NEK2A Interacts with MAD1 and Possibly Functions as a Novel Integrator of the Spindle Checkpoint Signaling*

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Chromosome segregation in mitosis is orchestrated by protein kinase signaling cascades. A biochemical cascade named spindle checkpoint ensures the spatial and temporal order of chromosome segregation during mitosis. Here we report that spindle checkpoint protein MAD1 interacts with NEK2A, a human orthologue of the Aspergillus nidulans NIMA kinase. MAD1 interacts with NEK2A in vitro and in vivo via a leucine zipper-containing domain localized at the C terminus of MAD1. Like MAD1, NEK2A is localized to HeLa cell kinetochore of mitotic cells. Elimination of NEK2A by small interfering RNA does not arrest cells in mitosis but causes aberrant premature chromosome segregation. NEK2A is required for MAD2 but not MAD1, BUB1, and HEC1 to associate with kinetochores. These NEK2A-eliminated or -suppressed cells display a chromosome bridge phenotype with sister chromatid inter-connected. Moreover, loss of NEK2A impairs mitotic checkpoint signaling in response to spindle damage by nocodazole, which affected mitotic escape and led to generation of cells with multiple nuclei. Our data demonstrate that NEK2A is a kinetochore-associated protein kinase essential for faithful chromosome segregation. We hypothesize that NEK2A links MAD2 molecular dynamics to spindle checkpoint signaling.

Chromosome movements during mitosis are governed by the interaction of spindle microtubules with a specialized chromosome domain located within the centromere. This specialized region, called the kinetochore (1, 2), is the site for spindle microtubule-centromere association. In addition to providing a physical link between chromosomes and spindle microtubules, the kinetochore has an active function in chromosomal segregation through microtubule motors and spindle checkpoint sensors located at or near it (3–5). Several lines of evidence have implicated the kinetochore in generation of a diffusible checkpoint signal that can block cell cycle progression into anaphase until all kinetochores have successfully attached to spindle microtubules. Delayed attachment of one or more chromosomes to the spindle is correlated with a corresponding delay in the onset of anaphase. For mutants that fail to arrest the cell cycle in mitosis after disassembly of microtubules in budding yeast, genetic screen has identified three MAD (mitotic arrest deficiency) and three BUB (budding uninhibited by benomyl) genes (7). Vertebrate homologues of MAD1 (8), MAD2 (9–10), BUB3 (11–12), BUB1, and BUBR1 (13–15) are spindle checkpoint components transiently associated with kinetochores. Expression of the kinetochore binding domain of murine BUB1 (13) or injection of antibodies against BUBR1 (15) results in premature onset of anaphase, presumably by replacement of the endogenous proteins at kinetochores. Collectively, these data indicate that binding of these spindle checkpoint components at the kinetochores may generate a signal in response to spindle defects and/or aberrant kinetochore-protein-protein interactions.

Mitosis is orchestrated by signaling cascades that coordinate mitotic processes and ensure accurate chromosome segregation. The key switch for the onset of mitosis is the archetypal cyclin-dependent kinase Cdc2. Besides the master mitotic kinase Cdc2, there are three protein serine/threonine kinase families as follows: the Polo kinases, Aurora kinases, and the NEK (NIMA-related kinases) (16, 17). The latter family has proven the most enigmatic in function, although recent advances from several sources are beginning to reveal a common functional theme.

NIMA (never in mitosis A) is vital in Aspergillus nidulans for entry into mitosis (17, 18). Mutation of NIMA arrests cells in G2 without interfering with p34CDC28 activation, suggesting that the NIMA protein has a central role in the G2/M transition. Moreover, if the G2 arrest of nimA mutants is bypassed by additional mutations, the resulting mitotic cells show aberrant spindle and nuclear envelope organization (17–19), pointing to functions of NIMA beyond the control of mitotic entry. NEK2A, the homologue in human cells with the greatest structural similarity to NIMA within the catalytic domain, is regulated in yeast in a manner similar to regulation of NIMA in A. nidulans; its expression and serine/threonine kinase activity is highest during late G2 phase, when NEK2A is expected to function critically (20). Furthermore, a portion of NEK2A localizes to centromeres and in mammalian cells appears to play a role similar to NIMA in controlling entry into mitosis (16, 17). NEK2A may have more diverse roles during several phases of the cell cycle, from S phase to multiple phases of mitosis, based on its dynamic expression and subcellular localization in cytosol, nucleus, and chromosome portions other than the centromere (16, 17, 21). Although it has been proposed that NEK2A is associated with centromere (17), it was not clear whether NEK2A is indeed localized to the kinetochore and whether NEK2A functions during chromosome movements in mitosis.
The mitotic spindle checkpoint is a chromosome segregation surveillance mechanism that prevents chromosome from premature segregation in the presence of mis-aligned chromosomes. This surveillance system is highly sensitive as a single unaligned chromosome or perturbation of kinetochores protein interactions is sufficient to block the transit of cells from metaphase to anaphase (22). The initial description of the mitotic checkpoint in yeast defined a nonessential pathway invoked only in response to spindle damage. But in metazoans, the checkpoint has evolved into an essential feature of normal mitoses and meioses (23). Microinjection of antibodies to MAD2 into cultured animal cells first revealed premature anaphase onset and chromosome mis-segregation (24). In budding yeast, the spindle checkpoint depends on a tight complex between the MAD1 and MAD2 proteins (25). Although MAD1 is required for MAD2 localization to the kinetochores (25), it is unknown how MAD1 function is integrated into the mitotic regulation.

