이학박사학위논문

중심체 단백질인 CEP215 의 세포주기에 따른 기능 연구

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지도교수 이 건 수

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ABSTRACT

Studies on Biological Roles of CEP215, a Centrosome Component, during the Cell Cycle

Seongjae Kim

In most animal cells, the centrosome functions as a major microtubule organizing center and controls cellular morphology, migration, subcellular transport and cell division. Understanding the functional mechanisms of this mysterious and interesting organelle at the molecular level has been a great topic in molecular and cellular biology. Meanwhile, proteomic analysis revealed that human centrosome is composed of several hundred kinds of proteins. The composition of the centrosome changes dynamically during the cell cycle. Among the centrosomal proteins, I focused on the functional mechanisms of CEP215, an important pericentriolar material component, for microtubule nucleation and centrosome maturation during interphase and mitosis,

respectively.

In chapter 1, I investigated the knockdown phenotypes of CEP215 during interphase. It has been reported that CEP215 is involved in several centrosome behaviors such as centrosome cohesion, microtubule nucleation and centrosome maturation. However, the precise mechanisms of these functions have not been thoroughly explored. Thus, I focused on biological roles of CEP215 for microtubule nucleation in interphase. The results revealed that the physical interaction of CEP215 with γ-tubulin is essential for the microtubule nucleation in interphase cells.

In chapter 2, I investigated roles of CEP215 in mitotic cells. From chapter 1, I found that CEP215 physically interacts with γ-tubulin in interphase cells. Here, I hypothesized that the interaction of CEP215 with γ-tubulin also contributes to the centrosome maturation during mitosis. It was unexpected that the physical interaction of CEP215 with γ-tubulin is not necessary for the centrosome maturation. Rather, physical interaction of CEP215 with pericentrin is critical for the centrosome maturation and subsequent proper bipolar spindle formation during mitosis. The present results provide an insight into how pericentriolar material components are assembled to form a spindle pole during mitosis.

Key Words: Microtubule nucleation, Centrosome maturation, CEP215, γ-tubulin, Pericentrin, Protein-protein interaction

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AND

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Background

1. Centrosome

Centrosome is a small organelle which functions as microtubule organizing center (MTOC) in most animal cells. The importance of this organelle was realized at the end of the 19th century by the zoologist Theodor Boveri. He observed the abnormal presence of four centrosomes in a fertilized egg leads to an unequal distribution of chromosomes in the daughter cells. It allows for the interpretation of the interplay of centrosomes, spindle and chromosomes during cell division (Satzinger, 2008). From these observations, centrosome has been considered as an especial organ of cell division. So far, later researcher revealed that this organelle functions in many subcellular processes such as cell shape, cell migration, microtubule network, cell cycle progression and mitotic spindle formation.

1.1. Centrosome Structure

The centrosome is comprised of two centrioles that are surrounded by an electron-dense matrix, the pericentriolar material (PCM) (Figure. 1)

(Bettencourt-Dias and Glover, 2007). The two centrioles are orthogonally linked to each other, and the older one named as mother centriole specially has distal/subdistal appendage structures which dock cytoplasmic MTs and might anchor centrioles to the cell membrane where they serve as basal bodies (Bettencourt-Dias and Glover, 2007). The pericentriolar material (PCM), the microtubule-organizing component of the centrosome, contains a multitude of proteins and is commonly described as an amorphous cloud surrounding the centrioles (Luders, 2012).

1.2. Centrosome Functions

The centrosome is the primary microtubule-organizing centre (MTOC) in animal cells, and so it regulates cell motility, adhesion and polarity in interphase. During mitosis, the centrosome has a crucial role as mitotic spindle poles which robustly emanate the bipolar spindles for the accurate segregation of duplicated chromosomes to each daughter cell (Bettencourt-Dias and Glover, 2007). Abnormalities in the spindle-pole-organization function occur in many cancers and can be associated with genomic instability because extra and often irregular centrosomes can give rise to aberrant cell division (Bettencourt-Dias and Glover, 2007). Moreover, the mother centriole, also referred as basal body, has another distinct function as a seed for the growth of cilia and flagella, which

have crucial roles in physiology, development and disease (Bettencourt-Dias and Glover, 2007) (Figure 2).

1.3. Centrosome Cycle

The centrosome cycle is tightly linked with the cell cycle (Figure 3). The centrosome is strictly duplicated once per cell cycle, as DNA replication (Nigg, 2006). In cycling cells, exactly one new centriole forms adjacent to each preexisting centriole, reminiscent of the replication of DNA (Nigg and Stearns, 2011). There are four consecutive steps have been described: (1) centriole disengagement, (2) cartwheel structure formation of the procentrioles, (3) elongation of the procentrioles and (4) separation of the duplicated centrosomes (Bettencourt-Dias and Glover, 2007) (Figure 3). In the step of centriole disengagement, at mitotic exit–early G1 phase, mother and daughter centrioles in a centrosome loose their orthogonal configuration. Disengagement requires the action of PLK1 as well as separase, the protease responsible for sister chromatid separation at the metaphase-to-anaphase transition. There might be an intercentriole link established with C-Nap1, rootletin as well as CEP68 at this stage. Next, centriole duplication starts in late G1-S phase with the formation of cartwheel structure composed of hSAS-6, a basal portion of procentrioles. The procentrioles elongate fully by late G2 phase to mitosis. In

this elongation step depends on several proteins such as CEP135, CPAP, hPOC5 and CP110. Last, maturation and separation of the two centrosomes occur at the G2-M transition by the acquisition of maturation markers, the recruitment of pericentriolar material and an increase in microtubule-organizing center (MTOC) activity. The mitotic kinase NEK2 and kinesin motor protein Eg5 are critical for the centrosome separation during G2-M transition. The centrosome maturation represented with robust recruitment of γ-TuRC complex is accomplished with various PCM components such as pericentrin, CEP215, CEP192 and NEDD1 as well as mitotic kinases such as PLK1 and Aurora A (Bettencourt-Dias and Glover, 2007; Nigg and Stearns, 2011).

2. Microtubule Nucleation

Microtubules are hollow cylindrical polymers that are assembled from heterodimers composed of α- and β-tubulin (Teixido-Travesa et al., 2012). The longitudinal orientation of the tubulin dimmers provides microtubules with an intrinsic polarity, with α-tubulin facing the minus end and β-tubulin facing the plus end (Teixido-Travesa et al., 2012). In vivo the minus end is relatively stable, whereas the plus end is highly dynamic (Jiang and Akhmanova, 2011).

Microtubule nucleation is typically spatially restricted to microtubule-organizing centers (MTOCs) (Luders and Stearns, 2007). The main MTOC in animal cells is the centrosome, a small spherical structure that comprises a central pair of centrioles surrounded by the pericentriolar material (PCM) (Azimzadeh and Bornens, 2007; Bornens, 2012).

2.1. γ-Tubulin Ring Complex (γ-TuRC)

The pericentriolar material is the protein mass that is responsible for MT nucleation (Gould and Borisy, 1977). In agreement with its microtubule nucleation activity, it has been reported that γ-tubulin localized to the pericentriolar region of mammalian centrosome (Stearns et al., 1991). γ-Tubulin is a universal component of MTOCs involved in MT nucleation, suggests a molecular basis for this common MT nucleation activity of MTOCs (Horio et al., 1991; Liu et al., 1994; Lopez et al., 1995; Marschall et al., 1996; Oakley et al., 1990; Spang et al., 1996; Stearns et al., 1991; Zheng et al., 1991). In *Drosophila*, *Xenopus* and humans γ-tubulin assembles into γ-TuRCs, which are the main cellular microtubule nucleators (Moritz et al., 1995; Murphy et al., 2001; Oegema et al., 1999; Zheng et al., 1995). In addition to nucleation, these complexes have also been implicated in microtubule stabilization by capping the minus ends (Anders and Sawin, 2011; Wiese and Zheng, 2000) and in the

modulation of microtubule-plus-end dynamics (Bouissou et al., 2009).

Previous work has suggested that, apart from γ-tubulin, all γ-TuRC core subunits, termed γ-tubulin complex proteins (GCPs), belong to a conserved protein family (Gunawardane et al., 2000; Murphy et al., 2001). Highly conserved sequences in GCPs 2-6 were initially described as γ-tubulin ring protein (Grip) motifs. γ-TuRCs are formed by the helical arrangement of smaller Y-shaped subcomplexes, the so-called γ-tubulin small complexes (γ-TuSCs), which are composed of two molecules of γ-tubulin and one molecule each of GCP2 and GCP3. Recent work has suggested that GCP4, GCP5 and GCP6 might be part of the γ-TuRC ring structure by substituting for GCP2 or GCP3 at specific positions to function as ring assembly initiators or terminators (Figure 4) (Guillet et al., 2011; Kollman et al., 2011).

2.2. Regulation of the γTuRC

Several γ-TuRC-associated proteins have been implicated in γ-TuRC regulation, frequently by mediating subcellular targeting of the complex to specific MTOCs (Teixido-Travesa et al., 2012). It has been proposed that several centrosomal proteins, including pericentrin (Zimmerman et al., 2004), AKAP450 (Takahashi et al., 2002) and CEP215 (Fong et al., 2008) recruit γ-TuRC to centrosomes. In human cells, the γ-TuRC subunit GCP-WD is the

attachment factor that lies most proximal to the γ-TuRC. GCP-WD is indispensible for the centrosomal localization of γ-tubulin in interphase and mitosis (Haren et al., 2006; Luders et al., 2006).

During mitosis, the γ-TuRC is also targeted to spindle microtubules, and expression of a GCP-WD mutant that specifically disrupts targeting of γ-TuRC to mitotic spindles, but not to centrosomes, interferes with proper spindle assembly and reduces microtubule density in the spindle (Luders et al., 2006). The identification of augmin, a multi-subunit protein complex that recruits γ-TuRC to spindle microtubules through the adaptor GCP-WD, has provided important molecular insight into this pathway (Goshima and Kimura, 2010).

Another non-centrosomal MTOC is the Golgi complex. Interestingly, AKAP45 and CEP215, which have both been described as γ-TuRC-tethering factors at the centrosome, also localize to the Golgi, and AKAP450 has been shown to recruit the γ-TuRC to the cis-Golgi compartment (Rivero et al., 2009; Wang et al., 2010).

3. Centrosome Maturation

From S phase, like as the replicated chromosomes, the two duplicated

centrosomes further progress the cell cycle, G2-M phase. During this relatively short period, the centrosome undergoes drastic changes which called as centrosome maturation. In this process, the amount of pericentriolar material (PCM) of centrosome including γ-tubulin but not centriole structure increases 3-5 fold, consequent microtubule nucleating activity is peak during late prophase to metaphase (Khodjakov and Rieder, 1999). The highest microtubule nucleating activities of centrosomes directly contribute to the formation of robust bipolar mitotic spindle, which executes the task of separating the duplicated genome (Figure 5).

3.1. Pericentriolar Material (PCM)

The pericentriolar material (PCM), the microtubule organizing component of the centrosome, contains a multitude of proteins and is commonly described as an amorphous cloud surrounding the centrioles (Luders, 2012). The PCM nucleates microtubules and enables centrosome to function as MTOC (Luders, 2012). The representative feature of PCM, microtubule nucleation, is accomplished by the PCM proteins including γ-tubulin, the principal microtubule nucleation factor in all eukaryotic cells. The structural components of PCM such as CEP215, pericentrin, CEP192 and NEDD1 involved in the recruitment and attachment of γ-tubulin ring complex (γ-TuRC)

to the centrosome.

Recently, super-resolution microscopy revealed that PCM proteins were organized into concentric layers that enclose the cylinder of the mother centriold in a tube or toroid-like arrangement (Figure 6). The authors classified PCM proteins according to their distance from the centriole wall (Lawo et al., 2012). At the onset of mitosis, centrosome is maturated by various mitotic kinase and structural PCM proteins such as PLK1, Aurora A, pericentrin, CEP192, CEP215 and GCP-WD. As a result, centrosome matures with dramatic increase in size and nucleation activity. Mitotic PCM consists of an inner, toroidal part and an outer, more expanded part, for which previously used terms such as ‘cloud’ or ‘matrix’ still apply. The inner toroidal part seems to be organized similarly to the interphase PCM. The outer matrix contains more expanded CEP192, pericentrin, CEP215 and γ-TuRC. The results suggest that within the extended mitotic matrix, CEP192, pericentrin, CEP215 and γ-TuRC organize the mitotic PCM systemically and have spatial relationships (Luders, 2012). The previous experimental evidence that these PCM components are interdependently involved in the recruitment of γ-tubulin to mitotic centrosome also supported the hypothesis (Haren et al., 2009).

3.2. Mitotic Bipolar Spindle Formation

It has been known that at least two independent microtubule nucleation pathways operate in mitotic animal cells (Figure 7). The centrosomal nucleation pathway depends on centrosome-bound γ-TuRCs and promotes the separation of the two duplicated centrosomes, their migration to opposite sides of the nucleus and the formation of two microtubule asters at the beginning of mitosis (Luders and Stearns, 2007). After nuclear envelope breakdown, a second nucleation pathway, which is controlled by the small GTPase Ran, becomes active (Gruss and Vernos, 2004). A chromatin-bound nucleotide-exchange factor for Ran generates a high local concentration of RanGTP, which activates spindle-assembly factors and promotes the formation and organization of microtubules in the vicinity of the chromosomes. This pathway also requires γ-tubulin *in vitro* and *in vivo*, as well as the targeting factor GCP-WD (Luders et al., 2006). Knockdown experiments in *Drosophila* S2 cells supported that γ-tubulin is also involved in the chromosome-mediated microtubule assembly (Mahoney et al., 2006).

In addition, it was also recently shown that the localization of γ-tubulin to the spindle is required for proper spindle formation and depends on mitotic phosphorylation of the γ-tubulin-targeting factor GCP-WD (Luders et al., 2006). This indicates that a mitosis-specific activity links the γ-TuRC to spindle microtubules. This spindle-bound γ-TuRC might be involved in an

amplification mechanism for spindle assembly. In this model, the forming spindle itself becomes a self-reinforcing MTOC. γ-TuRCs bound laterally to microtubules would contribute to the formation of an ordered, bipolar array by nucleating additional microtubules along existing microtubules, ensuring that new microtubules are made where needed (Luders and Stearns, 2007).

The spindle microtubules made by chromosome-dependent pathway are captured by astral microtubules and transported to the mitotic centrosome. In addition, γ-tubulin on spindle could nucleate microtubules along existing spindle microtubules (Mahoney et al., 2006). Therefore, the major role of mitotic centrosome is to make astral microtubules and to integrate preformed spindle microtubules into a common spindle. Although the mitotic spindle formation can be achieved by acentrosomal mechanisms, when centrosomes are removed from cells that normally contain them, the fidelity of mitosis is severely compromised under these conditions. As a result, centrosomes increase the fidelity of mitosis in animal cells (Wadsworth and Khodjakov, 2004).

Figure 1. The centrosome structure.

Schematic view of the centrosome. A centrosome is composed of two centrioles paired by interconnecting fiber and surrounding pericentriolar material (PCM) (Bettencourt-Dias and Glover, 2007).

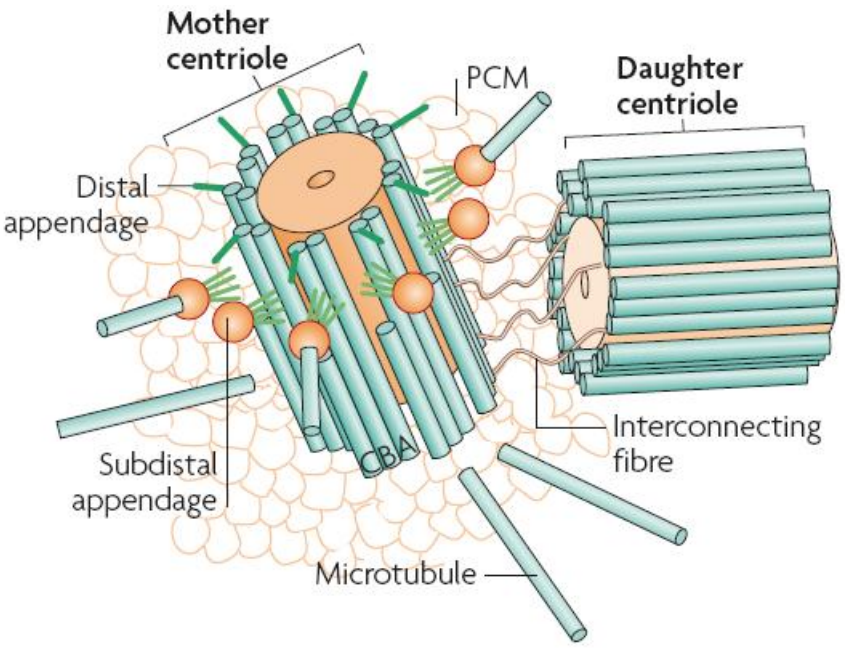

Mother centriole
PCM
Daughter centriole
Distal appendage
Interconnecting fibre
Subdistal appendage
CBA
Microtubule

Figure 2. The centrosome functions.

(A) During interphase, the centrosome has a role as a microtubule organizing center (MTOC) in cells which primarily establishes the microtubule network and regulates cell shape, cell migration and molecular trafficking. (B) During mitosis, the centrosome facilitates the formation of mitotic spindle poles and robust bipolar spindles for the accurate segregation of chromosomes to each daughter cell. (C) In early G1 phase or quiescent phase (G0), the centrosome has a role as basal body for the template of the primary cilia (Rieder et al., 2001).

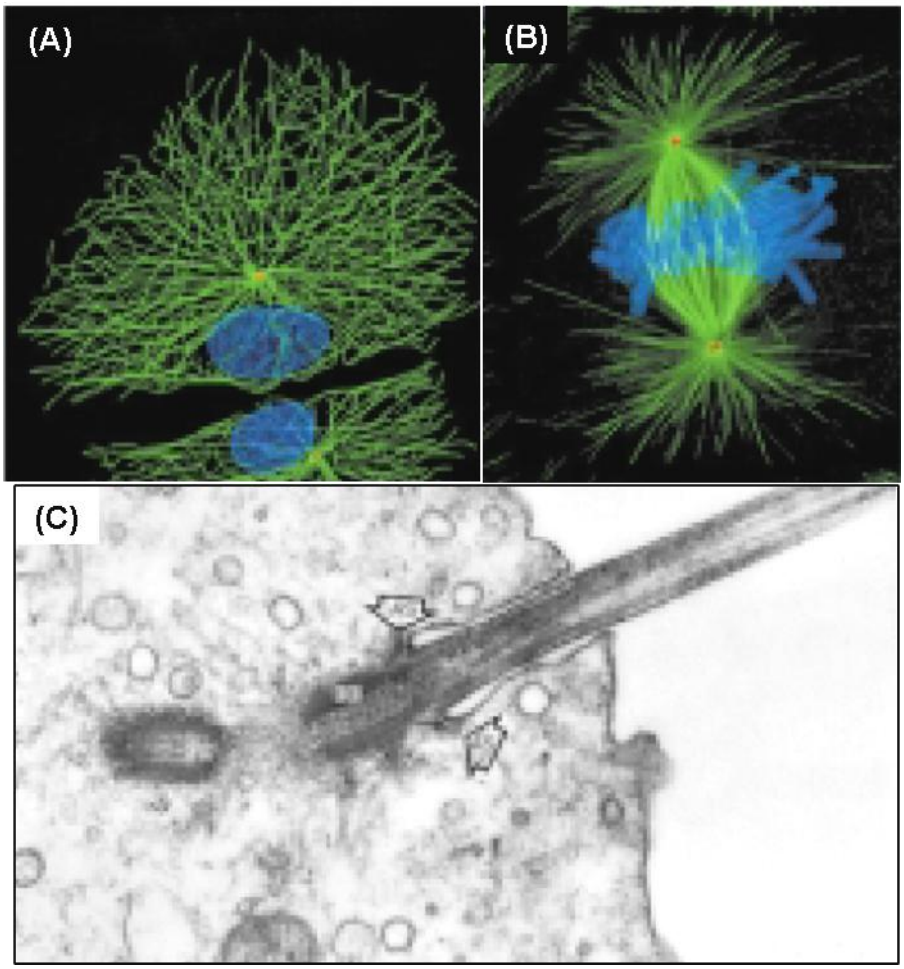

(A)
(B)
(C)

Figure 3. The centrosome cycle.

Electron microscopy and diagram show distinct steps during centrosome cycle. The mother centriole is represented in dark green showing appendages. Daughter centrioles are shown in light green (Bettencourt-Dias and Glover, 2007).

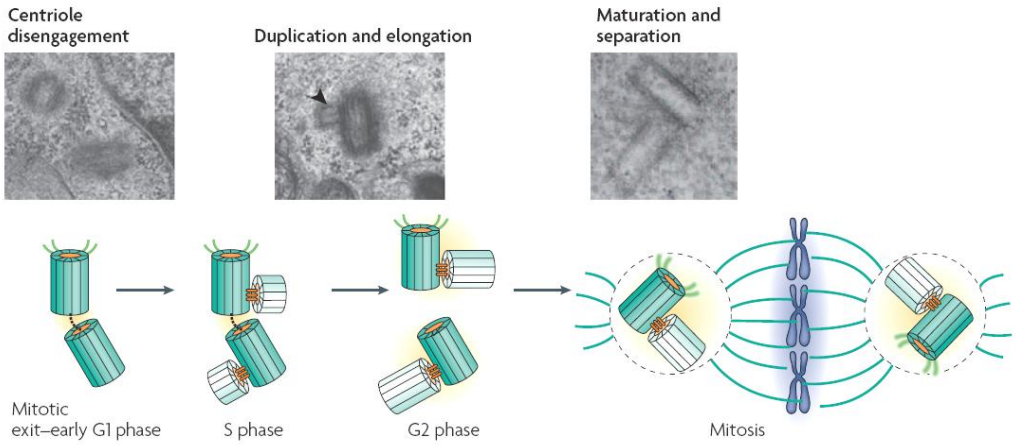

Centriole disengagement
Duplication and elongation
Maturation and separation
Mitotic exit–early G1 phase
S phase
G2 phase
Mitosis

Figure 4. Speculative model of γ-TuRC assembly.

