Centrosome-mediated microtubule nucleation is essential for spindle assembly during mitosis. Although γ-tubulin complexes have primarily been implicated in the nucleation process, details of the underlying mechanisms remain poorly understood. Here, we demonstrated that a member of the human transforming acidic coiled-coil (TACC) protein family, TACC3, plays a critical role in microtubule nucleation at the centrosome. In mitotic cells, TACC3 knockdown substantially affected the assembly of microtubules in the astral region and impaired microtubule nucleation at the centrosomes. The TACC3 depletion-induced mitotic phenotype was rescued by expression of the TACC3 C terminus predominantly consisting of the TACC domain, suggesting that the TACC domain plays an important role in microtubule assembly. Consistently, experiments with the recombinant TACC domain of TACC3 demonstrated that this domain possesses intrinsic microtubule nucleating activity. Co-immunoprecipitation and sedimentation experiments revealed that TACC3 mediates interactions with proteins of both the γ-tubulin ring complex (γ-TuRC) and the γ-tubulin small complex (γ-TuSC). Interestingly, TACC3 depletion resulted in reduced levels of γ-TuRC and increased levels of γ-TuSC, indicating that the assembly of γ-TuRC from γ-TuSC requires TACC3. Detailed analyses suggested that TACC3 facilitates the association of γ-TuSC-specific proteins with the proteins known to be involved in the assembly of γ-TuRC. Consistent with such a role for TACC3, the suppression of TACC3 disrupted localization of γ-TuRC proteins to the centrosome. Our findings reveal that TACC3 is involved in the regulation of microtubule nucleation at the centrosome and functions in the stabilization of the γ-tubulin ring complex assembly.

Conclusion: TACC3 is involved in microtubule nucleation and in stabilization of the γ-tubulin ring complex.

Significance: These results provide additional insights into the regulation of centrosomal microtubule nucleation.

The centrosome nucleates and organizes microtubules in most animal cells and plays key roles in the spatial and temporal control of microtubule networks (1, 2). This organelle comprises a pair of centrioles embedded in the pericentriolar material (PCM) (3). The centriole has a well defined structure that involves microtubule triplets, whereas the organization of the PCM is less precise (3, 4). During the cell cycle, microtubules undergo large scale reorganization from a few long radial arrays in interphase to shorter, but very dense, spindles in mitosis. During this reorganization, the PCM increases in volume, and microtubule nucleation exhibits a severalfold increase (5). The molecular basis of the microtubule nucleating activity of the PCM is poorly understood.

Microtubule nucleation is primarily mediated through the γ-tubulin ring complex (γ-TuRC), comprising γ-tubulin and the members of the conserved γ-tubulin complex family proteins (GCP) (6). The core structural unit is a tetrameric complex, the γ-tubulin small complex (γ-TuSC), which is composed of 2 units of γ-tubulin and 1 unit each of GCP2 and GCP3. Multiple γ-TuSC units are further assembled into a larger ring-shaped complex, γ-TuRC. In budding yeast, γ-TuRC consists of only γ-TuSC units, whereas in higher organisms, γ-TuRC contains additional GCP proteins, including GCP4, GCP5, and GCP6 (7).

Although γ-TuSCs have been shown to be critically important for microtubule nucleation in vivo, the same cannot be recapitulated in vitro using purified components, suggesting the involvement of yet unknown factors in this process (8). Consistent with this idea, structural analyses of purified γ-tubulin complexes from Saccharomyces cerevisiae have revealed that both γ-TuSC and γ-TuRC ring-like structures are defective in microtubule nucleation (9). Several groups attempted to identify these factors, and their studies implicated a number of pericentriolar proteins in the integration of the γ-tubulin complex to the centrosome but were unable to clearly define the

The abbreviations used are: PCM, pericentriolar material; GCP, γ-tubulin complex protein; TRITC, tetramethylrhodamine 5; γ-TuRC, γ-tubulin ring complex; γ-TuSC, γ-tubulin small complex; TACC, transforming acidic coiled-coil; MTOC, microtubule-organizing center; IP, immunoprecipitation; esiRNA, endonuclease-prepared siRNA; siRes, siRNA-resistant.
TACC3 Regulates Microtubule Nucleation

mechanisms of microtubule nucleation at the centrosome (10–22).

Earlier studies have demonstrated the presence of a stable salt-insoluble protein scaffold/matrix in the PCM, which is likely composed of coiled-coil proteins (23). Subsequently, proteomics analyses of purified centrosomes have demonstrated the abundance of coiled-coil proteins in the centrosome (24, 25). However, the functions of coiled-coil proteins in centrosome-mediated processes are poorly understood. Members of the transforming acidic coiled-coil (TACC) protein family are among the highly abundant coiled-coil proteins localized at the centrosomes. These proteins share an evolutionarily conserved long C-terminal coiled-coil region of ~200 amino acids, referred to as the TACC domain (26, 27). TACC3, the highly conserved TACC homolog in humans, is aberrantly expressed in a variety of human cancers and has been shown to promote tumor growth in vivo (28). Several lines of evidence have indicated that TACC3 is essential in mitosis. In Drosophila, D-TACC is required for normal spindle function and is critical for early embryonic development (29). In Caenorhabditis elegans, TAC-1 depletion leads to defects in spindle elongation in the one-cell-stage embryo (30). In Xenopus egg extracts, TACC has been shown to interact with microtubule polymerase XMAP215 and to promote microtubule stability (31). In human cells, TACC3 has been shown to be associated with the organization of the mitotic spindle poles (32). Subsequent studies revealed that TACC3 depletion induced chromosomal misalignment, reduced spindle stability, and triggered apoptosis in cells (33–35). However, the mechanism by which TACC3 regulates microtubule functions at the microtubule-organizing center (MTOC) has not been described.

