Human NUF2 Interacts with Centromere-associated Protein E and Is Essential for a Stable Spindle Microtubule-Kinetochore Attachment

Chromosome segregation in mitosis is orchestrated by dynamic interaction between spindle microtubules and the kinetochore, a multiprotein complex assembled onto centromeric DNA of the chromosome. Here, we show that Homo sapiens (Hs) NUF2 is required for stable kinetochore localization of centromere-associated protein E (CENP-E) in HeLa cells. HsNUF2 specifies the kinetochore association of CENP-E by interacting with its C-terminal domain. The region of HsNUF2 binding to CENP-E was mapped to its C-terminal domain by glutathione S-transferase pulldown and yeast two-hybrid assays. Suppression of synthesis of HsNUF2 by small interfering RNA abrogated the localization of CENP-E to the kinetochore, demonstrating the requirement of HsNUF2 for CENP-E kinetochore localization. In addition, depletion of HsNUF2 caused aberrant chromosome segregation. These HsNUF2-suppressed cells displayed reduced tension at kinetochores of bi-oriented chromosomes. Double knockdown of CENP-E and HsNUF2 further abolished the tension at the kinetochores. Our results indicate that HsNUF2 and CENP-E are required for organization of stable microtubule–kinetochore attachment that is essential for faithful chromosome segregation in mitosis.

The kinetochore is a supramolecular complex assembled at each centromere in eukaryotes. It provides a chromosomal attachment point for the mitotic spindle, linking the chromosome to the microtubule, and functions in initiating, controlling, and monitoring the movements of chromosomes during mitosis. The kinetochore of animal cells contains two regions: the inner kinetochore, which is tightly and persistently associated with centromeric DNA sequences throughout the cell cycle, and the outer kinetochore, which contains many dynamic protein components that interact with microtubules only during mitosis. The stable propagation of eukaryotic cells requires that each chromosome is accurately duplicated and faithfully segregated. During mitosis, attaching, positioning, and bi-orienting kinetochores with the spindle microtubules play essential roles in chromosome segregation and genomic stability (see Refs. 1–4).

Centromere-associated protein E (CENP-E) is a microtubule-based kinesin motor protein located on the outer kinetochore and is responsible for a stable microtubule–kinetochore attachment (5). CENP-E participates in the chromosome movements from prometaphase to anaphase (6). It moves toward and tethers kinetochores to microtubule “plus” ends (7), helping mono-oriented chromosomes align at the metaphase plate before bi-orientation (5–8). During this process, its location on the kinetochore depends on BUBR1 and CENP-F (9). Unattached kinetochores are also the sensing apparatus for the mitotic checkpoint, which delays anaphase onset until all kinetochores have properly attached to spindle microtubules (10, 11). In this context, CENP-E as a kinetochore attachment sensor is also involved in a spindle checkpoint signaling pathway. It activates BUBR1-related checkpoint signaling in a microtubule-dependent manner (12, 13), and CENP-E/BUBR1 interaction is quite important in controlling this spindle checkpoint (5, 12, 13). Thus, CENP-E is necessary for microtubule–kinetochore conjugation, chromosome congression, and spindle checkpoint activation.

Homo sapiens (Hs) NUF2 is a kinetochore protein that forms a stable complex with HEC1, HsSpc24, and HsSpc25 (named the HsNdc80 complex). Depletion of HsNUF2 by RNA interference results in a strong prometaphase block with an active

* This work was supported by Chinese 973 Project Grant 2002CB713700; Chinese Academy of Science Grants KSCX1-YW-R6S and KSCX2-YW-H-10; Chinese Natural Science Foundation Grants 39925018, 30270293, 3050183 (to X. D.), and 90508002; Chinese 863 Project Grant 2001AA15331; Chinese Ministry of Education Grant 20020358051; and a Georgia Cancer Coalition Grant and National Institutes of Health Grants DK56292, CA89019, and CA92080 (to X. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

‡ To whom correspondence may be addressed: Laboratory of Cellular Dynamics, University of Science and Technology of China, Hefei 230027, China. Tel.: 86-551-3657141; Fax: 86-551-3606304; E-mail: jincj@ustc.edu.cn.

§ Georgia Cancer Coalition Eminent Cancer Research Scholar. To whom correspondence may be addressed: Lab. of Cellular Dynamics, University of Science and Technology of China, Hefei 230027, China. Tel. 86-551-360660; Fax 86-551-360660; E-mail: yaoxb@ustc.edu.cn.

¶ The abbreviations used are: CENP-E, centromere-associated protein E; Hs, Homo sapiens; PBS, phosphate-buffered saline; GST, glutathione S-transferase; MBP, maltose-binding protein; GFP, green fluorescent protein; aa, amino acids; X-α-gal, 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside; siRNA, small interfering RNA; ACA, anti-centromere antibodies.

The kinetochore is a supramolecular complex assembled at each centromere in eukaryotes. It provides a chromosomal attachment point for the mitotic spindle, linking the chromosome to the microtubule, and functions in initiating, controlling, and monitoring the movements of chromosomes during mitosis. The kinetochore of animal cells contains two regions: the inner kinetochore, which is tightly and persistently associated with centromeric DNA sequences throughout the cell cycle, and the outer kinetochore, which contains many dynamic protein components that interact with microtubules only during mitosis. The stable propagation of eukaryotic cells requires that each chromosome is accurately duplicated and faithfully segregated. During mitosis, attaching, positioning, and bi-orienting kinetochores with the spindle microtubules play essential roles in chromosome segregation and genomic stability (see Refs. 1–4).

Centromere-associated protein E (CENP-E) is a microtubule-based kinesin motor protein located on the outer kinetochore and is responsible for a stable microtubule–kinetochore attachment (5). CENP-E participates in the chromosome movements from prometaphase to anaphase (6). It moves toward and tethers kinetochores to microtubule “plus” ends (7), helping mono-oriented chromosomes align at the metaphase plate before bi-orientation (5–8). During this process, its location on the kinetochore depends on BUBR1 and CENP-F (9). Unattached kinetochores are also the sensing apparatus for the mitotic checkpoint, which delays anaphase onset until all kinetochores have properly attached to spindle microtubules (10, 11). In this context, CENP-E as a kinetochore attachment sensor is also involved in a spindle checkpoint signaling pathway. It activates BUBR1-related checkpoint signaling in a microtubule-dependent manner (12, 13), and CENP-E/BUBR1 interaction is quite important in controlling this spindle checkpoint (5, 12, 13). Thus, CENP-E is necessary for microtubule–kinetochore conjugation, chromosome congression, and spindle checkpoint activation.

