이 학 박 사 학 위 논 문

# 중심체 복제 및 기능 조절에서의 Cdc6의 역할에 대한 연구

# Regulation of centrosome duplication and function by Cdc6

2015년 2월

서울대학교 대학원

생명과학부

김 광 수

# 중심체 복제 및 미세소관 형성에서 Cdc6의 역할에 대한 연구

# Novel functions of Cdc6 in centrosome duplication and microtubule organization

指導敎授 黃 德 秀

이 論文을 理學博士 學位 論文으로 提出함

2015 년 2 월

서울대학교 대학원 생명과학부

金 光 洙

金 光 洙 의 理學博士 學位 論文을 認准함

2015 년 2 월

委 員 長 이 건수

副 委 員 長 황 덕 수

委 員 이 준규

委 員 김 근필

委 員 최 희정

**Novel functions of Cdc6 in centrosome duplication and microtubule organization**

A dissertation submitted impartial fulfillments

of the requirements for the degree of

**DOCTOR OF PHILOSOPHY**

to the Faculty of the

School of Biological Sciences

at

**SEOUL NATIONAL UNIVERSITY**

by

**Gwang Su Kim**

**Date Approved**

_____

Kwon Ho

Deog Su Hwang

Joon Ho Lee

Kang Kim

Hee-Jung Choi

**ABSTRACT**

The centrosome functions as major microtubule organizing center. Microtubules play critical roles in chromosome segregation, cell shape and motility, vesicular transport, and signal transduction. The Cdc6 protein has been primarily investigated as a component of the pre-replicative complex for the initiation of chromosome replication, which contributes to maintenance of chromosomal integrity. I demonstrated a novel function of Cdc6 in controlling microtubule formation at the centrosomes. A conserved centrosome localization signal allowed Cdc6 to localize to S- and G2-phase centrosomes in a cell cycle-dependent manner. Cdc6 depletion increased the amounts of the integral proteins Cep215/CDK5RAP2 and Cep192 of the pericentriolar material (PCM), promoting the γ-tubulin ring complex (γ-TuRC) recruitment and microtubule formation, suggesting that Cdc6 modulates the PCM to control γ-TuRC recruitment. Furthermore, Cdc6 depletion increased cell adhesion and spreading, and Cdc6 overexpression had the opposite effect. These results demonstrate that Cdc6 contributes to control of cell cycle-regulated microtubule formation.

Centrosome is duplicated through cell cycle and its integrity is critical for chromosome segregation that is tightly linked to chromosome stability. I addressed that Cdc6 negatively regulated centrosome amplification and Grp78 was required for centrosome amplification. Whereas Cdc6 depletion induced centrosome amplification, Cdc6 overexpression suppressed hydroxyurea (HU)-induced centrosome amplification. The centrosome amplification arising by Cdc6 depletion was caused by centrosome over-duplication rather than centriole fragmentation. Amino acid residues 197 to 214 of Cdc6 (Cdc6-SCA: suppression of centrosome amplification) was responsible for suppression of centrosome amplification. The Cdc6-SCA contained a FHA domain protein binding motif and mediated interaction with Grp78, which is a molecular chaperon of HSP70 family. Grp78 localized to interphase centrosomes and was necessary for centrosome amplification by Cdc6 depletion or HU

treatment. My results suggest that interaction between Cdc6 and Grp78 is involved in controlling centrosome duplication.

Through my graduate works, I have demonstrated novel functions of Cdc6 on the regulation of microtubule formation and centrosome duplication. My achievements will contribute to understanding centrosome function and cell cycle control.

Key words : Cdc6, microtubule, centrosome, γ-TuRC, cell spreading, cell cycle, Grp78,

Student ID : 2008-22742

**TABLE OF CONTENTS**

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| APC | anaphase-promoting complex |
| ATP | adenosine triphosphate |
| BrdU | Bromodeoxyuridin |
| Cdc6 | cell division cycle 6 |
| CDK | cyclin-dependent kinase |
| DAPI | 4`,6-diamidino-2-phenylindole |
| DMEM | Dulbecco`s modified Eagle`s medium |
| FACS | fluorescence-activated cell sorting |
| FITC | fluorescein 5-isothiocyanate |
| Grp78 | Glucose regulted protein 78 |
| γ-TuRC | γ-tubulin ring complex |
| HU | hydoxyurea |
| MAPs | microtubule associated proteins |
| MTOC | microtubule-organizing center |
| NES | nuclear export signal |
| ORC | origin recognition complex |
| PBS | phosphate-buffered saline |

| | |
|---|---|
| PCM | pericentriolar material |
| PI | propodium iodide |
| pre-RC | pre-replication complex |
| RNAi | RNA interference |
| siRNA | small interfering RNA |

# CHAPTER 1

## Cdc6 Localizes to S and G2 Phase Centrosomes to Control Microtubule Formation

**Abstract**

The Cdc6 protein has been primarily investigated as a component of the pre-replicative complex for the initiation of chromosome replication, which contributes to maintenance of chromosomal integrity. Here, we show a novel function of Cdc6 in controlling microtubule formation at the centrosomes. A conserved centrosome localization signal allowed Cdc6 to localize to S- and G2-phase centrosomes in a cell cycle-dependent manner. Cdc6 depletion increased the amounts of the integral proteins Cep215/CDK5RAP2 and Cep192 of the pericentriolar material (PCM), promoting the γ-tubulin ring complex (γ-TuRC) recruitment and microtubule formation, suggesting that Cdc6 modulates the PCM to control γ-TuRC recruitment. Furthermore, Cdc6 depletion increased cell adhesion and spreading, and Cdc6 overexpression had the opposite effect. These results demonstrate that Cdc6 contributes to control of cell cycle-regulated microtubule formation.

## INTRODUCTION

Microtubule nucleation takes place at the γ-tubulin ring complex (γ-TuRC), followed by elongation (Moritz et al., 1995). α-and β-tubulin require GTP to form dimers and polymerize into microtubules. Microtubule-associated proteins (MAPs) and tubulin modification regulate microtubule dynamics (Janke and Bulinski, 2011). MAPs comprise not only microtubule-stabilizing proteins, including tau, MAP1, and MAP2, but also destabilizing proteins, including spastin, katanin, and kinesin-13 (Desai et al., 1999; Hunter et al., 2003; Zhang et al., 2007). Acetylation on tubulins stabilizes the microtubule (Maruta et al., 1986). Microtubules plays roles in diverse cellular functions, including chromosome segregation, cell motility, cell shape and polarity, vesicular transport, and signal transduction (Franker and Hoogenraad, 2013; Siegrist and Doe, 2007; Tanaka and Desai, 2008; Watanabe et al., 2005). In interphase, microtubules initiated at the centrosome form astral microtubules that interact with the nuclear envelope, which is essential for positioning of the nucleus and centrosome (Hulspas et al., 1994).

The centrosome functions as microtubule-organizing center (MTOC) for microtubule formation (Luders and Stearns, 2007) . Centrosomes consist of a pair of centrioles surrounded by pericentriolar material (PCM), which is composed of a meshwork of proteins. The two centrioles are distinguished as mother and daughter centrioles. The mother centriole possesses appendages at its distal end, where γ-TuRCs are anchored for microtubule formation. Microtubules are also nucleated at the γ-TuRC in the PCM. During interphase the centrosome is closely associated with the nucleus and is duplicated; the two centrosomes then separate and migrate to the poles to function as spindle poles for chromosome segregation during mitosis.

For the initiation of chromosome replication in eukaryotes, binding of the origin recognition complex (ORC) to the replication origins is followed by association of Cdc6 and Cdt1, recruiting the

helicase MCM2-7 complex to the origins, which results in the formation of the pre-replicative complex (pre-RC), in the G1 phase of the cell cycle (Yardimci and Walter, 2014). Formation of the pre-RC in G1 is critical to ensure that chromosomal replication occurs only once in each cell cycle (Costa et al., 2013). Cdc6, which is highly conserved within metazoans, interacts with DNA through the winged helix domain in its C-terminal region. Also, Cdc6 contains ATP-binding and hydrolytic activities, which are required for formation of the pre-RC (Fernandez-Cid et al., 2013; Randell et al., 2006). After Cdc6 participates in pre-RC formation in the nucleus during G1 phase, non–chromatin-bound Cdc6 translocates to the cytoplasm at the G1/S-phase transition (Alexandrow and Hamlin, 2004). These subcellular localizations through cell cycle progression are controlled by nuclear localization sequences (NLSs), nuclear export signals (NESs), and post-translational modifications, such as acetylation and phosphorylation (Delmolino et al., 2001; Paolinelli et al., 2009; Petersen et al., 1999).

Although Cdc6 functions in pre-RC formation in G1 phase, anaphase-promoting complex (APC)-CDH1 degrades Cdc6 in early G1, and Cdc6 mRNA and protein levels begin to increase in S phase (Petersen, 2000). Also, non–chromatin-bound Cdc6 is exported to the cytoplasm in S phase (Hook et al., 2007; Randell et al., 2006). This increase in expression level and export of Cdc6 to the cytoplasm in S and G2 phases suggests that Cdc6 may have another function in addition to its role as a component of the pre-RC. In this report, we demonstrate a novel function of Cdc6. Cdc6 localized to the centrosome in S and G2 phases, which negatively controlled microtubule formation.

## Material and Methods

### *DNA construction and transfection*

The wild-type or mutant form of the Cdc6 open reading frame was cloned into the vector pDsRed-Monomer-C1 (Clontech). The Cdc6 mutant L313A/I316A (LI/AA) was generated using the QuikChange Site-Directed Mutagenesis system (Stratagene). DNA constructs were transfected into cells using Lipofectamine 2000 transfection reagent (Invitrogen).

### *Short interfering RNA (siRNA) transfection*

siRNA oligonucleotides were purchased from Samchully Pharmaceutical. These were control GL3 siRNA, 5'-CUUACGCUGAGUA CUUCGATT-3', and Cdc6 siRNA, 5'-UAAGCCGGAUUCUGCAAGA-3'. siRNA oligonucleotides were transfected into cells using Oligofectamine (Invitrogen). Forty-eight hours after induction with doxycycline, U2OS Tet-On cells expressing FLAG-Cdc6 wild-type or FLAG-Cdc6(LI/AA) were transfected with siRNAs for 24 hr.

### *Cell culture and cell line construction*

U2OS human bone osteosarcoma cells, HeLa cervical adenocarcinoma cells, and HS68 human foreskin fibroblasts were cultured in Dulbecco`s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (penicillin/streptomycin). To construct the siRNA-resistant Cdc6, site-directed mutagenesis was performed by introducing three silent point mutations using the primers 5`- CTGCCTGCTTAAGTCGGATCCTGCAGGACCTCAAGAAGG-3` and 5`-CCTTCTTGAGGTCCTGCAGGATCCGACTTAAGCAGGCAG-3`. The Cdc6 siRNA-resistant FLAG-tagged wild-type or L313A/I316A mutant Cdc6 gene was cloned into the pTRE2hyg vector (Clontech) and transfected into U2OS Tet-On cells (Clontech). Hygromycin-resistant cells were

selected by culture in 200 μg/ml hygromycin for 2 weeks and then used in experiments. Expression of FLAG-tagged Cdc6 was induced by addition of 2 μg/ml doxycycline to culture medium for 48 h.

***Immunofluorescence microscopy***

Cells grown on coverslips were fixed with 4% paraformaldehyde for 15 min, followed by treatment with cold methanol for 10 min. Cells were permeabilized by incubation with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (PBST) for 15 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-Cdc6 antibody (Abcam), monoclonal anti–α-tubulin antibody (Sigma), rabbit anti-EB1 antibody (Millipore), rabbit anti–acetylated–α-tubulin antibody (Sigma), monoclonal anti-FLAG antibody (Sigma), or polyclonal anti–γ-tubulin antibody (Sigma). Anti-Cep215/CDK5RAP2 (Lee and Rhee, 2010), anti-pericentrin (Kim and Rhee, 2011), and anti-Cep192 (Kim and Rhee, 2014) antibodies were previously described. Cells were washed three times with PBST, incubated with Cy3- or FITC-conjugated anti-rabbit or anti-mouse secondary antibody, washed three times with PBST, and then mounted on glass slides with mounting media (Biomeda Corp.) containing 1 μg/ml 4',6-diamidino-2-phenylindole DAPI, Vectashield). Cells were viewed using an Olympus BX51 microscope.

***Microtubule regrowth assay***

Microtubule regrowth assay was performed as previously described (Lee and Rhee, 2010) with minor modifications. Cells were incubated for 1 h on ice with 2 μg/ml nocodazole to depolymerize microtubules. To allow microtubule regrowth, cells were washed with PBS and incubated in fresh medium at 25°C, followed by methanol fixation. For quantification of microtubule regrowth based on area after immunostaining, the fluorescence intensity in a 5-μm in diameter around centrosomes was measured using Image Pro Plus software.

***Fluorescence-activated cell sorting (FACS) analysis***

Cells were trypsinized and fixed in 70% ethanol at 4°C followed by incubation in 0.05% Nonidet P-40, 10 μg/ml RNase A, and 50 μg/ml propidium iodide for 1 h. For FACS analysis of BrdU staining, cells were incubated for 15 min in the presence of 10 μM BrdU. Harvested cells were fixed in 70% ethanol at 4°C and denatured in 2 N HCl, 0.5% Triton X-100 for 1 h. The cells were then neutralized with 0.1 M sodium borate (pH 8.5) and incubated with monoclonal rat anti-BrdU antibody (Abcam) and FITC-conjugated anti-rat secondary antibody in PBS containing 1% BSA and 0.5% Tween-20 for 1 h. Cells were stained with propidium iodide solution and then analyzed using the FACSCalibur instrument (BD Biosciences).

***Statistical analysis***

Groups were compared using two-tailed Student's *t*-test and Prism software (GraphPad). A p-value of below 0.05 was considered statistically significant.

## RESULTS

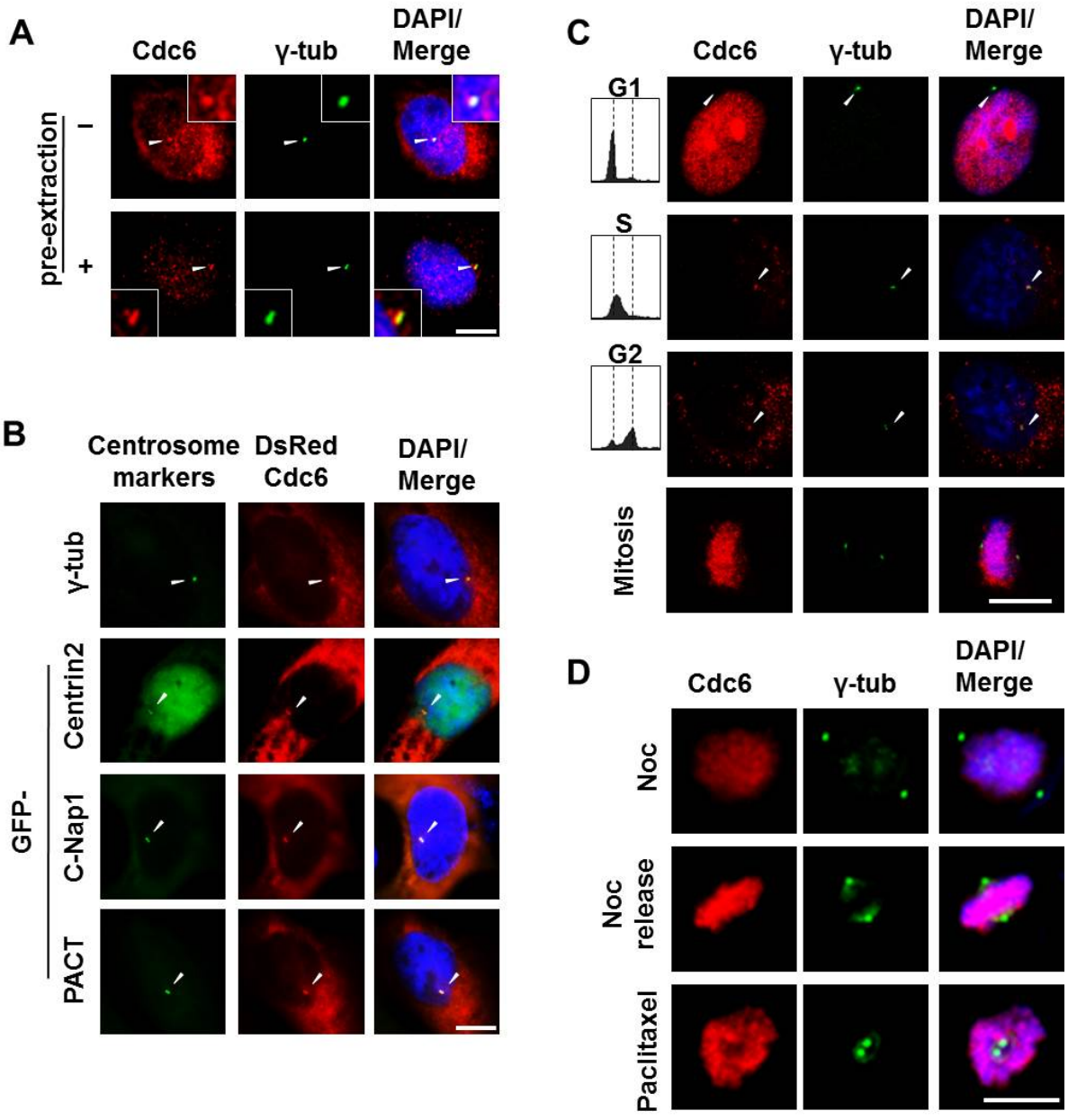
### *Cdc6 localizes to the centrosomes of S- and G2-phase cells*

A subpopulation of Cdc6 has been reported to exist at the centrosomes and spindle poles of mitotic cells (Narasimhachar et al., 2012; Yim and Erikson, 2010). In contrast, Cdc6 was also observed at the centrosomes of interphase cells (Narasimhachar et al., 2012). To clarify the difference in the centrosomal localization of Cdc6 during cell cycle progression, the centrosomal localization of Cdc6 was assessed by immunofluorescence analysis of U2OS cells using anti-Cdc6 monoclonal antibody (Fig. 1A). The morphology of the DAPI-stained nuclei indicated that the cells were in interphase. Colocalization of Cdc6 and γ-tubulin, which is a centrosomal protein (Stearns et al., 1991), implied that Cdc6 localized to interphase centrosomes. Even after extraction of cells with 0.1% Triton X-100 prior to immunostaining, Cdc6 was detected in the centrosomes. Transiently expressed DsRed-tagged Cdc6 (DsRed-Cdc6) also colocalized with γ-tubulin, as well as with the GFP-tagged centrosomal proteins centrin2, C-Nap1, and the PACT domain of AKAP450 (Gillingham and Munro, 2000) (Fig. 1B). Non-chromatin-bound Cdc6 is exported from the nucleus to the cytoplasm at entry into S phase (Petersen et al., 1999). The presence of Cdc6 in the cytoplasm and colocalization with other centrosomal proteins suggested that Cdc6 exists in the centrosomes during S and G2 phases of the cell cycle.