In this study, we aimed to investigate the mechanism of TACC3 in centrosome/spindle pole-mediated microtubule nucleation and to uncover its molecular links with the core microtubule-nucleating machinery, the γ-tubulin ring complex at the centrosome. We provide evidence that in mitotic cells, TACC3 primarily regulates microtubule nucleation and is essential for the nucleation of microtubules at the centrosome and spindle poles. We show that TACC3 interacts with proteins of the γ-tubulin complexes; it functions in stabilizing the assembly of γ-TuRC from γ-TuSC and integrates γ-TuRC proteins to the centrosome. Our results also demonstrate that the TACC domain of TACC3 possesses intrinsic microtubule nucleating activity. These results reveal for the first time the involvement of a TACC3-mediated mechanism in the regulation of microtubule nucleation at the centrosome.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, siRNAs—DAPI, fluorescein isothiocyanate (FITC), and thymidine were purchased from Sigma. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and antibiotic solutions were purchased from HiMedia, Inc. (Mumbai, India). Mouse monoclonal anti-TACC3, mouse polyclonal anti-GCP4, rabbit polyclonal anti-GCP6, anti-GCP5, anti-γ-tubulin, and anti-pericentrin were obtained from Abcam; mouse monoclonal anti-α-tubulin and mouse monoclonal anti-γ-tubulin were purchased from Sigma; rabbit monoclonal anti-TACC3 was purchased from Cell Signaling; mouse monoclonal anti-GCP3 were obtained from Santa Cruz Biotechnology, Inc.; and mouse monoclonal anti-actin was purchased from BD Biosciences. The secondary antibodies antimouse FITC and anti-rabbit TRITC were obtained from Jackson ImmunoResearch.

All esiRNAs were obtained from Sigma. The esiRNAs used were TACC3 esiRNA targeted against the 2166–2638-nucleotide region of TACC3 (NM_006342), catalog no. EUH063201 (Sigma), and control firefly luciferase esiRNA, catalog no. EHU-FLUC. All single siRNAs were purchased from Dharmaco. The sequence of the TACC3 single siRNA used for depletion of TACC3 in siRNA-resistant TACC3-GFP stable cells was AAGUACCGGAAAGACUGUC. The sequence of the TACC3–3’-UTR siRNA was UCUCUUAAGGUGUAGGUGUC and that of luciferase siRNA was GCGAUCAUCUCUAGGAGG. The full-length TACC3 DNA cloned into pCMV-6-AC (TACC3-GFP) with a C-terminal GFP tag, catalog no. RG210754, was obtained from Origene. The mammalian construct (pEGP-C1) of the GFP-tagged C-terminal region (amino acids 500–838) of TACC3 (GFP-TACC3(500–838)) (36) was generously provided by Stephen J. Royle, Warwick University, UK.

Cell Culture and Transfection—HeLa and MCF-7 cells, originally obtained from the ATCC, were cultured in DMEM containing 10% FBS at 37 °C under 5% CO2. The TACC3-siRNA-resistant TACC3-GFP-stable HeLa cell line (HeLa Kyoto 179), labeled as HeLa TACC3-GFP siRes, was generously provided by Drs. Alex Bird and Ina Poser, Max Planck Institute, Dresden, Germany, and are transgenic cells that stably express siRNA-resistant TACC3-GFP DNA in a bacterial artificial chromosome (37, 38). For the depletion of proteins, we used ribonuclease III-prepared small interfering RNAs (esiRNA), which ensure the efficient knockdown of proteins, with minimal off-target effects compared with single siRNAs (39, 40). For rescue experiments, we followed two methods. For the first method, we depleted TACC3 using TACC3 siRNA in the siRNA-resistant TACC3-GFP stable HeLa cells. For the second method, we co-transfected HeLa cells with TACC3–3’-UTR-siRNA and either TACC3-GFP or GFP-TACC3(500–838) that lacks the 3’-UTR. This method has been used for rescue experiments in other cellular studies (41). DNA, esiRNAs, or single siRNAs were transfected into cells using Lipofectamine 2000 (Invitrogen). For transfection in MCF-7 cells, Oligofectamine (Invitrogen) was used.

Microtubule Regrowth Assay—Microtubule regrowth assays were performed as described previously (42, 43). Briefly, mitotic cells were incubated in ice-cold DMEM containing 10% FBS for 3 h to depolymerize all cellular microtubules, and the medium was subsequently exchanged with growth medium that had been pre-warmed to 37 °C. The cells were fixed using 4% paraformaldehyde in 0.5% Triton X-100 at different time points over 15 min and then immunostained and imaged using a Leica SP5 confocal microscope. Similar methods were followed for regrowth assay in the interphase cells, except that the cold incubation time was for 2 h.

Immunofluorescence, Cell Synchronization, and Immunoprecipitation—Cells fixed in either methanol at −20 °C or paraformaldehyde in 0.5% Triton X-100 were washed with phos-
Cells were mitotically synchronized using a double thymidine block (44). Briefly, thymidine was added to 30% confluent cells, followed by an 18-h incubation (first block). Thymidine was again added to the cells after a 9-h release, followed by a 17-h incubation (second block). The cells were released from G1 arrest after the removal of thymidine and were collected at the mitotic stage. For experiments with synchronized interphase cells, the cells were collected under double thymidine-treated conditions. The synchronized cells were lysed using lysis buffer (4 °C) containing 20 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, 50 mM NaCl, 10 mM EGTA, phosphatase inhibitors 2 and 3, and a protease inhibitor mixture (Sigma). Subsequently, TACC3, GCP3, GCP6, and γ-tubulin were immunoprecipitated using the appropriate antibodies, followed by the addition of protein G-agarose beads. The beads were washed with lysis buffer and then boiled in SDS-PAGE sample buffer for immunoblot analysis. Membranes were developed for immunoblot using the Immobilon reagent (Millipore), followed by imaging using ChemiDoc XRS System (Bio-Rad).

