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Centlein, a novel microtubule-associated protein stabilizing microtubules and involved in neurite formation

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ABSTRACT

We have previously reported that the centriolar protein centlein functions as a molecular link between C-Nap1 and Cep68 to maintain centrosome cohesion [1]. In this study, we identified centlein as a novel microtubule-associated protein (MAP), directly binding to purified microtubules (MTs) via its longest coiled-coil domain. Overexpression of centlein caused profound nocodazole- and cold-resistant MT bundles, which also relied on its MT-binding domain. siRNA-mediated centlein depletion resulted in a significant reduction in tubulin acetylation level and overall fluorescence intensity of cytoplasmic MT acetylation. Centlein was further characterized in neurons. We found that centlein overexpression inhibited neurite formation in retinoic acid (RA)-induced SH-SY5Y and N2a cells. Taken together, we propose that centlein is involved in MT stability and neuritogenesis in vivo.

1. Introduction

MTs are one of the major cytoskeletal components that are composed of α-tubulin/β-tubulin heterodimer subunits [2,3] and serve many vital roles in diverse cellular functions, including intracellular transport, organelle positioning, chromosome segregation, neurite outgrowth, ciliogenesis, cell migration and cell morphogenesis [4]. The ability of MTs to fulfill its versatile cellular functions relies on its intrinsically dynamic polymer properties [3], a variety of MAPs [5] and post-translational modifications such as acetylation, detyrosination, polyglutamylation and polyglycylation [6–11]. It is well established that the dynamics and organization of the MT cytoskeleton are regulated largely by MAPs [12,13], particularly during neuronal morphogenesis, a process by which neurons extend dendrites and axons [14].

In this study, we identified a novel MAP, centlein. Centlein was previously characterized as a centriolar protein mediating an interaction between C-Nap1 and Cep68 to maintain centrosome cohesion [1]. However, the other functions of centlein, corresponding to its distinctly subcellular localization, await characterization. Here we have shown that overexpression of centlein bundles MTs resistant to cold shock and nocodazole treatment, while depletion of centlein leads to a marked reduction in the amount of acetylated tubulin and overall staining intensity of cytoplasmic MT acetylation. In line with these observations, neurite formation was suppressed in RA-induced SH-SY5Y and N2a cells upon centlein overexpression. Our data thus suggest that centlein is a novel MT-stabilizing protein and negative regulator of neurite formation.

2. Materials and methods

2.1. Plasmid construction and recombinant proteins

Plasmids EGFP tagged full-length and GST-centlein 901-1191aa was described previously [1]. The truncated mutants of centlein, full-length DDA3 and EB3 (obtained from HeLa cDNA)were subcloned into the vector pEGFP-C1. GST-centlein 901-1191aa were expressed in *Escherichia coli* strain BL21 (DE3) and purified with glutathione-Sepharose-4B (17-0757-01, GE Healthcare Life Sciences).

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2.2. Cell culture, transfections and siRNA

U2OS and HeLa cells were cultured in Ham's F12 and DMEM (Hyclone), respectively, and supplemented with 10% (v/v) fetal bovine serum (Hyclone). The SH-SY5Y and N2a cells were cultured in DMEM medium with 10% fetal bovine serum, 0.1 mM non-essential amino acids. All media were supplemented with 100 IU/ml penicillin and 100 mg/ml streptomycin. The cells were grown at

