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Ribosomal protein S3 localizes on the mitotic spindle and functions as a microtubule associated protein in mitosis

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ABSTRACT

The human ribosomal protein S3 (rpS3) has multi-functions such as translation, DNA repair and apoptosis. These multiple functions are regulated by post-translational modifications including phosphorylation, methylation and sumoylation. We report here a novel function of rpS3 that is involved in mitosis. When we examined localization of ribosomal proteins in mitosis, we found that rpS3 specifically localizes on the mitotic spindle. Depletion of the rpS3 proteins caused mitotic arrest during the metaphase. Furthermore, the shape of the spindle and chromosome movement in the rpS3 depleted cell was abnormal. Microtubule (MT) polymerization also decreased in rpS3 depleted cells, suggesting that rpS3 is involved in spindle dynamics. Therefore, we concluded that rpS3 acts as a microtubule associated protein (MAP) and regulates spindle dynamics during mitosis.

1. Introduction

Dramatic changes, including the condensation and alignment of the chromosomes and the reorganization of the cytoskeleton, in cellular organization occur when cells enter into mitosis for cell division. The nuclear envelop membrane also breaks down and nuclear envelop membrane proteins become dispersed throughout the ER membrane. MTs and MT-associated motors form the mitotic spindle to align chromosomes at the metaphase equator and segregate into each daughter cell [1]. The rearrangements of the intracellular organelles, such as the Golgi apparatus, endoplasmic reticulum (ER), and other organelles are less well understood. These organelles should be distributed to the newly divided daughter cells. During interphase, MTs play an important role in positioning, partitioning, and moving the intact ER network and other membranous organelles within the cytoplasm. When cells enter mitosis, the Golgi ribbon structure breaks down and the Golgi apparatus becomes fragmented and forms 100–200 Golgi fragments [2–5] whereas the ER network does not undergo fragmentation. However, it is unclear whether other organelles, such as ribosomes, undergo mitotic reorganization during mitosis.

Protein synthesis is coordinated by the ribosome in all cells. The ribosome is divided into two subunits; a large subunit that binds tRNA molecules and mediates peptidyl transfer and a small subunit that controls mRNA binding, decoding and translational fidelity. The eukaryotic ribosome consists of the small subunit (40S), containing an 18S rRNA and 33 proteins, and the large subunit (60S), containing a 28S rRNA, a 5.8S rRNA and 47 proteins. This central protein synthesis factory is often viewed as a finely tuned machine that functions as a static, reliable component of higher-order cellular processes [6]. In fact, the ribosome has a responsibility to correctly make newly synthesized protein efficiently in the cell. Recently, several ribosomal proteins have been reported to possess other functions aside from the traditional ribosomal function, including induction of apoptosis [7,8], suppression of tumors [9], regulation of development [10], and DNA repair [11–14]. It has also been reported that several ribosomal proteins including rpS3, rpS3a, rpS4, rpL11, and rpL12 were associated with mitotic spindle [15,16]. However, it is not clear whether ribosome itself or free ribosomal proteins bind to mitotic spindle, although microtubules act as paths of intracellular transport of ribosome in interphase [17,18].

Ribosomal protein S3 forms part of the domain on the ribosome, where initiation of translation occurs. It can be cross-linked to eukaryotic initiation factors eIF-2 [19] and eIF-3 [20], and it appears to be directly involved in ribosome–mRNA–aminoacyl tRNA interactions during translation [21]. Previous studies have reported that eukaryotic rpS3 has a DNA repair lyase activity on ariously damaged DNA lesions containing such as cyclobutane pyrimidine dimer, AP sites, 8-oxoG and so on [13,22,23]. Other groups found that *Drosophila* rpS3 has repair activities at sites of DNA damage, such as abasic site and 8-oxoguanine [24], a DNA deoxyribophosphodiesterase activity [25] and DNA glycosylase activity [26]. Therefore, rpS3 seems to have dual functions, both as a ribosome component and as a free ribosomal protein.

