TTK kinase is essential for the centrosomal localization of TACC2

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Abstract Chromosome segregation in mitosis is orchestrated by dynamic interaction between spindle microtubule and the kinetochore. Our recent ultrastructural studies demonstrated a dynamic distribution of TTK, from the kinetochore to the centrosome, as cell enters into anaphase. Here, we show that a centrosomal protein TACC2 is phosphorylated in mitosis by TTK signaling pathway. TACC2 was pulled down by wild type TTK but not kinase death mutant, suggesting the potential phosphorylation-mediated interaction between these two proteins. Our immunofluorescence studies revealed that both TTK and TACC2 are located to the centrosome. Interestingly, expression of kinase death mutant of TTK eliminated the centrosomal localization of TACC2 but not other centrosomal proteins such as γ-tubulin and NuMA, a phenotype seen in TTK-depleted cells. In these centrosomal TACC2-liberated cells, chromosomes were lagging and mis-aligned. In addition, the distance between two centrosomes was markedly reduced, suggesting that centrosomal TACC2 is required for mitotic spindle maintenance. The inter-relationship between TTK and TACC2 established here provides new avenue to study centrosome and spindle dynamics underlying cell divisional control.
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Keywords: Mitotic spindle; Kinetochore; Centrosome; TTK; TACC2

1. Introduction

Chromosome movements during mitosis are orchestrated by the interaction of spindle microtubules with a specialized chromosome domain located within the centromere (e.g., [1]). This specialized proteinous region, called the kinetochore, is responsible for mitotic microtubule-centromere association. Spatial and temporal control of microtubule dynamics is essential to ensure successful cell division. Centrosomes and kinetochores are primary regulators of microtubule dynamics in mitotic cells. Several recent studies revealed that protein kinase TTK interacts with kinetochore associated motor protein

CENP-E (e.g., [2,3]). In addition, our ultrastructural studies revealed the localization of TTK to corona fibers of the kinetochore in prometaphase cells but relocated to centrosome upon the chromosomal alignment at the equator [4]. Dynamic localization of TTK supports the notion in which TTK participates in centrosome-based spindle checkpoint (e.g., [4,5]).

TTK is a mammalian homolog of conserved Mps1 family members that are expressed in all proliferating cells and tissues [6,7], consistent with its function in cell cycle progression. In all these Mps1 family members, the C-terminal catalytic domains show a high degree of sequence similarity [8]. Furthermore, both yeast and mammalian Mps1 kinases phosphorylate serine/threonine as well as tyrosine residues [7,9,10]. It has been shown that TTK1 (hMPS1) is required for human cells to undergo checkpoint arrest in response to microtubule depolymerization. In contrast, centrosome (re-)duplication as well as cell division occurs in the absence of hMps1 [11]. While functional and ultrastructural studies support an essential role of TTK in mitotic checkpoint control, at both kinetochore and centrosome, in mammalian cells, the molecular mechanisms underlying TTK function in the mitotic centrosome are unknown.

Centrosomes are the main microtubule organizing centers in animal cells. In addition, centrosomes help to organize the poles of the mitotic spindle that is responsible for partitioning the chromosomes equally between the two daughter cells during cell division (e.g., [12]). It has been shown that the loss of a spindle checkpoint control could lead to both genetic instability and centrosomal abnormalities. However, it is unclear how spindle checkpoint signaling governs mitotic centrosomal dynamics.

Members of the transforming acidic coiled-coil (TACC) family of proteins have all been implicated in centrosome dynamics [12,13]. There are at least three members of TACC proteins in human cells. The three human TACC genes map to the chromosomal regions 8p11, 10q26 and 4p16, respectively (e.g., [13]). These chromosomal regions have been re-arranged in certain cancers, in particular breast cancer. Although there are three TACC proteins in human, their tissue-specific expression and distinct spatial–temporal regulation of subcellular distribution argue for functional specificity among the three proteins. Interestingly, TACC2 has also recently been identified as a potential tumor suppressor in a search for genes differentially expressed between malignant mammary epithelial cells and their immediate non-malignant progenitors [14].

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Abbreviations: TACC, transforming acidic coiled-coil; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; DAPI, 4,6-diamidino-2-phenylindole

TACC2 was often found to be downregulated as the cells progressed from a benign to a malignant phenotype of breast epithelial cells [14].

