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CDK5RAP2 Is a Pericentriolar Protein That Functions in Centrosomal Attachment of the γ-Tubulin Ring Complex

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Microtubule nucleation and organization by the centrosome require γ-tubulin, a protein that exists in a macromolecular complex called the γ-tubulin ring complex (γTuRC). We report characterization of CDK5RAP2, a novel centrosomal protein whose mutations have been linked to autosomal recessive primary microcephaly. In somatic cells, CDK5RAP2 localizes throughout the pericentriolar material in all stages of the cell cycle. When overexpressed, CDK5RAP2 assembled a subset of centrosomal proteins including γ-tubulin onto the centrosomes or under the microtubule-disrupting conditions into microtubule-nucleating clusters in the cytoplasm. CDK5RAP2 associates with the γTuRC via a short conserved sequence present in several related proteins found in a range of organisms from fungi to mammals. The binding of CDK5RAP2 is required for γTuRC attachment to the centrosome but not for γTuRC assembly. Perturbing CDK5RAP2 function delocalized γ-tubulin from the centrosomes and inhibited centrosomal microtubule nucleation, thus leading to disorganization of interphase microtubule arrays and formation of anastral mitotic spindles. Together, CDK5RAP2 is a pericentriolar structural component that functions in γTuRC attachment and therefore in the microtubule organizing function of the centrosome. Our findings suggest that centrosome malfunction due to the *CDK5RAP2* mutations may underlie autosomal recessive primary microcephaly.

INTRODUCTION

In animal cells, the centrosome is the primary microtubule (MT) organizing center (MTOC), which plays a key role in the control of the temporal and spatial distribution of MT networks (Ou and Rattner, 2004; Doxsey *et al.*, 2005; Luders and Stearns, 2007). Typically, centrosomes are positioned at the focus of a radial array of MTs during interphase and are incorporated into spindle poles during mitosis. Interphase centrosomes are composed of a pair of centrioles embedded in a cloud of electron-dense pericentriolar material (PCM). The centriole has a well-defined structure with MT triplets arranged into a cylinder, whereas the organization of the PCM is less apparent. Early studies have shown the existence of a salt (2 M KI)-insoluble scaffold/matrix underlying the PCM (Moritz *et al.*, 1998; Schnackenberg *et al.*, 1998). This matrix, formed with a high content of large coiled-coil proteins, provides binding sites for the tubulin family member γ-tubulin and other proteins associated with centrosomal functions, such as MT nucleation. Through the cell cycle, the PCM varies in volume and the MT-nucleating activity, which are smallest in G1-phase and biggest during mitosis. In addition, proteins have a precise and cell cycle–specific placement within the PCM that has been observed as a tubular structure located along the surface of the centriole and around its proximal end but not around its distal end (Ou and Rattner, 2000; Ou *et al.*, 2003). The outer surface of the PCM is dynamic and PCM proteins found in the cytoplasm transit to the PCM either by diffusion or via MTs.

Distributed throughout the PCM, γ-tubulin exists in a ring-shaped multiprotein complex, known as the γ-tubulin ring complex (γTuRC) that mediates MT nucleation (Zheng *et al.*, 1995). The γTuRC consists of at least six γ-tubulin complex proteins (GCPs) in addition to γ-tubulin (also known as GCP1; Wiese and Zheng, 2006; Luders and Stearns, 2007; Raynaud-Messina and Merdes, 2007). Among them, γ-tubulin, GCP2, and GCP3 form a structural unit that is repeated throughout the distinct ring structure of the γTuRC; other GCPs may form a cap on one side of the complex. There is a large cytoplasmic pool of the γTuRC, which is recruited to the centrosome and incorporated into the PCM (Felix *et al.*, 1994; Stearns and Kirschner, 1994; Meads and Schroer, 1995; Moudjou *et al.*, 1996). Several proteins, including pericentrin/kendrin, AKAP450 (also known as AKAP350, CG-NAP, and hyperion), ninein, Nlp (ninein-like protein), and GCP-WD/NEDD1 have been implicated in the integration of the γTuRC with the centrosome. Pericentrin, a scaffold component of the PCM, displays a mitosis-specific property of attaching γ-tubulin at spindle poles (Dictenberg *et al.*, 1998; Zimmerman *et al.*, 2004). Ninein localizes to specific sites within the PCM, whereas Nlp appears at the centrosome in certain stages of the cell cycle (Ou *et al.*, 2002; Casenghi *et al.*, 2003; Delgehyr *et al.*, 2005). GCP-WD/NEDD1 is a recently found γTuRC component that is required for γTuRC attachment to the centrosome and plays a role in centriole duplication and the

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Abbreviations used: MT, microtubule; MTOC, microtubule-organizing center; PCM, pericentriolar material; γTuRC, γ-tubulin ring complex; γTuC, γ-tubulin complex; GCP, γ-tubulin complex protein; MCPH, autosomal recessive primary microcephaly; Cnn, centrosomin; PDE4DIP, phosphodiesterase 4D-interacting protein.

organization of interphase and mitotic MT arrays (Gunawardane *et al.*, 2003; Haren *et al.*, 2006; Luders *et al.*, 2006).

CDK5RAP2 was discovered in a yeast two-hybrid screen to interact with the neuronal p35 activator of Cdk5 (Ching *et al.*, 2000). Although CDK5RAP2 is highly expressed in the brain, its RNA transcript has been detected in all human tissues examined (Ching *et al.*, 2000). Interestingly, CDK5RAP2 mutations cause autosomal recessive primary microcephaly (MCPH), a cell cycle disorder of neural progenitors during neurogenesis (Bond *et al.*, 2005). The CDK5RAP2 protein is found at centrosomes of the cultured human cells (Andersen *et al.*, 2003; Bond *et al.*, 2005), implicating its function at the centrosome. In this report, we show that CDK5RAP2 is present throughout the PCM and interacts with the γTuRC through a conserved region of CDK5RAP2, tethering γ-tubulin to the centrosome. The association of CDK5RAP2 with the γTuRC is essential for the MT nucleating and organizing function of the centrosome. Loss of CDK5RAP2 function resulted in disorganized interphase MT networks and anastral mitotic spindles. Therefore, through its interaction with the γTuRC, CDK5RAP2 plays a direct role in the MT-organizing function of the centrosome.

MATERIALS AND METHODS

DNA Constructs

The cDNA of human CDK5RAP2 (GenBank accession: AK122913) was obtained from the Biological Resource Center of the National Institute of Technology and Evaluation, Japan. The entire coding region was subcloned into pFLAG-CMV2 (Sigma, St. Louis, MO). CDK5RAP2 Fragments were amplified by PCR or generated by restriction digestions from the cDNA and then subcloned into pFlag-CMV2, pET28a (Novagen, Madison, WI), or pGEX-4T (GE Healthcare, Waukesha, WI). EGFP was engineered into CDK5RAP2(58-90)/pFlag-CMV2 for expression of amino acids (aa) 58-90, with consecutive Flag and GFP at the amino-terminus.

Antibodies

To generate CDK5RAP2 antibodies, aa 1-70 and 1486-1552 were expressed in *Escherichia coli* BL21(DE3) in fusion with either 6xHis or glutathione *S*-transferase (GST) and were purified by virtue of the binding of 6xHis to Ni^{2+}-nitrilotriacetic acid resin (Qiagen, Chatsworth, CA) or GST to GSH-beads (GE Healthcare). The 6xHis-tagged proteins were used to immunize rabbits. The GST proteins were immobilized onto CNBr-activated Sepharose (GE Healthcare) to generate two antigen-coupled columns. The antibodies were purified by absorption of the sera to respective antigen columns and elution at low pH. The generation of anti-CEP250 antibody has been described previously (Ou and Rattner, 2000). An anti-GCP3 antibody was a gift from Drs. M. Takahashi and Y. Ono (Kobe University, Japan; Takahashi *et al.*, 2002). We also generated a GCP3 antibody by immunizing rabbits with the 6xHis-tagged GCP3 fragment aa 1-240 and purified the antibody using immobilized GST-GCP3(1-240). A rabbit anti-pericentrin antibody (polyclonal M8) was obtained from Dr. Stephen J. Doxsey (University of Massachusetts; Dictenberg *et al.*, 1998). The mAb GT335 was from Dr. Bernard Edde (Centre de Recherches de Biochimie Macromoléculaire, France; Bobinnec *et al.*, 1998). An antibody recognizing centrin (mouse 20H5) was from Dr. Jeffrey L. Salisbury (Mayo Clinic; Sanders and Salisbury, 1994). Other primary antibodies used were: anti-γ-tubulin (GTU88, Sigma), anti-α-tubulin (Ab-1, Oncogene, Boston, MA), anti-β-tubulin (Sigma), anti-Flag (monoclonal M2 and polyclonal, Sigma), anti-myc (A-14, Santa Cruz Biotechnology, Santa Cruz, CA), anti-HA (Y-11, Santa Cruz), and anti-pericentrin2 (C-16, Santa Cruz).

Cell Culture, Transfection, and RNA Interference

HeLa, HEK293T, and U2OS (American Type Culture Collection, Manassas, VA) were maintained in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum and antibiotics. Human fetal lung fibroblasts MRC-5 (Health Science Research Resources Bank, Japan) were grown in MEM (Invitrogen) supplemented with 10% fetal bovine serum. Transient transfection of plasmids was performed using the Lipofectamine and Plus reagents (Invitrogen). Two small interfering RNA (siRNA) oligonucleotides were synthesized corresponding to nucleotides 467-485 (UGGAAGAUCUCCUAACUAA, siRNA-1) and nucleotides 1104-1122 (CUAUGAGACUGCUCUAUCA, siRNA-2) of human *CDK5RAP2*. Control was a scrambled sequence (CUAGACUCUAUGAUGCAUC). The siRNA duplexes were delivered into cells by transfection using Lipofectamine 2000 (Invitrogen). Cellular phenotypes observed by using siRNA-1 are presented here, and the depletion using siRNA-2 yielded identical results.

