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Nek2A kinase regulates the localization of numatrin to centrosome in mitosis

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Abstract Chromosome segregation in mitosis is orchestrated by the kinetochore and spindle microtubules stemming from two centrosomes. Our recent studies demonstrated the importance of Nek2A in faithful chromosome segregation during mitosis. Here, we report that Nek2A regulates the function of numatrin in mitosis. The biochemical interaction between Nek2A and numatrin in mitotic cells was revealed by a set of reciprocal immunoprecipitation experiments using Nek2A and numatrin antibodies, respectively. The interaction is validated by a pull-down assay using recombinant Nek2A and numatrin proteins. Moreover, our immunofluorescence studies demonstrate that numatrin becomes centrosome-associated as the cell enters into mitosis and depart from the centrosome after sister chromatid separation in anaphase. The co-localization of numatrin and Nek2A to the centrosome suggests their interaction with and involvement in centrosome function. Indeed, elimination of Nek2A kinase by siRNA diminished its association with the centrosome. Furthermore, we show that numatrin is phosphorylated by wild type but not kinase-death Nek2A. Our studies suggest that the Nek2A kinase cascade is essential for the localization of numatrin to the centrosome.

Keywords: Numatrin; Nek2A; Centrosome; Centromere; Mitotic spindle

1. Introduction

Mitosis is orchestrated by the interaction of the mitotic spindle, a dynamic microtubule array originating from the centrosome, microtubule organization center (e.g., [1,2]). In higher animal cells, duplication of centrosomes is triggered by CDK2/cyclin E-mediated phosphorylation while its maturation depends on Nek2A protein kinase, and the human homolog of never in mitosis A (NIMA), which is vital in *Aspergillus nidulans* for entry into mitosis (e.g., [3,4]). Mutation of NIMA arrests cells in G_2 without interfering with $p34^{cdc2}$ activation, suggesting that the NIMA protein has a central role in the G_2/M transition. Furthermore, a portion of Nek2A localizes to the centrosome of mammalian cells and appears to play a role similar to NIMA in controlling entry into mitosis (e.g., [5,6]). Nek2A appears to have diversified roles during several phases of the cell cycle, from S phase to multiple stages in mitosis, based on its dynamic expression and subcellular localization in the cytosol, nucleus, and mitotic structures other than the centrosome (e.g., [7]). In fact, our recent studies demonstrate the localization of Nek2A to the kinetochore of mitotic cells and its importance in faithful chromosome segregation [8].

Several lines of evidence demonstrate the requirement of Nek2A kinase in centrosome function during mitosis and the association of Nek2A with other centrosomal proteins such as C-Nap1 and phosphatase 1 [9,10]. The centrosomal protein C-Nap1 is thought to play an important role in centrosomal cohesion during interphase of the cell cycle (e.g., [1]). It has been shown that the dynamic association/dissociation of C-Nap1 with the centrosome is regulated by Nek2A kinase [11]. It remains elusive as to how molecular assembly and disassembly at the centrosome is regulated in mitosis as the chromosome undergoes dynamic and complex movements.

Mitosis in mammalian cells is accompanied by dramatic reorganization of cyto-architecture driven by protein kinase cascades (e.g., [1,2]). Among most organelles, the nucleus undergoes remarkable reorganization to facilitate the equal segregation of parental genome to two daughter cells. Numatrin, also called nucleophosmin/B23, a multifunctional nucleolar protein, has recently been identified as one of the substrates of CDK2/cyclin E in centrosome duplication [12]. Numatrin redistributes to the mitotic spindle upon entry into mitosis when the nucleous disassembles. It has been shown that numatrin is a substrate of casein kinase II and Cdk1/cyclin B in interphase and mitosis, respectively [13,14]. However, it remains unclear how numatrin redistribution to the centrosome is regulated by cell cycle machinery.

To further explore the molecular regulation of Nek2A in mitotic progression, we carried out immunoprecipitation

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Abbreviations: NIMA, never in mitosis A; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; DAPI, 4,6-diamidino-2-phenylindole

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assays and identified a novel Nek2A interacting partner, numatrin. Our studies show that Nek2A interacts with numatrin at the centrosome during early mitosis. In addition, we show that Nek2A is required for numatrin localization to the centrosome in mitosis. Thus, we proposed that Nek2A kinase links mitotic regulation to numatrin molecular dynamics.

