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이 학 석 사 학 위 논 문

미세소관 형성을 조절하는
중심체 **Cdc6**의 기능

Cdc6 negatively modulates the microtubule

organizing activity of the centrosome

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Abstract

Cdc6 negatively modulates the microtubule organizing activity of the centrosome

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The centrosome serves as a major microtubule-organizing center (MTOC). The Cdc6 protein, which is a component of pre-replicative complex and a licensing factor for the initiation of chromosome replication, localizes to centrosomes during the S- and G2-phases of the cell cycle of human cells. We found that Cdc6 negatively regulates MTOC activity and the amounts of the integral proteins composing the pericentriolar material (PCM). Whereas Cdc6 depletion increased microtubule nucleation at the centrosomes, induction of Cdc6 in Cdc6-depleted cells reduced this increase. This increase and reduction correlated with the centrosomal intensities of PCM proteins, such as γ-tubulin, pericentrin, CDK5RAP2, and Cep192. These regulations required the ATPase activity as well as the centrosomal localization of Cdc6. These results suggest a novel Cdc6 function that regulates centrosome assembly and function.

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List of Abbreviations

BrdU   Bromodeoxyuridin

Cdc6   cell division cycle 6

CLS   centrosome localization signal

DAPI   4',6-diamidino-2-phenylindole

DMEM   Dulbecco's modified Eagle's medium

FACS   fluorescence-activated cell sorting

GFP   green fluorescent protein

LI/AA   L313A/I316A

MTOC   microtubule-organizing center

NES   nuclear export signal

PBS   phosphate-buffered saline

PBST   phosphate-buffered saline containing 0.1% Triton X-100

PCM   pericentriolar material

pre-RC   pre-replicative complex

siRNA   small interfering RNA

Introduction

The centrosome, which plays roles in diverse cellular and developmental processes, consists of a pair of centrioles surrounded by pericentriolar material (PCM) (1-3). PCM is not a membrane-bound, amorphous mass, but rather contains a highly ordered hierarchical organization. The integral scaffold proteins, such as pericentrin, CDK5PAP2, CEP 192, CEP152, and NEDD1, exhibit concentric toroidal distributions in interphase PCM (4,5). PCM is matured and expanded at the onset of mitosis to form bipolar spindles for chromosome segregation in mitosis. During interphase, centrosomes associated closely with the nucleus are duplicated; they then separate and migrate to the poles of the dividing cell, to function as spindle poles during mitosis (6).

The centrosome serves as major microtubule-organizing center (MTOC). Microtubules are nucleated at the γ-tubulin ring complexes (γ-TuRC) embedded in the PCM (7-9). γ-tubulin in the γ-TuRC functions as a nucleation core for microtubule polymerization. α- and β-tubulins polymerize into microtubules (10-12). Tubulin modifications and microtubule-associated proteins (MAPs) regulate microtubule dynamics (13,14). During interphase, microtubules originating from centrosomes form astral microtubules that position the nucleus (15). Microtubules play important roles in diverse cellular functions, including chromosome segregation, vesicular transport, cell motility, cell shape, and motility (16-19).

To initiate eukaryotic chromosome replication during the G1 phase of the cell cycle, the association of Cdc6 with an origin recognition complex (ORC), bound to the origin of replication, recruits Cdt1/MCM2-7 complexes to form the pre-replicative complex (pre-RC) (20,21). Cyclin A/Cdk2 phosphorylation and nuclear export signals promote non-chromatin bound Cdc6 to translocate to the cytoplasm during the G1/S transition phase (22-24). Pre-RC formation during the G1 phase is essential to ensuring that chromosomal replication occurs only once per cell cycle. As a member of AAA+ family of ATPases, Cdc6 possesses the Walker A phosphate-binding loop (P-loop) and the Walker B motif, both of which are essential for ATP binding and hydrolysis. Cdc6 is highly conserved within metazoans; its ATPase activity is required for pre-RC assembly and for other biological functions (25-28). p21 and p27 bound to Cdk2 are eliminated by Cdc6 to activate Cdk2 for cell cycle progression (29,30). Studies have also shown that the formation of stable complexes of Cdc6 with Apaf-1, activated by cytochrome c, inhibit the apoptosome assembly required for cell death (31).

Although pre-RC formation occurs during G1 phase, Cdc6 protein levels are minimal during this phase, and its mRNA and protein levels don't begin to increase until S phase (32-34). Non-chromatin bound Cdc6 is exported from the nucleus to the cytoplasm during S phase (32,35). Furthermore, Cdc6 localizes to centrosomes during both the S and G2 phases (36,37). In this work, we address Cdc6 function at centrosomes during the S and G2 phases.

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Materials and Methods

DNA construction and transfection

FLAG-tagged full-length wild type or the L313A/I316A (LI/AA) mutant form of the cdc6 open reading frame was cloned into the pTRE2hyg vector (Clontech) Deleted or/and mutated cdc6 DNA were cloned into the p3XFLAG-CMV7 vector (Sigma). GFP-tagged Cdc6 fragment 75–366-PACT, as wild type or in mutant form, were cloned into the PCDNA5/FRT/TO vector (62). DNA constructs were transfected into cells using Lipofectamine 2000 transfection reagent (Invitrogen).

Short interfering RNA (siRNA) transfection

siRNA oligonucleotides were purchased from ST Pharm. GL3 siRNA, the control, had the following sequence: 5′-CUU ACG CUG AGU ACU UCG ATT-3′. Cdc6 siRNA had the following sequence: 5′-UAA GCC GGA UUC UGC AAG A-3′. siRNA oligonucleotides were transfected into cells using Oligofectamine (Invitrogen).

Cell culture and cell line construction— U2OS and HeLa cells were cultured in Dulbecco`s modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (penicillin/streptomycin). To generate the siRNA-resistant Cdc6, site-directed mutagenesis was performed by using the primers 5′- CTG CCT GCT TAA GTC GGA

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TCC TGC AGG ACC TCA AGA AGG-3′ and 5′-CCT TCT TGA GGT CCT GCA GGA TCC GAC TTA AGC AGG CAG-3′. The Cdc6 siRNA-resistant, FLAG-tagged, wild type or mutant Cdc6 DNA was cloned into the pTRE2hyg vector (Clontech) and transfected into U2OS Tet-On cells (Clontech). GFP-tagged Cdc6 fragment 75-366-PACT wild-type or mutant form was transfected into FRT/TO HeLa cells (gift from Dr. H. Lee). Hygromycin-resistant cells were selected by culturing in 200 μg/mL hygromycin for two weeks, and were then used in experiments. Expression of Cdc6 was induced by addition of 2 μg/mL doxycycline to the culture medium and allowing it to incubate for 48 h.

Microtubule regrowth assay

The microtubule regrowth assay was performed as previously described (63), with minor modifications. Cells were incubated for 1 h on ice to depolymerize microtubules. To allow microtubule regrowth, cells were washed with PBS and incubated for 15 sec, unless indicated, in fresh medium at 37 °C, followed by fixation in PEM buffer (80 mM PIPES, 5 mM EGTA, 1 mM MgCl2, 0.5% Triton X-100, and 4% PFA). Microtubule regrowth was quantified via immunostaining; the fluorescence intensity of a 5 μm diameter area around the centrosomes was measured using ImageJ software.

Immunofluorescence microscopy

Cells grown on coverslips were fixed with cold methanol for 10 min. Cells were permeabilized by incubation with 0.5% PBST (PBS containing 0.5% Triton X-100) for 10 min. After a 30 min incubation in blocking solution (PBS containing 3% bovine serum albumin [BSA] and 0.1% Triton X-100), cells were immunostained with monoclonal anti–α-tubulin antibodies (Sigma), rabbit anti-EB1 antibodies (Millipore), anti-β-tubulin antibody (Santa Cruz), anti-cyclin E (Santa Cruz), anti-cyclin A (Santa Cruz) or polyclonal anti–γ-tubulin antibodies (Sigma). Anti-Cep215/CDK5RAP2 (63), anti-pericentrin (64), and anti-Cep192 (65) antibodies were previously described. Cells were washed three times with 0.1% PBST, incubated with Cy3- or FITC-conjugated anti-rabbit or anti-mouse secondary antibodies, washed three times with 0.1% PBST, and then mounted on glass slides with mounting media (Biomeda Corp.) containing 1μg/mL 4′,6-diamidino-2-phenylindole (DAPI, Vectashield). Cells were viewed using an Olympus BX51 microscope.

BrdU incorporation and fluorescence-activated cell sorting (FACS) analysis

BrdU incorporation was performed by pulsing cells with 10 μM of BrdU for 45 min and then staining with BrdU Flow Kits (BD Biosciences). Cell cycles were analyzed using the FACS Calibur instrument (BD Biosciences).

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Statistical analysis

Groups were compared using two-tailed Student's t-test and Prism software (GraphPad). A p-value below 0.05 was considered statistically significant. At least three independent experiments were performed for statistical analysis.

Results

Cdc6 depletion increases microtubule regrowth originating from centrosomes

Cdc6 localizes to the centrosomes during the S and G2 phases of the human cell cycle (Fig. 1A and B) (36,37). Because the centrosome functions as a MTOC, we examined whether Cdc6 plays a role in microtubule formation. siRNA-mediated partial depletion of Cdc6 in U2OS cells was performed under conditions that minimized its incidental influence on chromosome replication and cell cycle progression (Fig. 2). Partial depletion with a Cdc6-specific siRNA for 24 h caused cells to contain approximately 25% of the Cdc6 protein amounts seen in cells treated with control siRNA GL3. This partial depletion also marginally affected DNA synthesis, as determined by BrdU incorporation followed by fluorescence-activated cell sorting (FACS) analysis.

Cdc6-depleted cells were incubated on ice for 1 h to depolymerize microtubules, followed by incubation at 37 °C to allow microtubule regrowth (38). Microtubule regrowth originating from the centrosomes was measured by immunostaining cells with either anti-α-tubulin or anti-EB1 antibodies to detect microtubule formation (Fig. 3A). α-tubulin is a structural subunit of microtubules (12). EB1, which is a microtubule plus-end tracking protein (39), produces "comets" that indicate microtubules emanating from the centrosome (40). The microtubule regrowth occurred in a time-dependent manner (Fig. 3). The intensities of both α-tubulin and EB1 were near 50% higher in Cdc6-depleted cells than in

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control cells (Fig. 4A). Depletion of Cdc6 did not significantly influence the amounts of α-tubulin, β-tubulin, or other proteins that are involved in microtubule formation (Fig. 4B). Therefore, the increased microtubule regrowth in Cdc6-depleted cells suggests that Cdc6 inhibits microtubule formation, rather than affecting (perhaps by downregulating) the expression of the proteins that participate in microtubule formation.