Immunofluorescence Microscopy

Cells grown on glass coverslips were fixed with methanol at −20°C for 10 min or 4% paraformaldehyde/phosphate-buffered saline at room temperature for 15 min. After fixation, the coverslips were subjected to immunostaining with antibodies as indicated. Secondary antibodies were AlexaFluor488, Alexa Fluro594, and AlexaFluro647 conjugates (Invitrogen). Hoechst 33258 (Sigma) was used as a nuclear counterstain. Quantification of fluorescence intensity at centrosomes was performed using the MetaMorph software (Universal Imaging, West Chester, PA) and was shown as a representative experiment from five repetitions.

Electron Microscopy

HeLa centrosomes were isolated according to a reported procedure (Ou and Rattner, 2000). The centrosome preparations contained 95% interphase cells. Isolated centrosomes were deposited on coverslips by centrifugation and reacted sequentially with CDK5RAP2 antibodies described above and a secondary antibody conjugated to 6-nm gold. The samples were then processed, embedded, and sectioned for microscopy as described by Salina *et al.* (2002). Sections were examined in a Hitachi H-600 electron microscope (Brisbane, CA). In acquired micrographs, 86% immunogold particles were found to associate with centrosomes (120 particles in 10 centrosomes and 20 particles in 10 similar areas in size that lack centrosomes), indicating the antibody staining specificity.

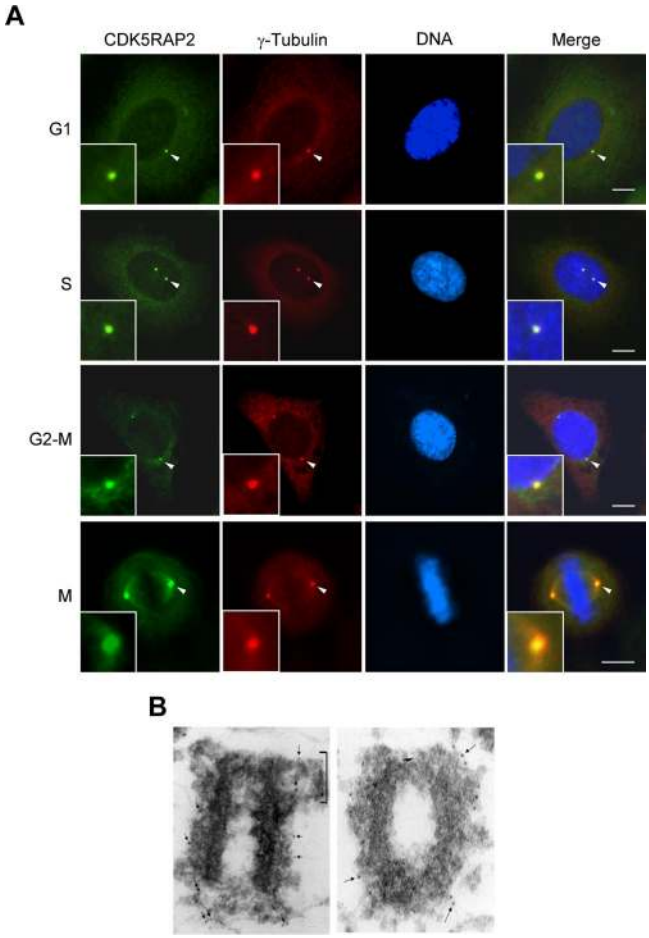

Figure 1. Centrosome localization of CDK5RAP2. (A) HeLa cells were double labeled for CDK5RAP2 and γ-tubulin. Arrow-pointed centrosomes are enlarged in insets. Bars, 10 μm. (B) Electron micrographs representative of isolated HeLa centrosomes after reaction with an antibody to CDK5RAP2 and a gold-labeled secondary antibody. The arrows point to CDK5RAP2 labels, and the bracket indicates a centriolar appendage. Magnification, ×80,000.

MT Regrowth Assay

Cells grown on coverslips were treated with 5 μM nocodazole in DMEM for 1 h at 37°C. After nocodazole removal by washing several times with DMEM, the cells were incubated at 37°C to allow MT regrowth and were fixed at various times for immunostaining.

Immunoprecipitation

Cell lysates were prepared at 4°C in lysis buffer containing 25 mM Tris-HCl, pH 7.4, 0.5% NP-40, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM NaF, 20 mM β-glycerophosphate, 1 mM dithiothreitol (DTT), and the Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN). Anti-Flag immunoprecipitation was performed with anti-Flag M2-coupled beads (Sigma). After immunoprecipitation, the beads were washed with the lysis buffer and boiled in the SDS-PAGE sample buffer for immunoblotting analysis. Alternatively, proteins were eluted with 100 mM glycine (pH 2.5) and separated by SDS-PAGE for identification by mass spectrometry. The gels were stained with SYPRO Ruby (Bio-Rad, Richmond, CA) according to the supplier's protocol and scanned using the Molecular Imager PharosFX Plus System (Bio-Rad).

Mass Spectrometry

Protein bands excised from SDS-PAGE gels were reduced, alkylated, and in-gel digested with trypsin (Shevchenko *et al.*, 1996). Recovered peptides were analyzed on a quadrupole/time-of-flight mass spectrometer (QSTAR-Pulsar, Applied Biosystems/Sciex, Thornhill, ON, Canada) equipped with a nanoelectrospray ion source. Peptide sequence tags generated from tandem mass spectrometry data were used to search sequence databases for protein identification.

Sucrose Gradient Sedimentation of the Cytosolic γTuRC

HEK293T cells, grown to confluence, were washed with HBS (50 mM HEPES, pH 7.4, and 150 mM NaCl) and were then lysed at 4°C in HBS supplemented with 1 mM DTT, 1 mM EGTA, 1 mM $MgCl_2$, 0.25 mM GTP, 0.5% Triton X-100, and the Protease Inhibitor Cocktail. Lysates were precleared by spinning in a microcentrifuge at full speed for 15 min and were further clarified by centrifugation at 100,000 × *g* (30 min; 4°C). 0.2 ml of the lysates was layered onto a sucrose gradient (4 ml of continuous 5–40%) for centrifugation at 120,000 × *g* (18 h; 4°C). After centrifugation, fractions were collected at 0.3 ml from the top to the bottom of the gradient.

RESULTS

Centrosomal Localization of CDK5RAP2

We generated two antibodies specifically recognizing either the amino- or carboxy-terminal region of CDK5RAP2. Antibody reactivity was confirmed by immunoblotting HeLa extracts with and without siRNA-mediated CDK5RAP2 depletion (see Figure 3D). Immunostaining of HeLa cells fixed in cold methanol revealed that CDK5RAP2 localized to one or two bright spots peripheral to the nuclei along with weak general staining in the cytoplasm (Figure 1A). Staining with either of the CDK5RAP2 antibodies yielded the same immunoreactive patterns, which could be eliminated by preincubation of the antibodies with corresponding recombinant protein antigens. Double immunostaining with a γ-tubulin antibody verified that the perinuclear spots of CDK5RAP2 were indeed centrosomes. Similarly, when centrosomes isolated from a HeLa extract were deposited on coverslips and then reacted with the CDK5RAP2 antibodies, the reactivity was observed in all of the centrosomes (data not shown). Furthermore, CDK5RAP2 exhibited centrosomal staining patterns similar to that of γ-tubulin throughout the cell cycle. In interphase cells with duplicated centrosomes, the CDK5RAP2 antibodies stained both centrosomes homogeneously (Figure 1A). The intensity of centrosomal labeling increased during mitosis (Figure 1A). In addition, CDK5RAP2 was detected in small amounts along spindle MTs in metaphase cells (Figure