2. Materials and methods

2.1. Cell culture

HeLa cells, from American Type Culture Collection (Rockville, MD), were maintained as subconfluent monolayers in DMEM (Invitrogen; Carlsbad, CA) with 10% FCS (Hyclone, UT) and 100 U/ml penicillin plus 100 μg/ml streptomycin (Invitrogen; Carlsbad, CA).

2.2. cDNA construction

For mammalian expression of the full-length NEK2A, both wild type and kinase death NEK2A cDNAs were cloned into pEGFP-C1 vector (Clontech, Palo Alto, CA) as described elsewhere (e.g., [8]).

2.3. Recombinant protein production

GST–Nek2A and histidine-tagged numatrin proteins were expressed in bacteria as fusion proteins. Briefly, the full length of Nek2A (K37R, kinase-death) cDNA was cloned into pGEX-4T vector (Amersham Biosciences), whereas numatrin was cloned into pET-22b vector (Novagen). Due to the insufficiency of expressing wild type Nek2A protein in bacteria, a fusion protein without kinase activity (kinase-death) was produced for assessing a possible direct contact between Nek2A and numatrin.

Purification of recombinant proteins was carried out as described previously (e.g., [8]). Briefly, 1 liter of LB media was inoculated with bacteria transformed either with Nek2A or numatrin. 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.

2.4. Affinity precipitation of NEK2A and numatrin

His-tagged numatrin-transformed bacterial cell lysate was used as a source of numatrin protein. The purified soluble GST-fused Nek2A (kinase death) protein was pre-bound to glutathione–agarose (Sigma Chemical). GST–NEK2A protein bound beads were washed with 5× column volume using wash buffer (10 mM phosphate buffer, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, and 0.01% phenylmethylsulfonyl 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, and 0.01% phenylmethylsulfonyl fluoride). The his-tagged numatrin bacterial cell lysates were loaded on the GST–NEK2A affinity column and incubated for 1 h. The column was washed with 10× volume of incubation buffer. Finally, extensively washed glutathione–agarose beads were boiled in 1× SDS–poly-acrylamide gel electrophoresis (SDS–PAGE) sample buffer. GST protein-bound glutathione–agarose beads were used as negative control. Protein samples were resolved on SDS–PAGE and were analyzed by immunoblot analysis using a mouse 6× histidine antibody (Cell Signaling Co.).

2.5. Transient transfection and immunoprecipitation

HeLa cells grown to ~75% confluency were exposed to 100 ng/ml nocodazole (Sigma Chemical, Inc.) for 20 h. Mitotic shake-off was conducted to collect mitotic cells as described previously (e.g., [15]). These mitotic cells were then lysed in buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EGTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml pepstatin A). Lysates were clarified by centrifugation at 16 000×*g* for 10 min at 4 °C and then incubated with anti-numatrin antibody (Affinity BioReagent) bound protein-A/G beads (Pierce Chemical, IL). After rotating at 4 °C for 2 h, beads were washed five times with lysis buffer and then boiled in protein sample buffer for 2 min. After SDS–PAGE, the proteins were transferred to a nitrocellulose membrane. The membrane was divided into three strips and probed with antibodies against the numatrin, Nek2A (BD Biosciences), and tubulin, respectively. Immunoreactive signals were detected with ECL kit (Pierce Chemical, IL) and visualized by autoradiography on Kodak BioMAX film.

2.6. SiRNA mediated depletion of Nek2A

The siRNA sequence used for silencing Nek2A corresponds to the coding region 260–280 (relative to the start codon) as described [8]. The 21-mer oligonucleotide RNA duplexes were synthesized by Dharmacon Research, Inc. (Boulder, CO, USA). An oligonucleotide with scrambled sequence was used as a control [8].

2.7. Immunofluorescence microscopy

For immunofluorescence, cells were seeded onto sterile, acid-treated 18-mm coverslips in 6-well plates (Corning Glass Works, Corning, New York). 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 or scrambled (control) oligonucleotides, HeLa cells were rinsed for 1 min with PHEM buffer (100 mM PIPES, 20 mM HEPES, pH 6.9, 5 mM EGTA, 2 mM $MgCl_2$ and 4 M glycerol) and were permeabilized for 1 min with PHEM plus 0.2% Triton X-100 as described [8,16,17]. Extracted cells were then fixed in freshly 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 Chemical Co). 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 γ-tubulin and Nek2A were visualized using fluorescein-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR), while binding of numatrin and NuMA was visualized using Texas Red-conjugated donkey anti-rabbit IgG (Molecular Probes, Eugene, OR). DNA was stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma Chemical Co.). Slides were examined with a Zeiss LSM510 confocal scanning fluorescence microscope and images were collected and analyzed with Image-5 (Carl Zeiss, Germany).