Centrosomal localization of Cdc6 protein reduces microtubule formation

We determined whether centrosomal localization of Cdc6 is required to inhibit microtubule formation. Amino acid residues 311–366 of Cdc6 were determined to be the centrosomal localization signal (CLS) region of the protein (therefore indicated as Cdc6-CLS) (36). The Cdc6-CLS allowed fused tag proteins, such as DsRed, GFP, and FLAG, to locate at the centrosome. Substitutions of Leu-313, Ile-316, or both, which are highly conserved within metazoans, with Ala prevented localization of the substituted Cdc6 to the centrosome (36; Fig. 5A). Cdc6(LI/AA) contained substitutions of both Leu-313 and Ile-316 with Ala. Cdc6 siRNA-resistant, [FLAG-Cdc6 wild type] or [FLAG-Cdc6(LI/AA)] coding sequences were introduced into a U2OS Tet-On cell line. Addition of doxycycline to the cultures induced expression of the corresponding Cdc6 protein (Fig. 6B). Induction of wild type or mutant protein expression in Cdc6- depleted cells did not significantly alter the cell cycle progression. Cdc6 depletion increased microtubule

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Figure 1. Centrosomal localization of Cdc6

FRT/TO HeLa cells were transfected with constructs expressing the indicated GFP-tagged Cdc6 derivatives. Cells were immunostained with anti-γ-tubulin and anti-Cyclin A or anti-Cyclin E antibodies. (A) Cyclin E is a marker of the G1 phase, and cyclin A is a marker of both the S and G2 phases (43,44). (B) Centrosomal localization of GFP-tag in Cyclin E- or Cyclin A-positive cells was monitored and quantified. Cyclin E-positive FL, N = 0/42; 75–366, N = 0/38; +PACT, N = 34/36. Cyclin A-positive FL, N = 35/45; 75–366, N = 19/30.

A

Cyclin E	Cyclin A	DAPI /Merge	
			58%
			42%

B

	Cyclin E	GFP	γ-tub	Merge	Centrosome Localization (%)
FL					0
75 - 366					0
+ PACT					94

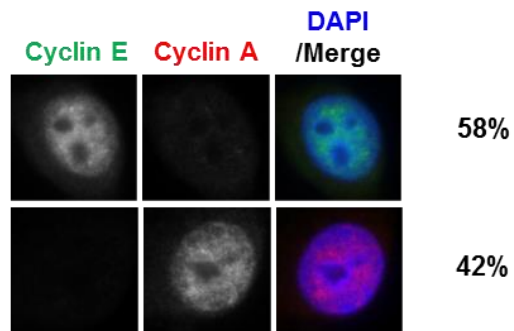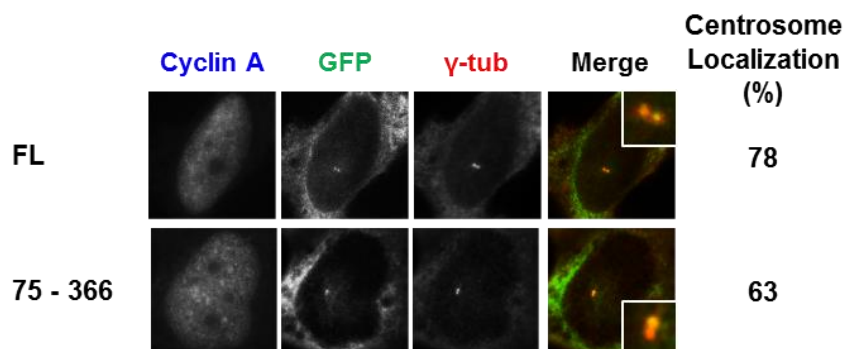|	Cyclin A	GFP	γ-tub	Merge	Centrosome Localization (%)
FL					78
75 - 366					63

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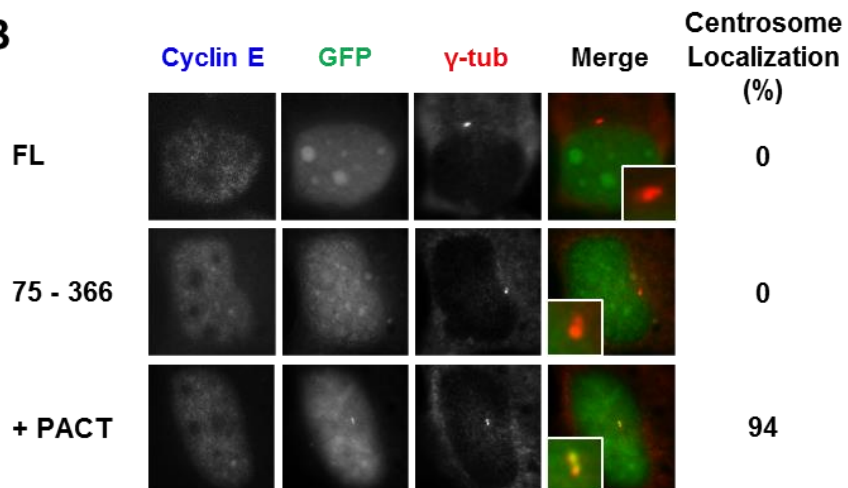
Figure 2. siRNA-mediated partial depletion of Cdc6

U2OS cells were transfected with control GL3 or Cdc6-specific siRNA for 24 h (upper left) or the indicated times (upper right), followed by immunoblot analysis. The lysates of the control siRNA-treated cells were loaded with the indicated, relative volumes (upper left). Actin served as an internal control. FACS analysis was performed with BrdU (lower top) and propidium iodide (lower bottom) staining of the Cdc6-depleted cells for the indicated time periods after transfection. S-phase cells are indicated in the dashed boxes. The proportions of replicating S-phase cells are shown below the FACS profiles. siGL3, control siRNA GL3; siCdc6, Cdc6-specific siRNA.

siGL3 siCdc6

	0.25	0.5	1	1	kDa
Cdc6					60
actin					45

siGL3 siCdc6

		24	48	72	(hr)
Cdc6					60
actin					45

siCdc6

	siGL3	24	48	72 (hr)
FITC				
Counts				

% of
S phase cells

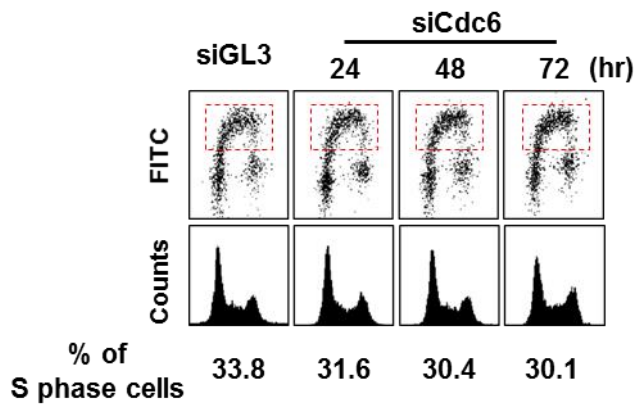| 33.8	31.6	30.4	30.1
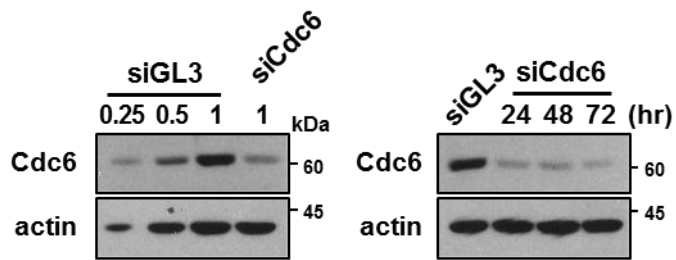
Figure 3. Time course of microtubule regrowth.

Time course microtubule regrowth assays were performed using cells treated with the indicated siRNA for 24 h. Microtubule regrowth assays were performed with cells treated with the indicated siRNA for 24 h, as described in Materials and Methods. Cells were fixed at 0, 10, 20, and 40 sec after transferring to 37 °C. (A) Microtubules were immunostained with antibodies specific to α-tubulin or EB1. (B) Centrosomal intensities of α-tubulin and EB1 were densitometrically determined, and fluorescent intensities of α-tubulin and EB1 were plotted.

A

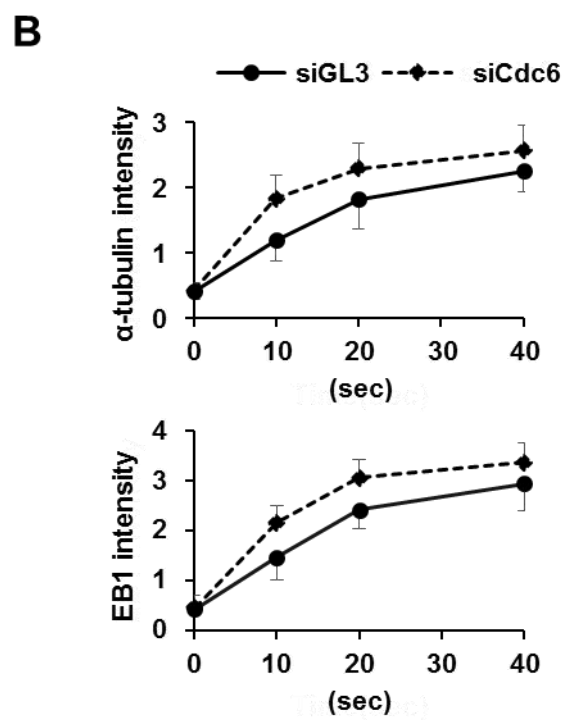|	siGL3		siCdc6	
(sec)	α-tubulin	EB1	α-tubulin	EB1
0				
10				
20				
40				

B

siGL3 — siCdc6

Figure 4. Cdc6 depletion increases microtubule regrowth from centrosomes.

(A) Microtubule regrowth assays were performed with cells treated with the indicated siRNA for 24 h. Microtubules were immunostained with antibodies specific to α-tubulin or EB1. Centrosomal intensities of α-tubulin and EB1 were densitometrically determined, and relative fluorescent intensities of α-tubulin and EB1 were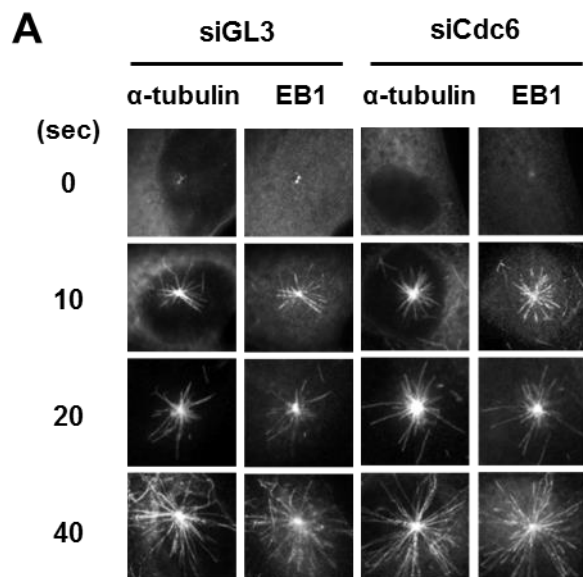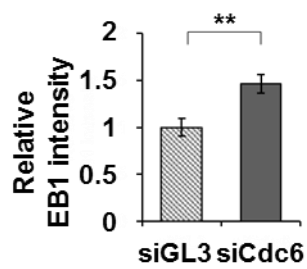 plotted (bottom). Values represent mean ± SD of at least 100 cells in each of three independent experiments (p > 0.05; **, p < 0.01; ***, p < 0.001). (C) The indicated proteins were detected in immunoblots with antibodies specific to each protein.

