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# ANKRD53 interacts with DDA3 and regulates chromosome integrity during mitosis

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ABSTRACT

Spindle dynamics drives chromosome movement and mitotic progression during mitosis. Microtubule (MT)-associated proteins (MAPs) regulate MT stabilization/destabilization and MT polymerization/depolymerization for congression of sister chromatids at the mitotic equator and subsequent segregation toward the spindle poles. Here, we identified ANKRD53 as a novel DDA3-interacting protein through proteomic analysis. Based on expression profiles, ANKRD53 is phosphorylated by mitotic kinases during mitosis. In ANKRD53-depleted HeLa cells, the progression of mitosis was delayed and the number of unaligned chromosomes increased substantially. In addition, spindle MT polymerization decreased and the spindle assembly checkpoint (SAC) was concomitantly activated by the decreased spindle dynamics in ANKRD53-depleted cells. Although ANKRD53 is recruited to the mitotic spindle by DDA3, it counteracts the activity of DDA3 for spindle MT polymerization. Furthermore, ANKRD53 depletion increased the number of bi-nuclei and polylobed nuclei. Thus, ANKRD53 is recruited to the mitotic spindle by DDA3 and acts as a regulator of spindle dynamics and cytokinesis.



## 1. Introduction

To maintain genetic stability, cells have evolved biochemical pathways that ensure completion of phase-specific events before passage into the next phase of the cell cycle [1]. Animal cells have several checkpoints, such as the G1/S and G2/M checkpoints [2]. These systems perceive damaged or incompletely replicated DNA and function to prevent transmission of damaged DNA to daughter cells. In addition to the DNA damage checkpoints, cells also possess a spindle assembly checkpoint (SAC). In all eukaryotic cells, bipolar spindle formation is required for the accurate division of the duplicated genome between the two daughter cells [3]. During prometaphase, microtubules emerge from centrosomes to seek and capture the kinetochores (KTs) of condensed chromosomes. At the same time, outer KT proteins concentrate at the KTs, providing a platform for KT–MT attachment. When all chromosomes are aligned at the metaphase plate with amphitelic attachment, chromosome segregation is initiated and each sister chromatid moves toward opposite spindle poles in anaphase. During this process, the SAC monitors defects in spindle orientation and interaction between KTs and spindle MTs and prevents the precocious separation of sister chromatids [4]. While Mad2 surveils KT–MT attachment, BubR1 acts as a sensor for inter-KT tension, which is generated by appropriate spindle bi-orientation [5]. Therefore, SAC activity is triggered by unattached KTs or insufficient tension between sister KTs resulted from monotelic, syntelic, or merotelic attachment. The activation of checkpoint proteins inhibits the anaphase-promoting complex/cyclosome (APC/C), which can initiate anaphase by degrading the separase inhibitor, securin, and concomitantly degrading cohesin between sister chromatids. When correct spindle orientation is achieved, the checkpoint is cleared, checkpoint proteins such as Mad2 and BubR1 are released from KTs, and sister chromatid segregation is triggered. In this respect, appropriate spindle MT dynamics is essential for execution of KT–MT attachment and establishment of spindle bi-orientation. Spindle dynamics is controlled by MT nucleators, MT depolymerases, and MT-associated proteins (MAPs). MAPs can be classified into MT-stabilizing factors and destabilizing factors [3,6–8]. Although several motor proteins and regulators for spindle dynamics have been extensively investigated, many aspects of the regulatory mechanism remain unclear.

ANKRD53 is a 530-amino acid protein that contains three ankyrin (ANK) repeats, which is one of the most common protein motifs [9]. The ANK repeat, which consists of ~33 residues, forms

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antiparallel α-helices stacked side-by-side and linked by β-hairpin motifs. Because 24 copies of this sequence are present in the cytoskeletal protein ankyrin [10], this motif has been named the ANK repeat. Although it does not recognize specific sequence motifs or protein modifications, the ANK repeat containing proteins have diverse biological roles and specific functions. ANK repeats function as scaffolds to facilitate protein–protein interactions involved in signal transduction, cell cycle regulation, vesicular trafficking, inflammatory response, cytoskeleton integrity, and transcriptional regulation [11]. To date, the ANK repeat motif has been identified in more than 400 proteins, including Cdk inhibitors, transcriptional regulators, cytoskeletal organizers, developmental regulators, and toxins [12,13]. For instance, the β-hairpin motifs of repeats 4, 5, and 6 of IκBα interact with the dimerization interface of NF-κB and inhibit its transcription by sequestering it in the cytoplasm. Cells with ankyrin repeat and KH Domain Containing 1 (ANKHD1) depletion showed decreased cell growth and delayed cell cycle progression at the S phase [14–16]. However, the functions of ANK repeat proteins in the eukaryotic cell cycle remain unknown.

In this study, we discovered that ANKRD53, which is an ANK repeat protein, interacts with DDA3 and regulates spindle stability during mitosis. ANKRD53 is phosphorylated by mitotic kinases and regulates spindle attachment to KTs by facilitating MT polymerization. In addition, ANKRD53 is involved in maintaining nuclear integrity by preventing formation of multi-nuclei such as bi-nuclei and polylobed nuclei.