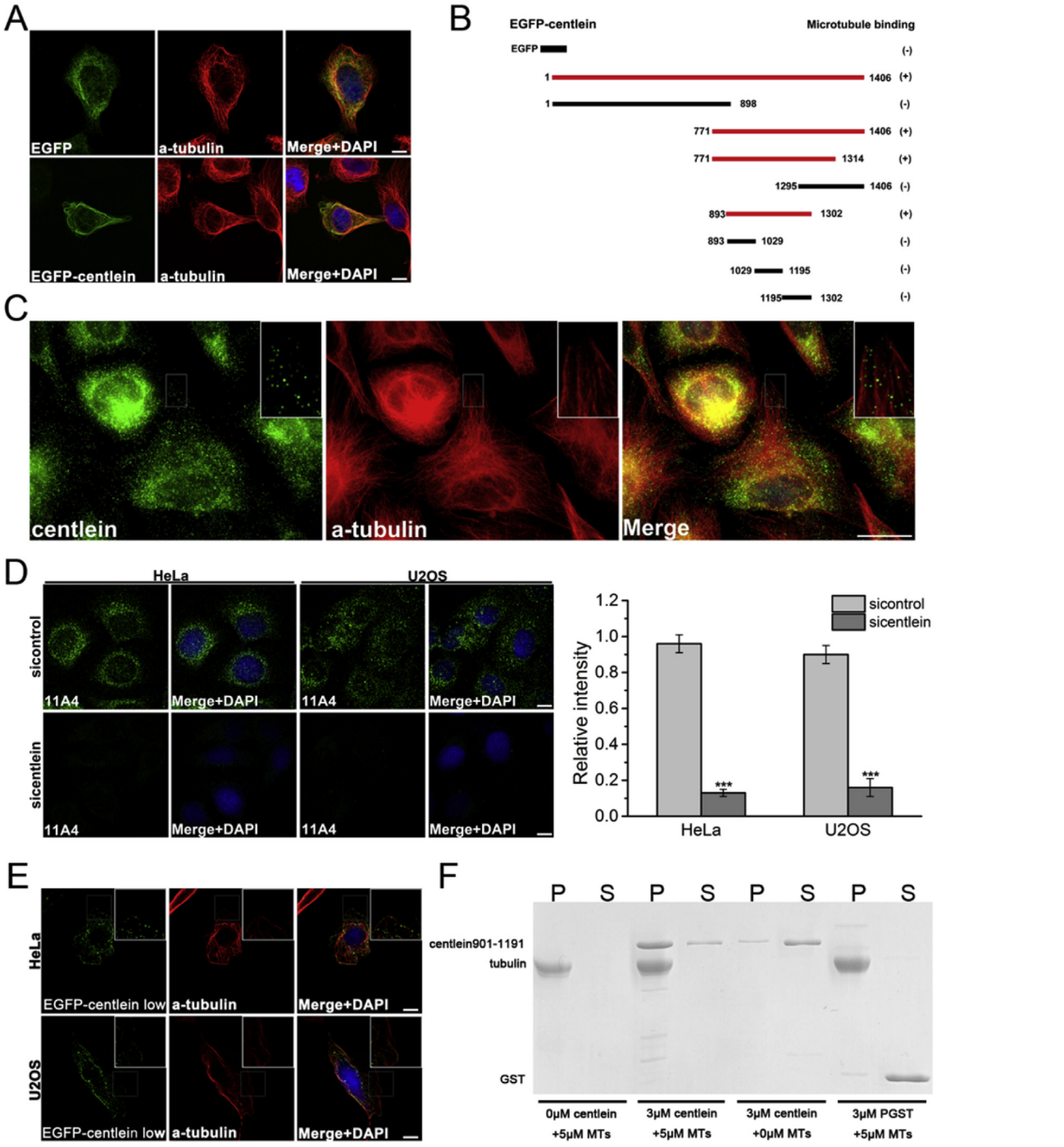

Fig. 1. Centlein is a MAP and induces MT bundle formation. (A) EGFP-centlein were transfected into U2OS cells. The cells were fixed and co-stained with antibodies against GFP (green) and α-tubulin (red) after transfected 24 h. As shown, EGFP-centlein (bottom panel) but not EGFP (top panel) induced MT bundles. (B) The shortest region, inducing bundling of cytoplasmic MTs, was mapped to aa 893–1302 of centlein. The numbers represent amino acid residues. +, red, bundles; −, black, no bundle. (C) Distribution of endogenous centlein in U2OS cells. Immunofluorescence staining using anti-centlein antibody (green) and anti-α-tubulin antibody (red) were shown. The insets show enlarged views of immuno-localization of endogenous centlein and MTs. (D) U2OS and HeLa cells were transfected for 72 h with a nonspecific siRNA (sicontrol) or specific siRNA for centlein (sicentlein), then stained with the indicated antibodies (green) and observed using confocal microscope (left). (D, right) Quantification of the normalized fluorescence intensity of cytoplasmic centlein. Bar graphs represent mean ± sd, n > 200 in three independent experiments (***P < 0.001). (E) Low expression of EGFP-centlein displayed staining pattern similar to endogenous protein. (F) MT co-sedimentation assay. The supernatant (S) and the pellet (P) were subjected to SDS-PAGE and visualized by Coomassie blue staining. Scale bars: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