The localization of Cdc6 to centrosomes was determined during cell cycle progression in HeLa cells (Fig. 1C). In G1-phase cells, Cdc6 was present in the nucleus, but was not detected in centrosomes. When Cdc6 was present in the cytoplasm, FACS analysis indicated that the cells were in S or G2 phase, as previously reported (Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998). In these cells, Cdc6 was detected in centrosomes. However, Cdc6 was not detected at the spindle poles of mitotic cells, but was associated with chromosomes, as shown previously (Clijsters et al., 2013). Nocodazole or paclitaxel treatment arrests cells at prometaphase (Andreu et al., 1992). In nocodazole- and

**Figure. 1-1. Cdc6 localizes to S- and G2-phase centrosomes**

(**A**) Immunostaining of U2OS cells for Cdc6 and γ-tubulin (γ-tub) with or without permeabilization in PBST (pre-extraction). Nuclei were counterstained with DAPI. Arrowheads indicate centrosomes. Fields containing centrosomes are shown at higher magnification in insets. (**B**) U2OS cells were cotransfected with DNA constructs encoding DsRed-Cdc6 and the indicated GFP-tagged centrosomal markers. Arrowheads indicate centrosomes. Nuclei were counterstained with DAPI. (**C**) HeLa cells were synchronized by double-thymidine block and release. Cell cycle progression was analyzed by FACS analysis. (**D**) Localization of Cdc6 and γ-tubulin in U2OS arrested with paclitaxel or nocodozole (Noc) and released from nocodozole arrest. Scale bar: 10 μm.


A
Cdc6
γ-tub
DAPI/
Merge
pre-extraction
-
+
B
Centrosome
markers
DsRed
Cdc6
DAPI/
Merge
γ-tub
GFP-
Centrin2
C-Nap1
PACT
C
Cdc6
γ-tub
DAPI/
Merge
G1
S
G2
Mitosis
D
Cdc6
γ-tub
DAPI/
Merge
Noc
Noc
release
Paclitaxel

paclitaxel-treated cells, Cdc6 was not detected at the spindle pole (Fig. 1D), and metaphase cells, which were released from nocodazole arrest, did not contain Cdc6 at the spindle poles. These results support the idea that Cdc6 localizes to centrosomes during S and G2 phase in a cell cycle-dependent manner.

***Cdc6 centrosome localization signal***

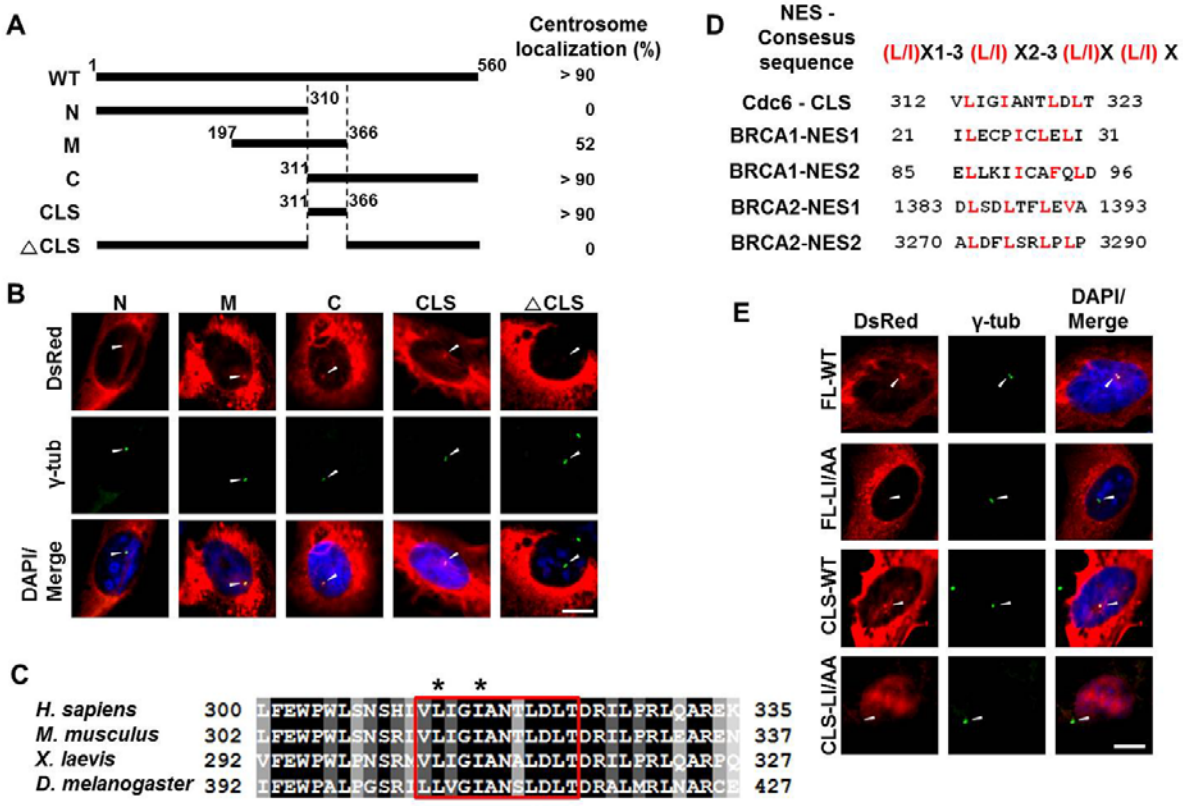
To identify the region responsible for localization of Cdc6 to the centrosome, we generated deletion mutants fused to the C-terminus of the DsRed-monomer-C1 expression vector and expressed the proteins in U2OS cells (Fig. 2A and B). Whereas the N-terminal fragment containing amino acid residues 1 to 310, did not localize to the centrosomes, both the C-terminal fragment, 311-560, and the fragment containing amino acid residues 197-366 did. Fragment 311-366, which overlapped both fragments 311-560 and 197-366, also localized to the centrosomes. Deletion of amino residues 311-366 abolished Cdc6 localization to centrosomes. The ability to localize the DsRed-tagged Cdc6 fragment to centrosomes indicated that a centrosome localization signal (CLS) is contained within amino acid residues 311-366 .

The Cdc6 CLS region was conserved in the Cdc6 homologues of other eukaryotes and contained a leucine-rich domain (Fig. 2C). The CLS region contains a putative nuclear export signal (NES), $\Phi X_{1-3}\Phi X_{2-3}\Phi X\Phi X$ (Fig. 2D); Φ indicates a large hydrophobic residue, such as Leu, Ile, Val, Met, and X indicates any amino acid (Han et al., 2008). This leucine-rich domain is found in other proteins, including BRCA1 and BRCA2, that localize to the centrosome (Fig. 2D). NES1 of BRCA1 was shown to participate in centrosomal localization of BRCA1 (Fabbro and Henderson, 2003; Thompson et al., 2005), and NES1 and NES2 of BRCA2 are involved in the centrosomal localization of BRCA2 (Han et al., 2008).

Leu313 and Ile316 of DsRed-Cdc6 were substituted with Ala (L313A/I316A, LI/AA) in DsRed-tagged Cdc6 and Cdc6-CLS fragment. Like full-length wild-type Cdc6, full-length mutant Cdc6

**Figure 1-2. Cdc6 centrosomal localization signal (CLS)**

(**A**) Schematic representation of deletion mutant constructs used to identify CLS. Numbers represent positions of amino acid residues. The indicated Cdc6 fragments were fused to the C-terminus of vector DsRed-monomer-C1. Centrosomal localization of each fragment was quantified with at least 50 DsRed-positive cells. WT, wild type; N, N-terminal; M, middle; C, C-terminal; ΔCLS, CLS-excised (**B**) Representative fluorescence micrographs of U2OS cells expressing the indicated deletion mutants. Arrowheads indicate centrosomes. (**C**) Amino acid sequences of human and homologous CLSs. Asterisks indicate amino acids substituted to inactivate CLS. Nuclear export signal (NES) consensus sequences are boxed in red. (**D**) Comparison of amino acid sequences of Cdc6 CLS and NESs of BRCA1 and BRCA2 (Han et al., 2008). X indicates any amino acid. (**E**) Fluorescence images of cells expressing full-length (FL) Cdc6 and the CLS fragment. LI/AA indicates mutant carrying substitutions L313A and I316A in the CLS.


A
Centrosome localization (%)
WT
N
M
C
CLS
△CLS
1
560
310
197
366
311
311
366
> 90
0
52
> 90
> 90
0
B
N
M
C
CLS
△CLS
DsRed
γ-tub
DAPI/ Merge
C
H. sapiens 300 LFEWPWLSNSHIVLIGIANTLDLTDRILPRLQAREK 335
M. musculus 302 LFEWPWLSNSRIVLIGIANTLDLTDRILPRLEARER 337
X. laevis 292 VFEWPWLPNSRNVLIGIANALDLTDRILPRLQARPQ 327
D. melanogaster 392 IFEWPALPGSRILLVGIANSLDLTDRALMRLNARCE 427
D
NES - Consesus sequence
(L/I)X1-3 (L/I) X2-3 (L/I)X (L/I) X
Cdc6 - CLS 312 VLIGIANTLDLT 323
BRCA1-NES1 21 ILECPICLELI 31
BRCA1-NES2 85 ELLKIICAFQLD 96
BRCA2-NES1 1383 DLSDLTFLEVA 1393
BRCA2-NES2 3270 ALDFLSRLPLP 3290
E
DsRed
γ-tub
DAPI/ Merge
FL-WT
FL-LI/AA
CLS-WT
CLS-LI/AA

(Cdc6-LI/AA) was present in the cytoplasm, but did not localize to centrosomes (Fig. 2E). In contrast, the mutant CLS fragment (CLS-LI/AA) did not localize to either the cytoplasm or centrosome, but remained in the nucleus. The NESs in other regions of Cdc6 were shown to be responsible for export of Cdc6 to the cytoplasm (Delmolino et al., 2001). Therefore, the Cdc6-ΔCLS and Cdc6-LI/AA mutant proteins, bearing absent and mutated CLS regions, respectively, did localize to the cytoplasm (Fig. 2A and E). These results suggest that the Cdc6 CLS functions in centrosomal localization of Cdc6 and not in nuclear export of the full-length protein.

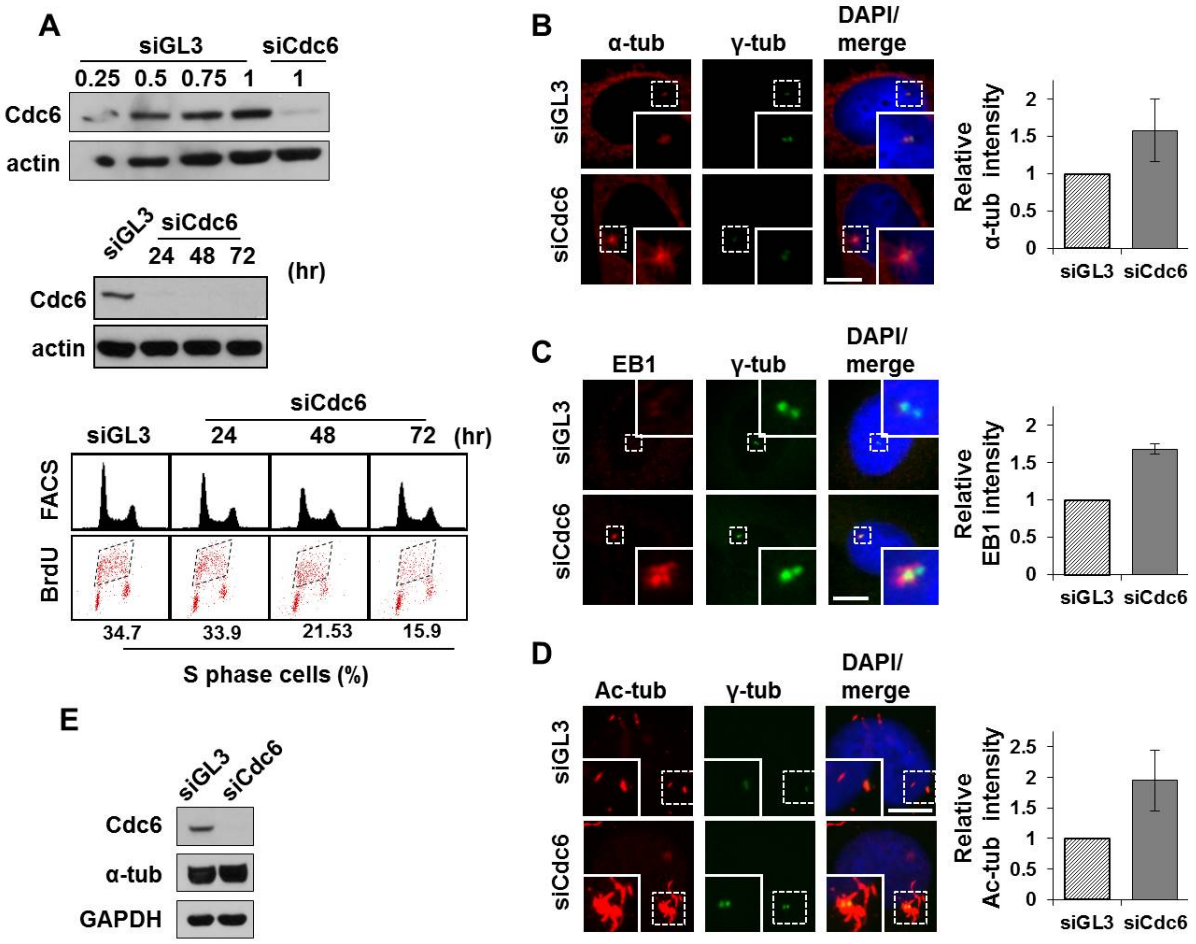
***Cdc6 depletion increases regrowth of centrosomal microtubules***

Cdc6 localizes to the centrosomes during S and G2 phase. One of the important functions of the centrosome is to serve as an MTOC (Luders and Stearns, 2007). To examine whether Cdc6 is involved in microtubule formation, we carried out siRNA-mediated Cdc6 knockdown in U2OS cells (Fig. 3A). Although Cdc6 depletion for 24 h did not significantly affect DNA synthesis, as determined by BrdU incorporation, or cell cycle progression, prolonged depletion for 48 or 72 h reduced BrdU incorporation, implying that cell cycle progression was arrested.

The Cdc6-depleted cells were incubated on ice for 1 h to depolymerize microtubules (Cassimeris et al., 1986), followed by incubation at 25°C for 5 min to allow microtubule regrowth. Microtubule regrowth emanating from centrosomes was measured by co-immunostaining cells with anti–γ-tubulin and anti-α-tubulin antibodies to detect microtubule formation from centrosomes (Fig. 3B). Microtubule regrowth was at least 50% higher in Cdc6-depleted cells than in cells transfected with control GL3 siRNA, based on intensity of α-tubulin staining (Fig. 3B), EB1 staining (Fig. 3C), and acetylation of α-tubulin (Fig. 3D). Because EB1 is a microtubule plus-end capping protein (Morrison et al., 1998), the intensity of staining of EB1 comets around the centrosome is related to the number of microtubules emanating from centrosomes. The increased microtubule regrowth and intensity of EB1

**Figure 1-3. Cdc6 depletion facilitates microtubule regrowth from centrosomes**

(**A**) Immunoblot analysis of Cdc6 in lysates of U2OS cells transfected with (upper) siRNA targeting Cdc6 (siCdc6) or the indicated dilutions of control GL3 siRNA (siGL3) for 24 h and (middle) siGL3 or siCdc6 for the indicated times. Actin served as internal control. (Lower) FACS analysis of propidium iodide (top) and BrdU (bottom) staining of cells transfected with siGL3 or siCdc6 for the indicated times. Replicating S-phase cells are indicated in the dashed boxes. The proportions of replicating S-phase cells are shown below the FACS profiles. (**B–D**) Fluorescence analysis of microtubule regrowth in cells treated with the indicated siRNAs for 24 h. Microtubules emanating from centrosomes were detected by immunostaining of α-tubulin (**B**), EB1 (**C**), or acetylated tubulin (**D**). Nuclei were counterstained with DAPI. Relative fluorescence intensities of the respective proteins in Cdc6-depleted cells and GL3 siRNA-treated cells are shown in graphs. Values represent mean±standard deviation (SD) of at least 100 cells in each of three independent experiments. Dashed boxes indicate centrosomes; these fields are shown at higher magnification in insets. (**E**) Immunoblot analysis of Cdc6 and α-tubulin in cells transfected with the indicated siRNAs for 24 h. GAPDH served as internal control. α-tub, α-tubulin; γ-tub, γ-tubulin; Ac-tub, acetylated tubulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.