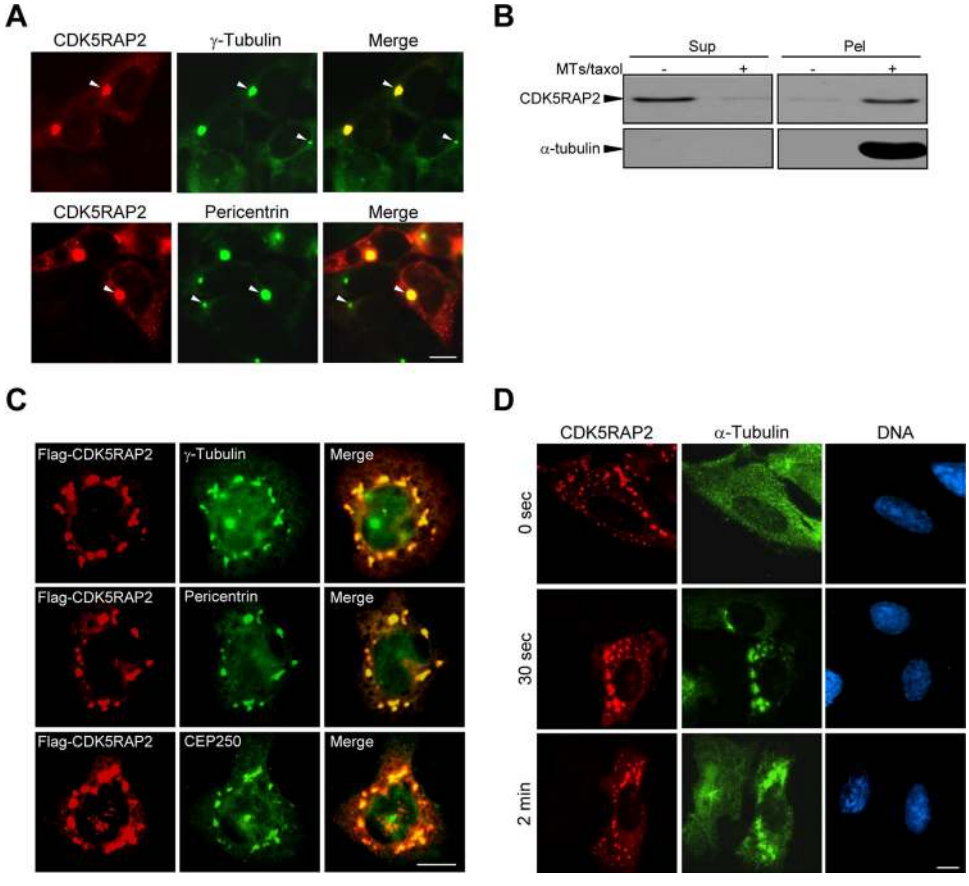

Figure 2. Subcellular localization of ectopically expressed CDK5RAP2. (A) HEK293T cells were transiently transfected with Flag-CDK5RAP2 and fixed for staining with antibodies against the Flag-tag and γ-tubulin or pericentrin. Fluorescence intensity of γ-tubulin or pericentrin was measured from arrow-pointed centrosomes to quantify the staining relative to those of untransfected cells. (B) Cell lysate was incubated with or without taxol-stabilized MTs. After MT sedimentation by centrifugation through a glycerol cushion, both the supernatant (Sup) and the pellet (Pel) were immunoblotted for CDK5RAP2 and α-tubulin. (C) Cells transfected with Flag-CDK5RAP2 were treated with 5 μM nocodazole for 1 h to disrupt MTs. The cells were then fixed and labeled with antibodies against the Flag-tag, γ-tubulin, pericentrin, and CEP250. (D) After the nocodazole treatment, the cells were incubated in the fresh medium without nocodazole at 37°C for times as indicated and then immunostained with anti-Flag and anti-α-tubulin antibodies. Bars, 10 μm.

1A), similar to γ-tubulin. Staining of synchronized populations of HeLa cells showed identical CDK5RAP2 patterns (data not shown).

Next, we studied the distribution of CDK5RAP2 in isolated centrosomes by immunoelectron microscopy. Figure 1B illustrates two examples observed in both longitudinal and cross-sectional profiles. CDK5RAP2 labels were found in the PCM region adhering to the surface of the centrosomes and the region of the centrosomal appendages (Figure 1B). Taken together, our light and electron microscopy data indicate that CDK5RAP2 is distributed throughout the PCM, is incorporated into the PCM as it forms in concert with centrosome duplication, and is a prominent component of mitotic spindle poles.

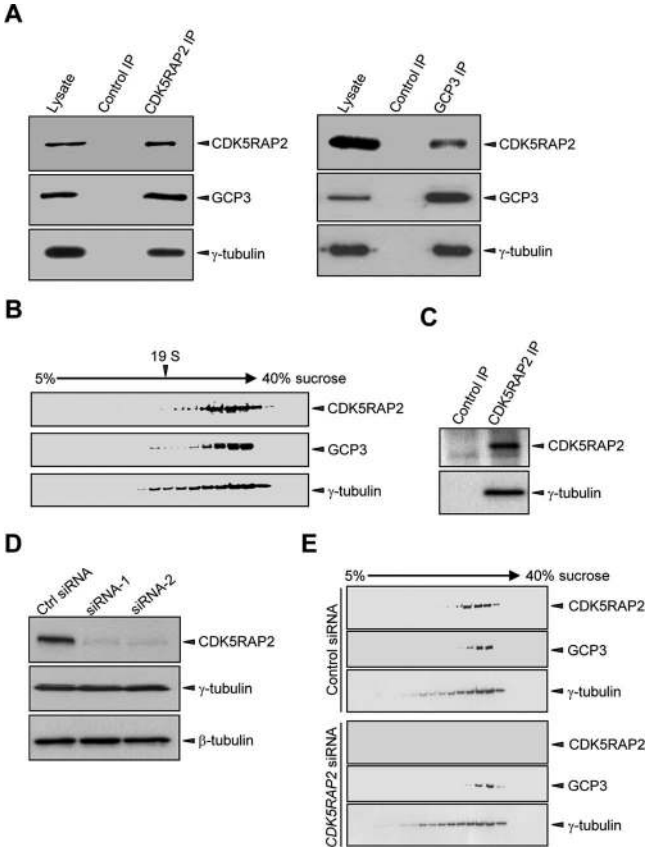

Figure 3. CDK5RAP2 associates with the γTuRC. (A) Immunoprecipitation was performed on HEK293T extracts with anti-CDK5RAP2 (left panels) and anti-GCP3 (right panels) antibodies. Nonspecific rabbit IgG was used in the control immunoprecipitation (Control IP). Immunoprecipitates were immunoblotted for CDK5RAP2, GCP3, and γ-tubulin. (B) Cytoplasmic extracts were fractionated over a continuous 5–40% sucrose gradient. Each gradient fraction was subjected to immunoblotting for CDK5RAP2, GCP3, and γ-tubulin. The S-value marker is thyroglobulin (19.4 S). (C) CDK5RAP2 was immunoprecipitated (CDK5RAP2 IP) from the peak fraction of the gradient. The immunoprecipitates were probed for CDK5RAP2 and γ-tubulin. (D) Cells transfected with siRNA targeting CDK5RAP2 (siRNA-1 or siRNA-2) or the control siRNA (Ctrl siRNA) were collected 48 h after transfection and analyzed for CDK5RAP2, γ-tubulin, and β-tubulin. (E) Cytoplasmic extracts transfected with control or *CDK5RAP2* siRNA were fractionated over the sucrose gradient to examine distributions of CDK5RAP2 and γTuRC components.

Assembly of Centrosomal Proteins by CDK5RAP2 Overexpression

We also examined subcellular localization of ectopically expressed CDK5RAP2. At low expression levels, the ectopically expressed protein displayed staining patterns identical to that of the endogenous protein (data not shown), further confirming the localization data shown above. When over-

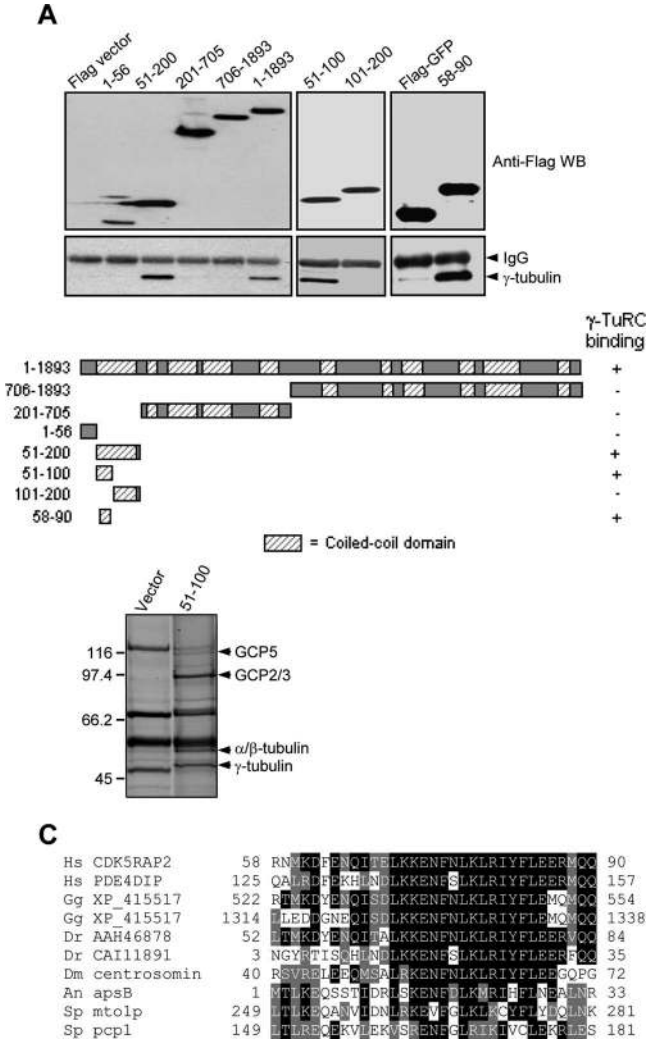

Figure 4. CDK5RAP2 contains a conserved γTuRC-binding sequence. (A) CDK5RAP2 and its fragments were transiently expressed in HEK293T with a tag (Flag or Flag-GFP). Anti-Flag immunoprecipitates were immunoblotted with anti-Flag and anti-γ-tubulin antibodies. Below the immunoblots is shown a summary of the binding results. (B) Cell extracts expressing Flag-CDK5RAP2(51-100) or the vector were clarified by centrifugation at 100,000 × *g* before anti-Flag immunoprecipitation. Immunoprecipitated proteins were visualized on a SDS-PAGE gel by staining with SYPRO Ruby. Marked protein bands were identified by mass spectrometry. (C) Alignment of CDK5RAP2(58-90) with sequences of various taxonomical classes. Hs, *Homo sapiens*; Gg, *Gallus gallus*; Dr, *Danio rerio*; Dm, *D. melanogaster*; An, *A. nidulans*; and Sp, *S. pombe*. Marked residues are conserved (black) or conservatively substituted (gray) in at least five of the sequences. GenBank sequences used for characterized proteins are CDK5RAP2, Q96SN8; PDE4DIP, BAA32299; centrosomin, NP_725298; ApsB, CAA05918; Mto1p, NP_588284; and Pcp1p, Q92351. Proteins predicted from *G. gallus* and *D. rerio* are named by accession numbers. *G. gallus* XP_415517 contains two regions homologous to aa 58-90 of CDK5RAP2.

