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NEK7 is a centrosomal kinase critical for microtubule nucleation

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Abstract

NIMA in *Aspergillus nidulans* is a mitotic kinase for chromosome condensation and segregation. We characterized NEK7, a human homologue of *Aspergillus* NIMA. NEK7 was located at the centrosome throughout the cell cycle. Temporal localization of NEK7 at midbody of the cytokinetic cell was also observed. NEK7 knockdown by RNAi caused a prometaphase arrest of the cell cycle with monopolar or disorganized spindle. We propose that such defects in spindle assembly are resulted from reduction in microtubule nucleation activity at the centrosome. In consistent to the proposal, we observed a decrease in the centrosomal γ-tubulin levels and reduction of the microtubule re-growth activity in the *NEK7*-suppressed cells. In addition, it was evident that NEK7 was directly involved in cytokinesis.

Keywords: NEK7; Mitotic kinases; Centrosome; Spindle assembly; Microtubule nucleation; Cytokinesis

Phosphorylation is one of the key regulatory mechanisms for execution of a series of mitotic events. Once Cdc2 is activated at the onset of mitosis, several other serine/threonine kinases, such as Aurora, NIMA-related (NEK) and Polo-like (PLK) kinases are accompanied for mitotic progression. Such mitotic kinases are often multifunctional. For example, PLK1 is implicated in diverse mitotic functions, including centrosome maturation, bipolar spindle assembly, sister chromatid cohesion, anaphase-promoting complex/cyclosome activation, and cytokinesis [1]. To carry out these functions, PLK1 phosphorylates multiple substrates at the sites of action, including centrosome, kinetochore, and midbody. Furthermore, the mitotic kinases are functionally interactive so that a kinase is activated by phosphorylation with another kinase, or that two kinases share a substrate for an action [2]. Mitotic kinases are often linked to tumorigenesis as their overexpression was frequently observed in solid human tumor tissues [3].

NEK kinases were initially identified as human homologues of the *Aspergillus* NIMA, and so far 11 *NEK*s have been identified in the human genome [4,5]. As *Apsergillus* NIMA was known as a mitotic kinase for chromosome condensation and segregation [6,7], human NEKs have been also suspected to be involved in mitotic events [5]. Perhaps NEK2 is most extensively studied among the NEK family proteins. NEK2 is centrosomal and promotes the splitting of duplicated centrosomes at the onset of mitosis [8]. NEK2 is also present outside of the centrosome, and involved in other mitotic events, such as spindle assembly and checkpoint [9–12]. Other NEK family proteins have been investigated to some extents. Cumulative works suggested that NEK6, NEK7, and NEK9 are involved in mitosis while NEK1 and NEK8 are linked to polycystic kidney disease [13–17].

In this paper, we investigated biological functions of NEK7. The results showed that NEK7 was centrosomal and critical for spindle assembly at prometaphase and for cytokinesis.

Materials and methods

Antibodies. Polyclonal NEK7 antiserum was raised against the GST-NEK7 fusion protein. The NEK7 antibody was affinity purified by incubation of the antiserum with a strip of nitrocellulose membrane blotted with the NEK7 fusion protein and eluted with 100 mM glycine, pH 2.5.

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The antibodies against α-tubulin (Sigma), β-tubulin (Sigma), γ-tubulin (Sigma), the Myc tag (Cell Signaling), GFP (Cell Signaling), Aurora B (BD Biosciences), NEK2 (Transduction Laboratories), PLK1 (Zymed), and phospho-histone H3 (Upstate) were purchased.

Cell culture, transfection, flow cytometry, immunoblot, and immunocytochemistry. HeLa, U2OS, and 293T cells were cultured in DMEM with 10% fetal bovine serum. The cells were treated with 2 mM thymidine and 100 ng/ml nocodazole for S and M phase arrest, respectively. Transient transfection into HeLa or 293T cells was carried out using Lipofectamine Plus reagent (Invitrogen). Flow cytometry, immunoblot and immunocytochemistry were carried out as described previously [18].

Microtubule re-growth assay. Cells were placed on ice for 30 min to disrupt cellular microtubule organization, and then transferred to a 37 °C environment to initiate microtubule growth. Twenty seconds later, the cells were fixed and subjected to immunocytochemistry.

RNA interference. For RNAi suppression, siRNAs specific to *NEK7* (CTCCGACAGTTAGTTAATATT), *NEK2* (AAGCGGGACTTCCGCACATAC), and *PLK1* (GCGGGACTTCCGCACATACTT) were transfected into HeLa cells with oligofectAMINE (Invitrogen) according to the manufacture's instructions. Forty-eight hours after transfection, the cells were fixed for immunocytochemistry or prepared for immunoblot or FACS analysis. Non-specific control siRNA (AAGTAGCCGAGCTTCGATTGC) was also prepared.