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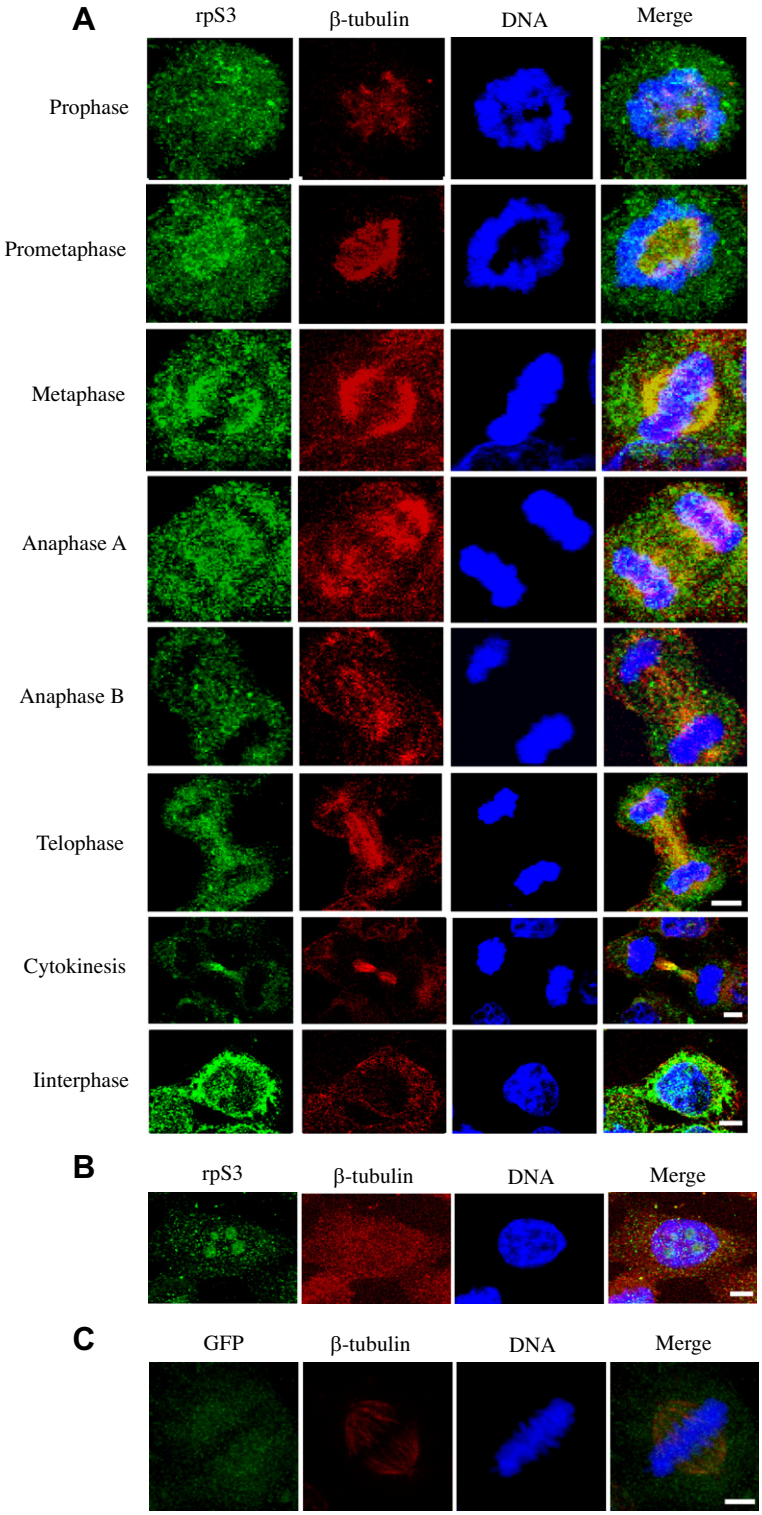

Fig. 1. rpS3 localizes on the spindle during mitosis. (A and B) HeLa cells were fixed with MeOH (A) or paraformaldehyde (B) and stained with antibodies as indicated. Images are maximum projections from z stacks of representative cells stained for rpS3 (green), β-tubulin (red), and DNA (blue). (C) HeLa cells were transfected with GFP-rpS3 and fixed with MeOH. Images are maximum projections from z stacks of representative cells that were stained for GFP (green), β-tubulin (red), and DNA (blue). Scale bars, 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In the present study, we demonstrated that rpS3 localizes onto the mitotic spindle during mitosis. Moreover, our data indicated that rpS3 is involved in MT-spindle polymerization, spindle formation, and chromosome movement. This function is responsible for the chromosome stability in dividing cells.