Homo sapiens (Hs) NUF2 is a kinetochore protein that forms a stable complex with HEC1, HsSpc24, and HsSpc25 (named the HsNdc80 complex). Depletion of HsNUF2 by RNA interference results in a strong prometaphase block with an active
spindle checkpoint, which is correlated with low but detectable MAD2 at kinetochores that have no or few stable kinetochore microtubules (14). HEC1 was originally identified as a retinoblastoma protein-associated protein. Microinjection of anti-HEC1 antibodies into cultured cells disrupts mitotic progression (15). Both the Nuf2-Ndc80 complex and its human homolog, NUF2-HEC1, have been shown to be important for stable microtubule-kinetochore attachment, chromosome alignment, and also spindle checkpoint activation in mitosis (14, 16–19).

Although it has been demonstrated that depletion of HsNUF2-HEC1 abrogates the localization of CENP-E to the kinetochore (18, 24), the underlying mechanism remains elusive. Given our recent success in obtaining the full-length human CENP-E and HsNUF2 cDNAs (14, 16–19), we conducted a new search for kinetochore proteins that specify the kinetochore localization of CENP-E using the new cDNA clone. We chose its C terminus as bait and conducted a yeast two-hybrid assay in which the kinetochore protein HsNUF2 was identified as one of several dozen positive clones. Our biochemical characterization validated the interaction between HsNUF2 and CENP-E. In addition, our study shows that HsNUF2 specifies the kinetochore localization of CENP-E and is essential for a stable microtubule-kinetochore attachment.

MATERIALS AND METHODS

Cell Culture and Synchronization—HeLa and 293T cells (American Type Culture Collection, Manassas, VA) were maintained as subconfluent monolayers in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (HyClone, Logan, UT) and 100 units/ml penicillin plus 100 µg/ml streptomycin (Invitrogen) at 37 ºC in 10% CO2. Cells were synchronized at G1/S with 5 mM thymidine for 16 h, washed three times with phosphate-buffered saline (PBS), and cultured in thymidine-free medium for 12 h for release. After another round of thymidine treatment for 12 h, cells were released for 9–10 h to initiate prometaphase. In some cases, 100 nM nocodazole was added to the cell culture to synchronize cells in prometaphase.

Antibodies—Affinity purification of rabbit anti-CENP-E antibody was as described previously (22). Mouse monoclonal antibody to HsNUF2 was purchased from Abcam and used at a 1:1000 dilution for Western blotting and a 1:300 dilution for immunofluorescence. Mouse monoclonal antibody to HEC1 was purchased from Abcam and used at a 1:1000 dilution for Western blotting at a 1:500 dilution for immunofluorescence. Mouse monoclonal antibodies to glutathione S-transferase (GST) and maltose-binding protein (MBP) were purchased from Cell Signaling Technology (Beverly, MA) and used at a 1:1000 dilution for Western blotting. Mouse monoclonal antibody to green fluorescent protein (GFP) was obtained from BD Biosciences. Rabbit antibody against the C-terminal tail of HsNUF2 was purchased from Abcam and used at a 1:1000 dilution for Western blotting and at a 1:300 dilution for immunofluorescence. Mouse monoclonal antibody to HEC1 antibodies into cultured cells disrupts mitotic progression. Given our recent success in obtaining the full-length human CENP-E cDNA clone and the finding that the original CENP-E clone missed 38 amino acids (Ref. 20), we conducted a new search for kinetochore proteins that specify the kinetochore localization of CENP-E using the new cDNA clone. We chose its C terminus as bait and conducted a yeast two-hybrid assay in which the kinetochore protein HsNUF2 was identified as one of several dozen positive clones. Our biochemical characterization validated the interaction between HsNUF2 and CENP-E. In addition, our study shows that HsNUF2 specifies the kinetochore localization of CENP-E and is essential for a stable microtubule-kinetochore attachment.

Expression and Purification of Recombinant Proteins—GST-CENP-E-(2167–2701), GST-HsNUF2, and MBP-CENP-E-(2167–2701) were expressed in E. coli strain BL21 (DE3)pLysS and purified using glutathione (GST-CENP-E-(2167–2701) and GST-HsNUF2) and amylase (MBP-CENP-E-(2167–2701) beads as described previously (23).

Pulldown Assays—in vitro pulldown assays were performed with bacterially expressed and purified GST-CENP-E-(2167–2701). GST-CENP-E-(2167–2701) with beads was combined with cell lysates of 293T cells transfected individually with a GFP-HsNUF2 series for 24 h at 37 mm NaCl, 2.7 mm KCl, 10 mm Na2HPO4, and 2 mm KH2PO4, 0.20% Tween 20 (TPBS) for 4 h at 4 ºC. The beads were washed three times with ice-cold TPBS and then boiled for 5 min in Laemmli sample buffer. Proteins were resolved by SDS-PAGE for Coomassie Blue staining or transferred onto nitrocellulose membrane for Western blotting.

To demonstrate a physical interaction between recombinant HsNUF2 and CENP-E, GST-tagged HsNUF2 immobilized on following plasmids were used for in vitro and in vivo studies: BD-CENP-E-(2167–2701) (where BD represents the Gal4 DNA-binding domain) and BD-CENP-E-(2131–2701) in pGBK7T; AD-HsNUF2-(1–464) (where AD represents the Gal4 DNA activation domain), AD-D1 (aa 1–341), AD-D2 (aa 424–464), AD-D3 (aa 341–424), and AD-D4 (aa 212–424) in pGADT7; GST-CENP-E-(2167–2701) and GST-HsNUF2 in pGEX4T3; MBP-CENP-E-(2167–2701) in pMAL-C2; and GFP-HsNUF2, GFP-D1 (HsNUF2-(281–341)), GFP-D2 (HsNUF2-(126–281)), GFP-D3 (HsNUF2-(246–341)), and GFP-D5 (HsNUF2-(281–464)) in pEGFP-C1. All constructs were sequenced in full.