Results

Expression and subcellular localization of NEK7

We raised a polyclonal antibody against bacterially expressed GST-NEK7 fusion protein and affinity-purified. Immunoblot analysis was carried out with the HeLa cells in which Myc-NEK7 or GFP-NEK7 was ectopically expressed. The results showed that the NEK7 antibody detected not only the ectopic NEK7 proteins but also the endogenous NEK7 protein of 34 kDa in size (Fig. 1A).

The NEK7 protein levels were determined in the HeLa cells whose cell cycle was arrested at S and M phases with treatment of thymidine and nocodazole, respectively. The results showed that the cellular NEK7 levels were more or less constant throughout the cell cycle (Fig. 1B).

When the NEK7 antibody was used for immunostaining U2OS cells, it detected the centrosome distinctly. NEK7 was present at centrosome throughout the cell cycle (Fig. 1C). In addition, the NEK7 signal was also detected at spindle midzone of the anaphase cells and eventually concentrated at the midbody (Fig. 1C, Supplementary Fig. 1). Presence of NEK7 in cytoplasm was also evident, since its diffused cytoplasmic signal disappeared in the *NEK7*-suppressed cells (Fig. 2A). Such subcellular localization of NEK7 suggests its functional link to the centrosome and mitosis.

NEK7 is required for the microtubule nucleation activity of the centrosome

We transfected siRNAs specific to *NEK7* or other related kinases, such as *PLK1* and *NEK2*, and observed that their expression was suppressed efficiently (Supplementary Fig. 2). At the same time, we observed increases

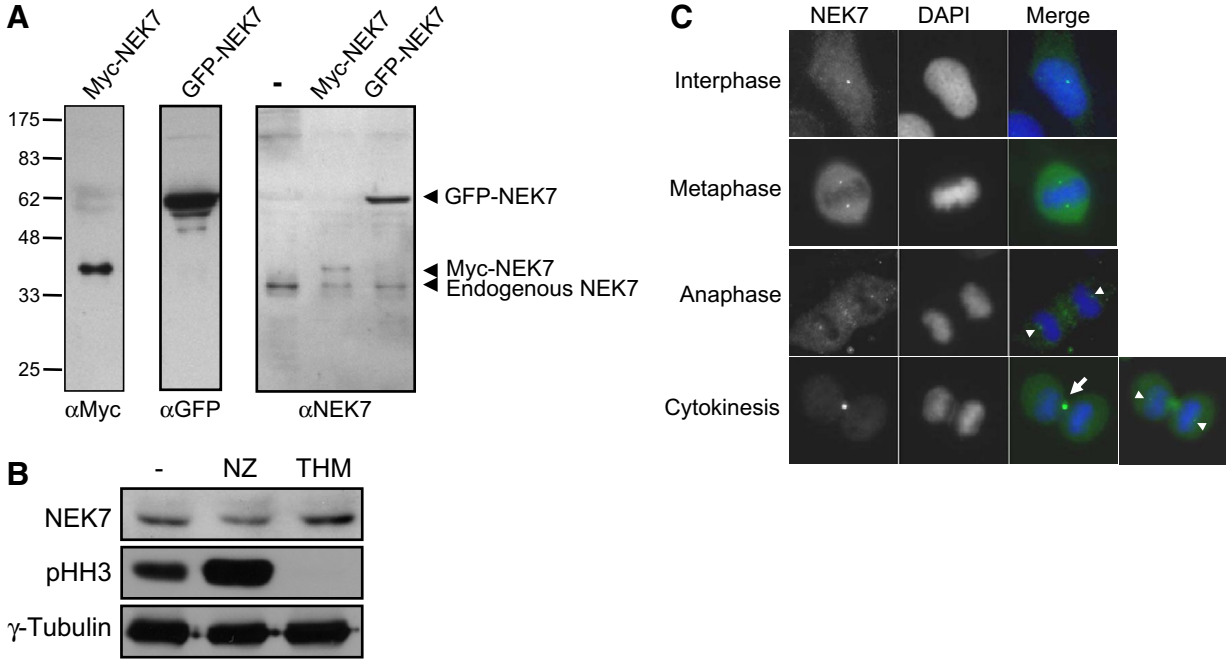

Fig. 1. Expression and subcellular localization of NEK7. (A) HeLa cells were transfected with *pMyc-NEK7* or *pGFP-NEK7*. Twenty-four hours after transfection, the cell lysates were prepared and subjected to immunoblot analysis with antibodies specific to NEK7, GFP, and the Myc tag. The endogenous NEK7 was detected along with the ectopic NEK7 proteins. (B) The HeLa cells were treated with 100 ng/ml nocodazole (NZ) for M phase arrest or with 2 mM thymidine (THM) for S phase arrest. The cell lysates were then prepared and subjected to immunoblot analysis with antibodies specific to NEK7, phospho-histone H3 (pHH3), and γ-tubulin. (C) The U2OS cells were immunostained with the NEK7 antibody (green). DNA was stained with DAPI. Arrowheads and an arrow indicated spindle poles and the spindle midzone, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)