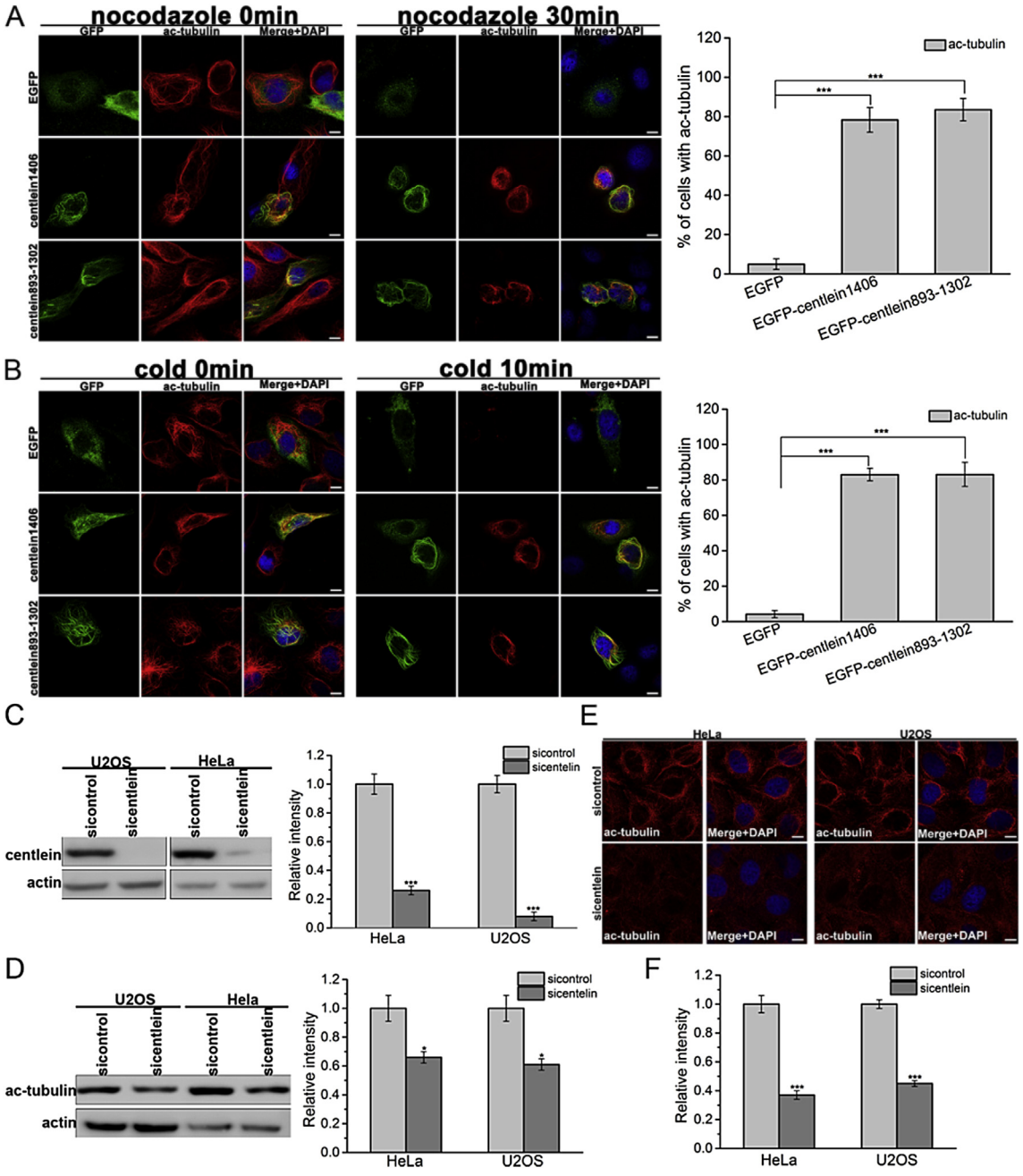

Fig. 2. Centlein enhances MT stability. (A, B) Overexpression of centlein stabilized MTs. U2OS cells overexpressing EGFP, EGFP tagged full-length or truncated centlein aa 893–1302 were treated with 10 μM nocodazole (A, left) for 30 min or on ice (B, left) for 10 min, then fixed and stained with antibodies against GFP and acetylated tubulin. (A, B, right) Quantification of the transfected cells with acetylated tubulin. Bar graphs represent mean ± sd, n > 50 in three independent experiments (***P < 0.001). (C, D) U2OS and HeLa cells were transfected for 72 h with sicontrol or sicentlein. Cell lysates were prepared and analyzed by immunoblotting with anti-centlein (C) or anti-acetylated tubulin (D) antibody. (C, D, right) Quantification of the protein bands. Bar graphs represent mean ± sd in three independent experiments (***P < 0.001, *P < 0.05). (E) U2OS and HeLa cells were transfected for 72 h with sicontrol or sicentlein, then stained with anti-acetylated tubulin (red) antibody and all images have been scaled identically for fluorescence intensity. (F) Quantification of the normalized fluorescence intensity of acetylated tubulin in U2OS and HeLa cells from (E). Bar graphs represent mean ± sd, n > 200 in three independent experiments (***P < 0.001, *P < 0.05). Scale bars: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