2. Materials and methods

2.1. Plasmids and antibodies

Full-length wild-type (WT) rpS3 was subcloned into pCS2+, which contains a N-terminal GFP tag.

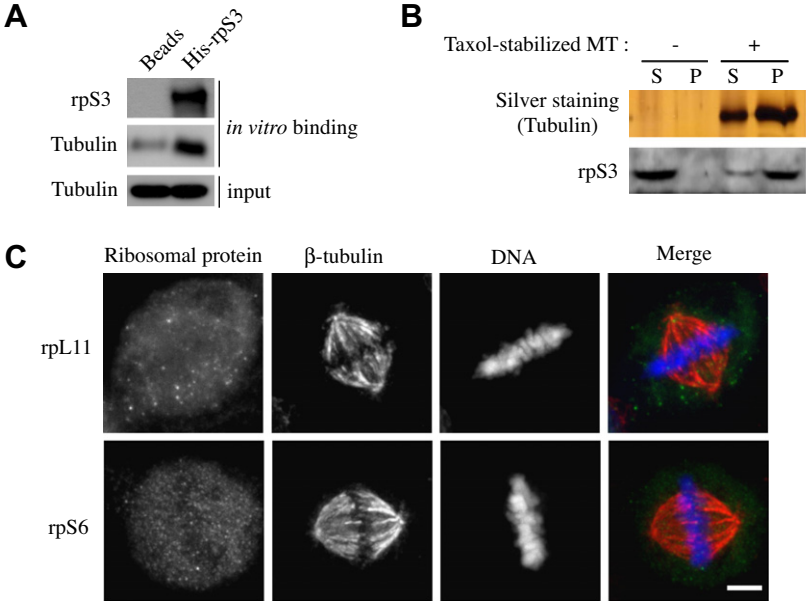

Fig. 2. rpS3 directly interacts with microtubule. (A) Recombinant His-rpS3 was incubated with pure α/β tubulin that had been purified from bovine brain. His-rpS3 was then purified by Ni^{+}-beads and associated α/β tubulin analyzed by Western blotting. (B) Recombinant His-rpS3 was incubated with taxol-stabilized MT and MT sedimentation assay was performed. Proteins present in supernatant (S) and pellet (P) factions were examined by silver staining or Western blotting. (C) HeLa cells were fixed with MeOH and stained with antibodies as indicated. Shown are maximum projections from deconvolved z stacks of representative cells stained for ribosomal proteins (green), β-tubulin (red), and DNA (blue). Scale bar, 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Anti-rpS6 (2217) antibody was purchased from Sigma (USA). Anti-rpL11 (sc-25931), anti-rpL13 (sc-100829) and anti-RACK1 (sc-10775) antibodies were purchased from Santa Cruz Biotechnology (USA). Anti-rpL26 (ab-59567) antibody was purchased from Abcam (UK). Anti-rpS3 antibody was obtained from AdipoGen (South Korea). Anti-β-tubulin E7 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank (USA).

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 sequence targeting rpS3 (siRpS3-203 and siRpS3-635) was 5′-CGGGAACTGACTGCTGTCGTACAG-3′ and 5′-AGCATTGTGGAACCCAAGGACGAG-3′. The control siRNA (siGL2) was 5′-CGTACGCGGAATACTTCGATT-3′. 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 the 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 (1X 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 using an LSM image examiner (Carl Zeiss, Germany) under a Zeiss LSM510 confocal microscope and 63× oil immersion lens. Some 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 an HRm CCD camera. Deconvolved images were obtained using AutoDeblur v9.1 and AutoVisualizer v9.1 (AutoQuant Imaging).