To better address the function of TTK in human cells, we have searched for proteins downstream from TTK signaling in HeLa cells. We show that the protein phosphorylation of TACC2 was minimized when TTK kinase death mutant was expressed. Our immunofluorescence study verified the co-localization of TTK with TACC2 in the centrosomes of mitotic cells. Significantly, loss of TTK activity nails down the centrosomal localization of TTK. The loss of TACC2 shortened the distance between the two centrosomes and perturbed chromosome segregation. Thus, we proposed that TTK exerts its centrosome-based function by modulating TACC2 in mitosis.

2. Materials and methods

2.1. Cell culture

HeLa and 293T cells, from American Type Culture Collection (Rockville, MD), were maintained as subconfluent monolayers in DMEM (Invitrogen; Carlsbad, CA) with 10% FBS (Hyclone, UT) and 100 U/ml penicillin plus 100 μg/ml streptomycin (Invitrogen).

2.2. Transient transfection and immunoprecipitation

HeLa cells were grown to ~75% confluency in DMEM with 10% FBS at 37 °C in 10% CO_2 and were exposed to 100 ng/ml nocodazole (Sigma Chemical Inc.) for 20 h. Mitotic shake-off was conducted to collect mitotic cells as described previously (e.g., [15,16]). In some cases, mitotic cells were collected and plated in nocodazole-free media for 4 h to enter early G1 (as judged by loss of cyclin B protein). These mitotic and G1 cells were then lysed in buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EGTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml pepstatin A), respectively. Lysates were clarified by centrifugation at $16\,000 \times g$ for 10 min at 4 °C and then incubated with anti-GFP antibody (BD Biosciences) bound protein-A/G beads (Pierce Chemical, IL). After rotating at 4 °C for 2 h, beads were washed five times with lysis buffer and then boiled in protein sample buffer for 2 min. After SDS–polyacrylamide gel electrophoresis (SDS–PAGE), proteins were transferred to nitrocellulose membrane. The membrane was divided into three strips and probed with antibodies against the TACC2, TTK (Zymed), and β-tubulin (Sigma Chemicals), respectively. Immunoreactive signals were detected with ECL kit (Pierce Chemical) and visualized by autoradiography on Kodak BioMAX film.

2.3. Suppression of TTK activity by SiRNA mediated protein knock-down or kinase death mutant

RNA interference was carried out as described previously (e.g., [17]). A 21-mer siRNA oligonucleotide, corresponding to nucleotides 1077–1097 from the start codon of TTK, was synthesized by Dharmacon Inc. (Lafayette, CO). SiRNA was delivered into cells by pre-mixing Lipofectamine 2000 (Invitrogen) with siRNA oligonucleotide in Opti-MEM (Invitrogen). The plates were replaced with fresh medium 16 h later and coverslips were usually harvested 36–48 h after transfection.

A kinase-death version of TTK (TTK-KD) was constructed by introducing a point mutation (D664A) in subdomain VII of the catalytic domain (by analogy with Mps1p; [18]) in pEGFP vector (Clonetch, Pala Alto, CA) as described [19]. Western blotting analyses using TTK monoclonal antibody show that exogenously expressed GFP-TTK protein is 2.3-fold higher than that of endogenous protein. In some cases, DNA profiles of the GFP-TTK (wild type and KD) expressing cells were determined by FACS analysis 48 h after the transfection as described [17].

2.4. Immunofluorescence microscopy

For immunofluorescence, cells were seeded onto sterile, acid-treated 18-mm coverslips in 6-well plates (Corning Glass Works, Corning, New York). Double thymidine blocked and released HeLa cells were transfected with 2 μg/ml lipofectamine 2000 pre-mixed with various siRNA oligonucleotides as described above. In general, 36–48 h after transfection with siRNA or scrambled (control) oligonucleotides, HeLa cells were rinsed for 1 min with PHEM buffer (100 mM Pipes, 20 mM HEPES, pH 6.9, 5 mM EGTA, 2 mM $MgCl_2$ and 4 M glycerol) and were permeabilized for 1 min with PHEM plus 0.2% Triton X-100 as described (e.g., [15,16]). Extracted cells were then fixed in freshly prepared 4% paraformaldehyde plus 0.05% glutaraldehyde in PHEM, and rinsed three times in PBS. Cells on the coverslips were blocked with 0.05% Tween 20 in PBS (TPBS) with 1% BSA (Sigma Chemicals). These cells were incubated with various primary antibodies in a humidified chamber for 1 h and then washed three times in TPBS. Monoclonal antibodies bound to γ-tubulin and TTK were visualized using fluorescein-conjugated goat anti-mouse IgG, respectively, while binding of TACC2 was visualized using Texas Red-conjugated donkey anti-rabbit IgG. DNA was stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma Chemicals). Slides were examined with a Zeiss LSM510 confocal scanning fluorescence microscope and images were collected and analyzed with Image-5 (Carl Zeiss, Germany).