Yeast Two-hybrid Screen—Yeast two-hybrid assays were performed as described (21). Briefly, two CENP-E baits containing aa 2167–2701 and 2131–2701 were inserted into the BamHI-EcoRI sites of pGBK7T individually to create a fusion with aa 1–147 of the Gal4 DNA-binding domain. The resultant pGBK7T/CENP-E C termini were transformed into strain AH109 along with the GAL4 reporter plasmid pCL and the negative control plasmid pGBK7T-Lam. Protein expression was validated by Western blotting using Gal4 and an anti-CENP-E antibody that reacts with the C-terminal tail. Transformants did not activate the HIS3 reporter gene and were transformed with the HeLa cDNA library. Transformants were selected on Leu +/Trp +/His +/Ade – 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (Xα-gal)-positive synthetic dropout plates. Almost 400 colonies were picked out on Leu +/Trp +/His +/Ade – X-α-gal-positive synthetic dropout plates. Minipreparation plasmids were isolated from Escherichia coli DH5α, and PCR products were digested with several restriction enzymes and grouped according to their digestion patterns. Specificity of the interaction was independently verified by re-transforming the candidate cDNAs back into strain AH109 along with pGBK7T/CENP-E C termini. Those cDNAs that could form colonies on Leu +/Trp +/His +/Ade – synthetic dropout plates were sent for sequencing.
glutathione-agarose beads (Sigma) was used as an affinity matrix for incubation with purified MBP-CENP-E-(2167–2701). The agarose beads were washed five times with PBS before being boiled in SDS-PAGE sample buffer. The proteins were then fractionated on SDS-polyacrylamide gel, and the gel was stained with Coomassie Brilliant Blue.

Interaction Domain Mapping by Yeast Two-hybrid Assay—A series of AD-HsNUF2 deletion plasmids were cotransformed with BD-CENP-E-(2167–2701) into yeast strains. After 3 days of incubation, those colonies that grew on Leu−/Trp−/His−/Ade− X-α-gal-positive synthetic dropout plates were inoculated onto Leu−/Trp−/His−/Ade− X-α-gal-positive synthetic dropout plates and turned blue. Strains transformed with AD-D1 (aa 1–341) and AD-D2 (aa 424–464) did not grow, indicating that these two fragments cannot bind to the CENP-E C terminus.

Co-immunoprecipitation—HeLa cells were synchronized at prometaphase as described above. The cells were then lysed with 0.5% Nonidet P-40, 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.02% sodium azide, and 1 mM phenylmethylsulfonyl fluoride (which was added prior to use). Anti-CENP-E polyclonal antibody HpX and anti-HsNUF2 monoclonal antibody were incubated for 2 h with protein A/G beads and washed three times with wash buffer (0.1% Nonidet P-40, 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.02% sodium azide, and phenylmethylsulfonyl fluoride (added just before use)) to prepare for the co-immunoprecipitation experiment. These antibody-bound protein A/G beads were added to the cell lysate and incubated for 4 h at 4°C. After incubation, the beads were washed four times with wash buffer and once with PBS. Samples were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membrane to perform Western blotting.

Small Interfering RNA (siRNA)—For the siRNA studies, the 21-mers of the siRNA duplexes against HsNUF2 (target sequence, 5′-AAGCATGGGCTGAAACTATA-3′) (14), HEC1 (target sequence, 5′-AAGTTCAGAAGCTGAAGTCTT-3′) (18), and CENP-E (target sequence, 5′-AAACACATCTACTCTCCAGTTT-3′) (18) were synthesized by Dharmacon Research Inc. (Lafayette, CO). After trial experiments using a series of concentrations and time course assays, treatment at 100 nM for 48 h was finally selected as the most efficient conditions for repressing target proteins.

Transfection and Immunofluorescence—Cells were transfected with siRNA and GFP-tagged plasmids in a 24-well plate using Oligofectamine reagent and Lipofectamine 2000 (Invitrogen), respectively, according to the manufacturer’s recommendations. For immunofluorescence, HeLa cells were seeded onto sterile acid-treated 12-mm coverslips in 24-well plates (Corning Corp., Corning, NY). Single thymidine-blocked and thymidine-released cells were transfected with HsNUF2, HEC1, or CENP-E siRNA and GFP fusion plasmids as described above. Fixation and staining were done as described previously (8). Antibody HpX and antibodies against GFP, HsNUF2, HEC1, and ACA were used at dilutions of 1:500, 1:300, 1:300, 1:500, and 1:1000, respectively. Images were acquired using a Zeiss Axiovert 200 inverted microscope with AxioVision Version 3.0 software.

Fluorescence Intensity Quantification and Kinetochore Distance Measurement—The fluorescence intensity of kinetochore protein labeling was measured using a Zeiss LSM 510 NLO confocal microscope scan head mounted transversely on an Axiovert 200 inverted microscope with a 100 × 1.3 numerical aperture PlanApo objective. The images from double labeling were collected using a dichroic filter set with Zeiss LSM 5 image processing software. The distance between sister kinetochores marked with ACA was measured as the distance between the peak fluorescence as described previously (5). To test whether double repression of HsNUF2 and CENP-E eliminates the tension across the sister kinetochore, we added 100 nM nocodazole to the aliquots of siRNA-treated cells 30 h after the transfection for additional 18 h (5). The distance between sister kinetochores marked with ACA was measured as described above.

Quantification of the level of kinetochore-associated protein was conducted as described by Johnson et al. (25). In brief, the average pixel intensities from at least 50 kinetochore pairs from five cells were measured, and background pixel intensities were subtracted. The pixel intensities at each kinetochore pair were then normalized against ACA pixel values to account for any variations in staining or image acquisition. The values of specific siRNA-treated cells were then plotted as a percentage of the values obtained from cells transfected with a control siRNA duplex.

RESULTS

HsNUF2 Is a Novel CENP-E-binding Partner—CENP-E contains an N-terminal microtubule-based motor domain, a central coiled-coil domain, and a C-terminal kinetochore-targeting domain. It has been reported the C terminus of CENP-E interacts with known kinetochore proteins BUBR1 and CENP-F, both of which are believed to be involved in anchoring CENP-E on kinetochores (9). Further research indicated that the interaction between CENP-E and BUBR1 is very important in spindle checkpoint activation (5). It has been proposed that CENP-E binds BUBR1 and activates its kinase activity and that the spindle checkpoint signal is silenced as CENP-E captures a microtubule (12, 13). These findings indicate that CENP-E is a key kinetochore protein in the spindle checkpoint signaling pathway and possibly links tension production to spindle checkpoint signal activation.