A

	siGL3	siCdc6
α-tubulin		
EB1		

B

	siGL2	siCdc6	kDa
Cdc6			60
α-tub			45
β-tub			45
EB1			35
γ-tub			45
actin			45

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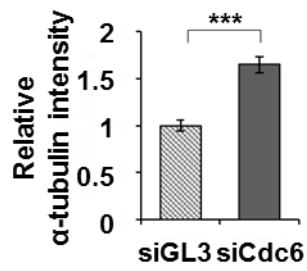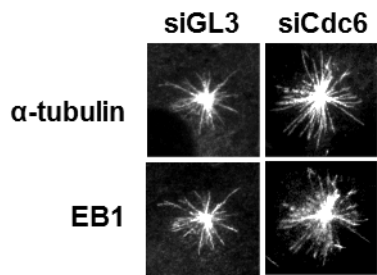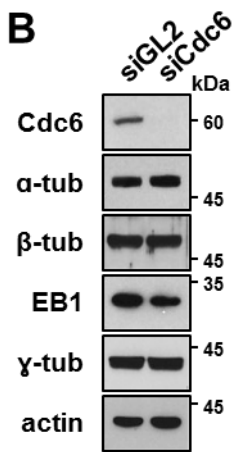
regrowth detected by α-tubulin and EB1 intensities, as shown in Figures 4A and 6B. Whereas induction of wild type Cdc6 in Cdc6-depleted cells reduced α-tubulin and EB1 intensities, the CLS-mutant Cdc6(LI/AA), which was defective in centrosomal localization, did not display these reductions (Fig. 5C, Fig. 6). The inability of Cdc6(LI/AA) to rescue the Cdc6 depletion suggests that Cdc6 needs to locate at centrosomes to reduce microtubule formation.

To determine the region(s) of the Cdc6 protein participating in the inhibition of microtubule formation, U2OS cells were transfected with deletion constructs of Cdc6 (Fig. 7A). Endogenous Cdc6 protein within transfected cells was depleted using Cdc6-specific siRNA; this was followed by a microtubule regrowth assay for detecting α-tubulin (Fig. 7B). Centrosome localization of each Cdc6 deletion-construct was detected via fluorescent microscopy (Fig. 7C, Fig. 8) and each Cdc6 constructs were confirmed by immunoblot analysis (Fig. 7D). Although deletion of the C-terminal 367–560 or the N-terminal 1–74 amino acid residues reduced α-tubulin intensity, further deletions of the N-terminus or C-terminus residues had a negative effect on this α-tubulin intensity reduction. Fragment 75–366, which was generated by deletion of both the C-terminal 367–560 and the N-terminal 1–74 amino acid residues, maintained all the inhibitory activity of the full-length protein. Because fragment 75–310, which did not contain Cdc6-CLS, could not localize to the centrosome, this deletion mutant lacked the inhibitory effect. However, attachment of either the PACT domain or the CycE-CLS—which facilitate the

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centrosomal localization of AKAP-450 (41) and Cyclin E (42), respectively—onto fragment 75–310 restored the inhibitory activity. These results suggest that fragment 75–310, upon localization to the centrosome, is able to reduce microtubule formation; furthermore they support that this reduction of microtubule formation requires Cdc6 to localize to the centrosome.

ATPase activity of Cdc6 is necessary for the reduction of microtubule formation

Fragment 75–366 of Cdc6 possesses an ATPase domain that harbors the Walker A motif or P-loop (amino acid residues 202-209), and the Walker B motif (284-287); these are essential for ATP binding and hydrolysis, respectively (Fig. 9A) (26). Both activities are indispensable for the Cdc6 protein's role in the initiation of chromosome replication (26-28). Substitutions of an amino acid residue on either the Walker A or the Walker B motif, which were accomplished via the substitution of either Lys-208 or Glu-285 with Ala or Gly, respectively, were introduced into FLAG-tagged fragment 75–366. U2OS Inducible Tet-On cell lines were then generated in which either the Cdc6 siRNA-resistant FLAG-Cdc6 wild type (WT), Walker A (K208A), or Walker B (E285G) mutant proteins could be induced. Centrosomal localization of Cdc6 mutant proteins was detected by fluorescent microscopy (Fig. 9B). The *in vitro* ATP hydrolytic activities of the Walker A and B mutant proteins were near background levels, but the activity of the CLS mutant

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Figure 5. Centrosomal localization of Cdc6 is required to reduce microtubule formation.

(A) Centrosomal localization of Cdc6. FRT/TO HeLa cells were transfected with constructs expressing the indicated GFP-tagged Cdc6 FL wild-type (FL WT) and LI/AA mutant (FL LI/AA). Cells were immunostained with anti-γ-tubulin. Centrosomal localization of GFP-tag was monitored and quantified. FL WT, N = 45/63; FL LI/AA, N = 0/70. (B) U2OS Tet-On cells expressing Cdc6-siRNA resistant FLAG-Cdc6 wild type or FLAG-Cdc6(LI/AA) were transfected with the indicated siRNA for 24 h. The proteins were induced by addition of 2 μg/mL of doxycycline, 24 h prior to siRNA treatment. Replicating S phase cells are indicated in dashed boxes. Proportions of replicating S-phase cells are shown below the FACS profiles. (C) Microtubule regrowth assays, quantifications, and statistical analyses were performed as described in Figure 4. Tet-Ctrl, control U2OS Tet-On cells; WT, FLAG-Cdc6 wild-type; LI/AA, FLAG-Cdc6(LI/AA).
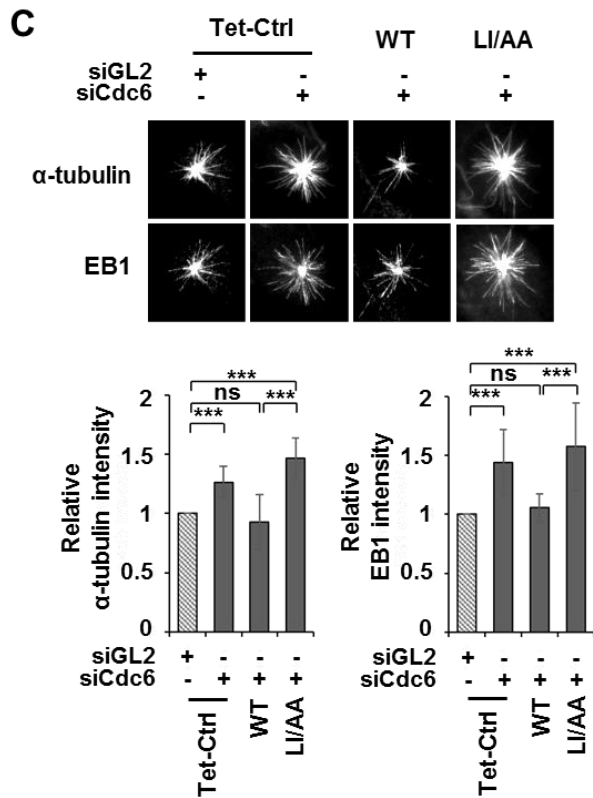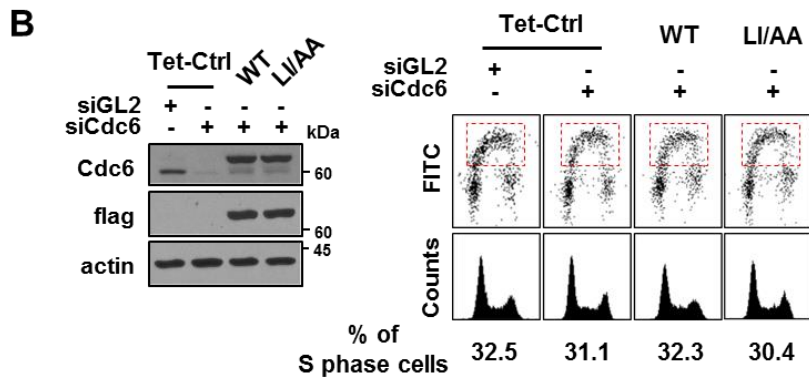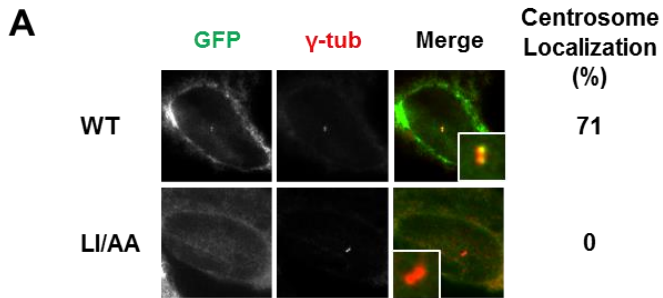
Figure 6. Time course of microtubule regrowth.