2.5. Western blot

Samples were subjected to SDS–PAGE on 6–16% gradient gel and transferred onto nitrocellulose membrane. Proteins were probed by appropriate primary and secondary antibodies and detected using ECL (Pierce Chemical). The band intensity was then scanned using a Phosphoimager (Amersham Bioscience, NJ).

3. Results and discussion

3.1. TACC2 is a phospho-protein associated with TTK

Requirement of TTK in spindle checkpoint signaling propelled us to search for its downstream effectors. We adopted GFP immunoprecipitation from HeLa cells expressing GFP-TTK (both wild type and kinase death) followed by mass spectrometric identification of in-gel digested peptide fingerprint [2,19]. Our preliminary mass spectrometric identification suggested that a polypeptide with approximately 120 kDa is a TACC2-like protein. To validate if TACC2 is associated with TTK protein, we probed GFP-TTK immunoprecipitates with affinity-purified TACC2 antibody. As shown in Fig. 1A, TACC2 Western blot verified that the 120 kDa protein in GFP immunoprecipitates is indeed TACC2. Careful examination of the TACC2 Western blot revealed that TACC2 migrates slower from cells expressing wild type TTK compared to that of kinase death TTK expressing cells. In addition, fewer TACC2 was recovered from kinase death TTK expressing cells compared to that of wild type TTK expressing cells. Probing same blot with a β-tubulin antibody indicates that TACC2 is specifically pulled down by TTK protein. Since TTK protein remains with diminished kinase activity in early G1 [7], we sought to validate if TACC2-TTK interaction depends on the kinase activity. Immunoprecipitation of TTK protein from G1 cell lysates did not pull down any TACC2 protein, indicating that the TTK-TACC2 interaction is a function of TTK kinase activation.

To validate if mobility shift of TACC2 is related to TTK kinase activity, we carried out Western blot of cell lysates from HeLa cells treated with TTK siRNA oligonucleotide and a scrambled oligonucleotides. As shown in Fig. 1B, Western blot with TTK antibody verified the suppression of endogenous TTK protein level. Western blotting of TACC2 confirmed that its mobility shift is a function of TTK protein kinase activity. Thus, we conclude that the interaction between TACC2 and TTK is likely a downstream component of the kinase cascade.
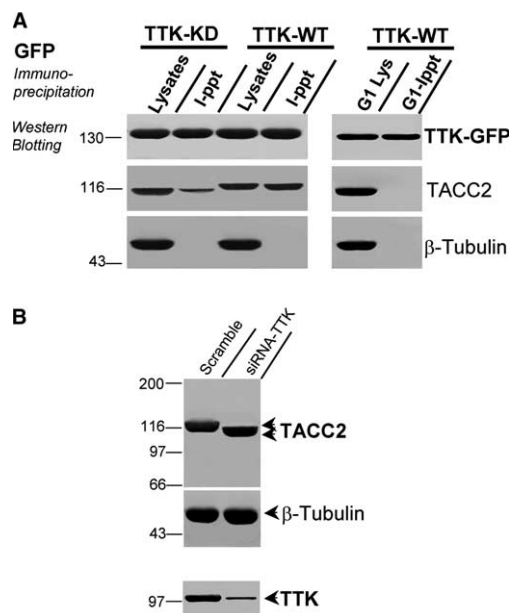
Fig. 1. TACC2 is a downstream effector protein of TTK kinase. (A) Immunoprecipitation of GFP pull down TACC2 from mitotic, but not G1, HeLa cells expressing GFP-TTK. HeLa cells transfected with GFP-TTK plasmids (wild type, referred to TTK-WT, and kinase death mutant, denoted as TTK-KD) for 24 h followed by treatment with 100 nM nocodazole for another 20 h as described in Section 2. After extraction in lysis buffer and clarification by centrifugation, lysates (~5 mg) were incubated with 10 μg GFP mouse IgG pre-bound protein A/G beads. Proteins from starting lysates and Nek2A immunoprecipitates were analyzed by SDS–PAGE and immunoblotting using a GFP rabbit antibody, TACC2 affinity-purified antibody, and a β-tubulin antibody, respectively. In the experiment shown in (A), Western blotting verifies co-immunoprecipitation of TACC2 and wild type TTK. The mobility shift of TACC2 seen in SDS–PAGE may reflect the possibility of protein phosphorylation retained from wild type TTK-expressing cells. No tubulin was detected in GFP immunoprecipitates. In a separated experiment (right panels in A), Western blotting verifies that TTK did not bring down any TACC2 protein from interphase cells (G1 Lys). The mobility shift of TACC2 (G1 vs. mitotic) seen in SDS–PAGE may reflect a diminished TTK kinase activity in interphase cells and kinase death mutant expressing cells, respectively. (B) Mobility shift of TACC2 protein band is a function of TTK kinase activity. HeLa cells treated with siRNA oligonucleotide for TTK and scrambled sequence control, respectively, followed by nocodazole treatment and solubilization in SDS–PAGE sample buffer as described in Section 2. Aliquots of total cell lysates (35 μg protein) from treated samples were fractionated on a 6–16% gradient SDS–PAGE followed by Western analyses with antibodies against TACC2, β-tubulin, and TTK, respectively. Western blotting with TTK antibody verified an effective knock-down in TTK protein accumulation, while probing with TACC2 antibody validated that the mobility shift of TACC protein is the function of TTK kinase.