We recently succeeded in generating a full-length human cDNA clone of CENP-E, and we discovered a deletion of 38 amino acids in the original clone (20) and corrected this deletion. To identify proteins that associate with the CENP-E C-terminal tail, aa 2167−2701 of CENP-E cDNA were used as bait to screen a HeLa cDNA library using the Gal4 yeast two-hybrid system as described previously (21). Screening was performed on a total 1 × 10⁶ clones, and we identified 43 positive clones. Following analysis by HaelIII and Alul restriction digests, we identified several novel interacting partners of CENP-E. Nucleotide sequencing revealed that one of these interactors encodes the C terminus of HsNUF2 (aa 212–424). Its sequence matches that of HsNUF2 (GenBank™ accession number NP_663735).
To verify the specificity of interaction between HsNUF2 and CENP-E and to define further the domain(s) that mediates this interaction, we took advantage of yeast genetics and sought to perform additional yeast two-hybrid screens to map the binding interface(s). To this end, we used reverse transcription-PCR to obtain the full-length cDNA of HsNUF2 from a HeLa cell library, and we cloned it into the pGADT7 vector. To better define the domain responsible for the specific interaction between HsNUF2 and CENP-E, we performed a computational structure prediction analysis of HsNUF2 to search for protein/protein interaction modules (supplemental Fig. 1). Among various binding modules searched, we found that both proteins contain coiled-coil domains. The coiled coil of HsNUF2 is located between aa 245 and 340, whereas the extended coiled coil of CENP-E is located between aa 581 and 2415. Because coiled coils mediate protein/protein interactions, we sought to test whether coiled coils mediate the association between CENP-E and HsNUF2-(1–341) (D1). To this end, a series of deletion mutants were generated as illustrated in Fig. 1A. The HsNUF2 deletion mutant plasmids were each cotransformed with the cDNA of the CENP-E C terminus into yeast cells. We used β-galactosidase activity (lacZ reporter) as a measure for protein/protein interaction. As shown in Fig. 1B, yeast genetic assay indicated that a region adjacent to the coiled coils of HsNUF2 bound to the CENP-E C-terminal tail. Thus, our yeast two-hybrid assay clearly demonstrated that the C terminus of CENP-E (aa 2167–2701) interacts directly with HsNUF2.

FIGURE 1. Identification and characterization of the HsNUF2/CENP-E interaction. A, the schematic drawing shows HsNUF2 and its fragments that encode various bait clones used for yeast two-hybrid assay for CENP-E-binding activity. B, yeast cells were cotransformed with a CENP-E bait construct (CENP-E-(2167–2701)) and the indicated prey constructs. An example of such an experiment in which cells were selected on supplemented minimal plates lacking uracil, tryptophan, leucine, and histidine is presented. C, the schematic drawing shows GFP-tagged HsNUF2 and a series of deletion mutants used for the in vitro pulldown assays. D, an in vitro pulldown assay was used to map the domain of HsNUF2 binding to CENP-E. Bacterial recombinant GST-CENP-E-(2167–2701) (GST-534) bound to glutathione beads was used as an affinity matrix to absorb GFP-HsNUF2 proteins from 293T cells. Note that only GFP-tagged full-length HsNUF2 and GFP-D5 could be pulled down by the GST-tagged CENP-E C-terminal tail (GST-CENP-E-(2167–2701)). First lane, molecular mass markers; second through seventh lanes, cell lysates of GFP-HsNUF2 and GFP-D1-D5; eight through thirteenth lanes, proteins retained on GST-CENP-E-(2167–2701) affinity beads. E, shown is reconstitution of the HsNUF2/CENP-E interaction using recombinant fusion proteins. Aliquots of GST-tagged recombinant HsNUF2 proteins (full-length (lane 2), D5 (lane 4), and GST (lane 6)) purified on glutathione-agarose beads were used as affinity matrices to absorb purified MBP-tagged CENP-E protein purified from bacteria (lane 2). Aliquots of GST fusion proteins and MBP-CENP-E were used as loading controls (lanes 1, 3, 5, and 7). CB, Coomassie Blue.
Mapping the CENP-E-binding Interface in HsNUF2—To define the precise binding region of HsNUF2, we generated additional deletion constructs of HsNUF2 based on the bioinformatics analysis. Deletion mutant cDNAs of HsNUF2 were cotransformed with CENP-E C-terminal tail cDNA into yeast cells. As expected, the region containing aa 341–424 of HsNUF2 bound to the CENP-E C-terminal tail (AD-D3) (Fig. 1, A and B), but its interaction efficiency seemed to be equal to that of aa 212–424 (AD-D4) (Fig. 1, A and B), which suggests that the coiled coil of HsNUF2 (aa 246–340) is not required for CENP-E binding (Fig. 1B).

To validate the interaction between CENP-E and HsNUF2 observed in our yeast two-hybrid assay, we carried out a pull-down assay using bacterial recombinant GST-CENP-E protein as an affinity matrix to score the biochemical binding activity of the HsNUF2 protein and its deletion mutants. To this end, GFP-tagged HsNUF2 and its deletion mutant proteins were expressed in 293T cells to obtain soluble HsNUF2 proteins as illustrated in Fig. 1C. Aliquots of mitotic 293T cell lysates containing GFP-HsNUF2 and its deletion mutants were incubated with GST-CENP-E-(2167–2701). As shown Fig. 1D, only full-length HsNUF2 and D5 (aa 281–464) could bind to CENP-E based on their retention on the GST-CENP-E affinity matrix as judged by Western blot analysis of the GFP tag. Deletion of the region containing aa 341–424 (D4) (Fig. 1, C and D) abolished the binding of HsNUF2 to GST-CENP-E. Thus, our biochemical study confirmed that the HsNUF2 C terminus (aa 281–464) binds to CENP-E and that aa 341–424 of HsNUF2 are essential for such binding activity.

To examine whether the recombinant HsNUF2 protein binds to the CENP-E protein directly, we carried out a pull-down assay in which GST-tagged recombinant HsNUF2 was purified on glutathione-agarose beads and used as an affinity matrix to absorb purified MBP-CENP-E1 in test tubes. As shown in Fig. 1E (lanes 2 and 4), GST-tagged HsNUF2 and D5 pulled down MBP-CENP-E, indicating a physical interaction between CENP-E and HsNUF2 in vitro. MBP-CENP-E was never pulled down by GST-glutathione beads (Fig. 1E, lane 6), indicating a specific physical interaction between HsNUF2 and CENP-E. Thus, we conclude that HsNUF2 directly interacts with CENP-E via its C-terminal tail.

HsNUF2 Forms a Cognate Complex with the Mitotic Kinesin CENP-E—To confirm the interaction between CENP-E and HsNUF2 observed in our pull-down assay and test whether CENP-E forms a complex with HsNUF2, we used anti-CENP-E antibody to immunoprecipitate soluble CENP-E and potential partner proteins from lysates of mitotically arrested HeLa cells. Immunoblotting with anti-CENP-E antibody confirmed a successful precipitation of CENP-E, and immunoblotting against HsNUF2 demonstrated that HsNUF2 was coprecipitated (Fig. 2A, lane 8). Immunoprecipitation of HsNUF2 using a mouse antibody confirmed the presence of a complex of HsNUF2 and CENP-E, although the proportion of HsNUF2 relative to CENP-E in the precipitate (Fig. 2A, lane 4) was 2–3-fold that seen with anti-CENP-E antibody (lane 8), suggesting a greater accumulation of HsNUF2. Neither CENP-E nor HsNUF2 was precipitated with control IgG (Fig. 2B, lanes 3 and 7), and no tubulin was detected in any of the immunoprecipitates. Thus, we conclude that HsNUF2 and CENP-E can form a complex in vitro.

