이학박사학위논문

중심립 유리과정에서 pericentrin 역할 연구

Studies on pericentrin in regulation of centriole separation

2017년 8월

서울대학교 대학원

생명과학부

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이 논문을 이학박사 학위논문으로 제출함
2017년 6월

서울대학교 대학원
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김재연의 이학박사 학위논문을 인준함
2017년 6월

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Studies on pericentrin in regulation of centriole separation

A dissertation submitted in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

to the Faculty of
School of Biological Sciences
at
Seoul National University
by

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Date Approved:

June, 2017

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ABSTRACT

A centrosome is composed of a centriole surrounded by protein matrix, called pericentriolar materials (PCM). The microtubule organization is the prime function of the centrosome. Cilia formation is another important function of the centrosome in quiescent cells. Duplication and segregation of centrioles occur in tight link to cell cycle. A daughter centriole is assembled next to the mother centriole during S phase, and remained in an engaged state until the cell exits M phase. New daughter centrioles may be generated only after the mother and daughter centrioles in the previous cycle are separated. Therefore, centriole separation is considered a licensing step for centriole duplication. However, it is largely unknown how centriole engagement is maintained and disrupted during the cell cycle.

Pericentrin (PCNT) is a PCM protein which is important for maturation process of centrosome to become spindle poles during mitotic entry. PCNT is also involved in induction of centriole separation during mitotic exit. PCNT is specifically cleaved, which is considered an essential step for centriole separation during mitotic exit. The purpose of my research is to elucidate mechanistic aspects of PCNT functions in centriole engagement and separation during M phase.

In chapter 1, I report that PCNT has to be phosphorylated by PLK1 in order to be a suitable substrate of separase. The phospho-resistant mutants of PCNT are not cleaved by separase and eventually inhibit centriole separation.

Furthermore, phospho-mimetic PCNT mutants rescue centriole separation even in the presence of BI2536. Based on these results, I propose that PLK1 phosphorylation is a priming step for separase-mediated cleavage of PCNT and eventually for centriole separation. PLK1 phosphorylation of PCNT provides an additional layer of regulatory mechanism to ensure the fidelity of centriole separation during mitotic exit.

In chapter 2, I generated *PCNT* knockout cell lines and analyzed the phenotypes in relation to PCM assembly and centriole association. Deletion of *PCNT* hardly affected interphase centrosomes but conferred defects in centrosome maturation in cells entering M phase. The centrioles in *PCNT*–deleted cells were prematurely separated in early phase of mitosis and frequently amplified in M phase-arrested cells. Abnormal multi-nuclear cells repeatedly appeared in *PCNT*-deleted cells at interphase. My results confirmed that PCNT is critical for centriole association during M phase.

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Background

1. Structure of centrosome

1.1 Discovery of centrosome

The centrosome was firstly observed by Edouard Van Beneden in 1883 and reported by Theodor Boveri in 1888 (Scheer, 2014) (Fig. 1). The centrosome consists of centrioles surrounded by pericentriolar material (PCM) (Bornens and Gonczy, 2014). The main function of centrosome is microtubule nucleation.

1.2 Centrioles

The centriole is a core component of centrosome. In human cells the centriole is a cylindrical structure composed of microtubule nine triplets (Fig. 2) (Gonczy, 2012). The length and diameter of centriole are approximately 500 nm and 250 nm, respectively (Gonczy, 2012).

Depending on the number of times that centrioles undergo mitosis, there are three types of centrioles; mature mother, mother, and daughter centrioles. Daughter centriole is the youngest centriole that does not undergo mitosis. At the beginning of S phase, daughter centriole stars to grow next to pre-existing centriole in a perpendicular angle. Once daughter centrioles undergo mitosis, they are converted to mother centrioles. Mother centrioles newly obtain capacities to recruit PCM and duplicate daughter centriole. If

these mother centrioles undergo mitosis once again, they obtain appendages at the distal end. Appendages allow the mature mother centriole to adhere to the plasma membrane during ciliogenesis.

1.3 Pericentriolar material (PCM)

The pericentriolar material (PCM) is a protein complex surrounding the centrioles. Pericentrin (PCNT), CEP215, CEP192, and γ-tubulin are considered core components of PCM (Lawo et al., 2012). Especially, γ-tubulin forms γ-tubulin ring complex (γ-TuRC) with several GCP proteins which functions a template for microtubule formation (Kollman et al., 2011). As a result, the bigger PCM size is, the stronger microtubule nucleating activity becomes (Feng et al., 2017).

PCM was believed as an amorphous electron dense material. However, super-resolution microscopies, such as structured illumination microscopy (SIM) or stochastic optical reconstruction microscopy (STORM), revealed that PCM is a highly organized toroidal structure in interphase (Fig. 3) (Fu and Glover, 2012; Lawo et al., 2012; Sonnen et al., 2012).

