mass spectrometry analysis of these proteins revealed that p70 and p50 were human HEC1 (the homolog of yeast Ndc80) and hNUF2, respectively (data not shown). Analysis of the p25 band yielded three peptides belonging to a novel coiled-coil protein (called AD024; Entrez protein data base accession number NP_065726) with unknown function and a predicted molecular mass of 26.2 kDa (Fig. 1C). The p24 protein was identified as another novel coiled-coil protein with a predicted molecular mass of 22.4 kDa (accession number NP_872319). Neither of these proteins shares any significant sequence similarity with the *Sacccharomyces cerevisiae* Spe25 and Spe4 proteins. However, database searches did allow us to identify homologs of p25 in mouse and *Schizosaccharomyces pombe* (accession number NP_588208) (Fig. 1C) and p24 homologs in mouse (accession number NP_080558). We chose to further characterize p25 (see below) and confirmed that it is a component of the human NDC80 complex and a functional homolog of the yeast Spe25 protein. Thus, we will henceforth refer to p25 as hSPC25.

**hSPC25 Binds to HEC1 in Vitro and Interacts with HEC1 throughout the Cell Cycle in Vivo**—To confirm the interaction between HEC1 and hSPC25, we transfected HeLa cells with plasmids encoding Myc-tagged HEC1 and HA-tagged hSPC25. The lysates of the transfected cells were then immunopurified using anti-Myc antibody and blotted with anti-HA antibody (Fig. 2A). The HA-hSPC25 protein was present in the anti-Myc immunoprecipitates (Fig. 2A), indicating that HA-hSPC25 interacted with Myc-HEC1 in these cells. In a comple-
mentary experiment, plasmids encoding Myc-hSPC25 and HA-HEC1 were cotransfected into HeLa cells. The cell lysates were again immunoprecipitated with anti-Myc antibody and blotted with anti-HA antibody. HA-HEC1 was again found to interact with Myc-hSPC25 (Fig. 2B). These results demonstrated that, when overexpressed, HEC1 and hSPC25 formed a complex in living cells. In another experiment, we cotranslated plasmids encoding HA-HEC1 and Myc-hSPC25 in rabbit reticulocyte lysate in vitro in the presence of [35S]methionine and subjected to immunoprecipitation with anti-Myc antibody. The immunoprecipitates were analyzed by SDS-PAGE, followed by autoradiography. Twenty percent of the input proteins were included for comparison. D, lysates of log-phase or nocodazole-arrested HeLa cells were subjected to immunoprecipitation with anti-GST or anti-HEC1 antibody. The immunoprecipitates were then resolved by SDS-PAGE and blotted with anti-hSPC25 antibody.

To further characterize hSPC25, we raised an antibody against the N-terminal 150 residues of this protein. After purification with the corresponding antigen, the anti-hSPC25 antibody recognized a 25-kDa band in HeLa cell lysates, which disappeared in lysates of cells subjected to hSPC25 RNAi treatment (see Fig. 4A). This indicated that this antibody selectively detected the hSPC25 protein at its endogenous levels. Immunoprecipitation with anti-HEC1 antibody followed by immunoblotting with anti-hSPC25 antibody showed that the endogenous HEC1 and hSPC25 proteins interacted with each other (Fig. 2D). This interaction was also observed in log-phase cells (Fig. 2D), suggesting that the NDC80 complex exists throughout the cell cycle.

hSPC25 Localizes to Kinetochores during Mitosis—HEC1 and hNUF2, two known components of the human NDC80 complex, localize to kinetochores during mitosis (29, 30). We
next examined whether hSPC25 exhibits a similar localization pattern. To determine the cellular localization of hSPC25, we transfected HeLa cells with plasmids encoding Myc-tagged hSPC25 and stained the transfected cells with anti-Myc antibody and a human autoimmune serum (CREST) that labeled the kinetochores (Fig. 3, A–C). Myc-hSPC25 exhibited a punctate staining pattern that matched that of CREST during all stages of mitosis, including prometaphase, metaphase, and anaphase (Fig. 3, A–C). This indicated that Myc-hSPC25 localized to kinetochores in mitosis. We next determined the cellular localization of the endogenous hSPC25 protein during mitosis. As shown in Fig. 3D, the endogenous hSPC25 protein also showed a punctate staining pattern that was similar to that of CREST, indicating that endogenous hSPC25 also localized to kinetochores during mitosis.