Image Analysis—All immunofluorescence images were captured using a ×63 (1.4 N.A.) oil-immersion objective on a Leica SP 5 confocal microscope. The same image acquisition and analysis settings were used for both control and depleted conditions. The maximum intensity images were produced by projecting the images (Z-stacks) captured in three-dimensional optical sections at 0.3-μm intervals. The images were analyzed using Leica Application Suite Advanced Fluorescence Lite 2.8.0 software. To analyze microtubule intensity (shown in Fig. 1C), a region of interest line of 19.83 μm in length was drawn across the spindle poles in the mitotic cell to measure microtubule intensity across the spindle pole axis (as shown in Fig. 1B). Intensities were analyzed using Leica Application Suite Advanced Fluorescence Lite software.

To quantify the microtubule regrowth in mitotic cells (as shown in Fig. 3), the microtubule intensities of the areas up to which the microtubules had grown around the spindle pole were quantified. The intensity of a microtubule-free background region of similar area was subtracted from the sample data to obtain the net intensity of the microtubules. The numbers of microtubules that had been nucleated from the centrosome in the interphase cells were counted by visual inspection (as shown in Fig. 4). To analyze the intensities of γ-tubulin and GCP6 at the spindle poles (as shown in Fig. 7), the total intensity within an area spanning 1.83 μm² around the spindle poles was quantified in each case.

Analysis of γ-TuScs and γ-TuRCs by Sucrose Gradient—A 5–40% sucrose gradient was prepared as described previously (8, 42). The cell lysates were overlaid onto the gradient and centrifuged at 30,000 rpm (150,000 × g) for 4 h at 4 °C using a Beckman SW32Ti rotor. The protein fractions (0.5 ml each) were collected from the top of the gradient and were then separated via SDS-PAGE followed by Western blotting to detect the γ-tubulin complex proteins and TACC3. Markers with known sedimentation coefficients (S), blue dextran (2000 kDa, 52.6 S), β-amylase (200 kDa, 8.9 S), and alcohol dehydrogenase (150 kDa, 7.4 S), were run to determine the size of the unknown protein complexes sedimented in the gradient.

Protein Purification—The TACC domain (residues 638–838) of TACC3 was obtained from human TACC3 cDNA (Origene) by PCR and subcloned into the pET15b vector, containing an N-terminal His₆ tag; the resulting plasmid was expressed in BL21 (DE3) cells. To purify His₆-TACC, a Ni²⁺-nitrilotriacetic acid column was employed, followed by separation using an ion exchanger, Mono Q. Tubulin was purified from goat brains through cycles of assembly and disassembly in vitro (41). EB1 was purified as described elsewhere (45). The resultant protein concentrations were estimated using the Bradford assay, with BSA as a standard (46).

Microtubule Polymerization Assay—The time-dependent microtubule assembly of tubulin (15 μM) in the absence and presence of gradients of TACC concentrations was assessed at 37 °C by measuring the turbidity at 360 nm using a Varian Cary 50 BIO UV-visible spectrophotometer (47).

Electron Microscopy—Tubulin (20 μM) was polymerized at 37 °C for 30 min in PEM (50 mM PIPES, 1 mM EGTA, and 0.5 mM MgCl₂) buffer at pH 7.0 with 1 mM GTP in the presence of TACC (15 μM) or 10% DMSO. The assembled microtubules were fixed using 0.1% glutaraldehyde, negatively stained with 0.1% uranyl acetate, and imaged using a transmission electron microscope (jeol, model 1101).

In Vitro Microtubule Imaging—The FITC-labeled TACC (638–838) domain was prepared by covalently linking the purified TACC domain with a 20-fold molar excess of FITC followed by separating the labeled protein on a Superdex 200 size-exclusion column (Akta 10, GE Healthcare) (48). For microtubule polymerization, a mixture of rhodamine-labeled and unlabeled tubulin (1 + 14 μM) was polymerized in the presence of the FITC-labeled TACC domain (5 μM) in PEM buffer, pH 7.0, with 1 mM GTP for 10 min at 37 °C. The microtubules were fixed with 1% glutaraldehyde and then sedimented onto polylysine-coated coverslips after passing through a 15% glycerol cushion followed by centrifugation (30,000 rpm). The microtubules were imaged on a Leica SP5 laser confocal microscope.

Size-exclusion Chromatography—Tubulin, TACC, or the mixture of tubulin and TACC were loaded onto a Superose-12 column (Akta 10, GE Healthcare) equilibrated and run at 4 °C. The elution profiles were obtained by measuring the absorbance at 280 nm. The molecular weights of tubulin and the complex between tubulin and the TACC domain were determined from the plot of the molecular weights of the standards versus the retention volume (elution volume/void volume) (Vₑ/Vᵥ). Vᵥ (void column) for the column was 8.4 ml. Vₑ refers to the elution volume. The molecular weights of the unknowns were determined by plotting their retention volumes on the standard curve (49). The eluted proteins were collected in fractions of 300 μl each, with 30 μl of each fraction subsequently loaded onto SDS-polyacrylamide gels and stained with Coomassie Blue for visualization.
Statistical Analysis—The normality of the data was assessed using the Shapiro-Wilk test. The normally distributed data were analyzed with Student’s t test at the 99% confidence level. All data analyses were performed using R software. The data were plotted using Origin 8 or GraphPad Prism 6 software. The figures were organized using Adobe Photoshop and Adobe Illustrator.