3.2. TACC2 is co-localized with TTK to centrosome of mitotic cells

Previous studies of TTK suggest its primary association with the kinetochore (e.g., [2,11]) in addition to centrosome. TACC2 is a centrosomal protein whose function is implicated in spindle stability (e.g., [20]). To explore the spatial–temporal relationship of TTK and TACC2 distribution in mitotic cells, we carried out immunocytochemical studies of HeLa cells using confocal scanning fluorescence microscope.

To better visualize molecular localization, we adopted a pre-extraction procedure that allows effective labeling of kinetochore and centrosomal proteins while preserving fine

cyto-structure of mitotic cells [8,15,16]. As shown in Fig. 2, pre-extracted HeLa cells were stained with a TTK monoclonal antibody followed by a fluorescein-conjugated secondary antibody, while TACC2 is labeled with an affinity-purified rabbit antibody followed by a Texas Red-conjugated secondary antibody. In metaphase cells shown in Fig. 2(c), it is readily apparent that TACC2 becomes as bright centrosomal staining located to two opposite poles, which is consistent with a previous report (e.g., [20]). Double labeling of the same cell with TTK mouse antibody displayed typical centrosome and kinetochore stainings as marked by arrowheads and arrows, respectively (Fig. 2(a)). The centrosomal protein staining of TTK is reminiscent of TACC2 labeling, which becomes apparent as two merged images generated yellow color (Fig. 2(d), arrowheads). The superimposition of TACC2 labeling onto that of TTK at the centrosome validates the early biochemical interaction between these two proteins.

As we reported in our immuno-electron microscopic studies [4], TTK dissociates from the kinetochore and traffics to the centrosomes as sister chromatids separate (Fig. 2(e), arrowheads). Labeling of TACC2 from the same cell displays a typical centrosomal labeling (Fig. 2(g), which is superimposed onto that of TTK. Given the co-distribution profile of these two proteins to the centrosome during mitosis and their association revealed by the immunoprecipitation, we conclude that TTK interacts with TACC2 at the centrosome in mitotic cells.

3.3. TTK kinase activity is essential for the centrosomal localization of TACC2

TACC2 is implicated in maintaining the stability of mitotic spindle (e.g., [20]). Since TACC2 mobility shift in SDS–PAGE is a function of TTK activity, we set to test whether the centrosomal association of TACC2 is dependent on TTK activity. To this end, we introduced wild type and kinase death TTK into HeLa cells by transfection of GFP-tagged TTK cDNA constructs. Thirty-six hours after the transfection, cells were collected and stained for GFP, TACC2 and DNA, respectively. As shown in Fig. 3A, expression of GFP-tagged TTK revealed a typical centrosomal and kinetochore staining identical to those of endogenous. As predicted, exogenously expressed TTK was superimposed to that of TACC2 at the centrosome of mitotic cells (arrowheads; Fig. 3A(d)), suggesting that expression of exogenous TTK did not interfere with the localization of TACC2 in HeLa cells.