PCNT is a major PCM protein, which spreads out from the centriole like the spoke of a wheel (Fig. 3) (Lawo et al., 2012). At the onset of mitosis, PCNT is phosphorylates by PLK1, which is an initiation step for mitotic PCM assembly (Lee and Rhee, 2011).

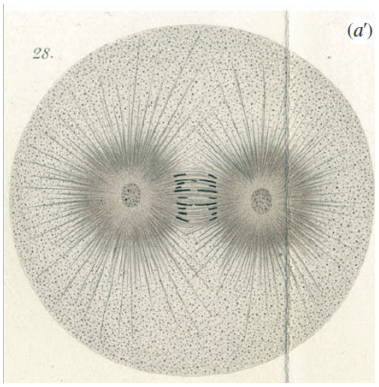

Figure 1. Boveri's drawing of the centrosome Theodor Boveri observed and described the centrosome in *Echinus microtuberculatus* during first cleavage. The centrosomes are seen in each spindle pole. (Scheer, 2014)

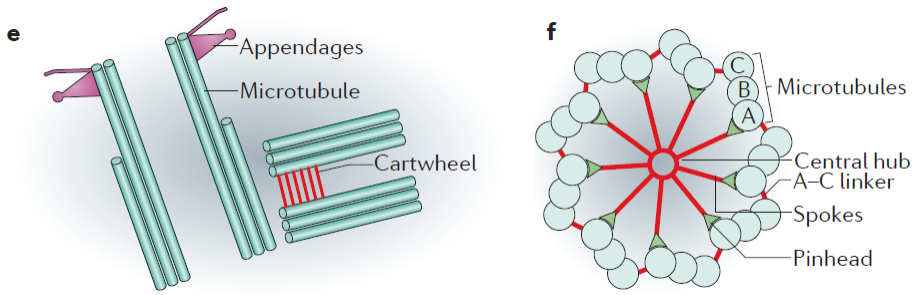

Figure 2. Structure of human centriole Centriole is a cylindrical structure composed of nine triplets. One triplet is composed of A, B, and C microtubules. Mature mother centrioles has appendages at the end of them. Cartwheel structure is only seen in daughter centriole. (Gonczy, 2012)

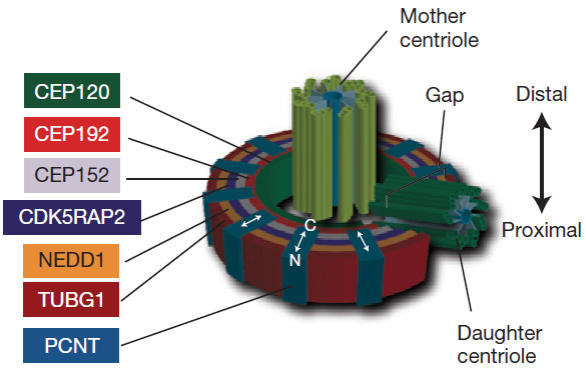

Figure 3. PCM structure observed with SIM Pericentriolar material (PCM) encircles the proximal end of mother centriole like donut shape. Each PCM proteins are localized at specific position. While other PCM proteins encompass centrioles, PCNT spreads out from the core. (Lawo et al., 2012)

2. Functions of centrosome

2.1 Microtubule network formation in interphase

Microtubules nucleated from centrosome from microtubule network in interphase cells (Handin et al., 2012) (Fig. 4). This microtubule network plays an important role in maintaining cell shape, cell migration, and intracellular transport (Handin et al., 2012) (Fig. 5). Especially, when cell migrates in particular direction, the centrosome moves with Golgi apparatus in the direction of movement and rearranges microtubule network for efficient migration (Rios, 2014).

2.2 Spindle formation during mitosis

At G2/M transition, the centrosome is matured and separated to each side of cell (Handin et al., 2012). Matured centrosome nucleates spindle fibers which can bind to chromosomes. During mitotic exit, spindle fibers segregate chromosome into two daughter cells (Wang et al., 2014) (Fig. 4, 6).

2.3 Primary cilia formation in quiescent cells

In quiescent cells, mature mother centriole is converted into the basal body which can form primary cilia (Avidor-Reiss and Gopalakrishnan, 2013) (Fig. 7). Primary cilia was considered a vestigial organelles until the 1990s, although it was firstly observed in 1898. However, recent findings suggests that primary cilia is sensory cellular antennae that coordinates several cellular signaling pathways, such as sonic SHH (sonic hedgehog) (Nachury, 2014).