To explore the nature of MAD1 function in mitotic spindle checkpoint signaling, we carried out yeast genetic screening and identified the novel MAD1-interacting partner NEK2A. Our studies show that NEK2A interacts with MAD1 via the leucine zipper motif on the MAD1 molecule. In addition, we show that both NEK2A and MAD1 are co-localized to the kinetochores of mitotic cells. Given the involvement of MAD1 in spindle checkpoint surveillance and novel function of NEK2A revealed in this study, we proposed that NEK2A links mitotic checkpoint signaling to chromosome segregation dynamics.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Assay**—Full-length human MAD1 cDNA, a gift from Dr. Dong-Yan Jin (University of Hong Kong), was employed to screen a HeLa cDNA library made in fusion with the GAL4 activation domain in the vector pGAD-T7 (Clontech, Palo Alto, CA) by using yeast strain AH109 containing the reporter genes his3 and lacZ. About 1 million co-transformants were screened for the two reporter genes on the plates containing media with amino acid selection.

To map the region(s) of MAD1, which interact with NEK2A, four deletion mutants of MAD1 were selected based on the secondary structure prediction (cubic.bioc.columbia.edu/predictprotein/). MAD1 bait deletion constructs were PCR-amplified and obtained by digestion of the bait with BamHI and EcoRI followed by gel purification of respective bands and sub-cloning into pGAD-T7 vector. In addition, six deletion mutants of NEK2A were selected to map the interface(s) between NEK2A and MAD1. NEK2A deletion constructs were generated by digesting the prey with BamHI and XhoI followed by the gel purification of respective bands and then sub-cloning into pGADT7 vector.

**cDNA Construction**—Total RNAs, isolated from HeLa cells by using standard protocol, were treated with RNase-free DNase I (Takara Bio-technology, Dalian, China) for 15 min at room temperature before addition of 25 mM EDTA and were heated to 65 °C for 10 min to stop the reaction. DNase I-treated RNAs were reverse-transcribed at 42°C for 200 min using oligo(dT) and Moloney murine leukemia virus-reverse transcriptase RNase H (Promega, Madison, WI). By using this cDNA as template, PCR was performed with related primer. The resulting fragment was cloned into TA cloning vector (Takara Biotechnology, Dalian, China) and completely sequenced.

For mammalian expression of the full-length NEK2A, NEK2A cDNA was digested with EcoRI and BamHI and cloned into pEGFP C1 vector (Clontech, Palo Alto, CA), whereas the full length of MAD1 cDNA was digested with EcoRI and BamHI and cloned into pEGFP C2 vector (Clontech, Palo Alto, CA).

**Recombinant Protein Production**—NEK2A, MAD1, and their fragments were expressed in bacteria as fusion proteins. Briefly, the full length of MAD1 cDNA was cloned into pMal C2 vector (New England Biolabs, Beverly, MA), whereas a BamHI/EcoRI fragment (amino acids 480–680) was cloned into pGEX-2T vector (Amersham Biosciences). The partial cDNA sequence of NEK2A (amino acids 305–448) was obtained from yeast screen. After digestion with NotI and Sall, it was fused in-frame with His6 at the C terminus (pET22b; Novagen) andGST at the N terminus (pGEX-5X-3), respectively.

Purification of recombinant proteins was carried out as described previously (26). Briefly, 1 liter of LB media was inoculated with bacteria transformed either with NEK2A or MAD1. The expression of protein was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. Bacteria were harvested by centrifugation 3 h after the induction, re-suspended in phosphate-buffered saline (PBS) containing proteinase inhibitors (leupeptin, pepstatin, and chymostatin; 5 μg/ml), and sonicated for four bursts of 10 s each by using a probe-tip sonicator. The lysis solution was clarified by centrifugation for 20 min at 10,000 × g. The soluble fraction was applied to a column packed with glutathione-agarose beads, followed by extensive washes with PBS.

**Affinity Precipitation of MAD1 and NEK2A**—His-tagged NEK2A-fragments was used as a source of NEK2A protein. The purified soluble GST-fused MAD1-(380–680) protein was pre-bound to glutathione-agarose (Sigma). GST-MAD1-(380–680) bound beads were washed with 5× column volume using wash buffer (10 mM phosphate buffer, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.01% phenylmethylsulfonfyl fluoride) and equilibrated with 10× column volume of incubation buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Triton X-100, 0.01% phenylmethylsulfonfyl fluoride). The purified His-fused NEK2A-(305–448), re-suspended in incubation buffer, was loaded on the column and incubated for 1 h, and the flow-through fraction was collected. The column was washed with 10× volume of incubation buffer, and wash fraction was collected. Finally, the column was eluted with elution buffer (50 mM Tris-HCl, pH 7.5, 1.5 mM NaCl, 0.1% Triton X-100, 0.01% phenylmethylsulfonfyl fluoride) and elution fraction was collected. Non-bound glutathione-agarose beads and GST protein-bound glutathione-agarose beads were used as negative control. All collected fractions were resolved on SDS-PAGE and were analyzed by immunoblot analysis using GST mouse antibody (NeoMarkers).