Time course microtubule regrowth assays were performed using cells treated with the

indicated siRNA for 24 h, as described in Figures 3 and 5 (B). Tet-Ctrl, control U2OS

Tet-On cells; WT, FLAG-Cdc6 wild-type; LI/AA, FLAG-Cdc6(LI/AA).
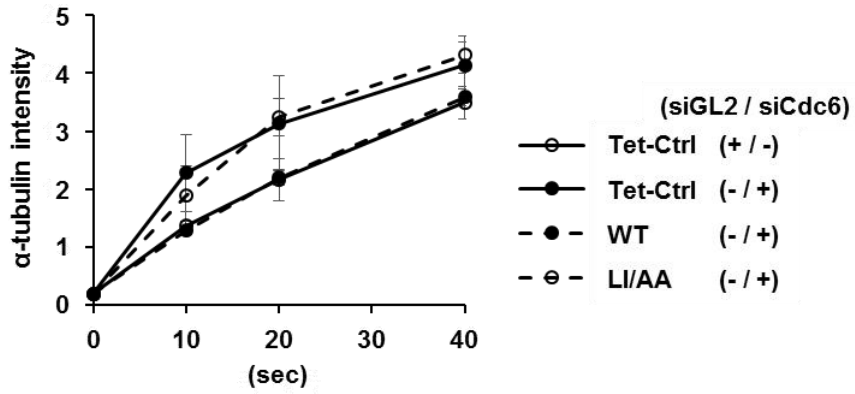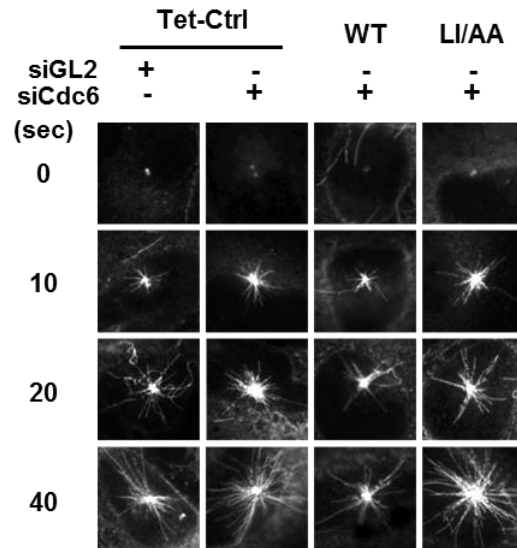
Figure 7. Amino acid residues 75–366 of Cdc6 participates in the reduction of microtubule formation.

(A) Schematic structures of Cdc6 motifs and domains as shown previously (66) are described (top). The N-termini of indicated fragments were fused to FLAG-tag. Numbers represent the amino acid residues. A, Walker A motif; B, Walker B motif; CLS, centrosome localization signal; NES, nuclear export signal; triangles, phosphorylation sites by CDKs; WHD, wing helix domain. (B) At 48 h after transfection with each DNA construct, U2OS cells were treated with Cdc6-specific siRNA; microtubule regrowth assays were performed 24 h after siRNA treatment. (C) Centrosomal localizations of each construct are described. (D) Expression of Cdc6 constructs. FRT/TO HeLa cells were transfected with constructs expressing the indicated GFP-tagged Cdc6 proteins that were described in Figure 3. Cells were subjected to immunoblotting, followed by detection using anti-GFP antibody.

Figure 8. Centrosomal localization of Cdc6 derivatives.

FRT/TO HeLa cells were transfected with constructs expressing the indicated GFP-tagged Cdc6 derivatives. Cells were immunostained with anti-γ-tubulin and anti-Cyclin A or anti-Cyclin E antibodies. Centrosomal localization in Cyclin-A positive cells co-immunostained with anti-γ-tubulin and anti-Cyclin A antibodies. Percentages of centrosomal localization were quantified in Cyclin A-positive cells. 75–310, N = 0/66; +PACT, N = 72/75; +CycE CLS,

	Cyclin A	GFP	γ-tub	Merge	Centrosome Localization (%)
75 - 310					0
+ PACT					96
+ CycE CLS					86

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protein Cdc6(LI/AA) was comparable to the corresponding wild type protein (Figs. 10).

Under conditions of endogenous Cdc6 depletion, induction of fragment 75–366 containing

either the K208A or E285G substitution did not significantly influence cell cycle

progression when compared to the induction of the wild type fragment (Fig. 9C). Although

the induced wild type fragment restored the inhibition of microtubule regrowth, the

corresponding Walker A and Walker B mutant fragments failed to accomplish this (Fig.

11). The inactivity of the Walker A and Walker B mutant fragments suggested that both

the ATP binding and hydrolysis activities of Cdc6 are involved in the reduction of

microtubule formation.

Cdc6 reduces microtubule formation in S- and G2-phase centrosomes

We determined whether the reduction of microtubule formation by Cdc6 is in concert

with the cell-cycle dependent localization of Cdc6 to the centrosome during the S and G2

phases. Cyclin E is a marker of the G1 phase, and cyclin A is of both the S and G2 phases

(43,44). In Cdc6-depleted G1 phase cells that were detected by cyclin E-positive

immunostaining, the differences between the induced FLAG-Cdc6 75–366 fragment wild

type and the corresponding Walker A mutant K208A, as well as between the control and

Cdc6-depleted cells, exhibited statistical insignificance in microtubule regrowth assays

(Fig. 12A). The lack of differences can be explained by the non-existence of Cdc6 at the

Figure 9. Walker A and Walker B mutant of Cdc6 fragment 75-366.
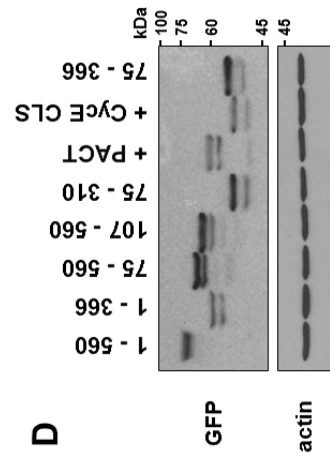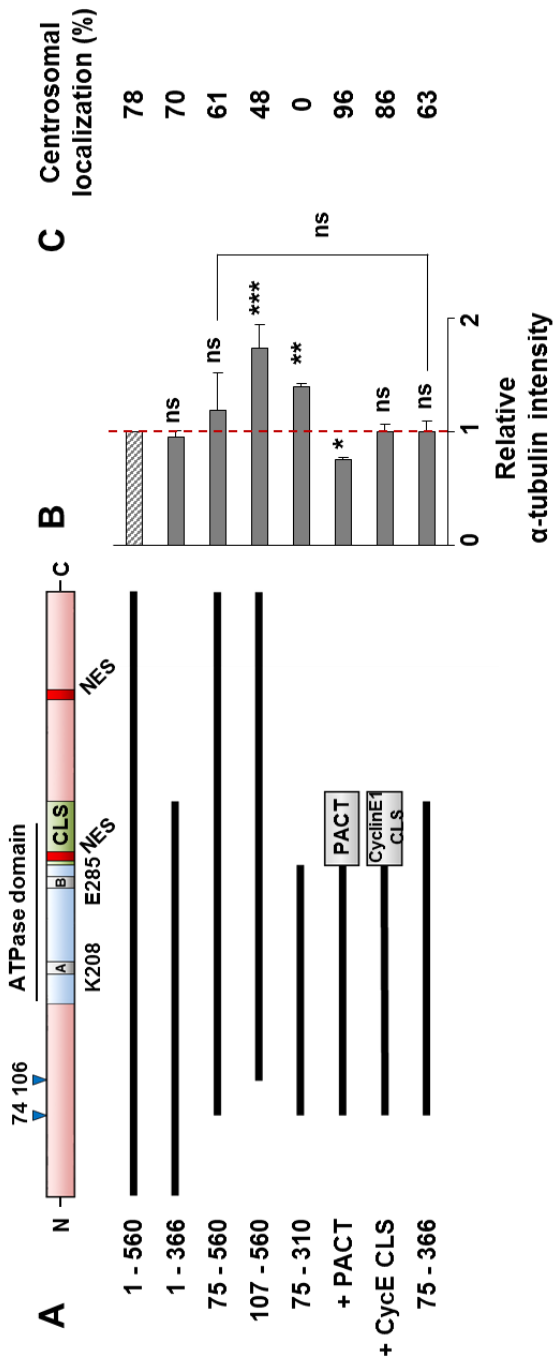
(A) Schematic structures of Cdc6 75-366 motifs and mutations. (B) Centrosomal localization of Cdc6 75-366 mutants. FRT/TO HeLa cells were transfected with constructs expressing the indicated GFP-tagged Cdc6 75-366 K208A (75-366 KA) and E285G (75-366 EG). Centrosomal localization in Cyclin-A positive cells co-immunostained with anti-γ-tubulin and anti-Cyclin A antibodies. Percentages of centrosomal localization were quantified in Cyclin A-positive cells. 75–366 KA, N = 22/35; 75–366 EG. (C) Depletions of endogenous Cdc6 and inductions of the indicated FLAG-Cdc6 fragments in U2OS Tet-On cell lines were performed as described in Figure 5. Immunoblot (left) and FACS (right) analyses were also performed as described in Figure 5. Tet-Ctrl, U2OS Tet-On control cells; WT, FLAG-Cdc6 fragment 75–366 wild type: K208A, FLAG-Cdc6 fragment 75–366(K208A); E285G, FLAG-Cdc6 fragment 75–366(E285G).

A

74 106

ATPase domain

N — A B CLS — C

K208   E285   NES   NES

75 - 366   A   B

K208A

E285G

B

	Cyclin A	GFP	γ-tub	Merge	Centrosome Localization (%)
75 – 366 KA					62
75 – 366 EG					63

C

Tet-Ctrl   WT   K208A   E285G

siGL2	+	-	-	-	-	kDa
siCdc6	-	+	+	+	+	
Cdc6						60
flag						35
actin						45

	Tet-Ctrl		WT	K208A	E285G
siGL2	+	-	-	-	-
siCdc6	-	+	+	+	+

FITC

Counts

% of S phase cells   34.2   33.7   33.6   32.3   32.8

36

Figure 10. ATPase activity of Cdc6 derivatives.

(A) The indicated FLAG-tagged Cdc6 wild type and derivatives, induced in U2OS Tet-On inducible cell lines were detected via immunoblotting, using anti-FLAG antibody.   (B) FLAG-Cdc6 proteins induced in U2OS Tet-On inducible cell lines were immunoprecipated using anti-FLAG antibody, followed by detection via immunoblotting. (C, D) Immunoprecipitated Cdc6 proteins were used to measure ATP hydrolysis activity for the indicated time, using the ADP-Glo™ Kinase Assay kit (Promega), according to the manufacturer's protocols. Values represent mean ± SD of data from each of three independent experiments. Tet-Ctrl, U2OS Tet-On control cells; FL WT, FLAG-Cdc6 wild type; FL LI/AA, FLAG-Cdc6(LI/AA); 75–366 WT, FLAG-Cdc6 fragment 75–366 wild type; 75–366 KA, FLAG-Cdc6 fragment 75–366(K208A); 75–366 EG, FLAG-Cdc6 fragment 75–366(E285G).