RESULTS

TACC3 Is Required for Astral Microtubule Assembly and Microtubule Nucleation—We first determined the effect of TACC3 depletion on the assembly and organization of spindle microtubules in HeLa cells. After 72 h, the level of TACC3 was reduced by ~80% in cells transfected with TACC3 esiRNA compared with control cells treated with luciferase esiRNA (Fig. 1A). As siRNA-mediated depletion varies between experiments and within individual cells, the level of TACC3 depletion was also verified in individual cells through immunofluorescence imaging (Fig. 1B). In TACC3-depleted cells, the mitotic spindles were typically bipolar (Fig. 1B). However, several characteristic defects were observed. The density of the astral microtubules emanating from the spindle poles in mitotic cells was substantially reduced under TACC3-depleted conditions (enlarged images, Fig. 1B). This effect was further confirmed by quantifying the microtubule intensity across the spindle pole axis (Fig. 1C). Although there was an overall decrease in microtubule density throughout the regions surrounding the spindle poles; the loss of microtubules in the astral region was much more prominent. Overall, the microtubules were loosely focused at the poles and diffused throughout a relatively wider area around the poles compared with control cells. Furthermore, the characteristic node-shaped morphology of the poles typically observed in control cells was lost in TACC3-depleted cells (Fig. 1B). Approximately 70 ± 0.6% of metaphase cells displayed the loss of astral microtubules at the
spindle poles under the TACC3 esiRNA-treated condition (number of cells counted was ~200). The control esiRNA-treated cells did not show any defects in astral microtubule assembly. To eliminate any off-target effects of TACC3 esiRNA on the expression of γ-tubulin complex components, we examined the expression levels of γ-TuSC and γ-TuRC proteins in TACC3 esiRNA-transfected cells. Transfection of TACC3 esiRNA did not alter the expression levels of any of the γ-TuSC and γ-TuRC proteins, indicating that the levels of the γ-tubulin complex components were not affected (Fig. 1D).

We also rescued TACC3 depletion-induced phenotypes using multiple methods to ensure that the phenotype was not caused by off-target effects. We transfected TACC3 single siRNA into HeLa cells stably expressing a TACC3-GFP mutant that is resistant to the siRNA (37, 38). As shown by Western blot in Fig. 2A, TACC3-siRNA suppressed the levels of endogenous TACC3 without interfering with the expression of TACC3-GFP. The transfection with TACC3-siRNA in control HeLa cells led to defects in astral microtubule assembly that mirrored the effects of esiRNA transfection (compare Figs. 1B and 2B, lower panels). The loss of astral microtubules due to TACC3 depletion was rescued in cells expressing the TACC3-GFP mutant (Fig. 2, B and C). Although ~70% of control HeLa metaphase cells displayed the phenotype in response to TACC3 depletion, the siRNA-resistant TACC3-GFP cells showed rescue of the phenotype in about 70% metaphase cells (number of cells counted was ~90). In another method, we transfected HeLa cells with a single siRNA targeted to the 3'-UTR of TACC3, TACC3–3'-UTR-siRNA, along with a TACC3-GFP construct lacking the 3'-UTR. As shown by Western blot in Fig. 2D, the co-transfected cells showed significant suppression of the endogenous TACC3 level along with considerable expression of TACC3-GFP. Transfection with TACC3–3'-UTR-siRNA led to defects in astral microtubule assembly similar to the effects of esiRNA transfection (compare Figs. 1 and 2F). The loss of astral microtubules due to treatment with TACC3–3'-UTR-siRNA was rescued in cells co-transfected with exogenous TACC3-GFP (Fig. 2, F and G). Approximately 68% recovery of the number of metaphase cells with normal astral microtubules was observed under this condition (Fig. 2H). We next examined the role of the TACC domain of TACC in this rescue effect. We used a GFP-tagged C-terminal construct consisting of amino acid residues 500–838 of TACC3 (GFP-TACC3(500–838), which largely consists of the TACC(638–838) domain and has been shown to localize much more efficiently to the spindle poles compared with the TACC-only domain (36). We transfected HeLa cells with TACC3–3'-UTR-siRNA along with the GFP-TACC3(500–838) that lacked the 3'-UTR. As expected, the co-transfected cells showed a considerable increase in the level of GFP-TACC3(500–838) expression but with efficient suppression of the level of endogenous TACC3 (Fig. 2E). Transflecting cells with GFP-TACC3(500–838) together with TACC3–3'-UTR-siRNA efficiently rescued the astral spindle defects, indicating that the TACC domain plays a critical role in the assembly of astral microtubules (Fig. 2, F–H).

The aforementioned mitotic defects implied that TACC3 might play an essential role in spindle pole-mediated microtubule nucleation. The effect of TACC3 depletion on microtubule nucleation was examined using a microtubule regrowth assay in MCF-7 cells. We selected MCF-7 cells for this assay because of their large size and flat morphology. We also confirmed that TACC3 depletion-induced microtubule defects in MCF-7 cells were similar to those observed in HeLa cells (data not shown). After 48 h of control or TACC3-siRNA treatment, the cells were fixed at different times during microtubule regrowth. In TACC3-depleted mitotic cells fixed after 0–15 min of microtubule regrowth, the nucleation of microtubules at the spindle poles was severely impaired (Fig. 3A). We could not determine the exact numbers of microtubules emanating from the spindle poles because of high spindle density near the poles. However, a comparison of the total microtubule intensity after 1 min of microtubule regrowth showed that the overall microtubule density around the poles was substantially reduced in TACC3-depleted cells compared with control cells (Fig. 3B). The intensity of microtubules grown in an area around the spindle poles at different time points of regrowth was quantified and plotted (Fig. 3B). There was a clear correlation between the observed microtubule regrowth defects and the level of TACC3 depletion in the cells (Fig. 3A). In TACC3-depleted interphase cells, the densities of the microtubules emanating from the centrosome were visibly reduced after 30 s to 5 min of regrowth (Fig. 4A). TACC3-depleted cells showed an ~40% reduction in the number of microtubules emanating from the centrosome compared with control interphase cells after 1 min of regrowth (Fig. 4B). These results indicate that TACC3 is essential for the nucleation of centrosomal microtubules.