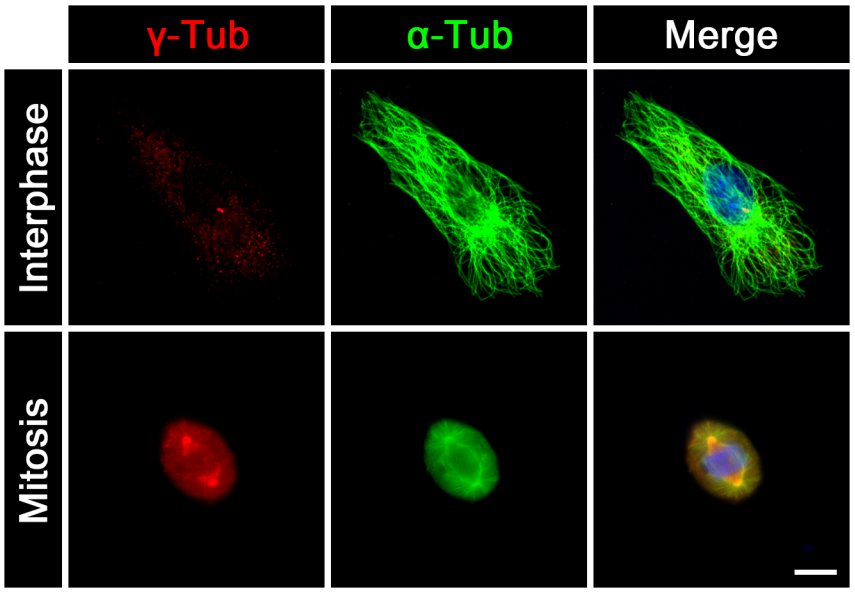

Figure 4. Microtubule organizing center Cells were stained with antibodies against γ -tubulin (red, γ-Tub; marker for centrosomes) and α-tubulin (green, α-Tub; marker for microtubule). The centrosome is a major microtubule organizing center (MTOC). In interphase, the centrosome forms microtubule network which is important for maintaining cell shape. When cells enter mitosis, the centrosome is converted to spindle poles and makes spindle fibers.

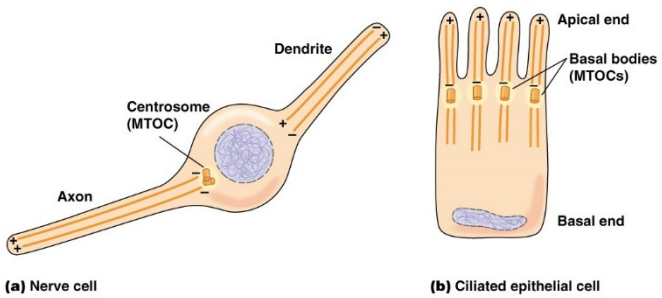

Figure 5. The functions of the centrosome in interphase The centrosome forms microtubule network which is important for maintaining cellular morphology. Unusually, several centrioles are observed in ciliated epithelial cells. (Handin et al., 2012)

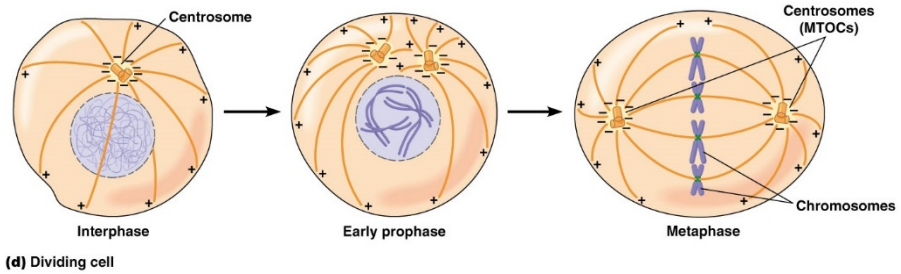

Figure 6. Bipolar spindle formation during mitosis At the onset of mitosis, the centrosome moves to each side of the cell and nucleates spindle fibers. Spindle fibers binds to chromosome in order to segregate it. (Handin et al., 2012)

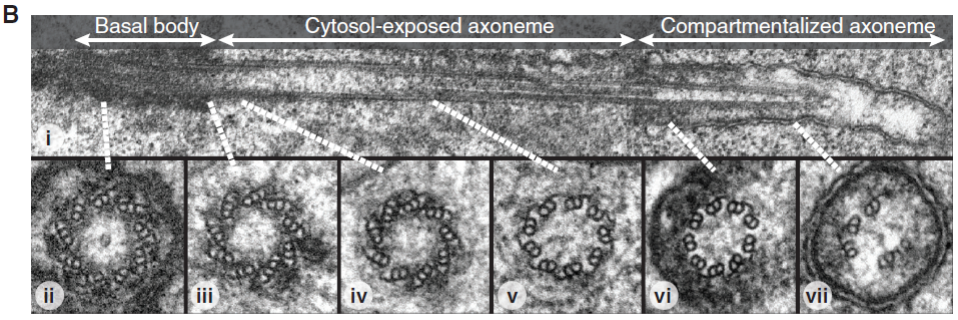

Figure 7. Structure of primary cilia The structure of primary cilia were observed by electron microscopy. The centrosome converts basal body in resting cell. Basal body moves and docks to plasma membrane through appendages of mother centriole. Primary cilia is grown from the basal body. (Avidor-Reiss and Gopalakrishnan, 2013)

3. Centrosome cycle

The centrosome is duplicated only once per cell cycle (Firat-Karalar and Stearns, 2014). Because the centrosome functions as spindle pole bodies during mitosis, the centrosome number is important to ensure precise mitosis. Abnormality of the centrosome number is one of mechanisms to cause genomic instability (Bakhoum et al., 2014; Holland and Cleveland, 2009; Vitre and Cleveland, 2012). Like DNA replication, the centrosome duplicates at S phase and segregated during mitotic exit (Fig. 8).