We next examined whether expression of kinase death TTK (GFP-TTK-KD) would alter the localization of TACC2 on the centrosome. Thirty-six hours after the transfection, cells were collected and stained for GFP, TACC2 and DNA, respectively. As shown in Fig. 3A(e and i), kinase death TTK remains largely associated with the centrosomes with slightly lower level deposition onto the kinetochores (arrow). However, double labeling of the same cells with TACC2 revealed no centrosomal but diffused spindle staining (Fig. 3A(g)), suggesting the possible liberation of centrosomal TACC in the absence of TTK. To test if there was gross alteration of centrosomal structure arisen from the loss of TTK activity, we doubly stained the HeLa cells expressing kinase death TTK for γ-tubulin, a founding component for the centrosome biogenesis. As shown in Fig. 3A(k), γ-tubulin marked a typical centrosomal staining (arrowheads), suggesting that loss of centrosomal TACC2 is a specific consequence of TTK loss. In
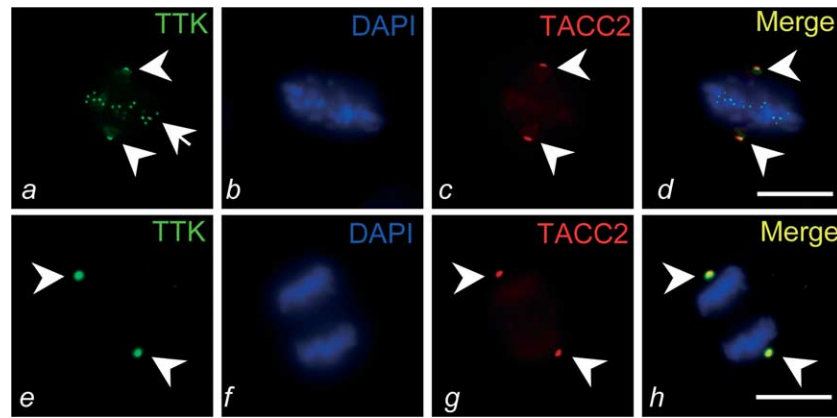
Fig. 2. Co-localization of TTK with TACC2 to the centrosomes. This set of montage represents optical images collected from prometaphase and anaphase of HeLa cells triply stained for rabbit TACC antibody (red), DAPI (blue), mouse TTK antibody (green), and their merged images. In the prometaphase cell (a–d), TTK displayed a typical centrosomal staining (a, arrowheads) in addition to characteristic kinetochore labeling (arrow). The chromosome configuration was marked by DAPI staining (b). The TACC2 labeled two bright spots situated at the opposite pole across almost-aligned chromosomes. Merge indicated that TACC2 is a centrosomal protein superimposed onto that of TTK. Bar: 10 μm. In anaphase cells (e–h), TTK dissociated from centromeres and concentrated to the centrosomes (arrowheads, e). A merge shows a superimposition of TACC2 to that of TTK staining at the centrosome but not kinetochore. Bar: 10 μm.

fact, elimination of TTK protein by siRNA-mediated knockdown validated our observed dislocation of TACC2 in kinase death TTK expressing cells, while localization of other centrosomal proteins such as NuMA remained unaltered (data not shown). Significantly, the distance between the two centrosomes was markedly reduced in these TTK kinase eliminated cells (8.1 ± 2.6 μm for kinase death; 7.9 ± 2.5 μm for siRNA-treatment) than those in control cells (12.5 ± 1.7 μm; listed in Table 1), consistent with the notion in which TACC2 is important for maintaining spindle stability [20,21]. Therefore, we conclude that TTK is essential for localization of TACC2 to the centrosome.

