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Depletion of tumor suppressor Kank1 induces centrosomal amplification via hyperactivation of RhoA

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ABSTRACT

Chromosome instability, frequently found in cancer cells, is caused by a deficiency in cell division, including centrosomal amplification and cytokinesis failure, and can result in abnormal chromosome content or aneuploidy. The small GTPase pathways have been implicated as important processes in cell division. We found that knockdown of a tumor suppressor protein Kank1 increases the number of cells with a micronucleus or bi-/multi-nuclei, which was likely caused by centrosomal amplification. Kank1 interacts with Daam1, known to bind to and activate a small GTPase, RhoA, in actin assembly. Knockdown of Kank1 or overexpression of Daam1, respectively, hyperactivates RhoA, potentially leading to the modulation of the activity of Aurora-A, a key regulator of centrosomal functions, eventually resulting in centrosomal amplification. Kank1 is also associated with contractile ring formation in collaboration with RhoA, and its deficiency results in the interruption of normal daughter cell separation, generating multinucleate cells. Such abnormal segregation of chromosomes may cause further chromosomal instability and abnormal gene functions, leading to tumorigenesis. Thus, Kank1 plays a crucial role in regulating the activity of RhoA through retrieving excess Daam1 and balancing the activities of RhoA and its effectors.

1. Introduction

Aneuploidy is a common feature in cancer and is caused by abnormal chromosomal segregation through deregulation at the mitotic checkpoint [1]. Dysfunctions at the mitotic checkpoint often result in multipolar spindles due to centrosomal amplification, a state with supernumerary centrosomes. Centrosomes serve as a microtubule organizing center (MTOC) and act as a regulator of cell-cycle progression, but they are frequently altered in cancer cells, often as supernumerary centrosomes, caused partly by loss of the tumor-suppressor protein p53 [2,3] or by deregulation of proteins such as BRCA1 [4]. Centrosomal amplification can be caused by failures during cell division, such as deregulation of the cell cycle, defective DNA or chromatin metabolism, or a failure in the spindle checkpoint [5,6]; however, less is known about cell signaling pathways leading to tumorigenesis through centrosomal amplification.

Rho GTPase pathways have been implicated as key regulatory processes in the progression of mitosis [7]. Rho GTPase members, RhoA and Cdc42, are related to centrosomal positioning, chromosomal alignment and/or cytokinesis. As a major role of Rho GTPases is actin polymerization, the involvement of Rho GTPases in cell division was initially thought to be limited to cytokinesis [7]. However, evidence has been accumulated of their roles in overall cell division, such as in the microtubule attachment to centrosomes at prometaphase and to chromosomes at metaphase. At telophase, RhoA is recruited to the site of cleavage and activated by ECT2, a Rho-guanine-nucleotide-exchange factor (Rho-GEF) [8], where RhoA stimulates actin polymerization by activating formins, and myosin II by phosphorylating its light chain to facilitate cleavage furrow ingression and contractile ring progression with the help of a Rho kinase, ROCK [9]. Constitutive activation of the RhoA pathway signals promotes centrosomal amplification and chromosomal instability by aberrant activation of its effectors [10], such as ROCK2 [11]; however, there is no report regarding the involvement of tumor suppressors in controlling the activity of Rho GTPases in centrosomal amplification and thus aneuploidy in cancer cells. We therefore show here, for the first time, that RhoA acts as an important regulator of centrosomal amplification through the interaction with a tumor suppressor and as a modulator of RhoA, Kank1 and Daam1, which were previously identified as regulators of other cellular functions.

Kank1 is a tumor suppressor of renal cell carcinoma and is related to a variety of cell functions, cell growth, cytoskeleton, cell motility and nuclear/cytoplasmic transport [12,13]. Kank1 functions in collaboration with Rho GTPases, such as Rac1, Cdc42 and RhoA. Depletion of

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Kank1 results in strengthened actin fibers and increased cell movement. Kank1 is involved in the directed orientation of the MTOC and Golgi apparatus in cell movement and intracellular trafficking in cooperation with BIG1, a GEF of Arf GTPases, and Kif21A [14]. On the other hand, Daam1 (Dishevelled-associated activator of morphogenesis 1) is a formin, a group of proteins which play important roles in actin assembly [15,16]. Daam1 has several domains including a GTPase-binding domain (GBD) and three formin-homology (FH) domains (FH1 to FH3). Daam1 is located downstream of Wnt signaling and is associated with cell migration and polarity through the interaction with Rho GTPases such as RhoA and Cdc42 [14]. Recently, Daam1 was found to be a key factor in centrosome polarity [17] and in planar cell polarity [18].

2. Materials and methods

2.1. Cell culture

NIH3T3 and HEK293T cells were maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM; GIBCO/Life Technologies) supplemented with 10% fetal bovine serum (FBS). Mouse IMCD cells were cultured in DMEM/Hams F-12 containing 10% FBS. NIH3T3 cells expressing tetracycline (Tet)-inducible GFP-Kank1 were described previously [19].

2.2. Antibodies and regents

Polyclonal and monoclonal antibodies against Kank1 were described previously [20,21]. Polyclonal antibody against Daam1 was raised in a rabbit with GST-fused full-length Daam1. For imaging, antibodies against Daam1 (Santa Cruz Biotechnology), α-tubulin, γ-tubulin, Rho and β-actin (Cell Signaling Technology) were used.