Centrosome cycle is divided into five stages: Initiation of centriole duplication, centriole elongation, centrosome maturation, centrosome separation, bipolar spindle formation, and centriole disengagement.

3.1 Initiation of centriole duplication

PLK4 is a master regulator of centriole duplication (Kleylein-Sohn et al., 2007). When cells enter S phase, PLK4 is concentrated on a single spot of mother centriole wall (Kim et al., 2013). Concentrated PLK4 phosphorylates several proteins and initiates centriole duplication (Dzhindzhev et al., 2014; Wang et al., 2014) (Fig. 9).

Daughter centriole is tightly and orthogonally attached to mother centriole during duplication. I call this arrangement centriole engagement. Additional round of centriole duplication is blocked when centrioles are

engaged (Nigg, 2007; Tsou and Stearns, 2006b) (Fig. 8).

3.2 Centriole elongation

After initiation, daughter centrioles are elongated by accumulating centriolar tubulins from S to M phase (Fig. 9). In human cells, centrioles are elongated to about 500 nm (Gonczy, 2012). Centriole elongation is antagonistically controlled by two centrosomal proteins, CPAP and CP110. CPAP enhances the accumulation of centriolar tubulins, while CP110 inhibits (Schmidt et al., 2009).

3.3 Centrosome maturation

Centrosome maturation is accumulation of PCM proteins at G2/M transition (Fig. 10). At the onset of mitosis, PLK1 phosphorylates PCNT at S1235 and S1231 residues which is initiation step for centrosome maturation (Lee and Rhee, 2011). After phosphorylation, volume of PCM was three to five times larger than interphase (Lee and Rhee, 2011). As a result, microtubule nucleating activity is dramatically improved and this is necessary step for spindle formation during mitosis.

3.4 Centrosome separation

Centrosome separation means a movement of two centrosomes to each spindle pole in prophase (Wang et al., 2014) (Fig. 11). Two centrosomes are loosely connected with fibrous linker proteins, such as C-Nap1, and rootletin during mitosis, and these linkers are removed by Nek2 at G2/M transition (Mardin et al., 2010). And then, two centrosomes are moved to each pole by motor proteins, such as Eg5 (Bertran et al., 2011).

3.5 Bipolar spindle formation

When centrosome maturation and centrosome separation are completed, two centrosomes are functions as spindle pole bodies which nucleate spindle fibers (Wang et al., 2012) (Fig. 6). Spindle fibers bind to chromosome and segregate them into two daughter cells during mitotic exit.

3.6 Centriole disengagement and separation

Centriole disengagement and separation is a detachment of daughter centriole from mother centriole which is considered licensing step for centriole duplication (Nigg, 2007) (Fig. 12). In general, centriole separation happens during mitotic exit (Tsou and Stearns, 2006b). Detailed descriptions are given in the following section.

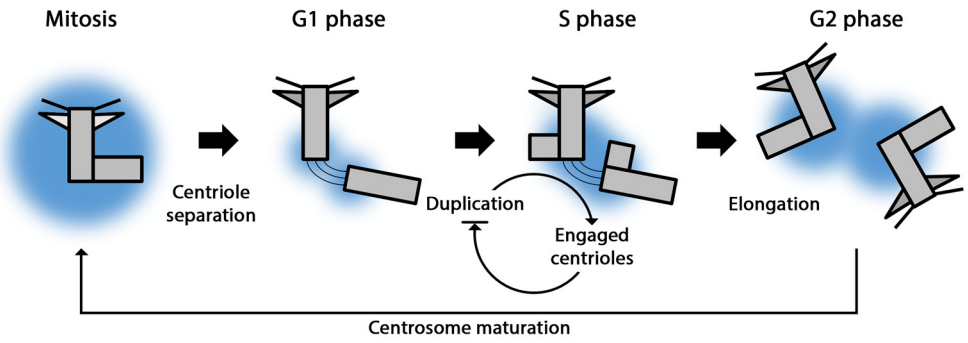

Figure 8. Overview of centrosome cycle The centrosome duplicated only once per cell cycle. In G1 phase, there are two centrosomes which contain only centriole. When cells enter S phase, daughter centrioles are nucleated from pre-exiting centrioles. During mitotic exit, daughter centrioles are disengaged from mother centrioles. (Wang et al., 2012)