2.4. In vitro binding assay

In vitro binding assay was performed as described [27]. His-rpS3 was bound to His-Bind Resin (Novagen, USA), and 5 μg of purified tubulin was incubated with rpS3-bound resin in 100 μl of the BRB80 buffer (80 mM PIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) at 4 °C for 2 h. After a brief centrifugation, the beads were washed three times with 1 ml of BRB80 buffer and resuspended in elution buffer (1 M imidazole, 500 mM NaCl, and 20 mM Tris–HCl [pH 7.9]). The samples were subjected to SDS–PAGE and immunoblotting with a primary anti-tubulin antibody and a secondary peroxidase-conjugated monoclonal anti-rabbit IgG (Sigma).

2.5. MT pelleting assay

MT pelleting assay was performed as described [28]. Recombinant His-rpS3 was incubated with 2 mM GTP, 10 μg/ml each of leupeptin, pepstatin, and chymostatin, 20 μM taxol, and 3.6 μM taxol-stabilized MTs in BRB80 buffer (80 mM PIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) at room temperature for 30 min and then pelleted through a 150 μl 40% glycerol cushion containing 20 μM taxol and protease inhibitors in the BRB80 buffer at 100,000g for 20 min at 30 °C. Pellets were washed three times with the BRB80 buffer and analyzed by SDS–PAGE, followed by silver staining and Western blotting.

3. Results and discussion

3.1. rpS3 localizes onto the spindle in mitosis

To investigate whether ribosome itself associates with mitotic spindle during mitosis, human HeLa cells were stained with several specific antibodies against ribosomal proteins. Among spindle associated ribosomal proteins [15,16], we tested the localization