**Immunofluorescence Microscopy**—For immunofluorescence, cells were grown to ~50% confluency in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 37 °C in 10% CO2 and were co-transfected with GFP-NEK2A and FLAG-MAD1 by GeneJammer (Stratagene, La Jolla, CA), according to the manufacturer’s protocol. Cells were collected 24–36 h after transfection and proteins were solubilized in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.1% EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonfyl fluoride, 10 μM leupeptin, and 10 μg/ml peptide A). Lysates were clarified by centrifugation at 16,000 × g for 10 min at 4 °C. GFP-tagged fusion proteins were incubated with anti-GFP monoclonal antibody bound to protein-A/G beads (Pierce). Beads were washed five times with lysis buffer and then boiled in protein sample buffer for 2 min. After SDS-PAGE, proteins were transferred to nitrocellulose membrane. The membrane was divided into three strips and probed with antibodies against the GFP epitope, MAD1, and tubulin, respectively. Immunoreactive signals were detected with ECL kit (Pierce) and visualized by autoradiography on Kodak BioMax film.

**siRNA Treatment and Assay for Knock-down Efficiency**—The siRNA sequence used for silencing of NEK2A corresponds to the coding region 260–280 (relative to the start codon). As a control, either a duplex targeting cyclophilin or scramble sequence was used (5). The 21-mer oligonucleotide RNA duplexes were synthesized by Dharmaco Research, Inc. (Boulder, CO). In the trial experiments, different concentrations of siRNA oligonucleotides were used for different time intervals of treatment as detailed previously (5). In brief, HeLa cells were synchronized and transfected with 21-mer siRNA oligonucleotides or control scramble oligonucleotide, whereas the efficiency of the siRNA oligonucleotide on NEK2A protein knock-down was judged by Western blotting and FACS analysis (5).

**Immunofluorescence Microscopy**—For immunofluorescence, cells were seeded onto sterile, acid-treated 18-mm coverslips in 6-well plates (Corning Glass). Double thymidine-blocked and -released HeLa cells were transfected with 2 μg/ml LipofectAMINE 2000 pre-mixed with various siRNA oligonucleotides as described above. In general, 36 h after transfection with siRNA, cells were scrambled (control). HeLa cells were rinsed for 1 min with PHEM buffer (100 mM PIPES, 20 mM HEPES, pH 6.9, 5 mM EGTA, 2 mM MgCl2, and 4 mM glycerol) and were permeabilized for 2 min with PHEM plus 0.2% Triton X-100 as described previously (5, 27). Extracted cells were then fixed in freshly prepared 4% paraformaldehyde in PHEM (pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.1% EDTA). The fixed cells were pre-incubated for 1 h with 5% normal horse serum followed by incubation in primary antibody at 4 °C overnight and then incubation in secondary antibody conjugated to Alexa-Fluor 488 or Alexa-Fluor 555 for 1 h at room temperature. After washing, coverslips were mounted in 1:1 mixture of PBS/glycerol (pH 8.0) and observed under a confocal laser scanning microscope (Zeiss 108, Thornwood, NY).

The abbreviations used are: GST, glutathione S-transferase; GFP, green fluorescent protein; RNA, RNA interference; PBS, phosphate-buffered saline; siRNA, small interfering RNA; PIPES, 1,4-piperazinediethanesulfonic acid; BSA, bovine serum albumin; DAPI, 4,6-diamidino-2-phenylindole; ACA, anti-centromere antibody.
prepared 4% paraformaldehyde plus 0.05% glutaraldehyde in PHEM and rinsed three times in PBS. Cells on the coverslips were blocked with 0.05% Tween 20 in PBS (TPBS) with 1% BSA (Sigma). These cells were incubated with various primary antibodies in a humidified chamber for 1 h and then washed three times in TPBS. Monoclonal antibodies bound to MAD1, HEC1, and NEK2A were visualized using fluorescein-conjugated goat anti-mouse IgG (H+L), respectively, whereas binding of anti-centromere antibody was visualized using Texas Red-conjugated goat anti-human IgG + IgM. DNA was stained with DAPI (Sigma), and MAD2 was labeled with an affinity-purified rabbit antibody as described previously (5). Slides were examined with a Zeiss Axiosvert-200 fluorescence microscope, and images were collected and analyzed with Image-5 (Carl Zeiss, Germany).

In some cases, aliquots of oligonucleotide-treated HeLa cells were exposed to 100 ng/ml nocodazole for a period of 18 h to determine whether elimination of NEK2A abrogates the mitotic spindle checkpoint. These cells were then fixed and stained with DAPI for scoring the cell fate profiling under microscope.

**For Western Assay**—The purified soluble GST-fused MAD1-(305–680) was resolved on 10% SDS-PAGE and transferred onto nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with 2% BSA in PBS containing Tween 20 and incubated with ~10 μg of His-tagged NEK2A-(305–446) as a probe and BSA as a control. After a 2-h incubation, the membrane was washed four times with PBS and incubated with histidine antibody (Cell Signaling Co.) for 1 h. Horseradish peroxidase-conjugated secondary antibody was used to detect primary antibody, and the blot was developed using an ECL kit (Amersham Biosciences).

**Cell Culture and Transfection**—HeLa and 293T cells, from American Type Culture Collection (Manassas, VA), were maintained as subconfluent monolayers in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal calf serum (HyClone, UT) and 100 units/ml penicillin plus 100 μg/ml streptomycin (Invitrogen). In some cases, thymidine-blocked and -released HeLa cells were transfected with 2 μg/ml LipofectAMINE 2000 pre-mixed with 2 μg/ml cDNA plasmid encoding GFP and GFP-NEK2A-KD (kinase-death mutant K37R), respectively, as described previously (28). In general, 36 h after transfection, HeLa cells were pro- or treated with PHEM plus 0.2% Triton X-100 followed by fixation and immunocytochemistry as described above.