2.8. In vitro phosphorylation of numatrin by Nek2A isolated from HeLa cells

Histidine-tagged numatrin protein was expressed and purified using Ni-beads as described previously (e.g., [8]). The fusion protein bound to the Ni- beads was eluted with 250 mM imidazole, dialyzed and re-suspended in phosphorylation buffer prior to use.

To verify whether numatrin is a substrate for Nek2A in vitro, 10 μg of purified histidine-tagged numatrin fusion protein was incubated with GFP antibody-bound protein A/G beads absorbed with wild type and kinase-death Nek2A in phosphorylation buffer containing 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol, 10 nM ATP, and 5 μCi of [γ-^{32}P] ATP (PerkinElmer Life Sciences) in a total volume of 60 μl. Kinase reaction was carried out at room temperature for 15 min and terminated by the addition of 20 μl of 4× SDS–PAGE sample buffer and separated by 6–16% gradient SDS–PAGE. The gel was stained with Coomassie brilliant blue, dried, and quantified by a PhosphorImager (Amersham Biosciences).

2.9. Western blot

Samples were subjected to SDS–PAGE on 6–16% gradient gel and transferred onto a nitrocellulose membrane. Proteins were probed by appropriate primary and secondary antibodies and detected using ECL (Pierce, IL). The band intensity was then scanned using a Phosphorimager (Amersham Bioscience, NJ).

3. Results and discussion

3.1. Numatrin is a Nek2A-associated protein

Our recent experiment demonstrated the localization of Nek2A at the kinetochore in addition to its distribution at the centrosome [8]. The involvement of Nek2A in faithful

chromosome segregation indicates its role in modulating mitotic spindle dynamics. To elucidate the molecular mechanisms underlying Nek2A function in mitosis, we sought to identify proteins associated with Nek2A from mitotic cells. To this end, we carried out Nek2A immunoprecipitation using mitotic HeLa cell lysates and then resolve these proteins in SDS–PAGE followed by Western blotting analysis with a battery of centrosomal protein antibodies. As shown in Fig. 1A, Western blot analysis using numatrin antibody identified the possibility of an association between Nek2A and numatrin. Probing the same blot with a α-tubulin antibody indicates that numatrin is specifically pulled down by Nek2A antibody.

To validate this interaction, we performed a reciprocal pull-down experiment using numatrin antibody. As shown in Fig. 1B, Western blot Nek2A monoclonal antibody confirmed that Nek2A is pulled down by numatrin immunoprecipitation. No tubulin was detected in any of the immunoprecipitates. In addition, similar immunoprecipitation using NuMA antibody revealed no co-precipitation of numatrin and Nek2A with NuMA (Fig. 1C), indicating the specificity of the complex between Nek2A and numatrin.

To evaluate whether numatrin directly binds to Nek2A, we expressed GST–Nek2A kinase death fusion protein and used GST–Nek2A fusion protein as an affinity matrix to pull down histidine-tagged numatrin from bacteria lysates. As shown in Fig. 1D, histidine-tagged numatrin was absorbed by GST–Nek2A fusion protein but not GST tag. Thus, we conclude that numatrin interacts with Nek2A.