γ-TuSCs are composed of GCP2 and GCP3 (shades of orange) and two molecules of γ-tubulin (blue). γ-TuSC-like complexes are assembled by replacement of GCP2 and/or GCP3 with GCP4, GCP5 and/or GCP6 (shades of green). Half complexes are composed of a single molecule of GCP4, GCP5 or GCP6 interacting with γ-tubulin. All complexes participate in the formation of the γ-TuRC ring structure. Nucleation of microtubule polymerization involves longitudinal interactions of α-tubulin - β-tubulin heterodimers with γ-tubulin in the γ-TuRC (template nucleation model) (Teixido-Travesa et al., 2012).

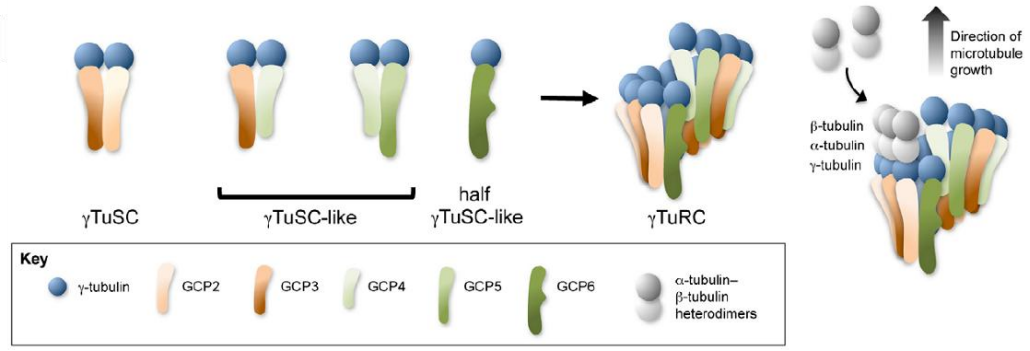

γTuSC
γTuSC-like
half
γTuSC-like
γTuRC
Direction of
microtubule
growth
β-tubulin
α-tubulin
γ-tubulin
Key
γ-tubulin
GCP2
GCP3
GCP4
GCP5
GCP6
α-tubulin–
β-tubulin
heterodimers

Figure 5. Centrosome maturation.

(A) Duplicated centrosomes start to recruit pericentriolar material (PCM) for centrosome maturation during G2 phase. (B) G2/M transition, two separated centrosomes are highly matured and form asters through enhanced microtubule nucleation activity. (C) After nuclear envelop breakdown (NEBD), the centrosome functions as a spindle pole and its maturation state reaches to maximum level at metaphase. (D) As cells exit mitosis, PCM amount and MT nucleation activity decrease. (E) During S phase of next cell cycle, the centrosome is duplicated (Rusan and Rogers, 2009).

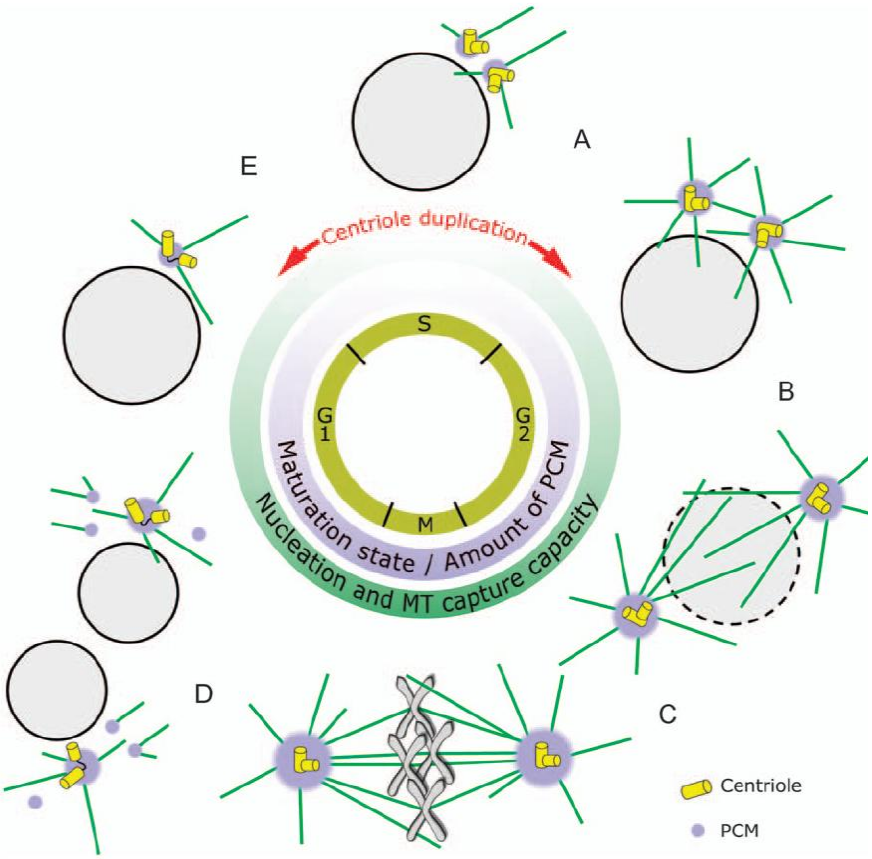

A
B
C
D
E
Centriole duplication
S
G1
G2
M
Maturation state / Amount of PCM
Nucleation and MT capture capacity
Centriole
PCM

Figure 6. Organization of the PCM at human centrosomes.

(a) During interphase, PCM proteins are organized in concentric toroids around mother centrioles. Proteins involved in microtubule nucleation (including the γ-tubulin ring complex (γ-TuRC) and CEP215) are found in the outer layers, whereas PCM components such as CEP192 and CEP120 are found closer to the wall of the mother centriole, which is decorated by CPAP. Some PCM proteins such as pericentrin have an extended conformation and are organized radially, with one end close to the centriole wall and the other end extending outwards. (b) Upon entry into mitosis, centrosomes acquire additional PCM, which forms an extended outer matrix around the toroidal PCM. The extended mitotic matrix contains subdomains formed by pericentrin, CEP192, CEP215 and γ-TuRC. The spatial relationships between these proteins in the mitotic matrix are similar to their arrangement in the interphase toroids (Luders, 2012).

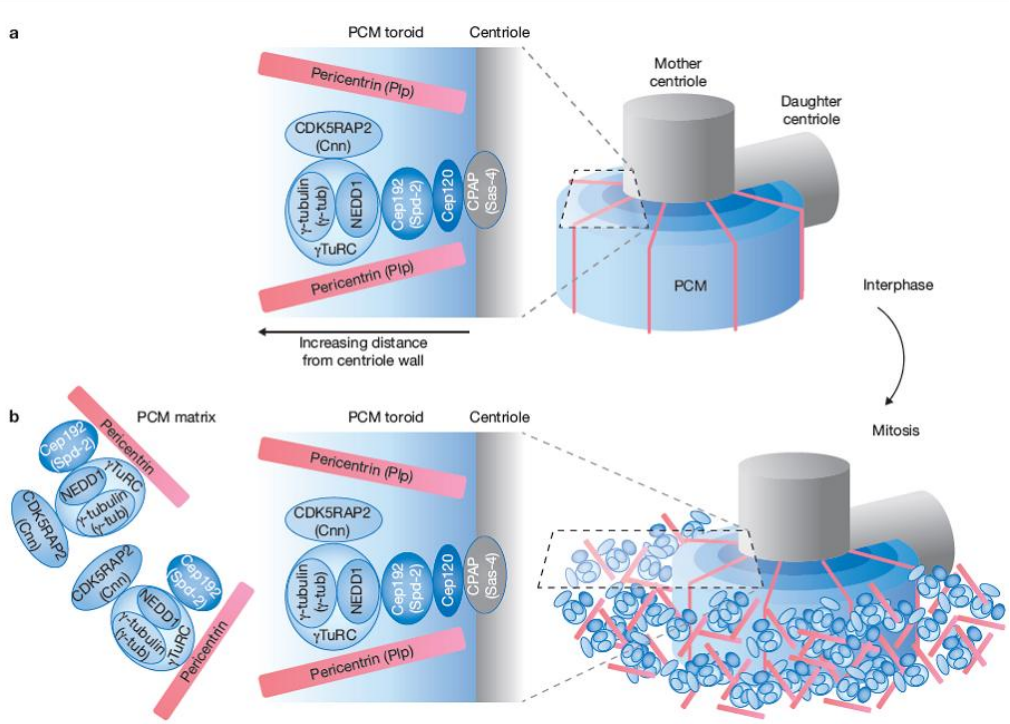
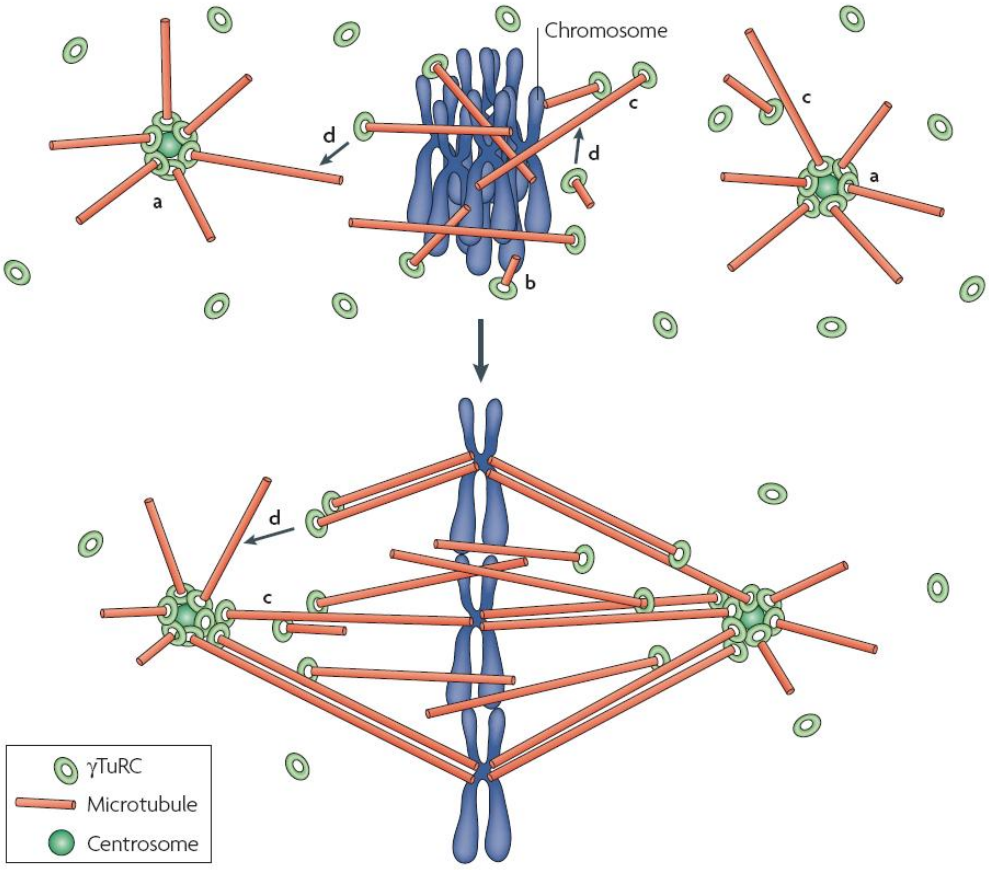

a
PCM toroid
Centriole
Pericentrin (Plp)
CDK5RAP2 (Cnn)
γ-tubulin (γ-tub)
NEDD1
γTuRC
Cep192 (Spd-2)
Cep120
CPAP (Sas-4)
Pericentrin (Plp)
Increasing distance from centriole wall
Mother centriole
Daughter centriole
PCM
Interphase
Mitosis
b
PCM matrix
Pericentrin
Cep192 (Spd-2)
NEDD1
γTuRC
γ-tubulin (γ-tub)
CDK5RAP2 (Cnn)
PCM toroid
Centriole

Figure 7. Spindle assembly by γ-tubulin ring complex (γ-TuRC).

During mitosis, microtubules are nucleated at the centrosomes (a) and near the chromosomes (b). γ-TuRC on spindle microtubules also nucleates additional microtubules (c). The minus ends of free microtubules are captured by astral microtubules emanating from centrosomes (d) (Luders and Stearns, 2007).


Chromosome
a
b
c
d
γTuRC
Microtubule
Centrosome

Purpose

In most animal cells, centrosome is the major microtubule-organizing center in interphase and also functions as a bipolar spindle pole during mitosis. The functions of centrosome in interphase are involved in cell shape, migration, migration, polarity and microtubule nucleation. During mitosis, centrosome forms a robust bipolar spindle poles to segregate the duplicated chromosomes to the two daughter cells. Although this organelle executes a lot of subcellular functions along with cell cycle, the molecular mechanisms regulating its structure and function are largely unknown. About a decade ago, proteomic analysis revealed that human centrosome consists of several hundred kinds of proteins. From this report, the characterization of novel centrosomal proteins and their knockdown phenotypes have been reported. However, the molecular mechanisms of their functions are still remains obscure. In this context, I believed that it is the very next question to investigate the precise mechanisms of their functions. Thus, I attempt to investigate the functional mechanisms of CEP215, one of the important pericentriolar material components, along with the cell cycle.

CHAPTER 1.
The physical interaction of CEP215 with γ-tubulin is required for microtubule nucleation in interphase cells

Abstract

Centrosome is the primary microtubule organizing center in most animal cells, so it nucleates and anchors the microtubules for the microtubule network formation in interphase. γ-Tubulin may be the most important molecule for microtubule nucleation. It has been known that CEP215, a pericentriolar material component, regulates the microtubule nucleation activity. However, a precise mechanism remains largely unknown. In this study, I revealed that CEP215 depletion resulted in reduction of the microtubule nucleating activity both in centrosome and in cytoplasm. Moreover, I investigated that CEP215 physically interacts with γ-tubulin. This interaction is important for the γ-tubulin recruitment to interphase centrosome and subsequent microtubule nucleation activity in interphase cells. This is the first time to demonstrate that CEP215 interacts with γ-tubulin, thereby indicating an important role in the microtubule nucleating activity in cytoplasm.

Introduction

The centrosome, the main microtubule organizing center (MTOC) in animal cells, consists of two principal components, centrioles and pericentriolar material (PCM) (Luders, 2012). The PCM, the microtubule organizing component of the centrosome, contains a multitude of proteins and is commonly described as an amorphous cloud surrouding the centrioles (Luders, 2012). Among these PCM molecules, γ-tubulin is well known for most important for the microtubule nucleation. Analysis of centrosomes by electron microscopy has revealed in great detail the structure of centrioles (Chretien et al., 1997) and identified within the PCM ring-like structures of ~25-30 nm in diameter, which represent microtubule nucleation sites formed by γ-tubulin ring complexes (γ-TuRCs) (Moritz et al., 1995; Vogel et al., 1997; Schnackenberg et al., 1998). At centrosome, γ-tubulin mediates microtubule nucleation and anchoring of the radial microtubule network (Choi et al., 2010). Thus, the proper recruitment of γ-tubulin to the centrosome is prerequisite for the formation and maintenance of microtubule network.

The Drosophila *centrosomin* (Cnn) is a core component of centrosome has a function for proper recruitment of PCM proteins including γ-tubulin at the mitotic spindle poles (li and Kaufman, 1996; Megraw et al., 1999; Zhang and

Megraw, 2007). Cnn has two highly conserved domains, called as Cnn motif 1 (CM1) and Cnn motif 2 (CM2). It has been reported that the CM1 of Cnn is essential for the interaction with γ-tubulin to the centrosome (Zhang and Megraw, 2007).

CEP215, the human ortholog of Cnn, is also known that has two conserved domains, CM1 and CM2, and is involved in the centrosomal attachment of γ-tubulin (Fong et al., 2008). It has been reported that the interaction of CEP215 with γ-tubulin through its CM1 is critical for the cytoplasmic microtubule nucleation in interphase cells (Choi et al., 2010). However, there are several limitations to interpret whether this interaction contributes to the microtubule nucleation at the centrosome. First, the previous report used only the CM1 domain (51-100a.a) for the microtubule nucleation assay. The cytoplasmic localization of CM1 is quite different from endogenous CEP215 which localize to the centrosome predominantly. Hence, I tried to investigate the functional significance of the interaction for microtubule nucleation using full-length of CEP215. In this study, I fundamentally confirmed the knockdown phenotypes of CEP215, and revealed that the physical interaction of CEP215 with γ-tubulin is essential for the microtubule nucleation activity in interphase cells.

Materials and Methods

Antibodies and plasmids

The CEP215 antibody has been previously described (Lee and Rhee, 2010). Antibodies specific for γ-tubulin (sc-7396; Santa Cruz Biotechnology), FLAG (F3165; Sigma-Aldrich), FLAG (#2368; Cell Signaling), α-tubulin (ab18251; Abcam), β-tubulin (T0198; Sigma-Aldrich) were commercially purchased. Alexa Fluor 488-, 555- and 594-conjugated secondary antibodies (Invitrogen) were used for immunostaining. Anti-mouse IgG-HRP (A9044; Sigma-Aldrich) and anti-rabbit IgG-HRP (AP132P; Millipore) were used as secondary antibodies for immunoblotting.

The wild type CEP215 (WT) and F75A mutant CEP215 (F75A) constructs were subcloned into the p3×FLAG-CMV10 vector. For knockdown-rescue experiments, the plasmids encoding CEP215 were silently mutated by using PCR technique.

Cell culture and transfection

HeLa cells and HEK293T cells were grown in DMEM supplemented with 10% FBS at 37°C. U2OS cells were grown in McCoy's 5A medium supplemented with 10% FBS at 37°C. The siRNAs were transfected into cells

using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. The plasmids were transfected into cells using FugeneHD (Promega). The siRNAs used in this study were *siCTL* (5'-GCA AUC GAA GCU CGG CUA CTT-3'), *siCEP215* (5'-GUG GAA GAU CUC CUA ACU AAA-3'; Lee and Rhee, 2010).

Rescue experiments and microtubule regrowth assay

For the rescue experiments, cells were initially transfected with siRNAs; 24 hrs later, the cells were transfected with siRNA-resistant DNA constructs using FugeneHD. The cells were cultured for additional 24 hrs, and incubated on ice for 1 hr 15 minutes to depolymerize the microtubules completely. Then, the cells were incubated with pre-warmed fresh medium for 30 seconds. The cells were fixed with MtOH or PEM fixative (80 mM PIPES [pH7.0], 5 mM EGTA, 1 mM $MgCl_2$, 4% Paraformaldehyde, 0.5% Triton X-100) and coimmunostained with the indicated antibodies.

Immunocytochemistry and image processing

Cells were grown on 12-mm coverslips. The cells were fixed with MtOH or PEM fixative at -20°C for 10 minutes, washed with PBS, blocked in 3% bovine serum albumin (BSA) in PBS for 20 minutes, and incubated with the

indicated primary antibodies for 1 hr. The cells were washed with PBS with 0.3% Triton X-100 (PBST) and subsequently incubated with Alexa Fluor 488-, Alexa Fluor 555-, or Alexa Fluor 594-conjugated secondary antibodies (Invitrogen). The DNA was counterstained with DAPI. The samples were mounted in ProLong Gold antifade reagent (P36930; Invitrogen) and viewed on a fluorescence microscope (Olympus IX51) equipped with a CCD (Qicam fast 1394, Qimaging) camera. The images were analyzed using ImagePro 5.0 (Media Cybernetics, Inc.). The intensity of a specific protein was determined based on the sum of fluorescence intensity, and background intensity was subtracted. All statistical data were analyzed with SigmaPlot (Systat Software, Inc.).

Immunoprecipitation and immunoblotting experiments

For coimmunoprecipitation experiments, HEK293T cells were transfected with DNA constructs using PEI. After 24 hrs, the cells were lysed on ice for 20 minutes with a lysis buffer (25 mM Tris-HCl [pH 7.4], 100 mM NaCl, 5 mM $MgCl_2$, 5 mM NaF, 20 mM β-glycerophosphate and 0.5% NP-40) containing an appropriate amount of protease inhibitor cocktail (P8340; Sigma-Aldrich). The lysates were centrifuged at 12,000 rpm for 20 minutes at 4°C, and the supernatants were incubated with FLAG-M2 AffinityGel (A2220; Sigma-

Aldrich) for 1.5 hrs at 4°C. The immunoprecipitates were then separated by SDS-PAGE, blotted onto a nitrocellulose membrane (Protran BA85; GE), and incubated with the indicated primary antibodies. HRP-conjugated secondary antibodies were used at a 1:10,000 dilution. The immunoreactive bands were visualized using ECL solution.

Results

CEP215 regulates interphase microtubule network

So far, it has been reported that CEP215 is involved in several centrosome behaviors such as microtubule nucleation, centrosome cohesion and cell division (Fong et al., 2008; Lee and Rhee, 2010; Graser et al., 2007; Choi et al., 2010). However, the precise mechanisms of CEP215 functions are largely unknown. Among these phenotypes, I decided to investigate the molecular mechanism of CEP215 function as a regulator of interphase microtubule network. As previously reported, I observed that control group cells (*siCTL*) exhibit well-organized microtubule network from centrosome, the major MTOC, whereas, CEP215-depleted cells (*siCEP215*) showed the disorganized microtubule in interphase (Figure 8) (Fong et al., 2008). I also observed the severely impaired cytoplasmic microtubules in CEP215-depleted cells (Figure 9). These results suggest that CEP215 functions in the regulation of microtubule formation both in centrosome and cytoplasm in interphase cells.