**Western Blot**—Samples were subjected to SDS-PAGE on 6–16% gradient gel and transferred onto nitrocellulose membrane. Proteins were probed by appropriate primary and secondary antibodies and detected using ECL (Pierce). The band intensity was then scanned using a PhosphorImager (Amersham Biosciences).

**RESULTS**

**NEK2A Is a Novel Interactor for MAD1**—To identify proteins that associate with MAD1, the full length of MAD1 cDNA was used as bait to screen a HeLa cDNA library by using GAL4 yeast two-hybrid system. Screening was performed on a total 1 × 10^9 colonies with 31 positive clones. Following analysis by restriction digests of HaeIII and AluI, we got several novel interacting partners of MAD1. Nucleotide sequencing revealed that one of these interactors encodes the C-terminus of NEK2A (amino acids from 305 to 446). Its sequence matches human NEK2A (GenBank™ accession number XM001878).

To verify the specificity of interaction between NEK2A and MAD1 and to define further the domain(s) that mediates the interaction between MAD1 and NEK2A, we took advantage of yeast genetics and sought to perform additional yeast two-hybrid screens to map the binding interface(s) between NEK2A and MAD1. To this end, we used reverse transcriptase-PCR to obtain the full-length cDNA of NEK2A from a HeLa cell line, and we cloned it into pGAD T7 vector.

**To define better the domain responsible for the specific interaction between MAD1 and NEK2A, we employed a two-dimensional structure prediction analysis to search for protein-protein interaction modules in the two proteins. We found that both proteins contained a leucine zipper motif. The leucine zipper of MAD1 is located between amino acids 500 and 522, and the leucine zipper of NEK2A is located between amino acids 306 and 328. Since leucine zipper mediates protein-protein interaction, we sought to test if leucine zippers mediate the association between MAD1 and NEK2A-(305–446) (NEK2AD3). To this end, a series of deletion mutants were generated as illustrated in Fig. 1A. The NEK2A deletion mutant plasmids were co-transformed with full-length MAD1 cDNA into yeast cells, respectively. We used β-galactosidase activity (LacZ reporter) as a measure for protein-protein interaction. As shown in Fig. 1B, yeast genetic assay indicates full-length MAD1 binds to NEK2A C-terminus (amino acids 329–446), a region adjacent to the leucine zipper. However, full-length NEK2A does not interact with MAD1, suggesting that a conformational change of NEK2A, which exposed its C-terminus, may be required for exposing its MAD1 binding domain.

To map the NEK2A binding interface on MAD1, deletion mutant cDNAs of MAD1 were co-transformed with NEK2AD3 into yeast cells. As shown in Fig. 1, C and D, NEK2A binds to...
the MAD1 central region (amino acids 380–532), which contains a leucine zipper motif. Thus, our yeast genetic assay indicates that the leucine zipper of MAD1 directly binds to the C-terminal 117 amino acids of NEK2A.

**NEK2A Forms a Complex with the Mitotic Checkpoint Component MAD1**—To validate the interaction between MAD1 and NEK2A, we observed in our yeast two-hybrid assay and test if NEK2A forms a complex with MAD1, we carried out immunoprecipitation in 293T cells using epitope tag antibodies. As shown in Fig. 2A, Western blot using GFP antibody confirmed that NEK2A is pulled down by FLAG immunoprecipitation of MAD1 (lane 8; FLAG intraperitoneal). Conversely, Western blot using FLAG antibody revealed that MAD1 is pulled down by GFP-NEK2A (lane 4; GFP intraperitoneal). No FLAG-tagged MAD1 was precipitated with control IgG (lane 3), and no actin was detected in any of the immunoprecipitates. Thus, we conclude that the interaction between MAD1 and NEK2A is specific.

To test if NEK2A directly binds to MAD1 in vitro and to confirm the binding interface from the yeast screen, we employed GST-MAD1 D3 as affinity matrix and loaded equal protein for 2 h as described under Experimental Procedures. As shown in Fig. 2A, Western blot using GST-MAD1 pull-down, indicating that MAD1D3 absorbs NEK2AD3 protein. In addition to a same size band from GST-MAD1 pull-down, but not GST pull-down, a 21-kDa protein band of NEK2A D3 in isolated HeLa cell chromosome fraction. As shown in Fig. 3A, Western blot analysis revealed that the NEK2A antibody specifically recognized a single protein band of ~46 kDa in whole mitotic HeLa cell extracts, which closely matched the calculated molecular weight of NEK2A (Fig. 3A, left panel). To determine if NEK2A is a centromere-associated protein, we compared NEK2A protein levels in the interphase and mitotic cell lysates with that of isolated chromosome scaffold fraction by using Western blot analysis. As shown in Fig. 3A (right panel), the NEK2A signal is intensified in isolated chromosome scaffolds compared with that of mitotic cell lysates, a similar profile to CENP-E, indicating that NEK2A is enriched in the isolated chromosome fraction.