hSPC25 Is Required for Proper Progression through Mitosis—To further demonstrate that hSPC25 is a functional homolog of yeast Spc25, we depleted hSPC25 from HeLa cells using RNAi and compared the phenotypes of the hSPC25 RNAi cells with those of the HEC1 RNAi cells (Fig. 4). The protein levels of hSPC25 and HEC1 were significantly reduced in HeLa cells transfected with the corresponding siRNA oligonucleotides (Fig. 4A). We first examined the cell cycle status of the hSPC25 RNAi cells by FACS. At 48 h after RNAi treatment, ~30% of these cells possessed 4N DNA contents, whereas only 15% of the control cells had 4N DNA contents (Fig. 4B). About 30% of the hSPC25 RNAi cells possessed less than 2N DNA contents (Fig. 4B), suggesting that they had undergone cell death. Thus, depletion of hSPC25 from HeLa cells caused an accumulation of cells in G2/M and cell death. To determine whether the hSPC25 RNAi cells accumulated in G2 or mitosis, we examined the cellular and DNA morphology of these cells using a fluorescence microscope. Cells in mitosis were typically round with condensed DNA. At 24 h after RNAi treatment, we noticed an accumulation of hSPC25 RNAi cells in mitosis. The mitotic index of the hSPC25 RNAi cells was 32%, compared with a mitotic index of 10% in control cells (Fig. 4C). At 48 h after RNAi treatment, the mitotic index was ~28% (Fig. 4C). This indicated that the hSPC25 RNAi cells accumulated in mitosis and that the majority of the 4N cells as determined by FACS represented cells in mitosis, rather than G2 (Fig. 4, B and C).

Although the mitotic index of the hSPC25 RNAi cells did not
increase at later time points, there was a dramatic increase in cell death (Fig. 4C). For example, at 72 h after transfection, ~75% of the hsPC25 RNAi cells had undergone cell death, whereas <10% of the control cells had died (Fig. 4C). Therefore, our results are consistent with the notion that inactivation of hsPC25 causes a delay or arrest in mitosis, followed by cell death. Very similar phenotypes were observed with cells depleted of HEC1 by RNAi (Fig. 4C) (29). This indicated that inactivation of either hsPC25 or HEC1 had similar effects on the cell cycle, further supporting that hsPC25 is a component of the NDC80 complex.

Mitotic Arrest in hsPC25-depleted Cells Depends upon a Functional Spindle Checkpoint—The mitotic arrest of hsPC25 cells can be explained by two equally plausible scenarios. First, inactivation of hsPC25 causes a defect in kinetochore function and arrests cells in prometaphase. Second, the hsPC25 RNAi cells undergo mitotic arrest mediated by an active spindle checkpoint. One way to distinguish between these two possibilities is to determine the protein level of cyclin A1 in these cells. As shown previously, the level of cyclin A1 is high in prometaphase, and it is already degraded by the metaphase-anaphase transition (34). Interestingly, although an active spindle checkpoint blocks the APC/C-mediated degradation of cyclin B1 and securin, it does not prevent cyclin A1 degradation (34). Therefore, mitotic cells arrested with an active spindle checkpoint are expected to contain low levels of cyclin A1 and high levels of cyclin B1 and securin. On the other hand, cells in prometaphase are expected to contain high levels of all three proteins. To determine the status of the spindle checkpoint and the stage of mitotic arrest of mitotic hsPC25 RNAi cells, we examined the protein levels of cyclin A1, cyclin B1, and securin in these cells by immunostaining with the corresponding antibodies. As shown in Fig. 5, the levels of cyclin B1 and securin were high in mitotic hsPC25 RNAi cells, whereas the levels of cyclin A1 were low. This suggests that these cells might be arrested in mitosis by an active spindle checkpoint, instead of a mechanical slowdown in prometaphase.