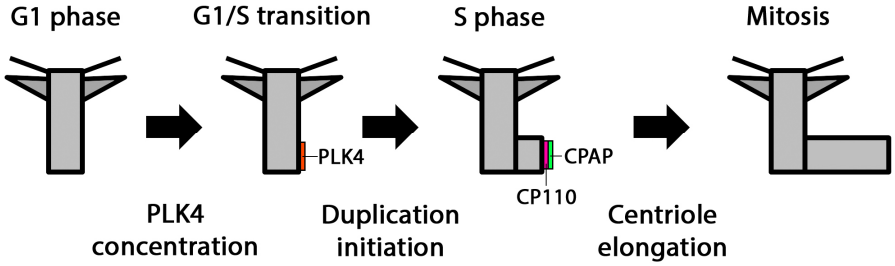

Figure 9. Molecular mechanisms of daughter centriole formation At G1/S transition, PLK4 is concentrated on a spot in wall of mother centriole. Concentrated PLK4 recruits building blocks of daughter centriole. As a result, centriole duplication initiates. From S phase to mitosis, centriole is elongated. CPAP and CP110 antagonistically regulate centriole elongation.

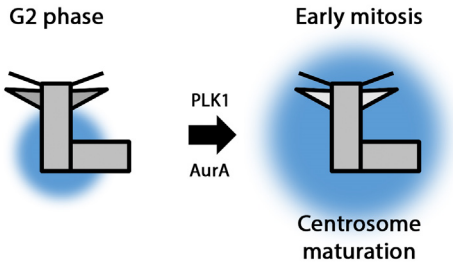

Figure 10. Centrosome maturation When cells enter mitosis, PCM (blue) is dramatically enlarged. PLK1 and Aurora A (AurA) are key regulators for centrosome maturation. Especially, PLK1 initiates centrosome maturation by phosphorylating pericentrin (Lee and Rhee, 2011).

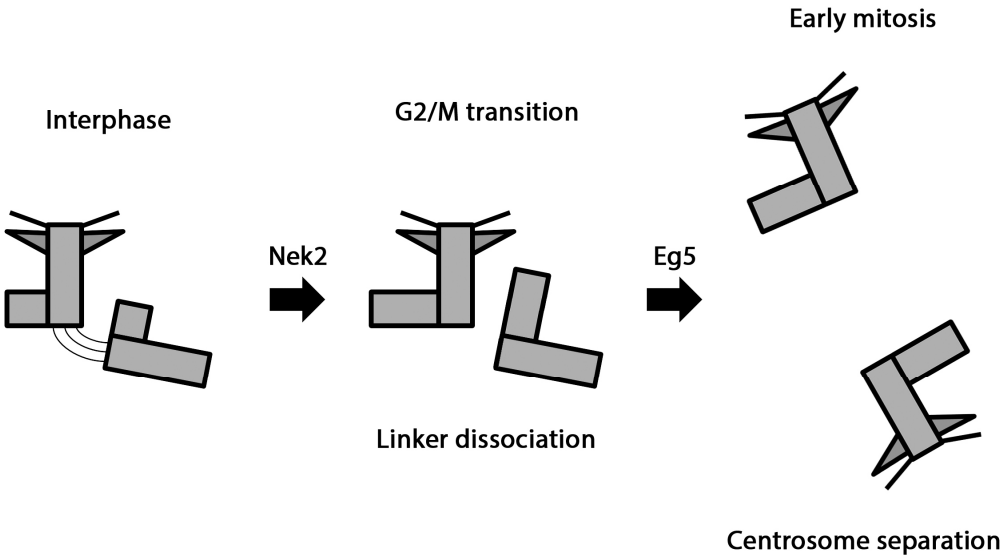

Figure 11. Two step for centrosome separation Two centrosomes are separated to form bipolar spindle formation. In interphase, two mother centrioles are connected by linker proteins, such as C-Nap1 and rootletin. At G2/M transition, C-Nap1 and rootletin are phosphorylated by Nek2. After this phosphorylation, linker protines are dissociated from the centrosome. Eg5, which is a motor protein, moves two centrosomes to each spindle pole during mitosis.

4. Licensing mechanism for centriole duplication: Centriole engagement and separation

Centrosome should be duplicated only once per cell cycle because an abnormality of centrosome number causes genomic instability (Vitre and Cleveland, 2012). Centriole engagement and disengagement is considered licensing mechanism for limiting centriole duplication only once per cell cycle (Nigg, 2007).

Centriole engagement is considered an intrinsic block mechanism of centriole reduplication (Tsou and Stearns, 2006a, b). Several experimental results suggest that mother centriole can continually duplicate daughter centriole, but its ability is blocked when daughter centrioles are engaged. For example, engaged daughter centriole is ablated by laser, mother centriole duplicates additional new daughter centriole (Loncarek et al., 2008).

Mother centriole duplicates only one centriole per cell cycle, because daughter centriole is disengaged during mitotic exit. When centrioles are disengaged before mitosis, centriole overduplication is frequently observed (Nakamura et al., 2009; Thein et al., 2007). Therefore, centriole disengagement is considered a licensing step for regulating centriole duplication.