2.3. Cloning of full-length Daam1 and Daam1 mutants

A full-length cDNA clone encoding *Daam1* (KIAA0666) was a gift from the Kazusa DNA Research Institute (Chiba, Japan). A full-length *Daam1* cDNA was cloned into cytomegalovirus (CMV) promoter-driven vectors, pcDNA 3.1(+) (Invitrogen/Life Technologies) or pEGFP (Clontech Laboratories), for gene expression or for constructing tagged deletion mutants. *Daam1* cDNA was subjected to amplification with a left primer containing a *Kpn*I site just before the ATG sequence and a right primer containing the *Pvu*II site of *Daam1* cDNA. A DNA fragment generated by restriction digestion with *Kpn*I and *Pvu*II of the PCR product was ligated with a fragment of *Pvu*II/*Xho*I from the clone KIAA0666 and cloned into the pcDNA 3.1(+) vector. To generate GFP-tagged Daam1 (GFP-Daam1), a DNA fragment containing an ORF generated with *Kpn*I/*Xba*I restriction digestion was cloned into the pEGFP vector. Deletion mutants lacking coiled-coil domain 1 (CC-1; 430–544 aa) or coiled-coil domain 2 (CC-2; 961–1020 aa) were generated by PCR-mediated recombination and cloned into pEGFP-C1 vector. The *Apa*I fragment from GFP-Daam1, which contained the N-terminal/CC-1 region, was subcloned in pEGFP-C2 vector with proper orientation to generate GFP-ΔC-Daam1. All the constructs were verified by sequencing. Kank1 constructs were described previously [22].

Coiled-coil domains in Daam1 were predicted with a software found at http://www.ch.embnet.org/software/COILS_form.html as described previously [23]. The amino acid sequence of Daam1 was scanned in this software and it was found that 430–544 aa and 961–1020 aa are potential coiled-coil domains in Daam1.

2.4. Treatment of cells with siRNA

Cells were transfected with control, Kank1 or Daam1 siRNA (100 nM; Santa Cruz Biotechnology) using Lipofectamine 2000 (Invitrogen/Life Technologies).

2.5. Cell cycle synchronization

NIH3T3 cells were cultured in medium containing 0.5% FBS for 2 days, and then cultured in 10% DMEM or 0.5% DMEM in the presence or absence of Wnt3a for 24 h. To obtain cells arrested at the M phase, exponentially growing cells were treated with 400 ng/ml nocodazole for 16 h. They were collected by either pipetting or by gentle shaking, washed twice with phosphate-buffered saline (PBS) and further cultivated for 15 min in complete DMEM without nocodazole. To synchronize cells at the G_1/S boundary, a double thymidine block was made by incubating cells in culture medium containing 2 mM thymidine for 16 h. The cells were washed with PBS and then incubated for 8 h in fresh complete DMEM without thymidine. The cells were incubated again for 13 h in a medium containing 2 mM thymidine. After washing twice with PBS, the cells were cultured in fresh medium and the cell cycle was observed.

2.6. Immunoprecipitation and immunoblotting

HEK293T and NIH3T3 cells were cultured in DMEM supplemented with 10% FBS. Mouse IMCD cells were cultured in DMEM/Hams F-12 with 10% FBS. For the co-immunoprecipitation experiment, IMCD cells were lysed in buffer A (50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 10% glycerol, 1% Nonidet P-40, 100 mM NaF, 200 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml chymotrypsin). Transfection was performed using Lipofectamine 2000 (Invitrogen/Life Technologies) according to the manufacturer's instructions. After 36 h, cells were harvested, washed with ice-cold PBS, and lysed for 15 min on ice in buffer A. The cell lysates were clarified by centrifugation, and the proteins were immunoprecipitated, washed in lysis buffer and boiled in SDS-PAGE loading buffer. Proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride membranes (Immobilon; Millipore), and detected with respective antibodies. Signals were enhanced with a secondary antibody against AP (Sigma-Aldrich) or HRP (GE Healthcare) and developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT; Sigma-Aldrich) or ECL (GE Healthcare).

GST-RBD (GST-fused RhoA-binding domain of rhotekin) pull-down assay was performed to quantify the amount of active RhoA (GTP-RhoA) as described previously [19].

2.7. Confocal microscopy

Cells were fixed with 4% (v/v) formaldehyde for 20 min at room temperature and then permeabilized with 0.3% (v/v) Triton X-100. The cells were then treated with 3% (w/v) bovine serum albumin (BSA) in PBS to block non-specific binding and were stained with the first antibodies and then with Alexa Fluor 488 or Alexa Fluor 546-conjugated second antibodies (Molecular Probes/Life Technologies). Nuclei were stained with DAPI (Molecular Probes/Life Technologies). The stained cells were analyzed using a confocal laser microscope (LSM510; Carl Zeiss) using AxioVision software. Images were obtained using a 63X Plan-Apo/1.4 NA Oil objective lens.

3. Results

3.1. Knockdown of Kank1 causes abnormal chromosomal segregation

We first investigated the role of Kank1 in cell division (Fig. 1). When Kank1 was knocked-down in NIH3T3 cells (Fig. 1A), the cells exhibited abnormal chromosomal segregation, resulting in cells with a micronucleus (MN) or bi-/multi-nuclei (BMN), or cells containing both MN and BMN (MN+BMN) (Fig. 1B and Fig. S1). The rates of cells with