38
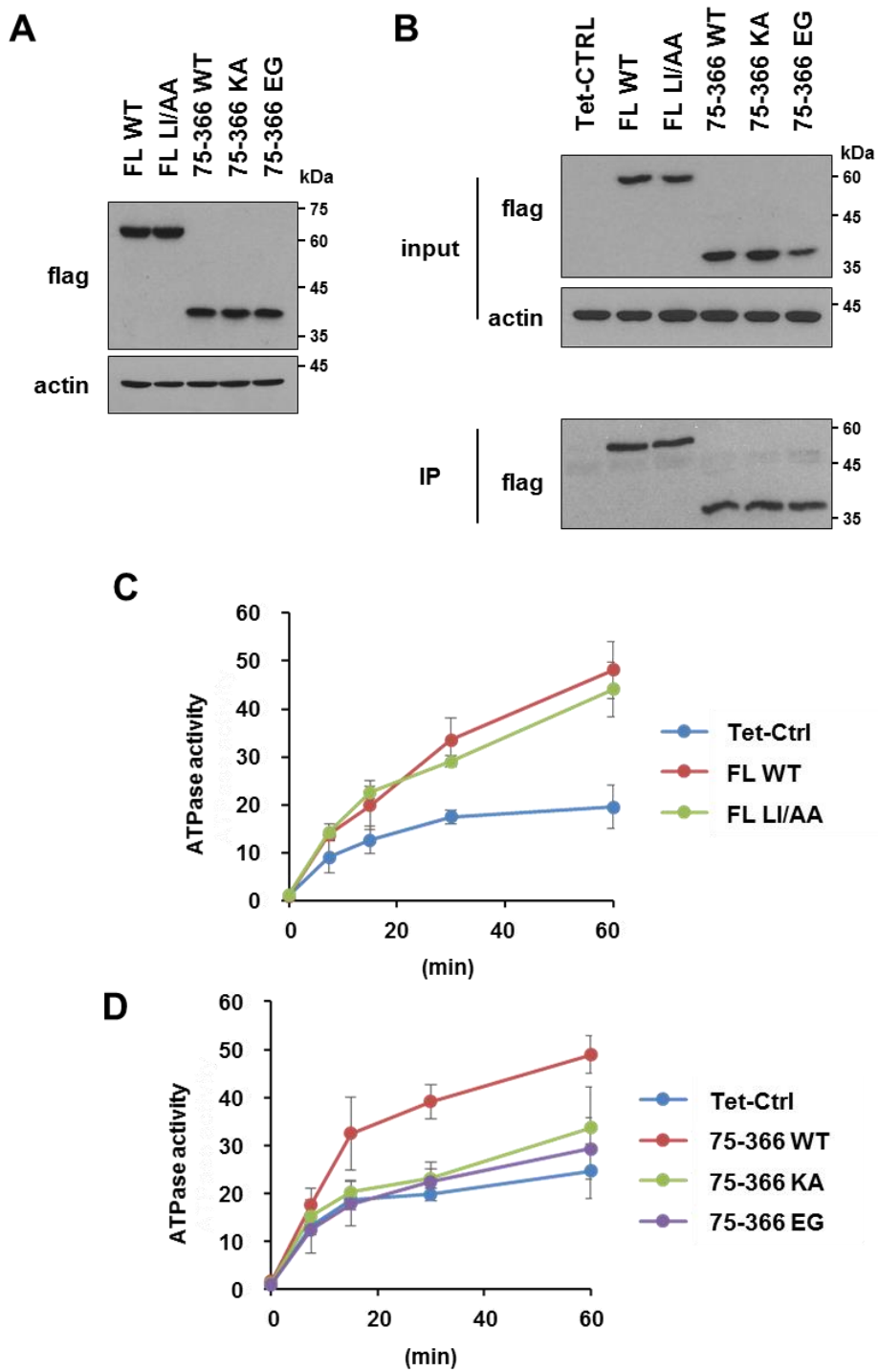
Figure 11. Both ATP binding and hydrolysis activities of Cdc6 are required to reduce microtubule formation.

Microtubule regrowth assay analyzed at least 100 cells in each of three independent experiments, as described in Figure 5B. Tet-Ctrl, U2OS Tet-On control cells; WT, FLAG-Cdc6 fragment 75–366 wild type: K208A, FLAG-Cdc6 fragment 75–366(K208A); E285G, FLAG-Cdc6 fragment 75–366(E285G).
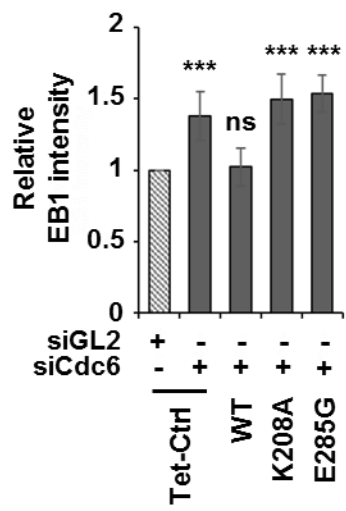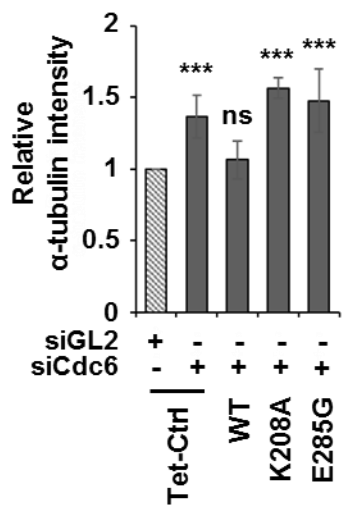
G1 phase centrosome (Fig 1B). In contrast, the induced wild type protein decreased microtubule regrowth in S- and G2-phase cells (Fig. 12A). Because Walker B mutant E285G behaved similarly to Walker B mutant K208A in the microtubule regrowth assay and in other assays described here, we did not include the results obtained with the K208A mutant in the following results.

Fusion of the PACT domain onto a protein causes the fused protein to localize to the centrosomes throughout the cell cycle (41). The fusion of the PACT domain to the 75–366 fragment allowed it to localize to the G1-phase centrosomes, in contrast to the 75–366 fragment when not fused to the PACT domain (Fig. 8). The induced FLAG-Cdc6-75–366-PACT wild type protein reduced microtubule regrowth, but the corresponding Walker A mutant did not (Fig. 12B). These results imply that the Cdc6-dependent inhibition of microtubule formation, which requires centrosomal localization, occurs during the S and G2 phases.


Cdc6 controls microtubule nucleation

The association of EB1 protein with growing microtubule tips (39) suggested that the reduction of EB1 intensities occurred through Cdc6-mediated control of microtubule nucleation. Therefore, the number of microtubules originating from the centrosome was measured by counting EB1 comet numbers (Fig. 13). Depletion of Cdc6 resulted in

41

Figure 12. Cdc6 negatively regulates microtubule formation in S- and G2-phase centrosomes.

Cdc6 negatively regulates microtubule formation in S- and G2-phase centrosomes. Microtubule regrowth assays were performed with asynchronously grown cells. Cells were then immunostained with anti-cyclin E antibodies (top panel) or anti-cyclin A antibodies (bottom panel). Induction of the indicated Cdc6 proteins, and detection and quantification of relative α-tubulin intensities, were carried out as described in Figure 11. (*A*) U2OS Tet-On inducible cell lines used are described in Figure 11. Tet-Ctrl, U2OS Tet-On control cells; WT, FLAG-Cdc6 fragment 75–366 wild type: K208A, FLAG-Cdc6 fragment 75–366(K208A). (*B*) FLAG-Cdc6 fragment 75–366-PACT wild type (WT) or Walker A mutant protein (K208A) was induced in FRT/TO HeLa cell lines.

A
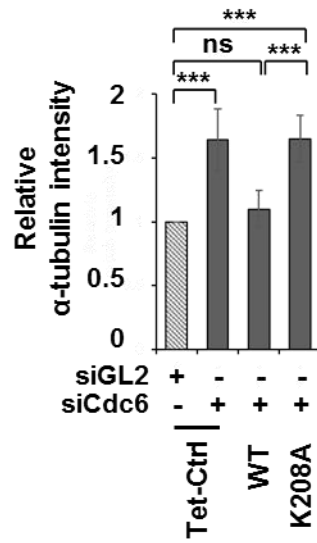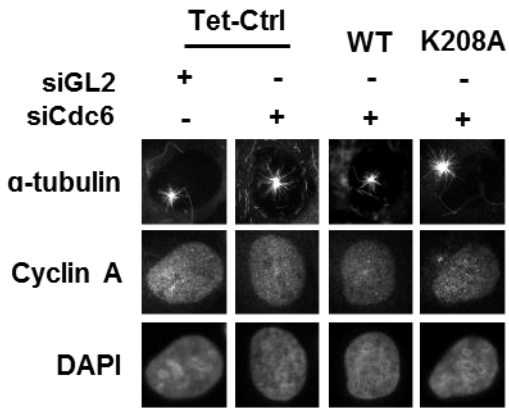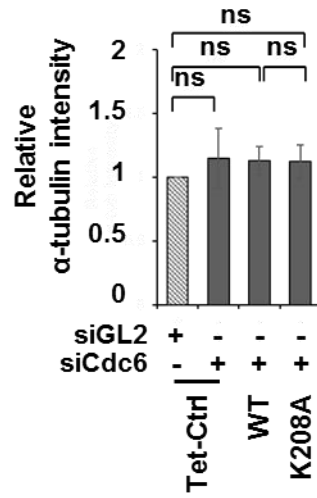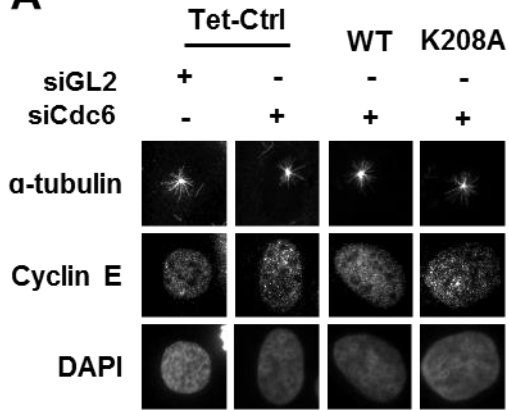
B
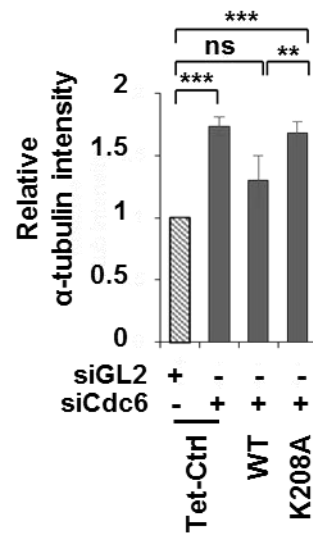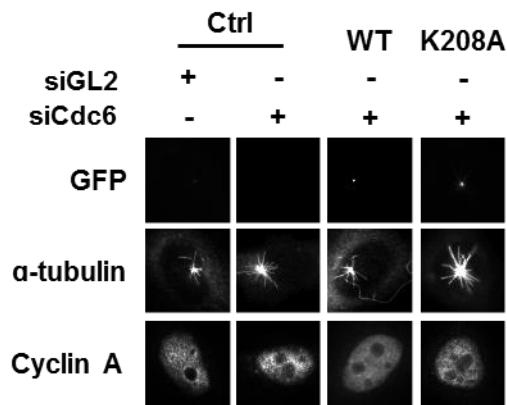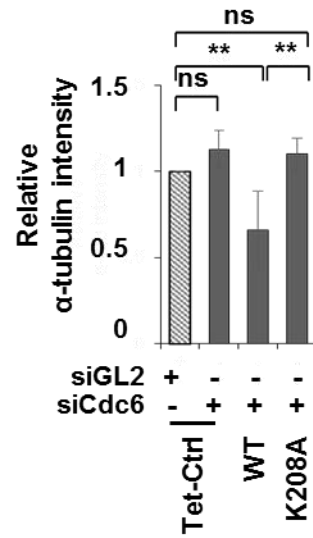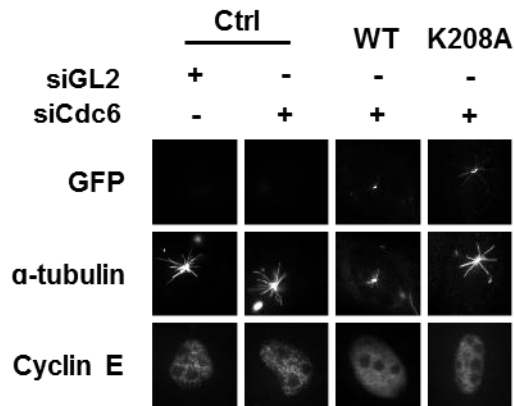
increased microtubule numbers. Transfection of an mCherry-tagged Cdc6 wild-type-expressing DNA construct into Cdc6-depleted cells decreased the microtubule numbers compared to those of control GL2 siRNA-treated cells, whereas the corresponding Walker A mutant did not show this effect.