CEP215 is required for microtubule nucleation in interphase cells

γ-Tubulin is most well-known molecule which is representative for the

activity of microtubule nucleating and anchoring at the centrosome. I then examined whether the CEP215 knockdown phenotypes are due to the reduction of γ-tubulin at interphase centrosome. The depletion of CEP215 is confirmed by immunostaining (Figure 10A) and immunoblotting (Figure 10B). The immunostaining result showed that depletion of CEP215 results in the reduction of γ-tubulin at interphase centrosomes (Figure 10A, C). The level of γ-tubulin is critical for the microtubule nucleation capacity, I next examined the microtubule nucleation activity in CEP215-depleted cells through microtubule regrowth assay (Figure 11). The results showed that the control group cells (*siCTL*) exhibits both of strongly emanated microtubules from the centrosome and newly formed microtubules at cytoplasm. However, the CEP215-depleted cells (*siCEP215*) significantly reduced the microtubule nucleating activity both in centrosome and cytoplasm (Figure 11). These results indicate that CEP215 is important not only for centrosomal microtubule nucleation but also for cytoplasmic microtubule nucleation.

The physical interaction of CEP215 with γ-tubulin is important for the γ-tubulin recruitment to interphase centrosome

I then decided to investigate how CEP215 regulates the microtubule nucleation activity in interphase cells. It has been reported that CEP215

physically interacts with γ-tubulin through its CM1, and the F75 residue is critical for the interaction. At first, I confirmed that full-length of CEP215 physically interacts with γ-tubulin in asynchronous HEK293T cells, and the substitution of F75 to alanine completely abolish the interaction (Figure 12). To investigate the functional significance precisely, I performed the knockdown rescue experiment using the FLAG-tagged full-length of CEP215 (WT) and F75A mutant CEP215 (F75A). The knockdown and ectopic expression of CEP215 were confirmed by immunoblotting (Figure 13). The results showed that both the wild type and F75A mutant CEP215 successfully localized at the interphase centrosome (Figure 14A). Depletion of CEP215 results in significantly reduced γ-tubulin at the interphase centrosome, and it was successfully rescued with ectopic CEP215 (WT), however, F75A mutant CEP215 (F75A) can not rescue the reduced γ-tubulin at the interphase centrosome (Figure 14A, B). These results suggested that the physical interaction of CEP215 with γ-tubulin is important for the γ-tubulin recruitment to interphase centrosome.

The physical interaction of CEP215 with γ-tubulin is important for microtubule nucleating activity not only in centrosome but also in cytoplasm

Then, I also performed microtubule regrowth assay with each knockdown-rescued groups (Figure 15). The knockdown-rescued cells were incubated on ice for 1hr 15minutes to depolymerize the cellular microtubules completely. Then, the cells were re-incubated with pre-warmed media for 30 seconds to allow the microtubules regrow, and immunostained with FLAG and α-tubulin antibodies. The results showed that the cells rescued with wild type CEP215 (WT) exhibits vigorously enhanced microtubule nucleating activity both in centrosome and cytoplasm much more than control (*siCTL*) group cells (Figure 15, 16). It is thought to be due to the level of rescued CEP215 is much higher than endogenous CEP215. On the other hand, the cells rescued with F75A mutant CEP215 (F75A) showed that dramatically diminished microtubule nucleating activity (Figure 15, 16). From the microtubule regrowth assay with knockdown-rescued groups, I realized that wild type CEP215 (WT) successfully localizes along with the newly formed microtubules, whereas F75A mutant CEP215 (F75A) can not localize on the microtubules (Figure 15). Thus, I examined whether F75A mutant of CEP215 lose the ability of localization onto the microtubules in unperturbed condition (Figure 17). To eliminate the effect of remaining endogenous CEP215, I tried to deplete the endogenous CEP215 using knockdown-rescue system. The results showed that the abilities of localization onto microtubules between wild type CEP215 (WT)

and F75A mutant CEP215 (F75A) are not distinguishable (Figure 17). Taken together, these results proposed that the function of CEP215 for microtubule nucleation is mediated by the physical interaction with γ-tubulin.

The physical interaction of CEP215 with γ-tubulin is not essential for the γ-tubulin recruitment to mitotic centrosome and centrosome maturation

The centrosome maturation is thought to be prerequisite step for formation of mitotic bipoles. In this process, centrosome undergoes the drastic expansion of PCM, and subsequent 3-5 fold increase γ-tubulin at mitotic centrosome (Khodjakov and Rieder, 1999). Like as interphase, γ-tubulin is thought to be most important molecule, representative for microtubule nucleating activity, for the assembly of robust mitotic bipolar spindle during mitosis. Thus, I investigated whether the interaction of CEP215 with γ-tubulin contributes to the γ-tubulin recruitment to mitotic centrosome, subsequent centrosome maturation. At first, I examined whether CEP215 interacts with γ-tubulin through F75 residue during mitosis. The mitotic CO-IP results showed that CEP215 also interacts with γ-tubulin during mitosis, and F75 residue is critical for the interaction (Figure 18). I next performed the CEP215 knockdown-rescue experiments to examine the contribution of the interaction to γ-tubulin recruitment to mitotic centrosome. The CEP215-depleted cells were

rescued with FLAG-tagged CEP215 (WT) and F75A mutant CEP215 (F75A) and coimmunostained with FLAG and γ-tubulin antibodies. The results showed that both the wild type and F75A mutant CEP215 were successfully localized at the spindle poles (Figure 19A, B). Concurrently, γ-tubulin was also effectively recruited to the mitotic spindle poles with the F75A mutant as well as wild-type CEP215 (Figure 19A, C). These results suggest that the physical interaction of CEP215 with γ-tubulin is dispensable for γ-tubulin recruitment to spindle poles during mitosis. Therefore, I searched for other roles of CEP215 in γ-tubulin recruitment during centrosome maturation.

Figure 8. Depletion of CEP215 results in disorganized interphase microtubule network.

HeLa cells were transfected with control siRNA (*siCTL*) and CEP215 specific siRNA (*siCEP215*). 48hrs after the transfection, the cells were fixed, and coimmunostained with CEP215 (red) and β-tubulin (green). DNAs are stained with DAPI (blue).

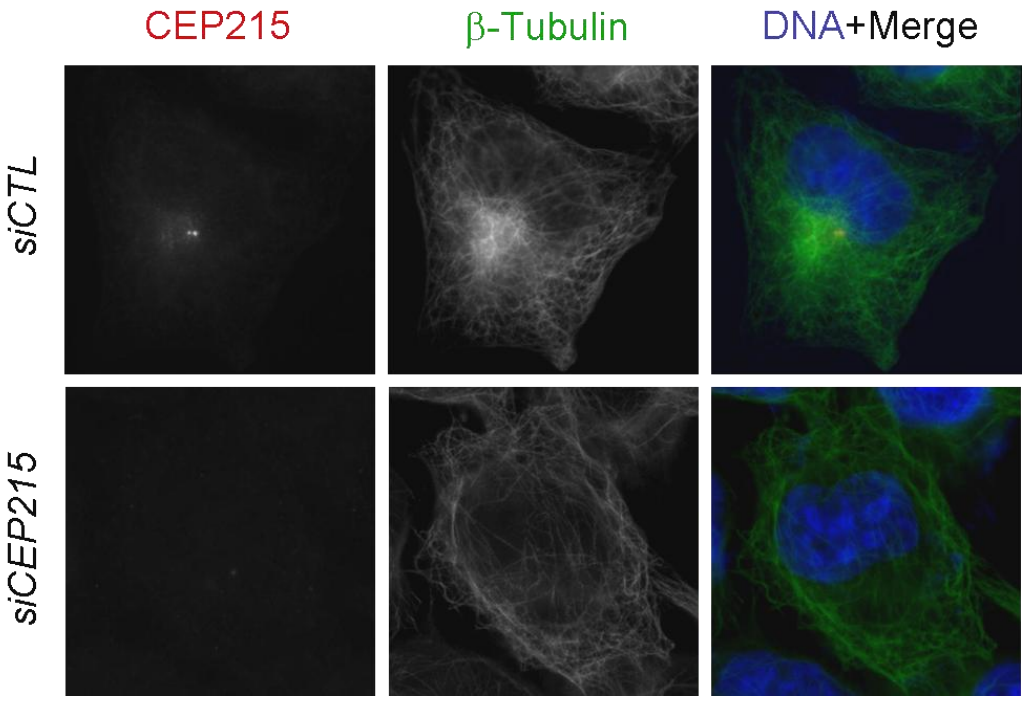

CEP215
β-Tubulin
DNA+Merge
siCTL
siCEP215

Figure 9. Depletion of CEP215 results in impaired cytoplasmic microtubules in RPE-1 cells.

RPE-1 cells were transfected with control siRNA (*siCTL*) and CEP215 specific siRNA (*siCEP215*). 48hrs after the transfection, the cells were fixed, and coimmunostained with CEP215 (red) and β-tubulin (green). DNAs are stained with DAPI (blue).

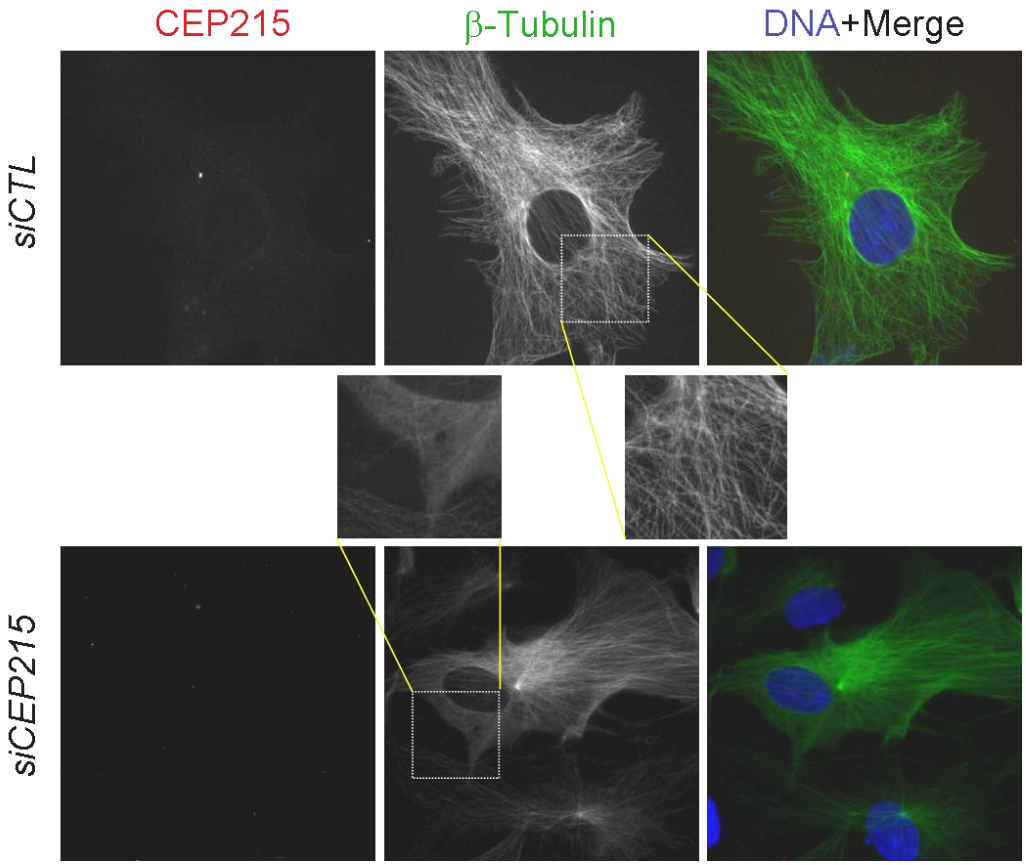

CEP215
β-Tubulin
DNA+Merge
siCTL
siCEP215

Figure 10. Depletion of CEP215 results in the reduction of γ-tubulin at the interphase centrosome.

HeLa cells were transfected with control siRNA (*siCTL*) and CEP215 specific siRNA (*siCEP215*). 48hrs after the transfection, the cells were analyzed with (A) coimmunostaining with γ-tubulin (red) and CEP215 (green) and (B) immunoblotting with indicated antibodies. Scale bar; 10μm. (C) The fluorescence intensities of γ-tubulin at the interphase centrosome were quantified in more than 30 cells per group in three independent experiments. Error bars; SEM.

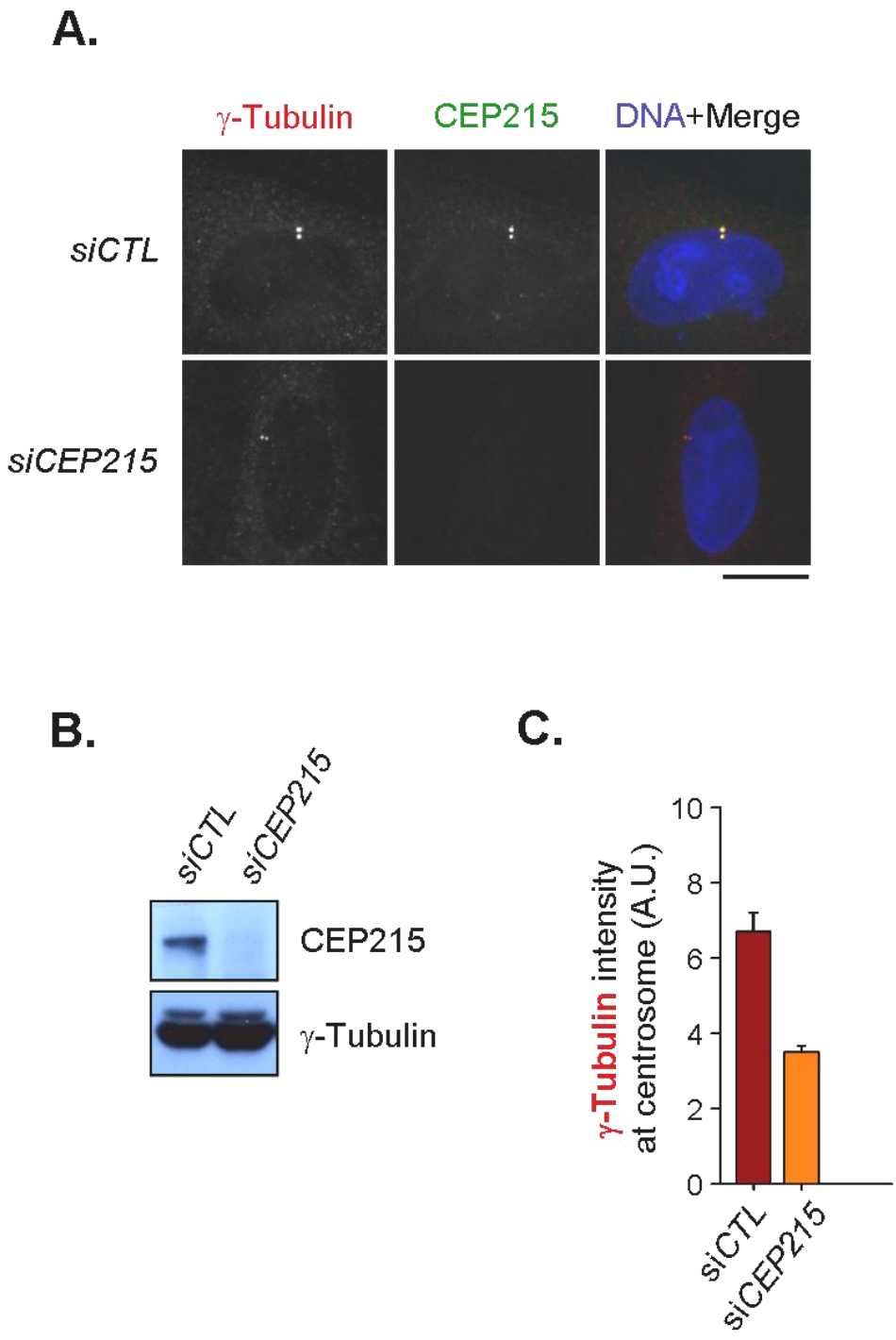

A.
γ-Tubulin
CEP215
DNA+Merge
siCTL
siCEP215
B.
siCTL
siCEP215
CEP215
γ-Tubulin
C.
γ-Tubulin intensity at centrosome (A.U.)
10
8
6
4
2
0
siCTL
siCEP215

Figure 11. Microtubule regrowth assay in CEP215-depleted U2OS cells.

U2OS cells were transfected with control siRNA (*siCTL*) and CEP215 specific siRNA (*siCEP215*). 48hrs after the transfection, the cells were incubated on ice for 1hr 15minutes to depolymerize the microtubules completely. The cells were re-incubated with pre-warmed media for 1 minute, and coimmunostained with γ-tubulin (red) and α-tubulin (green).

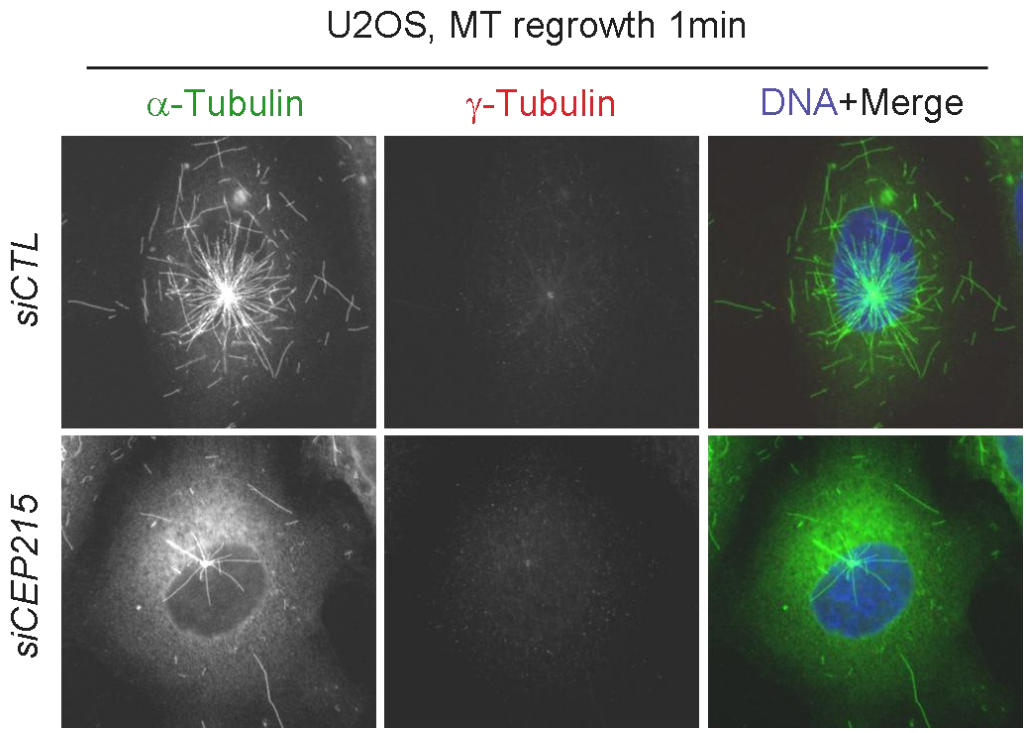

U2OS, MT regrowth 1min
α-Tubulin
γ-Tubulin
DNA+Merge
siCTL
siCEP215

Figure 12. The physical interaction between CEP215 and γ-tubulin during interphase.

HEK293T cells were transfected with FLAG-tagged GFP (Con), wild-type CEP215 ($CEP215^{WT}$) and F75A mutant CEP215 ($CEP215^{F75A}$). 24hrs after the transfection, the cells were lysed, the cell lysates were subjected to immunoprecipitation with FLAG resin followed by immunoblot analysis with γ-tubulin and FLAG antibodies.

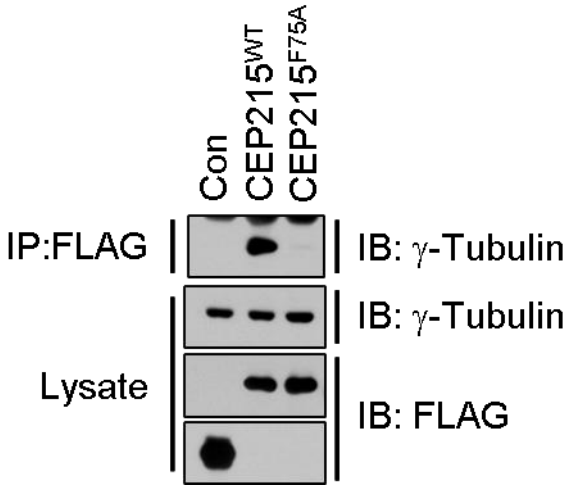

Con
CEP215WT
CEP215F75A
IP:FLAG
IB: γ-Tubulin
IB: γ-Tubulin
Lysate
IB: FLAG

Figure 13. Immunoblot analyses to confirm the knockdown and rescue of CEP215.

Initially, cells were transfected with *siCTL* or *siCEP215*. 24hrs after the siRNA transfection, the cells were rescue-transfected with FLAG-tagged GFP (GFP), wild type CEP215 (WT) and F75A mutant CEP215 (F75A). After the additional 24hrs, the cells were subjected to immunoblot analysis with indicated antibodies.

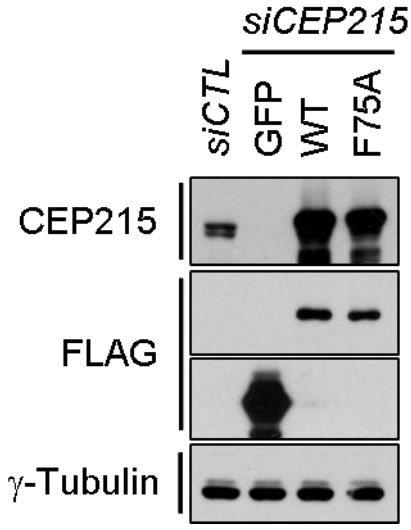

siCEP215
siCTL
GFP
WT
F75A
CEP215
FLAG
γ-Tubulin

Figure 14. The γ-tubulin intensities at centrosomes in CEP215 knockdown-rescued groups.

(A) The CEP215-depleted cells were rescued with FLAG-tagged wild type CEP215 (WT) and F75A mutant CEP215 (F75A). The cells were coimmunostained with γ-tubulin (red) and FLAG (green) antibodies. Scale bar; 10μm. (B) The intensities of γ-tubulin signal at centrosomes were quantified in more than 30 cells per group in three independent experiments. Error bars; SEM.