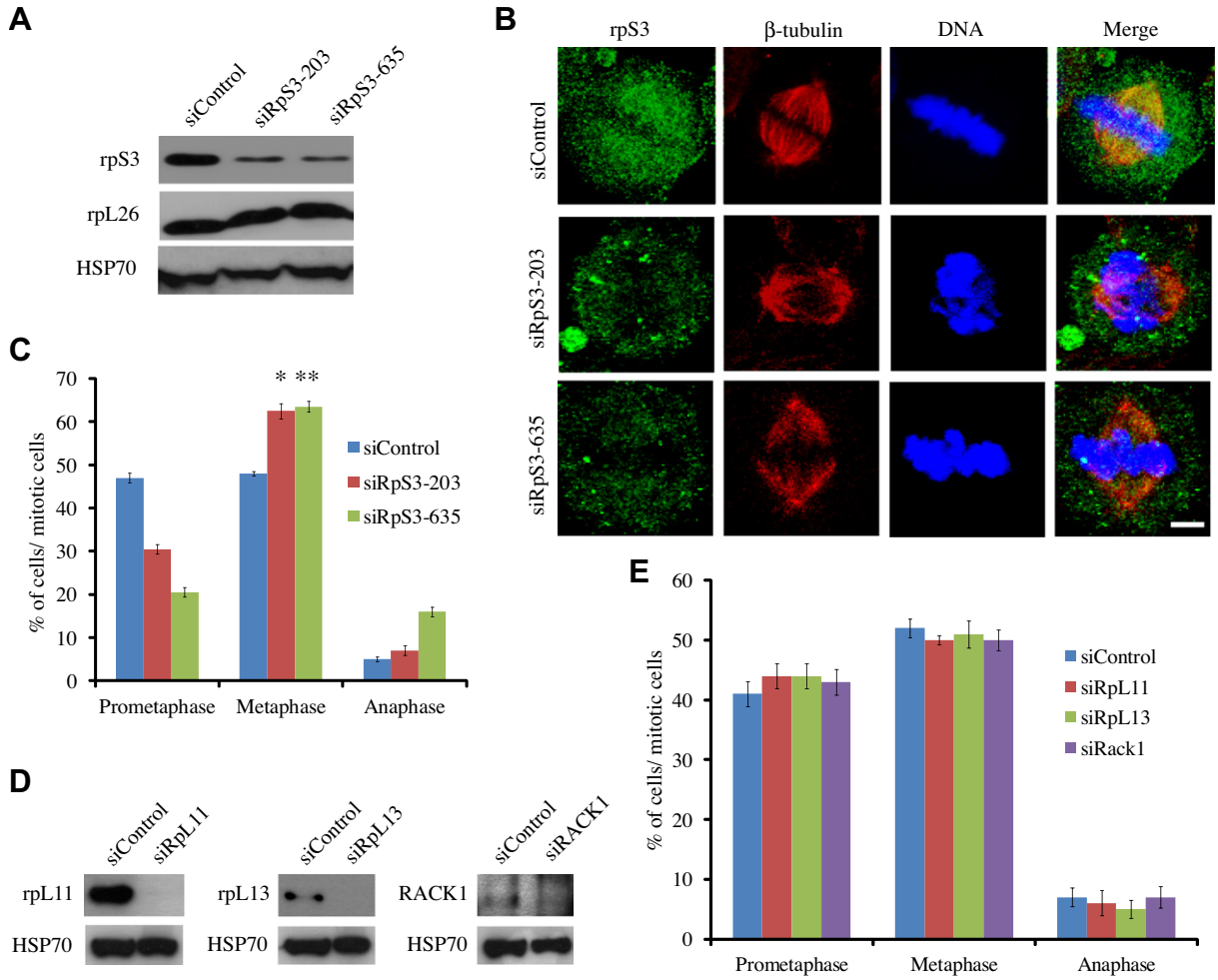

Fig. 3. rpS3 has mitotic functions involved in spindle formation and chromosome movement. (A) HeLa cells were transfected with control (siControl) or rpS3-specific siRNAs (siRpS3–203 and siRpS3–635). Cells were harvested at 72 h post-transfection and lysates were analyzed by Western blotting against the indicated antibodies. (B) Shown are maximum projections from z stacks of representative control or rpS3-depleted cells stained for rpS3 (green), β-tubulin (red), and DNA (blue). Scale bar, 5 μm. (C) Mitotic population of control or rpS3-depleted cells were counted in three independent experiments ($n = 100$ for each quantification). Error bars show SEM. $^{*}p < 1.212 \times 10^{-3}$; $^{**}p < 2.34 \times 10^{-4}$ (two tailed t-test). (D) HeLa cells were transfected with control (siControl) or siRNAs for indicated ribosomal proteins. Cells were harvested at 72 h post-transfection and lysates were analyzed by Western blotting against the indicated antibodies. (E) Mitotic population of control or ribosomal protein-depleted cells were counted in three independent experiments ($n = 100$ for each quantification). Error bars show SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of rpS3 during mitosis, as it has extra-ribosomal function. Ribosomal proteins were localized in the cytoplasm during the interphase when cells were fixed with methanol (Fig. 1A). Also, ribosomal proteins were observed in the nucleolus when cells were extracted with detergent and fixed with paraformaldehyde (Fig. 1B). Interestingly, ribosomal protein S3 localized to the mitotic spindle from the prometaphase to cytokinesis (Fig. 1A). Other ribosomal proteins, such as rpL11 and rpS6, were diffusely spread out in mitotic cells (Fig. 2C), even-though the signals from abundant ribosomal proteins slightly overlapped with spindle. This indicates that free form of rpS3, but not ribosome itself, bind to the mitotic spindle during mitosis. To confirm whether this localization is specific to rpS3, GFP fused wild type rpS3 was transfected into HeLa cells. As expected, GFP-rpS3 also localized onto the mitotic spindle in metaphase cells (Fig. 1C), suggesting that rpS3 is associated with spindle MT during mitosis. Next, to test whether rpS3 directly interacts with tubulin, we purified pure tubulins and recombinant His-rpS3 and performed His pulldown assay. When compared with beads control, we observed that His-rpS3 efficiently pulled down pure tubulins (Fig. 2A). To further verify the interaction between rpS3 and spindle MT, we performed MT co-sedimentation assay. Purified recombinant His-rpS3 was incubated with taxol-stabilized MT, and then the mixture was loaded onto glycerol cushion and pelleted by centrifugation. In the absence of MT, rpS3 was found in the supernatant fraction. When MT was added, rpS3 was mainly found in the pellet fraction compared with the supernatant fraction (Fig. 2B). Therefore, we suggested that rpS3 is a new microtubule associated protein (MAP) that appears to regulate the mitotic spindle dynamics during mitosis.