Martin-Lluesma et al. (29) have shown that the mitotic delay observed in HEC1 RNAi cells is dependent on the spindle checkpoint, as simultaneous depletion of HEC1 and MAD2 by RNAi allows cells to escape from this mitotic arrest. To examine whether the mitotic arrest observed in the hsPC25 RNAi cells also depends on the spindle checkpoint, we depleted both hsPC25 and MAD2 simultaneously in HeLa cells by RNAi. Both the mitotic arrest and cell death phenotypes observed in the cells subjected to hsPC25 siRNA alone were mostly reversed in cells that received both hsPC25 and MAD2 siRNAs. For example, at 24 and 48 h after RNAi treatment, only 15% of the double RNAi cells were arrested in mitosis, whereas ~30% of the hsPC25 RNAi cells were mitotic (Fig. 4C). At 72 h after RNAi treatment, the majority of the hsPC25 RNAi cells had undergone cell death, whereas >70% of the hsPC25/MAD2 double RNAi cells were viable (Fig. 4C). It is worth noting that many of the hsPC25/MAD2 double RNAi cells escaped from mitosis abnormally, as a vast majority of the hsPC25/MAD2-depleted interphase cells contained multiple or aberrant multilobed nuclei (data not shown). Therefore, similar to HEC1-depleted cells (29), the mitotic delay or arrest caused by the inactivation of hsPC25 also requires a functional spindle checkpoint.

Inactivation of the Human NDC80 Complex Causes Apoptosis after Transient Mitotic Arrest—To determine the timing of cell death in response to the loss of NDC80 function, we followed HEC1 siRNA-transfected cells after synchronization at the G1/S boundary by thymidine treatment. The percentage of cell death was significantly lower in thymidine-treated HEC1 RNAi cells (12%) compared with untreated RNAi samples (28%), suggesting that blocking cells at G1/S reduces cell death in HEC1-depleted cells. We next washed off the mitotic and dead cells that had accumulated during the 18-h thymidine treatment and examined HEC1-depleted cells and mock-transfected cells for 16 h after release from thymidine arrest (Fig. 6). As expected, a major increase in the mitotic index was observed at ~9–10 h after thymidine release in both samples (Fig. 6A). Comparison of the mitotic index at later time points revealed that mitotic progression was much slower in the HEC1-depleted cells compared with the mock-transfected cells (Fig. 6A). After 14 h of thymidine release, only 10–15% of the control cells were in mitosis, whereas ~30–35% of the HEC1-depleted cells were mitotic (Fig. 6A). There was no significant cell death in the HEC1 siRNA-treated cells until 14 h after thymidine release, at which time a sharp increase in cell death was observed (Fig. 6B). By 16 h, ~50% of the HEC1-depleted cells were dead (Fig. 6B). This indicates that HEC1-depleted cells die following transient arrest in mitosis. During the same period, no significant cell death was observed for the control cells. These results are consistent with time lapse video microscopy by us (data not shown) and DeLuca et al. (30). A large percentage of the cells with a compromised NDC80 function died following a 5–8-h arrest in mitosis.

To determine whether the cell death observed in cells without proper NDC80 function was due to apoptosis or necrosis, we stained the dead HEC1 RNAi cells for the commonly used apoptotic markers annexin V and PARP1. The HEC1 RNAi cells were first synchronized at G1/S with thymidine and released for 18 h. Most of the dead HEC1 RNAi cells stained positive for annexin V and negative for PI (Fig. 6B), indicating that the HEC1-depleted cells underwent apoptosis. This was further confirmed by the detection of cleaved PARP1 in immunoblots of HEC1 RNAi samples (Fig. 6C) and positive immu-
nositaining of these dead cells with an antibody specific for the cleaved form of PARP1 (Fig. 6D). Staurosporine is known to trigger apoptosis in HeLa cells, and staurosporine-treated cell lysate was used as a positive control in the anti-PARP1 immunoblot (Fig. 6C). Interestingly, we found that treatment of HEC1 RNAi cells with nocodazole did not prevent cell death (data not shown). This suggests that apoptosis of the HEC1 RNAi cells is not a secondary consequence of cells attempting to undergo mitosis in the presence of a functional spindle but dysfunctional kinetochores. It is possible that kinetochores with a compromised NDC80 function trigger an apoptotic pathway directly only following a 5–8-h mitotic arrest. Cell death can be avoided if cells do not undergo this mitotic arrest due to the inactivation of the spindle checkpoint (as in HEC1/MAD2 RNAi cells). To examine whether the cell death in response to hSPC25/HEC1 RNAi is specific to HeLa cells and other cell lines with defective p53/Rb pathways, we depleted hSPC25 from HCT116 cells, which contain wild-type p53. About 20% of the HCT116 cells underwent apoptosis after 48 h of hSPC25 siRNA transfection, compared with 5% in mock-transfected cells (see Supplemental Fig. S1). This clearly indicates that hSPC25 RNAi also induces cell death of a cell line with wild-type p53, albeit to a lesser extent.