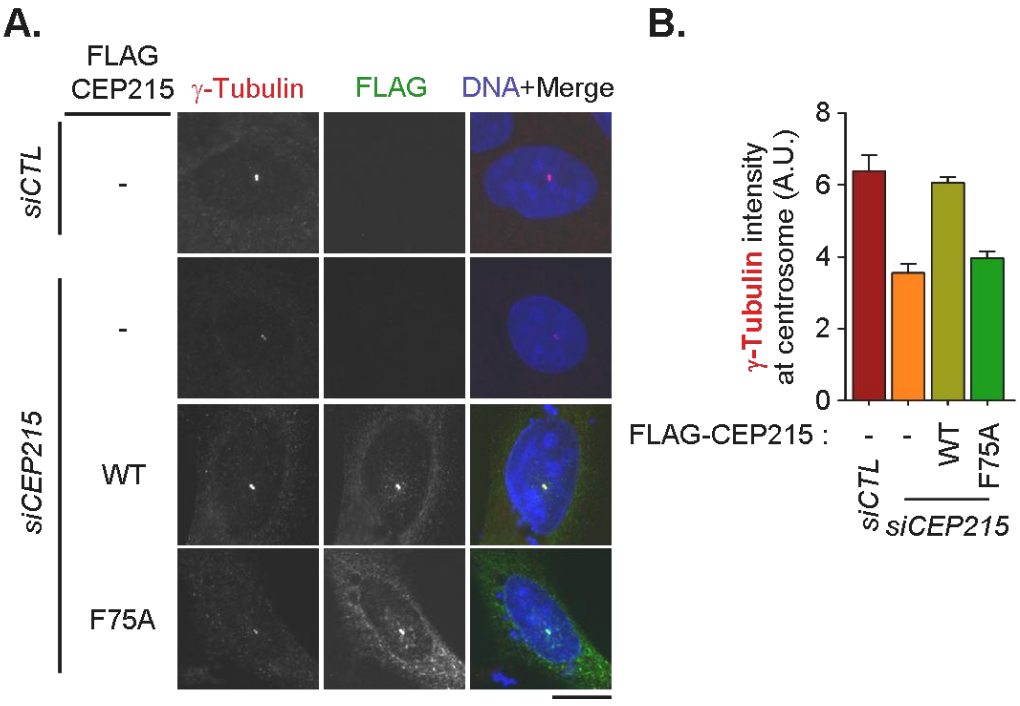

A.
FLAG
CEP215
γ-Tubulin
FLAG
DNA+Merge
siCTL
-
siCEP215
-
WT
F75A
B.
γ-Tubulin intensity
at centrosome (A.U.)
8
6
4
2
0
FLAG-CEP215 :
-
-
WT
F75A
siCTL
siCEP215

Figure 15. Microtubule regrowth assay with CEP215 knockdown-rescued cells.

The CEP215-depleted cells were rescued with wild type CEP215 (WT) and F75A mutant CEP215 (F75A). The cells were incubated on ice for 1hr 15minutes to depolymerize the microtubules completely. The cells were re-incubated with pre-warmed media for 30 seconds, and coimmunostained with α-tubulin (red) and FLAG (green) antibodies. Scale bar; 10μm.

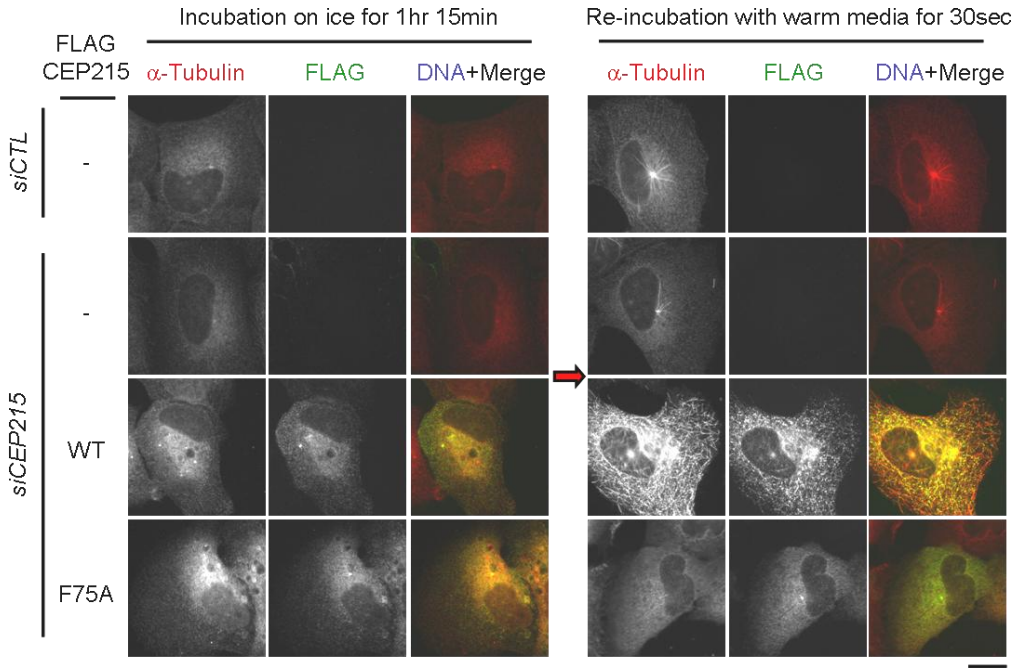

Incubation on ice for 1hr 15min
Re-incubation with warm media for 30sec
FLAG
CEP215
α-Tubulin
FLAG
DNA+Merge
α-Tubulin
FLAG
DNA+Merge
siCTL
-
siCEP215
-
WT
F75A

Figure 16. The quantification of the aspects of microtubule regrowth in CEP215 knockdown-rescued groups.

After the microtubule regrowth assay with the CEP215 knockdown-rescued groups, the cells were by categorized by α-tubulin staining patterns.

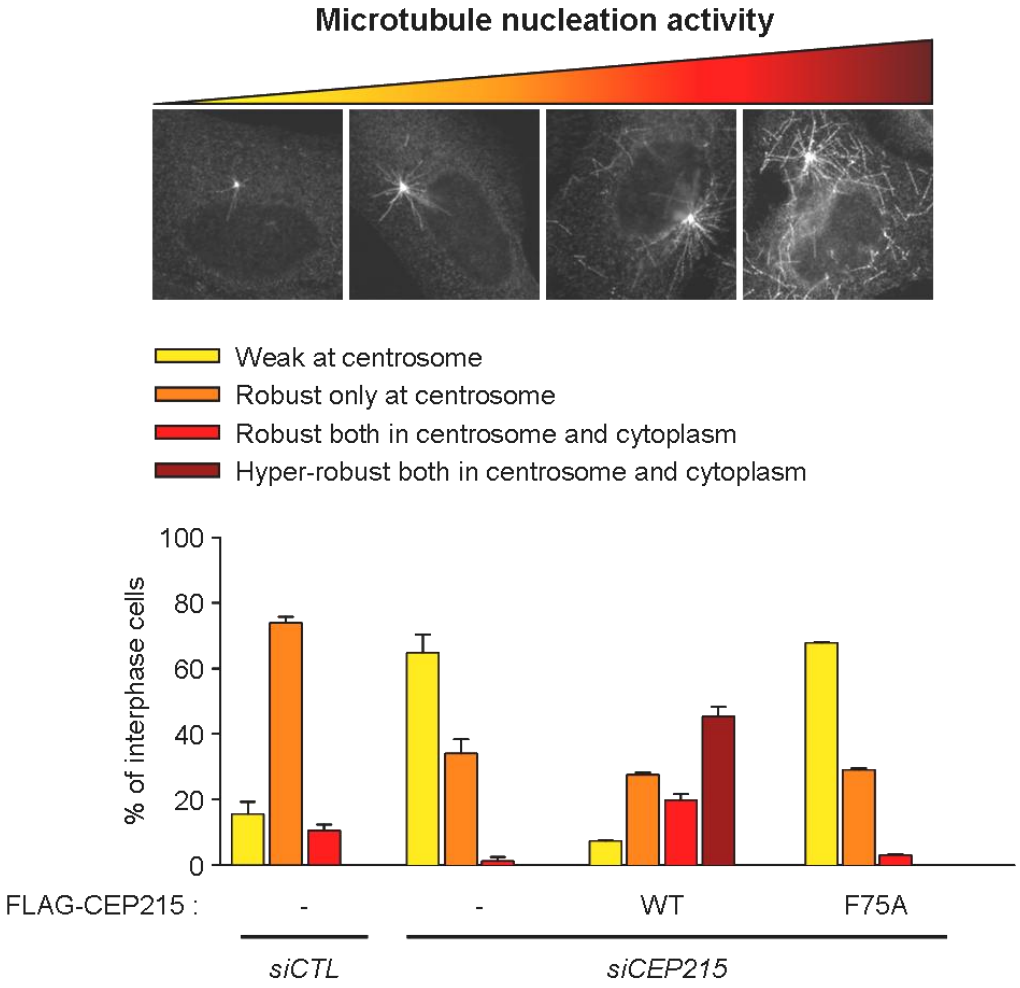

Microtubule nucleation activity
Weak at centrosome
Robust only at centrosome
Robust both in centrosome and cytoplasm
Hyper-robust both in centrosome and cytoplasm
% of interphase cells
100
80
60
40
20
0
FLAG-CEP215 :
-
-
WT
F75A
siCTL
siCEP215

Figure 17. Abilities of the localization onto microtubules between wild type CEP215 (WT) and F75A mutant CEP215 (F75A).

CEP215-depleted cells were ectopically transfected with FLAG-tagged wild type CEP215 (WT) and F75A mutant CEP215 (F75A). Then, the cells were coimmunostained with α-tubulin (red) and FLAG (green) antibodies. Scale bar; 10μm.

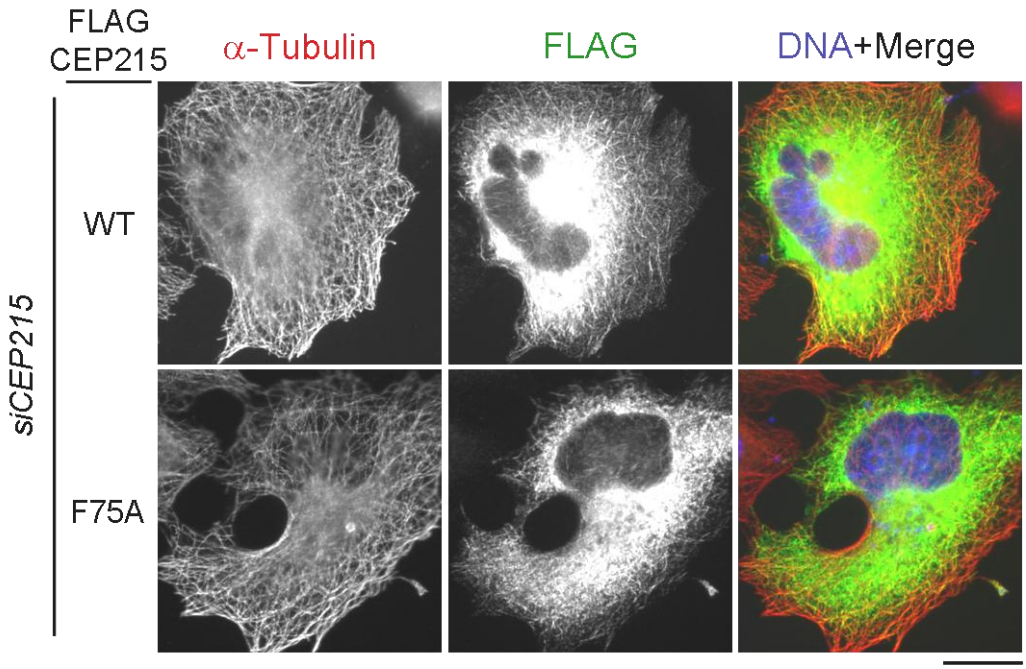

FLAG
CEP215
α-Tubulin
FLAG
DNA+Merge
siCEP215
WT
F75A

Figure 18. The physical interaction of CEP215 with γ-tubulin during mitosis.

HEK293T cells transfected with FLAG-tagged GFP (Con), CEP215 ($CEP215^{WT}$) and F75A mutant CEP215 ($CEP215^{F75A}$) were treated with STLC for 16 hrs to synchronize the cells at mitosis. The mitotic cell lysates were subjected to immunoprecipitation with FLAG resin followed by immunoblot analysis with γ-tubulin and FLAG antibodies.

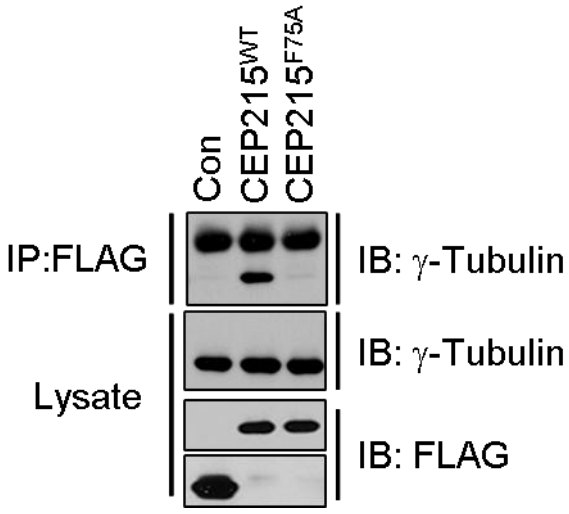
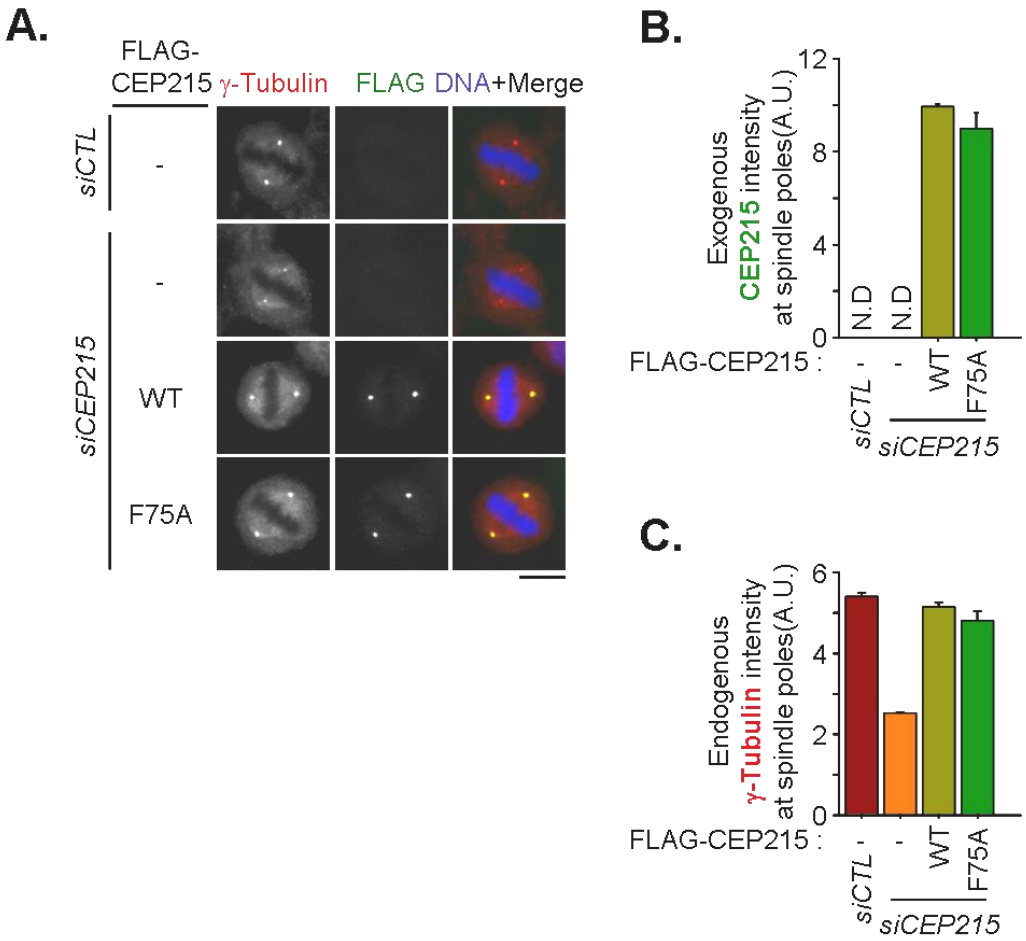

Con
CEP215WT
CEP215F75A
IP:FLAG
IB: γ-Tubulin
Lysate
IB: γ-Tubulin
IB: FLAG

Figure 19. The γ-tubulin intensities at the spindle poles in CEP215 knockdown-rescued groups.

(A) The CEP215-depleted cells were rescued with FLAG-tagged CEP215 (WT) and F75A mutant CEP215 (F75A). The cells were treated with RO3306 for 16 hrs then removed for 40 minutes to allow accumulation of mitotic cells. The cells were coimmunostained with γ-tubulin (red) and FLAG (green) antibodies. Scale bar; 10 μm. (B, C) The intensities of ectopic CEP215 (B) and endogenous γ-tubulin (C) at the spindle poles were quantified in more than 40 cells per group in three independent experiments. Error bars; SEM.


A.
FLAG-
CEP215
γ-Tubulin
FLAG DNA+Merge
siCTL
siCEP215
-
-
WT
F75A
B.
Exogenous
CEP215 intensity
at spindle poles(A.U.)
12
8
4
0
N.D
N.D
FLAG-CEP215 :
siCTL
-
-
WT
F75A
siCEP215
C.
Endogenous
γ-Tubulin intensity
at spindle poles(A.U.)
6
4
2
0
FLAG-CEP215 :
siCTL
-
-
WT
F75A
siCEP215

Discussion

As the principal nucleator of microtubules, γ-TuRC plays a pivotal role in microtubule organization. Basically, it has been known that the level of centrosomal γ-tubulin directly affect the microtubule nucleating activity. Thus far, it has been reported that several centrosomal proteins, including pericentrin, AKAP450, CEP215 and GCP-WD recruit γ-TuRC to centrosomes. However, the molecular mechanisms responsible for the regulation of its microtubule nucleating activity remain largely unknown. In this study, I revealed the fact that an important PCM component, CEP215 physically interacts with γ-tubulin. I also revealed that CEP215 recruit the γ-tubulin to interphase centrosome through this interaction, and the interaction facilitates the microtubule nucleation activity both in centrosome and cytoplasm.

In fact, it has been reported that CEP215 knockdown resulted in significant reduced of microtubule nucleating activity both in centrosome and cytoplasm (Fong et al., 2008; Choi et al., 2010). From these two reports, the researchers revealed the fact that CEP215 physically interacts with γ-tubulin, and the F75 residue is critical for the interaction. However, they could not investigate precisely whether the physical interaction of CEP215 with γ-tubulin contributes to the centrosomal microtubule nucleating activity. Because of their

technical limitations using only CM1 (51-100aa) of CEP215, the significance of the interaction for centrosomal microtubule nucleation has been not determined. Thus, I thoroughly determined the functional significance of the physical interaction of CEP215 with γ-tubulin in interphase cells. I observed that CEP215 depletion leads to significant reduced γ-tubulin level at interphase centrosome, and wild-type CEP215 successfully rescue the phenotype. On the other hand, F75A mutant CEP215 can not rescue the decreased γ-tubulin at interphase centrosome. Furthermore, microtubule regrowth assay with CEP215 knockdown-rescued cells suggested that ectopically expressed CEP215 greatly increase the microtubule nucleating activity both in centrosome and cytoplasm. However, the cells rescued with F75A mutant CEP215 exhibits significantly diminished the microtubule nucleating activity both in centrosome and cytoplasm. From these results, I suggested that CEP215 recruits the γ-tubulin to interphase centrosomes and it also might facilitate the microtubule nucleating activity of γ-tubulin through the physical interaction.

In interphase, centrosomes are thought to nucleate and anchor microtubules to form a radial array that provides cells with support and rigidity. This array also establishes intracellular polarity, with microtubule minus ends concentrated at the centrosomes in the middle of the cell and plus ends extending toward the cell cortex. Microtubule polarity then provides

directionality to traffic a variety of cargo toward and away from the nucleus (Rusan and Rogers, 2009). Other examples of centrosome-based intracellular polarity include the apical to basal microtubules found in columnar epithelial cells and the microtubule arrays oriented toward the leading edge of migrating cells (Rusan and Rogers, 2009). From these important aspects of interplay between centrosome and microtubule network, understanding the molecular mechanism of its regulation is remaining issues. In this context, my research may provide insights into molecular mechanism of microtubule formation.

During mitosis, centrosome undergoes the process called as centrosome maturation and becomes to mitotic bipoles. Also in this process, the robust γ-tubulin recruitment to the mitotic centrosome is prerequisite event. In this study, I also investigated whether the physical interaction of CEP215 with γ-tubulin contribute to the γ-tubulin recruitment to mitotic centrosome and centrosome maturation. However, the results suggested that there would be another functional mechanism of CEP215 for centrosome maturation rather than the physical interaction with γ-tubulin during mitosis. I mechanistically investigated this issue in chapter 2.

CHAPTER 2.
The physical interaction of CEP215 with pericentrin is critical for centrosome maturation during mitosis

Abstract

At the onset of mitosis, the centrosome undergoes maturation, which is characterized by a drastic expansion of the pericentriolar material and a robust increase in microtubule-organizing activity. CEP215 is one of the major pericentriolar material components which accumulate to the centrosome during mitosis. The depletion phenotypes indicate that CEP215 is essential for centrosome maturation and bipolar spindle formation. From chapter 1, I found that the physical interaction of CEP215 with γ-tubulin is not essential for the γ-tubulin recruitment to mitotic centrosome. Thus, I tried to find another functional interacting partner of CEP215 for centrosome maturation. The results indicate that the CEP215-pericentrin interaction is essential for centrosome maturation and subsequent bipolar spindle formation during mitosis. These results provide an important insight how pericentriolar material components are assembled during centrosome maturation to form a spindle pole during mitosis.

