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γ-Taxilin temporally regulates centrosome disjunction in a Nek2A-dependent manner

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ABSTRACT

Never in mitosis A-related kinase 2A (Nek2A), a centrosomal serine/threonine kinase, is involved in mitotic progression by regulating the centrosome cycle. Particularly, Nek2A is necessary for dissolution of the intercentriole linkage between the duplicated centrosomes prior to mitosis. Nek2A activity roughly parallels its cell cycle-dependent expression levels, but the precise mechanism regulating its activity remains unclear. In this study, we found that γ-taxilin co-localized with Nek2A at the centrosome during interphase and interacted with Nek2A in yeast two-hybrid and pull-down assays and that γ-taxilin regulated centrosome disjunction in a Nek2A-dependent manner. γ-Taxilin depletion increased the number of cells with striking splitting of centrosomes. The precocious splitting of centrosomes induced by γ-taxilin depletion was attenuated by Nek2A depletion, suggesting that γ-taxilin depletion induces the Nek2A-mediated dissolution of the intercentriole linkage between the duplicated centrosomes nevertheless mitosis does not yet begin. Taken together with the result that γ-taxilin protein expression levels were decreased at the onset of mitosis, we propose that γ-taxilin participates in Nek2A-mediated centrosome disjunction as a negative regulator through its interaction with Nek2A.

1. Introduction

The centrosome is the major microtubule-organizing center in animal cells and consists of a pair of centrioles and a surrounding protein meshwork, the so-called pericentriolar material [1–3]. Similarly to DNA replication, the centrosome duplicates once per cell cycle [3,4]. During interphase, microtubules radiating from the centrosome contribute to the cell shape, cell polarity, maintenance of the compartmentalization of cells, and intracellular vesicle trafficking, while during mitosis, bipolar spindles radiating from the duplicated centrosomes participate in segregation of chromosomes, leading to genetic stability [2,5–7]. Centrosome duplication occurs during S phase, and the duplicated centrosomes connect to each other via a proteinaceous linker between the two pairs of centrioles until mitosis begins [1,2]. The proteinaceous linker includes at least C-Nap1, rootletin, and LRRC45 [2,8,9]. Rootletin and LRRC45 form a fiber-like structure to link the two pairs of centrioles through their interaction with C-Nap1 localized at the proximal ends of the centriole [8,9]. Dissolution of this intercentriole linkage prior to mitosis allows bipolar spindle formation. Accumulating evidence has demonstrated that this dissolution is restrictedly regulated by multiple kinases including cyclin-dependent kinase 1, Aurora-A, Polo-like kinase, and Nek2 [2,10–16]. Among these kinases, Nek2A has been demonstrated as a pivotal player to directly phosphorylate and subsequently dissolve linker proteins.

Nek2A is a spliced isoform of Nek2, a serine/threonine protein kinase [17–19]. In addition to Nek2A, Nek2 has two other spliced variants including Nek2B and Nek2C [17–19]. Nek2A is mainly present in the nucleus and cytoplasm, while Nek2B and Nek2C are predominantly present in the cytoplasm and nucleus, respectively [19]. Although Nek2A and Nek2B exhibit similar cell cycle-dependent expression

Abbreviations: C-Nap1, centrosomal never in mitosis A-related kinase-associated protein 1; Nek2, never in mitosis A-related kinase 2; APC/C, anaphase promoting complex/cyclosome; ER, endoplasmic reticulum; PCR, polymerase chain reaction; siRNA, small interfering RNA; PBS, phosphate buffered saline; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; RT, reverse transcription; Sf9, *Spodoptera frugiperda* 9; GST, glutathione S-transferase; GFP, green fluorescent protein; CEP, centrosomal protein; PCM-1, pericentriolar material-1; RNAi, RNA interference; ATF4, activating transcription factor 4

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patterns prior to mitosis, Nek2A, but not Nek2B, is rapidly degraded at the onset of mitosis owing to APC/C-mediated ubiquitylation [16,20–22]. These findings imply that these spliced variants have distinct functions. In centrosome disjunction, several lines of evidence have demonstrated that Nek2A phosphorylates linker proteins, such as C-Nap1, rootletin, and LRRC45, and dissolves the intercentriole linkage to allow duplicated centrosomes to separate at G2/M transition [8,13–15]. Nek2A kinase activity roughly parallels its expression levels that are mainly regulated at the transcriptional level prior to mitosis [14,17,18,20,22]. Expression levels of Nek2A are very low in G1 phase, significantly increase at G1/S transition, and remain high throughout S and G2 phases [14,18]. Considering that Nek2A kinase activity should remain low during interphase to prevent centrosome disjunction, the expression patterns of Nek2A imply certain mechanisms inhibiting Nek2A kinase activity until mitosis begins.

The taxilin family was initially identified as a binding partner of the syntaxin family that is involved in intracellular vesicle trafficking [23]. The taxilin family consists of at least three members, including α-, β-, and γ-taxilins, which share a C-terminal long coiled-coil region [23,24]. α- and γ-Taxilins are expressed ubiquitously, while β-taxilin is restrictedly expressed in the heart and skeletal muscle [23,25,26]. α-Taxilin plays a role in the recycling pathway of the transferrin receptor through an interaction with sorting nexin 4 [27] and participates in intracellular trafficking of hepatitis B virus DNA-containing particles [28]. Moreover, α-taxilin is expressed in parallel with cell proliferation in the mouse gastrointestinal tract [25] and is overexpressed in hepatocellular carcinoma, renal cell carcinoma, and glioblastoma [29–31], implying that α-taxilin participates in cell proliferation, probably by regulating the intracellular trafficking of certain signalling molecules related to cell proliferation. β-Taxilin interacts with and prevents dysbindin from inhibiting the differentiation of C2C12 myoblasts into myotubes [26]. It has been reported that γ-taxilin participates in ER stress responses in hypoxic cells exhibiting a decrease in the expression levels of γ-taxilin protein [32]. Thus, the physiological significance of taxilin family members has been gradually revealed in the past decade, but elucidation of the functions of γ-taxilin remains poor. In this study, we demonstrate that γ-taxilin localizes at the centrosome during interphase, and its protein expression levels decrease at G2/M transition through proteasomal degradation. Furthermore, we propose that γ-taxilin temporally regulates Nek2A-mediated centrosome disjunction through its interaction with Nek2A.