**TACC3 Interacts with γ-Tubulin Complex Components and Is Required for γ-TuRC Assembly**—We next examined whether TACC3 interacts with the components of the γ-tubulin complex. Co-immunoprecipitation (co-IP) experiments were performed in mitotic synchronized HeLa cell lysates to determine the interaction of γ-tubulin complex proteins with TACC3. γ-Tubulin, GCP3, GCP4, GCP5, and GCP6 were co-precipitated with TACC3 (Fig. 5A). These interactions were also confirmed through reverse IP experiments in which TACC3 was co-precipitated with GCP3, GCP6, and γ-tubulin (Fig. 5A). In each of the co-IPs of the γ-tubulin complex proteins (GCP3, GCP6, or γ-tubulin), other γ-tubulin complex components were also present together with TACC3 (Fig. 5A). Similar to mitotic cells, the interaction of TACC3 with GCP3, GCP4, and γ-tubulin were detected in co-IP experiments with synchronized interphase cells (Fig. 5B). We could not detect the association of GCP6 with TACC3 in the TACC3 co-IP of interphase cells, although it was detected in the reverse co-IP.

Then, we investigated whether TACC3 interacts with the soluble γ-tubulin complexes. The unsynchronized HeLa cell lysate was fractionated by sucrose gradient centrifugation, and the protein fractions were analyzed by immunoblot for the presence of γ-TuSC-specific proteins, γ-TuRC-specific proteins, and TACC3. γ-Tubulin, GCP3, and GCP4 were sedimented at a peak sedimentation coefficient of ~30 S, which corresponded to the γ-TuRC complex (Fig. 5C). The immunoblot also showed another peak with increased levels of γ-tubulin and GCP3, but not GCP4, at ~10 S, which corresponded to the γ-TuSC complex (Fig. 5C). The sedimentation coefficients
of γ-TuRC and γ-TuSC obtained in this study were in good agreement with the previously reported values (42). TACC3 was present in the protein fractions of both the 30 S and 10 S peaks, indicating that it was associated with both γ-TuRC and γ-TuSC (Fig. 5C). The immunoblot also showed that a relatively greater amount of TACC3 was associated with the γ-TuSC than with the γ-TuRC. To determine whether TACC3 is essential for the stability and assembly of γ-TuRC, we
assessed the relative abundance of the γ-TuRCs versus γ-TuSCs in TACC3-depleted versus control cells. The levels of γ-TuRC proteins (at 30 S) were significantly reduced, and those of γ-TuSC proteins (at 10 S) were increased in the TACC3-depleted cells compared with control cells (Fig. 5C). The plot of the ratio of γ-tubulin levels in γ-TuSC versus γ-TuRC showed an ∼40% increase of γ-tubulin in TACC3-depleted cells compared with control cells (Fig. 5D). These results indicated that TACC3 depletion inhibited the assembly of γ-TuRC from γ-TuSC.

Next, we determined the effect of TACC3 depletion on the molecular interactions between the γ-tubulin complex proteins during the assembly of γ-TuSC versus γ-TuRC. We performed co-IPs directed against GCP3, γ-tubulin, and GCP6 in TACC3-depleted cells, and we assessed the relative levels of both γ-TuSC- and γ-TuRC-specific proteins associated with these IP complexes with those found in IP complexes from control esiRNA-treated cells. The amounts of GCP3 present in the γ-tubulin IPs, as well as the amounts of γ-tubulin present in the GCP3 IP complexes, were comparable in control and TACC3-depleted cells, indicating that the molecular association between the γ-TuSC-specific proteins was not significantly affected (Fig. 6, A and B). However, the amounts of GCP4, GCP5, and GCP6 present in either γ-tubulin or GCP3 IP complexes were markedly reduced in TACC3-depleted cells compared with control cells. Similarly, the amounts of γ-tubulin and GCP3 detected in the GCP6 IP complexes were significantly reduced in TACC3-depleted cells compared with control cells (Fig. 6, A and B). However, the amounts of GCP4 and GCP5 associated with the GCP6-IP were apparently similar. Therefore, these results indicate that the depletion of TACC3 interferes with the molecular interactions between the γ-TuRC-specific proteins and the γ-TuSC during the assembly of γ-TuRC, but it does not affect the molecular interactions between the γ-TuSC proteins involved in the formation of γ-TuSC.

TACC3 Is Required for the Integration of γ-TuRC at the Centrosome—As TACC3 is involved in the molecular assembly of γ-TuRC, we then asked whether it is required for the integration of γ-TuRC to the centrosome. We assessed the local-  