Introduction

The centrosome in most animal cells functions as a major microtubule organizing center and controls cellular morphology, migration and subcellular transport. The centrosome consists of a pair of centrioles surrounded by pericentriolar material (PCM), in which the minus ends of microtubules are embedded. The amount of PCM and microtubule organizing activity of the centrosome fluctuate in parallel during the cell cycle. At the onset of mitosis, the centrosome matures with a robust increase in centrosomal γ-tubulin. The failure of this process causes defects in bipolar spindle formation and chromosome congression. Select PCM components, including pericentrin (Zimmerman et al., 2004; Lee and Rhee, 2011), CEP215/CDK5RAP2 (Fong et al., 2008; Lee and Rhee, 2010), NEDD1/GCP-WD (Haren et al., 2006; Luders et al., 2006) and CEP192 (Gomez-Ferreira et al., 2007), are known to be critical for γ-tubulin recruitment to the centrosome. However, the means by which these PCM components accumulate and organize to form a mature centrosome is not fully understood.

CEP215 is one of the major PCM components that accumulate at spindle poles during centrosome maturation. In fact, CEP215-depleted cells failed to recruit γ-tubulin into the centrosome, and bipolar spindle poles were

not properly formed as a consequence (Fong et al., 2008; Haren et al., 2009; Lee and Rhee, 2010). Loss-of-function mutants of *centrosomin*, a *Drosophila* homolog of *CEP215*, might recruit PCM components but cannot maintain proper attachment to the centrioles (Lucas and Raff, 2007). As a result, the centrioles moved rapidly at the spindle poles and often lost their connection to the spindle poles during mitosis (Lucas and Raff, 2007). Detachment of the centrioles from the spindle poles was also observed in *CEP215* mutant chicken DT40 cells and CEP215-depleted HeLa cells (Barr et al., 2010; Lee and Rhee, 2010). Involvement of CEP215 in other centrosomal behaviors, such as centrosome cohesion and centriole engagement, has been reported (Graser et al., 2007; Barrera et al., 2010).

Physical interactions among the centrosomal proteins are considered to be important factors for the organization of the PCM lattice. In fact, *Drosophila* centrosomin interacts with Asl, DSpd-2 and D-PLP for PCM establishment and accumulation (Conduit et al., 2010). CEP215 includes two conserved regions for protein-protein interactions. The first is the γ-tubulin binding domain, referred to as CM1, which is considered to play an important role in establishing microtubule organizing activity during interphase (Fong et al., 2008; Choi et al., 2010). The second domain, referred to as CM2, is located at the C-terminus and is known to interact with pericentrin and AKAP450

(Buchman et al., 2010; Wang et al., 2010). However, it is unclear whether these interactions contribute to PCM accumulation during centrosome maturation.

Pericentrin is a large coiled-coil protein that serves as a scaffold for recruiting a number of PCM proteins (Zimmerman et al., 2004; Haren et al., 2009). Pericentrin is required for centrosome maturation because its centrosomal level increases at the onset of mitosis, and its depletion results in a significant reduction of PCM components at spindle poles and leads to monopolar spindles (Zimmerman et al., 2004; Haren et al., 2009; Lee and Rhee, 2011). Recently, super-resolution microscopic observations have suggested that pericentrin plays a pivotal role in the formation of the toroidal structure in interphase PCM and the expansion of the PCM lattice during mitosis (Lawo et al., 2012; Mennella et al., 2012). However, the means by which pericentrin functions as a scaffold and specifically interacts with PCM components for centrosome maturation remains obscure.

In this study, I investigated the molecular mechanisms of CEP215 function during centrosome maturation. Importantly, I attempted to link the protein-protein interaction properties of CEP215 with its biological functions. The results indicate that CEP215 interaction with pericentrin is critical for centrosome maturation in cells entering mitosis.

Materials and Methods

Antibodies and plasmids

The CEP215 and pericentrin antibodies have been previously described (Lee and Rhee, 2010; Kim and Rhee, 2011). Antibodies specific for NEDD1 (ab57336; Abcam), CEP192 (A302-324A; Bethyl Laboratories), γ-tubulin (sc-7396; Santa Cruz Biotechnology), FLAG (F3165; Sigma-Aldrich), FLAG (#2368; Cell Signaling), α-tubulin (ab18251; Abcam), NuMA (#NA09L; Calbiochem) and γ-tubulin (GTU-88; Sigma-Aldrich) were commercially purchased. Alexa Fluor 488-, 555- and 594-conjugated secondary antibodies (Invitrogen) were used for immunostaining. Anti-mouse IgG-HRP (A9044; Sigma-Aldrich) and anti-rabbit IgG-HRP (AP132P; Millipore) were used as secondary antibodies for immunoblotting.

The CEP215 (WT) and CEP215$^{\Delta 1726\text{-}1893}$ (ΔC) constructs were subcloned into the p3×FLAG-CMV10 vector. The PACT domain used in this study is the C-terminal region of the human pericentrin cDNA (9337-10011 bp). The wild-type and mutant pericentrin constructs (PCNT (WT), PCNT$^{1235/1241AA}$ (AA) and PCNT$^{\Delta 2390\text{-}2406}$ (Δ17)) were subcloned into the p3×FLAG-CMV10-GFP-myc vector. For knockdown-rescue experiments, the plasmids encoding CEP215 were silently mutated by using PCR technique.

Cell culture and transfection

HeLa cells and HEK293T cells were grown in DMEM supplemented with 10% FBS at 37°C. The siRNAs were transfected into HeLa cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. The plasmids were transfected into HeLa cells using FugeneHD (Promega). The siRNAs used in this study were *siCTL* (5'-GCA AUC GAA GCU CGG CUA CTT-3'), *siCEP215* (5'-GUG GAA GAU CUC CUA ACU AAA-3'; Lee and Rhee, 2010) and *siPCNT* (5'-GCA GCU GAG CUG AAG GAG ATT-3'; Srsen et al., 2006).

Rescue experiments and cell synchronization

For the rescue experiments, HeLa cells were initially transfected with siRNAs; 4 hrs later, the cells were transfected with siRNA-resistant DNA constructs using FugeneHD. The cells were cultured for 24 hrs, treated with RO3306 (5 μM) for 16 hrs to arrest the cells at the G2-M boundary, and removed for 40 minutes. Most of the cells were at prometaphase to anaphase. To observe the spindle morphology, the cells were removed from RO3306 and subsequently treated with STLC for 1 hr to arrest the cells at prometaphase. Then, the cells were washed three times with PBS and re-incubated in a medium containing 20 μM MG132 for 1.5 hrs to block the exit from mitosis.

Immunocytochemistry and image processing

HeLa cells were grown on 12-mm coverslips. The cells were fixed in methanol at -20°C for 10 minutes, washed with PBS, blocked in 3% bovine serum albumin (BSA) in PBS for 20 minutes, and incubated with the indicated primary antibodies for 1 hr. The cells were washed with PBS with 0.3% TX-100 (PBST) and subsequently incubated with Alexa Fluor 488-, Alexa Fluor 555-, or Alexa Fluor 594-conjugated secondary antibodies (Invitrogen). The DNA was counterstained with DAPI. The samples were mounted in ProLong Gold antifade reagent (P36930; Invitrogen) and viewed on a fluorescence microscope (Olympus IX51) equipped with a CCD (Qicam fast 1394, Qimaging) camera. The images were analyzed using ImagePro 5.0 (Media Cybernetics, Inc.). The intensity of a specific protein was determined based on the sum of fluorescence intensity, and background intensity was subtracted. All statistical data were analyzed with SigmaPlot (Systat Software, Inc.).

Immunoprecipitation and immunoblotting experiments

For coimmunoprecipitation experiments, HEK293T cells were transfected with DNA constructs using PEI. After 24 hrs, the cells were treated with STLC for 16 hrs and lysed on ice for 20 minutes with a lysis buffer (25 mM Tris-HCl [pH 7.4], 100 mM NaCl, 5 mM $MgCl_2$, 5 mM NaF, 20 mM β-

glycerophosphate and 0.5% NP-40) containing an appropriate amount of protease inhibitor cocktail (P8340; Sigma-Aldrich). The lysates were centrifuged at 12,000 rpm for 20 minutes at 4°C, and the supernatants were incubated with FLAG-M2 AffinityGel (A2220; Sigma-Aldrich) for 1.5 hrs at 4°C. The immunoprecipitates were then separated by SDS-PAGE, blotted onto a nitrocellulose membrane (Protran BA85; GE), and incubated with the indicated primary antibodies. HRP-conjugated secondary antibodies were used at a 1:10,000 dilution. The immunoreactive bands were visualized using ECL solution.

Results

Depletion of CEP215 causes a reduction of PCM components at mitotic spindle poles

To investigate the function of CEP215 during mitosis, we depleted the endogenous CEP215 protein via transfection of HeLa cells with a specific siRNA. CEP215 depletion was confirmed with immunoblot and immunostaining analyses (Figure 20A, B). Subsequently, we determined the intensities of the selected PCM proteins at the spindle poles of CEP215-depleted mitotic cells. The results showed that the relative intensities of selected PCM proteins (γ-tubulin, pericentrin, NEDD1 and CEP192) were significantly reduced in the CEP215-depleted mitotic cells (Figure 20C, D). However, the total cellular amounts of the PCM proteins were not affected by CEP215 depletion (Figure 20A; Haren et al., 2009). These results confirm that CEP215 is essential for PCM accumulation at spindle poles in cells at the onset of mitosis (Lee and Rhee, 2010).

Physical interaction of CEP215 with pericentrin is essential for pericentrin recruitment to mitotic spindle poles

In chapter 1, I revealed that the physical interaction of CEP215 with γ-

tubulin is not responsible for the γ-tubulin recruitment to mitotic centrosome. Thus, I decided to find another functional interacting partner of CEP215 for centrosome maturation during mitosis. Pericentrin was previously shown to interact with the CM2 domain (1726-1893) of CEP215 (Buchman et al., 2010; Wang et al., 2010). In fact, I observed that pericentrin coimmunoprecipitated with CEP215 in mitotic 293T cell lysates (Figure 21A). I also observed that pericentrin did not coimmunoprecipitate with CM2-truncated CEP215 ($CEP215^{\Delta C}$) (Figure 21B). I then examined the subcellular localization of $CEP215^{\Delta C}$. The results showed that $CEP215^{\Delta C}$ was barely detectable at the centrosomes of both interphase and mitotic cells (Figure 22A, B). These results indicate that the C-terminal domain of CEP215 is important for its centrosomal localization. It is not likely that $CEP215^{\Delta C}$ overexpression causes a dominant-negative effect on the recruitment or accumulation of PCM components because centrosomal γ-tubulin and pericentrin levels were not altered in $CEP215^{\Delta C}$-expressing cells (Figure 22A, B).

Pericentrin contains a strong centrosome-targeting domain referred to as the pericentrin-AKAP450 centrosomal targeting (PACT) domain (Gillingham and Munro, 2000). On the basis of this feature, we examined whether ectopic pericentrin can accumulate at mitotic spindle poles in CEP215-depleted cells. The results showed that despite the PACT domain, ectopic

pericentrin was barely detectable at the spindle poles of CEP215-depleted cells (Figure 23A, B). Thus, CEP215 is critical for pericentrin accumulation at the spindle pole during centrosome maturation. I next performed rescue experiments to determine the importance of the interaction between CEP215 and pericentrin for centrosome maturation. CEP215-depleted HeLa cells were rescued with FLAG-tagged CEP215 or CEP215$^{\Delta C}$, and their cell cycle was synchronized at M phase with RO3306 treatment and removal. Depletion and ectopic expression of CEP215 were confirmed with immunoblot analyses (Figure 24). The results showed that ectopic CEP215 was successfully localized to the spindle poles, whereas ectopic CEP215$^{\Delta C}$ was not (Figure 25A, B). Centrosomal pericentrin levels were significantly reduced upon CEP215 depletion and rescued with wild-type CEP215 (Figure 25A, C). However, the CM2-truncated CEP215 (CEP215$^{\Delta C}$) could not rescue the accumulation of pericentrin at the spindle poles (Figure 25B, C). Because I revealed that CEP215$^{\Delta C}$ itself cannot properly accumulate at the spindle poles, it is possible that pericentrin may not be recruited to the centrosome as a result. To rule out this possibility, I generated the PACT domain-linked CEP215$^{\Delta C}$ (CEP215$^{\Delta C}$-PACT), which is held at the centrosome. A coimmunoprecipitation assay revealed that CEP215$^{\Delta C}$-PACT still could not interact with endogenous pericentrin in mitotic 293T cells (Figure 26). Immunostaining analysis revealed

that the level of CEP215$^{\Delta C}$-PACT accumulation at the spindle poles was greater than that of CEP215$^{\Delta C}$ (Figure 25A, B). However, the centrosomal pericentrin levels were not effectively rescued (Figure 25A, C). These results indicate that the CM2 of CEP215 is necessary for the interaction and subsequent recruitment of pericentrin to the spindle poles. To compare the expression level of ectopically rescued CEP215 with endogenous CEP215, I measured the CEP215 intensities at spindle poles in each CEP215 knockdown-rescued groups (Figure 27A, B). The results indicated that the level of ectopically rescued CEP215 is very similar to that of endogenous CEP215 (Figure 27A, B). It suggested that the condition of CEP215 knockdown-rescue experiments is ideal to interpret the results.

The physical interaction of CEP215 with pericentrin is critical for centrosome maturation and bipolar spindle formation during mitosis

The centrosomal level of γ-tubulin reflects the centrosome maturation status. Therefore, I determined the centrosomal γ-tubulin levels in CEP215-rescued mitotic cells. As expected, the centrosomal γ-tubulin levels were rescued with wild-type CEP215 (Figure 28A-C). On the other hand, both CEP215$^{\Delta C}$ and CEP215$^{\Delta C}$-PACT did not rescue the centrosomal γ-tubulin levels (Figure 28A-C). I also determined the mitotic spindle formation activity in

CEP215-rescued cells. The morphology of mitotic spindles was categorized as bipole, small bipole or monopole based on the immunostaining pattern of NuMA (Figure 29A-C). The results showed that CEP215 and CEP215-PACT successfully rescued the defects in bipolar spindle formation whereas $CEP215^{\Delta C}$ and $CEP215^{\Delta C}$-PACT did not (Figure 29C). Based on these results, I concluded that the physical interaction between CEP215 and pericentrin is critical for centrosome maturation and bipolar spindle formation during mitosis.

Pericentrin also needs the interaction with CEP215 for its proper accumulation to mitotic spindle poles

I further examined the functional significance of the physical interaction between CEP215 and pericentrin for centrosome maturation using a truncated mutant of pericentrin. I determined that the removal of 17 amino acids (2390-2406, $PCNT^{\Delta 17}$) prevents pericentrin interaction with CEP215 during mitosis (Figure 30). I also utilized a phospho-resistant pericentrin with mutations at S1235 and S1241 ($PCNT^{AA}$); in this mutant, centrosome maturation is prohibited (Lee and Rhee, 2011). A coimmunoprecipitation assay revealed that wild-type PCNT and the $PCNT^{AA}$ mutant physically interacted with CEP215 during mitosis (Figure 30). Pericentrin-depleted HeLa cells were rescued with wild-type and mutant pericentrin proteins, and their localization to

the centrosome was observed. Depletion and ectopic expression of pericentrin were confirmed with immunoblot analyses (Figure 31). The results demonstrated that the accumulation levels of $PCNT^{AA}$ and wild-type pericentrin at the centrosome were similar (Figure 32A, B). In contrast, the $PCNT^{\Delta 17}$ mutant was barely detectable at the spindle poles (Figure 32A, B). I then determined the intensity of CEP215 at the spindle poles in pericentrin-rescued cells. First, CEP215 was not properly recruited to the spindle poles in pericentrin-depleted cells; however, this phenotype was effectively rescued with ectopic pericentrin (FLAG-PCNT; Figure 32A, C). Second, endogenous CEP215 was recruited to the spindle poles of the $PCNT^{AA}$-rescued cells, but not to those of the $PCNT^{\Delta 17}$-rescued cells (Figure 32A, C). These results reveal that pericentrin must interact with CEP215, not only for its own accumulation at the spindle poles but also for centrosomal recruitment of CEP215 during centrosome maturation.

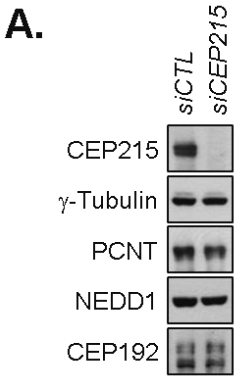
Pericentrin should physically interact with CEP215 for centrosome maturation and mitotic spindle formation

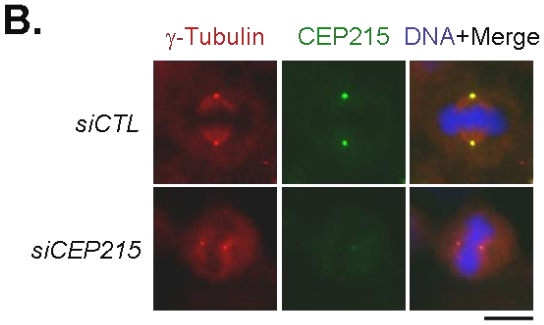
To investigate the functional significance of the CEP215-pericentrin interaction for centrosome maturation, I determined the centrosomal γ-tubulin levels in pericentrin-rescued mitotic cells. The centrosomal γ-tubulin levels

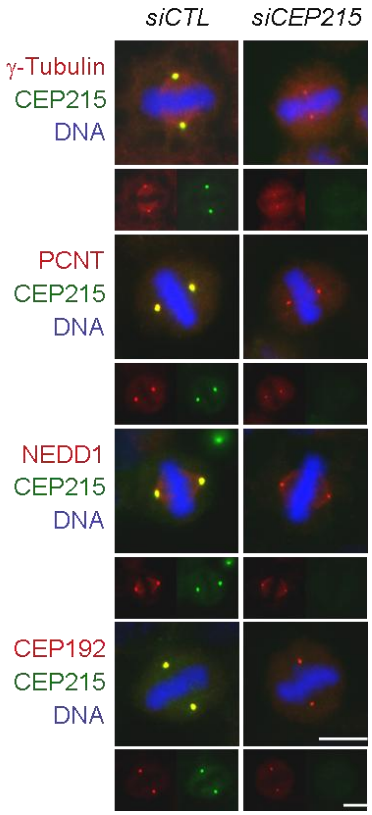
were significantly reduced in pericentrin-depleted cells and were effectively rescued with wild-type pericentrin (Figure 33A, C). The ectopic $PCNT^{AA}$ localized properly at the centrosome but did not recruit γ-tubulin to the centrosome, as reported previously (Figure 33A-C, Lee and Rhee, 2011). On the other hand, the $PCNT^{\Delta 17}$ mutant did not localize at the spindle poles and did not recruit γ-tubulin to the spindle poles (Figure 33A-C). I further determined the mitotic spindle formation activity in the pericentrin-rescued cells. Depletion of pericentrin induced abnormalities in bipolar spindle formation, including small bipole and monopole phenotypes based on NuMA staining patterns (Figure 34A-C). Wild-type pericentrin successfully rescued the defects in bipolar spindle formation (Figure 34C), however, these phenotypes could not be rescued with either the $PCNT^{AA}$ or $PCNT^{\Delta 17}$ mutant (Figure 34C). These results reveal that pericentrin must interact with CEP215 and must be phosphorylated by PLK1 to ensure centrosome maturation and subsequent bipolar spindle formation during mitosis.

Figure 20. Reduction of PCM proteins at the spindle poles of CEP215-depleted mitotic cells.

(A) HeLa cells were transfected with nonspecific control (*siCTL*) or CEP215-specific (*siCEP215*) siRNAs for 48 hrs. The cell lysates were then harvested for immunoblot analysis with the indicated antibodies. (B) The CEP215-depleted HeLa cells were co-immunostained with γ-tubulin (red) and CEP215 (green) antibodies. Scale bar; 10 μm. (C) The CEP215-depleted mitotic cells were coimmunostained with CEP215 antibody (green) along with antibodies for γ-tubulin, pericentrin (PCNT), NEDD1 and CEP192 (red). Scale bar; 10 μm. (D) Relative intensities of γ-tubulin, pericentrin, NEDD1 and CEP192 at the spindle poles of CEP215-depleted cells were quantified in more than 40 cells per group in three independent experiments. Error bars; SEM.