To ascertain if NEK2A is indeed a kinetochore protein, we adopted a pre-extraction procedure that allows better labeling of kinetochore protein while preserving fine cyto-structure of mitotic cells (5, 27). As shown in Fig. 3B, pre-extracted HeLa cells were stained using the NEK2A monoclonal antibody and a fluorescein-conjugated goat anti-mouse secondary antibody, whereas a human CREST anti-centromere antibody that reacts primarily with CENP-B followed by a rhodamine-conjugated goat anti-human secondary antibody was used to identify the actual centromere (Fig. 3B, red). In the prometaphase shown in Fig. 3B, NEK2A staining appears as pairs of clearly resolved double dots (green, arrow), whereas CREST centromere antigens are often present as pairs of unresolved dots (Fig. 3B, arrowhead). The merged image from three channels demonstrates that NEK2A is located at the centromere outer domain relative to CENP-B. As shown in Fig. 4C (upper panel), MAD1 labeling demonstrates typical centromere localization relative to ACA, similar to that of NEK2A, which is consistent with previous reports of human MAD1 (8, 29). To test whether exogenously express NEK2A can target to kinetochore, we...
transfected HeLa cells with GFP-NEK2A plasmids and assayed for its intracellular distribution by using immunofluorescence microscopy. As shown in Fig. 3C, NEK2A fusion protein displays a distribution pattern similar to that of endogenous protein, which is overlapped with centromere marker CENP-E staining in the merge (Fig. 3C-d). These results indicate that exogenously expressed GFP-NEK2A bears a characteristic centromere distribution profile similar to endogenous NEK2A. Given the fact that NEK2A is located at the centromere and is co-precipitated with MAD1, we conclude that NEK2A interacts with MAD1 at the kinetochore.

**NEK2A Involved in the Spindle Checkpoint**—To investigate the possible influence of NEK2A on the localization of MAD1 to the kinetochore and on the mechanism of mitotic-checkpoint signaling, we introduced RNA interference (RNAi) oligonucleo-
To determine an optimal concentration of the RNAi oligonucleotide for knocking down NEK2A protein, we transfected HELa cells with a series of concentrations of oligonucleotides, and we collected cells 36 h post-transfection. Immunoblotting with a NEK2A antibody revealed that 100 nM NEK2A RNAi oligonucleotide caused a remarkable 6–7-fold suppression of NEK2A protein without altering the levels of other proteins such as actin. Further increases of the RNAi oligonucleotide did not significantly improve the efficiency of NEK2A suppression. As NEK2A synthesis in the ∼25% of un-transfected cells with little or no oligonucleotide was unlikely to be markedly diminished, the observed 6–7-fold inhibition at 100 nM must represent almost complete inhibition of NEK2A in 75 ± 3% of successfully transfected cells.

We next examined whether knock-down of NEK2A protein alters localization of MAD1 to the kinetochore, and we treated HEla cells with 100 nM RNAi, and either scrambled oligonucleotide or a control cyclophilin oligonucleotide, respectively. Thirty six hours after the transfection, cells were collected and stained for MAD1, ACA, and DNA, respectively. As shown in stained for CENP-E (CENP-E, red), DAPI (DNA, blue), GFP antibody (GFP-NEK2A-KD, green), and their merged images. Kinase death NEK2A (c) can target to kinetochore as wild type NEK2A as shown in Fig. 3C. As shown in a, expression of NEK2A-KD induced cells entered into anaphase with chromosome bridges (b, arrow). In addition, a pair of sister chromatids (c, arrowhead) failed to separate. Bars, 10 µm. C, depletion of NEK2A by siRNA effected mitotic defects in chromosome segregation. HELa cells were transfected with NEK2A siRNA oligonucleotide and control oligonucleotide for 48 h followed by fixation and DNA staining. Cells were then examined under a fluorescence microscope to sort out the phenotypes. We categorized the phenotypes as normal mitosis (prometaphase/metaphase and anaphase/telophase) and those that carried lagging chromosomes and that prematurely exited from mitosis with unseparated sister chromatids and chromosome bridges. They were quantified and expressed as percentage of total mitotic cells. An average of 150 cells from three separate experiments were counted for HELa cells treated with NEK2A siRNA oligonucleotides compared with those treated with control scramble oligonucleotides. Error bars represent S.E.; n = 3 preparations. *, p < 0.001. D, NEK2A kinase activity is required for faithful mitotic progression. HELa cells were transfected with GFP-NEK2A-KD and GFP constructs and for 48 h followed by fixation and DNA staining. Cells were then examined under fluorescence microscopy to determine the phenotypes. Cells in mitosis (prometaphase/metaphase and anaphase/telophase) and those that carried lagging chromosomes and that prematurely exited from mitosis with unseparated sister chromatids and chromatid bridges were quantified and expressed as percentage of total mitotic cells. An average of 150 cells from three separate experiments were counted for HELa cells transfected with NEK2A-KD construct compared with those transfected with GFP only. Error bars represent S.E.; n = 3 preparations. *, p < 0.001.
cell cycle-related defects in HeLa cells, we collected control—
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essential for the localization of HEC1 to the kinetochore. As
shown in Fig. 4D, HEC1 labeling displays a typical kinetochore
localization in both control and NEK2A-depleted cells, suggest-
ing that HEC1 localization to kinetochore is independent of
NEK2A.
NEK2A Is Essential for Faithful Chromosome Segreg-
tion—To explore whether knock-down of NEK2A causes any
cell cycle-related defects in HeLa cells, we collected control
oligo-treated and RNAi-treated cells, stained with DAPI, ACA, and
MAD1. Control anaphase cells treated with scramble oligo-
nucleotide exhibited a total separation of two sets of sister
chromatids. However, cells treated with NEK2A siRNA display a
typical chromosome bridge phenotype, in which the majority of
sister chromatids are separated with one or more sister
chromatids entangled (Fig. 5A, b, arrow), a phenotype reported
recently (30) in U2OS cells overexpressing kinase-dead
NEK2A. Careful examination of these NEK2A-suppressed cells
also revealed that some sister chromatids failed to separate as
the characteristic double-dot labeling marked by MAD1 and
ACA, respectively (Fig. 5A, a and c, arrowheads). The failure in
separating sister chromatids prior to entry into anaphase re-
sulted in unequal distribution of chromatids, which leads to
aneuploidy. We surveyed approximately 150 mitotic cells, in
which both sets of sister chromatids were in the same focal
plane, from NEK2A-siRNA oligonucleotide-treated and control
oligonucleotide-treated cells. We counted the number of cells
displaying typical chromosome bridge phenotype, and we ex-
pressed it as the percentage of the total cell population that is
in mitosis. As shown in Fig. 5C, a summary from three different
experiments show that the depletion of NEK2A resulted in
significant increases in cells bearing lagging chromatomes
(25.7 ± 5.5%) and chromosome bridges (45.1 ± 5.4%). These
data demonstrate that loss of NEK2A is responsible for defects
of chromosome segregation associated with premature entry
into anaphase.
To validate if NEK2A kinase is essential for integrity of spindle
checkpoint and for mitotic progression, we expressed kinase-
death mutant of NEK2A, and we assayed for its relevance in
chromosome segregation. Western blotting analysis carried out
using transfected cells showed that exogenously expressed GFP-
NEK2A proteins (both wild type and kinase death mutant) were
about three times the level of endogenous NEK2A (data not
shown). Microscopic examination revealed that the NEK2A-KD
mutant is targeted to the centromere of HeLa cells, suggesting
the kinase activity is not required for its association with kine-
tochore. However, examination of these transfected cells revealed
the presence of lagging chromosome and chromosome bridges
(Fig. 5B, b, arrow) between sister chromatids in premature an-
aphase cells, similar to those seen in NEK2A-depleted cells (Fig.
5A). Moreover, some sister chromatids failed to separate in
NEK2A-KD-expressing cells (Fig. 5B, a and c, arrowheads). We
surveyed approximately 150 mitotic cells of each preparation,
from three different experiments in which both sets of sister
chromatids were in the same focal plane. We counted the number
of cells, either expressing GFP alone or GFP-NEK2A-KD mu-
tant, displaying typical chromosome bridge phenotype and ex-
pressed the percentage of mitotic cells. As shown in Fig. 5D,
expression of the mutant NEK2A resulted in significant in-
creases in cells with lagging chromosomes (26.7 ± 6.1%; com-
pared with 7.8 ± 0.8% of control) and chromosome bridges
(53.7 ± 7.3%; compared with 6.7 ± 0.8% of control), validating
the outcome from our early NEK2A knock-down experiment us-
ing siRNA (Fig. 5C), which is also consistent with the phenotype
reported by a recent study (30). Therefore, we conclude that
NEK2A kinase is essential for faithful chromosome segregation
during metaphase-anaphase transition.
To test whether NEK2A kinase is essential for spindle check-
point in the presence of spindle disrupters, we transfected
HeLa cells with NEK2A-KD mutant followed by nocodazole