We carried out time-lapse imaging of live cells in the GFP-tagged EB1-expressing U2OS cell-line. A microtubule regrowth assay was performed at 25°C, following incubation on ice to slow microtubule formation. The number of microtubules was determined by counting GFP fluorescent comets of GFP-EB1 (Fig. 14A and B). Cdc6 depletion increased microtubule numbers as shown in Figure 13. Transfection of mCherry-Cdc6 wild-type-expressing DNA construct into the Cdc6-depleted cells significantly reduced microtubule numbers, but the corresponding Walker A mutant did not. In contrast, either Cdc6 depletion or transfection of the cdc6 wild-type construct did not significantly change the rates of microtubule growth (Fig. 14C). The increase of microtubule numbers by Cdc6 depletion, together with the reduction by Cdc6 expression in Cdc6-depleted cells, suggests that Cdc6 negatively controls microtubule nucleation at the centrosome.

Cdc6 negatively regulates the levels of PCM proteins

Because centrosomal localization of Cdc6 negatively controls microtubule nucleation, we investigated whether Cdc6 also affects γ-tubulin level at the centrosomes. γ-tubulin of

Figure 13. Cdc6 controls microtubule nucleation.

The DNA construct expressing Cdc6 siRNA-resistant mCherry-tagged Cdc6 fragment 75–366 wild-type or Walker A mutant (K208A) was transfected, 24 h prior to siRNA treatment. At 24 h after siRNA treatment, (A) immunoblot analyses were performed with the indicated antibody. (B) Microtubule regrowth assays were performed. EB1 comets (green) immunostained with anti-EB1 antibody and fluorescence of mCherry-Cdc6 (red) were detected by using DeltaVision. (C) Fields containing centrosomes are shown at higher magnifications in sets. The number of EB1 comets emanated from centrosomes of 30 cells per each treatment was quantified.

A

	siGL2	siCdc6	siCdc6 +WT	siCdc6 +KA	
siGL2	+	-	-	-	
siCdc6	-	+	+	+	
mCherry-WT	-	-	+	-	
mCherry-KA	-	-	-	+	kDa

Cdc6 — 60

mCherry — 60

actin — 45

C

MT number

siGL2 siCdc6 siCdc6 +WT siCdc6 +KA

*** ns *** ***

B

siGL2   siCdc6   siCdc6 +WT   siCdc6 +KA

EB1

Merge
EB1 /
DAPI /
mCherry
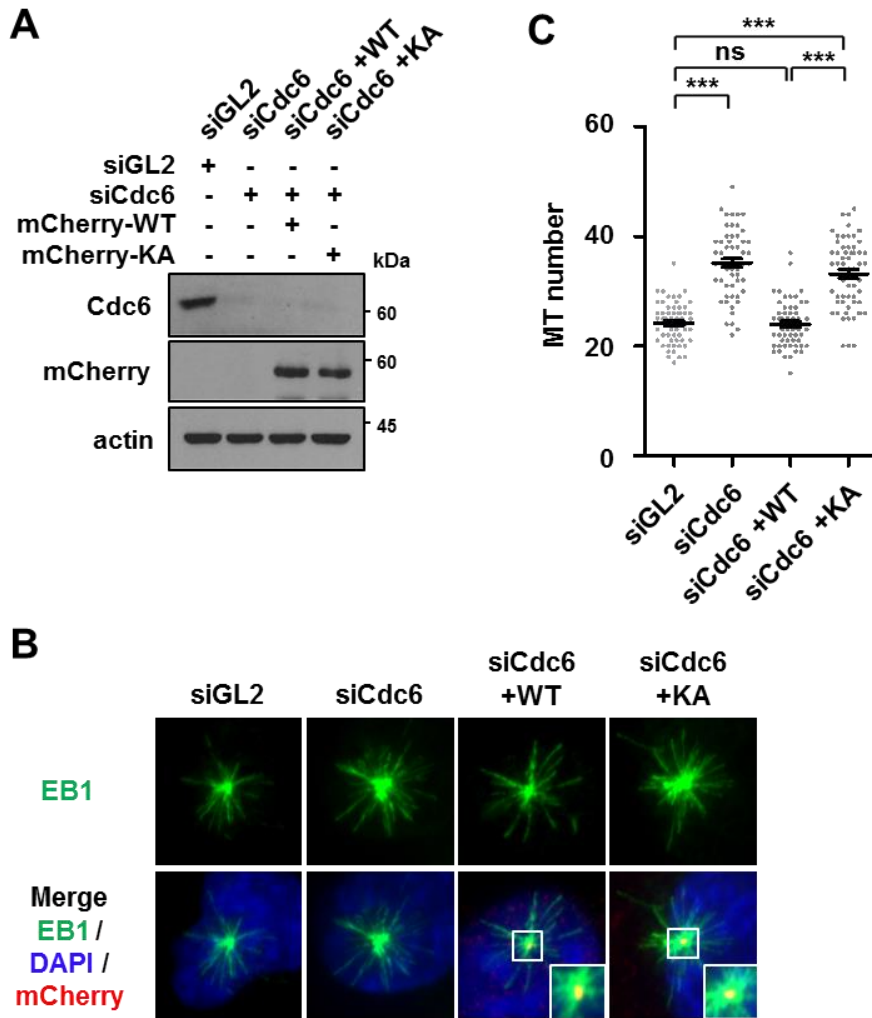
Figure 14. Cdc6 controls microtubule nucleation in live cell.

The indicated DNA constructs were transfected into EB1-GFP-expressing U2OS stable cells as described in Figure 13. (A) To perform live cell time-lapse imaging of microtubule regrowth, depolymerized cells on ice were transferred to the chamber, pre-warmed at 25°C, of DeltaVision. Single frames of video for 10 cells per each treatment were collected at 30-sec intervals (Supplementary Movie). Representative images are displayed. EB1, GFP-EB1; mCherry, mCherry-Cdc6. (B) Rates of microtubule nucleation were determined at 30-sec intervals for 4 min by counting GFP-EB1 comets emerging from the centrosome. (C) Rates of microtubules growth were quantified by measuring the length of each EB1-GFP comet from centrosomes. The length was manually tracked using SoftWoRx. At least 5 microtubules at early time of regrowth were tracked in 10 cells. Scatter plots were drawn using Graphpad software.

A

Regrowth (sec)	30	90	150	210

EB1/mCherry

siGL2

siCdc6

siCdc6 +WT

siCdc6 +KA

B

C
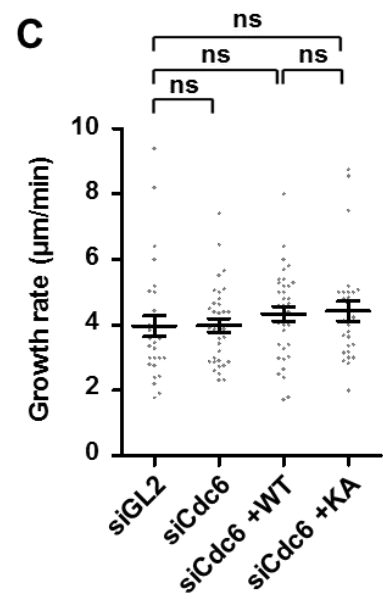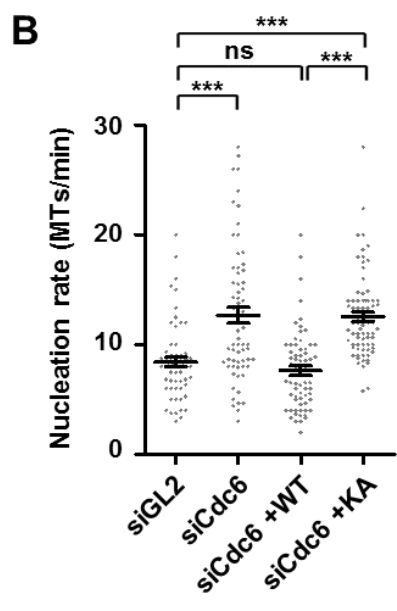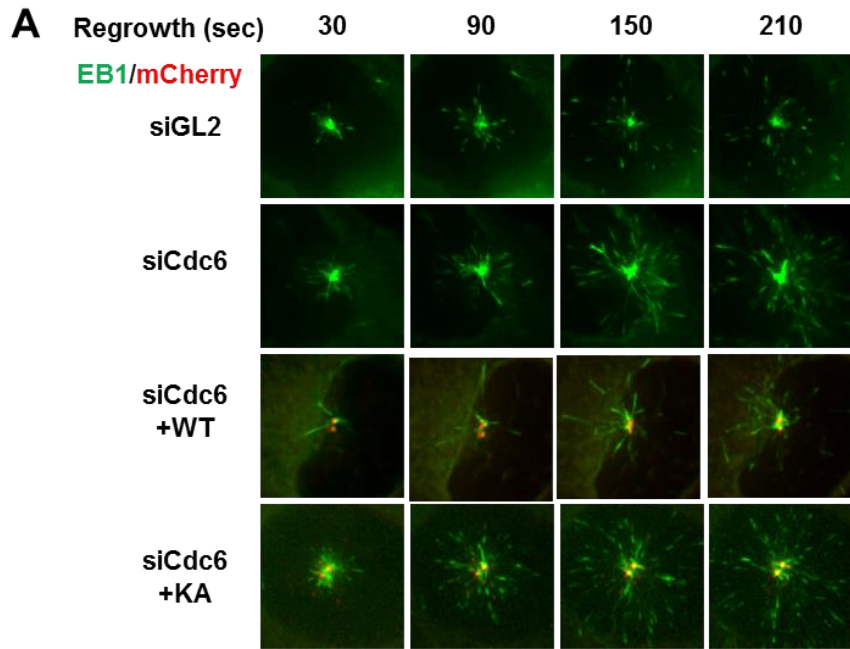
the γ-TuRC functions as a nucleation core for microtubule polymerization (7-9). Because