Centriole disengagement is regulated by mitotic kinase of PLK1 and cysteine protease of separase (Tsou et al., 2009) (Fig. 12). Centriole disengagement happens during mitotic exit. Furthermore, PCNT and Scc1

were identified as substrates of separase for centriole disengagement (Lee and Rhee, 2012; Matsuo et al., 2012; Schockel et al., 2011). However, PLK1 substrate remains to be elucidated.

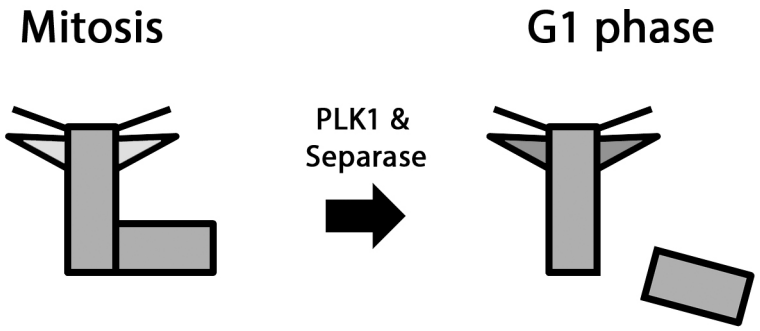

Figure 12. Centriole disengagement and separation Daughter centriole is tightly and orthogonally attached to mother centriole in interphase. During mitotic exit, daughter centriole is detached and separated from mother centriole. PLK1 and separase is known to be key regulators of centriole disengagement.

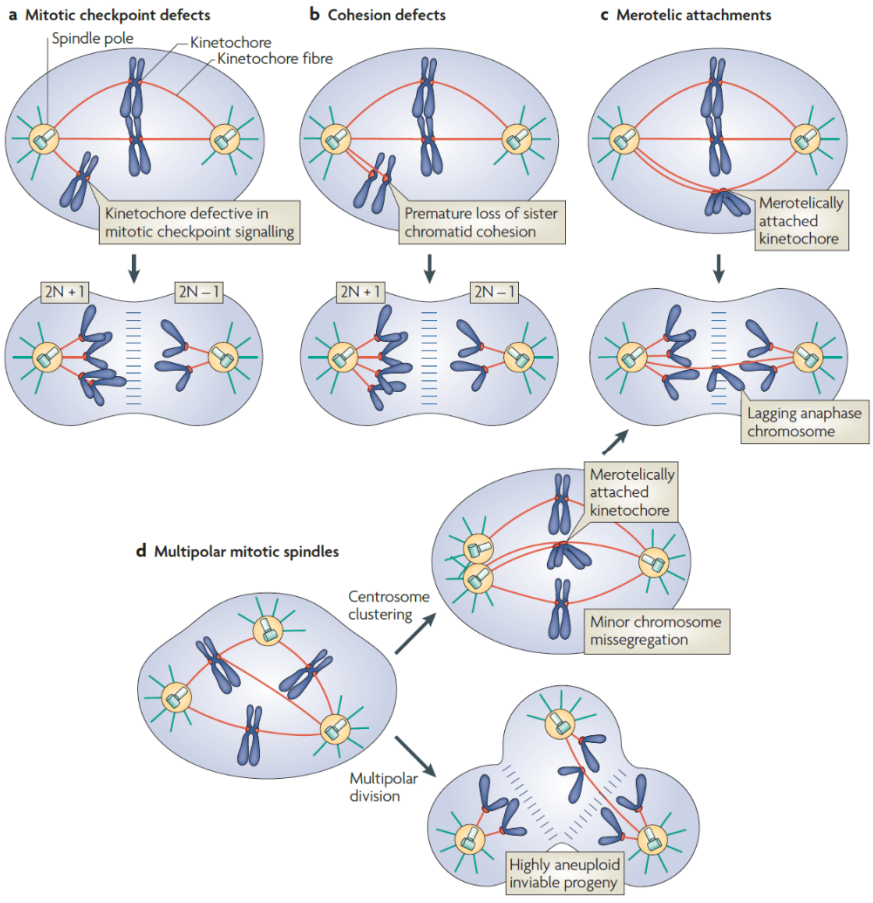

Figure 13. Four pathways to the generation of aneuploidy Mitotic checkpoint defects, cohesion defects, merotelic attachments, and multipolar mitotic spindles were suggested as the causes of aneuploidy. Premature centriole disengagement can induce multipolar mitotic spindles. (Holland and Cleveland, 2009)

PURPOSE

The centrosome duplicates only once per cell cycle. When centrioles are engaged, additional round of centriole duplication is blocked. This observation suggests that centriole disengagement is a licensing mechanism for centriole duplication. Consistent with this notion, misregulation of centriole disengagement frequently causes centrosome overduplication, resulting in chromosomal instability and eventually cancers (Fig. 13). Therefore, centriole disengagement should be precisely regulated.