treatment for 18 h. As shown in Fig. 5F, there was a 1.9-fold
decrease in the number of mitotic cells that expressed GFP-
NEK2A-KD mutant compared with cells transfected with only
GFP. Conversely, there was an 8.6-fold increase in the number
of cells with multiple nuclei, which was a hallmark of check-
point failure. Given the heterogeneity in exogenous protein
expression among transfected cells, we reasoned that the pop-
ulation of cells expressing lower level mutant protein was un-
able to compete for endogenous NEK2A kinase and to overcome
spindle checkpoint set by endogenous protein.
NEK2A Is Essential for MAD2 to Associate with the Kineto-
chore—Depletion of NEK2A by siRNA treatment did not alter
the localization of MAD1 and HEC1 to kinetochores (Fig. 4, C
and D). To examine the relationship between MAD1 and other
spindle checkpoint proteins such as MAD2 and BUB1, we used
siRNA to suppress NEK2A expression. We surveyed promo-
aphase cells for MAD2 labeling as spindle checkpoint proteins
can be easily marked at the kinetochore at this stage of cell
cycle. As shown in Fig. 6A, MAD2 labeling displayed a typical
kinetochore localization in control HeLa cells. However, MAD2
is absent from most kinetochores of NEK2A-depleted cells,
suggesting that the NEK2A regulates the association between
MAD2 and kinetochore, perhaps via interaction with MAD1.
Further examination of other spindle checkpoint protein local-
ization in the NEK2A-depleted cells revealed no alteration in
either the assembly of BUB1 onto kinetochores of promo-
aphase cells or the disassembly of BUB1 from kinetochores of
anaphase cells (Fig. 6B). Therefore, we conclude that NEK2A is
required for the assembly of MAD2, but not BUB1 and MAD1,
to kinetochores.