Inactivation of hSPC25 Leads to Multiple Spindle Abnormalities—Several groups have observed various mitotic defects in vertebrate cells depleted of HEC1 or NUF2 (29–32). We also analyzed the DNA and spindle morphology of the hSPC25 RNAi cells. As shown in Fig. 7, the hSPC25 RNAi cells also exhibited defects in spindle morphology and chromosome congression. First, ~18% of the mitotic hSPC25 RNAi cells contained multipolar spindles (Fig. 7A). In the mitotic cells with bipolar spindles, there were also clear defects: these spindles were more elongated and often distorted (Fig. 7A). Second, despite the presence of bipolar spindles, the chromosomes in most mitotic hSPC25-depleted cells were scattered throughout the cell, indicating failure of the chromosomes to align at the metaphase plate. In a few cells, although most of the chromosomes were aligned at the metaphase plate, some chromosomes were still lagging behind (Fig. 7A). These results indicate that hSPC25 is required for the proper execution of many mitotic processes, again consistent with its being a subunit of the NDC80 complex.

To examine whether the spindle defects in cells lacking proper NDC80 function are an indirect consequence of prolonged mitotic arrest, HEC1 RNAi cells were synchronized by thymidine treatment for 18 h. Cells were then allowed to enter mitosis at 9–10 h after thymidine release. The morphology of the mitotic spindle was monitored by immunostaining cells for tubulin and DNA at 1-h intervals after 9 h of thymidine release. Surprisingly, even at 9 h after thymidine release, most mitotic cells exhibited an elongated spindle, and ~25% of these mitotic cells contained multipolar spindles (see Supplemental Fig. S2). There was no significant increase in the number of cells with multipolar spindle at later time points (see Supplemental Fig. S2). This indicates that these spindle defects occur
shortly after the entry into mitosis and are unlikely to be a non-specific consequence of prolonged mitotic arrest. However, we did observe an increase in the number of cells with fractured spindles at later time points (see Supplemental Fig. S2), suggesting that this defect might be an indirect consequence of prolonged mitotic arrest.

**hSPC25-deficient Kinetochores Retain the Ability to Form Microtubule Attachments**—The chromosome congression defect observed in hSPC25-depleted cells might be due to their inability to form functional kinetochore-microtubule connections. The presence of kinetochore-bound microtubules can be specifically detected by incubating cells for a short period with ice-cold medium or by permeabilizing them in the presence of high levels of calcium. Both of these treatments destabilize non-kinetochore microtubules, whereas kinetochore-bound microtubules are preserved. Microtubules appeared to be stable under both of these circumstances in hSPC25-deficient cells (Fig. 7B), suggesting that the NDC80 complex is not completely indispensable for the formation of kinetochore-microtubule attachments. However, we do not know whether these kinetochore-microtubule attachments formed in hSPC25 RNAi cells are functional.

**Depletion of hSPC25 Leads to the Loss of Kinetochore Localization of MAD1 in a Microtubule-dependent Manner**—Martin-Lluesma et al. (29) showed that HEC1 interacts with MAD1 in yeast two-hybrid assays and is essential for the localization of MAD1 to kinetochores during mitosis. We next tested whether hSPC25 is also required for the kinetochore recruitment of MAD1. We examined the kinetochore localization of three spindle checkpoint proteins (MAD1, BUB1, and BUBR1) in the hSPC25-depleted cells (Fig. 8). In control cells, MAD1 was clearly observed at the kinetochores during prometaphase (Fig. 8). However, the kinetochore staining of MAD1 was undetectable in prometaphase cells depleted of hSPC25 (Fig. 8). Depletion of hSPC25 did not adversely affect the integrity of the kinetochores in a general way, as CREST staining at the kinetochores was normal. The kinetochore staining of other checkpoint proteins, such as BUB1 and BUBR1, was also largely unaffected by the RNAi-mediated depletion of hSPC25 (Fig. 8). This indicates that inactivation of hSPC25 specifically abolishes the kinetochore localization of MAD1. Martin-Lluesma et al. (29) showed that the intensity of the kinetochore staining of BUB1 is reduced by 50% in HEC1 RNAi cells. In our hands, the BUB1 and BUBR1 kinetochore staining did not appear to be weaker in either HEC1 or hSPC25 RNAi cells. We do not know the underlying reason for this discrepancy, although it may result from the use of different antibodies and different fixation protocols.