2. Materials and methods

2.1. cDNA constructs

Standard recombinant DNA techniques were used to prepare the following expression constructs: pEGFP-γ-taxilin (full length), pEGFP-γ-taxilin (aa 1–132), pEGFP-γ-taxilin (aa 133–528), pEGFP-γ-taxilin (aa 40–172), pEGFP-γ-taxilin (aa 190–309), pEGFP-γ-taxilin (aa 343–528), pGBKT7-γ-taxilin (full length), pEGFP-Nek2A (full length), pGADT7-Nek2A (full length), pGADT7-Nek2A (aa 1–300), pGADT7-Nek2A (aa 301–455), pGADT7-Nek2A (aa 370–455), pGADT7-Nek2A (aa 1–369), pGADT7-Nek2A (aa 301–369), and pFastBac HT-Nek2A (full length). The constructs were prepared by inserting fragments generated by PCR or restriction enzyme digestion into the vectors. The inserts were sequenced and the structures of all plasmids were confirmed by restriction analysis.

2.2. Cell culture, transfection, and siRNAs

HeLaS3 and U2OS cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2 incubator. Transfection of expression vectors into cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Targeted proteins were depleted by siRNA using RNAiMAX (Invitrogen) according to the manufacturer's protocol. siRNA sequences are listed in Supplementary Table S1.

2.3. Fluorescence microscopy

Cells grown on coverslips were fixed in PBS containing 3.7% formaldehyde (1% formaldehyde for endogenous γ-taxilin and C-Nap1) for 10 min at room temperature, followed by fixation with methanol at −20 °C for 20 min. The permeabilized cells were blocked in PBS containing 1% BSA and 1% goat serum at room temperature for 1 h. The cells were treated with the indicated antibodies in PBS containing 1% BSA and 1% goat serum for 1 h at room temperature. After washing twice with PBS, the cells were treated with the secondary antibody in PBS containing 1% BSA and 1% goat serum for 1 h at room temperature. The cells were observed using an FV10i confocal laser-scanning fluorescence microscope (Olympus, Tokyo, Japan) or an LSM 710 Laser-scanning confocal microscope (Carl Zeiss, Thornwood, NY, USA). The distance between two centrioles and the fluorescence intensity of each protein at the centrosome were measured in at least 30 cells using FV10-ASW 3.0 software or ZEN blue software. Commercial antibodies used for immunofluorescence staining are listed in Supplementary Table S2.

2.4. Cell treatments

HeLaS3 and U2OS cells synchronized at the G1/S boundary by thymidine double block (2 mM) were released for the indicated periods. To synchronize cells at the G2/M boundary, cells were treated with 2 mM thymidine for 16 h, followed by incubation with 9 μM RO-3306 (Sigma-Aldrich, St. Louis, MO) for 20 h. To synchronize cells in prometaphase, cells were treated with 2 mM thymidine for 16 h, followed by incubation with 40 ng/ml nocodazole (Sigma-Aldrich) for 16 h. To inhibit the proteasome pathway, synchronized cells at the indicated phases were treated with 10 μM MG132 (Calbiochem, San Diego, CA) for 1 h before harvesting. To depolymerize and stabilize microtubules, cells were treated with 5 μg/ml nocodazole and 5 μM taxol (Sigma-Aldrich) for 4 h at 37 °C, respectively. To observe microtubule regrowth, the cells were incubated in ice-cold PBS for 30 min to depolymerize microtubules and subsequently incubated in fresh culture medium at 37 °C for the indicated periods.

2.5. Subcellular fractionation

The following procedures were performed at 0–4 °C. HeLaS3 cells were suspended in buffer A (10 mM Tris/HCl [pH 7.5], 1 mM EDTA, 250 mM sucrose, and a protease inhibitor cocktail (Roche Diagnostics, Basal, Switzerland)) and then homogenized by 20 strokes in a ball homogenizer (clearance: 0.012 nm). The homogenate was centrifuged at 1000 × *g* for 10 min, and the supernatant was used as the post-nuclear fraction. The supernatant was further centrifuged at 100,000 × *g* for 1 h. The pellet was resuspended in a comparable amount of buffer A and used as the membrane fraction, while supernatant was used as the cytosol fraction.

2.6. Isolation of centrosomes

HeLaS3 cells were treated with 0.2 μM nocodazole and 1 μg/ml cytochalasin B (Sigma-Aldrich) for 1 h at 37 °C. The treated cells were lysed in centrosome lysis buffer (1 mM HEPES [pH 7.5], 0.5% NP-40, 0.5 mM $MgCl_2$, and 1 mM 2-mercaptoethanol) and then centrifuged at 2500 × *g* for 10 min at 4 °C. The supernatant was treated with 2 U/ml DNase I (Takara, Shiga, Japan) for 30 min at 37 °C, and the treated supernatant (2 ml) was loaded on 11 ml sucrose gradients consisting of 4.9 ml of 40%, 2.4 ml of 50%, and 3.7 ml of 70% (w/v) sucrose solutions. The gradient was centrifuged at 130,000 × *g* for 2 h at 4 °C, and