A.
siCTL
siCEP215
CEP215
γ-Tubulin
PCNT
NEDD1
CEP192


B.
γ-Tubulin
CEP215
DNA+Merge
siCTL
siCEP215


C.
siCTL
siCEP215
γ-Tubulin
CEP215
DNA
PCNT
CEP215
DNA
NEDD1
CEP215
DNA
CEP192
CEP215
DNA

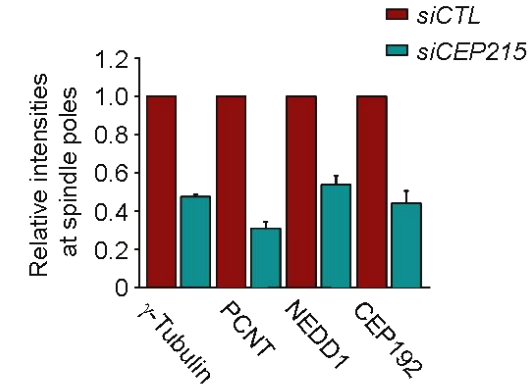

D.
siCTL
siCEP215
Relative intensities at spindle poles
1.2
1.0
0.8
0.6
0.4
0.2
0
γ-Tubulin
PCNT
NEDD1
CEP192

Figure 21. Physical interaction of CEP215 with pericentrin during mitosis. (A) Mitotic HEK293T cells were subjected to immunoprecipitation with pre-immune (PI) or CEP215-specific (CEP215) serum followed by immunoblot analysis with antibodies specific to CEP215, pericentrin and NEDD1. (B) HEK293T cells transfected with FLAG-tagged GFP (Con), CEP215 ($CEP215^{WT}$) and $CEP215^{\Delta 1726\text{-}1893}$ ($CEP215^{\Delta C}$) were treated with STLC for 16 hrs to synchronize the cells at mitosis. The mitotic cell lysates were subjected to immunoprecipitation with FLAG resin followed by immunoblot analysis with pericentrin and FLAG antibodies

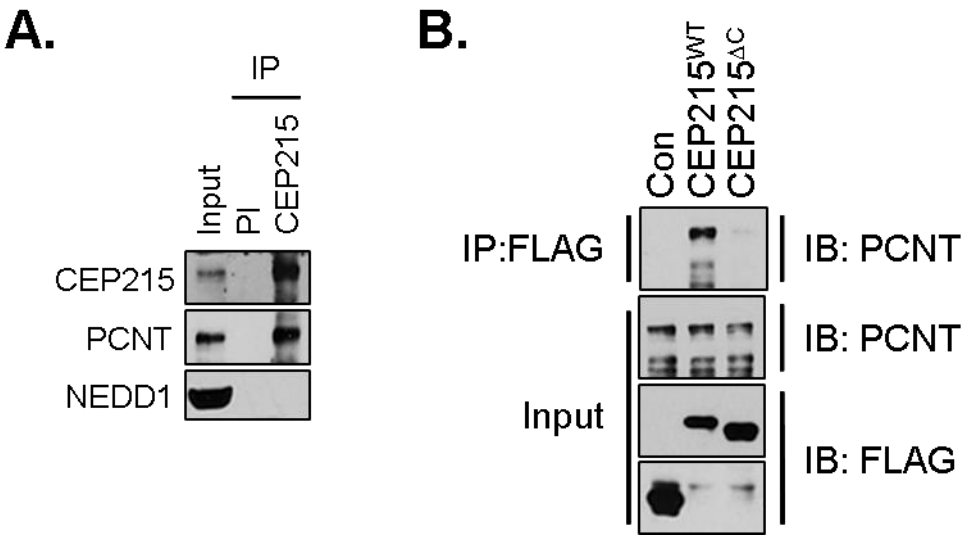

A.
IP
Input
PI
CEP215
CEP215
PCNT
NEDD1
B.
Con
CEP215WT
CEP215ΔC
IP:FLAG
IB: PCNT
IB: PCNT
Input
IB: FLAG

Figure 22. The subcellular localization of CEP215 (WT) and CEP215$^{\Delta 1726-1893}$ (ΔC) during cell cycle.

HeLa cells were transfected with FLAG-tagged CEP215 (WT) and CEP215$^{\Delta 1726-1893}$ (ΔC). The cells were coimmunostained with (A) γ-tubulin (red) or (B) pericentrin (PCNT, red) antibody along with the FLAG (green) antibody. Scale bar; 10 μm.

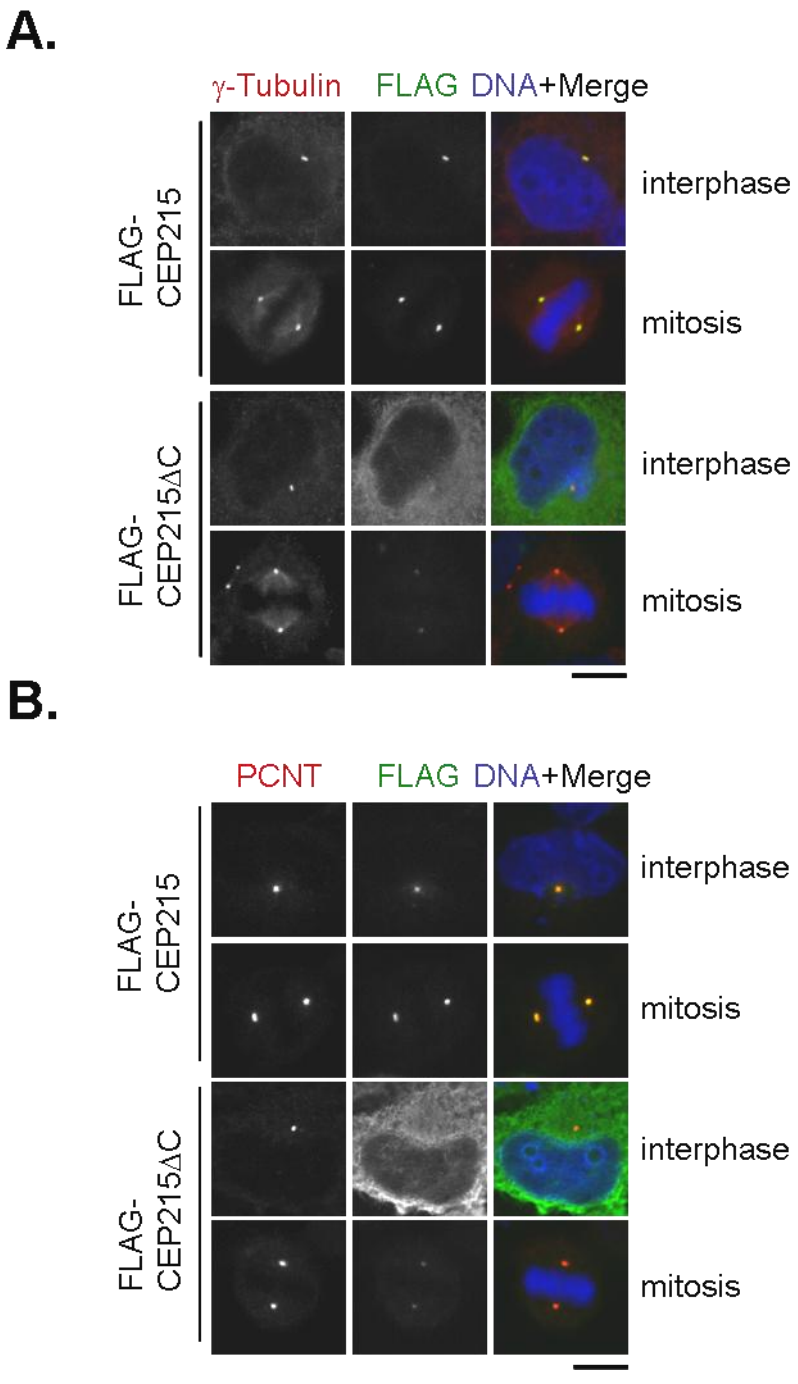

A.
γ-Tubulin
FLAG
DNA+Merge
FLAG-CEP215
interphase
mitosis
FLAG-CEP215ΔC
interphase
mitosis
B.
PCNT
FLAG
DNA+Merge
FLAG-CEP215
interphase
mitosis
FLAG-CEP215ΔC
interphase
mitosis

Figure 23. Ectopic expression of pericentrin in CEP215-depleted cells.

(A, B) Ectopic FLAG-PCNT was expressed in the CEP215-depleted HeLa cells. Scale bar; 10 μm. The intensity of FLAG-PCNT at the spindle poles was determined in more than 40 cells per experimental group in two independent experiments. Error bars; SEM.

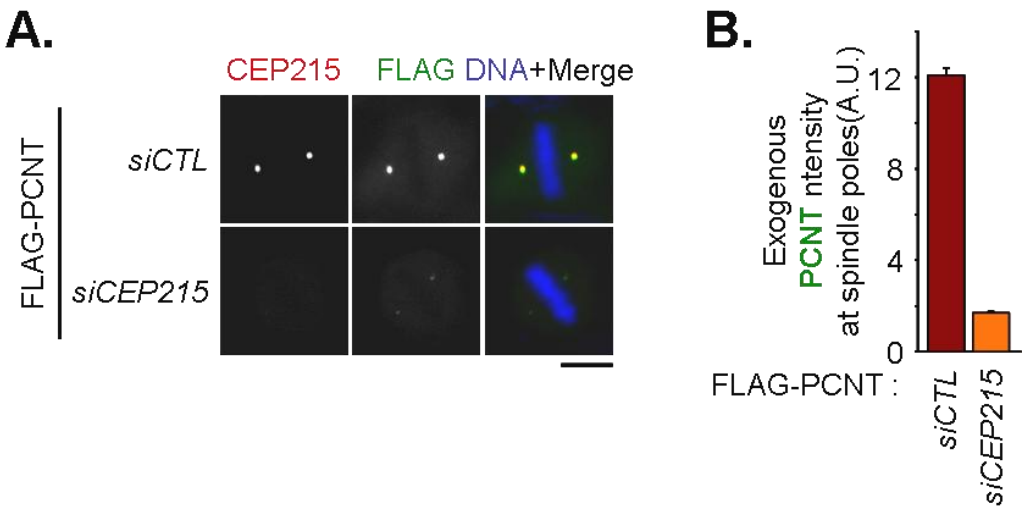

A.
CEP215
FLAG DNA+Merge
FLAG-PCNT
siCTL
siCEP215
B.
Exogenous PCNT ntensity at spindle poles(A.U.)
12
8
4
0
FLAG-PCNT :
siCTL
siCEP215

Figure 24. Immunoblot analyses to confirm the knockdown and rescue of CEP215.

CEP215-depleted HeLa cells were rescued with FLAG-tagged GFP (GFP), CEP215 (WT), CEP215$^{\Delta 1726\text{-}1893}$ (ΔC), CEP215-PACT (WT-PT) and CEP215$^{\Delta 1726\text{-}1893}$-PACT (ΔC-PT). Then, the cells were subjected to immunoblot analysis with indicated antibodies.

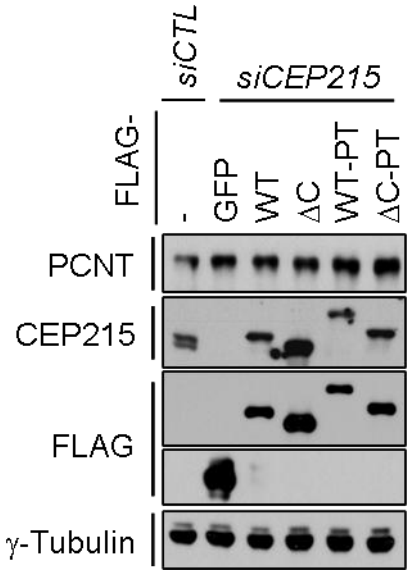

siCTL
siCEP215
FLAG-
-
GFP
WT
ΔC
WT-PT
ΔC-PT
PCNT
CEP215
FLAG
γ-Tubulin

Figure 25. The physical interaction of CEP215 with pericentrin is critical for the pericentrin recruitment to mitotic spindle poles.

(A-C) CEP215-depleted HeLa cells were rescued with FLAG-tagged CEP215 (WT) and CEP215$^{\Delta 1726\text{-}1893}$ (ΔC), CEP215-PACT (WT-PACT) and CEP215$^{\Delta 1726\text{-}1893}$-PACT (ΔC-PACT). The cells were treated with RO3306 for 16 hrs and subsequently removed for 40 minutes to allow accumulation of mitotic cells. (A) The cells were coimmunostained with pericentrin (red) and FLAG (green) antibodies. Scale bar; 10 μm. The intensities of ectopic CEP215 (B) and endogenous pericentrin (C) at the spindle poles were quantified in more than 40 cells per group in three independent experiments. Error bars; SEM.

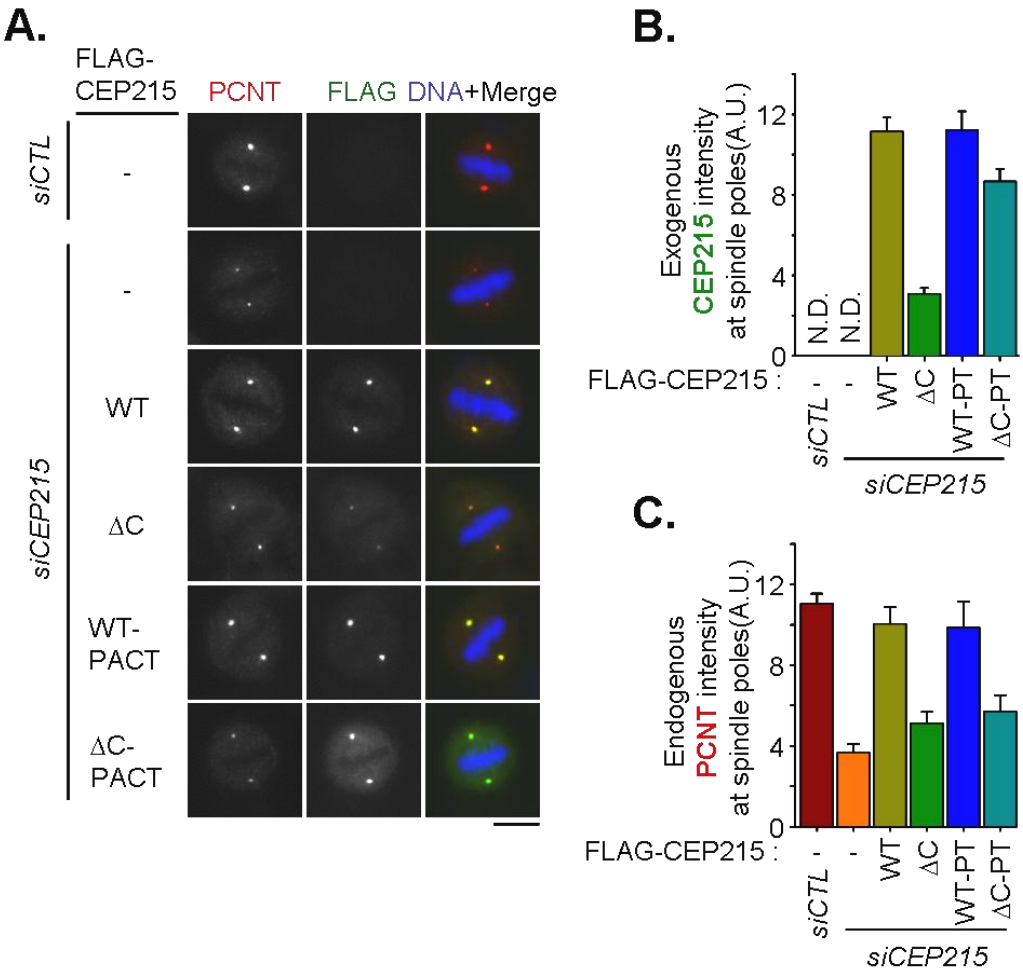

A.
FLAG-CEP215
PCNT
FLAG
DNA+Merge
siCTL
siCEP215
-
-
WT
ΔC
WT-PACT
ΔC-PACT
B.
Exogenous CEP215 intensity at spindle poles(A.U.)
12
8
4
0
N.D.
N.D.
FLAG-CEP215 :
siCTL
-
-
WT
ΔC
WT-PT
ΔC-PT
siCEP215
C.
Endogenous PCNT intensity at spindle poles(A.U.)
12
8
4
0
FLAG-CEP215 :
siCTL
-
-
WT
ΔC
WT-PT
ΔC-PT
siCEP215

Figure 26. The physical interaction of the each CEP215 constructs with pericentrin during mitosis.

HEK293T cells transfected with FLAG-tagged GFP (Con), CEP215 ($CEP215^{WT}$) and $CEP215^{\Delta 1726\text{-}1893}$ ($CEP215^{\Delta C}$), CEP215-PACT ($CEP215^{WT\text{-}PACT}$) and $CEP215^{\Delta 1726\text{-}1893}$-PACT ($CEP215^{\Delta C\text{-}PACT}$) were treated with STLC for 16 hrs to synchronize the cells at mitosis. The mitotic cell lysates were subjected to immunoprecipitation with FLAG resin followed by immunoblot analysis with pericentrin and FLAG antibodies.

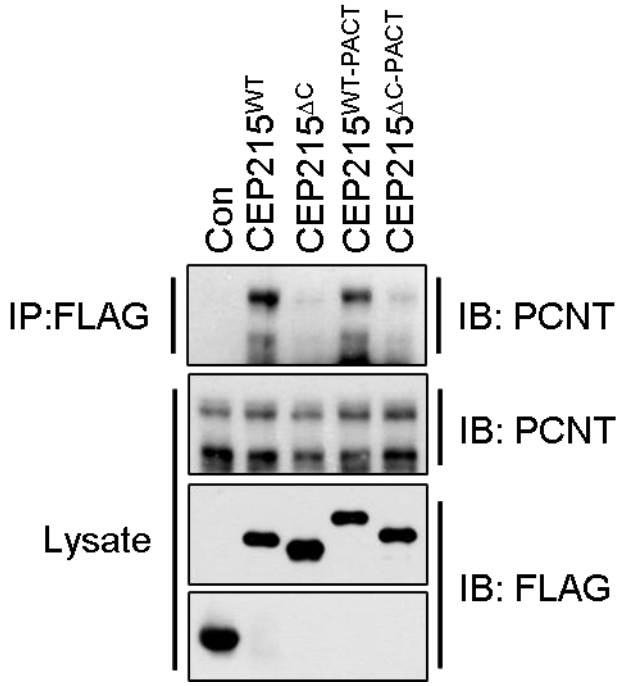

Con
CEP215WT
CEP215ΔC
CEP215WT-PACT
CEP215ΔC-PACT
IP:FLAG
IB: PCNT
IB: PCNT
Lysate
IB: FLAG

Figure 27. The comparison of the expression level of ectopically rescued CEP215 with the endogenous CEP215 at the spindle poles.

(A) CEP215-depleted HeLa cells were rescued with FLAG-tagged CEP215 (WT), F75A mutant CEP215 (F75A), CEP215$^{\Delta 1726\text{-}1893}$ (ΔC), CEP215-PACT (WT-PACT) and CEP215$^{\Delta 1726\text{-}1893}$-PACT (ΔC-PACT). The cells were treated with RO3306 for 16 hrs and subsequently removed for 40 minutes to allow accumulation of mitotic cells. The cells were coimmunostained with CEP215 (red) and FLAG (green) antibodies. Scale bar; 10 μm. (B) The intensities of CEP215 signal at the spindle poles were quantified in more than 20 cells per group in three independent experiments. Error bars; SEM.

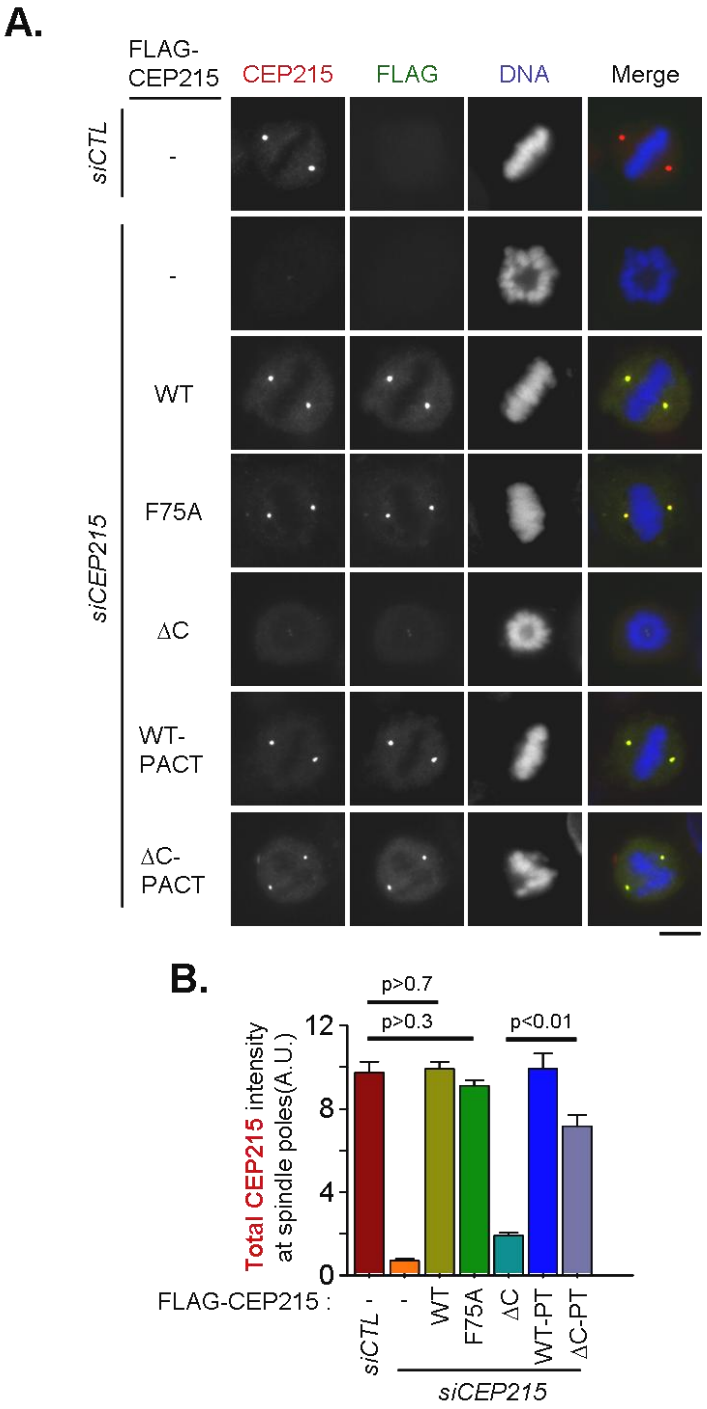

A.
FLAG-CEP215
CEP215
FLAG
DNA
Merge
siCTL
-
siCEP215
-
WT
F75A
ΔC
WT-PACT
ΔC-PACT
B.
p>0.7
p>0.3
p<0.01
Total CEP215 intensity at spindle poles(A.U.)
12
8
4
0
FLAG-CEP215 :
siCTL
-
WT
F75A
ΔC
WT-PT
ΔC-PT
siCEP215

Figure 28. The physical interaction of CEP215 with pericentrin is critical for γ-tubulin recruitment to mitotic spindle poles.

(A-C) CEP215-depleted HeLa cells were rescued with FLAG-tagged CEP215 (WT), CEP215$^{\Delta 1726\text{-}1893}$ (ΔC), CEP215-PACT (WT-PACT) and CEP215$^{\Delta 1726\text{-}1893}$-PACT (ΔC-PACT). The cells were treated with RO3306 for 16 hrs and subsequently removed for 40 minutes to allow accumulation of mitotic cells. The cells were coimmunostained with γ-tubulin (red) and FLAG (green) antibodies. Scale bar; 10 μm. The intensities of ectopic CEP215 (B) and endogenous γ-tubulin (C) at the spindle poles were quantified in more than 40 cells per group in three independent experiments. Error bars; SEM.