We also tested whether hSPC25 is required for the kinetochore localization of HEC1 in mitosis. As shown in Fig. 8, HEC1 localized to the kinetochores in control mitotic cells. The intensity of the kinetochore staining of HEC1 was greatly diminished in mitotic hSPC25-depleted cells. This indicates that the kinetochore localization of HEC1 depends on hSPC25. This is again consistent with hSPC25 being a component of the NDC80 complex.

We next tested whether the NDC80 complex is also required for the kinetochore localization of MAD1 in nocodazole-arrested mitotic cells. As shown in Fig. 9, MAD1 localized to kinetochores in mock-transfected mitotic cells treated with nocodazole. As expected, the kinetochore localization of MAD1 was absent in mitotic HEC1 RNAi cells in the absence of nocodazole (Fig. 9). Surprisingly, the kinetochore localization of MAD1 was restored in HEC1 RNAi cells upon nocodazole treatment (Fig. 9). Although we could not determine whether a particular cell indeed received RNAi, we did analyze >100 mitotic cells, and the vast majority of them exhibited positive MAD1 staining. Therefore, it appears that the NDC80 complex is not absolutely required for MAD1 kinetochore localization in nocodazole-arrested mitotic cells that lack a functional spindle.

**DISCUSSION**

We have identified two novel components of the human NDC80 complex through immunoaffinity purification. Despite the lack of significant sequence homology, hSPC25 is the functional homolog of the yeast Spc25 protein based on the following observations. First, hSPC25 was isolated based on its physical interaction with HEC1, a known component of the human NDC80 complex. It also interacted with HEC1 in vitro. Second, similar to HEC1 and hNUF2 (two known components of human NDC80 complex), hSPC25 localized to kinetochores during mitosis. Third, depletion of hSPC25 in HeLa cells by RNAi caused similar mitotic defects, as did RNAi-mediated depletion of HEC1 or hNUF2. Although we have not performed a detailed characterization of the 24-kDa protein identified in the HEC1 immunoprecipitates, it is very likely the human ortholog of the yeast Spc24 protein because it was present in the same stoichiometry as the other NDC80 complex components and because it is also a coiled-coil protein, similar to the yeast Spc24 protein.

**Roles of the NDC80 Complex in Chromosome Congregation and Spindle Morphology**—The Ndc80 complex has now been characterized in several organisms, including *S. cerevisiae*, *Xenopus*., chicken, and mammals (28–32). In all the systems studied so far, the NDC80 complex proteins localize to kinetochores in mitosis,
and their inactivation leads to a defect in chromosome congression. We have observed a similar phenotype for HeLa cells transfected with either hSPC25 or HEC1 siRNA. The exact function of the NDC80 components in this process is not yet understood. The simplest explanation for the chromosome congression defect in these cells would be their inability to form functional kinetochore-microtubule attachments. But the resistance of microtubules to cold- or calcium-mediated destabilization in these cells indicates that these cells can achieve a certain degree of kinetochore-microtubule attachment. Moreover, some degree of chromosome alignment can be observed in many mitotic hSPC25/HEC1-depleted cells. The inability of these cells to align their chromosomes might then stem from their unstable or improper kinetochore-microtubule connections.

Another important aspect of the kinetochore-microtubule connection is that these cells can achieve a certain degree of kinetochore-microtubule attachment. This phenomenon allows the cells to undergo chromosome congression. However, failure to align chromosomes might be due to their unstable or improper kinetochore-microtubule connections.

Moreover, we have observed that these cells are still able to form functional spindle formation. This suggests that HEC1-depleted cells can achieve a certain degree of kinetochore-microtubule attachment.

Our experiments with synchronized cells treated with HEC1 RNAi support the second possibility. Elongated and multipolar spindles could be observed at early stages of mitosis in HEC1-depleted cells, and no significant increase in the frequency of these abnormal spindles was observed at later time points. In addition, functional disruption of other kinetochore components, such as the NDC80 complex, is essential to maintain the integrity of the spindle. Our experiments with synchronized cells treated with HEC1 RNAi support the second possibility. Elongated and multipolar spindles could be observed at early stages of mitosis in HEC1-depleted cells, and no significant increase in the frequency of these abnormal spindles was observed at later time points. In addition, functional disruption of other kinetochore components, such as the NDC80 complex, is essential to maintain the integrity of the spindle.