expressed, CDK5RAP2 formed large protein clusters onto the centrosomes and also appeared in cytoplasmic granules (Figure 2A), which were probably protein aggregates. The centrosomal staining of γ-tubulin and pericentrin was markedly expanded, up to eight times compared with those in adjacent untransfected cells, and their centrosomal staining patterns correlated with that of expressed CDK5RAP2 (Figure 2A). The centrosomes appeared to expand because of CDK5RAP2 overexpression. In this and the following experiments, our immunoblotting data indicated that CDK5RAP2 overexpression did not affect the expression levels of the centrosomal proteins examined and that the overexpression increased the amount of these proteins in the Triton X-100-insoluble particulate fraction (data not shown). Therefore, overexpressed CDK5RAP2 sequestered the proteins probably from the cytoplasmic pools. In an MT sedimentation experiment, we observed that cytoplasmic CDK5RAP2 associated with MTs (Figure 2B). When MTs were disrupted by nocodazole treatment, cells overexpressing CDK5RAP2 displayed CDK5RAP2 clusters of various sizes in the cytoplasm (Figure 2C), suggesting that MTs prevent aggregation of the protein. The protein aggregates were found to contain three centrosomal proteins γ-tubulin, pericentrin, and CEP250 (Figure 2C). Moreover, these clusters formed MT asters during the incubation after nocodazole was removed (Figure 2D). Together, CDK5RAP2 can assemble centrosomal proteins including γ-tubulin onto the centrosome or into cytoplasmic clusters with the MT-nucleating capability. The cytoplasmic clusters did not display any specific pattern when stained with the antibody GT335 (data not shown), which labels glutamylated centriole α-tubulin (Bobinnec *et al.*, 1998). Therefore, centrioles were not formed within the clusters.

CDK5RAP2 Associates with the γTuRC

It has been reported that CDK5RAP2 shares homology at the amino terminus with several eukaryotic proteins, including *Schizosaccharomyces pombe* Mto1p (also known as Mbo1p and Mod20p) and Pcp1p, and *Drosophila melanogaster* centrosomin (Cnn), which have been shown to function in the recruitment of γ-tubulin to MTOCs through interacting with γTuCs (Flory *et al.*, 2002; Terada *et al.*, 2003; Sawin *et al.*, 2004). We set out to investigate whether CDK5RAP2 interacts with the γTuRC. Immunoprecipitation of CDK5RAP2 specifically coprecipitated γTuRC components, as detected on immunoblots of γ-tubulin and GCP3 (Figure 3A). Immunoprecipitation of GCP3 confirmed the association with CDK5RAP2 in addition to γ-tubulin (Figure 3A). The cytoplasmic γTuRC is a large molecular complex that sediments at ~32S on sucrose gradients (Stearns and Kirschner, 1994; Zheng *et al.*, 1995). We fractionated cell extracts by centrifugation over a continuous sucrose gradient to examine the distribution of CDK5RAP2 and the γTuRC. CDK5RAP2 cosedimented with γ-tubulin and GCP3 as heavy complexes that displayed the sedimentation coefficient of ~32S (Figure 3B). Moreover, γ-tubulin coimmunoprecipitated with CDK5RAP2 from the CDK5RAP2 and γ-tubulin peak fraction of the gradient (Figure 3C). These results revealed physical association of CDK5RAP2 with the γTuRC in the cytoplasm.

Next, we examined γTuRC assembly after suppression of CDK5RAP2 expression. Two nonoverlapping siRNA sequences were designed to silence CDK5RAP2 expression. Transfection of either sequences effectively reduced the protein expression and showed similar silencing efficiencies (86.8% for siRNA-1 and 83.4% for siRNA-2; Figure 3D). The silencing effects were specific, as the levels of γ-tubulin and β-tubulin were unchanged by the siRNA transfections (Figure 3D). In the sucrose-gradient centrifugation experiment, γ-tubulin and GCP3 from *CDK5RAP2*-silenced cells displayed sedimentation characteristics similar to those from the control cells (Figure 3E), indicating that CDK5RAP2 depletion does not disassemble the γTuRC.

To determine the γTuRC-binding site, several CDK5RAP2 fragments were constructed and used in immunoprecipitation. γ-Tubulin was readily detected in the immunoprecipitates of aa 58-90 and fragments containing this region, but not in those of the other CDK5RAP2 fragments (Figure 4A). To further explore the γTuRC association, a cytoplasmic

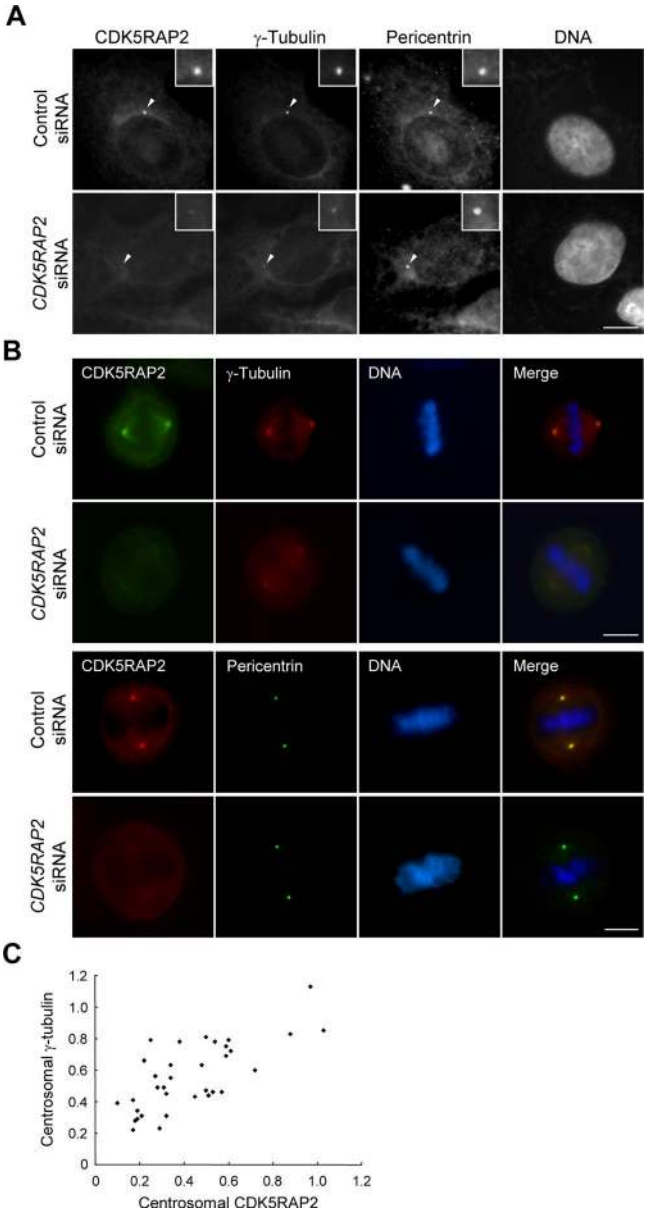

Figure 5. CDK5RAP2 is required for γTuRC association with the centrosome. HeLa cells transfected with siRNAs were immunostained for CDK5RAP2, γ-tubulin, and pericentrin. (A) Immunofluorescence micrographs of interphase cells. Centrosomes are enlarged in insets. (B) Images of transfected mitotic cells. Bars, 10 μm. (C) Quantitative analysis of centrosomal CDK5RAP2 and γ-tubulin in *CDK5RAP2* siRNA-transfected cells. Fluorescence intensities of CDK5RAP2 and γ-tubulin relative to that of pericentrin were determined at centrosomes (n = 34).

extract transiently expressing the CDK5RAP2 fragment aa 51-100 was prepared. After immunoprecipitation of the fragment, bound proteins were separated by SDS-PAGE and stained with a fluorescent dye (Figure 4B). The major protein bands were identified by mass spectrometry to be the γTuRC components γ-tubulin, GCP2, GCP3, and GCP5, as well as α/β-tubulin (Figure 4B), confirming the specific binding of the γTuRC to the CDK5RAP2 fragment. Together, we conclude that aa 58-90 is responsible for the association of CDK5RAP2 with the γTuRC. This segment is the most conserved region within the 64-aa sequence that shows homology to corresponding regions in Mto1p, Pcp1p, Cnn, *Aspergillus nidulans* ApsB, human myomegalin/phosphodiesterase 4D-interacting protein (PDE4DIP), and several unknown sequences of other species (Sawin *et al.*, 2004; see Figure 4C of this article). Searching sequence databases also identified sequences equivalent to this CDK5RAP2 region in two putative proteins of zebrafish and one sequence of chicken (Figure 4C). These sequences encode for putative large proteins with multiple coiled-coil structures, which are features of centrosomal proteins. Potentially, they are functional counterparts of CDK5RAP2 in their respective organisms.