Elimination of TTK, either by overexpression of kinase death mutant or siRNA-mediated suppression, revealed typical phenotypes consistent with failures in both centrosome and kinetochore including chromosome mis-alignment at metaphase (e.g., Fig. 3A(f and f'), arrows) and lagging chromosomes during anaphase (data not shown). These mis-aligned chromosomes, close to either spindle poles, are marked by BubR1 (Fig. 3A(h'), arrows) while these centrally positioned chromosomes were free of BubR1 (Fig. 3A(h'), double-arrow), indicating that these BubR1-marked chromosomes were not aligned (e.g., [16]). Even these highly disorganized cells, however, were capable of eventually exiting mitosis, and we observed many cells in interphase with an aberrant DNA content (Fig. 3B), a phenotype seen in TACC3-depleted mammalian cells (e.g., [13]). These observations demonstrate that while association of TACC2 with the centrosome is essential for faithful mitosis in HeLa cells, phenotypical change related to the elimination of centrosomal TACC2 may be compensated by other TACC proteins given their overlapped localization to the centrosome. Given the role of TTK in centrosome and spindle stability maintenance [5], the failure to recruit TACC2 to mitotic centrosome presumably explains, at least in part, the function of TTK in centrosome dynamics. Future experimentation will clarify the specific role of TTK-TACC2 interaction in cell cycling.

Recent studies from Glover and colleagues demonstrated that centrosomal localization of Drosophila TACC (denoted as D-TACC; the only TACC protein in flies) to the mitotic spindle and centrosome could occur via distinct mechanisms [21]. They have showed that inactivation of Aurora-A kinase, another mitotic kinase located to the centrosomes, diminished the assembly of D-TACC onto the centrosome but not to the spindle. In addition, the mutation of D-TACC also nailed down the localization of MSPS, a microtubule-binding protein also located to the centrosome [22]. Since MSPS interacts with D-TACC in vitro and in vivo, it remains to be established whether loss of centrosomal localization of D-TACC is the secondary consequence associated with the dislocation of MSPS arisen from Aurora-A inactivation. Given the fact that Drosophila Aurora-A interacts with and phosphorylates D-TACC, it would be important to know whether human Aurora-A kinase interacts with TACC2 and whether it is required for the localization of TACC2 to the centrosome.

Bissell and colleagues have recently isolated TACC2/Azu-1 in a screen aimed for identification of stage-specific tumor suppressors during tumorigenesis of human breast cancer [14]. They found that TACC2/Azu1 was downregulated not only in tumorigenic mammary epithelial cells, but also in primary tumors. The inability to assemble TACC2 protein onto the centrosome in TTK kinase suppressed cells demonstrated in our studies suggests that Nek2A kinase may form a link between TACC2 protein phosphorylation and its role in the centrosome dynamics and chromosome segregation fidelity. Given the aberrant expression profile of TTK in human breast cancer, it would be of great interest to further elucidate the molecular interaction between TTK and TACC2, and the precise function of TTK in the centrosome dynamics given its importance in maintaining chromosome stability.