## 2. Materials and methods

### 2.1. Antibodies

Anti-GFP (Green Fluorescence Protein) sera were raised against full-length recombinant GFP and affinity-purified. Anti-β-tubulin E7 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank (USA). The following antibodies were obtained from commercial sources: anti-Hsp90, anti-ANKRD53, anti-p38MAPK, and anti-Cyclin B1 (Santa Cruz Biotechnology); anti-Mad2 (Thermo Scientific Pierce Antibodies); anti-BubR1 (LifeSpan BioSciences); anti-CREST (Antibodies Incorporated); and anti-Hec1 (Gentex).

### 2.2. Cell culture and transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, WelGENE Inc.) supplemented with 10% fetal bovine serum (FBS, Invitrogen), penicillin (100 units/mL), and 100 μg/mL streptomycin (Invitrogen). The cells were maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$. siRNAs were synthesized by Bioneer, Inc. (South Korea). The sequences of the siRNAs targeting ANKRD53 were 5′-ACCUUGAUACUCAAUCAGG-3′ (siANKRD53-1) and 5′-GGAGAAUGUUGAAACACAAUU-3′ (siANKRD53-2). The sequence of the siRNA targeting BubR1 was 5′-GAUGGUGAAUUGUGGAAUA-3′. siRNA (siGL2) with the sequence 5′-UGAUAGCAGCUUCUCUGAG-3′ was used as the control. siRNAs were transfected into HeLa cells using DharmaFect 1 (Dharmacon, Inc.).

### 2.3. Immunofluorescence

HeLa cells on coverglasses were fixed with methanol at −20 °C for 30 min. Alternatively, cells were extracted with BRB80-T buffer (80 mM PIPES, pH 6.8, 1 mM $MgCl_2$, 5 mM EGTA, and 0.5% Triton X-100) and then fixed with 4% paraformaldehyde for 15 min at room temperature (Fig. 1B). The fixed cells were then permeabilized and blocked with PBS-BT (1 × PBS, 3% BSA, and 0.1% Triton X-100) for 30 min at room temperature. Coverslips were then incubated in primary and secondary antibodies diluted in PBS-BT. Images were acquired with AxioVision 4.8.2 (Carl Zeiss) under a Zeiss Axiovert 200M microscope using a 1.4 NA plan-Apo 100 × oil immersion lens and a HRm CCD camera. Deconvolved images were obtained using AutoQuant X3 (AutoQuant Imaging). All images are maximum projections from z stacks of representative cells stained for the indicated antigens. Images for quantification were acquired under a constant exposure in each channel for all of the cells.

### 2.4. Live cell images

For time-lapse microscopy, HeLa cells stably expressing GFP-H2B were cultured in Leibovitz's L-15 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 2 mM L-glutamine (Invitrogen). Cells were placed into a sealed growth chamber heated to 37 °C and observed on a Zeiss Axiovert 200 M microscope with a 20 × lens. Images were acquired every 3 min for 5 h with AxioVision 4.8.2 (Carl Zeiss).

### 2.5. Statistical analysis

Data were analyzed using Student's *t*-test. Error bars represent the SE of several independent experiments. A p-value < 0.05 (two-tailed) was considered statistically significant. *, $p < 0.05$; **, $p < 0.01$ (two-tailed *t*-test relative to control cells).

## 3. Results and disscussion

### 3.1. ANKRD53 interacts with DDA3 and localizes at the spindle around the spindle poles in mitosis

DDA3 acts as a novel MT-destabilizing factor by recruiting the MT depolymerase, Kif2a, and regulates spindle dynamics to facilitate spindle attachment to KTs and chromosome movement [17]. To investigate the precise function of DDA3 in spindle dynamics, the mitotic complex of DDA3 was purified [17,18]; ANKRD53 was identified as a DDA3-interacting protein through proteomic analysis (Fig. 1A). To further investigate this interaction, HeLa S3 cells stably expressing GFP-DDA3 were treated with thymidine-nocodazole (TN) and released into fresh media. Lysates of mitotic cells were immunoprecipitated with anti-GFP antibodies. Western blot analysis revealed that GFP-DDA3 and endogenous ANKRD53 interacted at 1 h after release from TN arrest (TN1) (Fig. 1B), suggesting that ANKRD53 interacts with DDA3 after prometaphase. To confirm whether ANKRD53 acts as a mitotic protein, we examined the expression profile of ANKRD53 during mitosis. HeLa S3 cells were synchronized at G1/S phase by double thymidine (TT) treatment and released into fresh media. Cells were harvested at the indicated time points and analyzed by western blot. Similar to the expression pattern of cyclin B1, levels of ANKRD53 increased from the G2 phase to mitotic exit (Fig. 1C). Interestingly, ANKRD53 was up-shifted in cells synchronized with thymidine-nocodazole (TN0) and at 1 h after release from TN (Fig. 1D). The shifted ANKRD53 corresponded to the phosphorylated protein because the slower migrating band of endogenous ANKRD53 disappeared in response to the addition of λ-phosphatase (λ-PPase) (Fig. 1E). To determine which mitotic kinase is responsible for ANKRD53 phosphorylation, we used inhibitors of mitotic kinases such as Aurora B inhibitor, Hesperadin, Aurora A inhibitor, VX680, Cdk1 inhibitor, RO-3306, and Plk1 inhibitor, BI2536. As shown in Fig. 1F, only the Plk1 inhibitor did not block ANKRD53 phosphorylation, suggesting that