CDK5RAP2 Functions in γTuRC Association with the Centrosome

We explored CDK5RAP2 function by suppressing its expression with siRNAs. In interphase and mitotic cells depleted of CDK5RAP2, γ-tubulin was dramatically diminished at centrosomes and dispersed into the cytoplasm (Figure 5, A and B). In contrast, every control cells (scrambled siRNA-transfected) displayed normal centrosomal γ-tubulin patterns, with one or two perinuclear foci of γ-tubulin in each cell (Figure 5, A and B). CDK5RAP2 depletion did not affect the centrosomal staining of pericentrin (Figure 5, A and B) and centrin (data not shown). Thus, the loss of CDK5RAP2 resulted in the removal of γ-tubulin from the centrosomes. To quantitatively analyze the effect, cells were triple-labeled for CDK5RAP2, γ-tubulin, and pericentrin to determine fluorescence intensities of centrosomal CDK5RAP2 and γ-tubulin

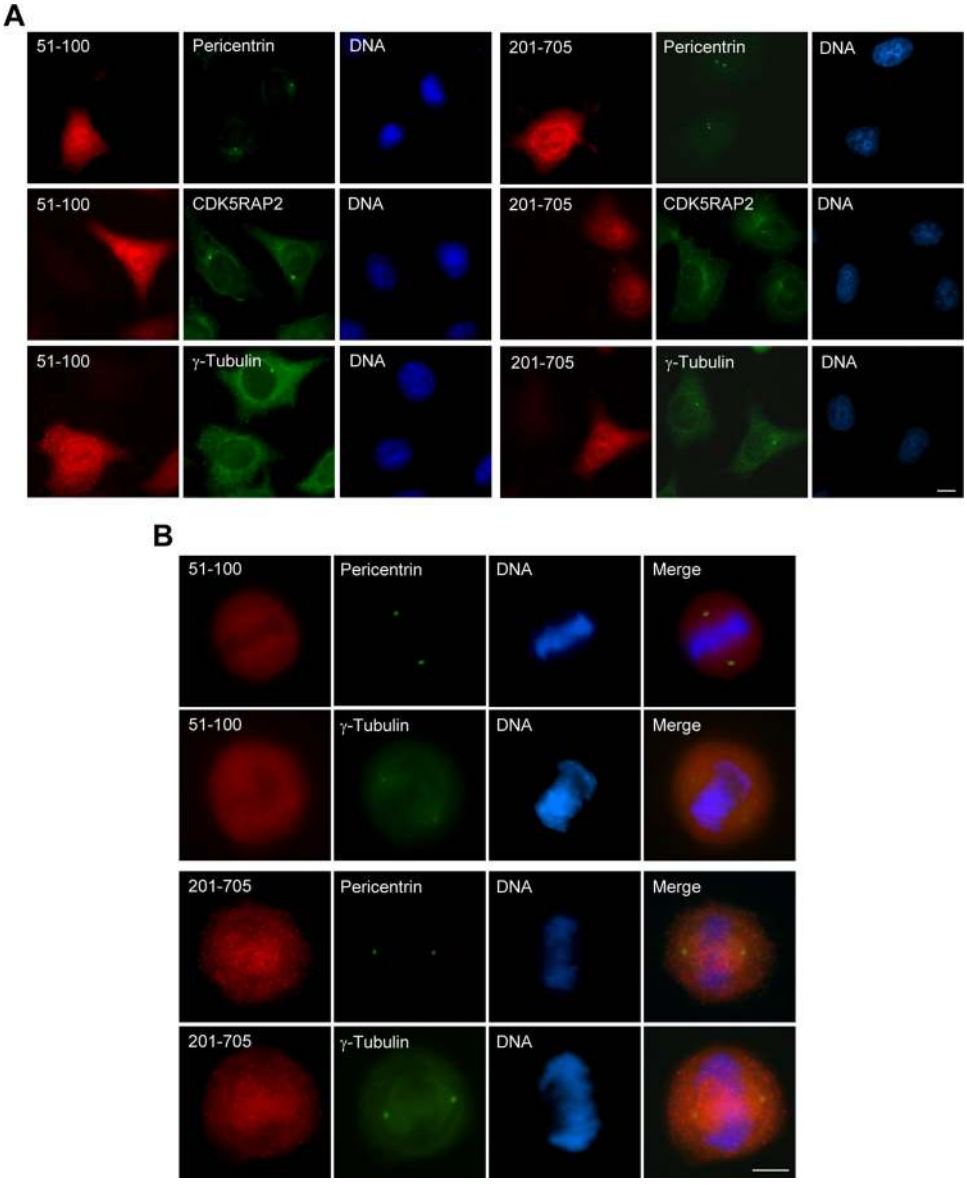

Figure 6. Overexpression of the γTuRC-binding domain delocalizes the γTuRC from the centrosome. HeLa cells transiently transfected with Flag-tagged CDK5RAP2(51-100) or CDK5RAP2(201-705) were stained for the transfected proteins (anti-Flag), CDK5RAP2, pericentrin, and γ-tubulin as indicated. (A) Interphase cells; (B) mitotic cells that were enriched by a nocodazole treatment. Bars, 10 μm.

relative to that of pericentrin. It was found that the reduction of γ-tubulin correlated with that of CDK5RAP2 at the centrosomes (Figure 5C). In addition to our studies with the malignant cell line HeLa, we also examined the function of CDK5RAP2 in the primary human fibroblast cell line MRC-5. In these cells, a moderate level reduction of CDK5RAP2 caused γ-tubulin reduction to a similar level (Supplementary Figure 1). Further, a severe knockdown of CDK5RAP2 yielded almost complete loss of γ-tubulin at the centrosomes (Supplementary Figure 1). Therefore, CDK5RAP2 is required for γTuRC attachment to the centrosomes in both the malignant and nonmalignant cells.

We also attempted to interfere with CDK5RAP2 function by transient expression of the CDK5RAP2 fragment aa 51-100, which contains the γTuRC-binding domain. This fragment displayed diffused patterns without centrosome-localizing property, and the expression did not affect the centrosomal localization of pericentrin and endogenous CDK5RAP2 (n = 45; Figure 6). In interphase cells overexpressing the fragment, centrosomal γ-tubulin was dramatically reduced or even undetectable (reduced to <10% in 76% of the cells and to 15 ± 3% in the rest of the cells, n = 50; Figure 6A). In mitotic cells transfected with the fragment, centrosomal γ-tubulin was also significantly reduced but to a lesser extent (reduced to 28–52%, n = 30; Figure 6B). The lesser reduction may imply the involvement of alternative mechanisms in anchoring the γTuRC during mitosis. The transfection of a control fragment, CDK5RAP2(201-705) which does not contain the γTuRC-binding domain, neither affected the centrosomal localization of γ-tubulin nor those of pericentrin and CDK5RAP2 in both interphase and mitotic cells (n = 45; Figure 6). Taken together, our data indicate that γTuRC assembly to the centrosome requires the function of CDK5RAP2.

CDK5RAP2 Function Is Required for MT Organization by the Centrosome

We investigated whether depletion of CDK5RAP2 or expression of the γTuRC-binding domain affects organization of the MT cytoskeleton. Interphase U2OS cells usually display a well-focused MT network, which was observed in cells transfected with control siRNA or CDK5RAP2(201-705); see Figure 7. In cells either depleted of CDK5RAP2 or expressing CDK5RAP2(51-100), MTs exhibited unfocused patterns and were no longer arranged into a radial array (Figure 7). It is noteworthy that neither CDK5RAP2 depletion nor CDK5RAP2(51-100) expression changed the density of MTs in the cytoplasm (Figure 7). We proceeded to test centrosomal MT nucleation after MT depolymerization. In untransfected cells and cells transfected with control siRNA or CDK5RAP2(201-705), well-defined single MT asters were formed by 2 min of MT regrowth, and the asters had developed into robust radial arrays of MTs after 10 min (Figure 8). In cells transfected with *CDK5RAP2* siRNA or CDK5RAP2(51-100), MT asters were not observed even after prolonged incubation, and MTs grew from the cytoplasm instead of the centrosomes (Figure 8). Eventually, MTs appeared unfocused and disorganized in these cells (Figure 8). Similar phenotypes were observed when silencing of CDK5RAP2 expression was performed on MRC-5 fibroblasts (Supplementary Figure 2). Therefore, the inhibition of CDK5RAP2 function interfered with the MT nucleating and organizing properties of the centrosomes.

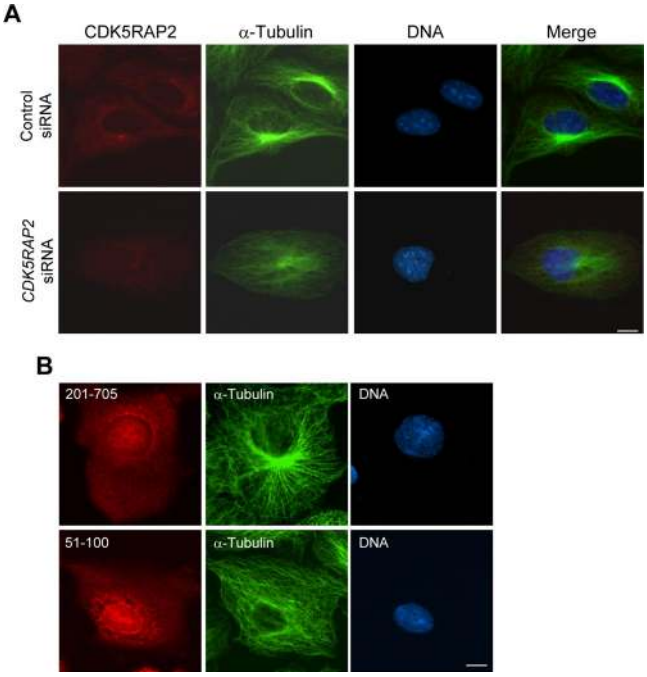

Figure 7. Perturbing CDK5RAP2 function disrupts interphase MT networks. (A) siRNA-transfected U2OS cells were labeled for α-tubulin and CDK5RAP2. (B) U2OS cells expressing Flag-tagged CDK5RAP2(51-100) or CDK5RAP2(201-705) were stained with anti-Flag and anti-α-tubulin antibodies. Bars, 10 μm.