3.2. rpS3 is involved in chromosome movement and spindle formation

There are many MAPs that regulate spindle formation and spindle dynamics [1]. Microtubules are highly dynamic polymers that switch between growing and shrinking phases, known as dynamic instability [29]. Mitotic regulators, such as microtubule nucleators, microtubule depolymerases and MAPs, regulate spindle dynamics. Each MAP plays different roles during spindle dynamics. For example, MT-stabilizing factors, such as TPX2, HURP and ch-TOG, directly bind to MT and enhance its stability [1,30,31]. MT-destabilizing MAPs, such as katanin and Op18, destabilize the

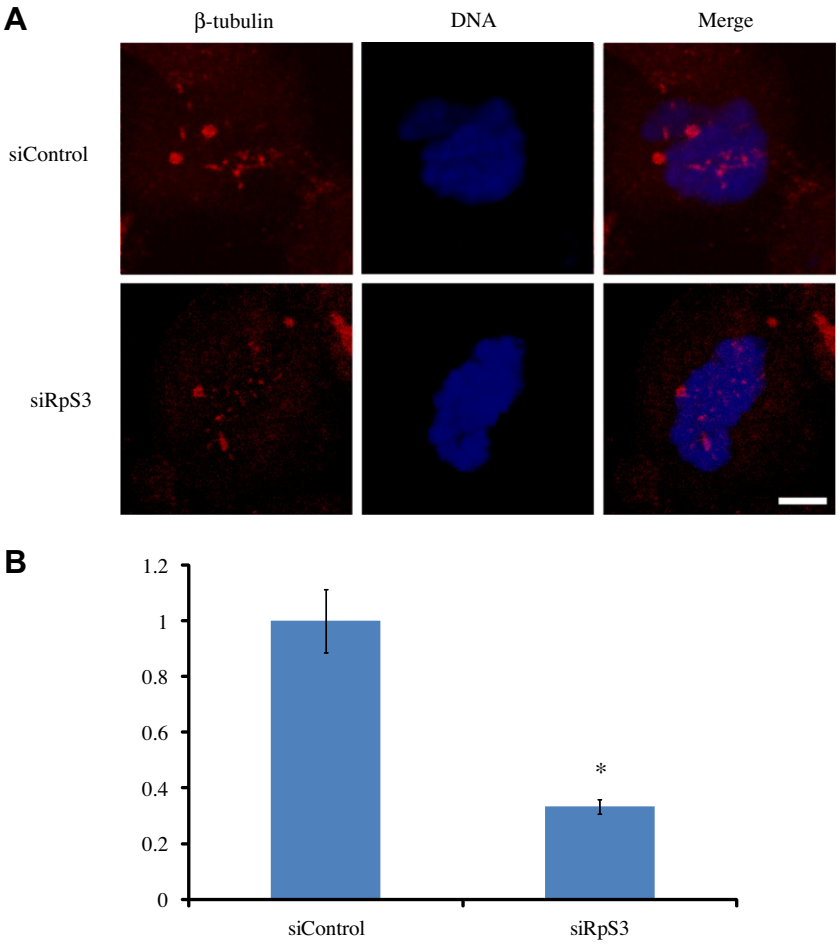

Fig. 4. rpS3 regulates spindle microtubule polymerization. (A) HeLa cells were transfected with a siControl or siRpS3–635. Control or rpS3-depleted cells were treated with 1 μg/ml nocodazole for 10 min at 37 °C, washed, released into fresh media, and fixed with MeOH at the 3 min timepoints. Cells were analyzed by immunofluorescence staining of β-tubulin. Scale bar, 5 m. (B) Images for β-tubulin in (A) were acquired under a constant exposure time. β-tubulin immunofluorescence intensity on spindles was quantified and normalized to control sample ($n = 10$ cells for each quantification). $^{*}p < 2.267 \times 10^{-5}$ (two-tailed t test relative to control cells). Error bar, SEM.