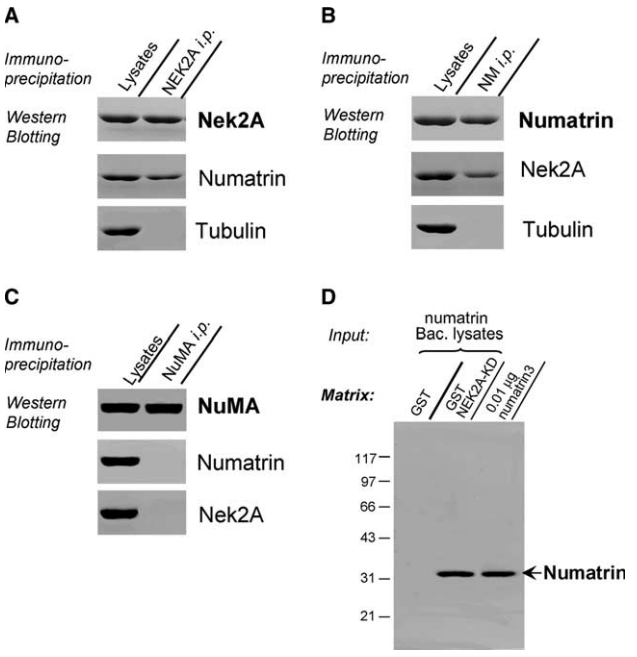

Fig. 1. Biochemical characterization of Nek2A–numatrin interaction. (A) Immunoprecipitation of Nek2A pull down numatrin from mitotic HeLa cells. HeLa cells were treated with nocodazole followed by extraction in lysis buffer as described in Section 2. Clarified lysates (~5 mg) were incubated with 10 μg Nek2A antibody pre-bound protein A/G beads. Proteins from starting lysates and Nek2A immunoprecipitates were analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting using Nek2A antibody, numatrin antibody, and a tubulin antibody, respectively. In the experiment shown in A, Western blotting verifies co-immunoprecipitation of numatrin and Nek2A. No tubulin was detected in Nek2A immunoprecipitates. (B) Reciprocal immunoprecipitation of numatrin verified Nek2A–numatrin association. HeLa cells were treated with nocodazole followed by extraction in lysis buffer as described in Section 2. Clarified lysates (~5 mg) were incubated with 5 μg numatrin antibody pre-bound protein A/G beads. Proteins from starting lysates and Nek2A immunoprecipitates were analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting using numatrin antibody, Nek2A antibody, and a tubulin antibody, respectively. In the experiment shown in B, Western blotting verifies an association between numatrin and Nek2A. No tubulin was detected in numatrin immunoprecipitates. (C) NuMA immunoprecipitation revealed no interaction with either Nek2A or numatrin. In brief, clarified lysates (~5 mg) were incubated with 5 μg NuMA rabbit antibody pre-bound protein A/G beads. Proteins from starting lysates and NuMA immunoprecipitates were analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting using numatrin antibody, Nek2A antibody, and NuMA antibody, respectively. Neither numatrin nor Nek2A was detected in NuMA immunoprecipitates. (D) Reconstitution of Nek2A–numatrin association using bacterially expressed fusion protein. GST–Nek2A recombinant protein purified on glutathione–agarose beads (GST–Nek2A–KD) was used as affinity matrix for absorbing histidine-tagged numatrin protein as described in Section 2. GST protein-bound agarose beads (GST) were used as a control. After washing, proteins bound to agarose beads were boiled in sample buffer and fractionated by SDS–PAGE followed by transferring onto a nitrocellulose membrane. An aliquot of purified histidine-tagged numatrin fusion protein was loaded on an adjacent well as a positive control. Histidine antibody reacts with a 33-kDa protein band of numatrin in addition to a same size band from GST-MAD1 pull-down, but not GST pull-down, indicating that Nek2A interacts with numatrin protein.

3.2. *Numatrin is co-localized with Nek2A to the centrosome of mitotic cells*

Previous studies suggest that Nek2A is primarily located at the centrosomes and involved in centrosomal maturation. Numatrin is a nucleolar protein of interphase cells, which becomes associated with the centrosome in mitotic cells and has been implicated in the centrosome duplication. To explore the spatial–temporal relationship of Nek2A and numatrin distribution, we carried out immunocytochemical studies of HeLa cells using confocal scanning fluorescence microscopy.

To better visualize molecular localization, we adopted a pre-extraction procedure that allows effective labeling of the kinetochore and centrosomal proteins while preserving fine cyto-structure of mitotic cells [8,16,17]. As shown in Fig. 2, pre-extracted HeLa cells were stained with a Nek2A monoclonal antibody followed by a fluorescein-conjugated secondary antibody, while numatrin is labeled with a rabbit antibody followed by a rhodamine-conjugated secondary antibody. In the interphase cells shown in Fig. 2(a), numatrin staining appears as 3–4 clearly resolved dots in the nucleus (red, arrows) while Nek2A staining shows typical nuclear staining (c). The nucleolar localization becomes readily apparent when numatrin image is merged with DAPI staining, where red spots are superimposed onto nucleoli, DAPI stain-free areas, indicating that numatrin is a nucleolar protein of the interphase cells.

The mitotic spindle forms as the cell enters into mitosis. As shown in Fig. 2(e), it is readily apparent that numatrin appears as bright centrosomal staining located at two opposite poles, which is consistent with a previous report (e.g., [18]). Double labeling with a Nek2A mouse antibody displayed a typical centrosomal and kinetochore staining as marked by arrowheads and arrow, respectively (Fig. 2(g)). The centrosomal staining of Nek2A is identical to the numatrin labeling, which becomes apparent when the two images merge (arrowheads; Fig. 2(h)). The co-localization of numatrin labeling onto that

of Nek2A at the centrosome validates that these two proteins form a complex.