CDK5RAP2 Function Is Required for Astral MT Formation during Mitosis

We examined the role of CDK5RAP2 in the formation of mitotic spindles in U2OS cells. At metaphase, control cells transfected with scrambled siRNA or CDK5RAP2(201-705) displayed a typical bipolar spindle organization with radial arrays of astral MTs at the spindle poles, as determined by immunofluorescence and linescans (95% of the spindles, n = 100; Figure 9). In cells transfected with siRNA against *CDK5RAP2*, the spindles maintained bipolar symmetry with chromosomes congressed to the metaphase plate (Figures 5B and 9A). However, an obvious defect was the pronounced diminution of both astral MTs and α-tubulin at the spindle poles (Figure 9A). Astral MTs were present on much fewer spindles (20% of the spindles, n = 90) and when present, both the length and number of astral MTs were reduced. Densitometry scans along the pole-to-pole axis also confirmed the reduction of fluorescent tubulin at the spindle poles (Figure 9A). The same phenotypes were observed in mitotic cells overexpressing CDK5RAP2(51-100; 75% of the spindles, n = 60; Figures 6B and 9B). Therefore, the functional abrogation of CDK5RAP2 did not affect spindle assembly but gave rise to defective mitotic centrosomes, resulting in the formation of spindle poles with altered MT asters. When we examined the mitotic index, we found that CDK5RAP2 depletion or CDK5RAP2(51-100) expression did not increase the number of mitotic cells. Further, as with control cells, cells transfected with *CDK5RAP2* siRNA or CDK5RAP2(51-100) could be effectively arrested in mitosis using nocodazole, showing that the spindle checkpoint function was intact. This suggests that the centrosomal and spindle defects caused by CDK5RAP2 depletion or CDK5RAP2(51-100) expression did not activate the spindle assembly checkpoint.

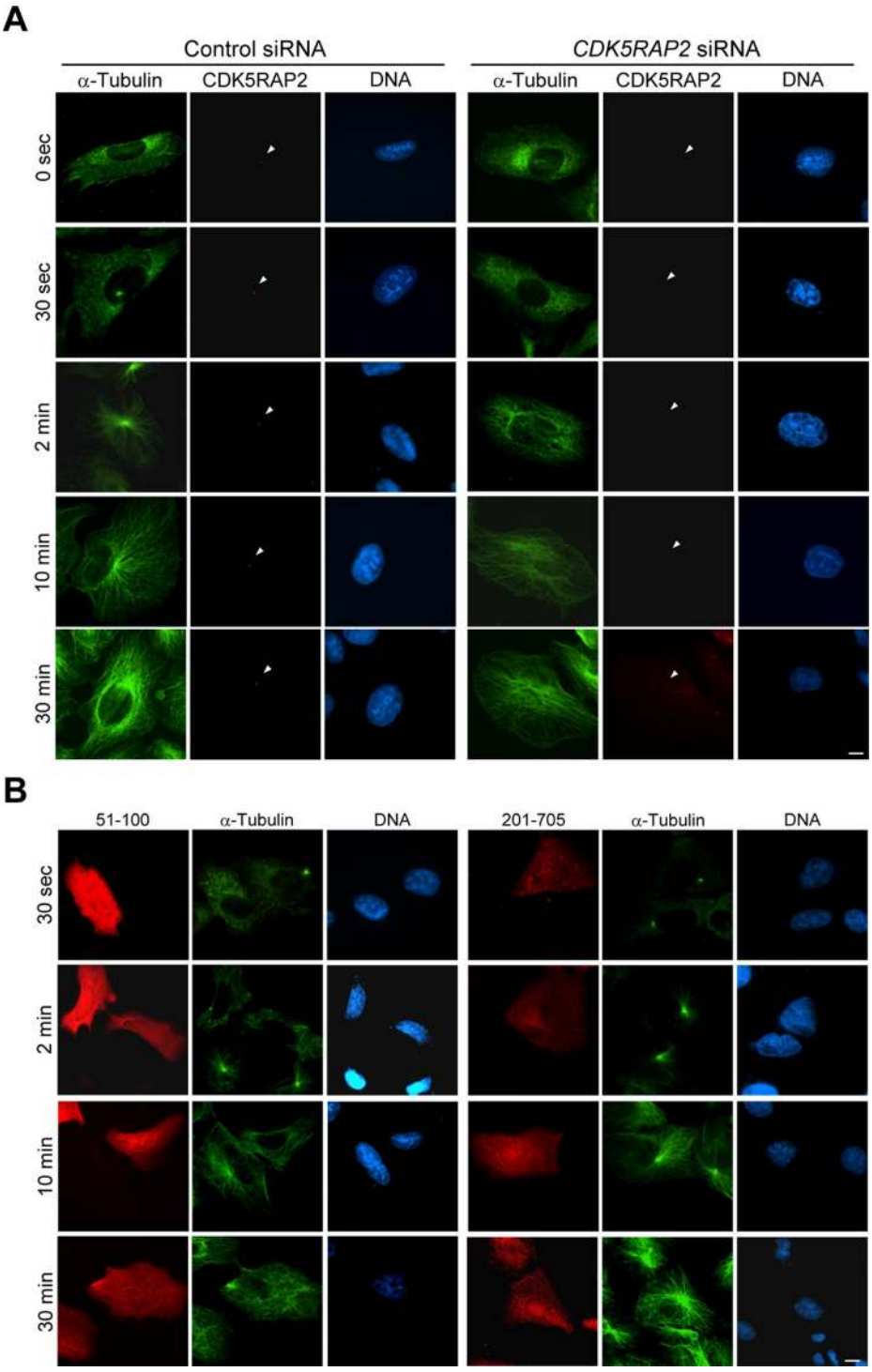

Figure 8. CDK5RAP2 function is required for centrosomal MT nucleation. (A) MT regrowth was performed on siRNA-transfected U2OS cells. After the nocodazole treatment, MTs were allowed to grow for various times. The cells were fixed and stained with anti-α-tubulin and anti-CDK5RAP2 antibodies. Arrowheads denote centrosomes. (B) MT regrowth assay of cells transfected with CDK5RAP2(51-100) or CDK5RAP2(201-705). Bars, 10 μm.

DISCUSSION

The major function of the centrosome is to act as a center for the organization of MTs and this function depends on the collection, tethering, and proper interaction of proteins required for this function at the centrosome. One protein that is essential to MT nucleation is γ-tubulin, which is found both at the centrosome and within the cytoplasm. We have found that CDK5RAP2 is required for docking γ-tubulin at the centrosome. CDK5RAP2 is a ubiquitously expressed protein that stably associates with the centrosome (Ching *et al.*, 2000; Andersen *et al.*, 2003). Structure prediction using the method of Lupas *et al.* (1991) revealed that CDK5RAP2 contains predominantly coiled-coil domains and may form a filamentous structure, which is a characteristic of PCM scaffold proteins. In fact, CDK5RAP2 is present throughout the PCM in all stages of the cell cycle. The centrosomal localization does not require association with the γTuRC, because a high-salt condition (2 M KI) that extracts centrosomal γ-tubulin does not remove CDK5RAP2 from centrosomes (Andersen *et al.*, 2003). In addition, CDK5RAP2 overexpres-