If endogenous HsNUF2 forms a complex with CENP-E, exogenously expressed HsNUF2 should also interact with CENP-E in vivo. To test this rationale and to ascertain that the HsNUF2 domain (D5) responsible for CENP-E binding in vitro also mediates HsNUF2/CENP-E interaction in vivo, we used rabbit anti-GFP antibody to immunoprecipitate wild-type GFP-HsNUF2 proteins and potential partner proteins from lysates of mitotically arrested HeLa cells transiently transfected to express various GFP-HsNUF2 proteins were incubated with anti-GFP antibody (lanes 2, 4, and 6), and immunoprecipitates were resolved by SDS-PAGE. Western blotting verified immunoprecipitation (Immunoprep.) of GFP-HsNUF2 (lower panel) and co-purification of CENP-E with full-length HsNUF2 and D5 (upper panel). No CENP-E was detected in GFP-D4 immunoprecipitates. Lanes 1, 3, and 5 are starting materials.

FIGURE 2. CENP-E forms a complex with HsNUF2 in vivo. A, co-immunoprecipitation of CENP-E and HsNUF2 from mitotic HeLa cells. Extracts from nocodazole-synchronized mitotic HeLa cells were incubated with antibodies against HsNUF2 (lanes 1 and 4) and CENP-E (lanes 5 and 8), and immunoprecipitates (IP) were resolved by SDS-PAGE. Lanes 3 and 7, immunoprecipitation with nonspecific IgG antibody from the same mitotic extracts. Western blotting verified co-immunoprecipitation of HsNUF2 (middle panel) and CENP-E (upper panel). No tubulin was detected in either HsNUF2 or CENP-E immunoprecipitates (lower panel). Lanes 2 and 6 are non-binding materials. B, co-immunoprecipitation of exogenously expressed GFP-D5 with CENP-E from mitotic HeLa cells. Extracts from mitotic HeLa cells transiently transfected to express various GFP-HsNUF2 proteins were incubated with anti-GFP antibody (lanes 2, 4, and 6), and immunoprecipitates were resolved by SDS-PAGE. Western blotting verified immunoprecipitation (Immunoprep.) of GFP-HsNUF2 (lower panel) and co-purification of CENP-E with full-length HsNUF2 and D5 (upper panel). No CENP-E was detected in GFP-D4 immunoprecipitates. Lanes 1, 3, and 5 are starting materials.

HsNUF2 and HEC1 Affect CENP-E Localization to the Kinetochore to Different Extents—HsNUF2 is an important component of the HsNDC80 centromere core complex (e.g. Ref. 14).
CENP-E Interacts with HsNUF2

To investigate the possible influence of HsNUF2 on the localization of CENP-E to the kinetochore and on the mechanism of mitotic checkpoint signaling, we introduced siRNA oligonucleotide duplexes to HsNUF2 and HEC1 by transfection into HeLa cells. Trial experiments revealed that treatment of HeLa cells with 100 nM siRNA for 48 h produced optimal suppression of the target proteins. As shown in Fig. 3A, Western blotting with anti-HsNUF2 antibody revealed that the siRNA oligonucleotide caused remarkable suppression of HsNUF2 protein levels at 48 h. This suppression was relatively specific, as it did not alter the levels of other proteins such as tubulin. A similar suppression of HEC1 and CENP-E was also achieved using a similar approach as judged by Western blot analyses (Fig. 3A).

Because HsNUF2 and HEC1 form a stable and evolutionarily conserved complex, we next examined the effect of repressing HEC1 and HsNUF2 on the localization of CENP-E to the kinetochore. HeLa cells were subcultured on coverslips in 24-well plates and transfected with siRNA. Cells were synchronized with thymidine to increase the number of prometaphase cells. Because addition of nocodazole enhances the signal of some kinetochore proteins due to the depolymerization of microtubules (14, 24), we used thymidine to synchronize HeLa cells to avoid the possible artifacts in kinetochore protein detection. After releasing cells from thymidine for 9–10 h, we collected the coverslips and carried out an immunofluorescence study.

In control cultures, HsNUF2 and HEC1 localized with CENP-E at the prometaphase kinetochores (Fig. 3B, Control panels). In cells in which HEC1 had been suppressed, the levels of kinetochore-bound HsNUF2 appeared reduced (Fig. 3B, Hec1 siRNA panels). Quantitation of normalized pixel intensities showed that, when HEC1 was reduced to <10% of its control value, HsNUF2 levels were reduced to ~37%, indicating that HEC1 is required for efficient kinetochore localization of HsNUF2, consistent with a previous study (27). However, in cells in which HEC1 had been repressed, the levels of CENP-E detectable at kinetochores appeared largely unaffected (Fig. 3B, Hec1 siRNA CENP-E panels). Quantitation of normalized pixel intensities showed that, when HEC1 was reduced to <10% of its control value, CENP-E levels were slightly reduced to ~62%.

Next, we examined the effect of repressing HsNUF2. In cells in which HsNUF2 had been suppressed, the levels of kinetochore-bound HsNUF2 appeared reduced (Fig. 3B, Nuf2 siRNA panels). Quantitation of normalized pixel intensities showed that, when HsNUF2 was reduced to <10% of its control value, HEC1 levels were reduced to ~13%, whereas CENP-E levels were reduced to ~21%, indicating that HsNUF2 is required for efficient kinetochore localization of CENP-E and HEC1.

We then examined the effect of repressing CENP-E on the kinetochore localization of HsNUF2 and HEC1. In control cultures, HsNUF2 and HEC1 localized with CENP-E at the prometaphase kinetochores (Fig. 3B, Control panels). In cells in which CENP-E had been suppressed, the levels of kinetochore-bound HEC1 and CENP-E appeared largely unaffected (Fig. 3B, CENP-E siRNA panels). Quantitation of normalized pixel intensities showed that, when CENP-E was reduced to <10% of its control value, HEC1 levels were ~95% of the control levels, whereas the levels of kinetochore-bound HsNUF2 were slightly reduced to ~81% (Fig. 3C), indicating that CENP-E is not required for efficient kinetochore localization of HsNUF2 and HEC1, consistent with a previous study (27). Thus, we conclude that both HEC1 and HsNUF2 are required for stable association of CENP-E with the kinetochore and that HsNUF2 contributes more than HEC1 to such an association.