ization of individual γ-TuRC- and γ-TuSC-specific compo- 
nts using immunofluorescence imaging in TACC3-depleted cells. The obtained images of TACC3 esiRNA-treated mitotic cells showed that the localization of GCP6 to the poles was completely disrupted (Fig. 7A). We also observed a considerable reduction in the level of γ-tubulin at the spindle poles (Fig. 7C). There was a strong correlation between the loss of these proteins and the reduced levels of TACC3 at the poles. Intensity analyses of a large population of cells revealed an ∼80% reduction of GCP6 and an ∼50% reduction of γ-tubulin from the poles in TACC3-depleted mitotic cells (Fig. 7, B and D). The loss of GCP6 and γ-tubulin was also observed in cells transfected with TACC3–3′-UTR-siRNA (Fig. 7, A and C), albeit to a slightly lesser extent compared with esiRNA transfection (Fig. 7, B and D, bar graphs). To determine the specificity of the TACC3 depletion-induced effects, we examined whether the γ-TuRC proteins were able to re-localize to the spindle poles after the introduction of TACC3-GFP into cells following endogenous TACC3 depletion by TACC3–3′-UTR-siRNA. The disruption of the localization of both GCP6 and γ-tubulin due to treatment with TACC3–3′-UTR-siRNA was rescued to a significant extent, by ∼76 and ∼100%, respectively, in cells co-transfected with TACC3-GFP (Fig. 7, A and B, and C and D). Similar rescue of GCP6 and γ-tubulin was also observed in cells co-transfected with TACC3–3′-UTR-siRNA and GFP-TACC3(500–838) (data not shown). Immunolocalization of other γ-TuRC proteins could not be tested because of the unavailability of antibodies suitable for immunofluorescence imaging. In contrast to the γ-TuRC proteins, the levels of pericentrin at the spindle poles were not significantly reduced in response to TACC3 depletion (data not shown). We also examined the localization of γ-tubulin complex proteins in the TACC3-depleted inter- phase cells. Similar to mitotic cells, the localization of γ-tubulin was markedly reduced, and the localization of GCP6 was com- pletely abolished, at the centrosomes in TACC3-depleted inter- phase cells (Fig. 8, A and B).

TACC Domain of TACC3 Possesses Microtubule Nucleating Activity—Next, we examined whether TACC3 itself possesses any intrinsic microtubule nucleating activity. As the recombinant full-length TACC3 protein could not be purified because of poor solubility, we purified the TACC(638–838) domain of TACC3 and determined the ability of this region to nucleate microtubule assembly in vitro. We obtained ∼99% pure TACC protein (Fig. 9A). Microtubule polymerization was analyzed using a turbidity

FIGURE 2. Rescue of TACC3 depletion-induced mitotic defects. A, Western blot to detect the expression levels of endogenous TACC3 and TACC3-GFP in siRNA-resistant TACC3-GFP-stable HeLa cells, labeled as TACC3-GFP-siRes. The levels of endogenous TACC3 expression in normal HeLa cells treated with TACC3 siRNA (48 h) and control siRNA are also shown. B, representative confocal images of mitotic TACC-GFP-siRes HeLa cells treated with control siRNA or TACC3 siRNA (48 h). Representative image of normal HeLa cells treated with TACC3 siRNA (48 h) is shown in the bottom panel. Microtubules were stained with mouse monoclonal anti-α-tubulin (red), and endogenous TACC3, labeled as TACC3-endo, was stained with rabbit monoclonal anti-TACC3 (green). TACC3-GFP expression levels in TACC3-GFP-siRes cells are shown in the panels as indicated. DNA was stained with DAPI. Scale bar, 5 μm. C, analysis of α-tubulin fluorescence intensities along the pole-to-pole spindle axis of control siRNA-treated (blue line) and TACC3 siRNA-treated (green line) TACC3-GFP siRes HeLa cells. Intensity plot for the same in TACC3 siRNA-treated normal HeLa cells is shown by red line. The solid arrows represent the relative positions of the spindle poles. D, lysates of HeLa cells transfected with TACC3–3′-UTR-siRNA or co-transfected with TACC3–3′-UTR-siRNA and TACC3-GFP plasmid DNA for 48 h were analyzed by Western blot to detect the expression levels of endogenous TACC3 and TACC3-GFP. Actin was probed as a control. Quantification of the proteins as in the Western blot is also shown, data are mean ± S.E. (three experiments). E, Western blot showing the expression levels of endogenous TACC3 and GFP. TACC3(500–838) (~64 kDa) after co-transfection with TACC3–3′-UTR-siRNA and GFP-TACC3(500–838) plasmid DNA for 48 h in HeLa cells. F, representative confocal images of mitotic HeLa cells treated with control luciferase siRNA (48 h), TACC3–3′-UTR-siRNA, TACC3–3′-UTR-siRNA plus TACC3-GFP or TACC3–3′-UTR-siRNA plus GFP-TACC3(500–838). Microtubules (red), endogenous TACC3 (green), and DNA were stained similarly as B. Exogenously expressed TACC3-GFP or GFP-TACC3(500–838) levels are shown (green) in the panels as indicated. Scale bar, 5 μm. G, analysis of α-tubulin fluorescence intensities along the pole-to-pole spindle axis direction of control siRNA-treated (black line), TACC3–3′-UTR-siRNA-treated (red), TACC3–3′-UTR-siRNA plus TACC3-GFP-treated (green), and TACC3–3′-UTR-siRNA + GFP-TACC3(500–838)–treated (cyan line) cells. The solid arrows represent the relative positions of the spindle poles. H, bar graph shows the quantification of metaphase cells with astral microtubule assembly defects in response to TACC3–3′-UTR-siRNA, TACC3–3′-UTR-siRNA plus TACC3-GFP, and TACC3–3′-UTR-siRNA plus GFP-TACC3(500–838) treatments (48 h). The bars represent mean ± S.E. n = number of metaphase cells.
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A

|          | control esiRNA | TACC3 esiRNA |
|----------|----------------|--------------|
| α-tubulin| TACC3         | DAPI         |
| 30 sec   |                |              |
| 1 min    |                |              |
| 2 min    |                |              |
| 5 min    |                |              |
| 10 min   |                |              |
| 15 min   |                |              |

B

1 min

MT intensity (a.u.)