It is well known that PLK1 and separase are essential for centriole disengagement during mitotic exit. Pericentrin were identified as substrates of separase for centriole disengagement, but PLK1's target remains to be elucidated.

The purpose of my research is to understand molecular mechanisms of centriole disengagement. In particular, I focused on roles of PLK1 and PCNT in centriole disengagement during mitosis.

CHAPTER 1

PLK1 regulation of PCNT cleavage ensures fidelity of centriole separation during mitotic exit

ABSTRACT

Centrioles are duplicated and segregated in close link to the cell cycle. During mitosis, daughter centrioles are disengaged and eventually separated from mother centrioles. New daughter centrioles may be generated only after centriole separation. Therefore, centriole separation is considered a licensing step for centriole duplication. It was previously known that separase specifically cleaves pericentrin (PCNT) during mitotic exit. Here, I report that PCNT has to be phosphorylated by PLK1 in order to be a suitable substrate of separase. The phospho-resistant mutants of PCNT are not cleaved by separase and eventually inhibit centriole separation. Furthermore, phospho-mimetic PCNT mutants rescue centriole separation even in the presence of BI2536. Based on these results, I propose that PLK1 phosphorylation is a priming step for separase-mediated cleavage of PCNT and eventually for centriole separation. PLK1 phosphorylation of PCNT provides an additional layer of regulatory mechanism to ensure the fidelity of centriole separation during mitotic exit.

INTRODUCTION

The centrosome is a non-membrane-bound organelle which consists of centrioles surrounded by pericentriolar material (PCM) (Bettencourt-Dias and Glover, 2007). Centrioles are duplicated and segregated in a close link to the cell cycle (Mardin and Schiebel, 2012). In the beginning of S phase, a daughter centriole starts to grow next to the mother centriole in a perpendicular angle and remains engaged until mitosis. Once the cells undergo mitosis, daughter centrioles are disengaged and eventually separated from the mother centrioles. A new round of centriole biogenesis is inhibited, as far as the daughter centriole is closely associated to the mother centriole (Cabral et al., 2013; Tsou and Stearns, 2006b). Therefore, centriole separation is a licensing step for centriole duplication. A premature separation of centrioles may result in multipolar spindles during mitosis, which is one of the main causes for chromosome aneuploidy (Ganem et al., 2009).

The centrosome is a major microtubule organizing center in dividing cells and functions as spindle poles during mitosis. The amount of microtubules emanated from the centrosome is correlated with the amount of PCM, due to the fact that a majority of γ-TuRC is anchored to PCM (Haren et al., 2006; Manning et al., 2010; Zimmerman et al., 2004). In fact, a significant amount of PCM becomes accumulated in the centrosome of cells entering mitosis (Woodruff et al., 2014). Defects in PCM accumulation often result in mitotic arrest with monopolar spindles (Lee and Rhee, 2011). It is interesting that mutations in PCM protein genes cause congenital brain defects, such as

microcephaly (Bond et al., 2005). It was proposed that such mutations affect neuronal stem cell division, and lead to reduction of stem cell population and apoptosis (Thornton and Woods, 2009).

Pericentrin (PCNT) is a major PCM protein which is involved in mitotic spindle organization, DNA damage checkpoint, and primary cilia formation (Jurczyk et al., 2004; Tibelius et al., 2009; Zimmerman et al., 2004). Mutations in PCNT gene cause pleiotropic defects, including primordial dwarfism, cancer, mental disorder, and ciliopathies (Delaval and Doxsey, 2010; Rauch et al., 2008; sChen et al., 2014). Studies with super-resolution microscopy revealed that PCNT spreads out like the spokes of a wheel with the C-terminal domain at the centriole wall (Lawo et al., 2012; Mennella et al., 2012; Sonnen et al., 2012). PCNT is also crucial for mitotic spindle pole formation and disintegration. When cells enter mitosis, PCNT is phosphorylated by PLK1 and recruits other PCM proteins to assemble spindle poles with a high microtubule organizing activity (Lee and Rhee, 2011). At the end of mitosis, PCNT is specifically cleaved by separase and removed from the centrosome (Lee and Rhee, 2012; Matsuo et al., 2012). This event is considered to be important for centriole separation during mitotic exit.

PLK1 is a mitotic kinase which participates in diverse mitotic events, such as sister chromatid separation, spindle assembly checkpoint, and cytokinesis (Barr et al., 2004; Petronczki et al., 2008). PLK1 is also localized at the centrosome and involved in multiple centrosomal functions (Kong et al., 2014; Liu and Erikson, 2002; Loncarek et al., 2010; Tsou et al., 2009).