depletion or induction of Cdc6 did not significantly affect the intensities of centrin, which

is located in the centrioles and pericentriolar lattice of the centrosome (Fig. 15B and 17A),

the intensities of PCM proteins were comparable to the intensities of centrin (Fig. 15). Cdc6

depletion increased the intensity of γ-tubulin at the centrosomes (Fig. 15C). This increase

was reduced by induction of the Cdc6 full-length wild-type protein, but not by induction

of Cdc6(LI/AA), which is defective in centrosomal localization (Fig. 5A). Similarly, Cdc6

depletion increased while induction of the wild type protein reduced the intensity of

pericentrin, which is a scaffold protein within the PCM (45). Increases of γ-tubulin and

pericentrin were pronounced during the S and G2 phases in Cdc6-depleted cells (Fig. 16).

The control of pericentrin levels by Cdc6 led us to examine whether Cdc6 controls the

levels of two other PCM scaffold proteins, Cep215 (also known as CDK5RAP2) and

Cep192, at the centrosome. As seen with full-length Cdc6 (Fig. 15), induction of the Cdc6

fragment 75–366 wild type protein reduced the intensities of γ-tubulin and pericentrin (Fig.

17). Whereas depletion of Cdc6 increased the intensities of those PCM proteins, induction

of Cdc6 fragment 75–366 wild type reduced their intensities. However, the corresponding

Walker A mutant, K208A, failed to reduce these PCM protein intensities. Pericentrin,

CDK5RAP2, and Cep192 have been shown to be involved in the recruitment of γ-TuRC

to the centrosome (4,46-49). The increased levels of these PCM scaffold proteins and of γ-

tubulin caused by Cdc6 depletion, and conversely the reduction of their levels by the

presence of Cdc6, together suggest that Cdc6 negatively controls recruitment of PCM

proteins to the centrosomes and thereby modulates nucleation for microtubule formation

(Fig. 18).

Figure 15. Centrosomal localization of Cdc6 is required to regulate the amounts of PCM proteins at the centrosome.

(A) Cells were co-immunostained with the indicated antibodies. Fields around the centrosomes containing 4 dots of centrin are shown at higher magnifications in sets. (B, C) U2OS Tet-On inducible cell lines used and induction of the indicated proteins are shown in Figure 5. Relative fluorescence intensities of centrin are determined in (B). Ratios are described as relative intensities of the indicated protein to relative intensity of centrin in (C). γ-tub, γ-tubulin; PCNT, pericentrin; Tet-Ctrl, control U2OS Tet-On cells; WT, FLAG-Cdc6 wild-type; LI/AA, FLAG-Cdc6(LI/AA).
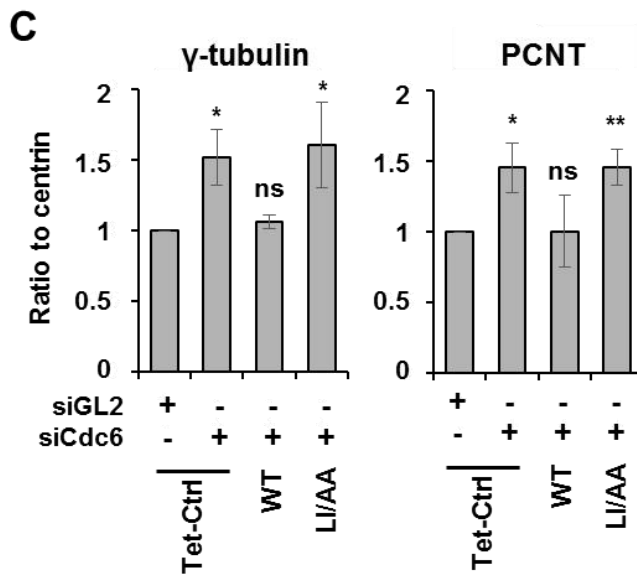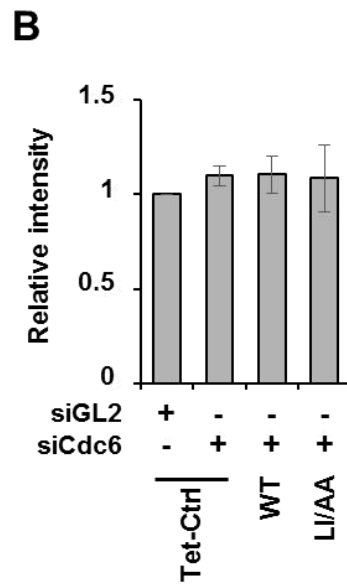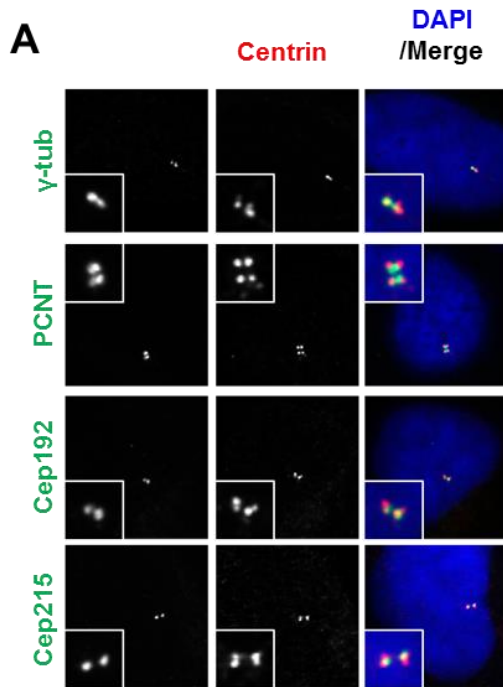
Figure 16. Cdc6 regulates recruitment of PCM proteins in S and G2 phase cells.

(A-C) HeLa cells were synchronized by double thymidine block, and were released into fresh media for the indicated time. (A) Cell cycle distribution was verified by FACS analysis. (B) siRNA transfections were performed when cells were released from the first thymidine block. Cell lysates obtained for the indicated time after the release from the double thymidine block were subjected to immunoblotting, and the indicated proteins were detected using the corresponding antibody. * indicates cleaved PCNT (Kim J. *et. al* (2015) *Nat. Commun.* 6:10076). (C) γ-tubulin or PCNT intensities at centrosomes were quantified at the indicated time point. Centrin was co-immunostained with γ-tubulin or PCNT to distinguish cell cycle-staged cells (Paloletti A. *et. al* (1996) *J. Cell Biol.* 109:3089-3102).

A

Hours after double thymidine release

Asy	0	3	6	9	12

Counts

G1/S   S   S/G2   G2/M   G1

B

siGL2   siCdc6

(hr) 0 3 6 9 12   0 3 6 9 12   kDa

Cdc6 — 60

γ-tub — 45

intact ► — 500
cleaved ► — 279
         251

actin — 45

C

55
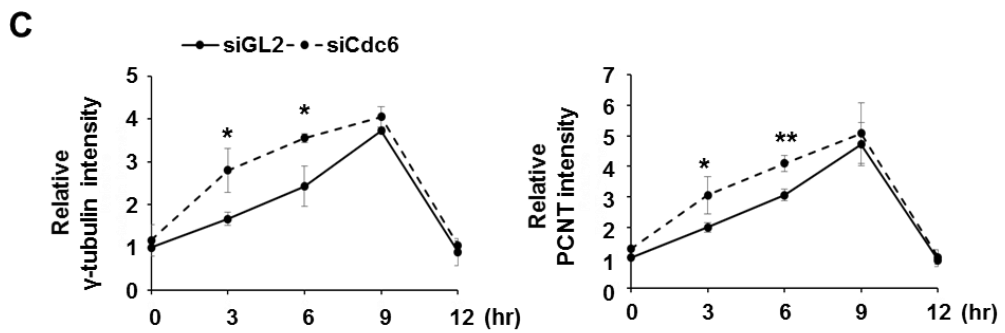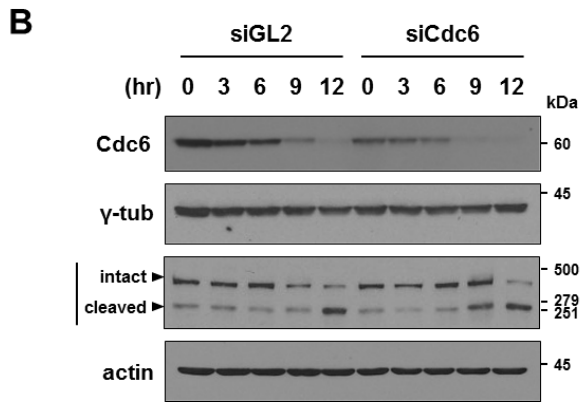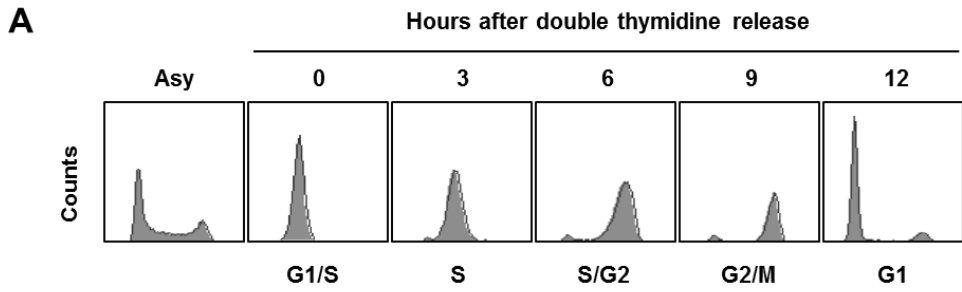
Figure 17. ATPase activity of Cdc6 is required to regulate the amounts of PCM proteins at the centrosome.