mitotic spindle by severing MTs or by sequestrating the α/β-tubulin dimers. Microtubule plus end proteins (+TIPs), including CLIP-170, CLASP, APC, and EB1, also regulate spindle dynamics [32–35]. Next, we examined whether rpS3 has mitotic functions to regulate spindle formation and chromosome movement. We first evaluated the mitotic function of rpS3 by depletion of rpS3 protein with siRNA. As shown in Fig. 3A, more than 70% of rpS3 protein was depleted with two different specific siRNAs (siRpS3-203 and siRpS3-635) against rpS3. Furthermore, rpS3 almost disappeared from the mitotic spindle in rpS3 depleted cells (Fig. 3B). In order to confirm the function of human rpS3 in spindle dynamics, we observed mitotic spindle and mitotic chromosome in rpS3 depleted cells. Interestingly, the removal of rpS3 from the mitotic spindle decreased the density of MT-spindle and resulted in an abnormal spindle shape (Fig. 3B), suggesting that rpS3 is involved in spindle formation and spindle dynamics.

The mitotic spindle generates a driving force for chromosome movement, such as chromosome congression and segregation by polymerizing and depolymerizing MTs. To evaluate the function of rpS3 in chromosome movement, we therefore took a closer look at chromosome alignment in metaphase cells among the rpS3 depleted cells. As shown in Fig. 3B, the chromosome alignment at the metaphase plate in rpS3 depleted cells was not perfect, indicating that rpS3 is needed for chromosome congression to the metaphase plate.

Further analysis revealed a significant increase of metaphase cells in rpS3-depleted mitotic cells compared with the control samples (Fig. 3C). Furthermore, there was more than a tenfold increase in the number of metaphase cells with unaligned chromosomes when rpS3 was depleted, suggesting that spindle assembly checkpoint is activated by unaligned chromosomes generated by the depletion of rpS3 and arrests of mitotic progress at metaphase. In contrast, depletion of other ribosomal proteins including rpL11, rpL13, and RACK1 did not affect mitotic population and chromosome alignment (Fig. 3D and E). Based on these findings, we concluded that rpS3 has a novel mitotic function that is essential for chromosome congression and mitotic progression.

3.3. rpS3 is involved in MT polymerization

Several mitotic regulators, such as DDA3, HURP and EB1, regulate spindle dynamics by increasing MT-polymerization or MT-depolymerization. The spindle dynamics are essential for chromosome movement, including chromosome congression and chromosome segregation. Therefore, a decrease in the spindle dynamics can cause defects in chromosome movement. To explore the exact mitotic function of rpS3, we analyzed the MT-repolymerization after removal of the mitotic spindle from mitotic cells using nocodazole treatment. As shown in Fig. 4A and B, MT-polymerization was slower in rpS3 depleted cells than control

cells, indicating that rpS3 is required for MT-polymerization during mitosis.

As a ribosomal protein, bacterial rpS3 homolog cross-links with several other ribosomal proteins, including S2, S4, S5, S9, S10, S12 and S13 in the 30S subunit and L7/L12 in 50S subunit [36]. Moreover, the putative path taken by the mRNA is composed of the components likely to interact with mRNA, such as S3, S4, S5, S7, S11, S12 and S18 [37], implying that the rpS3 is located around the decoding center and is involved in the decoding process which is responsible for the accuracy in translation. Several ribosomal proteins have been found to play roles in translation along with various extra-ribosomal functions, such as the induction of apoptosis and DNA repair [38]. Among these, rpS3 is involved in DNA repair including DNA repair endonuclease and translational function [13]. Moreover, these functions of rpS3 seem to be regulated by post-translational modifications, including phosphorylation, dephosphorylation and sumoylation [27,39,40]. Accordingly, the mechanism by which rpS3 regulates spindle dynamics through protein modification and protein interaction with other MAPs remains to be elucidated.

In summary, we demonstrated that rpS3 binds to the mitotic spindle throughout the mitosis. Furthermore, rpS3 functions as a MAP to control spindle dynamics by regulating MT polymerization. Our novel data clearly showed that rpS3 plays a role in maintaining chromosomal stability during mitosis. However, the mitotic reorganization of the ribosome or ribosomal proteins during mitosis remains to be elucidated.

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