Taken together, our finding of the interaction of TTK with TACC2 demonstrates a critical role of TTK in centrosome dynamics in addition to its function at mitotic checkpoint. The fact that suppression of TTK kinase activity disrupts assembly and/or retention of TACC2 to the centrosome demonstrates the function and importance of TTK kinase cascade in integrating kinetochore and centrosome dynamics with the mitotic checkpoint signaling. Our studies presented here open a new
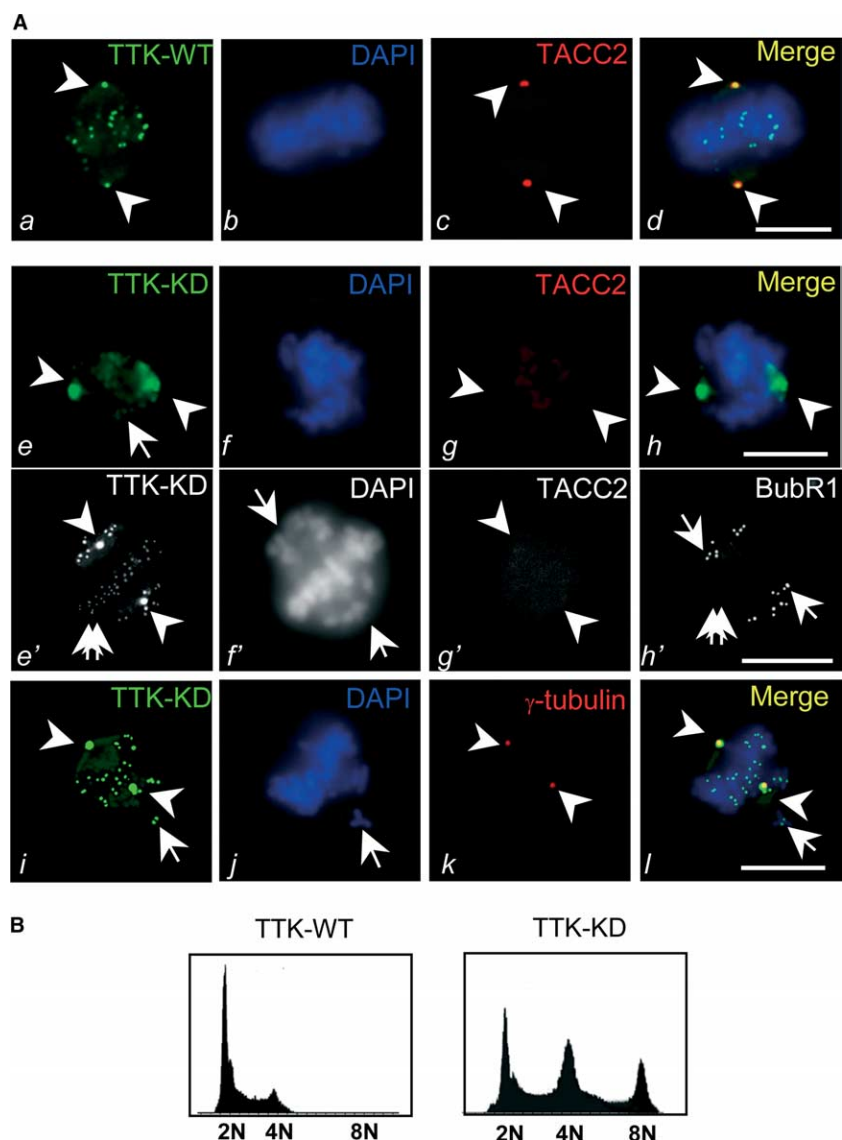
Fig. 3. The centrosomal localization of TACC2 is dependent on TTK kinase activity. (A) This set of montage represents optical images collected from HeLa cells triply stained for GFP-TTK wild type (green; TTK-WT, a) and kinase death mutant (green; TTK-KD, e and i), DAPI (blue; b, f, and j), an affinity rabbit TACC2 antibody (red; c and g), or γ-tubulin rabbit antibody (red; g), and their merged images (d, h, and l), respectively. These cells were transfected with GFT-TTK plasmids (wild type and kinase death) for 36 h followed by fixation and immunocytochemical staining. Wild type GFP-TTK marked the centromeres the centrosome (arrows) in mitotic cells (a). Overexpression of GFP-TTK kinase death mutant displays a typical centrosomal and kinetochore staining in addition to deposition onto spindle due to the high expression. Overexpression of TTK-KD caused chromosome segregation abnormality phenotype also seen in TTK depleted cells. These cells display chromosome mis-alignment and a reduced distance between two centrosomes. Overexpression of TTK-KD displaced centrosomal localization of TACC2 (arrowheads, g) without alteration of the centrosomal staining of γ-tubulin (arrowheads, k). Lagging and mis-aligned chromosomes were seen in TTK-KD expressing cells (arrow, f, f′ and j). Bars: 10 μm. (B) DNA profiles of the GFP-positive cells, transfected with wild type kinase and kinase death mutant, were determined by FACS analysis. Suppression of TTK kinase activity caused a shift of DNA contents. Cells expressing kinase death TTK accumulate with a 4N DNA content and a significant proportion of cells with polyploidy (25.7% compared with 3.5% in control cells expressing wild type TTK).

Table 1
Liberation of centrosomal TACC2 releases tension between sister centrosomes

Treatment	Distance (μm)[a]
Control	12.5 ± 1.7
SiRNA (TTK)	7.9 ± 2.5
TTK-GFP	13.5 ± 2.3
TTK-KD	8.1 ± 2.6

[a] Distance between γ-tublin-marked centrosomes. Data were obtained from >100 cells in which both centrosomes were in the same focal planes (see Section 2).

avenue to define the molecular mechanisms underlying centrosome dynamics in cell division control.

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