Interestingly, numatrin staining relocated to the midzone as cells enter into anaphase (Fig. 2(i)). Labeling of Nek2A from the same cell displays a diminished overall staining (Fig. 2(k)), which is consistent with a cell cycle regulated expression and destruction of Nek2A protein (e.g., [1]). Given the spatial–temporal distribution profile of these two proteins during the cell cycle and their co-precipitation, we conclude that Nek2A may interact with numatrin at the centrosome in early mitotic cells.

3.3. *Nek2A is essential for numatrin localization to the centrosome of mitotic cells*

Numatrin is a hyper-phosphoprotein regulated by kinase and phosphatase (e.g., [14]). To investigate the possible influence of Nek2A kinase cascade on the localization of numatrin to the centrosome and on the molecular mechanism of centrosome assembly, we introduced RNA interference (RNAi) oligonucleotides targeted to Nek2A by transfection into HeLa cells. As shown in Fig. 3A, Western blot with a Nek2A monoclonal antibody revealed that the 100 nM RNAi oligonucleotide caused remarkable 6–7-fold suppression of the Nek2A protein level at 36 h, while control cells treated with a scrambled oligonucleotide express normal level of Nek2A protein. This suppression is relatively specific since it does not alter the level of other proteins, such as tubulin and actin (e.g., [8]). As Nek2A synthesis in the ~25% of un-transfected cells with little or no oligonucleotide was unlikely to be markedly diminished, the observed 7-fold inhibition at 100 nM siRNA oligonucleotide must represent almost complete inhibition of Nek2A protein in $72 \pm 3\%$ of successfully transfected cells.

We next examined whether suppression of Nek2A protein would alter the localization of numatrin to the centrosome. To this end, we treated HeLa cells with 100 nM RNAi oligonucleotide to knock down Nek2A protein, and with a scrambled oligonucleotide for control, respectively. Thirty-six hours after the transfection, cells were collected and stained for numatrin, γ-tubulin and DNA, respectively. As shown in Fig. 3B(i)–(l), elimination of Nek2A caused a typical chromosome segregation defect. Examination of numatrin staining in these Nek2A-depleted cells revealed an apparently dispersed localization (Fig. 3B(i), arrows) in contrast to its well-defined centrosomal localization in scrambled oligonucleotide-treated control cells (e.g., Fig. 3B(a) and (e)). In these Nek2A-depleted cells, the levels of numatrin present at the centrosome were diminished (Fig. 3B(k), arrowheads). Quantitation of normalized pixel intensities shows that when Nek2A was reduced to less than 3% of its control value, numatrin was reduced to 5%. Double staining with γ-tubulin antibody marked a characteristic centrosomal location (Ref. [19]; Fig. 3(o)), indicating that elimination of Nek2A did not alter the centrosomal distribution of γ-tubulin. A merged image of numatrin and γ-tubulin labeling verifies a dislocation of numatrin from the centrosome marked by γ-tubulin staining (Fig. 3B(p)). Thus, Nek2A appears to be required for efficient centrosomal localization of numatrin.

To determine whether the elimination of Nek2A causes a gross alteration of centrosomal protein localization, we carried out a double labeling experiment in which Nek2A-suppressed