The existence of kinetochore-microtubule attachment in these cells as detected by cold- or calcium-sensitive staining prompted us to examine MAD1 localization in hSPC25/HEC1-depleted cells after treatment with a microtubule-depolymerizing agent, such as nocodazole. Surprisingly, MAD1 localization to kinetochores remained unaffected in HEC1-depleted cells treated with nocodazole. Our findings are consistent with those of DeLuca et al. (30, 37). This suggests that HEC1-deficient kinetochores might still retain the ability to recruit MAD1.

Role of NDC80 in the Spindle Checkpoint—Martin-Lluesma et al. (29) have reported that HEC1 interacts with MAD1 in yeast two-hybrid assays and is required for the localization of MAD1 to kinetochores during mitosis. These observations have been confirmed by our findings in this study and by Hori et al. (32) using Nuf2-deleted chicken DT40 cell lines. There are two possible explanations for the lack of MAD1 kinetochore localization in NDC80-deficient cells. The NDC80 complex might form the docking surface through which MAD1 latches onto the kinetochores. Alternatively, it could be a secondary consequence of cells attempting to divide in the presence of a defective kinetochore but functional spindle. In the future, it will be interesting to determine whether and how the NDC80-deficient kinetochores actively trigger apoptosis.
attachment might be sufficient for the dissociation of MAD1 from kinetochores. A similar situation has been observed in PtK1 cells treated with hypothermia or in HeLa cells treated with microtubule-stabilizing agents, such as Taxol (38, 39). In all these situations, kinetochores are attached to microtubules, but lack tension. These kinetochores also lack MAD1 and MAD2 staining, suggesting that microtubule attachment to kinetochores is sufficient to trigger the dissociation of MAD1 from the kinetochores, even in the absence of tension. Despite the lack of MAD1 and MAD2 localization to kinetochores, these cells maintain mitotic arrest in a spindle checkpoint-dependent manner. Although the kinetochore localization of MAD1 and MAD2 appears to be dispensable for mitotic arrest in these cells, the cytoplasmic pool of MAD2 is absolutely necessary for the checkpoint activity. Microinjection of anti-MAD2 antibodies results in escape from mitotic arrest in all these circumstances. Similarly, the spindle checkpoint is active in NDC80-deficient cells, as indicated by low levels of cyclin A1 and cyclin B1 and securin; and this checkpoint activity is dependent upon proper MAD2 function. Simultaneous depletion of hSPC25/HEC1 and MAD2 from HeLa cells abolishes the mitotic arrest observed with the depletion of hSPC25/HEC1 alone.

Studies on the NDC80 complex and all the above-mentioned scenarios, where kinetochore attachment is established without tension, pose an important question: how do these cells maintain mitotic arrest despite a lack of MAD1 and MAD2 localization to the kinetochores? We suggest two possible explanations. First, it is possible that small amounts of MAD1, undetectable by our microscopy techniques, are still present on the kinetochores in the NDC80-deficient cells and are responsible for an active checkpoint. Alternatively, it is possible that MAD1 recruitment to kinetochores is required for the initial generation of the APC/C inhibitory checkpoint complexes and the establishment of the spindle checkpoint, but not for its maintenance. Other checkpoint proteins are then responsible for the maintenance of these APC/C inhibitory signals through mechanisms that do not require the kinetochore localization of MAD1 and MAD2. Clearly, more studies are needed to clarify these issues.

In conclusion, we have identified two novel components of the human NDC80 complex. Detailed characterization of hSPC25 revealed that it plays a pivotal role in chromosome congression and the maintenance of spindle integrity. Our studies also suggest that loss of NDC80 function in cells might serve as a direct signal to trigger apoptosis. Finally, we have also demonstrated that HEC1 depletion results in microtubule-dependent dissociation of MAD1 from kinetochores, reinforcing the notion that kinetochore localization of MAD1 and MAD2 is dependent primarily upon the microtubule occupancy status of kinetochores.

Acknowledgments—We thank Feras Gadmasi and Yue Chen for mass spectrometry and Todd Stukenberg for communicating results prior to publication.

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