37 °C under 5% CO_2. U2OS, HeLa, N2a and SH-SY5Y cells were transfected using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's protocol. Cells were analyzed 24 h after transfection. The siRNA experiments were performed as described previously [1].

2.3. Immunofluorescence microscopy

Immunofluorescence assay was performed as described previously [1]. Antibodies used were as follows: mouse anti-α-tubulin (1:5000, T6199, Sigma), mouse anti-acetylated tubulin (1:1500, T7451, Sigma), rabbit anti-GFP (1:2000, 50430-2-AP, ProteinTech), rabbit anti-βIII-tubulin (1:1000, T2200, Sigma). Centlein antibody mouse clone 11A4 and rat Clone 7H2 were used as described previously [1]. The secondary antibodies used were Alexa Fluor 488 donkey anti-rabbit IgG (1:1500, A21206, Invitrogen), Alexa Fluor 594 goat anti-mouse IgG (1:1500, A11012, Invitrogen), Alexa Fluor 488 goat anti-mouse IgG (1:1500, A11029, Invitrogen),

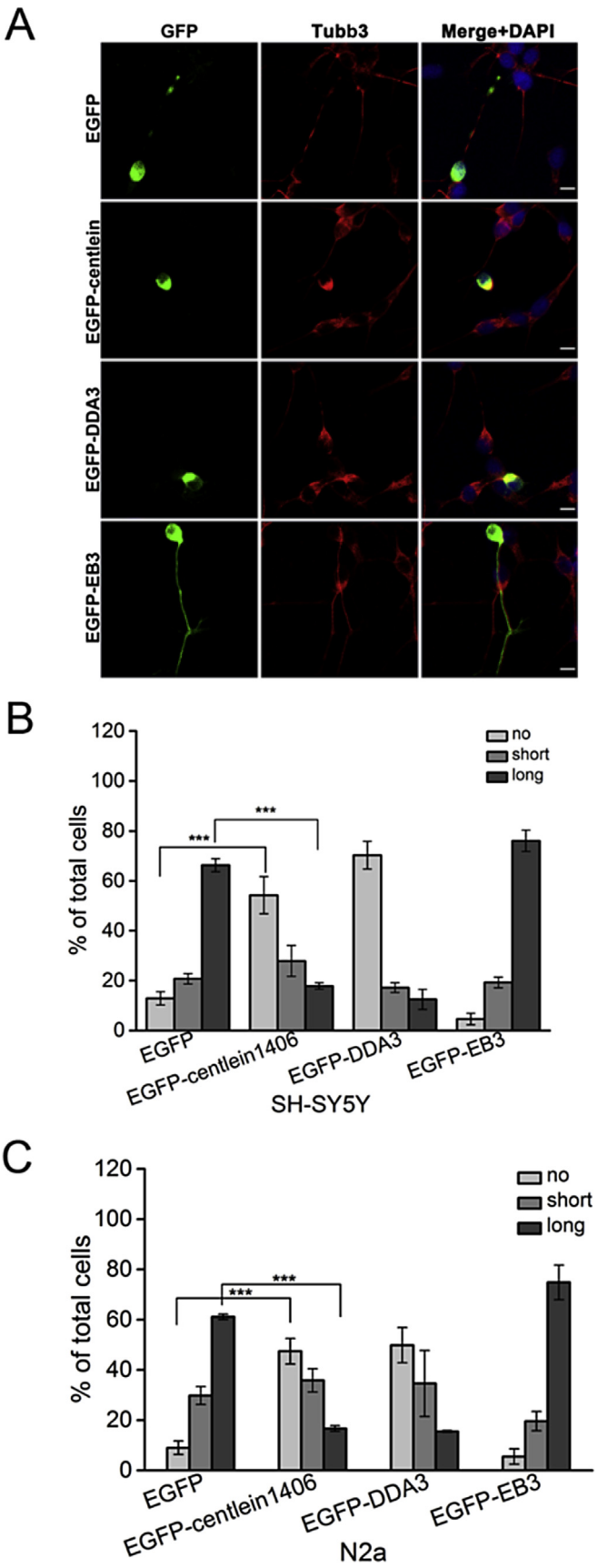

Fig. 3. Overexpression of centlein inhibits neurite formation in SH-SY5Y cells. (A) SH-SY5Y cells overexpressing EGFP (first row), EGFP-centlein (second row), EGFP-DDA3 (third row), EGFP-EB3 (forth row), after 6 h transfection, were induced for differentiation in DMEM containing 10% FBS and 20 μM RA for 4d. The cells were stained with antibodies against Tubb3 (red) and GFP (green). (B) Quantification of the results from (A). Neurites with lengths of 1–2 cell bodies were counted as short neurites; those shorter than one cell body were counted as no neurites; those longer than two cell bodies were categorized as long neurites. Bar graphs represent mean ± sd, n > 50 in three independent experiments (***P < 0.001). (C) The same experiment was performed in N2a cells. Bar graphs represent mean ± sd, n > 50 in three independent experiments (***P < 0.001). Scale bars: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Alexa Fluor 594 goat anti-rabbit IgG (1:1500, A11012, Invitrogen). To simultaneously visualize centlein (11A4) and α-tubulin, a mouse anti-α-tubulin antibody was covalently coupled to Alexa Fluor 594, using an APEX Antibody Labeling Kit (A10474, Invitrogen). Immunofluorescence microscopy was performed using a ZEISS LSM780 confocal microscope.

2.4. Immunoblotting

Immunoblot analysis was performed as previous reported [1]. Antibodies were as follows: mouse anti-α-tubulin (1:5000), mouse anti-acetylated tubulin (1:1200), rabbit anti-GFP (1:2000). Secondary antibodies were (HRP)-conjugated goat anti-mouse IgG (1:10000 GAM0072, Liankebio), swine anti-rabbit IgG (1:10000, P0399, DakoCytomation).