DISCUSSION
NEK2A Is a Kinetochore Protein Interacting with MAD1—
We have identified and characterized NEK2A as a MAD1-
binding partner. Most interesting, NEK2A is localized to the
kinetochore in addition to its centrosomal distribution estab-
lished previously (16, 20). These results are consistent with the
distribution of other mitotic kinasas such as TTK (31, 32).
Although centrosomal localization of NEK2A has been re-
ported, our novel finding of kinetochore-associated NEK2A dif-
ers from some previous reports (16, 20) that did not reveal any
localization of NEK2A to kinetochore. We believe that this
discrepancy is probably a result of difference in specificity of
the antibodies that were used in the respective studies (16, 20).
It is possible that the epitope recognized by their antibody was
not accessible at kinetochores.
We found that NEK2A exhibits a dynamic pattern of distri-
bution. NEK2A is not associated with kinetochore in the inter-
phase but became associated with kinetochore upon the break-
down of the nuclear envelope. Our studies also revealed the
novel interaction between MAD1 and NEK2A and their co-
distribution to kinetochores of the mitotic cells. Most interest-
ing, NEK2A localization is not required for MAD1 localization
to the kinetochore and vice versa. In addition, elimination of
NEK2A did not alter localization of HEC1 and BUB1, suggest-
ing that kinetochore assembly involves several independent
but perhaps inter-related pathways, and NEK2A controls the
NEK2A Is an Integral Component of the Mitotic Spindle Checkpoint—We demonstrated that NEK2A is an important component of the spindle checkpoint. Cells defective for NEK2A function failed to arrest mitosis in the presence of the spindle damage by the microtubule inhibitor nocodazole. In addition, NEK2A is critical for normal mitotic progression as cells without NEK2A exited mitosis with entangled chromosomes and un-separated sister chromatids, which led to formation of cells containing multinuclei and the genesis of aneuploidy phenotype. This is a hallmark of perturbation of mitotic checkpoint that forces cells to exit mitosis prematurely, before all the chromosomes are aligned correctly. This phenotype resembles those observed when functions of spindle checkpoint proteins MAD1, MAD2, BUB1, BubR1, ZW10, and MPS1 were disrupted (23, 33, 34). Recent studies (30) also revealed similar phenotypes arising from expression of the kinase death mutant NEK2A in U2OS cells. Although these authors tried to link the chromosome segregation defects to the centrosomal abnormality due to the loss of NEK2A activity, our experimentation provides the mechanistic view in which NEK2A may serve as a novel mitotic checkpoint integrator by retaining MAD2 function at kinetochore to ensure full separation of all sister chromatids before anaphase starts.

MAD2 is a spindle checkpoint sensor preferentially localized to the unattached kinetochores in mitosis, which marks the “anaphase-wait” signal (9, 23, 24). It has been shown that localization of MAD2 to kinetochores requires MAD1 (35, 36). In addition, MAD2 is phosphorylated in mitosis by unknown protein kinase(s), and this phosphorylation regulates its association with MAD1 and Cdc20 (37). The inability to assemble MAD2 protein onto kinetochores in NEK2A-depleted cells as demonstrated in our studies suggests that NEK2A kinase may form a link between MAD2 protein phosphorylation and its role in spindle checkpoint signaling. In fact, MAD2 contains the NEK2A phosphorylation motif consensus (F/K/R)(K/R)(S/T); see Ref. 17. However, our attempt to reconstitute MAD2 protein phosphorylation in vitro by using recombinant NEK2A kinase from bacteria was blocked by the inability of recombinant NEK2A protein to catalyze ATP transfer. Nevertheless, it would of great interest to evaluate whether MAD2 is a true substrate for NEK2A in vitro and in vivo. The identification of kinase(s) that phosphorylates MAD2 will be important for a better understanding of the MAD2-dependent mitotic checkpoint cascade.

Cancer cells are often defective in maintaining mitotic block in response to microtubule disrupters such as nocodazole and taxol, which leads to mis-segregation of chromosomes and biogenesis of aneuploidy (23). It has been demonstrated that haploinsufficiency (loss of an allele) of MAD2 protein resulted in an aberrant mitotic checkpoint in mouse embryonic fibroblasts and an increased susceptibility to lung cancer in mice (37). The inability to assemble MAD2 onto kinetochores in NEK2A-depleted cells and the subsequent occurrence of aneuploidy in the presence of nocodazole mirrored the phenotype seen in the MAD2 haploinsufficiency in genetically manipulated mouse cells (37). Thus, aberrant NEK2A cascade signaling could lead to insufficient assembly of MAD2 to kinetochores, which provides another mechanism to compromise the mitotic spindle checkpoint. It would be of great interest to explore whether any cancer cells bear disrupted NEK2A activity. Our experimentation of manipulation of NEK2A expression and activity shown here may provide a useful system to dissect the signaling cascade underlying the NEK2A-MAD2 signaling cascade, which will aid in elucidating the pathogenesis of chromosome instability and identifying the other components that are involved in mitotic checkpoint signaling.

Recent studies (21) showed that NEK2A interacts with a highly conserved kinetochore protein HEC1, a protein homologous to the NDC80 protein located at the budding yeast kinetochore during mitosis. Furthermore, these authors showed that NEK2A phosphorylates HEC1, and this phosphorylation is required for faithful chromosome separation in yeast. We confirmed the critical role of NEK2A in chromosome segregation of HeLa cells. In addition, we showed that elimination of NEK2A does not affect kinetochore localization of HEC1, suggesting that NEK2A-mediated protein phosphorylation of HEC1 at Ser-165 is not required for localization of HEC1 to the
kinetochore. It has been shown that HEC1 (33), MPS1 (31), and CENP-I (34) are involved in association of MAD1 to the kinetochore of mammalian cells. However, it remains unclear as to how their dynamics are regulated by mitotic kinase cascades. Our finding that MAD1 interacts with NEK2A at the kinetochore adds an additional layer of complexity underlying the protein-protein interaction network at the kinetochore. Therefore, it would be of great interest to evaluate precisely the respective contribution of the multiprotein complex of MAD2-MAD1-NEK2A-HEC1-Mps1 in kinetochore assembly dynamics and chromosome movements by using real time analysis.