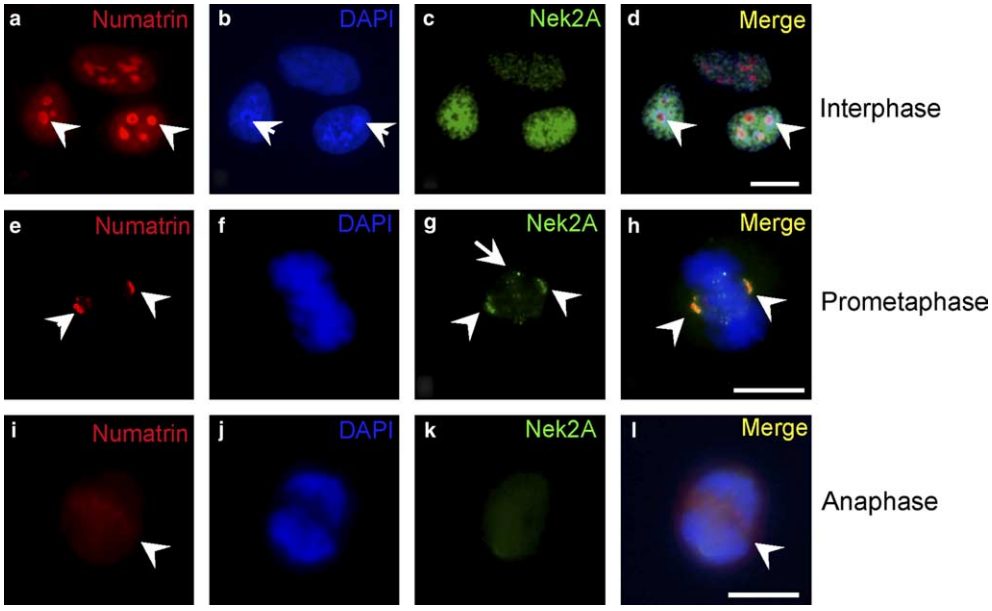

Fig. 2. Co-localization of Nek2A with numatrin to the centrosome of mitotic HeLa cells. This set of optical images was collected from interphase and mitotic HeLa cells triple stained with rabbit numatrin antibody (red), DAPI (blue), mouse Nek2A antibody (green), and their merged images. In interphase cells (a)–(d), numatrin displays a typical 3-5 nucleolar dots localization (a, arrowheads). The nucleolar localization was highlighted by DAPI staining, where nucleoli were unstained (b, arrowheads). Merged image indicates that numatrin is a nucleolar protein in interphase cells. Bar: 10 μm. In prometaphase cells (e–h), Nek2A marked centromeres of mitotic HeLa cells as pairs of unresolved double dots (arrow, g) in addition to bright centrosome staining (arrowheads). Numatrin staining also appears as centrosome-like staining located at opposite poles (arrowheads, e). A merged image shows a superimposition of numatrin to that of Nek2A staining at the centrosome but not kinetochore. Bar: 10 μm. In anaphase cells (e–h), Nek2A departed from the mitotic spindle (centrosome and kinetochore) as evidenced by much diminished staining (k). Numatrin staining also appears as faint dot-like staining around the midline of mitotic spindle (arrowhead, i). A merged image verified the distribution of numatrin between pairs of separated sister chromatids (arrowhead, l). Bar: 10 μm.