2.5. Microtubule co-sedimentation assay

This experiment was performed as described [15]. In brief, purified GST-centlein 901-1191 fusion protein was incubated with MTs, 10 mM taxol, and 2 mM ATP in PEM buffer (80 mM PIPES, 1 mM $MgCl_2$, and 1 mM EGTA, pH 6.8). After incubation at room temperature, the reaction was centrifuged at 55,000 g. The supernatant and pellet fraction were analyzed by SDS-PAGE individually. Proteins of the supernatant and pellet were visualized by Coomassie Brilliant Blue staining. This assay was repeated three times.

2.6. Neurite outgrowth assay

Differentiation was assessed in SH-SY5Y cells using the previously reported neurite outgrowth assay [16]. Immunofluorescence was performed using antibody against βIII-tubulin. Differentiation of N2a cells were induced as the previously reported neurite outgrowth assay [17,18]. Differentiation is expressed as percentage transfected cells showing differentiated morphology of total transfected cells. The cells were counted from each of three separate transfection experiments.

2.7. Measurements and statistical analysis

Image J software was used to measure the intensity and neurite length. The detailed methods of measurements are detailed elsewhere [19]. The statistical significance of the difference between two means was determined using a two-tailed Student's t-test. Differences were considered significant when $P < 0.05$.

3. Results and discussion

3.1. Centlein is a novel MAP

We have reported that centlein is localized to the proximal ends of centrioles and required for centrosome cohesion by mediating an interaction between C-Nap1 and Cep68 [1]. During the course of our investigation, we unexpectedly found that overexpression of

the full-length centlein induced a striking formation of filamentous structures in both U2OS and HeLa cells, identified as MTs by co-staining with α-tubulin (Fig. 1A and Fig. S1A). We further mapped the region responsible for induction of bundling of cytoplasmic MTs. A series of deletion mutants of centlein were generated and transfected into U2OS cells. As shown in Fig. 1B and Fig. S1B, a portion, amino acids (aa) 893-1302, containing the longest coiled-coil domain (aa 980-1311), was essential for bundling of MTs by centlein.