Taken together, our finding of the interaction of NEK2A with MAD1 demonstrates a critical role for NEK2A in kinetochore dynamics in addition to its function for centrosome regulation. The fact that elimination of NEK2A disrupts assembly of MAD2 to kinetochores, abrogates nocodazole-induced mitotic arrest, and induces chromosome cross-bridges in HeLa cells prematurely exited from anaphase demonstrates the importance of NEK2A in faithful chromosome segregation.

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REFERENCES
1. Brinkley, B. R., and Stubblefield, P. (1966) Chromosoma 19, 28–43
2. Roos, U. P. (1976) J. Cell Biol. 54, 363–385
3. Nicklas, R. B. (1989) J. Cell Biol. 109, 2245–2255
4. Rieder, C. L., and Alexander, S. P. (1990) J. Cell Biol. 110, 81–95
5. Yao, X., Abrieu, A., Zheng, Y., Sullivan, K. F., and Cleveland, D. W. (2000) Nat. Cell Biol. 2, 484–491
6. Li, R., and Murray, A. W. (1991) Cell 66, 519–531
7. Hoyt, M. A., Tota, L., and Roberts, B. T. R. (1990) Cell 66, 507–517
8. Jin, D. Y., Spencer, F., and Jeang, K. T. (1998) Cell 93, 81–91
9. Chen, R. H., Waters, J. C., Salmon, E. D., and Murray, A. W. (1996) Science 274, 242–246
10. Li, Y., and Benezra, R. (1996) Science 274, 246–248
11. Taylor, S. S., Ha, E., and McKeon, F. (1988) J. Cell Biol. 142, 1–11
12. Martinez-Exposito, M. J., Kaplan, K. B., Copeland, J., and Sorger, P. K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8489–8498
13. Taylor, S. S., and McKeon, F. (1997) Cell 89, 727–735
14. Cahill, D. P., Lengauer, C., Yu, J., Riggins, G. J., Wilson, J. K., Markowitz, S. D., Kinzler, K. V., and Vogelstein, B. (1998) Nature 392, 300–303
15. Chan, G. K., Jablonski, S. A., Sudakin, V., Hittle, J. C., and Yen, T. J. (1999) J. Cell Biol. 146, 941–954
16. Nigg, E. A. (2001) Nat. Rev. Mol. Cell. Biol. 2, 21–32
17. O’Connell, M. J., Krien, M. J., and Hunter, T. (2003) Trends Cell Biol. 13, 221–228
18. Oakley, B. R., and Morris, N. R. (1983) J. Cell Biol. 96, 1155–1158
19. Lu, K. P., and Roberts, B. T. S. (1990) J. Cell Biol. 110, 413–424
20. Fry, A. M., Meraldi, P., and Nigg, E. A. (1998) EMBO J. 17, 479–481
21. Chen, Y., Riley, D. J., Zheng, L., Chen, P. L., and Lee, W. H. (2002) J. Biol. Chem. 277, 49408–49416
22. Li, X., and Nicklas, R. B. (1997) J. Cell Sci. 110, 537–545
23. Shah, J. V., and Cleveland, D. W. (2000) Cell 103, 997–1000
24. Gorbsky, G. J., Chen, R. H., and Murray, A. W. (1998) J. Cell Biol. 141, 1193–1205
25. Chen, R. H., Brady, D. M., Smith, D., Murray, A. W., and Hardwick, K. G. (1999) Mol. Biol. Cell 10, 2607–2618
26. Zhou, R., Cao, X., Watson, C., Miao, Y., Guo, Z., Forte, J. G., and Yao, X. (2003) J. Biol. Chem. 278, 35651–35659
27. Yao, X., Anderson, K. L., and Cleveland, D. W. (1997) J. Cell Biol. 139, 435–447
28. Zhang, J., Fu, C., Miao, Y., Duo, Z., and Yao, X. (2002) Sci. Bull. 119, 345–353
29. Campbell, M. S., Chan, G. K., and Yen, T. J. (2001) J. Cell Sci. 114, 953–963
30. Faragher, A. J., and Fry, A. M. (2003) Mol. Biol. Cell 14, 2876–2889
31. Liu, S. T., Chan, G. K., Hittle, J. C., Fuji, G., Lees, E., and Yen, T. J. (2003) Mol. Biol. Cell 14, 1638–1651
32. Mao, Z., Sawaguchi, A., Zhang, J., Hong, L., Brako, L., and Yao, X. (2003) Cell Res. 13, 443–449
33. Martin-Lluesma, S., Stucke, V. M., and Nigg, E. A. (2002) Science 297, 2267–2270
34. Liu, S. T., Hittle, J. C., Jablonski, S. A., Campbell, M. S., Yoda, K., and Yen, T. J. (2003) Nat. Cell Biol. 5, 341–345
35. Chen, R. H., Shevchenko, A., Mann, M., and Murray, A. W. (1998) J. Cell Biol. 143, 283–295
36. Wassmann, K., Liberal, V., and Benezra, R. (2003) EMBO J. 22, 797–806
37. Michel, L. S., Libal, V., Chatterjee, A., Kirchwegger, R., Pasche, B., Gerald, W., Dobles, M., Sorger, P. K., Murty, V. V., and Benezra, R. (2001) Nature 409, 355–359
NEK2A Interacts with MAD1 and Possibly Functions as a Novel Integrator of the Spindle Checkpoint Signaling
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