Area of regrowth (µm²)

- Control
- TACC3 KD
The results showed that the TACC domain nucleated microtubule polymerization in vitro, independent of polymerization inducers. In control reactions, tubulin alone could not nucleate microtubule polymerization to a detectable level (Fig. 9B, control curve). However, the addition of the TACC domain to tubulin induced the nucleation of microtubule polymerization and increased the polymerization in a concentration-dependent manner. The nucleation lag phase decreased with increasing concentrations of the TACC domain (Fig. 9C). At a 1:5 molar ratio of TACC/tubulin, the nucleation lag phase was ~1.75 min, whereas at a 1:2 molar ratio of TACC/tubulin, the lag phase was reduced to 0.25 min, an ~7-fold reduction, and the turbidity was increased ~7-fold (Fig. 9C). Although the TACC domain was able to nucleate microtubule polymerization to a reasonable extent at a very low stoichiometric ratio (1:5 TACC/tubulin), we found that at higher concentrations the TACC domain increased the overall

FIGURE 3. TACC3 depletion inhibits microtubule nucleation in mitotic cells. Microtubule regrowth experiments were performed in MCF-7 cells treated with either control or TACC3 esiRNA after microtubule (shown as green) regrowth for the indicated time periods. Scale bar, 5 μm. B, bar charts show the number of microtubules emanating from the centrosomes of interphase cells in control versus TACC3 knockdown (TACC3-KD) conditions after 30 and 1 min of regrowth. Approximately 30–50 cells were analyzed in each of three independent experiments. The bars represent mean ± S.E. ***, p < 0.001.

FIGURE 4. TACC3 depletion inhibits microtubule nucleation in interphase cells. A, representative confocal images of interphase MCF-7 cells treated with either control or TACC3 esiRNA after microtubule (shown as green) regrowth for the indicated time periods. Scale bar, 5 μm. B, bar charts show the number of microtubules emanating from the centrosomes of interphase cells in control versus TACC3 knockdown (TACC3-KD) conditions after 30 and 1 min of regrowth. Approximately 30–50 cells were analyzed in each of three independent experiments. The bars represent mean ± S.E. ***, p < 0.001.
extent of microtubule polymerization in addition to its ability to stimulate nucleation (Fig. 9B). We verified that the TACC domain itself did not increase turbidity to a detectable level under similar reaction conditions in the absence of tubulin (data not shown). To avoid any nonspecific effect of the His$_6$ tag attached to the TACC domain on microtubule nucleation, we performed a control experiment with an unrelated His$_6$-tagged protein, EB1. Indeed, His$_6$-EB1 could not nucleate microtubule polymerization to a detectable extent under similar conditions (Fig. 9B). We also verified the cold instability of TACC-nucleated microtubules. TACC-nucleated microtubules were disassembled soon after incubation at 4 °C (data not shown). Electron microscopic (EM) images showed that the morphology of the TACC-nucleated microtubules was identical to that of control microtubules polymerized using DMSO as an inducer (Fig. 9D). We also confirmed that the TACC domain itself did not form any microtubule-like structures under similar experimental conditions. However, TEM images of the TACC domain revealed extended structures ranging from ~0.1 to 0.2 μm in size, indicating that the TACC domain could form small oligomeric structures under the reaction conditions (Fig. 9D). We next examined the localization of the TACC domain on TACC-nucleated microtubules in vitro using a fluorescence-based assay (see under “Experimental Procedures”). Microtubules were polymerized in vitro using a 3:1 mixture of purified rhodamine-labeled tubulin and FITC-labeled TACC domain and were sedimented onto glass coverslips for imaging (see under “Experimental Procedures”). A visibly greater amount of TACC was found to be localized at one end of the microtubules, which is presumably the nucleating end (Fig. 9E). Additionally, we also observed the localization of the TACC domain along the lengths of the microtubules, although this was visibly lower in intensity compared with the nucleating end.

To determine the molecular mechanism underlying the microtubule nucleating activity of TACC, we examined the
ability of TACC to bind the αβ-tubulin dimer using size-exclusion chromatography. To prevent the polymerization of microtubules in the protein mixtures, the size-exclusion experiments were performed under cold conditions (4 °C) and in the absence of GTP, and we verified that no polymerization was induced under these conditions. A mixture of tubulin and TACC was preincubated for 5 min and loaded onto a Superose-12 size-exclusion column, and the sizes of the eluted proteins were assessed. The results showed that the TACC domain bound to the tubulin dimer by forming a stable complex. Free tubulin dimer was eluted at 11.6 ml, whereas the mixture of 10 μM tubulin and 10 μM TACC showed an additional peak at 11.2 ml (Fig. 10A). Calibration using molecular mass standards revealed the molecular mass of the TACC-tubulin complex to be ~152 kDa, which corresponds to a 1 to ~1.7 tubulin-TACC complex (Fig. 10B). The molecular mass of the tubulin dimer was ~110 kDa. The peak corresponding to the unbound tubulin present in the mixture was not detectable from the 280-nm profile, most likely due to its low abundance as well as the small size difference between tubulin and the complex (Fig. 10A); however, this peak was detected from the profile obtained at 215 nm (data not shown). We also detected the presence of TACC and tubulin in the eluted fractions at 11.2 ml, corresponding to the TACC-tubulin complex, via SDS-PAGE analysis (Fig. 10C). The quantification of proteins from three independent SDS-polyacrylamide gels yielded a molar ratio of the tubulin dimer and TACC of 1 to ~2.1 ± 0.07, which was in the close range with the stoichiometry determined from the gel filtration data.

Unbound TACC was eluted at ~14 ml and was visible as a flat peak (red line, Fig. 10A). However, it was not detectable in the
SDS-PAGE (Fig. 10C), most likely because of its low quantity. The elution positions of TACC and tubulin were further confirmed by size-exclusion chromatography of the respective control proteins followed by SDS-PAGE analysis (Fig. 10, A, D, and E). Together, these results indicate that approximately two molecules of TACC associate with each tubulin dimer in the TACC-tubulin complex.