A
siGL3
siCdc6
0.25 0.5 0.75 1 1
Cdc6
actin
siGL3
siCdc6
24 48 72 (hr)
Cdc6
actin
siCdc6
siGL3 24 48 72 (hr)
FACS
BrdU
34.7 33.9 21.53 15.9
S phase cells (%)
E
siGL3
siCdc6
Cdc6
α-tub
GAPDH
B
α-tub
γ-tub
DAPI/ merge
siGL3
siCdc6
Relative α-tub intensity
2 1.5 1 0.5 0
siGL3 siCdc6
C
EB1
γ-tub
DAPI/ merge
siGL3
siCdc6
Relative EB1 intensity
2 1.5 1 0.5 0
siGL3 siCdc6
D
Ac-tub
γ-tub
DAPI/ merge
siGL3
siCdc6
Relative Ac-tub intensity
2.5 2 1.5 1 0.5 0
siGL3 siCdc6

staining in Cdc6-depleted cells suggested that Cdc6 inhibits microtubule formation in centrosomes. The α-tubulin of microtubules is acetylated by α-tubulin *N*-acetyltransferase 1 (Maruta et al., 1986). Acetylation of microtubules was measured by immunofluorescence analysis using anti–acetylated–α-tubulin antibody. Acetylation was pronounced in Cdc6-depleted cells compared with control cells. The increased acetylation appeared to be accompanied by an increase in microtubule formation. Depletion of Cdc6 did not significantly influence the amounts of α-tubulin (Fig. 3E). Therefore, the increased microtubule formation, EB1 comet intensity, and acetylation of microtubules in Cdc6-depleted cells indicated that Cdc6 negatively controls microtubule formation.

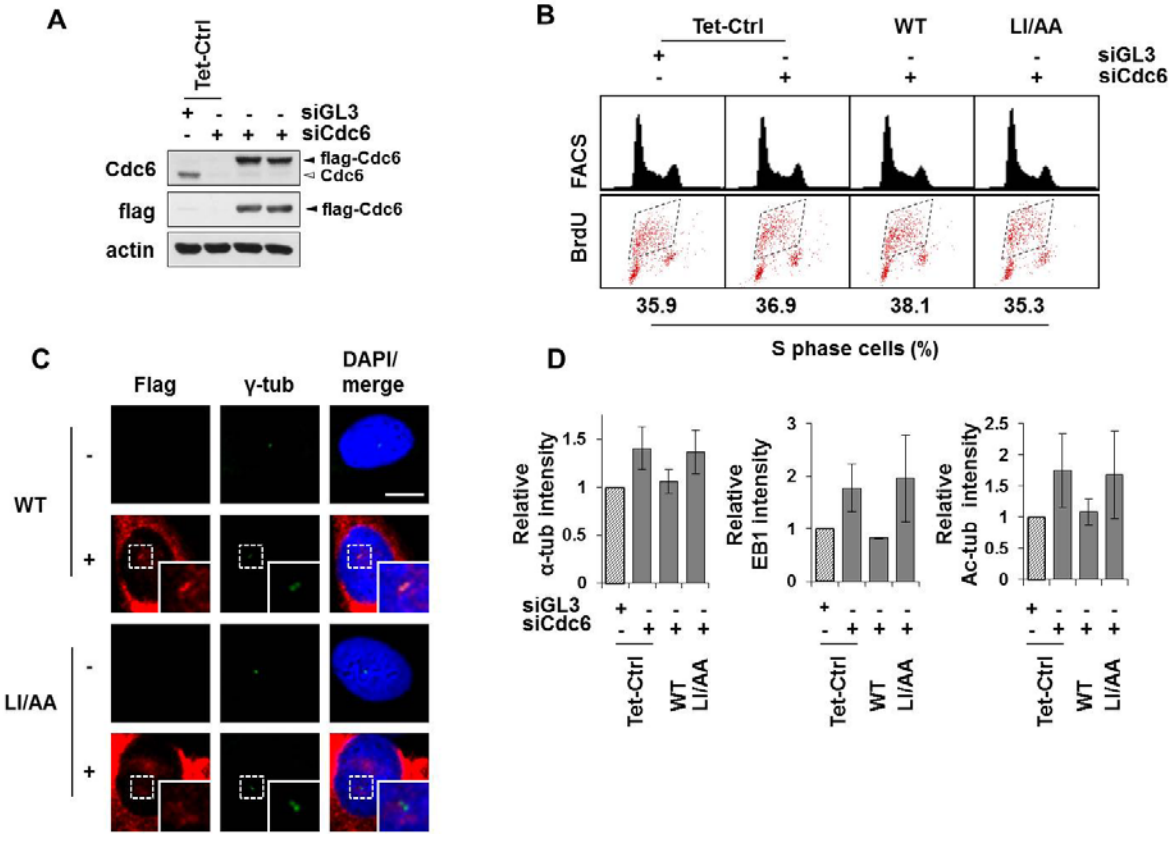
***Inhibition of microtubule formation by Cdc6 requires centrosomal localization of Ccd6***

Cdc6 existed in the cytoplasm and at the centrosome during S and G2 phase (Fig. 1). Therefore, we determined whether centrosomal localization of Cdc6 is required for inhibition of microtubule formation. Substitution of Leu313 and Ile316 of Cdc6 with Ala (LI/AA) prevented localization of mutated Cdc6 to centrosomes, although the mutant protein was present in the cytoplasm (Fig. 2E). The Cdc6 siRNA-resistant FLAG-Cdc6 wild-type or FLAG-Cdc6(LI/AA) coding sequences were introduced into a U2OS Tet-On cell line and selected in the presence of hygromycin. Addition of doxycycline to the cultures induced expression of the corresponding Cdc6 protein. In cells depleted of endogenous Cdc6 by siRNA-mediated knockdown (Fig. 4A), induction of wild-type or mutant protein expression did not significantly alter cell cycle progression (Fig. 4B).

The induced FLAG-Cdc6 wild-type protein was detected in both cytoplasm and centrosomes. In contrast, FLAG-Cdc6(LI/AA) localized to the cytoplasm but not to centrosomes, confirming that this mutant protein is defective in centrosomal localization (Fig. 4C). Depletion of Cdc6 in U2OS Tet-On cells increased microtubule regrowth, EB1 intensity, and acetylation of microtubules (Fig. 4D). Whereas induction of wild-type Cdc6 in Cdc6-depleted U2OS Tet-On cells reversed these changes,

**Figure 1-4. Centrosomal localization of Cdc6 is required to inhibit microtubule formation**

Immunoblot analysis of Cdc6 (**A**) and FACS analysis of propidium iodide (top) and BrdU (bottom) staining (**B**) in U2OS Tet-On cells expressing Cdc6-siRNA resistant FLAG-Cdc6 wild type or FLAG-Cdc6(LI/AA) and transfected with control GL3 siRNA or siRNA targeting Cdc6. Replicating S phase cells are indicated in dashed boxes. Proportions of replicating S-phase cells are shown below the FACS profiles. (**C**) Immunostaining demonstrates centrosomal localization of FLAG-Cdc6 (WT) and FLAG-Cdc6(LI/AA) in Tet-On cells without (-) or with (+) doxycycline induction. Dashed boxes indicate centrosomes; these fields are shown at higher magnification in insets. (**D**) Relative fluorescence intensities of α-tubulin, EB1, and acetylated tubulin in control cells and cells expressing WT and LI/AA Cdc6 and transfected with the indicated siRNAs. Values represent mean±SD of at least 100 cells in each of three independent experiments. Tet-Ctrl, control U2OS Tet-On cells; WT, FLAG-Cdc6 wild-type; LI/AA, FLAG-Cdc6(LI/AA).


A
Tet-Ctrl
siGL3
siCdc6
Cdc6
flag
actin
flag-Cdc6
Cdc6
flag-Cdc6
B
Tet-Ctrl
WT
LI/AA
siGL3
siCdc6
FACS
BrdU
35.9
36.9
38.1
35.3
S phase cells (%)
C
Flag
γ-tub
DAPI/
merge
WT
LI/AA
D
Relative
α-tub intensity
Relative
EB1 intensity
Relative
Ac-tub intensity
siGL3
siCdc6
Tet-Ctrl
WT
LI/AA

the mutant protein did not have this effect. The inability of FLAG-Cdc6(LI/AA), which was defective in centrosomal localization, to rescue the depletion of Cdc6 indicated that centrosomal localization of Cdc6 is necessary for inhibition of microtubule formation.

***Cdc6 participates in the control of γ-TuRC recruitment to the centrosomes***

Because Cdc6 negatively controlled centrosomal microtubule formation, we investigated whether Cdc6 affected recruitment of γ-TuRC to centrosomes. The γ-TuRC is composed of γ-tubulin, γ-tubulin complex proteins 2–6, and other associated proteins (Teixido-Travesa et al., 2012). Gamma-tubulin in the γ-TuRC functions as a nucleation core for microtubule polymerization. Cdc6 depletion increased intensity of γ-tubulin staining by 40% over that in control cells (Fig. 5A). This increase was reduced by induction of wild type-type Cdc6, but not by the mutant protein with statistical significances ($p$ value < 0.0001), which were similar to microtubule formation as shown in Figure 4D, suggesting that centrosomal Cdc6 inhibited γ-TuRC recruitment to centrosomes. PCM integral proteins, such as Cep215/CDK5RAP2 and pericentrin, have been reported to facilitate recruitment of γ-TuRC to centrosomes (Choi et al., 2010; Lee and Rhee, 2011). Whereas Cep215/CDK5RAP2 increased in Cdc6-depleted cells, pericentrin did not (Fig. 5B and C). Cep192, which is another PCM protein (Andersen et al., 2003), also increased in Cdc6-depleted cells (Fig. 5D). Depletion of Cep192 was reported to decrease microtubule formation (O'Rourke et al., 2014). The increases in Cep215/CDK5RAP2 and Cep192 along with the decreases by Cdc6 induction suggest that Cdc6 modulates PCM components, thereby controlling γ-TuRC recruitment to centrosomes.

***Cdc6 controls cell spreading***

Microtubules play roles in cell adhesion, spreading, and motility (Ganguly et al., 2012; Rhee et al., 2007). As depletion of Cdc6 increased microtubule formation, we determined whether the depletion

**Figure 1-5. Cdc6 controls γ-TuRC recruitment in centrosomes**

Relative fluorescence intensity of γ-tubulin (**A–D**), and Cep215/CDK5RAP2 (**B**), pericentrin (**C**), and Cep192 (**D**) in centrosomes of U2OS Tet-On cells expressing Cdc6-siRNA resistant FLAG-Cdc6 wild type or FLAG-Cdc6(LI/AA) treated with control GL3 or Cdc6-siRNA for 24 h. Values represent mean±SD of at least 50 cells in each of three independent experiments. Groups were compared using two-tailed Student's *t*-test. ns, not significant; ***, $P < 0.0001$.

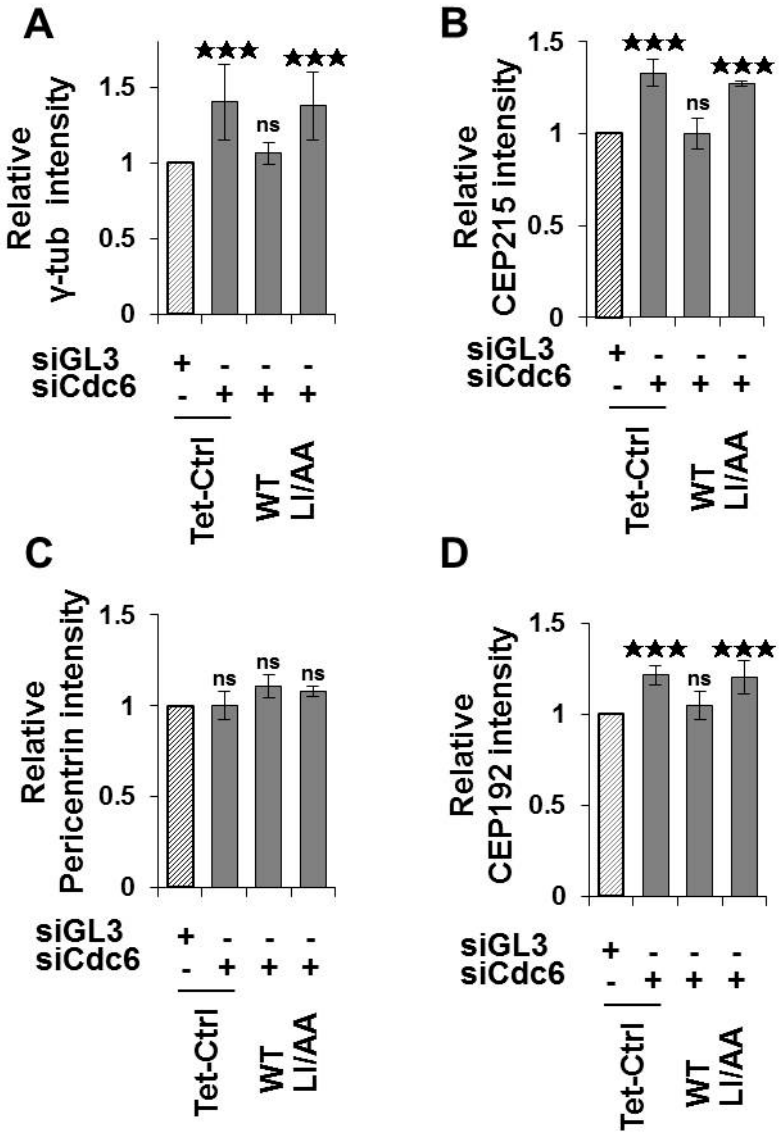
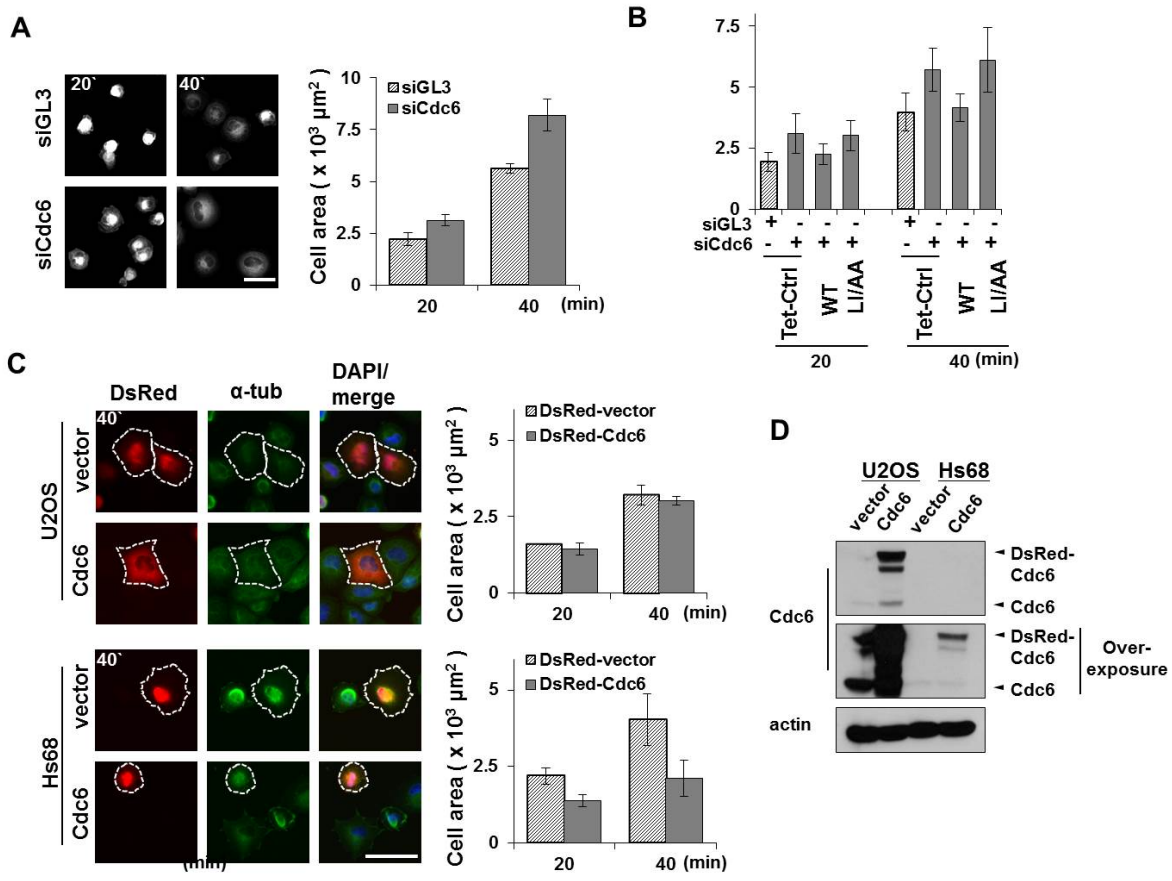

A
Relative γ-tub intensity
1.5
1
0.5
0
ns
★★★
★★★
siGL3
siCdc6
Tet-Ctrl
WT
LI/AA
B
Relative CEP215 intensity
1.5
1
0.5
0
ns
★★★
★★★
siGL3
siCdc6
Tet-Ctrl
WT
LI/AA
C
Relative Pericentrin intensity
1.5
1
0.5
0
ns
ns
ns
siGL3
siCdc6
Tet-Ctrl
WT
LI/AA
D
Relative CEP192 intensity
1.5
1
0.5
0
ns
★★★
★★★
siGL3
siCdc6
Tet-Ctrl
WT
LI/AA

**Figure 1-6. Cdc6 controls cell spreading**

(**A**) Immunofluorescence analysis of α-tubulin in U2OS Tet-On cells transfected with control GL3 and Cdc6 siRNAs, at the indicated times after seeding on fibronectin-coated coverslips (*left*) and quantification of cell area (*right*). (**B**) Quantification of areas of U2OS Tet-Ctrl and Tet-On cells expressing FLAG-Cdc6 wild-type (WT) or FLAG-Cdc6(LI/AA) for the indicated times. (**C**) Immunofluorescence analysis of spreading U2OS and Hs68 cells 40 min after transfection with DsRed-Cdc6-encoding construct or vector DNA for 48 h (*left*) and quantification of cell area (*right*) (dashed lines in images). (**D**) Immunoblotting of DsRed-Cdc6 and endogenous Cdc6 in U2OS and Hs68 cells transfected with vector or DsRed6-encoding DNA construct.