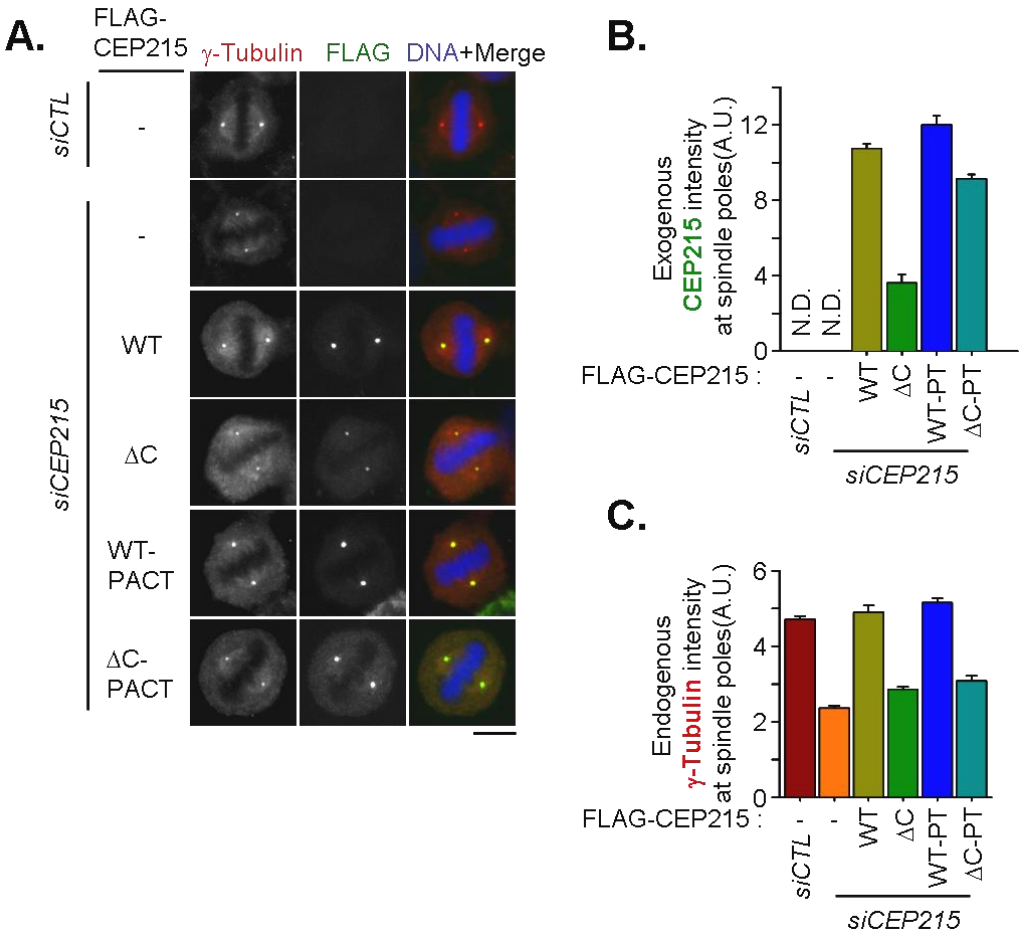

A.
FLAG-CEP215
γ-Tubulin
FLAG
DNA+Merge
siCTL
siCEP215
-
-
WT
ΔC
WT-PACT
ΔC-PACT
B.
Exogenous CEP215 intensity at spindle poles(A.U.)
12
8
4
0
N.D.
N.D.
FLAG-CEP215 :
siCTL
-
WT
ΔC
WT-PT
ΔC-PT
siCEP215
C.
Endogenous γ-Tubulin intensity at spindle poles(A.U.)
6
4
2
0
FLAG-CEP215 :
siCTL
-
WT
ΔC
WT-PT
ΔC-PT
siCEP215

Figure 29. The physical interaction of CEP215 with pericentrin is important for the bipolar spindle formation.

(A, B) HeLa cells were transfected with *siCTL* and *siCEP215*. After 48 hrs, the cells were coimmunostained with (A) CEP215 (red) and NuMA (green) antibodies. Scale bar; 10 μm. (B) CEP215-depleted HeLa cells were coimmunostained with α-tubulin (red) and NuMA (green) antibodies. The phenotype of the bipolar spindle was categorized as bipole, small bipole or monopole based on the NuMA staining patterns. Scale bar; 10 μm. (C) The CEP215-depleted HeLa cells were rescued with the FLAG-tagged CEP215 (WT), CEP215$^{\Delta 1726\text{-}1893}$ (ΔC), CEP215-PACT (WT-PACT) and CEP215$^{\Delta 1726\text{-}1893}$-PACT (ΔC-PACT). The cells were treated with RO3306 for 16 hrs and released with STLC for additional 1 hr to arrest the cells at prometaphase. Then, the cells were washed and re-incubated with MG132 for 1.5 hrs to block the exit from mitosis. The cells were coimmunostained with FLAG and NuMA antibodies. The phenotype of the bipolar spindle was categorized as bipole, small bipole or monopole based on the NuMA staining patterns. Error bars; SEM.

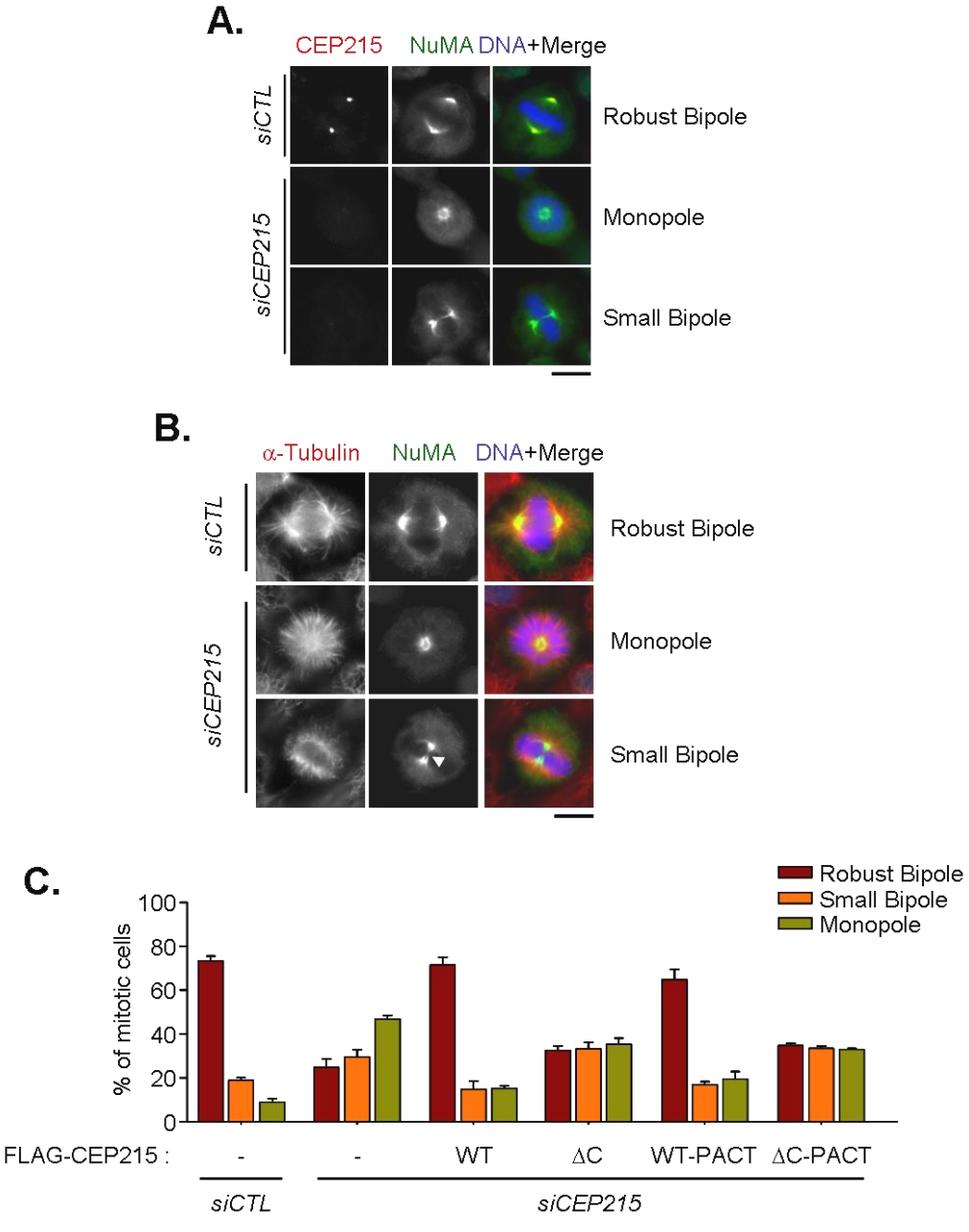

A.
CEP215
NuMA
DNA+Merge
siCTL
siCEP215
Robust Bipole
Monopole
Small Bipole
B.
α-Tubulin
NuMA
DNA+Merge
siCTL
siCEP215
Robust Bipole
Monopole
Small Bipole
C.
Robust Bipole
Small Bipole
Monopole
% of mitotic cells
100
80
60
40
20
0
FLAG-CEP215 :
-
-
WT
ΔC
WT-PACT
ΔC-PACT
siCTL
siCEP215

Figure 30. Confirmation of the physical interaction of pericentrin with CEP215 using pericentrin constructs.

HEK293T cells transfected with FLAG-tagged GFP (Con), PCNT ($PCNT^{WT}$), $PCNT^{1235,1241AA}$ ($PCNT^{1235,1241AA}$) and $PCNT^{\Delta 2390\text{-}2406}$ ($PCNT^{\Delta 17}$) were treated with STLC for 16 hrs to synchronize the cells at mitosis. The mitotic cell lysates were subjected to immunoprecipitation with FLAG resin followed by immunoblot analysis with CEP215 and FLAG antibodies.

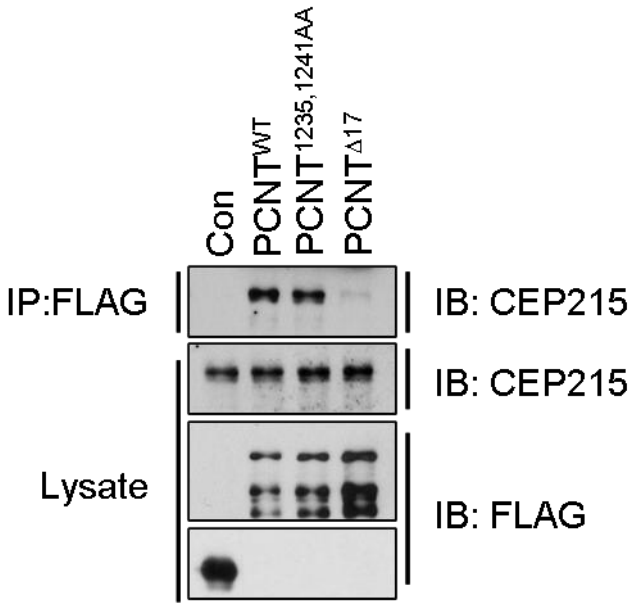

Con
PCNT^WT
PCNT^1235,1241AA
PCNT^Δ17
IP:FLAG
IB: CEP215
IB: CEP215
Lysate
IB: FLAG

Figure 31. Immunoblot analyses to confirm the knockdown and rescue of pericentrin.

Pericentrin-depleted HeLa cells were rescued with FLAG-tagged GFP (GFP), PCNT (WT), $PCNT^{1235,1241AA}$ (AA) and $PCNT^{\Delta 2390\text{-}2406}$ (Δ17). Then, the cells were subjected to immunoblot analysis with indicated antibodies.

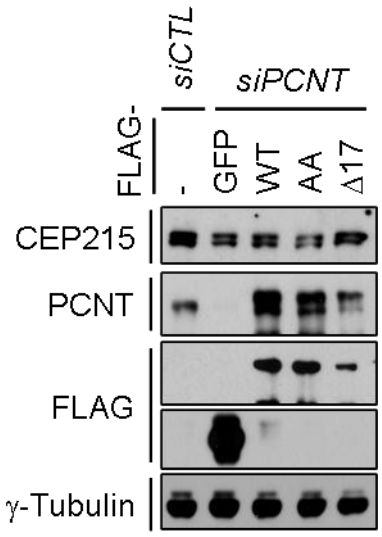

siCTL
siPCNT
FLAG-
-
GFP
WT
AA
Δ17
CEP215
PCNT
FLAG
γ-Tubulin

Figure 32. Pericentrin also needs the interaction with CEP215 for its proper accumulation and CEP215 recruitment to mitotic spindle poles.

(A-C) The pericentrin-depleted HeLa cells were rescued with FLAG-tagged PCNT (WT), $PCNT^{1235,1241AA}$ (AA) and $PCNT^{\Delta 2390\text{-}2406}$ (Δ17). The cells were treated with RO3306 for 16 hrs and subsequently removed for 40 minutes to allow accumulation of mitotic cells. The cells were coimmunostained with CEP215 (red) and FLAG (green) antibodies. Scale bar; 10 μm. The intensities of ectopic pericentrin (B) and endogenous CEP215 (C) at the spindle poles were quantified in more than 40 cells per group in three independent experiments. Error bars; SEM.

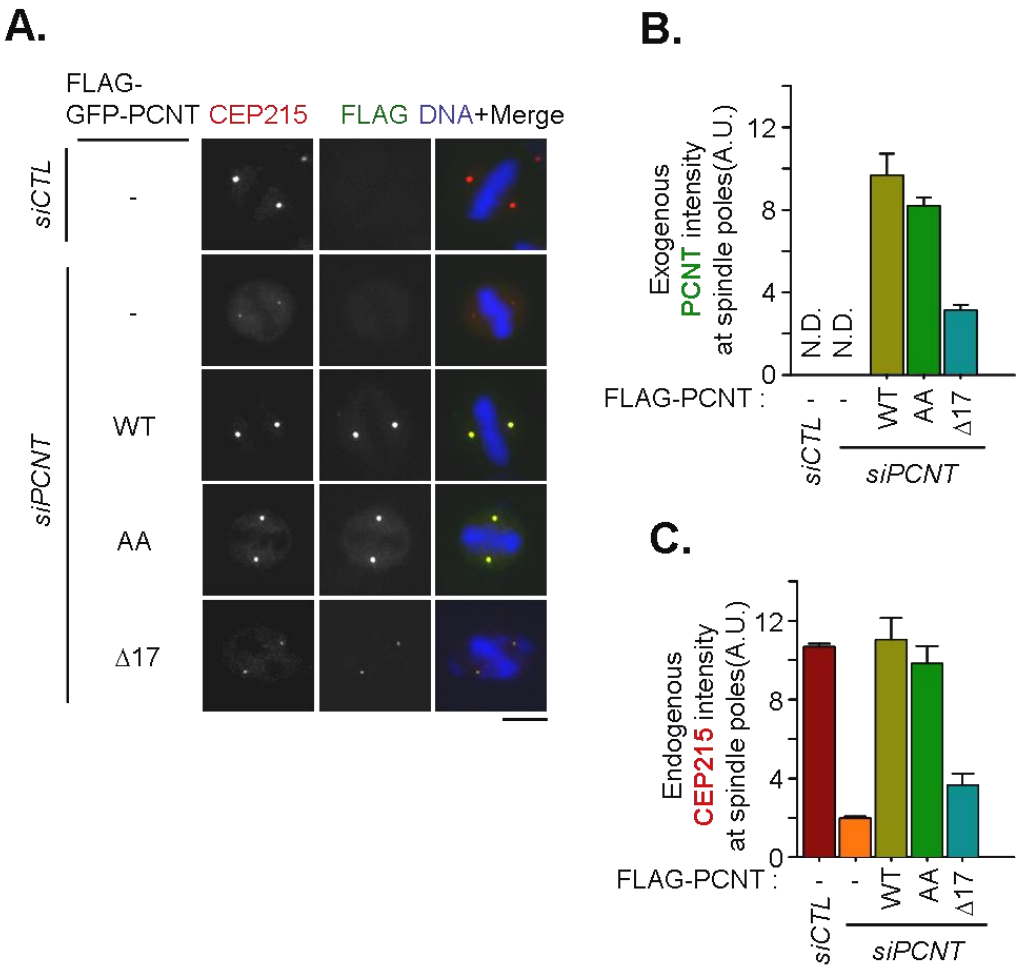

A.
FLAG-
GFP-PCNT
CEP215
FLAG
DNA+Merge
siCTL
siPCNT
-
-
WT
AA
Δ17
B.
Exogenous
PCNT intensity
at spindle poles(A.U.)
12
8
4
0
N.D.
N.D.
FLAG-PCNT :
-
-
WT
AA
Δ17
siCTL
siPCNT
C.
Endogenous
CEP215 intensity
at spindle poles(A.U.)
12
8
4
0
FLAG-PCNT :
-
-
WT
AA
Δ17
siCTL
siPCNT

Figure 33. The physical interaction of pericentrin with CEP215 is essential for the γ-tubulin recruitment to the spindle poles.

(A-C) Pericentrin-depleted HeLa cells were rescued with FLAG tagged PCNT (WT), $PCNT^{1235,1241AA}$ (AA) and $PCNT^{\Delta 2390\text{-}2406}$ (Δ17). The cells were treated with RO3306 for 16 hrs and subsequently removed for 40 minutes to allow accumulation of mitotic cells. The cells were coimmunostained with γ-tubulin (red) and FLAG (green) antibodies. Scale bar; 10 μm. The intensities of ectopic pericentrin (B) and endogenous γ-tubulin (C) at the spindle poles were quantified in more than 40 cells per group in three independent experiments. Error bars; SEM.

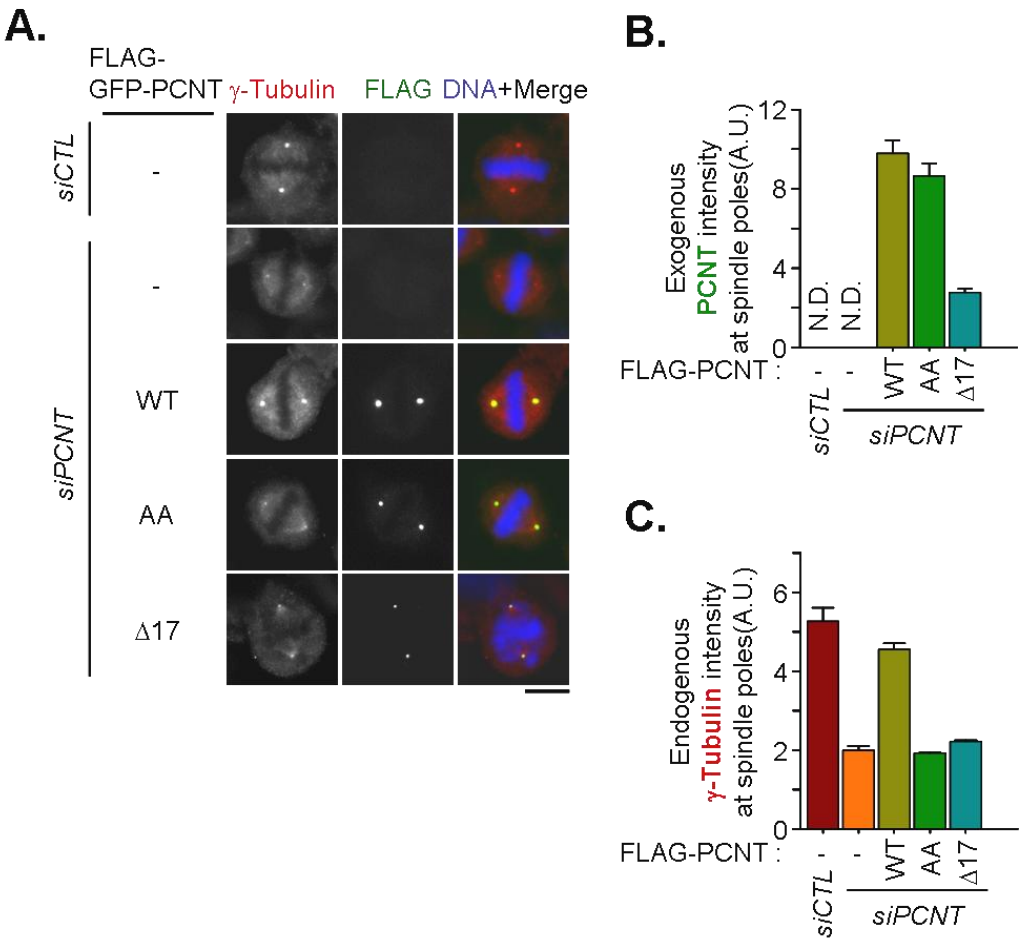

A.
FLAG-
GFP-PCNT
γ-Tubulin
FLAG
DNA+Merge
siCTL
siPCNT
-
-
WT
AA
Δ17
B.
Exogenous
PCNT intensity
at spindle poles(A.U.)
12
8
4
0
N.D.
N.D.
FLAG-PCNT :
-
-
WT
AA
Δ17
siCTL
siPCNT
C.
Endogenous
γ-Tubulin intensity
at spindle poles(A.U.)
6
4
2
0
FLAG-PCNT :
-
-
WT
AA
Δ17
siCTL
siPCNT

Figure 34. Pericentrin should interact with CEP215 for centrosome maturation and subsequent bipolar spindle formation.

(A, B) HeLa cells were transfected with *siCTL*, and *siPCNT*. After 48 hrs, the cells were coimmunostained with (A) pericentrin (red) and NuMA (green) antibodies. Scale bar; 10 μm. (B) The pericentrin-depleted cells were coimmunostained with α-tubulin (red) and NuMA (green) antibodies. The phenotype of the bipolar spindle was categorized as bipole (completely separated NuMA), small bipole (arrowhead; inter-bridged NuMA) or monopole (round-shaped NuMA) based on the NuMA staining patterns. Scale bar; 10 μm. (C) PCNT-depleted HeLa cells were rescued with FLAG-tagged PCNT (WT), $PCNT^{1235,1241AA}$ (AA) and $PCNT^{\Delta 2390\text{-}2406}$ (Δ17). The cells were treated with RO3306 for 16 hrs and released with STLC for additional 1 hr to arrest the cells at prometaphase. Then, the cells were washed and re-incubated with MG132 for 1.5 hrs to block the exit from mitosis. The cells were coimmunostained with FLAG and NuMA antibodies. The phenotype of the bipolar spindle was categorized as bipole, small bipole or monopole based on the NuMA staining patterns. Error bars; SEM.