We next used immunofluorescence microscopy to examine the association between cytoplasmic MTs and endogenous centlein. Centlein was detected as distinct speckles throughout the cytoplasm (Fig. 1C). The authenticity of these cytoplasmic centlein speckles was verified by using siRNA-mediated centlein depletion in HeLa and U2OS cells, this resulted in loss of speckles as well as loss of centrosomal labeling (Fig. 1D). Confocal immunolocalization of endogenous centlein and MTs displayed 82.6% (±3.9%, n > 50) centlein speckles on MTs (Fig. 1C) of U2OS cells, which was consistent with U2OS and HeLa cells transiently expressing centlein at low expression levels (Fig. 1E).

The aforementioned results led us to determine whether centlein interacted directly with MTs in a MT co-sedimentation assay. Purified GST-901-1191aa fusion protein was generated and incubated with porcine brain MTs. The fusion protein clearly co-sedimented with preassembled Taxol-stabilised MTs from purified tubulin whereas purified GST remained in the supernatant (Fig. 1F). We thus conclude that centlein is a novel MAP.

3.2. Centlein enhances MT stability

Given the critical roles of MAPs in maintaining and regulating MT stability, we asked whether ectopic expression of centlein was able to stabilize MTs against depolymerizing challenges. To this end, we overexpressed either the full-length or a truncated centlein containing the MT-binding domain tagged with GFP in U2OS or HeLa cells, and subsequently depolymerized the MTs with either addition of nocodazole for 30 min or on ice for 10 min. As shown in Fig. 2A, B and Fig. S2A, S2B, following either treatment, about 80% of the centlein overexpressing cells remained stable and MT network observed by labelling acetylated tubulin, a marker of stable MTs, whereas MTs completely disassembled in the adjacent cells without centlein overexpression. These results indicate that centlein overexpression significantly increases MT stability.

We then sought to examine the effect of centlein siRNA on the level of tubulin acetylation in both HeLa and U2OS cells. Upon depletion of centlein (Fig. 2C), the amount of acetylated tubulin was diminished by 40% (Fig. 2D), concurrent with a marked reduction in overall staining intensity of cytoplasmic MT acetylation (Fig. 2E and F). Altogether, our data suggest that centlein can stabilize MTs in vivo.

3.3. Overexpression of centlein inhibits neurite formation in SH-SY5Y and N2a cells

In neurons, MAPs modulate neuronal shape and neurite outgrowth by influencing the stability and organization of MTs [20,21]. Next, we used the RA-induced SH-SY5Y and N2a cells as a neuronal differentiation model system [22] to examine the effect of centlein overexpression on neurite formation. We first validated the experimental approach by assessment of two known protiens, DDA3 and EB3. It has been shown that overexpression of DDA3 suppressed neurite outgrowth [17], while EB3 promoted neurite formation [17,23]. SH-SY5Y cells expressing EGFP, EGFP-tagged DDA3 or EB3 were treated with RA for neuronal differentiation [16]. After 4d treatment, cells were fixed and stained with the antibodies against EGFP and neuron-specific β-Tubulin III (Tubb3 /TUJ-1) [24] (Fig. 3A). Neurite lengths were measured by Image J software and categorized as long, short and no neurite [17]. In accordance with previous studies, we indeed observed that the neurite lengths of SH-SY5Y cells expressing EGFP-DDA3 were 13.9% (long), 17.2% (short) and 68.9% (no), while the neurite lengths of EGFP-EB3 cells were 76.2% (long), 19% (short) and 4.8% (no), respectively (Fig. 3B).

We then examined the effect of EGFP-centlein on neurite formation. RA-induced neurite outgrowth could be suppressed by overexpression of centlein, but not EGFP itself. The percentage of cells bearing no neurites rose from 12.9% to 54.3%, compared with EGFP control, and this increase was accompanied by a parallel decrease in cells containing long neuritis from 66.3% to 17.8% (Fig. 3B). Similar results were obtained with N2a cells (Fig. 3C). Taken together, our data suggest that centlein may be a novel negative regulator of neurite formation.

Centlein was first identified from MT-cosedimented proteins prepared from rat brain followed by tandem mass spectrometry and ion exchange column chromatography [25]. We later reported that centlein was localized to the proximal ends of centrioles and required for centrosome cohesion by bridging an interaction between C-Nap1 and Cep68 [1]. In this study, we have shown that centlein is a novel MAP exerting its function by stabilizing MTs. We also found that overexpression of centlein inhibited neurite outgrowth, indicating that centlein is a novel negative regulator of neurite formation. Further studies need to be conducted to elucidate the functional association between centlein-induced MT stabilization and suppression of neurite formation, and its involvement in neuritogenesis in vivo.

Acknowledgments

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Transparency document

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.02.079.

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