HsNUF2 Specifies the Kinetochore Localization of CENP-E—HsNUF2 forms a cognate complex with HEC1, HsSPC24, and HsSPC25 (28, 29). If HsNUF2 is the determinant for CENP-E localization to the kinetochore, expression of GFP-D5, which contains CENP-E-binding activity, in the absence of endogenous HsNUF2 should retain the association of CENP-E with the kinetochore. In this regard, D5 serves as a “rescue,” as it does not contain the HsNUF2 siRNA target sequence. To this end, HeLa cells were transfected with HsNUF2 siRNA, followed by a second transfection of GFP-D5 after 8 h. After the double transfection, thymidine was used to synchronize cells in prometaphase as described above. As shown in Fig. 3D (Nuf2 siRNA panel), most of the CENP-E molecules had been removed from the kinetochore in HsNUF2 siRNA-transfected cells. Exogenously expressed GFP-D5 codistributed with CENP-E at the kinetochore (Fig. 3D, middle GFP-Nuf2-D5 panel), indicating that the HsNUF2 C terminus is sufficient for the kinetochore localization of HsNUF2. Significantly, expression of GFP-D5 retained the kinetochore localization of CENP-E in the cells depleted of endogenous HsNUF2 (Fig. 3D, Nuf2 siRNA + D5 panel). As shown in Fig. 3E, quantitation of normalized pixel intensities showed that expression of GFP-D5 in the HsNUF2-repressed cells increased the kinetochore-bound CENP-E level from ~21 to ~83% of its control value, indicating that HsNUF2/CENP-E interaction determines the kinetochore localization of CENP-E.

CENP-E Collaborates with HsNUF2 to Form a Stable Microtubule-Kinetochore Attachment—Previous studies have established that CENP-E and the HsNUF2-HEC1 complex are essential for stabilizing microtubule-kinetochore attachments (5, 16, 27). Distance between the sister kinetochores marked by ACA has been used as an accurate reporter for judging the tension developed across the kinetochore pair (e.g. Ref. 5). In this case, shortened distance often reflects aberrant microtubule attachment to the kinetochore, in which less tension is developed across the sister kinetochore. To test the functional activity of microtubule capturing in the cells depleted of HsNUF2, HEC1, CENP-E, or both HsNUF2 and HEC1, we measured this distance in 200 kinetochore pairs in which both kinetochores were in the same focal plane in both siRNA-treated cells and control cells (Fig. 4B).

As shown in Fig. 4A, depletion of HEC1, HsNUF2, or CENP-E resulted in errors in chromosome alignment at the equator, which is consistent with previous reports (e.g. Refs. 5, 14, and 18). Control kinetochores exhibited a separation of 1.73 ± 0.14 μm, whereas the distances between kinetochores were 1.27 ± 0.11 μm in HEC1-depleted cells, 1.16 ± 0.12 μm in HsNUF2-depleted cells, and 1.31 ± 0.13 μm in CENP-E-depleted cells. Although double depletion of HEC1 and HsNUF2 did not induce any further shortening of the inter-kinetochore distance (1.18 ± 0.15 μm), simultaneous depletion of HsNUF2 and CENP-E did result in dramatic shortening of the inter-
FIGURE 3. HsNUF2 and HEC1 determine the kinetochore localization of CENP-E to different extents. A, efficiency of siRNA treatments in HeLa cells. Aliquots of HeLa cells were transfected with 100 nm siRNA oligonucleotide duplexes for CENP-E, HsNUF2, and HEC1 and their controls (scrambled oligonucleotide) for 48 h and subjected to SDS-PAGE and immunoblotting. Upper panels, immunoblots against targeted proteins; lower panels, immunoblots against tubulin. B, localization of CENP-E to the kinetochore depends on HsNUF2 and HEC1. Aliquots of HeLa cells were transfected with oligonucleotides (control and siRNA for CENP-E, HEC1, and HsNUF2) for 48 h, followed by fixation and immunocytochemical staining as described under “Materials and Methods.” Optical images were collected from HeLa cells transfected with control siRNA (Control panels), HEC1 siRNA (Hec1 siRNA panels), HsNUF2 siRNA (Nuf2 siRNA panels), and CENP-E siRNA (CENP-E siRNA panels). Scale bars = 10 μm. C, quantitation of HsNUF2, CENP-E, and HEC1 levels at kinetochores of control and siRNA-treated cells. The pixel intensities of HsNUF2, CENP-E, and HEC1 (normalized to the ACA signal) in control (closed bars) and HsNUF2-repressed and HEC1-repressed (open bars) cells were measured. Values represent the means ± S.E. of at least 100 kinetochores in 10 different cells. D, exogenous expression of D5 restores the kinetochore localization of CENP-E in endogenous HsNUF2-repressed cells. Aliquots of HeLa cells were transfected with HsNUF2 siRNA oligonucleotides and GFP-D5 for 48 h, followed by fixation and immunocytochemical staining as described under “Materials and Methods.” Optical images were collected from HeLa cells transfected with HsNUF2 siRNA (Nuf2 siRNA panels), control siRNA plus GFP-D5 (GFP-D5 panels), and HsNUF2 siRNA plus GFP-D5 siRNA (Nuf2 siRNA + D5 panels). Scale bars = 10 μm. E, quantitation of CENP-E levels at kinetochores of control, HsNUF2 siRNA-treated, and HsNUF2 siRNA-treated and GFP-D5-expressing cells. The pixel intensities of HsNUF2, CENP-E, and HEC1 (normalized to the ACA signal) in control (black bar) and HsNUF2-repressed and HEC1-repressed (open bars) cells were measured. Values represent the means ± S.E. of at least 100 kinetochores in 10 different cells.
kinetochore distance (0.86 ± 0.13 μm), suggesting that double repression of CENP-E and HsNUF2 eliminates the tension across the sister kinetochore. To validate this hypothesis, we measured the inter-kinetochore distances in nocodazole-treated cells. The distance between sister kinetochores in nocodazole-treated cells, in which kinetochore pairs were presumably under no tension, was 0.85 ± 0.10 μm, whereas this distance in double CENP-E- and HsNUF2-repressed cells was 0.86 ± 0.13 μm (Fig. 4B), indicating that both CENP-E and HsNUF2 are required for stable microtubule-kinetochore association. Thus, we concluded that CENP-E cooperates with HsNUF2 in stabilizing microtubule-kinetochore association.