A
siGL3
siCdc6
20`
40`
siGL3
siCdc6
Cell area ( x 10³ µm² )
10
7.5
5
2.5
0
20
40
(min)
B
7.5
5
2.5
0
siGL3
siCdc6
Tet-Ctrl
WT
LI/AA
Tet-Ctrl
WT
LI/AA
20
40 (min)
C
DsRed
α-tub
DAPI/
merge
U2OS
vector
Cdc6
40`
DsRed-vector
DsRed-Cdc6
Cell area ( x 10³ µm² )
5
2.5
0
20
40 (min)
Hs68
vector
Cdc6
40`
DsRed-vector
DsRed-Cdc6
Cell area ( x 10³ µm² )
5
2.5
0
20
40 (min)
(min)
D
U2OS
Hs68
vector
Cdc6
vector
Cdc6
Cdc6
DsRed-
Cdc6
Cdc6
DsRed-
Cdc6
Cdc6
Over-
exposure
actin

affects cell adhesion and spreading on fibronectin-coated coverslips. Cells transfected with Cdc6-specific or control GL3 siRNA for 24 h were detached and transferred to fibronectin coated-coverslips, followed by measurement of isotropic spreading (Lafarga et al., 2012) (Fig. 6A). Cdc6-depleted cells exhibited increased cell areas compared with control GL3 siRNA-transfected cells (Fig. 6A). In contrast to FLAG-Cdc6 wild-type protein, FLAG-Cdc6(LI/AA), which was defective in both centrosomal localization and inhibition of microtubule formation (Fig. 4), did not reverse the increase in area of Cdc6-depleted U2OS Tet-On cells (Fig. 6B).

Although depletion of Cdc6 increased the spreading capability of cells as well as microtubule formation (Fig. 3), overexpression of Cdc6 did not significantly change the size of U2OS cells (Fig. 6C). U2OS, which is a cancer cell line, contained a much higher amount of Cdc6 than the fibroblast line Hs68 (Fig. 6D). When Cdc6 was overexpressed in Hs68 cells, cell spreading was reduced (Fig. 6C). The overexpression might not affect cell size or microtubule regrowth in U2OS cells, due to the relative abundance of Cdc6 in these cells. The reduction of spreading by Cdc6 overexpression in Hs68 cells support the idea that the reduced spreading is the result of inhibition of microtubule formation by Cdc6.

## DISCUSSION

Although Cdc6 participates in pre-RC formation in the nucleus during G1 phase, Cdc6 mRNA and protein levels increase in S phase, with a limited amount of Cdc6 present in G1 phase (Petersen, 2000). Also, non–chromatin-bound Cdc6 is exported from the nucleus in G1/ S transition phase . Our results demonstrated that the increased protein level and export from the nucleus of Cdc6 in S and G2 phases are involved in control of microtubule formation in in the centrosomes.

Cdc6 possesses a CLS sequence that is responsible for centrosomal localization of Cdc6 (Fig. 2). The Cdc6 CLS contains a leucine-rich domain, which is also preserved in NESs. The Cdc6 CLS fragment was present in both the cytoplasm and centrosomes. On the other hand, mutation of the leucine-rich domain of the Cdc6 CLS fragment resulted in defects in cytoplasmic as well as centrosomal localization, suggesting that this domain functions as a NES as well as a CLS. Cdc6 contains several putative NES sequences, which are responsible for transport of Cdc6 to the cytoplasm from the nucleus (Delmolino et al., 2001), and other NESs, distinct from the NES sequence in Cdc6-CLS, have been shown to contribute to the cytoplasmic localization of Cdc6. Therefore, mutation of the leucine-rich sequence of Cdc6-CLS of Cdc6 full-length protein did not affect the localization of the mutant Cdc6 to the cytoplasm; however, the mutant protein did not localize to the centrosome. These results suggest that the centrosomal localization of Cdc6 is carried out specifically by the Cdc6-CLS.

Cdc6 depletion increased microtubule formation, EB1 staining intensity, and α-tubulin acetylation (Fig. 3). The induced Cdc6 required localization to centrosomes to rescue Cdc6 depletion (Fig. 4). In centrosomes, microtubules are nucleated and elongated at the γ-TuRC in the PCM (Moritz et al., 1995). Also, the γ-TuRC is anchored to subdistal appendages of the mother centriole for elongation (Ibi et al., 2011; Mogensen et al., 2000). In addition to the γ-tubulin component of the γ-TuRC, Cdc6 depletion increased the amounts of Cep215/CDK5RAP2 and Cep192, which are integral proteins of

the PCM, in centrosomes (Fig. 6). These increases may contribute to promotion of the recruitment of γ-TuRC to the centrosome. The increased γ-TuRC recruitment upon Cdc6 depletion resulted in an increase in centrosomal microtubule formation. γ-TuRC recruitment to centrosomes increases at the G2/M transition to prepare formation of the mitotic spindle for chromosome segregation (Tanenbaum and Medema, 2010; Wang et al., 2014). The presence of Cdc6 in S- and G2-phase centrosomes, as well as delocalization from mitotic centrosomes, may contribute to controlling γ-TuRC recruitment for microtubule formation.

The increased microtubule formation upon Cdc6 depletion resulted in increased cell adhesion and spreading (Fig.6). Consistently, overexpression of Cdc6 reduced cell spreading in Hs68 cells. The Cdc6 depletion and overexpression results indicate that Cdc6 negatively controls microtubule formation in centrosomes, thereby decreasing cell spreading. In contrast to Cdc6, expression of pericentrin and Cep215, which are structural components of PCM and facilitate γ-TuRC recruitment, was shown to enhance cell polarity and migration by increasing microtubule nucleation (Endoh-Yamagami et al., 2010; Takitoh et al., 2012). BRCA1 has been reported to inhibit microtubule nucleation by ubiquitinating Lys344 of γ-tubulin (Starita et al., 2004). Because microtubules are critical for cell structure and function, microtubule formation in centrosomes must be under positive and negative control to accommodate diverse cellular requirements.

Centrosome duplication and chromosomal replication during the cell cycle share similarities in the following aspects. Both duplications take place in interphase in a cell cycle-dependent manner; these cell cycle-dependent duplication processes are commonly regulated by cyclins and cyclin-dependent kinases; and the duplicated centrosomes and chromosomes are equally segregated into daughter cells (Lacey et al., 1999; Nam and van Deursen, 2014; Tsou and Stearns, 2006; Vitre and Cleveland, 2012). Furthermore, the pre-RC forming and controlling proteins such as ORC subunits (Hemerly et al., 2009; Prasanth et al., 2004; Stuermer et al., 2007), MCM2-7 subunits (Ferguson and Maller, 2008; Ferguson

et al., 2010), and geminin (Lu et al., 2009) also exist in centrosomes to maintain centrosome integrity. Our results showed that Cdc6 that is a component of the pre-RC localizes to centrosomes in a cell cycle-dependent manner to modulate microtubule formation. The proteins that regulate both pre-RC and centrosome function may contribute to coordinating the interconnection of chromosome replication and centrosome function.

# CHAPTER 2

## Cdc6 contributes to maintaining centrosome integrity

**Abstract**

Centrosome is duplicated through cell cycle and its integrity is critical for chromosome segregation that is tightly linked to chromosome stability. Here, I addressed that Cdc6 negatively regulated centrosome amplification and Grp78 was required for centrosome amplification. Whereas Cdc6 depletion induced centrosome amplification, Cdc6 overexpression reduced hydroxyurea (HU)-induced centrosome amplification. The centrosome amplification arising by Cdc6 depletion was caused by centrosome over-duplication rather than centriole fragmentation. Amino acid residues 197 to 214 of Cdc6 (Cdc6-SCA: suppression of centrosome amplification) was responsible for suppression of centrosome amplification. The Cdc6-SCA contains a FHA domain binding motif and mediated interaction with Grp78, which is a molecular chaperon of HSP70 family. Grp78 localized to interphase centrosomes and was necessary for centrosome amplification by Cdc6 depletion or HU treatment. My results suggest that interaction between Cdc6 and Grp78 is involved in controlling centrosome duplication.

## Introduction

Centrosomes function as microtubule organizing center throughout the cell cycle, and thus contribute to formations of cell shape, polarity and motility, as well as mitotic spindle for chromosome segregation and cell division (Luders and Stearns, 2007). Centrosomes are composed of two centrioles and pericentriolar matrix (Nigg et al., 2014). Two centrioles of the mother centriole and daughter centriole are structurally and functionally distinct. Mother centriole possesses appendages at its distal end. Centriole duplication initiates at the G1/S transition of the cell cycle with the formation of one daughter centriole adjacent to each pre-existing mother centriole. This event is regulated by Plk4 and Cdk proteins (Mardin and Schiebel, 2012). Cyclin E/Cdk2, a known inducer of S-phase entry, has a role in the initiation of centrosome duplication. In late G1 phase, activation of cyclin E/Cdk2 coordinates the initiation of centrosome duplication and DNA replication (Matsumoto et al., 1999). Daughter centriole, once formed, elongates during S and G2 phase. This elongation process depends on several proteins, including SAS-4, OFD1, and CP110 (Kohlmaier et al., 2009; Schmidt et al., 2009; Singla et al., 2010). CP110 localizes to the distal ends of growing centrioles and functions as a cap to inhibit microtubule extension (Schmidt et al., 2009). Cyclin A/Cdk2 also has been implicated in the regulation of centrosome duplication in S and G2 phase. Centriole maturation is acquisition of distal and subdistal appendages. Appendage proteins are important to microtubule anchoring (Ibi et al., 2011).

Recent studies have described the critical roles played by centrosomes in cell cycle progression (Doxsey et al., 2005). Duplicated centrosomes are separated and migrate to each pole to function as spindle poles. Bipolar spindle assembly, which is attachment of mitotic spindles emanating from opposite spindle poles to sister chromatids, is critical for chromosome segregation equally during mitosis and bipolar division. Failure of proper control of centrosome function can lead to multipolar

spindles, aneuploidy, cell polarity disruption, and asymmetric cell division (Vitre and Cleveland, 2012).

Centrosome amplification is a hallmark of tumorigenesis. Several abnormal conditions, including centriole overduplication, *de novo* centriole assembly, cytokinesis failure, mitotic slippage, lead to centrosome amplification and subsequent formation of multipolar spindles (Marthiens et al., 2012). Although activation of cyclin E/Cdk2 by cyclin E overexpression alone do not efficiently induce centrosome amplification, this condition is required for generation of amplified centrosomes (Hanashiro et al., 2008). Cdk2 activity is negatively controlled by Cdk inhibitor p21, one of the major transactivation targets of the p53 tumor-suppressor protein (He et al., 2005). The involvement of p53 in the regulation of centrosome duplication is suggested by the observations that the cells from p53-defiecent mice show a high frequency of centrosome amplification (Tarapore and Fukasawa, 2002). When cells are exposed to DNA synthesis inhibitor, hydroxyurea (HU), centrosomes undergo reduplication without DNA replication, resulting in centrosome amplification (D'Assoro et al., 2004). Amplified centrosomes disrupt mitotic spindle bipolarity that is essential for faithful segregation of chromosomes. Supernumerary centrosomes that result from centrosome overduplication or cytokinesis failure are associated with multipolar spindles, which are often seen in human cancer cells (Vitre and Cleveland, 2012). Multipolar spindles divide in a multipolar manner, or generate merotelic attachments, which result in lagging chromosomes formed during anaphase and cause aneuploidy (Kwon et al., 2008). Aneuploidy, the gain or loss of chromosomes, is associated with malignancy and tumor progression (Manchado et al., 2012). Therefore, it is important to understand centrosome instability correlating with centrosome integrity.

Glucose regulated protein 78(Grp78) that is induced by the endoplasmic reticulum (ER) stress response has been recognized as a molecular chaperone in the ER (Li and Li, 2012). Grp78 belonging to the heat shock protein 70 (HSP70) family is also known as the immunoglobulin heavy chain

binding protein (BiP) (Li and Lee, 2006). Grp78 localizes in cell membrane, cytoplasm, mitochondria, and nucleus, besides in the ER. GRP78 is involved in tumor proliferation, survival, metastasis, and resistance to a wide variety of therapies (Li and Li, 2012). Thus, GRP78 has been considered to serve as a biomarker for tumor behavior and treatment response.

I found that Cdc6, which is a DNA replication licensing factor, regulates centrosome duplication. Cdc6 negatively controls centrosome duplication. Grp78 that interacts with Cdc6 is required for centrosome amplification. I consider that the interaction between Cdc6 and Grp78 participates in maintaining centrosome integrity.

## Materials and Methods

### *DNA construction and transfection*

The wild-type or mutant form of the Cdc6 open reading frame was cloned into the vector pDsRed-Monomer-C1 (Clontech). The Cdc6 mutants T209A and T209D were generated using the QuikChange Site-Directed Mutagenesis system (Stratagene). DNA constructs were transfected into cells using Lipofectamine 2000 transfection reagent (Invitrogen).

### *Short interfering RNA (siRNA) transfection*

siRNA oligonucleotides were purchased from Samchully Pharmaceutical. These were control GL3 siRNA, 5'-CUUACGCUGAGUA CUUCGATT-3', Cdc6 siRNA, 5'-UAAGCCGGAUUCUGCAAGA-3', and Grp78 siRNA, 5'- GGAGCGCAUUGAUACUAGA-3'. siRNA oligonucleotides were transfected into cells using Oligofectamine (Invitrogen).

### *Cell culture and cell line construction*

U2OS human bone osteosarcoma cells, HeLa cervical adenocarcinoma cells, 293T human embryonic kidney cells, and HEK293 human embryonic kidney cells were cultured in Dulbecco`s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (penicillin/streptomycin). To construct the siRNA-resistant Cdc6, site-directed mutagenesis was performed by introducing three silent point mutations using the primers 5`-CTGCCTGCTTAAGTCGGATCCTGCAGGACCTCAAGAAGG-3` and 5`-CCTTCTTGAGGTCCTGCAGGATCCGACTTAAGCAGGCAG-3`. The Cdc6 siRNA-resistant FLAG-tagged wild-type, T209A or T209D mutant Cdc6 gene was cloned into the pTRE2hyg vector (Clontech) and transfected into U2OS Tet-On cells (Clontech). Hygromycin-resistant cells were selected by culture in 200 μg/ml hygromycin for 2 weeks and then used in experiments. Expression of

FLAG-tagged Cdc6 was induced by addition of 2 μg/ml doxycycline to culture medium for 48 h. The Cdc6 SCA wild-type, T209A or T209D mutant Cdc6 gene was cloned into the pIRES2-EGFP vector (Clontech) and transfected into HEK293 cells. G-418-resistant cells were selected by culture in 500 μg/ml G-418 for 2 weeks and then isolated by single clonal selection.

***In vivo $^{32}$P-orthophosphate incorporation assay***

293T cells transfected with a vector containing Cdc6-SCA were incubated in phosphate-free medium for 2 h and then in medium containing 200 μCi/ml of $^{32}$P-orthophosphate (IZOTOP, 10 mCi/ml) for 4 h before harvesting. Cells were lysed for 15 min lysis buffer (50 mM Tris-HCl, pH 7.4, 0.3M NaCl, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% Nonidet P40, 200 μM $Na_3VO_4$, 10 mM NaF, and protease inhibitor cocktail). Cdc6 SCA mutant was purified by glutathione pull-down and electrophoresis was performed in 10% SDS-polyacrylamide gel followed by gel-dry. Radio-activity was measured by BAS2500 (Fujifilm).