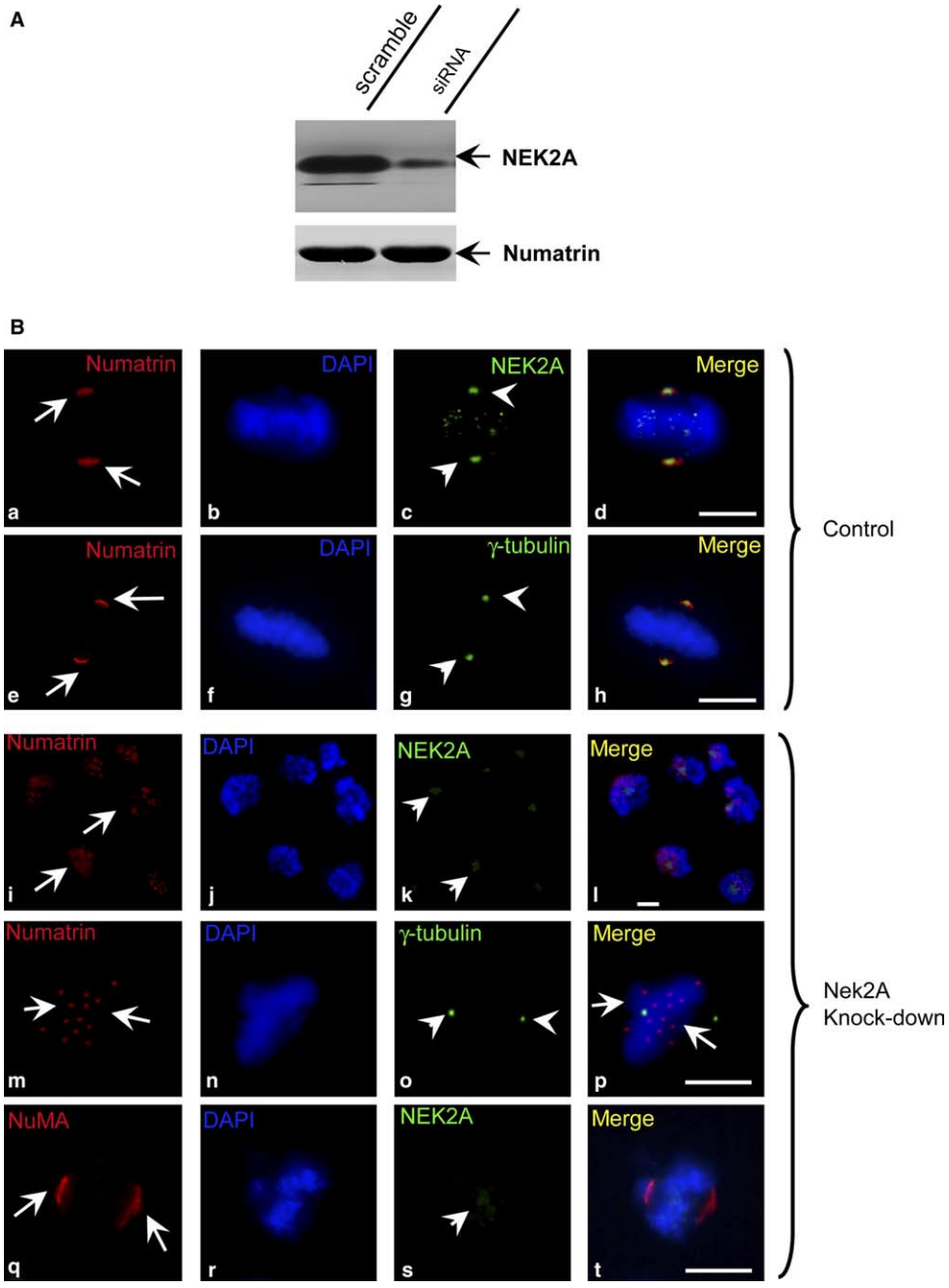

Fig. 3. Suppression of Nek2A protein accumulation eliminates the centrosomal localization of numatrin. (A) Suppresion of Nek2A protein using siRNA oligonucleotide. HeLa cells were transfected with 100 nM Nek2A siRNA oligonucleotides and a control oligonucleotide (scramble) for 36 h and subjected to SDS–PAGE and immunoblotting. Upper panel, immunoblot against Nek2A; lower panel, immunoblot against numatrin. (B) Localization of numatrin to the centrosome is dependent on Nek2A. This set of montage represents optical images collected from HeLa cells triply stained for a rabbit numatrin antibody (red; a, e, i and m), DAPI (blue, b, f, j, n and r), mouse Nek2A antibody (green, c, k, and s), or γ-tubulin antibody (green; g and o), and their merged images (d, h, l, p and t), respectively. These cells were transfected with oligonucleotides (control and siRNA for Nek2A) for 36 h followed by fixation and immunocytochemical staining. Nek2A stained the centromeres, the centrosome (arrows), in control oligonucleotide-treated cells (c). γ-Tubulin labeled the centrosome of mitotic cells (arrowheads, g). Treatment with siRNA markedly diminished the Nek2A level (e.g., k and o). Depletion of Nek2A protein by siRNA treatment does not alter the centrosomal staining of γ-tubulin (arrowheads, o) and NuMA (arrowheads, q). However, elimination of Nek2A caused dispersed distribution of numatrin (arrows; i, l, q and t). Bars: 10 μm.

cells were stained for NuMA, another centrosomal protein marker. While siRNA oligonucleotide efficiently suppressed Nek2A protein level (Fig. 3B(s), labeling of NuMA displayed a characteristic centrosomal localization (Fig. 3B(q)), suggesting that Nek2A is not required for targeting and/or association of NuMA to the centrosome. Therefore, we conclude that Nek2A kinase is essential for localization of numatrin to the centrosome.

Recent studies show that Cdc2-mediated protein phosphorylation regulates numatrin association with the centrosome [20]. To test whether numatrin is a substrate of PKA, we performed in vitro phosphorylation on recombinant histidine–numatrin fusion protein, using wild type and kinase-death Nek2A proteins immuno-isolated from HeLa cells. Recombinant histidine–numatrin migrates at about the predicted 33 kDa as shown in Fig. 4. Incubation of the fusion proteins with [^{32}P] ATP and the wild type GFP-Nek2A resulted in the incorporation of ^{32}P into wild type numatrin (Fig. 4; upper panel). This Nek2A-mediated phosphorylation is specific, since incubation of numatrin with [^{32}P] ATP in the presence of the kinase-death Nek2A resulted in no detectable incorporation of radioactivity into the numatrin protein. Thus, numatrin is a substrate for Nek2A. Overexpression of kinase-death Nek2A mutant in HeLa cells also caused dislocation of numatrin from the centrosome (data not shown), similar to those seen in Nek2A-suppressed cells (e.g., Fig. 3B). Therefore, we conclude that the association of numatrin with the centrosome is likely a function of Nek2A kinase signaling cascade.