DISCUSSION

The HsNDC80 complex is composed of four proteins: HEC1, HsNUF2, HsSPC24, and HsSPC25. HsNDC80 is an essential kinetochore core component highly conserved across species, with a crucial role in proper chromosome congression and segregation during mitosis (14, 17, 18, 27–29). The HsNDC80 complex is constructed of two discrete HsNUF2-HEC1 and HsSPC24-HsSPC25 subcomplexes (30, 31), each stabilized by a parallel heterodimeric coiled coil. In budding yeast and human, these two subcomplexes are joined through their coiled coils to form a rod-like heterotetrameric structure with a stoichiometry of 1:1:1:1 by an interaction of the C-terminal regions of HsNUF2-HsNDC80/HEC1 and the N-terminal regions of HsSPC24-HsSPC25 (30, 31). The combination of computational analysis of functional modules in HsNUF2 with our biochemical assay suggests that the HsNUF2/CENP-E interaction may not involve the coiled-coil structure of HsNUF2.

Previous studies have established that the localization of HEC1 and HsNUF2 is exterior to the inner kinetochore proteins (17, 18, 27) and that HEC1 localizes to the interior of CENP-E (This study and Ref. 27). Moreover, the HsNDC80 complex is indispensable for establishing microtubule-kinetochore attachments. Thus, the HsNDC80 complex is postulated to link microtubule-binding proteins and chromatin-bound centromere core proteins (30, 32). It has been reported that the main function of the HsNUF2-HEC1 complex is to stabilize the microtubule-kinetochore association, as cells lacking the HsNUF2-HEC1 complex often carry unstable microtubules (27). Consistent with this notion, recent studies have shown that the HsNDC80 complex binds microtubules weakly but that this binding is dramatically enhanced when the HsNDC80 complex is incubated with KNL proteins (33, 34). Our finding of an HsNUF2/CENP-E interaction provides a novel link between spindle microtubules and the kinetochore core complex via the mitotic kinesin CENP-E and confirms that the HsNDC80 complex may establish a bridge between microtubule-binding proteins and chromatin-bound proteins in addition to a cooperative action with CENP-E in microtubule association. Given our finding of an HsNUF2/CENP-E interaction established here, we reasoned that CENP-E links the kinetochore core component HsNUF2-HEC1 complex with the spindle microtubule.
CENP-E Interacts with HsNUF2

![Diagram A: Control cells](image)

![Diagram B: Nuf2 D5 overexpressing cells](image)

![Diagram C: Checkpoint signaling pathway](image)

**FIGURE 5. Working model accounting for the functional interaction between HsNUF2 and CENP-E in mitosis.** A, schematic illustration of the normal HsNUF2/CENP-E interaction in spindle microtubule (MT)-kinetochore attachment. B, schematic illustration of D5/CENP-E interaction in spindle microtubule-kinetochore attachment in which the inter-kinetochore tension is weakened because of lack of the HsNUF2 N terminal globular domain and likely alteration in the association of the HsNDC80 complex with spindle microtubules. C, both the HsNUF2-HEC1 complex and CENP-E localize at the outer plate of the kinetochore, but HsNUF2-HEC1 is closer to the inner plate and determines the kinetochore localization of CENP-E. The interactions between the HsNUF2-HEC1 complex and the CENP-E C terminus form a microtubule-kinetochore (KT) interface that is stabilized by BUBR1 and other CENP-E accessory proteins. The motor domain of CENP-E binds to the microtubule and provides additional affinity to stabilize the microtubule-kinetochore attachment. When all these proteins function well, there will be tension developed between sister kinetochores. CENP-E senses the tension and switches the spindle checkpoint on or off through a BUBR1-mediated biochemical cascade (13).

HsNUF2-depleted but GFP-D5-expressing cells indicated that the distance is $1.49 \pm 0.09 \mu m$, which is slightly reduced compared with that of control cells but greater than those of CENP-E, HEC1, or HsNUF2 siRNA-treated cells. This phenotype clearly indicates that microtubule-kinetochore attachment remains in GFP-D5-expressing cells in the absence of full-length HsNUF2. As GFP-D5 contains the binding module sequence required for establishment of the HsNDC80 complex, it is possible that D5 can still form a complex with other HsNDC80 components (31). However, it would be of great interest to evaluate whether D5 remains interacting with HEC1, HsSPC24, and HsSPC25 like full-length HsNUF2 and how D5 collaborates with CENP-E in microtubule-kinetochore attachment in real-time chromosome movements in mitosis.

Molecular analyses of HsNDC80 architecture indicated that the rod-like heterotetrameric HsNDC80 complex has two possible orientations (26, 30, 31). One interesting but unresolved question is how HsNDC80 components are oriented toward the centromere and toward the microtubule. A previous study demonstrated that CENP-H interacts with the HsNUF2-HEC1 complex (26), and we have also determined that CENP-H binds to the HsSPC24-HsSPC25 complex. These results suggest that CENP-H may play a role in anchoring the HsNDC80 complex to the kinetochore during mitosis. In our study, CENP-E is a kinetochore outer plate protein, and its N terminus can extend along the microtubule at least 50 nm (22). Thus, it can maintain the connection of the kinetochore and microtubule. The HsNUF2/CENP-E interaction established here indicates that the HsNUF2-HEC1 complex should be oriented toward the microtubule and stabilize the kinetochore localization of CENP-E. Notably, this orientation is consistent with previous studies showing that the kinetochore localization of HsNDC80 and HsNUF2 depends on both HsSPC24 and HsSPC25 but that the reverse does not (29, 35).

CENP-E is also an important protein in chromosome congress (36) and spindle checkpoint control pathways (5, 12, 13). Its interaction with BUBR1 activates its kinase activity, which is important in activating the spindle checkpoint (12). The capture of microtubules by CENP-E silences BUBR1-dependent checkpoint signaling (13), but CENP-E alone is not enough. Many studies indicate that the HsNUF2-HEC1 complex is also necessary for proper segregation of sister chromatids. Repression of HsNUF2 or HEC1 leads to a strong prometaphase block (14–19, 27). Future studies will be directed toward precisely analyzing the real-time chromosome segregation defects in D5-expressing cells so that the functional specificity of the HsNDC80 complex in mitotic chromosome movements can be delineated.

As we know, the distance between a kinetochore pair marked by ACA reflects the tension between sister kinetochores as defined by the microtubule-capturing process. If kinetochores are not correctly attached to microtubules, the spindle checkpoint will be activated, and the cell cycle will be stopped at prometaphase. We propose that CENP-E is a link between the inter-kinetochore tension and spindle checkpoint machinery in
CENP-E Interacts with HsNUF2

mitotic cells. It is possible that the interaction of CENP-E with the microtubule and HsNUF2 orchestrates its conformational change, thereby activating or silencing BUBR1 kinase activity, turning the spindle checkpoint on or off (Fig. 5C). Loss of CENP-E, HEC1, HsNUF2, or two of them will lead to the breakdown of microtubule-kinetochore attachment and the tension-sensing system and therefore activate the spindle checkpoint through the CENP-E/BUBR1 signal transduction pathway.