***Tandem affinity purification***

HEK293 stable-cells containing Cdc6-SCA WT, T209A or T209D were lysed in lysis buffer NETN buffer (100 mM NaCl, 0.5 mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.5% Nonidet P40) supplemented with 50 mM NaF, 100 μM $Na_3VO_4$, and protease inhibitor cocktail, then Cdc6 SCA protein was tandem affinity purification using Strepavidin-Sepharose and S beads followed by electrophoresis in a 10% SDS polyacrylamide gel. The protein band was excised and analyzed by **ProteomeTech Inc.**

***Immunofluorescence microscopy***

Cells grown on coverslips were fixed with 4% paraformaldehyde for 15 min, followed by treatment with cold methanol for 10 min. Cells were permeabilized by incubation with phosphate-buffered

saline (PBS) containing 0.1% Triton X-100 (PBST) for 15 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–γ-tubulin antibody (Sigma), poly clonal anti- γ-tubulin antibody (Sigma), monoclonal anti-cyclin E (Santa Cruz), polyconal anti-cyclin A antibody (Santa Cruz), monoclonal anti-cyclin B antibody (Santa Cruz), or monoclonal anti-Myc antibody (Santa Cruz). Anti-CP110 antibody was previously described. Cells were washed three times with PBST, incubated with Cy3- or FITC-conjugated anti-rabbit or anti-mouse secondary antibody, washed three times with PBST, and then mounted on glass slides with mounting media (Biomeda Corp.) containing 1 μg/ml 4',6-diamidino-2-phenylindole DAPI, Vectashield). Cells were viewed using an Olympus BX51 microscope.

## Results

### *Cdc6 prevents centrosome amplification*

I demonstrated that cell cycle-dependent localization of Cdc6 to the centrosomes of S and G2 phase decreased recruitment of PCM proteins and consequently reduced γ-TuRC formation, thereby interfering with microtubule formation (Chapter I). Depletion of Cdc6 with Cdc6-sepicfic siRNA (Cdc6-siRNA) resulted in increase of MT formation within 24 hrs. When Cdc6 depletion was prolonged for 48 or 72 hr, I observed increase of cell population containing prematurely separated centrosomes and amplified (>2) centrosomes (Fig. 2-1A and B). In normal cells, a G1-phase cell possesses one centrosome containing two centrioles and the centrosome is duplicated during S and G2 phase followed by being separated and migrated to opposite poles in prophase (Tanenbaum and Medema, 2010; Tsou and Stearns, 2006). Cdc6-siRNA treatment increased cells containing prematurely separated centrosome 1.5 fold more than control cells treated with GL3-siRNA. Furthermore, centrosome amplification was 10-fold more at 72 hr in Cdc6-siRNA-treated cells than GL3-siRNA-treated cells. For further analyses, I used U2OS Tet-on cell line containing Cdc6-siRNA-resistant FLAG-Cdc6, in which FLAG-Cdc6 is inducible by doxycycline (Fig 1-4). Cdc6-siRNA treatments to the U2OS Tet-On cells increased prematurely separated centrosomes and amplified centrosomes as shown in Figure 2-A (Fig. 2-1B). Induction of FLAG-Cdc6 reduced the prematurely separated centrosomes and amplified centrosomes. These results suggested that Cdc6 prevents premature separation and amplification of centrosomes.

Centrosome amplification results from either over-duplication or centriole fragmentation of the centrosomes (Hut et al., 2003; Loffler et al., 2013). To address a cause of centrosome amplification, centrosomes were immunostained with anti-CP110 antibody. Because CP110 localizes at subdistal appendage of centriole, each γ-tubulin spot was adjacent with two CP110 spots (Chen et al., 2002). In

**Figure 2-1. Cdc6 depletion induces centrosome amplification**

(**A**) U2OS cells transfected with Cdc6-siRNA (siCdc6) or control GL3-siRNA (siGL3) were, at the indicated time after transfection, analyzed in immunoblot analysis with indicated antibodies. Actin was served as an internal control. (**B**) The ratio of cells containing the indicated number of γ-tub spots was determined at the indicated time after siRNA transfection. (**C**) U2OS Tet-On cells-inducing FLAG-Cdc6, which was resistant to Cdc6-siRNA, upon addition of doxycycline were transfected with siRNA for 72 h, then doxycycline was added to culture medium for 48 h prior to colleting the cells. Ctrl, control U2OS Tet-On cell; + or -, with or without doxycycline, respectively; 1C, one γ-tub spot; 2C(L), linked two γ-tub spots; 2C(S), separated two γ-tub spots; 3≥C, more than two γ-tub spots. Values represent mean±standard deviation (SD) of at least 100 cells in each of three independent experiments.

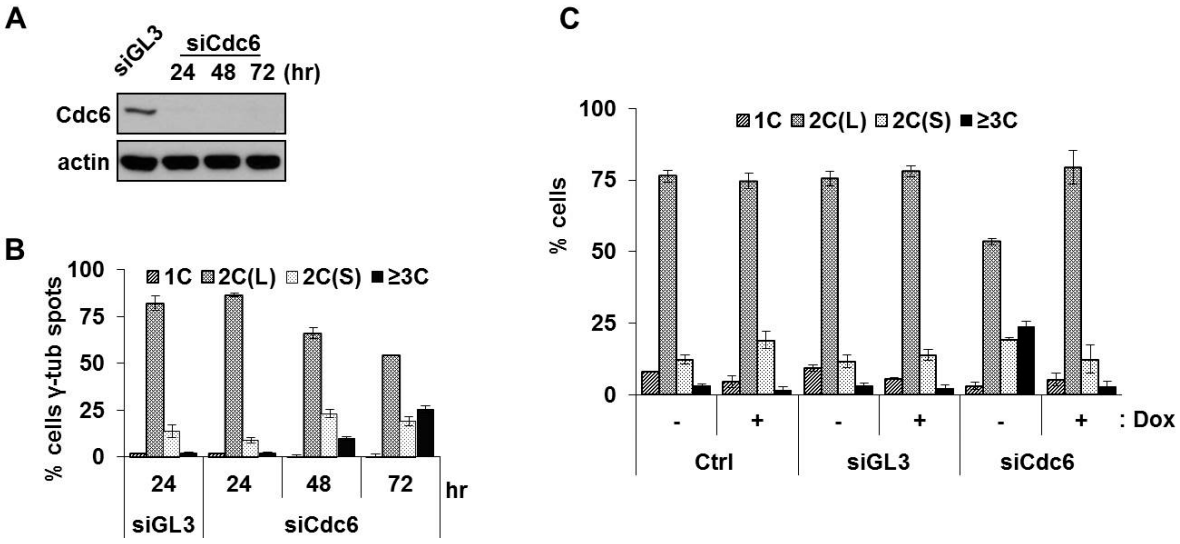
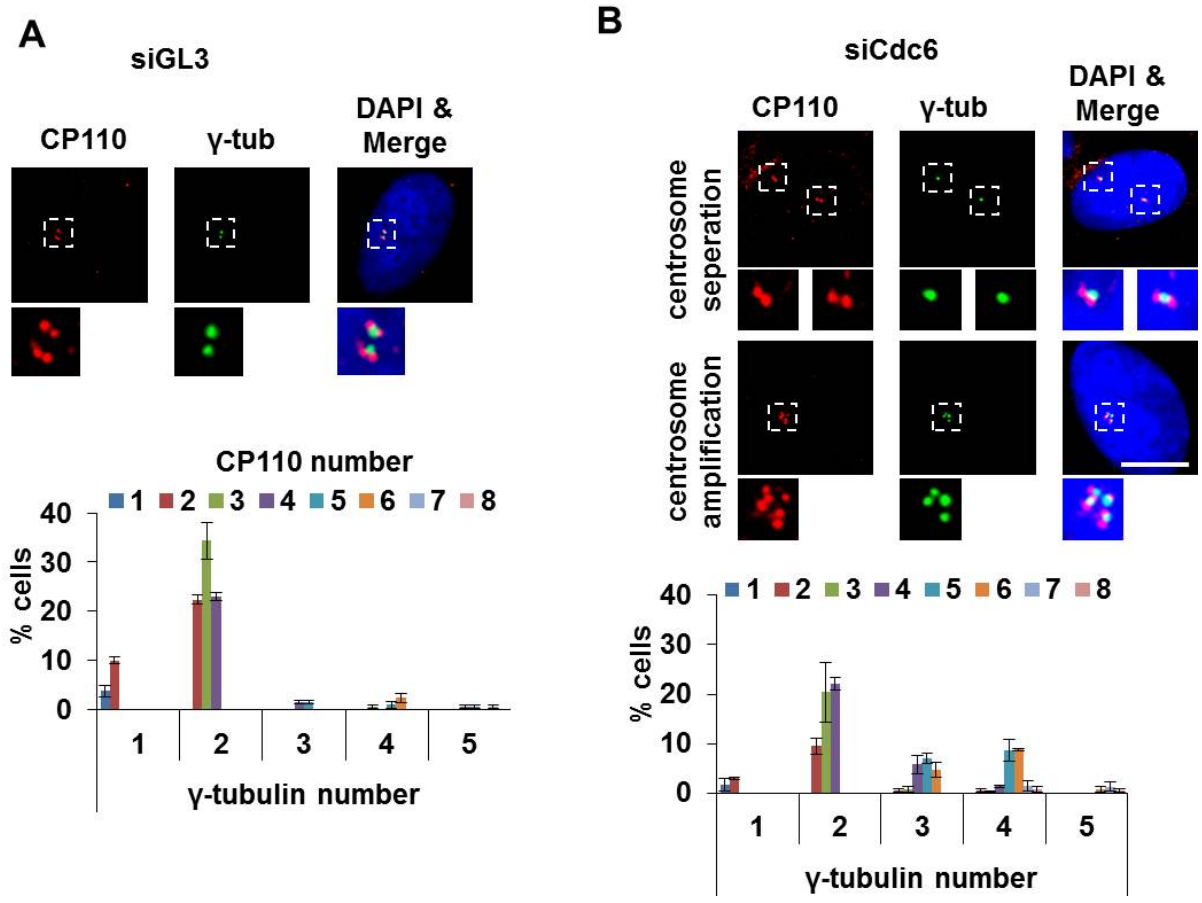

A
siGL3
siCdc6
24 48 72 (hr)
Cdc6
actin
B
% cells γ-tub spots
100
75
50
25
0
1C 2C(L) 2C(S) ≥3C
24
24
48
72
hr
siGL3
siCdc6
C
% cells
100
75
50
25
0
1C 2C(L) 2C(S) ≥3C
- + - + - + : Dox
Ctrl
siGL3
siCdc6

**Figure 2-2. Centrosome amplification by Cdc6 depletion is not caused by centrosome fragmentation**

U2OS cells were transfected with siGL3 (**A**) or siCdc6 (**B**) and representative images of cells immunostained with anti-γ-tubulin (green) or anti-CP110 (red) were displayed (*upper panel*). Quantifications of cells containing γ-tub and CP110 spots were shown at *lower pannel*. Insets showing centrosomes were at higher magnification. Scale bar: 10μm.


A
siGL3
CP110
γ-tub
DAPI &
Merge
CP110 number
1 2 3 4 5 6 7 8
40
30
20
10
0
% cells
1
2
3
4
5
γ-tubulin number
B
siCdc6
CP110
γ-tub
DAPI &
Merge
centrosome
seperation
centrosome
amplification
1 2 3 4 5 6 7 8
40
30
20
10
0
% cells
1
2
3
4
5
γ-tubulin number

control cells, each centrosome contained two CP110 spots (Fig. 2-2). A γ-tubulin spot in Cdc6 depleted cells was also observed with two adjacent CP110 spots. Amplified centrosomes of Cdc6-depleted cells contained more number of CP110 spots than γ-tubulin spots. If centrosome amplification is caused by centriole fragmentation, resulting centrosomes contain one or no CP110 spot. These results suggested that centrosome amplification of Cdc6-depleted cells was derived from centrosome over-duplication rather than centriole fragmentation.

Cell cycle arrest in G2 phase often causes centrosome amplification (Dodson et al., 2004). Because cell cycle arrest arising by Cdc6 depletion happened through interphase (Fig. 1-3), I examined whether centrosome amplification was limited in G2 phase of Cdc6-depleted cells. Cyclin E, Cyclin A and Cyclin B are cell cycle phase markers of G1, S and G2, and G2 phase, respectively (Moore, 2013). In control cells, Cyclin E positive cells possessed one or linked centrosomes, and Cyclin A and B positive cells possessed linked or separated centrosomes, which agreed with the centrosome status of each cell cycle phase (Fig.2-3). Each cyclin positive Cdc6-depleted cells contained amplified centrosomes relative to control cells, implying that centrosome amplification of Cdc6-depleted cells were not limited to the cell cycle arrest in a specific phase.

***Overexpression of Cdc6 suppresses hydroxyurea-induced centrosome amplification***

Treatment of hydoxyurea (HU) to U2OS cells induces centrosome amplification (D'Assoro et al., 2004). Whereas HU treatment for 72 hr to U2OS cells increased centrosome amplification to 34.8%, no treatment yielded 3.4% of cells containing amplified centrosomes (Fig.2-4), in agreement with previous report (D'Assoro et al., 2004). When cells were transfected with DsRed vector in the presence of HU treatment, centrosome amplification was observed in 31.5% of cells similar to un-transfected cells. In contrast, ectopic expression of Cdc6 reduced centrosome amplification to 10.9%

**Figure 2-3. Centrosome amplification by Cdc6 depletion is not limited to a specific cell cycle phase**

Representative fluorescence images displayed γ-tub spots, co-immunostained with cyclin E (**A**), cyclin A (**B**), and cyclin B (**C**) of U2OS cells transfected with siCdc6 or siGL3. Percentages of cells containing the numbers of γ-tub spots were determined with the indicated cyclin-positive (+) or negative (-) cells (*lower panel*).

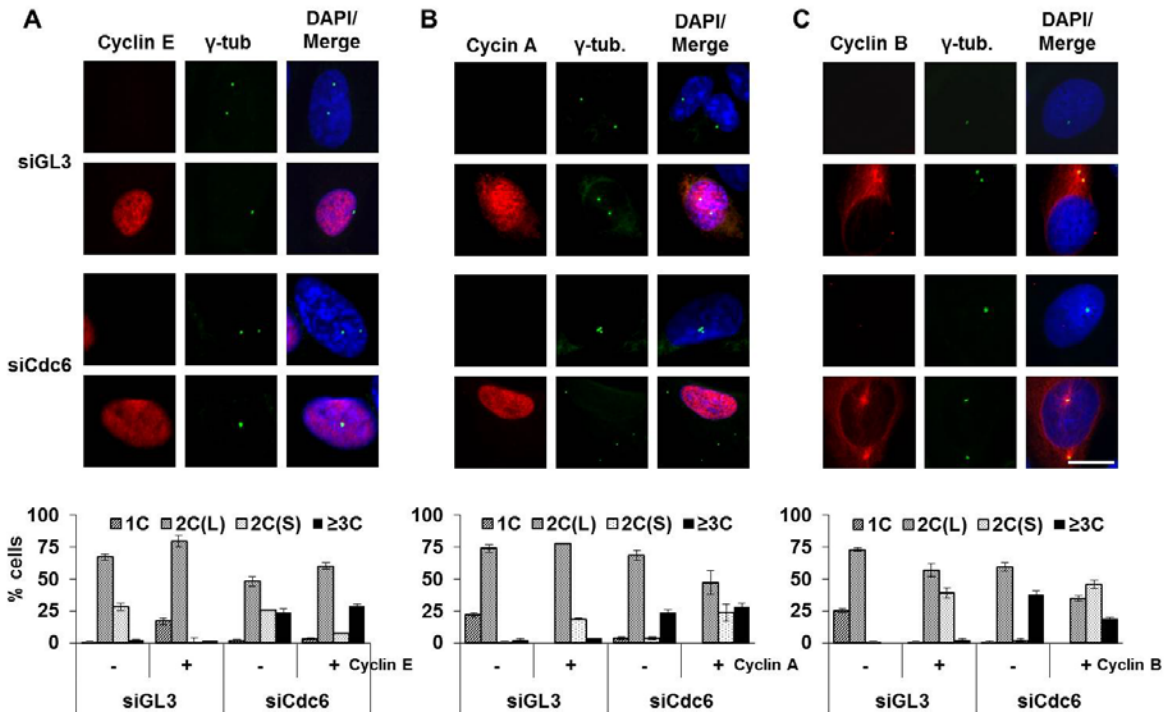
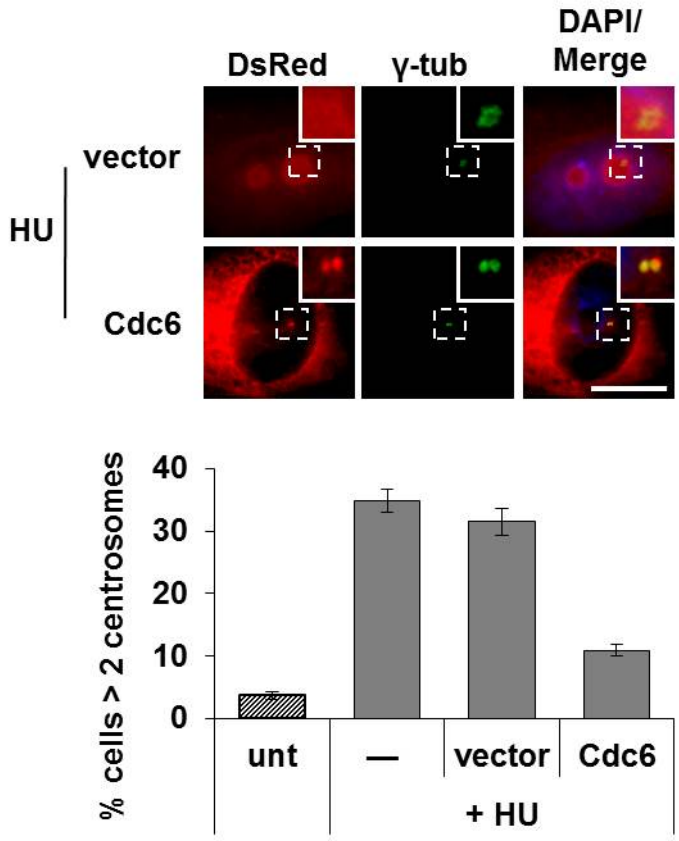

A
Cyclin E
γ-tub
DAPI/ Merge
siGL3
siCdc6
B
Cycin A
γ-tub.
DAPI/ Merge
C
Cyclin B
γ-tub.
DAPI/ Merge
% cells
100
75
50
25
0
1C
2C(L)
2C(S)
≥3C
-
+
+ Cyclin E
+ Cyclin A
+ Cyclin B
siGL3
siCdc6

**Figure 2-4. Overexpression of Cdc6 suppresses HU-induced centrosome amplification**

U2OS cells transfected with DsRed-Cdc6 or empty vector were treated with HU for 72 h. Representative fluorescence images (*upper panel*) and percentages of cells containing centrosome amplification (*lower panel*). unt, no HU treatment; -, no transfection; vector, empty vector; Cdc6, DsRed-Cdc6 vector.