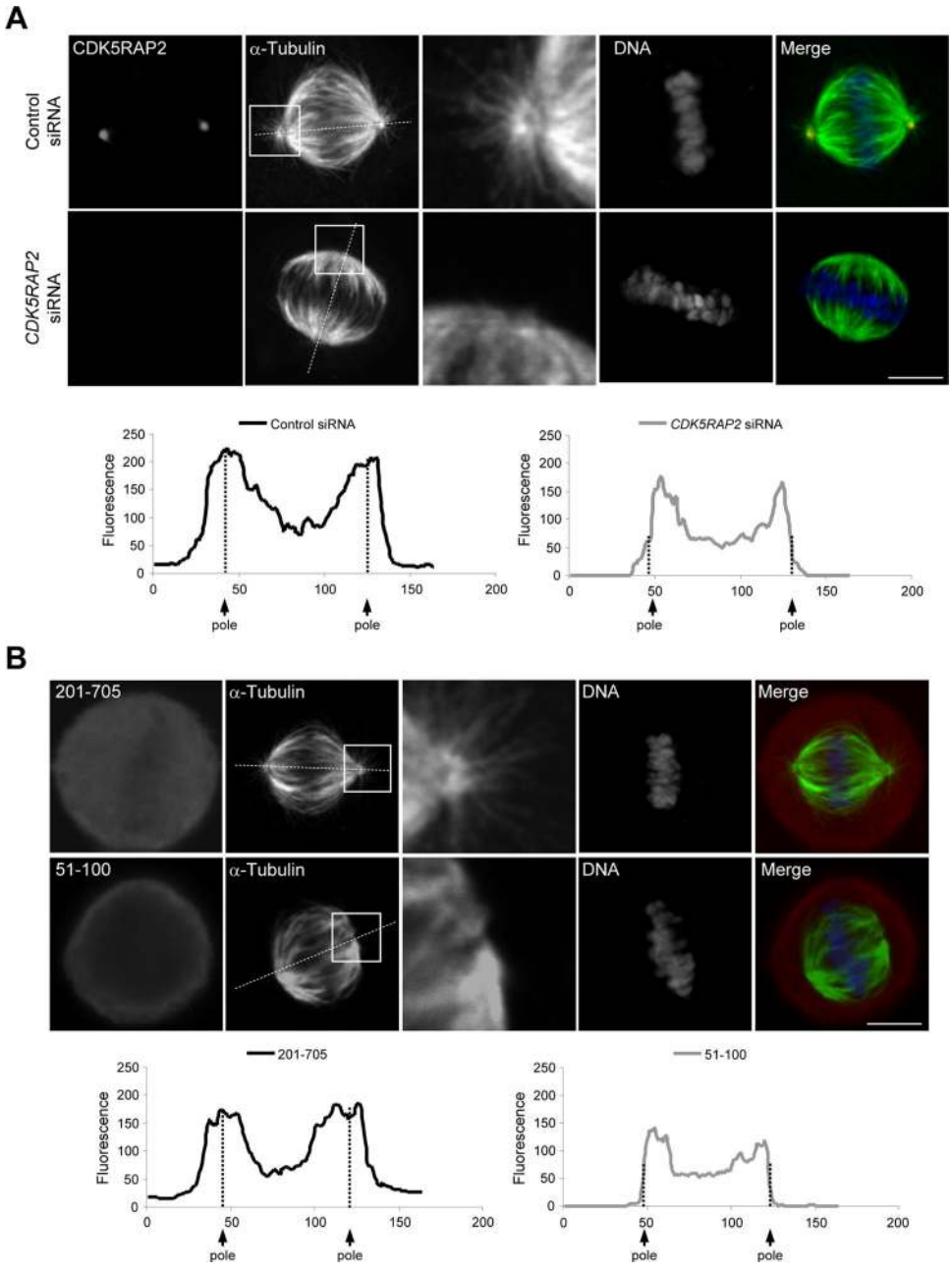

Figure 9. Effect of perturbing CDK5RAP2 function on mitotic spindle formation. (A) Spindle morphology of U2OS cells treated with control or *CDK5RAP2* siRNA. Below the images are linescans of α-tubulin fluorescence intensity along the pole-to-pole spindle axis. Boxed areas are enlarged. (B) Mitotic cells expressing Flag-tagged CDK5RAP2(51-100) or CDK5RAP2 (201-705). Shown here are fluorescence microscopy images and corresponding linescans of fluorescence intensity (α-tubulin) along the pole-to-pole spindle axis. Bars, 10 μm.

sion resulted in enhanced targeting of centrosomal proteins such as γ-tubulin to the centrosomes. Altogether, these observations suggest that CDK5RAP2 is a pericentriolar scaffold component that may play an important role in protein assembly onto the centrosome.

There is a large cytoplasmic pool of CDK5RAP2 that is not associated with the centrosome, but is present in large complexes with the γTuRC. Presumably, CDK5RAP2 transits together with the γTuRC from the cytoplasm to the centrosome where CDK5RAP2 is incorporated into the PCM along with the bound γTuRC. The amount of centrosomal CDK5RAP2 varies during the cell cycle, correlating with the amount of γ-tubulin and the MT-organizing capacity of the centrosome. This correlation is consistent with the idea that CDK5RAP2 is important in determining γ-tubulin level at the centrosome. Indeed, the functional abrogation of CDK5RAP2 by suppression of its expression or overexpression of the γTuRC-binding domain, which acts as a dominant-negative species, effectively removed γ-tubulin from the centrosomes and interfered with the MT-nucleating and organizing functions of the centrosomes. Therefore, a functional centrosome requires CDK5RAP2 for integration of the γTuRC and possibly other proteins onto the centrosome.

CDK5RAP2 contains a γTuRC-binding domain, which is conserved in several related eukaryotic proteins, including Mto1p, Pcp1p, and Cnn. This domain, predicted to be involved in the formation of an α-helical coiled-coil, represents a conserved γTuC-binding module. However, it does not display any homology to pericentrin, AKAP450, ninein, Nlp, and GCP-WD, which are centrosomal proteins exhibiting γTuRC-binding activity in mammals (Takahashi *et al.*, 2002; Casenghi *et al.*, 2003; Zimmerman *et al.*, 2004; Delgehyr *et al.*, 2005; Haren *et al.*, 2006; Luders *et al.*, 2006).

Among them, pericentrin is specifically required for mitotic spindle organization, whereas GCP-WD is required for centrosomal γTuRC attachment and MT nucleation throughout the cell cycle (Zimmerman *et al.*, 2004; Haren *et al.*, 2006; Luders *et al.*, 2006). We have preliminary data to indicate that CDK5RAP2 and pericentrin or GCP-WD do not depend on one another for γTuRC association (unpublished data). Therefore, γTuRC tethering to the centrosome may involve interaction with several centrosomal proteins; the γTuRC-binding mechanism adopted by CDK5RAP2 is distinct from those utilized by the other mammalian proteins. At present, it is unclear what interrelationship CDK5RAP2 has with the other γTuRC-binding proteins. It is tempting to propose that CDK5RAP2 cooperates with some of them in centrosomal attachment of the γTuRC and has separate and distinct functions from some others with respect to the centrosomal γTuRC. PDE4DIP, a mostly uncharacterized centrosomal/Golgi protein that is expressed primarily in muscle (Verde *et al.*, 2001), shares a high degree of homology with CDK5RAP2, especially in the γTuRC-binding region. This similarity raises the possibility that PDE4DIP and CDK5RAP2 share the γTuRC-anchoring function in muscle cells.

Perturbing CDK5RAP2 function led to the formation of mitotic spindles with few or no astral MTs. It has been shown by a number of studies that centrosomes are not essential for mitotic spindle assembly in animal cells (Sluder, 2005; Luders and Stearns, 2007); for example, somatic cells removed of centrosomes by microsurgery or laser ablation are able to form bipolar spindles but without astral MTs to undergo passable mitosis (Hinchcliffe *et al.*, 2001; Khodjakov and Rieder, 2001). In addition, centrosome-independent MT nucleation such as chromatin-based nucleation is involved in spindle assembly (Gruss and Vernos, 2004; Luders and Stearns, 2007). The function of γTuRC core components in the spindle assembly checkpoint does not depend on their localization to the centrosome and the integrity of the centrosome (Muller *et al.*, 2006). In fact, centrosomal defects caused by inhibiting CDK5RAP2 function are insufficient to activate the checkpoint control. Our study does not rule out the possibility that in cells depleted of CDK5RAP2 or expressing its γTuRC-binding fragment, the residual centrosomal γTuRC may eventually organize the spindle. In addition, the centrosomal levels of pericentrin, a γTuRC-anchoring protein that functions during mitosis (Zimmerman *et al.*, 2004), were not affected under these conditions. CDK5RAP2 appears to play a specialized role in spindle function. In fission yeast, Mto1p is essential for growth of astral MTs during mitosis, whereas Pcp1p has been implicated to function in spindle MT nucleation at the spindle pole body (Sawin *et al.*, 2004; Venkatram *et al.*, 2004). In *Drosophila*, loss of Cnn misplaces γ-tubulin from spindle poles and produces anastral bipolar spindles (Megraw *et al.*, 1999, 2001; Vaizel-Ohayon and Schejter, 1999); but somatic cells lacking Cnn have few mitotic defects (Megraw *et al.*, 2001; Mahoney *et al.*, 2006). Although the spindle function of CDK5RAP2 is reminiscent of those of Mto1p and Cnn, CDK5RAP2 is distinct from them in at least the following aspects: Unlike CDK5RAP2, Mto1p is not required for γ-tubulin complex localization to the spindle pole body, which is equivalent to the animal centrosome (Sawin *et al.*, 2004; Venkatram *et al.*, 2004); Cnn anchors γ-tubulin to the centrosome only during mitosis in most cell types and does not associate with the γTuRC in the cytoplasm (Li and Kaufman, 1996; Megraw *et al.*, 2001). Therefore, CDK5RAP2 has features distinct from Mto1p and Cnn in terms of γTuC localization and function.

Highly expressed in the neuroepithelium of the developing brain, CDK5RAP2 has been proposed to be involved in the control of brain size (Bond *et al.*, 2005). Two homozygous mutations of *CDK5RAP2* are identified from individuals with MCPH; both mutations cause early termination of the protein (Bond *et al.*, 2005). We found that these mutants do not have centrosome-localizing properties and that one of them loses γTuRC-binding activity (unpublished data), suggesting that the mutations cause defects in γTuRC-targeting to the centrosome.

During neurogenesis, the length of the cell cycle, the proportion of symmetric and cell fate-determining asymmetric divisions of neural progenitors crucially affect the brain size. Our findings suggest that loss of CDK5RAP2 function interferes with the ability of the centrosome to function as an MTOC; this altered function may deregulate neurogenic cell divisions and hence the production of neurons. Therefore, these findings provide additional evidence for the notion that centrosomes play a key role in controlling brain size and that this role at least in part relies on the proper function of CDK5RAP2.

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REFERENCES

Andersen, J. S., Wilkinson, C. J., Mayor, T., Mortensen, P., Nigg, E. A., and Mann, M. (2003). Proteomic characterization of the human centrosome by protein correlation profiling. Nature *426*, 570–574.