We propose that the functional collaboration of CENP-E and HsNUF2 is necessary in stabilizing microtubule-kinetochore attachment, maintaining tension, and silencing the spindle checkpoint. It is likely that all of the kinetochore outer plate proteins interact to orchestrate a functional kinetochore during chromosome segregation. The HsNUF2/HEC1 interaction established here is a core of this huge complex, which links kinetochore structure components to spindle microtubule attachment in the centromere. The interaction of HEC1 with Zwint1 and CENP-H that has been reported recently (26, 37) also supports our working model.

Acknowledgments—We thank members of our groups for insightful discussion during the course of this study.

REFERENCES

1. Fukagawa, T. (2004) Exp. Cell Res. 296, 21–27
2. Amor, D. J., Kalitsis, P., Sumer, H., and Choo, K. H. (2004) Trends Cell Biol. 14, 359–368
3. Cleveland, D. W., Mao, Y., and Sullivan, K. F. (2003) Nat. Cell Biol. 112, 407–421
4. Maito, H., DeLuca, J., Salmon, E. D., and Earnshaw, W. C. (2004) J. Cell Sci. 117, 5461–5477
5. Yao, X., Abrieu, A., Zheng, Y., Sullivan, K. F., and Cleveland, D. W. (2000) Nat. Cell Biol. 2, 484–491
6. Yen, T., Compton, D. A., Wise, D., Zinkowski, R. P., Brinkley, B. R., Earnshaw, W. C., and Cleveland, D. W. (1991) EMBO J. 10, 1245–1254
7. Wood, K. W., Sakowicz, R., Goldstein, L. S. B., and Cleveland, D. W. (1997) Cell 91, 357–366
8. Schaar, B. T., Chan, G. K., Maddox, P., Salmon, E. D., and Yen, T. J. (1997) J. Cell Biol. 139, 1373–1382
9. Chan, G. K., Schaar, B. T., and Yen, T. J. (1998) J. Cell Biol. 143, 49–63
10. Rieder, C. L., Schultz, A., Cole, R., and Sluder, G. (1994) J. Cell Biol. 127, 1301–1310
11. Li, X., and Nicklas, R. B. (1995) Nature 373, 630–632
12. Mao, Y., Abrieu, A., and Cleveland, D. W. (2003) Cell 114, 87–98
13. Mao, Y., Desai, A., and Cleveland, D. W. (2005) J. Cell Biol. 170, 873–880
14. DeLuca, J. G., Moree, B., Hickey, J. M., Kilmartin, J. V., and Salmon, E. D. (2002) J. Cell Biol. 159, 549–555
15. Chen, Y., Riley, D. J., Chen, P. L., and Lee, W. H. (1997) Mol. Cell. Biol. 17, 6049–6056
16. He, X., Rines, D. R., Espelin, C. W., and Sorger, P. K. (2001) Cell 106, 195–206
17. Wigge, P. A., and Kilmartin, J. V. (2001) J. Cell Biol. 152, 349–360
18. Martin-Bluesma, S., Stucke, V. M., and Nigg, E. A. (2002) Science 297, 2267–2270
19. Hori, T., Haraguchi, T., Hiraoka, Y., Hiroshi, K., and Fukagawa, T. (2003) J. Cell Sci. 116, 3347–3362
20. Xue, Y., Liu, D., Fu, C., Dou, Z., Zhou, Q., and Yao, X. (2006) Chin. Sci. Bull. 51, 1836–1847
21. Lou, Y., Yao, J., Zereshki, A., Dou, Z., Ahmed, K., Wang, H., Hu, J., Wang, Y., and Yao, X. (2004) J. Biol. Chem. 279, 20049–20057
22. Yao, X., Anderson, K. L., and Cleveland, D. W. (1997) J. Cell Biol. 139, 435–447
23. Cao, X., Ding, X., Guo, X., Zhou, R., Forte, J. G., Teng, M., and Yao, X. (2005) J. Biol. Chem. 280, 13584–13592
24. McCleland, M. L., Kallio, M. J., Barrett-Wilt, G. A., Kestner, C. A., Shabanowitz, J., Hunt, D. F., Gorbsky, G. J., and Stukenberg, P. T. (2004) Curr. Biol. 14, 131–137
25. Johnson, V. L., Scott, M. I. F., Holt, S. V., Hussein, D., and Taylor, S. S. (2004) J. Cell Sci. 117, 1577–1589
26. Mikami, Y., Hori, T., Kimura, H., and Fukagawa, T. (2005) Mol. Cell. Biol. 25, 1958–1970
27. DeLuca, J. G., Dong, Y., Hergert, P., Strauss, J., Hickey, J. M., Salmon, E. D., and McEwen, B. F. (2005) Mol. Cell Biol. 16, 519–531
28. McCleland, M. L., Gardner, R. D., Kallio, M. J., Daum, J. R., Gorbsky, G. J., Burke, D. J., and Stukenberg, P. T. (2003) Genes Dev. 17, 101–114
29. Bhardwaj, R., Qi, W., and Yu, H. (2004) J. Biol. Chem. 279, 13076–13085
30. Wei, R. R., Sorger, P. K., and Harrison, S. C. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 5363–5367
31. Ciferri, C., DeLuca, J., Monzani, S., Ferrari, K. J., Ristic, D., Wyman, C., Stark, H., Kilmartin, J., Salmon, E. D., and Musacchio, A. (2005) J. Biol. Chem. 280, 29088–29095
32. De Wulf, P., McAinsh, A. D., and Sorger, P. K. (2003) Genes Dev. 17, 2902–2921
33. DeLuca, J., Gall, W. E., Ciferri, C., Cinmini, D., Musacchio, A., and Salmon, E. D. (2006) Cell 127, 969–982
34. Cheeseman, I. M., Chappie, J. S., Wilson-Kubalek, E. M., and Desai, A. (2006) Cell 127, 983–997
35. Gillett, E. S., Espelin, C. W., and Sorger, P. K. (2004) J. Cell Biol. 164, 535–546
36. Kapoor, T. M., Lampson, M., Hergert, P., Cameron, L., Cinmini, D., Salmon, E. D., McEwen, B. F., and Khodjakov, A. (2006) Science 311, 388–391
37. Lin, Y. T., Chen, Y., Wu, G., and Lee, W. H. (2006) Oncogene 25, 6901–6914