DsRed
γ-tub
DAPI/
Merge
vector
HU
Cdc6
% cells > 2 centrosomes
40
30
20
10
0
unt
—
vector
Cdc6
+ HU

(Fig. 2-4). This reduction of HU-induced centrosome amplification by overexpression of Cdc6 agreed to the result that Cdc6 depletion induced centrosome amplification.

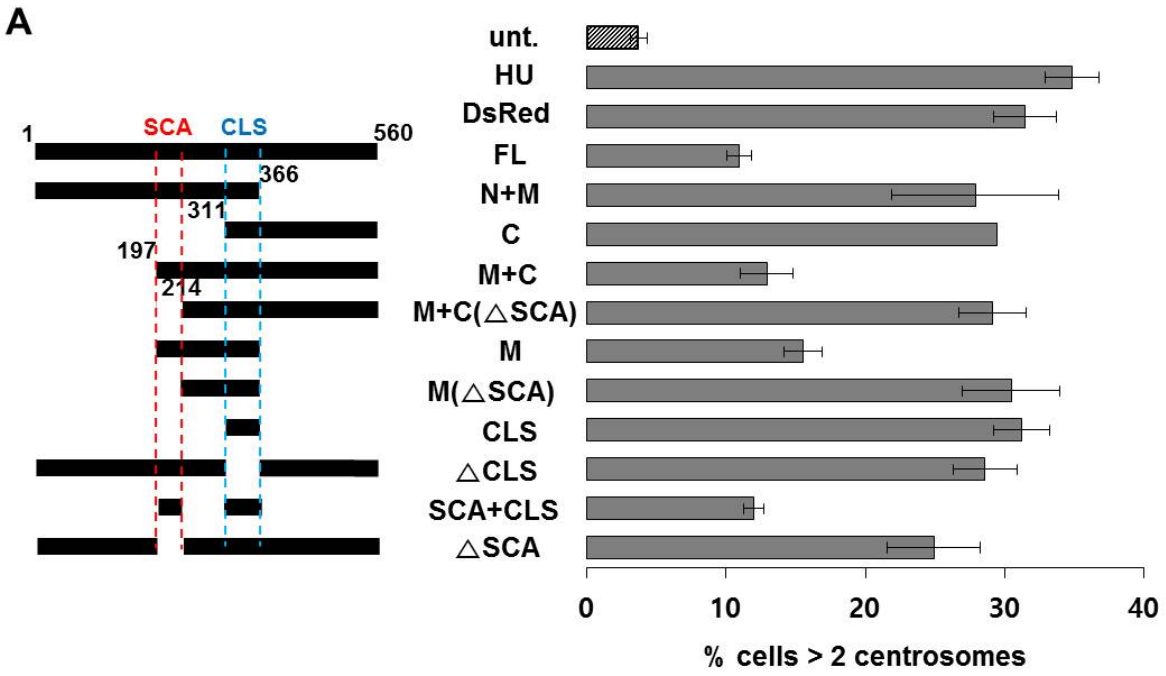
To find a domain responsible for suppressing HU-induced centrosome amplification, I generated Cdc6 serial deletion mutants. Deletions of amino acids residues 197-214 of Cdc6 reduced or abolished the ability of suppressing centrosome amplification, but deletions of other regions did not significantly affected the suppression (Fig. 2-5). Therefore, the amino acid residues 197-214 were suggested to be involved in suppression of centrosome amplification (SCA).

***FHA protein binding motif of Cdc6-SCA is involved in suppression of centrosome amplification***

Cdc6-SCA domain contained a putative Forkhead-Associated (FHA) protein binding motif, S/T-X-X-L, of which S/T, corresponding to Thr-209 of Cdc6, is phosphorylated for binding FHA-containing protein (Mahajan et al., 2008) (Fig. 2-6A). In order to examine a possibility of phosphorylation on Thr-209 of Cdc6, 293T cells were transfected with a vector expressing Cdc6-SCA wild type or phosphor-defective form T209A, containing substitution of Thr-209 with Ala, fused to GST tag and then incubated with $^{32}$P-orthophosphate for *in vivo* labeling (Fig. 2-6B). Whereas GST-pulldown of mock-transfected cells did not show $^{32}$P incorporation, wild-type protein did $^{32}$P incorporation. T209A SCA mutant protein exhibited reduced $^{32}$P incorporation compared to wild-type protein (Fig. 2-6B). To verify whether Thr-209 of Cdc6 was functionaly involved in centrosome amplification, centrosome amplification assay was performed with transfection of a vector expressing wild-type, phosphor-defective T209A, or phosphor-mimetic T209D, containing a substitution of Thr-209 with Asp, Cdc6 protein. Centrosome amplification was induced by Cdc6 depletion (Fig. 2-6C). Ectopically expressed wild-type or phosphor-mimetic T209D proteins inhibited centrosome amplification of Cdc6-depleted cells. On the other hand, phosphor-defective T209A protein could not inhibit centrosome amplification. These results suggested that FHA protein binding motif of Cdc6-SCA, possibly through

**Figure 2-5. Cdc6 contains a region for the suppression of centrosome amplification**

(**A**) Cdc6 deletion constructs used to identify SCA. Numbers represent the positions of amino acid residues. The indicated Cdc6 fragments were fused to the C-terminus of DsRed-monomer-C1 vector. (**B**) Centrosome amplification of U2OS cells transfected with each construct was quantified with at least 50 DsRed-positive cells. WT, wild type; N, N-terminal; M, middle; C, C-terminal; ΔCLS, CLS deletion; ΔSCA, SCA deletion.




**CLS: centrosome localization signal**
**SCA: suppression of centrosome amplification**

**Figure 2-6. Thr-209 of Cdc6 is involved in suppression of centrosome amplification**

(**A**) Cdc6-SCA amino acid sequence contained a putative FHA binding motif. (**B**) *In vivo* kinase assay of HEK293 cells transfected with DNA construct expressing Cdc6-SCA fragment. The $^{32}$P and CBB panel are autoradiogram and coomassie brilliant blue stained gel, respectively. $^{32}$P intensity was quantified with using Image J software. (**C**) U2OS-Tet-On cells inducing FLAG-Cdc6 wild type (WT), T209A, or T209D protein were treated with siRNAs. The ratios of cells containing the indicated number of centrosomes were determined.

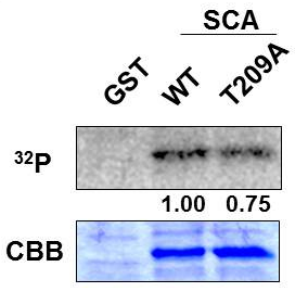
A

197 214

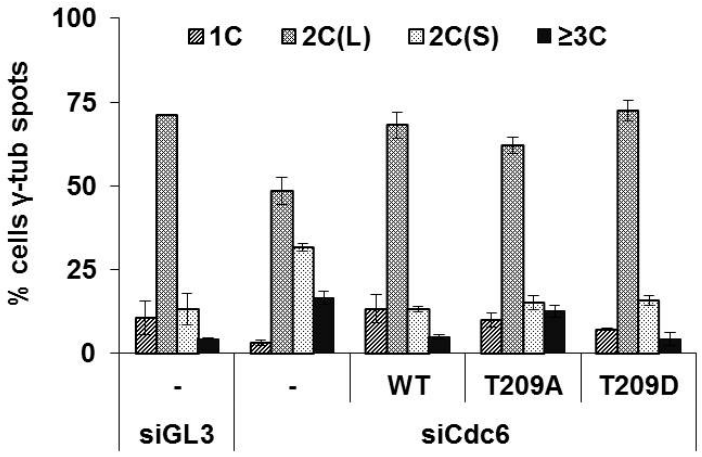
SLYLSGAPGTGKTACLSRILQDLKKELKGFKTIMI

pTXXL : FHA domain binding motif

B




C



phosphorylation on Thr-209, is participated in suppression of centrosome amplification.

***Cdc6 interacts with Grp78 through Cdc6-SCA motif***

To identify an interacting protein with Cdc6-SCA domain, tandem affinity purification was performed using Cdc6-SCA wild-type, T209A and T209D fragments (Fig. 2-7). A 75 kDa protein was identified with binding to wild-type and T209D fragment, but not to T209A (Fig. 2-7A). Mass spectrometric analysis of 75kDa protein revealed that the protein was Grp78 (Fig. 2-7B). Grp78, which is a chaperone protein hsp70 family**,** is involved in ER stress and signal transduction and functions as a chaperone (Li and Li, 2012).

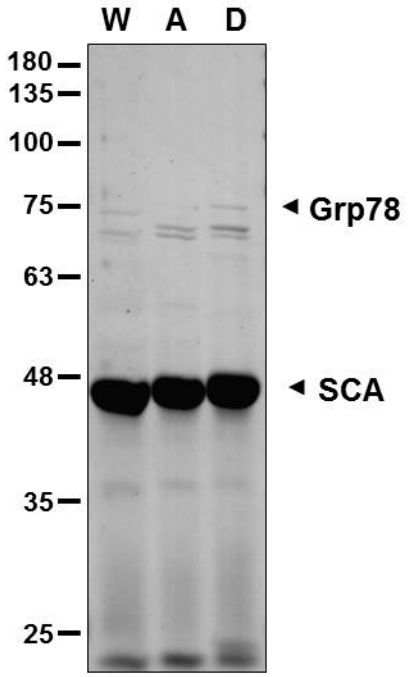
Immunoprecipitation analysis was performed to confirm an interaction between Cdc6 and Grp78. Immunoprecipitation of Cdc6 with monoclonal anti-Cdc6 antibody co-precipitated Grp78 (Fig. 2-8A). Furthermore, co-immunoprecipitation experiment of ectopically expressed FLAG-Cdc6- and Myc-Grp78 was performed with anti-flag and anti-Myc antibodies (Fig. 2-7B, *upper panel*). Reciprocal co-immunoprecipitations of FLAG-Cdc6 and Myc-Grp78 were detected. To investigate whether Cdc6-SCA motif is involved in interaction of Cdc6 with Grp78, I generated a FLAG-tagged SCA motif deletion mutant. Co-immunoprecipitation of the SCA deletion mutant with Grp78 was reduced compared with wild-type (Fig. 2-7B, *lower panel*). Furthermore, phosphor-defective Cdc6-T209A mutant displayed lesser co-immunoprecipitation of Grp78 than wild-type or phosphor-mimetic Cdc6-T209D protein (Fig. 2-7C). These results support that Cdc6 interacts with Grp78 through Cdc6-SCA motif. However, FHA motif did not exist on Grp78, suggesting that a protein containing FHA motif could mediate interaction between Cdc6 and Grp78.

***Grp78 participates in centrosome amplification***

**Figure 2-7. Identification of Cdc6 SCA interacting protein**

(**A**) A Cdc6-SCA interacting protein was identified by tandem-affinity purification of Cdc6-SCA wild type, T209A and T209D mutant proteins as described in **Materials and Methods**. (**B**) Identification of Cdc6-interacting protein was performed with Mass spectrometric analysis. W, wild type SCA; A, T209A-SCA; D, T-209D-SCA.

**A**




**B**

| No. | Protein name | Score |
|---|---|---|
| 1 | 78 kDa glucose-regulated protein precursor [Homo sapiens] | 921 |
| 2 | KIAA0003 [Homo sapiens] | 134 |
| 3 | Cartilage matrix protein, partial [Homo sapiens] | 63 |

**Figure 2-8. Cdc6 interacts with Grp78 through SCA motif**

(**A**) Cdc6 of 293T cells was immunoprecipitated by monoclonal anti-Cdc6 or anti-FLAG antibody and the indicated proteins were detected in immunoblot. Input was with 10% of lysate. (**B**)-(**C**) Indicated DNA constructs were co-transfected into 293T cells and co-immunoprecipitations were determined in immunoblot assay. Co-immunoprecipitated Grp78 was quantified by using Image J softwere (**C**, *lower pannel*). Values represent mean±standard deviation (SD) of three independent experiments.

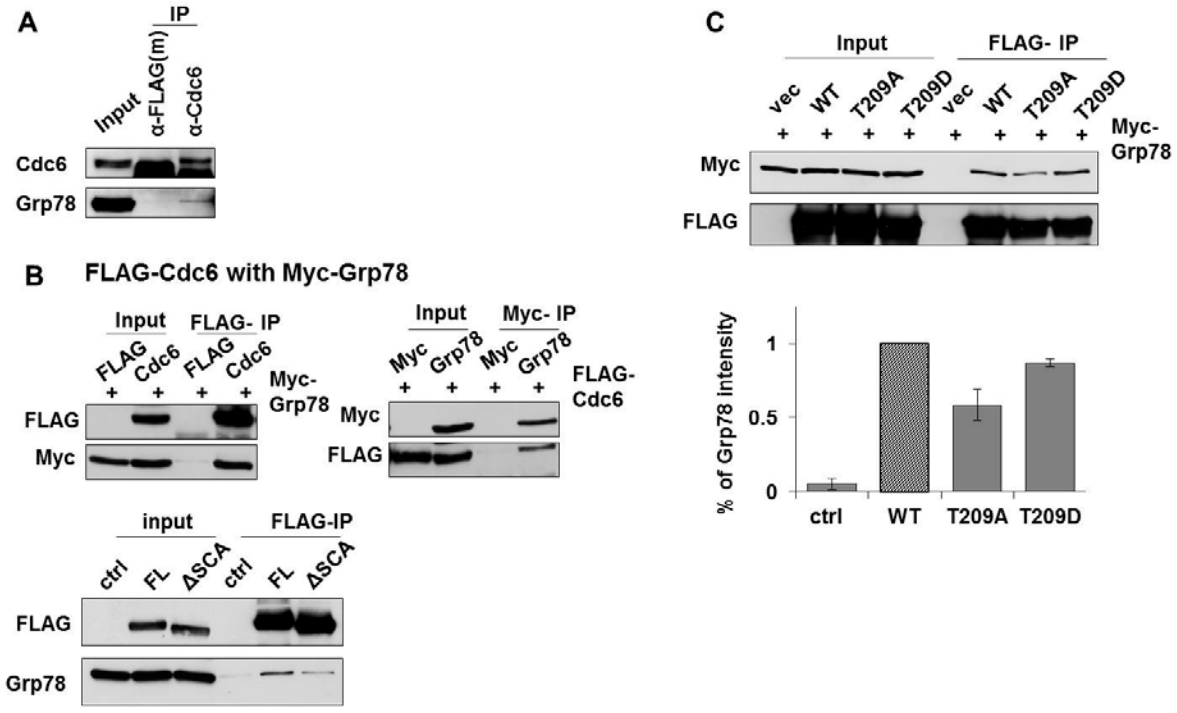
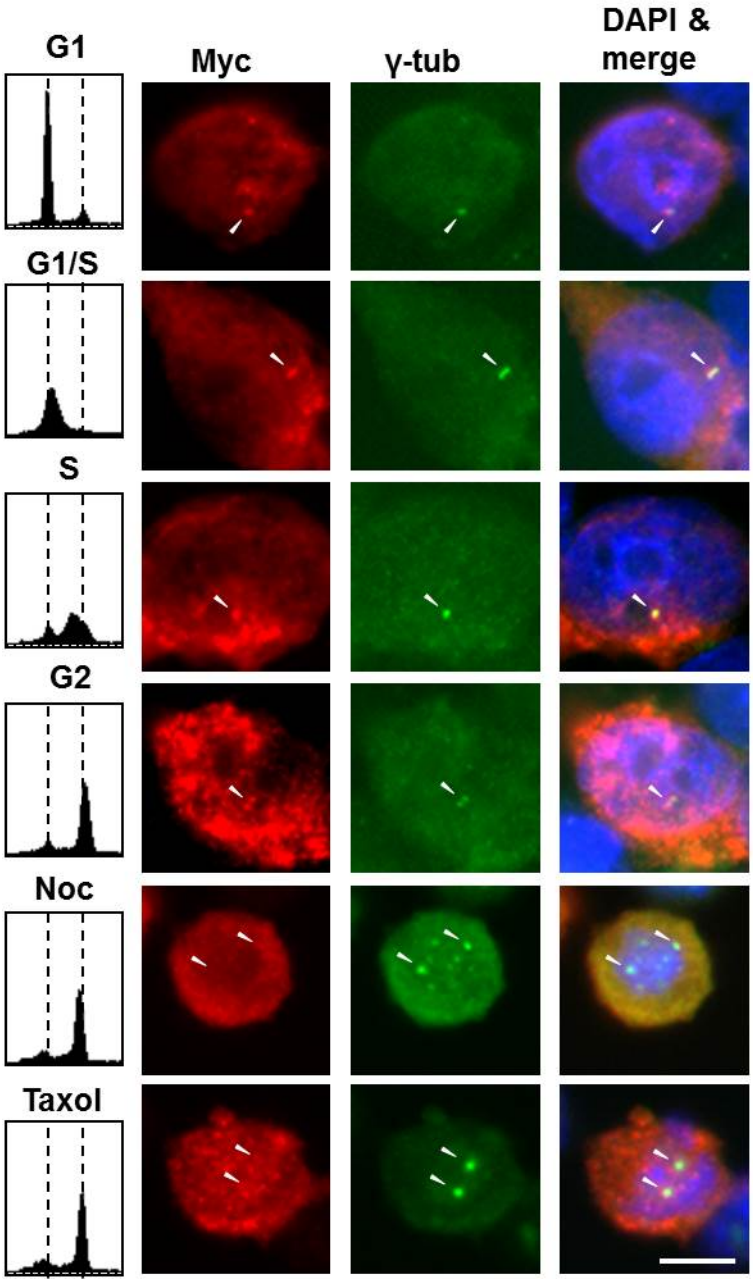

A
IP
Input
α-FLAG(m)
α-Cdc6
Cdc6
Grp78
B
FLAG-Cdc6 with Myc-Grp78
Input
FLAG- IP
FLAG
Cdc6
Myc-
Grp78
Myc
FLAG
Input
Myc- IP
Myc
Grp78
FLAG-
Cdc6
input
FLAG-IP
ctrl
FL
ΔSCA
FLAG
Grp78
C
Input
FLAG- IP
vec
WT
T209A
T209D
Myc-
Grp78
Myc
FLAG
% of Grp78 intensity
1
0.5
0
ctrl
WT
T209A
T209D

**Figure 2-9. Grp78 localizes to interphase centrosomes**

Centrosomal localization of MYC-Grp78 was determined with co-immunostaing of γ-tubulin through cell cycle progression of HeLa cells. Cell cycle profiles determined by FACS analysis were shown at the left-most panel.