Numatrin is an important nucleolar protein that has been proposed to shuttle proteins from the nucleolus to the cytoplasm [21,22]. In addition, numatrin is phosphorylated in mitosis by unknown mitotic protein kinase(s) other than p34^{Cdc2}. One recent study shows that the polo-like kinase interacts and phosphorylates numatrin on the serine residue at position 4 [23]. Interestingly, inhibition of polo-like kinase-mediated numatrin phosphorylation by mutagenesis induced centrosome instability and failure in cytokinesis. Despite the fact that both polo-like kinase and numatrin are localized at the centrosome in mitosis, it was unclear whether such non-phosphorylatable numatrin is liberated from the centrosome. Another recent study showed that re-association of numatrin with the centrosome during mitosis is regulated by Cdc2-mediated phosphorylation on threonine residues at positions 234 and 237 [20]. Moreover, blocking phosphorylation at these two residues reduced the association of numatrin to the centrosome by approximately 60%. It would be of great interest to know whether the remaining fraction of centrosome-associated numatrin is governed by Nek2A kinase activity as Nek2A and p34^{cdc2} function in parallel during mammalian cell division [4]. At any rate, the inability to assemble numatrin protein onto the centrosome in Nek2A-depleted cells demonstrated in our studies suggests that Nek2A kinase may form a link between numatrin protein phosphorylation and its role in the centrosome dynamics. Our reconstitution of numatrin protein phosphorylation in vitro using GFP-Nek2A kinase purified from HeLa cells will facilitate the precise mapping of the Nek2A-mediated phosphorylation site(s) on numatrin. It would be of great importance to pinpoint the Nek2A-mediated phosphorylation sites on numatrin so that we can elucidate the phospho-regulation of numatrin function in the centrosome and ascertain the respective roles of mitotic kinases in regulating numatrin dynamics at the centrosome. In addition, the identification of Nek2A kinase that phosphorylates numatrin will also be important for a better understanding of the numatrin-dependent shuttling between nucleus and mitotic spindle, and the maintenance of genomic stability in cell division.

Our finding of the Nek2A-mediated phospho-regulation of numatrin to the centrosome demonstrates a critical role of Nek2A in centrosome dynamics in addition to its function in the mitotic checkpoint. The fact that elimination of Nek2A disrupts assembly and/or retention of numatrin to the centrosome indicates the importance of Nek2A in the centrosome integrity and dynamics. Further studies will be required to define the molecular mechanisms underlying Nek2A-mediated regulation of numatrin dynamics in mitosis.

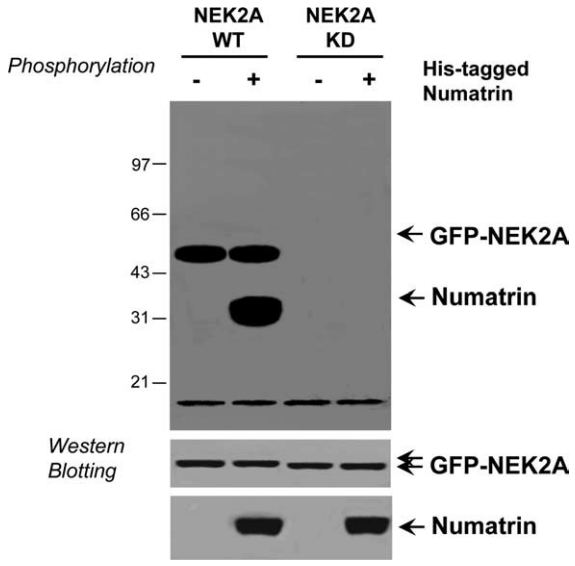

Fig. 4. Numatrin is a substrate of Nek2A in vitro. Bacterially expressed histidine–numatrin fusion protein was phosphorylated in vitro using [^{32}P] ATP and purified Nek2A as described in Section 2. Protein samples were separated by 6–16% gradient SDS–PAGE gel, dried and subjected to autoradiogram. Upper: The SDS–PAGE gel was dried and subsequently incubated with X-ray film. Note that in the presence of wild type Nek2A, there was dramatic incorporation of ^{32}P into numatrin protein. Lower: Western blotting validated the isolation of wild type and kinase-death Nek2A protein. Note that wild type GFP-Nek2A protein migrates slower compared to that of kinase-death mutant Nek2A. The level of numatrin added was also validated by histidine antibody blot.

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