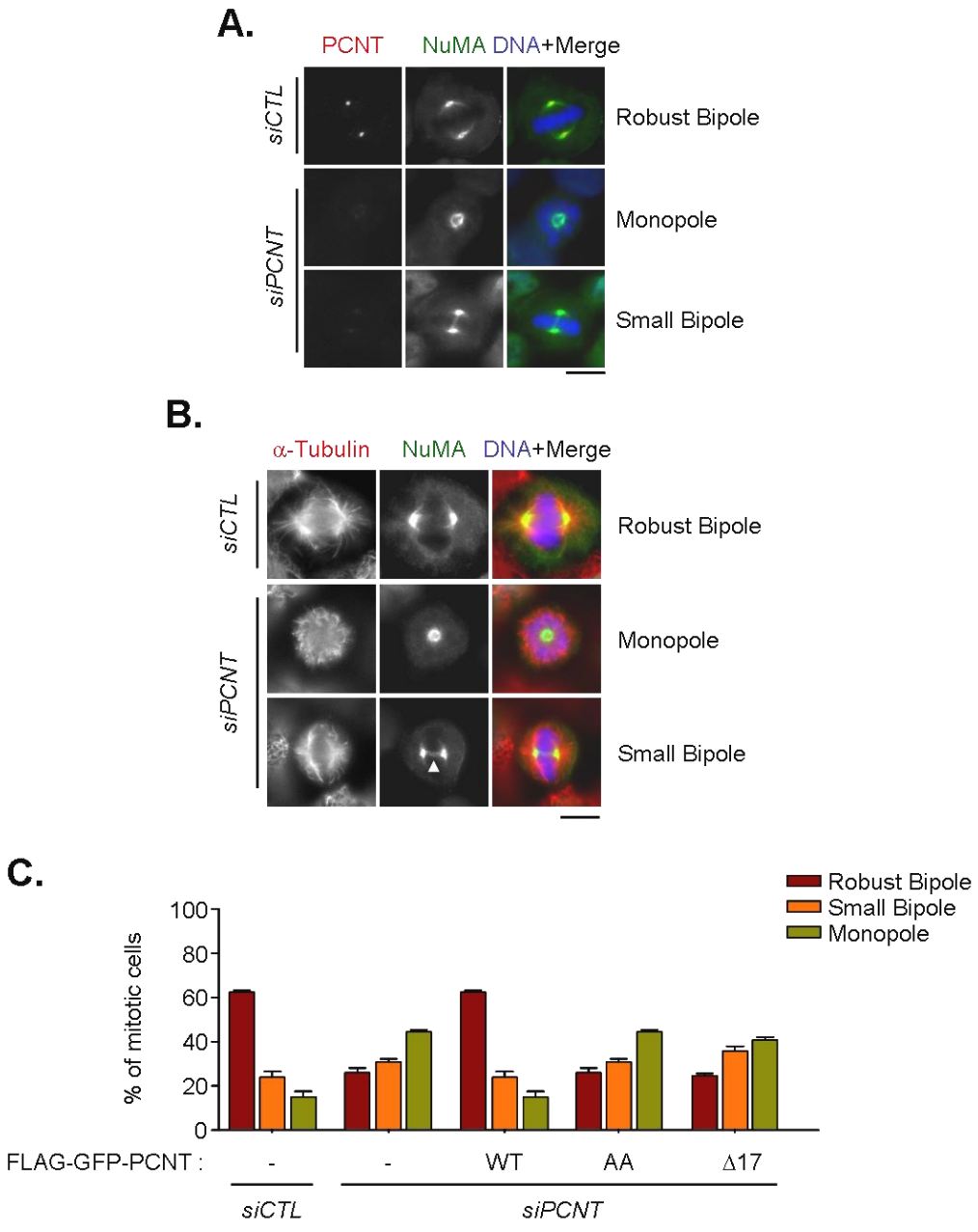
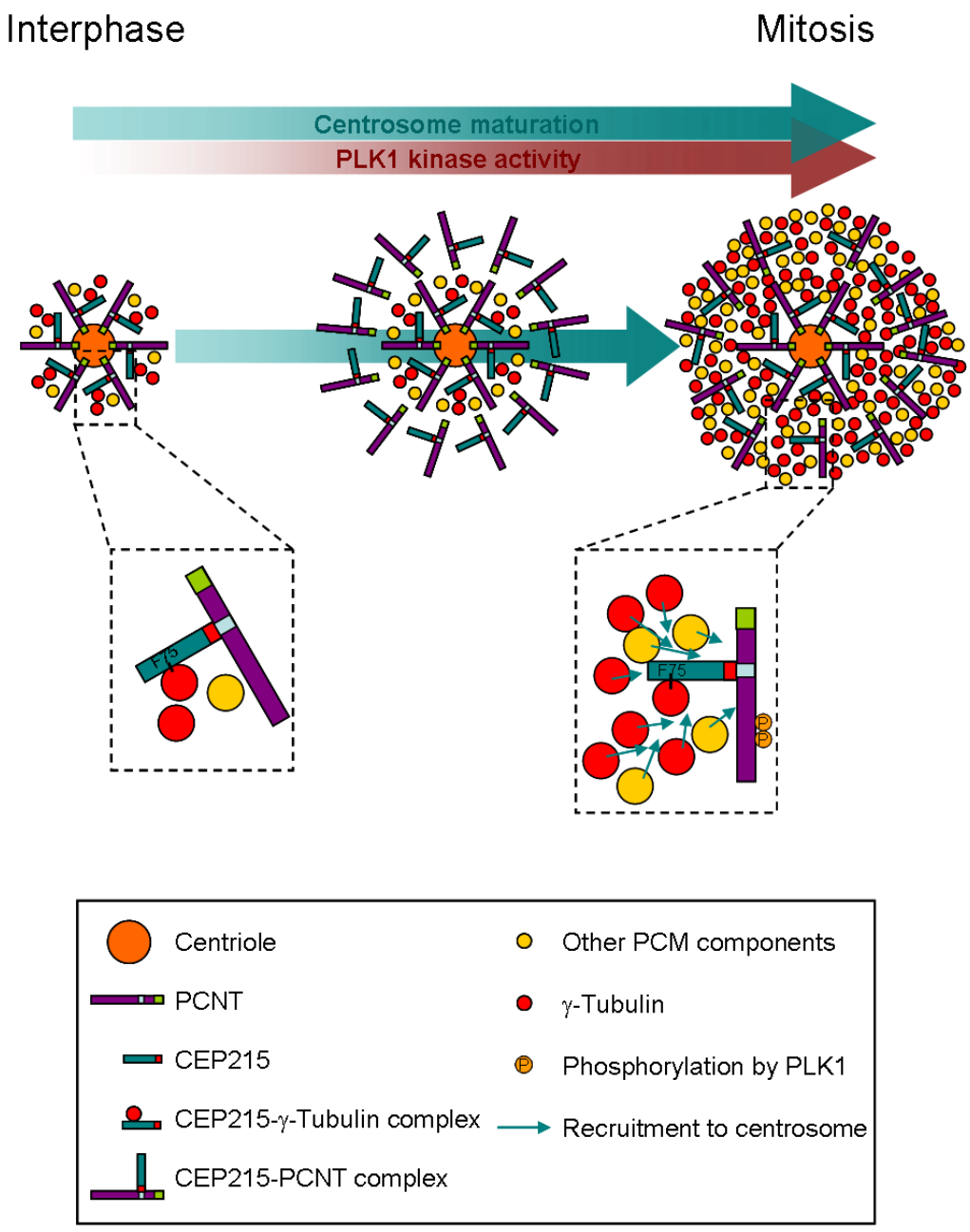

A.
PCNT
NuMA DNA+Merge
siCTL
siPCNT
Robust Bipole
Monopole
Small Bipole
B.
α-Tubulin
NuMA
DNA+Merge
siCTL
siPCNT
Robust Bipole
Monopole
Small Bipole
C.
Robust Bipole
Small Bipole
Monopole
% of mitotic cells
100
80
60
40
20
0
FLAG-GFP-PCNT :
-
-
WT
AA
Δ17
siCTL
siPCNT

Figure 35. An integrative working model.

In interphase, the PCM structure is basically maintained by pericentrin. CEP215 may be recruited to the centrosome through a specific interaction with pericentrin. And the γ-tubulin recruitment to interphase centrosome is sufficiently accomplished by the physical interaction with CEP215 through it F75 residue. On the other hand, PCM accumulation mechanisms in mitotic cells are quite different from those in interphase cells. The interaction of CEP215 with γ-tubulin seemed to be not enough or not critical for the centrosome maturation. As a result, CEP215 and pericentrin are interdependent for their proper accumulation to mitotic centrosome. They physically interact each other and cooperatively drive the centrosome maturation and subsequent bipolar spindle formation. In addition, once pericentrin is phosphorylated by PLK1, downstream PCM components would be recruited to the centrosomes, which become spindle poles with an eminent microtubule organizing activity.


Interphase
Mitosis
Centrosome maturation
PLK1 kinase activity
F73
F75
P
P
Centriole
PCNT
CEP215
CEP215-γ-Tubulin complex
CEP215-PCNT complex
Other PCM components
γ-Tubulin
Phosphorylation by PLK1
Recruitment to centrosome

Discussion

Physical interaction between CEP215 and pericentrin has been previously reported (Buchman et al., 2010; Wang et al., 2010). However, importance of this interaction during mitosis has not been clearly elucidated yet. In this study, we revealed that the CEP215-pericentrin interaction is essential for their interdependent accumulation to mitotic centrosomes and eventually for spindle pole formation in mitotic cells.

The organizational features of PCM have been examined with sub-diffraction fluorescence microscopy. The analyses of interphase centrosome revealed that the C-terminal end of pericentrin is positioned at the centriole wall and radiates outward into the matrix (Lawo et al., 2012; Mennella et al., 2012). The PACT domain at the C-terminal end might play an important role in pericentrin attachment to the centriole wall in interphase cells (Gillingham and Munro, 2000). Depletion of pericentrin results in reduction of CEP215 at the interphase centrosome (Graser et al., 2007; Buchman et al., 2010). However, CEP215 depletion did not affect the centrosomal pericentrin levels in interphase cells (Graser et al., 2007; Buchman et al., 2010). It was also reported that a CEP215 mutant without the pericentrin-interaction domain CM2 could not be

located on the interphase centrosome (Wang et al., 2010). These results suggest that the PCM structure in interphase centrosome is primarily maintained by pericentrin. CEP215 may be recruited to the centrosome through a specific interaction with pericentrin (Figure 35).

PCM accumulation mechanisms in mitotic cells are quite different from those in interphase cells. Pericentrin is still required for mitotic spindle pole formation as shown by the knockdown experiments (Lee and Rhee, 2011; Haren et al., 2009; Lawo et al., 2012). Unlike interphase cells, CEP215 is also critical for spindle pole formation in mitotic cells. First, we and others showed that CEP215 depletion results in reduction of the centrosomal pericentrin levels in mitotic cells (Haren et al., 2009; Lawo et al., 2012). Second, neither $CEP215^{\Delta C}$ nor $PCNT^{\Delta 17}$was sufficiently accumulated to the mitotic centrosome because they were not able to form CEP215-pericentrin complexes. Finally, the centrosomal pericentrin levels remained low in $CEP215^{\Delta C}$-rescued mitotic cells. The centrosomal CEP215 levels also remained low in $PCNT^{\Delta 17}$-rescued cells, indicating that the localization of CEP215 and pericentrin to spindle poles is interdependent during mitosis. Therefore, the CEP215-pericentrin complex is essential for centrosome maturation and eventually spindle pole formation during mitosis.

We previously demonstrated that PLK1 phosphorylation of pericentrin

is a critical step for PCM accumulation in centrosome maturation during mitosis (Lee and Rhee, 2011). CEP215 is already placed at the centrosome irrespective of the phosphorylation status of pericentrin (Lee and Rhee, 2011). Therefore, we propose a model in which the CEP215-pericentrin complex provides a proper environment for accumulation of the other PCM components in maturing centrosomes (Figure 35). Once pericentrin is phosphorylated by PLK1, additional PCM would be recruited to the centrosomes, which become spindle poles with an eminent microtubule organizing activity (Figure 35). If CEP215 was not properly positioned along with pericentrin, PCM components might not be recruited, and bipolar spindles might not be formed during mitosis (Fong et al., 2008; Lee and Rhee, 2010; Haren et al., 2009). The precise mechanisms by which the CEP215-pericentrin complex recruits PCM components to the spindle poles remain to be investigated. However, the physical interaction of CEP215 with γ-tubulin may not play a critical role in γ-tubulin recruitment to spindle poles because a CEP215 mutant that cannot interact with γ-tubulin ($CEP215^{F75A}$) effectively rescued the CEP215-depletion phenotypes in mitotic cells. Instead, we propose that γTuRC might specifically interact with a group of PCM components that are recruited during centrosome maturation (Figure 35). In fact, additional PCM components such as NEDD1 are known to be essential for centrosomal recruitment of γTuRC for spindle

pole formation (Haren et al., 2006; Luders et al., 2006; Zhu et al., 2008; Haren et al., 2009).

Mutations in *CEP215* are linked to primary microcephaly, a congenital disease that causes a substantial reduction in brain size at birth (Bond et al., 2005). Natural *CEP215* mutant mice also have a small cerebral cortex that results from an overall reduction of neuronal layers (Lizarraga et al., 2010). The neuronal progenitor pool in CEP215 mutant mice may be prematurely depleted due to defects in centrosome maturation and/or mitotic spindle orientation (Lizarraga et al., 2010). Furthermore, there is evidence that CEP215 interaction with pericentrin is critical for the microcephaly phenotypes. First, CEP215 depletion leads to a premature neuronal differentiation, and pericentrin depletion phenocopies this effect (Buchman et al., 2010). Second, mutant CEP215 proteins in microcephaly patients have lost large regions of their C-termini, including the pericentrin-interacting domain (Bond et al., 2005; Hassan et al., 2007; Pagnamenta et al., 2012). In fact, mutations in pericentrin are linked to a number of congenital disorders, including microcephalic osteodysplastic primordial dwarfism type II (Rauch et al., 2008; Delaval and Doxsey, 2010). Thus, our research may provide insights into the molecular mechanisms of congenital brain disease.

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국문초록

대부분의 동물세포에서의 중심체는 미세소관 형성의 중추기관으로 기능하며, 세포의 모양, 이동, 세포 내 물질 수송 및 세포 분열을 조절한다. 세포 소기관인 중심체의 기능과 그 메커니즘을 분자적 수준으로 이해하는 연구는 학계의 큰 화두였다. 최근, 인간 중심체를 이용한 단백질체학 연구 결과로 중심체가 수 백 종류의 단백질로 구성되어 있다는 사실이 밝혀졌다. 중심체를 구성하는 구성 인자들은 세포 주기에 따라 급변한다. 수 많은 중심체를 구성하는 단백질들 가운데, CEP215을 선택하여 이의 기능과 기전을 간기 및 세포분열기의 세포에서 규명하고자 연구를 수행했다.

1장에서는, 간기 세포에서의 CEP215의 발현 억제 표현형을 분석했다. CEP215는 이미 몇몇의 중심체의 특성, 예를 들면, 중심체 연결, 미세소관 형성 그리고 중심체 성숙과정에 관여되어 있다는 보고가 있었다. 그러나 이러한 기능에 존재하는 정확한 기작에 대해서는 분석된 바가 없었다. 따라서 본인은 간기 세포에서의

미세소관 형성에 대한 CEP215의 생물학적 기작을 분석했다. 그 결과, CEP215는 γ-tubulin과 물리적으로 결합하며, 이것이 간기 세포에서의 미세소관 형성에 필수적이라는 사실을 확인하였다.

2장에서는 세포분열기에서의 CEP215의 기능과 연관기전을 연구하였다. 앞서, CEP215와 γ-tubulin간의 물리적 결합을 확인했기 때문에 이 결합이 세포 분열기의 중심체 성숙과정에도 기여하는지 조사해 보았으나, 의외로 중심체 성숙과정에는 이들간의 물리적 결합이 결정적인 기여를 하지 않는 것으로 나타났다. 대신, CEP215는 세포 분열기에 pericentrin이라는 또 다른 중심체 단백질과 강력히 결합하며, 이는 중심체 성숙과정과 이어서 양극성 방추사 형성에 매우 중요함을 확인하였다. 이 연구는 세포 분열기 동안 PCM 구성 물질들이 어떻게 결합되어 방추극을 형성하는지에 대한 단초를 마련한 것이라 할 수 있다.

주요어: 미세소관 형성, 중심체 성숙과정, CEP215, γ-tubulin, pericentrin, 단백질간 결합

학번: 2005-20443

감사의 글

참 길고 긴 시간이었습니다. 생명 현상에 대한 막연한 호기심과 생물을 이해하는 것이야 말로 가장 흥미롭고 신비로운 일이라는 생각에서부터 학위 과정의 첫 발걸음을 내딛었습니다. 그 첫 단추를 끼우고 지금 마지막 단추까지 끼우는데, 벌써 8년이라는 시간이 걸렸네요. 이 8년이란 시간 동안, 부족한 저를 믿어주시고 기다려주시고, 따뜻한 위로와 격려를 해주시며 몇 번이나 쓰러져 그만 두고 싶었던 저를 일으켜 세워주신 저의 지도교수이신 이건수 교수님께 이 지면을 빌려 다시 한번 깊은 감사의 마음을 표현하고 싶습니다. 그리고, 아낌없이 저의 학위 논문 심사 및 지도를 해주신, 김경진 위원장님, 김재범 교수님, 설재홍 교수님, 그리고 선 웅 교수님께도 다시 한번 고개 숙여 감사드립니다.

박사 학위 과정 동안, 참 많은 것을 느끼고 배웠습니다. 실험의 어려움도, 해냈다는 성취감도, 실패의 쓰라림도, 지금 돌이켜보니 다 저를 튼튼하고 건강한 학자로 키우는 양분이었습니다. 앞으로 더 넓고 큰 무대가 열릴 것이며, 그 무대에서 제 능력을 마음껏 발휘한다는 것이 얼마나 어려운 일인지 잘 알고 있습니다. 그 어려움, 학위 과정 동안 건강하고 따뜻한 마음과 소신과 의지를 가지고 연구를 할

수 있게 도와주신 많은 분들 생각하며 이겨낼 것이고, 또 하나의 새로운 단추를 끼워보려고 합니다.

저에게도 이런 날이 오기는 하는군요. 언제쯤일까, 언제쯤 나도 학위 심사 발표를 하고, 축하를 받을까.. 한없이 이 순간을 그려왔습니다. 쉽게 그려지지 않더군요. 그런데, 지금 이렇게 지면을 빌려 감사의 글을 쓰고 있네요. 이렇게 끝이 보이지 않는 길을 가고 있는 것에 대해 고민을 하고 있을 후배들에게도 제가 하나의 좋은 본보기와 위로가 될 수 있다면 좋을 것 같습니다.

오늘이 있기까지, 저는 정말 많은 도움을 받아 어려움 없이 이 힘든 공부를 계속해 올 수 있었습니다. 그것을 누구보다도 제가 잘 알고 있지요. 세상의 모든 부모의 마음은 다 똑같다고들 합니다. 하지만, 저에게 엄마, 아빠, 할머니 그리고 할아버지는 세상의 그 어떤 부모의 마음보다도 크고 깊은 울림으로 다가옵니다. 아직도 저를 많이 부족하고 어린 아이처럼 여기시어 항상 걱정과 염려로 지내시는 부모님이 안쓰럽고 가끔은 화가 날 때도 있었습니다. 그 걱정과 염려를 조금이나마 덜어드릴 수 있다면 그것이 효도일까요.. 아무래도 부모님 마음과 은혜에 보답하기엔 아직도 한없이 작은 한걸음이지만, 조금씩 그 걸음도 내딛어보려 합니다.

저는 학위 과정 동안, 또 하나의 가족을 얻었습니다. 세상에서 제일 사랑스럽고 아름다운 다은이를 아내로 맞이했고, 그런 소중한

사람을 힘든 공부를 하는 저에게 기꺼이 허락해주신 어머님, 아버님이 계십니다. 세상에 둘도 없이 사랑스러운 딸을 궤도에 오르지 못한 학생이었던 저에게 허락하신다는 것이 얼마나 큰 결단인지 사위인 제가 잘 알고 있지요. 그 크신 결단과 둘도 없는 사랑스러운 사위로 저를 아껴주심에 오늘 이렇게 보답할 수 있어서, 제가 얼마나 뿌듯한지 모릅니다.

마지막으로, 저 하나만을 보고, 믿고, 의지하며, 기꺼이 사랑으로 저의 부족함을 채워주고, 같이 울고 웃는 제 아내 다은이를 위해 나머지 지면을 할애하고자 합니다. 저는, 아내를 만나고 참 많이 달라졌습니다. 주변 사람들이 느낄 정도였지요. 저의 그 변화 안에, 평생 절대로 잊지 못할 제 아내의 말 한마디가 있습니다. “영광의 순간이 언제든 당신은 분명 그 순간을 맞이할 것이다. 그 순간이 조금 일찍 오거나, 조금 늦게 올 뿐이다. 분명 그 순간은 오리라 믿기에 나는 걱정하지 않는다. 나는 당신을 믿는다. 그러니 약해지지 말아라.” 바로 이 말이 지금의 제가 있게 해준 말입니다. 앞으로도 함께 걸어갈 인생길이 쉽지만은 않겠지만, 그 길을 기꺼이 저와 손 꼭 잡고 걸어가고자 하는 제 아내에게 온 마음을 바쳐 고마움과 사랑으로 이 긴 글을 맺고자 합니다.

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