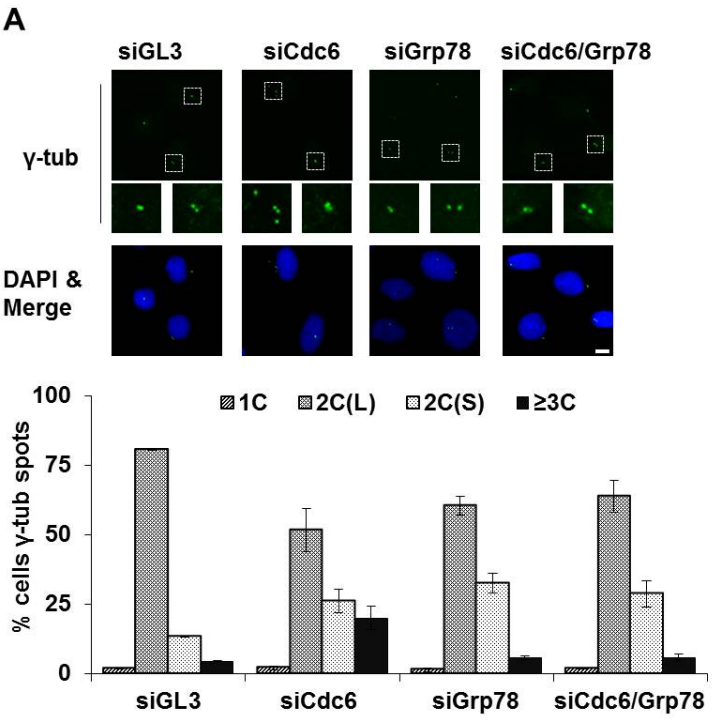

G1
Myc
γ-tub
DAPI &
merge
G1/S
S
G2
Noc
Taxol

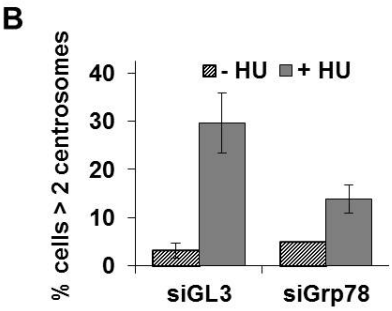
The localization of Grp78 to centrosomes was determined during cell cycle progression (Fig. 2-9A). Ectopically expressed myc-Grp78 in HeLa cells was monitored in each cell cycle phase with co-immunostaing of γ-tubulin, which is a centrosome marker. Cell cycle phases were determined by FACS analysis. Myc-Grp78 was co-localized with γ-tubulin through G1, S, and G2 phase cells. However, the co-localization was not detected in mitotic cells, which was also confirmed with nocodazole or taxol treatment arresting cell cycle progression at prometaphase of mitosis. On the other hand, Cdc6 localized to centrosomes in S and G2 phase (Fig. 1-1C).

Centrosomal function of Grp78 was investigated with regard to centrosome amplification. Grp78 depletion did not significantly affect centrosome amplification, which is similar to control depletion (Fig. 2-10A). However, centrosome amplification induced by Cdc6 depletion decreased upon co-depletion of Grp78. Also, Grp78 depletion decreased HU-induced centrosome amplification (Fig. 2-10B). These reduced centrosome amplification of Cdc6-depleted or HU-treated cells by Grp78 depletion suggested that Grp78 is necessary for centrosome amplification.

**Figure 2-10. Grp78 is required for centrosome amplification by HU or Cdc6 depletion**

(**A**) Immunostaining of γ-tub in U2OS cells transfected with indicated siRNA (*upper panel*) and quantification of cells containing the indicated numbers of γ-tub spots (*lower panel*). Dashed boxes indicate centrosomes with higher magnification. (**B**) Quantification of cells containing centrosome amplification in U2OS cells transfected with indicated siRNA without (-) or with (+) HU during 72 h.


A
siGL3
siCdc6
siGrp78
siCdc6/Grp78
γ-tub
DAPI &
Merge
100
75
50
25
0
% cells γ-tub spots
1C
2C(L)
2C(S)
≥3C
siGL3
siCdc6
siGrp78
siCdc6/Grp78


B
40
30
20
10
0
% cells > 2 centrosomes
- HU
+ HU
siGL3
siGrp78

**Discussions**

Cdc6 depletion for longer than 48 hr not only increased centrosome separation, but also induced centrosome amplification (Fig. 2-1 to 6). Cdc6 depletion-induced centrosome amplification was not caused by centriole fragmentation, suggesting that Cdc6 controls centrosome duplication through inhibiting centrosome amplification. The increases of separation and amplification arose through interphase rather than in a specific cell cycle phase. HU-induced centrosome amplification was inhibited by over-expression of Cdc6, mediated through Cdc6-SCA region containing FHA domain binding motif. These results confirmed that Cdc6 negatively controls centrosome duplication.

Because cell cycle progression is coordinated with centrosome cycle, aberrant cell cycle progression influences centrosome duplication and integrity and *vice versa* (Nigg et al., 2014; Vitre and Cleveland, 2012). Loss of centrosome integrity induces G1 cell cycle arrest depending upon p38-p53-p21 proteins (Mikule et al., 2007). G2 arrest due to DNA damage or defect on certain proteins gives rise to premature separation of centrosomes, which otherwise occurs in prophase of mitotic phase, and centrosome amplification (Dodson et al., 2004). The inactivation of cyclin A/CDK2, a cyclin-dependent kinase, by a cell-cycle checkpoint signaling pathway causes cells to arrest in G2 phase, which in turn leads to dysfunction of controlling centrosome duplication (Prosser et al., 2012). DNA damage-induced centrosome amplification is contributed by fragmentation of centrioles. Although Cdc6 depletion caused cell cycle arrest (Fig. 1-3), the following results argue against the possibility that cell cycle arrest upon Cdc6 depletion induced centrosome amplification. Centrosome amplification arouse through interphase, did not at a specific cell cycle phase. Cdc6-SCA, amino acid residues 197 to 214 of Cdc6, expression as well as Cdc6 overexpression inhibited HU-induced centrosome amplification. Therefore, I suggest that Cdc6 negatively controls centrosome duplication to maintain centrosome integrity.

Some proteins that regulate DNA replication also control centrosome duplication and function. The cell cycle regulators, cyclin E, cyclin A, and CDK2, play critical roles in both DNA replication and centrosome duplication (Tsou and Stearns, 2006). The origin recognition complex (ORC) initiates the assembly of pre-replicative complex onto replication origins. The Orc1 and Orc2 subunits of ORC localize to centrosomes through whole cell cycle and their depletions induce centrosome amplification (Hemerly et al., 2009; Prasanth et al., 2004). Also, depletion of Geminin, which binds to and inhibits Cdt1 that is a factor for the pre-replicative complex, causes centrosome amplification (Lu et al., 2009). Overexpression of Orc1 inhibits HU-induced centrosome amplification (Hemerly et al., 2009). Similarly to Orc1, Cdc6 overexpression suppressed HU-induced centrosome amplification (Fig. 2-4). The amino acids homology between Cdc6 and Orc1 is over 50 % (Kawakami and Katayama, 2010). Whereas Orc1 controls cyclin E/CDK2 activity through an interaction with CDK inhibitory domain (Hossain and Stillman, 2012), Cdc6 did not affect cyclin E/CDK2 activity (data not shown). Therefore, Cdc6 differently controls centrosome duplication from Orc1.

Cdc6 contains a Walker A motif, amino acid residues 202 to 209, for ATP binding and a Walker B motif, amino acid residues 284 to 287, for ATP hydrolysis (Speck et al., 2005). Both activities of the ATP binding and hydrolysis are required for licensing chromosomal replication initiation. Cdc6-SCA inhibited centrosome amplification by HU (Fig. 2-5). Cdc6-SCA contains Walker A motif, but no Walker B motif (Fig. 2-6). The FHA binding motif locates in the Walker A motif and Thr-209-Ala mutation, which abolished inhibition of centrosome amplification and reduced the interaction with Grp78. The Thr-209-Ala mutation may abolish ATP binding. From these results, I consider that inhibition of HU-induced centrosome amplification may require ATP binding, but no ATP hydrolysis activity of Cdc6.

Cdc6-SCA contains the FHA protein binding motif, TXXR, of which Thr needs to be phosphorylated for binding to FHA domain ( (Mahajan et al., 2008), Fig. 2-6). Whereas wild type- and its phosphor-

mimetic mutant Thr209Asp-containing SCAs inhibited centrosome amplification and interacted with Grp78, the phosphor-defective mutant Thr209Asp containing-SCA led to a failure to inhibition and exhibited reduced interaction. However, amino acid sequence analysis of Grp78 revealed no possession of FHA domain in Grp78. Also, Grp78 does not contain a kinase activity (Li and Li, 2012). These results suggested that a protein containing FHA domain may mediate the interaction between Cdc6 and Grp78.

Grp78 is a member of the HSP family of molecular chaperones and plays a central role in regulating the unfolded protein response (UPR) (Wei et al., 2012). Grp78 is required for endoplasmic reticulum integrity and is an obligatory component of stress-induced autophagy in mammalian cells.

FLAG-Grp78 localizes to centrosomes through interphase (Fig. 2-9). Unfortunately, I could not detect centrosome localization of endogenous Grp78 by immunostaining, because anti-Grp78 antibody was not probably suitable for centrosome immunostaining. Either depletion of Cd6 or Grp78 increased centrosome premature separation, but co-depletion did not show a synergetic or antagonistic effect on the separation (Fig. 2-10A). Over-expression of Cdc6 reduced HU-induced centrosome amplification (Fig. 2-4). Cdc6 depletion induced centrosome amplification, but co-depletion of Grp78 interfered with centrosome amplification (Fig. 2-10A). HU-induced centrosome amplification was reduced by Cdc6 over-expression (Fig. 2-4) or Grp78 depletion (Fig. 2-10B). These results suggested that Cdc6 inhibits centrosome amplification and Grp78 is necessary for centrosome amplification.

Through graduate works, I demonstrated novel functions of Cdc6 in centrosomes on the controls of microtubule formation and centrosome duplication. My achievements will contribute to understanding centrosome function and cell cycle control.

## REFERENCES


Alexandrow, M.G., and Hamlin, J.L. (2004). Cdc6 chromatin affinity is unaffected by serine-54 phosphorylation, S-phase progression, and overexpression of cyclin A. Molecular and cellular biology *24*, 1614-1627.

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

Andreu, J.M., Bordas, J., Diaz, J.F., Garcia de Ancos, J., Gil, R., Medrano, F.J., Nogales, E., Pantos, E., and Towns-Andrews, E. (1992). Low resolution structure of microtubules in solution. Synchrotron X-ray scattering and electron microscopy of taxol-induced microtubules assembled from purified tubulin in comparison with glycerol and MAP-induced microtubules. Journal of molecular biology *226*, 169-184.

Cassimeris, L.U., Wadsworth, P., and Salmon, E.D. (1986). Dynamics of microtubule depolymerization in monocytes. The Journal of cell biology *102*, 2023-2032.

Chen, Z., Indjeian, V.B., McManus, M., Wang, L., and Dynlacht, B.D. (2002). CP110, a cell cycle-dependent CDK substrate, regulates centrosome duplication in human cells. Developmental cell *3*, 339-350.

Choi, Y.K., Liu, P., Sze, S.K., Dai, C., and Qi, R.Z. (2010). CDK5RAP2 stimulates microtubule nucleation by the gamma-tubulin ring complex. The Journal of cell biology *191*, 1089-1095.

Clijsters, L., Ogink, J., and Wolthuis, R. (2013). The spindle checkpoint, APC/C(Cdc20), and APC/C(Cdh1) play distinct roles in connecting mitosis to S phase. The Journal of cell biology *201*, 1013-1026.

Costa, A., Hood, I.V., and Berger, J.M. (2013). Mechanisms for initiating cellular DNA replication. Annual review of biochemistry *82*, 25-54.

D'Assoro, A.B., Busby, R., Suino, K., Delva, E., Almodovar-Mercado, G.J., Johnson, H., Folk, C., Farrugia, D.J., Vasile, V., Stivala, F., *et al.* (2004). Genotoxic stress leads to centrosome amplification in breast cancer cell lines that have an inactive G1/S cell cycle checkpoint. Oncogene *23*, 4068-4075.

Delmolino, L.M., Saha, P., and Dutta, A. (2001). Multiple mechanisms regulate subcellular localization of human CDC6. The Journal of biological chemistry *276*, 26947-26954.

Desai, A., Verma, S., Mitchison, T.J., and Walczak, C.E. (1999). Kin I kinesins are microtubule-destabilizing enzymes. Cell *96*, 69-78.

Dodson, H., Bourke, E., Jeffers, L.J., Vagnarelli, P., Sonoda, E., Takeda, S., Earnshaw, W.C., Merdes, A., and Morrison, C. (2004). Centrosome amplification induced by DNA damage occurs during a prolonged G2 phase and involves ATM. The EMBO journal *23*, 3864-3873.

Doxsey, S., Zimmerman, W., and Mikule, K. (2005). Centrosome control of the cell cycle. Trends in cell biology *15*, 303-311.

Endoh-Yamagami, S., Karkar, K.M., May, S.R., Cobos, I., Thwin, M.T., Long, J.E., Ashique, A.M., Zarbalis, K., Rubenstein, J.L., and Peterson, A.S. (2010). A mutation in the pericentrin gene causes abnormal interneuron migration to the olfactory bulb in mice. Developmental biology *340*, 41-53.

Fabbro, M., and Henderson, B.R. (2003). Regulation of tumor suppressors by nuclear-cytoplasmic shuttling. Experimental cell research *282*, 59-69.

Ferguson, R.L., and Maller, J.L. (2008). Cyclin E-dependent localization of MCM5 regulates centrosome duplication. Journal of cell science *121*, 3224-3232.

Ferguson, R.L., Pascreau, G., and Maller, J.L. (2010). The cyclin A centrosomal localization sequence recruits MCM5 and Orc1 to regulate centrosome reduplication. Journal of cell science *123*, 2743-2749.

Fernandez-Cid, A., Riera, A., Tognetti, S., Herrera, M.C., Samel, S., Evrin, C., Winkler, C., Gardenal,

E., Uhle, S., and Speck, C. (2013). An ORC/Cdc6/MCM2-7 complex is formed in a multistep reaction to serve as a platform for MCM double-hexamer assembly. Molecular cell *50*, 577-588.

Franker, M.A., and Hoogenraad, C.C. (2013). Microtubule-based transport - basic mechanisms, traffic rules and role in neurological pathogenesis. Journal of cell science *126*, 2319-2329.
Ganguly, A., Yang, H., Sharma, R., Patel, K.D., and Cabral, F. (2012). The role of microtubules and their dynamics in cell migration. The Journal of biological chemistry *287*, 43359-43369.

Gillingham, A.K., and Munro, S. (2000). The PACT domain, a conserved centrosomal targeting motif in the coiled-coil proteins AKAP450 and pericentrin. EMBO reports *1*, 524-529.

Han, X., Saito, H., Miki, Y., and Nakanishi, A. (2008). A CRM1-mediated nuclear export signal governs cytoplasmic localization of BRCA2 and is essential for centrosomal localization of BRCA2. Oncogene *27*, 2969-2977.