**DISCUSSION**

MTOC-mediated microtubule nucleation is critical for proper spindle assembly during mitosis. However, the underlying mechanisms are poorly understood. In this study, we demonstrated that TACC3 plays a critical role in this process in human cells. TACC3 depletion not only substantially reduced the density of astral microtubules emanating from the spindle poles in mitotic cells (Figs. 1 and 2) but also disrupted microtubule nucleation at the spindle poles and centrosome (Figs. 3 and 4), indicating that TACC3 plays a major role in maintaining the structural integrity of the protein complex involved in centrosome-mediated microtubule nucleation. Consistent with such a role for TACC3, we found that TACC3 mediates interactions with the components of the γ-TuRC, the core microtubule nucleating protein assembly (Fig. 5). TACC3 depletion interfered with the assembly of γ-TuRC from γ-TuSC (Figs. 5 and 6), resulting in a high abundance of γ-TuSC. This could be the result of either failed assembly or disintegration of assembled γ-TuRC. Moreover, this effect of TACC3 on γ-TuRC assembly is not indirect because our data demonstrate that TACC3 inter-
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TACC3 demonstrates that the integration of spindle microtubules at the MTOC presumably requires TACC3. A recent study (50) reported that knocking down the GCP4, GCP5, and GCP6 and γ-TuSCs are critical for the stable assembly of γ-TuRC (54). Our observation that TACC3 associates with γ-TuRC proteins and that the suppression of TACC3 disrupts the assembly of these proteins into γ-TuRC indicates that TACC3 is a critical component for stable γ-TuRC assembly (Figs. 5 and 6). We also provide evidence that depleting TACC3 disrupted the localization of GCP6 to the site of γ-tubulin contact at the centrosome (Figs. 7 and 8). Taken together, these results suggest that the integration of GCP4, GCP5, and GCP6 to γ-TuRC and to the centrosome requires TACC3.

Interestingly, the TACC domain of TACC3 had previously been shown to be involved in kinetochore-microtubule (K-fiber) stabilization. This process is believed to involve the recruitment of the microtubule-associated protein X-Map215 to the K-fibers (36). Our finding that the TACC domain exhibits intrinsic microtubule nucleating activity (Fig. 9) implies that in addition to microtubule stabilization, TACC3 is involved in the formation of nucleation centers necessary for the initiation of microtubule polymerization. Coiled coils are unique motifs that can form extended protein structures or oligomers (55–57). Overexpressed TACC had been shown to form large oligomers in cells that were associated with microtubules (58). Recent structural studies with recombinant murine TACC3 have also suggested that it can form highly extended oligomeric structures ranging from dimer to hexamer (59). Consistently, our TEM analysis of the human recombinant TACC domain indicated the formation of small oligomeric structures by TACC itself in vitro (Fig. 9D). Given that the TACC domain is predicted to be a coiled-coil domain and that it can form extended protein structures or oligomers, it is logical to think that these extended structures or oligomers could act as nucleation centers for the initiation of microtubule polymerization and the formation of microtubule lattices. In support of this idea, the TEM images showed that the inclusion of the recombinant TACC domain in the reaction mixture with αβ-tubulin in vitro resulted in the nucleation of microtubule polymerization (Fig. 9, B, D, and E). Fluorescence-based imaging data showed an increase in the accumulation of the TACC domain at one end of the microtubules, presumably the nucleating minus ends (Fig. 9E), which further supports the idea that the TACC domain acts as the nucleating center for microtubule polymerization. Consistent with such a role for the TACC domain in microtubule assembly, we showed that the TACC domain binds to the αβ-tubulin dimer (Fig. 10). The 1 to ~2 stoichiometry of the tubulin dimer-TACC complex additionally suggests that the nucleation of microtubule assembly could be mediated through the TACC dimer form. Our turbidity data showed that in addition to nucleating microtubule polymerization, the TACC domain increased overall microtubule polymerization. Consistent with such a role for the TACC domain, the TEM images showed that the inclusion of the recombinant murine TACC3 has been shown to form large oligomers in cells that were associated with microtubules (58). Recent structural studies with recombinant murine TACC3 have also suggested that it can form highly extended oligomeric structures ranging from dimer to hexamer (59). 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The 1 to ~2 stoichiometry of the tubulin dimer-TACC complex additionally suggests that the nucleation of microtubule assembly could be mediated through the TACC dimer form. Our turbidity data showed that in addition to nucleating microtubule polymerization, the TACC domain increased overall microtubule polymerization when added to tubulin at higher stoichiometry ratios (Fig. 9B). This suggests that in addition to its role in nucleation, the TACC domain is involved in microtubule elongation (growth). In support of this idea, we showed that the TACC domain localized, albeit weakly, along the lengths of microtubules...
in addition to its localization at the nucleating end (Fig. 9E). The association of the TACC domain with the microtubule lattice, as well as its involvement in microtubule growth, was consistent with previous findings by other groups (30, 36).

In conclusion, our results indicate that TACC3 plays an important role in microtubule nucleation through stabilization of the γ-TuRC assembly and through its interaction with the αβ-tubulin dimer. However, the relative contribution of these two mechanisms to microtubule nucleation awaits further investigation. Because TACC3 disruption has previously been shown to suppress tumor growth in vivo (28), it would be interesting to explore the role of TACC3-mediated microtubule nucleation in tumorigenesis. Importantly, the loss of TACC3 has been shown to induce multipolarity in lymphoma cells (34).
Therefore, our study of the TACC3-mediated stabilization of γ-TuRC may provide clues to the molecular basis of spindle pole integrity in cells.

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