U2OS Tet-On inducible cell lines used and induction of the indicated proteins are shown in Figure 9. (A) Relative fluorescence intensities of centrin are determined. (B) Cells were co-immunostained with the indicated antibodies. Fields around the centrosomes containing 4 dots of centrin are shown at higher magnifications in sets. Ratios are described as relative intensities of the indicated protein to relative intensity of centrin.

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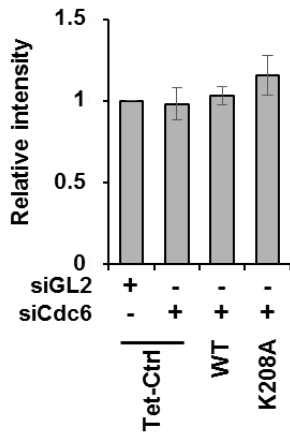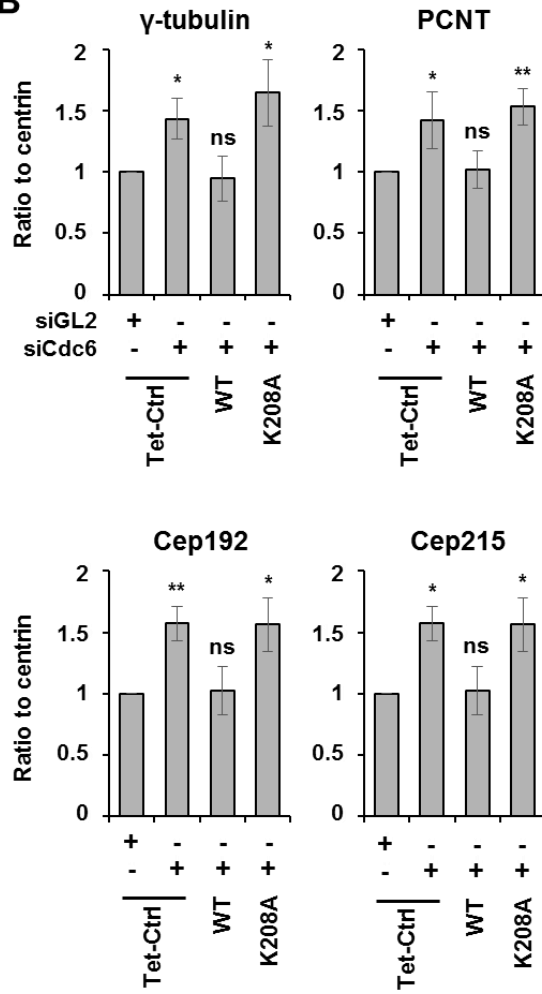
Figure 18. Cdc6 negatively controls PCM assembly and microtubule nucleation.

Cdc6 localizes to centrosomes during the S and G2 phases of cell cycle. Cdc6 negatively controls both microtubule nucleation for MTOC activity and the levels of PCM proteins by utilizing ATPase activity. The malfunction of Cdc6 causes PCM overgrowth result in an increase of microtubule nucleation.
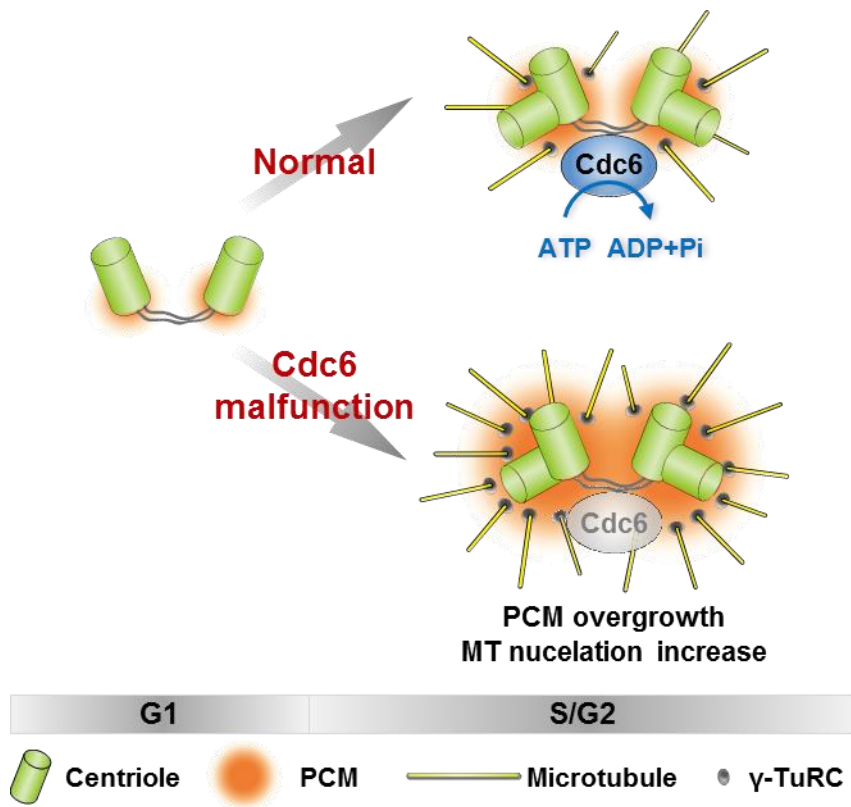
Discussion

Although Cdc6 participates in pre-RC formation in the nucleus during G1 phase, its protein levels are lowest during G1 phase, and its mRNA and protein levels begin to increase from S phase (32-34). In addition, non-chromatin-bound Cdc6 is exported from the nucleus during the G1/ S transition (22). These results suggest non-nuclear roles for Cdc6 in the S and G2 phases. Cdc6 localizes to centrosomes during the S and G2 phases (36,37). This work demonstrates a novel function for Cdc6 at S- and G2-phase centrosomes: that Cdc6, with its ATPase activity, negatively controls both microtubule nucleation for MTOC activity and the levels of PCM proteins (Fig. 24). MTOC regulation appears to be achieved via the modulation of PCM protein levels at the centrosomes.

Many centrosomal proteins are involved in promoting MTOC activity, but only a few have been identified as negative regulators of the MTOC prior to mitosis. BRCA1 protein localized to the centrosome decreases microtubule nucleation by ubiquitinating Lys344 of γ-tubulin (50). NEK2 phosphorylation of centrobin reduces microtubule networks during G2 phase, whereas PLK1 phosphorylation increases microtubule stability for bipolar spindle formation during M phase (51). In this study, we add that Cdc6 is a negative regulator of MTOC in interphase centrosomes. The functions of Cdc6 may differ from those of BRCA1 and centrobin, in that Cdc6 regulates the recruitment of the PCM proteins participating in MTOC assembly or activity.

The PCM in interphase centrosomes is organized in an orderly manner, with a concentric toroidal structure (4,5). Pericentrin is an elongated molecule extending away from the centriole, while other PCM proteins, such as CDK5Rap2, Cep192, and NEDD1, occupy separable concentric domains surrounding the centrioles. As the ATPase activity of Cdc6 in protein complexes participates in modulating the functions of proteins within the complex (25–31), Cdc6 at the centrosome may form complexes with regulatory proteins to control PCM assembly. The increased intensities of centrosomal pericentrin, CDK5RAP2, and Cep192 caused by Cdc6 depletion, along with the centrosomal localization and function of Cdc6, suggest that Cdc6 is an upstream regulator of PCM organization for maintenance of PCM proteins during interphase. As a result, the γ-TuRC may be limited to be recruited to the centrosome, in the presence of Cdc6, during S- and G2-phase cells. Delocalization of Cdc6 from the centrosomes during mitosis temporally coincides with the maturation and expansion of the PCM at the onset of mitosis (36,46).

Centrosome duplication and chromosomal replication share similarities in the following aspects. Both occur once per cell cycle, and in a cell cycle-dependent manner; these cell cycle-dependent processes are commonly regulated by cyclin-dependent kinases; and the duplicated centrosomes and chromosomes are equally segregated into daughter cells during mitosis (52-55). Furthermore, the pre-RC forming and controlling proteins, such as ORC subunits (56-58), MCM subunits (59,60), and geminin (61), also exist in centrosomes to maintain centrosomal integrity. Our results show that Cdc6, which is a component of

the pre-RC, localizes to centrosomes in a cell cycle-dependent manner, and that it modulates both the levels of PCM proteins and MTOC activity. The replicative proteins that regulate both the pre-RC and centrosomal functions may contribute to coordinating chromosome replication and centrosomal function.

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국 문 초 록

중심체는 미세소관을 형성하는 중심이 되는 세포소기관으로서 세포 골격, 세포 극성, 물질수송 및 세포 운동성에 관여한다. 중심체 미세소관은 중심구의 구조를 형성하는 중심립주변물질에 의해 형성된다. 중심립주변물질을 구성하는 Pericentrin, Cep192, CDK5RAP2 단백질은 규칙적인 환상 구조를 형성하며 미세소관 형성의 기반이 되는 γ-TuRC의 기능을 유도한다.

Cdc6는 DNA 복제전복합체 인자로서 세포주기 G1기에 염색체 복제를 개시한 후, S와 G2기에 중심체에 위치한다. 또한, Cdc6와 함께 염색체 복제에 관여하는 ORC, MCM, geminin 단백질도 중심체에 위치하여 중심체의 구조와 기능을 조절한다. 이를 통해, 중심체 주기와 염색체 복제 시스템을 통합적으로 조절하는 핵심 단백질의 역할이 중요하다는 것을 알 수 있다.

본 연구에서는 세포주기 S와 G2기 중심체에 위치하는 Cdc6가 중심체의 미세소관 형성을 조절할 수 있는지 알아보고자 한다. 중심체에서 Cdc6의 결핍은 미세소관 형성을 증가시키는 것으로 보아, Cdc6는 미세소관 형성을 억제하는 기능을 수행하는 것으로 확인하였다. 중심체 Cdc6가 미세소관 형성을 억제할 수 있는 핵심 도메인으로서 Cdc6의 아미노산 서열 75-366번을 발굴하였다. Cdc6의 아미노산 서열 75-366번에 존재하는 ATPase 도메인을 결함시킨 ATPase 활성이 없는 돌연변이 또한 미세소관 형성을 억제시키지 못했다. 중심체 미세소관은 중심구를 형성하는 중심립주변물질에 의해 조절되는

데, 중심체 Cdc6가 결핍된 세포나 ATPase 활성에 결함이 있는 세포에서는 중심체에서의 중심립주변물질 형광강도가 증가한 것을 확인하였다. 이를 통해 Cdc6는 S와 G2기에 중심체에 위치하여 ATPase 활성을 통해 중심구를 음성적으로 조절함으로써 미세소관 형성을 억제한다는 것을 밝혔다.