Hanashiro, K., Kanai, M., Geng, Y., Sicinski, P., and Fukasawa, K. (2008). Roles of cyclins A and E in induction of centrosome amplification in p53-compromised cells. Oncogene *27*, 5288-5302.

He, G., Siddik, Z.H., Huang, Z., Wang, R., Koomen, J., Kobayashi, R., Khokhar, A.R., and Kuang, J. (2005). Induction of p21 by p53 following DNA damage inhibits both Cdk4 and Cdk2 activities. Oncogene *24*, 2929-2943.

Hemerly, A.S., Prasanth, S.G., Siddiqui, K., and Stillman, B. (2009). Orc1 controls centriole and centrosome copy number in human cells. Science (New York, N.Y.) *323*, 789-793.

Hook, S.S., Lin, J.J., and Dutta, A. (2007). Mechanisms to control rereplication and implications for cancer. Current opinion in cell biology *19*, 663-671.

Hossain, M., and Stillman, B. (2012). Meier-Gorlin syndrome mutations disrupt an Orc1 CDK inhibitory domain and cause centrosome reduplication. Genes Dev *26*, 1797-1810.

Hulspas, R., Houtsmuller, A.B., Bauman, J.G., and Nanninga, N. (1994). The centrosome moves out

of a nuclear indentation in human lymphocytes upon activation. Experimental cell research *215*, 28-32.

Hunter, A.W., Caplow, M., Coy, D.L., Hancock, W.O., Diez, S., Wordeman, L., and Howard, J. (2003). The kinesin-related protein MCAK is a microtubule depolymerase that forms an ATP-hydrolyzing complex at microtubule ends. Molecular cell *11*, 445-457.

Hut, H.M., Lemstra, W., Blaauw, E.H., Van Cappellen, G.W., Kampinga, H.H., and Sibon, O.C. (2003). Centrosomes split in the presence of impaired DNA integrity during mitosis. Molecular biology of the cell *14*, 1993-2004.

Ibi, M., Zou, P., Inoko, A., Shiromizu, T., Matsuyama, M., Hayashi, Y., Enomoto, M., Mori, D., Hirotsune, S., Kiyono, T., *et al.* (2011). Trichoplein controls microtubule anchoring at the centrosome by binding to Odf2 and ninein. Journal of cell science *124*, 857-864.

Janke, C., and Bulinski, J.C. (2011). Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions. Nature reviews. Molecular cell biology *12*, 773-786.

Jiang, W., Wells, N.J., and Hunter, T. (1999). Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. Proceedings of the National Academy of Sciences of the United States of America *96*, 6193-6198.

Kawakami, H., and Katayama, T. (2010). DnaA, ORC, and Cdc6: similarity beyond the domains of life and diversity. Biochemistry and cell biology = Biochimie et biologie cellulaire *88*, 49-62.

Kim, K., and Rhee, K. (2011). The pericentriolar satellite protein CEP90 is crucial for integrity of the mitotic spindle pole. Journal of cell science *124*, 338-347.

Kim, S., and Rhee, K. (2014). Importance of the CEP215-pericentrin interaction for centrosome maturation during mitosis. PloS one *9*, e87016.

Kohlmaier, G., Loncarek, J., Meng, X., McEwen, B.F., Mogensen, M.M., Spektor, A., Dynlacht, B.D., Khodjakov, A., and Gonczy, P. (2009). Overly long centrioles and defective cell division upon excess

of the SAS-4-related protein CPAP. Current biology : CB *19*, 1012-1018.

Kwon, M., Godinho, S.A., Chandhok, N.S., Ganem, N.J., Azioune, A., Thery, M., and Pellman, D. (2008). Mechanisms to suppress multipolar divisions in cancer cells with extra centrosomes. Genes Dev *22*, 2189-2203.

Lacey, K.R., Jackson, P.K., and Stearns, T. (1999). Cyclin-dependent kinase control of centrosome duplication. Proceedings of the National Academy of Sciences of the United States of America *96*, 2817-2822.

Lafarga, V., Aymerich, I., Tapia, O., Mayor, F., Jr., and Penela, P. (2012). A novel GRK2/HDAC6 interaction modulates cell spreading and motility. The EMBO journal *31*, 856-869.

Lee, K., and Rhee, K. (2011). PLK1 phosphorylation of pericentrin initiates centrosome maturation at the onset of mitosis. The Journal of cell biology *195*, 1093-1101.

Lee, S., and Rhee, K. (2010). CEP215 is involved in the dynein-dependent accumulation of pericentriolar matrix proteins for spindle pole formation. Cell cycle *9*, 774-783.

Li, J., and Lee, A.S. (2006). Stress induction of GRP78/BiP and its role in cancer. Current molecular medicine *6*, 45-54.

Li, Z., and Li, Z. (2012). Glucose regulated protein 78: a critical link between tumor microenvironment and cancer hallmarks. Biochimica et biophysica acta *1826*, 13-22.

Loffler, H., Fechter, A., Liu, F.Y., Poppelreuther, S., and Kramer, A. (2013). DNA damage-induced centrosome amplification occurs via excessive formation of centriolar satellites. Oncogene *32*, 2963-2972.

Lu, F., Lan, R., Zhang, H., Jiang, Q., and Zhang, C. (2009). Geminin is partially localized to the centrosome and plays a role in proper centrosome duplication. Biology of the cell / under the auspices of the European Cell Biology Organization *101*, 273-285.

Luders, J., and Stearns, T. (2007). Microtubule-organizing centres: a re-evaluation. Nature reviews. Molecular cell biology *8*, 161-167.

Mahajan, A., Yuan, C., Lee, H., Chen, E.S., Wu, P.Y., and Tsai, M.D. (2008). Structure and function of the phosphothreonine-specific FHA domain. Science signaling *1*, re12.

Manchado, E., Guillamot, M., and Malumbres, M. (2012). Killing cells by targeting mitosis. Cell death and differentiation *19*, 369-377.

Mardin, B.R., and Schiebel, E. (2012). Breaking the ties that bind: new advances in centrosome biology. The Journal of cell biology *197*, 11-18.

Marthiens, V., Piel, M., and Basto, R. (2012). Never tear us apart--the importance of centrosome clustering. Journal of cell science *125*, 3281-3292.

Maruta, H., Greer, K., and Rosenbaum, J.L. (1986). The acetylation of alpha-tubulin and its relationship to the assembly and disassembly of microtubules. The Journal of cell biology *103*, 571-579.

Matsumoto, Y., Hayashi, K., and Nishida, E. (1999). Cyclin-dependent kinase 2 (Cdk2) is required for centrosome duplication in mammalian cells. Current biology : CB *9*, 429-432.

Mikule, K., Delaval, B., Kaldis, P., Jurcyzk, A., Hergert, P., and Doxsey, S. (2007). Loss of centrosome integrity induces p38-p53-p21-dependent G1-S arrest. Nature cell biology *9*, 160-170.

Mogensen, M.M., Malik, A., Piel, M., Bouckson-Castaing, V., and Bornens, M. (2000). Microtubule minus-end anchorage at centrosomal and non-centrosomal sites: the role of ninein. Journal of cell science *113 ( Pt 17)*, 3013-3023.

Moore, J.D. (2013). In the wrong place at the wrong time: does cyclin mislocalization drive oncogenic transformation? Nature reviews. Cancer *13*, 201-208.

Moritz, M., Braunfeld, M.B., Sedat, J.W., Alberts, B., and Agard, D.A. (1995). Microtubule nucleation by gamma-tubulin-containing rings in the centrosome. Nature *378*, 638-640.

Morrison, E.E., Wardleworth, B.N., Askham, J.M., Markham, A.F., and Meredith, D.M. (1998). EB1, a protein which interacts with the APC tumour suppressor, is associated with the microtubule cytoskeleton throughout the cell cycle. Oncogene *17*, 3471-3477.

Nam, H.J., and van Deursen, J.M. (2014). Cyclin B2 and p53 control proper timing of centrosome separation. Nature cell biology *16*, 538-549.

Narasimhachar, Y., Webster, D.R., Gard, D.L., and Coue, M. (2012). Cdc6 is required for meiotic spindle assembly in Xenopus oocytes. Cell cycle *11*, 524-531.

Nigg, E.A., Cajanek, L., and Arquint, C. (2014). The centrosome duplication cycle in health and disease. FEBS letters *588*, 2366-2372.
O'Rourke, B.P., Gomez-Ferreria, M.A., Berk, R.H., Hackl, A.M., Nicholas, M.P., O'Rourke, S.C., Pelletier, L., and Sharp, D.J. (2014). Cep192 controls the balance of centrosome and non-centrosomal microtubules during interphase. PloS one *9*, e101001.

Paolinelli, R., Mendoza-Maldonado, R., Cereseto, A., and Giacca, M. (2009). Acetylation by GCN5 regulates CDC6 phosphorylation in the S phase of the cell cycle. Nature structural & molecular biology *16*, 412-420.

Petersen, B.O. (2000). Cell cycle- and cell growth-regulated proteolysis of mammalian CDC6 is dependent on APC-CDH1. Genes & Development *14*, 2330-2343.

Petersen, B.O., Lukas, J., Sorensen, C.S., Bartek, J., and Helin, K. (1999). Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. The EMBO journal *18*, 396-410.

Prasanth, S.G., Prasanth, K.V., Siddiqui, K., Spector, D.L., and Stillman, B. (2004). Human Orc2

localizes to centrosomes, centromeres and heterochromatin during chromosome inheritance. The EMBO journal *23*, 2651-2663.

Prosser, S.L., Samant, M.D., Baxter, J.E., Morrison, C.G., and Fry, A.M. (2012). Oscillation of APC/C activity during cell cycle arrest promotes centrosome amplification. Journal of cell science *125*, 5353-5368.

Randell, J.C., Bowers, J.L., Rodriguez, H.K., and Bell, S.P. (2006). Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase. Molecular cell *21*, 29-39.

Rhee, S., Jiang, H., Ho, C.H., and Grinnell, F. (2007). Microtubule function in fibroblast spreading is modulated according to the tension state of cell-matrix interactions. Proceedings of the National Academy of Sciences of the United States of America *104*, 5425-5430.

Saha, P., Chen, J., Thome, K.C., Lawlis, S.J., Hou, Z.H., Hendricks, M., Parvin, J.D., and Dutta, A. (1998). Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase. Molecular and cellular biology *18*, 2758-2767.

Schmidt, T.I., Kleylein-Sohn, J., Westendorf, J., Le Clech, M., Lavoie, S.B., Stierhof, Y.D., and Nigg, E.A. (2009). Control of centriole length by CPAP and CP110. Current biology : CB *19*, 1005-1011.
Siegrist, S.E., and Doe, C.Q. (2007). Microtubule-induced cortical cell polarity. Genes Dev *21*, 483-496.

Singla, V., Romaguera-Ros, M., Garcia-Verdugo, J.M., and Reiter, J.F. (2010). Ofd1, a human disease gene, regulates the length and distal structure of centrioles. Developmental cell *18*, 410-424.

Speck, C., Chen, Z., Li, H., and Stillman, B. (2005). ATPase-dependent cooperative binding of ORC and Cdc6 to origin DNA. Nature structural & molecular biology *12*, 965-971.

Starita, L.M., Machida, Y., Sankaran, S., Elias, J.E., Griffin, K., Schlegel, B.P., Gygi, S.P., and Parvin, J.D. (2004). BRCA1-dependent ubiquitination of gamma-tubulin regulates centrosome number. Molecular and cellular biology *24*, 8457-8466.

Stearns, T., Evans, L., and Kirschner, M. (1991). Gamma-tubulin is a highly conserved component of the centrosome. Cell *65*, 825-836.

Stuermer, A., Hoehn, K., Faul, T., Auth, T., Brand, N., Kneissl, M., Putter, V., and Grummt, F. (2007). Mouse pre-replicative complex proteins colocalise and interact with the centrosome. European journal of cell biology *86*, 37-50.

Takitoh, T., Kumamoto, K., Wang, C.C., Sato, M., Toba, S., Wynshaw-Boris, A., and Hirotsune, S. (2012). Activation of Aurora-A is essential for neuronal migration via modulation of microtubule organization. The Journal of neuroscience : the official journal of the Society for Neuroscience *32*, 11050-11066.

Tanaka, T.U., and Desai, A. (2008). Kinetochore-microtubule interactions: the means to the end. Current opinion in cell biology *20*, 53-63.

Tanenbaum, M.E., and Medema, R.H. (2010). Mechanisms of centrosome separation and bipolar spindle assembly. Developmental cell *19*, 797-806.

Tarapore, P., and Fukasawa, K. (2002). Loss of p53 and centrosome hyperamplification. Oncogene *21*, 6234-6240.

Teixido-Travesa, N., Roig, J., and Luders, J. (2012). The where, when and how of microtubule nucleation - one ring to rule them all. Journal of cell science *125*, 4445-4456.

Thompson, M.E., Robinson-Benion, C.L., and Holt, J.T. (2005). An amino-terminal motif functions as a second nuclear export sequence in BRCA1. The Journal of biological chemistry *280*, 21854-21857.

Tsou, M.F., and Stearns, T. (2006). Mechanism limiting centrosome duplication to once per cell cycle. Nature *442*, 947-951.

Vitre, B.D., and Cleveland, D.W. (2012). Centrosomes, chromosome instability (CIN) and aneuploidy.

Current opinion in cell biology *24*, 809-815.

Wang, G., Jiang, Q., and Zhang, C. (2014). The role of mitotic kinases in coupling the centrosome cycle with the assembly of the mitotic spindle. Journal of cell science.

Watanabe, T., Noritake, J., and Kaibuchi, K. (2005). Regulation of microtubules in cell migration. Trends in cell biology *15*, 76-83.

Wei, P.C., Hsieh, Y.H., Su, M.I., Jiang, X., Hsu, P.H., Lo, W.T., Weng, J.Y., Jeng, Y.M., Wang, J.M., Chen, P.L., *et al.* (2012). Loss of the oxidative stress sensor NPGPx compromises GRP78 chaperone activity and induces systemic disease. Molecular cell *48*, 747-759.

Yardimci, H., and Walter, J.C. (2014). Prereplication-complex formation: a molecular double take? Nature structural & molecular biology *21*, 20-25.

Yim, H., and Erikson, R.L. (2010). Cell division cycle 6, a mitotic substrate of polo-like kinase 1, regulates chromosomal segregation mediated by cyclin-dependent kinase 1 and separase. Proceedings of the National Academy of Sciences of the United States of America *107*, 19742-19747.

Zhang, D., Rogers, G.C., Buster, D.W., and Sharp, D.J. (2007). Three microtubule severing enzymes contribute to the "Pacman-flux" machinery that moves chromosomes. The Journal of cell biology *177*, 231-242.

# 국문 초록

중심체는 세포 내에서 미세소관 형성 중심부위로 작용한다. 미세소관은 염색체의 분리, 세포형태와 이동, 세포 내 물질 수송 및 신호전달에 관여한다. Cdc6 단백질은 복제 개시 중합체의 구성요소로서, 염색체 복제를 시작하게 하며 염색체의 안정성을 유지하는데 기능이 있다. 이에 Cdc6 가 중심체에서 미세소관 형성을 조절하는 새로운 기능을 밝혔다. Cdc6 는 보존된 중심체 위치지정 부위에 의해 S-, G2 기의 중심체에 위치한다. Cdc6 결핍에 의해 중심체 구성 단백질이고, γ-tubulin ring complex 의 중심체로 위치하게 하며 미세소관 형성을 유도하는 Cep215/CDK5RAP2 과 Cep192 단백질의 중심체로의 유도가 증가하는 것을 확인하였다. 이는 Cdc6 에 의해 중심체 구성 단백질의 유도가 조절 되며, γ-TuRC 의 유도 또한 조절된다는 것을 의미한다. 나아가 Cdc6 결핍은 세포 부착성 및 세포 확장성을 증가하고, Cdc6 의 과량발현은 반대 작용을 한다. 이러한 결과를 바탕으로 Cdc6 는 세포주기에 따라 미세소관 형성을 조절 하는 기능을 지니고 있음을 알 수 있다.

중심체는 세포주기에 따라 복제되며, 염색체 분리에 중요한 작용을 하여, 염색체 안정성과 밀접한 연관이 있다. 이에 Cdc6 는 중심체 과량형성을 음성적으로 조절하며, Grp78 은 중심체 과량형성에 필요하였다. Cdc6 결핍은 중심체 과량형성을 유도하고, Cdc6 의 과량발현은 hydroxyurea (HU)에 의한 중심체 과량형성을 억제하였다. Cdc6 결핍에 의한 중심체 과량형성은 중심립 절편화에 의한 것이 아닌 중심체 과다복제에 의함이다. Cdc6 의 아미노산 서열 197-214 는 중심체 과량형성을 억제하는데 필요하며 이 부위를 SCA (suppression of centrosome amplification: 중심체 과량형성 억제 부위)로

명명하였다. Cdc6-SCA 는 FHA 단백질과 결합할 수 있는 단백질 서열을 지니고 있으며, Grp78 과 결합하였다. Grp78 은 간기의 중심체에 위치하며, Cdc6 결핍과 HU 에 의한 중심체 과량형성에 필요한 것으로 나타났다. 따라서 Cdc6 와 Grp78 의 결합은 중심체 복제 조절에 관여한다는 것을 의미한다.

중심어: Cdc6, 미세소관, 중심체, $\gamma$-TuRC, 세포 확장, 세포 주기, Grp78,

학번: 2008-22742