Bobinnec, Y., Khodjakov, A., Mir, L. M., Rieder, C. L., Edde, B., and Bornens, M. (1998). Centriole disassembly in vivo and its effect on centrosome structure and function in vertebrate cells. J. Cell Biol. *143*, 1575–1589.

Bond, J. *et al.* (2005). A centrosomal mechanism involving CDK5RAP2 and CENPJ controls brain size. Nat. Genet. *37*, 353–355.

Casenghi, M., Meraldi, P., Weinhart, U., Duncan, P. I., Korner, R., and Nigg, E. A. (2003). Polo-like kinase 1 regulates Nlp, a centrosome protein involved in microtubule nucleation. Dev. Cell *5*, 113–125.

Ching, Y. P., Qi, Z., and Wang, J. H. (2000). Cloning of three novel neuronal Cdk5 activator binding proteins. Gene *242*, 285–294.

Delgehyr, N., Sillibourne, J., and Bornens, M. (2005). Microtubule nucleation and anchoring at the centrosome are independent processes linked by ninein function. J. Cell Sci. *118*, 1565–1575.

Dictenberg, J. B., Zimmerman, W., Sparks, C. A., Young, A., Vidair, C., Zheng, Y., Carrington, W., Fay, F. S., and Doxsey, S. J. (1998). Pericentrin and gamma-tubulin form a protein complex and are organized into a novel lattice at the centrosome. J. Cell Biol. *141*, 163–174.

Doxsey, S., McCollum, D., and Theurkauf, W. (2005). Centrosomes in cellular regulation. Annu. Rev. Cell Dev. Biol. *21*, 411–434.

Felix, M. A., Antony, C., Wright, M., and Maro, B. (1994). Centrosome assembly in vitro: role of gamma-tubulin recruitment in *Xenopus* sperm aster formation. J. Cell Biol. *124*, 19–31.

Flory, M. R., Morphew, M., Joseph, J. D., Means, A. R., and Davis, T. N. (2002). Pcp1p, an Spc110p-related calmodulin target at the centrosome of the fission yeast *Schizosaccharomyces pombe*. Cell Growth Differ. *13*, 47–58.

Gruss, O. J., and Vernos, I. (2004). The mechanism of spindle assembly: functions of Ran and its target TPX2. J. Cell Biol. *166*, 949–955.

Gunawardane, R. N., Martin, O. C., and Zheng, Y. (2003). Characterization of a new gammaTuRC subunit with WD repeats. Mol. Biol. Cell *14*, 1017–1026.

Haren, L., Remy, M. H., Bazin, I., Callebaut, I., Wright, M., and Merdes, A. (2006). NEDD1-dependent recruitment of the gamma-tubulin ring complex to the centrosome is necessary for centriole duplication and spindle assembly. J. Cell Biol. *172*, 505–515.

Hinchcliffe, E. H., Miller, F. J., Cham, M., Khodjakov, A., and Sluder, G. (2001). Requirement of a centrosomal activity for cell cycle progression through G1 into S phase. Science *291*, 1547–1550.

Khodjakov, A., and Rieder, C. L. (2001). Centrosomes enhance the fidelity of cytokinesis in vertebrates and are required for cell cycle progression. J. Cell Biol. *153*, 237–242.

Li, K., and Kaufman, T. C. (1996). The homeotic target gene centrosomin encodes an essential centrosomal component. Cell *85*, 585–596.

Luders, J., Patel, U. K., and Stearns, T. (2006). GCP-WD is a gamma-tubulin targeting factor required for centrosomal and chromatin-mediated microtubule nucleation. Nat. Cell Biol. *8*, 137–147.

Luders, J., and Stearns, T. (2007). Microtubule-organizing centres: a re-evaluation. Nat. Rev. Mol. Cell Biol. *8*, 161–167.

Lupas, A., Van Dyke, M., and Stock, J. (1991). Predicting coiled coils from protein sequences. Science *252*, 1162–1164.

Mahoney, N. M., Goshima, G., Douglass, A. D., and Vale, R. D. (2006). Making microtubules and mitotic spindles in cells without functional centrosomes. Curr. Biol. *16*, 564–569.

Meads, T., and Schroer, T. A. (1995). Polarity and nucleation of microtubules in polarized epithelial cells. Cell Motil. Cytoskelet. *32*, 273–288.

Megraw, T. L., Kao, L. R., and Kaufman, T. C. (2001). Zygotic development without functional mitotic centrosomes. Curr. Biol. *11*, 116–120.

Megraw, T. L., Li, K., Kao, L. R., and Kaufman, T. C. (1999). The centrosomin protein is required for centrosome assembly and function during cleavage in *Drosophila.* Development *126*, 2829–2839.

Moritz, M., Zheng, Y., Alberts, B. M., and Oegema, K. (1998). Recruitment of the gamma-tubulin ring complex to *Drosophila* salt-stripped centrosome scaffolds. J. Cell Biol. *142*, 775–786.

Moudjou, M., Bordes, N., Paintrand, M., and Bornens, M. (1996). gamma-Tubulin in mammalian cells: the centrosomal and the cytosolic forms. J. Cell Sci. *109*, 875–887.

Muller, H., Fogeron, M. L., Lehmann, V., Lehrach, H., and Lange, B. M. (2006). A centrosome-independent role for gamma-TuRC proteins in the spindle assembly checkpoint. Science *314*, 654–657.

Ou, Y., and Rattner, J. B. (2000). A subset of centrosomal proteins are arranged in a tubular conformation that is reproduced during centrosome duplication. Cell Motil. Cytoskelet. *47*, 13–24.

Ou, Y., and Rattner, J. B. (2004). The centrosome in higher organisms: structure, composition, and duplication. Int. Rev. Cytol. *238*, 119–182.

Ou, Y. Y., Mack, G. J., Zhang, M., and Rattner, J. B. (2002). CEP110 and ninein are located in a specific domain of the centrosome associated with centrosome maturation. J. Cell Sci. *115*, 1825–1835.

Ou, Y. Y., Zhang, M., Chi, S., Matyas, J. R., and Rattner, J. B. (2003). Higher order structure of the PCM adjacent to the centriole. Cell Motil. Cytoskelet. *55*, 125–133.

Raynaud-Messina, B., and Merdes, A. (2007). Gamma-tubulin complexes and microtubule organization. Curr. Opin. Cell Biol. *19*, 24–30.

Salina, D., Bodoor, K., Eckley, D. M., Schroer, T. A., Rattner, J. B., and Burke, B. (2002). Cytoplasmic dynein as a facilitator of nuclear envelope breakdown. Cell *108*, 97–107.

Sanders, M. A., and Salisbury, J. L. (1994). Centrin plays an essential role in microtubule severing during flagellar excision in *Chlamydomonas reinhardtii.* J. Cell Biol. *124*, 795–805.

Sawin, K. E., Lourenco, P. C., and Snaith, H. A. (2004). Microtubule nucleation at non-spindle pole body microtubule-organizing centers requires fission yeast centrosomin-related protein mod20p. Curr. Biol. *14*, 763–775.

Schnackenberg, B. J., Khodjakov, A., Rieder, C. L., and Palazzo, R. E. (1998). The disassembly and reassembly of functional centrosomes in vitro. Proc. Natl. Acad. Sci. USA *95*, 9295–9300.

Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. *68*, 850–858.

Sluder, G. (2005). Two-way traffic: centrosomes and the cell cycle. Nat. Rev. Mol. Cell Biol. *6*, 743–748.

Stearns, T., and Kirschner, M. (1994). In vitro reconstitution of centrosome assembly and function: the central role of gamma-tubulin. Cell *76*, 623–637.

Takahashi, M., Yamagiwa, A., Nishimura, T., Mukai, H., and Ono, Y. (2002). Centrosomal proteins CG-NAP and kendrin provide microtubule nucleation sites by anchoring gamma-tubulin ring complex. Mol. Biol. Cell *13*, 3235–3245.

Terada, Y., Uetake, Y., and Kuriyama, R. (2003). Interaction of Aurora-A and centrosomin at the microtubule-nucleating site in *Drosophila* and mammalian cells. J. Cell Biol. *162*, 757–763.

Vaizel-Ohayon, D., and Schejter, E. D. (1999). Mutations in centrosomin reveal requirements for centrosomal function during early *Drosophila* embryogenesis. Curr. Biol. *9*, 889–898.

Venkatram, S., Tasto, J. J., Feoktistova, A., Jennings, J. L., Link, A. J., and Gould, K. L. (2004). Identification and characterization of two novel proteins affecting fission yeast gamma-tubulin complex function. Mol. Biol. Cell *15*, 2287–2301.

Verde, I., Pahlke, G., Salanova, M., Zhang, G., Wang, S., Coletti, D., Onuffer, J., Jin, S. L., and Conti, M. (2001). Myomegalin is a novel protein of the golgi/centrosome that interacts with a cyclic nucleotide phosphodiesterase. J. Biol. Chem. *276*, 11189–11198.

Wiese, C., and Zheng, Y. (2006). Microtubule nucleation: gamma-tubulin and beyond. J. Cell Sci. *119*, 4143–4153.

Zheng, Y., Wong, M. L., Alberts, B., and Mitchison, T. (1995). Nucleation of microtubule assembly by a gamma-tubulin-containing ring complex. Nature *378*, 578–583.

Zimmerman, W. C., Sillibourne, J., Rosa, J., and Doxsey, S. J. (2004). Mitosis-specific anchoring of gamma tubulin complexes by pericentrin controls spindle organization and mitotic entry. Mol. Biol. Cell *15*, 3642–3657.