However, it is largely unknown how PLK1 regulates centriole functions and what are critical substrates of PLK1 for centriole regulation. It has been previously reported that PLK1 phosphorylates PCNT at S1235 and S1241 residues for initiation of centrosome maturation (Lee and Rhee, 2011). In this report, I reveal that PLK1 phosphorylation is also essential for the separase-dependent cleavage of PCNT during mitotic exit. My results propose molecular mechanisms how PLK1 controls centriole separation and eventually how centriole biogenesis is controlled during the cell cycle.

MATERIALS AND METHODS

Plasmids, siRNA, and cell culture

All PCNT (NCBI reference sequence: NM_006031.5) constructs were subcloned into pLVX-IRES-Puro vector (Clontech, 632183) and tagged with 3×FLAG and Myc at 5'- and 3'- ends, respectively. In order to generate siRNA-resistant PCNT construct, I induced silent mutations of PCNT with the following primers: 5'-GCA GCA GAA CTC AAG GAG-3' and 5'-TGG ACC TCT TCG AAT GAG-3'.

In order to make PCNT sensor (3×FLAG-mCherry-PCNT$^{2059-2389}$-GFP-PACT), I linked the PCNT$^{2059-2389}$ fragment with mCherry and GFP at 5'- and 3'- ends, respectively. The PACT domain of PCNT$^{3112-3336}$ was joined at 3'-end of GFP. Addition, the 3×FLAG fragment was linked at 5'-end of mCherry.

For depletion of endogenous PLK1 and PCNT, siPLK1 (5'-AAG CGG GAC TTC CTC ACA TCA-3'; Lee and Rhee, 2010) and siPCNT (5'-UGG ACG UCA UCC AAU GAG A-3'; Jurczyk et al., 2004) were used. A scrambled siRNA sequence (siCTL; 5'-GCA AUC GAA GCU CGG CUA CTT-3') was used as a control. RNAiMAX (Invitrogen, 13778-075) was used for siRNA transfection according to the manufacturer's protocol.

The HeLa cells were cultured in DMEM (Welgene, LM 001-05) supplemented with 10% FBS (Welgene, S101-01) and antibiotics (Invivogen, ANT-MPT) at 37°C and 5% CO_2. To generate stable cell lines, I seeded

2.4×10^5 HeLa cells on 60 mm dish and 2.5 μg of plasmid DNA was transfected in the next day. The plasmids were transfected into the HeLa cells using Fugene HD (Promega, E2311). One day after the transfection, the cells were transferred to a 100 mm dish and treated with 1 mg/ml puromycin (Calbiochem, 540222) for 2-3 weeks. Monoclonal cell lines were established with the dilution cloning method and maintained with 0.5 mg/ml puromycin.

To synchronize cell cycle, I used 2 mM thymidine (Sigma, T9250), 5 μM paclitaxel (Sigma, T7402), and 2 μM ZM447439 (Cayman chemical company, 13601). For inhibition of PLK1 activity during mitosis, I treated the cells with 100 nM BI2536 (Selleck chemicals, S1109) for 3 h after the thymidine-paclitaxel block.

Antibodies

The CEP135 (ICC 1:5000; Jeong et al., 2007), CEP215 (ICC 1:5000, IB 1:5000; Lee and Rhee, 2010), C-Nap1 (ICC 1:20000; Lee and Rhee, 2010), PCNT (ICC 1:20000, IB 1:5000; Kim and Rhee, 2011), and pS1241PCNT (ICC 1:100; Lee and Rhee, 2011) antibodies were previously described. Antibodies specific to centrin-2 (Merck Millipore, 04-1624; ICC 1:1000), CEP192 (Bethyl Laboratories, A302-324A; ICC 1:20000), cyclin B1 (Santa Cruz Biotechnology, sc-254; IB 1:1000), GFP (Santa Cruz Biotechnology, sc-9996; ICC 1:500, IB 1:500), GAPDH (Life technologies, AM4300; IB 1:20000), FLAG (Sigma-Aldrich, F3165; ICC 1:2000, IB 1:10000), mCherry

(Abcam, ab167453; ICC 1:1000, IB 1:500), PLK1 (zymed, 33-1700; IB 1:500), γ-tubulin (Santa Cruz Biotechnology, sc-7396; ICC 1:200), and separase (abcom, ab16170; IB 1:500) were purchased. The secondary antibodies conjugated with fluorescent dye (Alexa-488, Alexa-594, and Alexa-647, Life Technologies; 1:1000) and for with HRP (Sigma-Aldrich or Millipore, 1:10000) were purchased.

The pS2259PCNT antibodies (ICC 1:1000, IB 1:100) were generated as described previously (Lee and Rhee, 2011). In brief, the KLH-conjugated 'CSADT(pS)LGDRAD' peptide (synthesized by abclon) was injected into rabbits with a complete Freud's Adjuvant (Sigma-Aldrich, F5881). To induce strong immune response, the same peptide with an incomplete Freud's Adjuvant (Sigma-Aldrich, F5506) was additionally injected twice. The immune serum was obtained and the phospho-antibody was affinity-purified. All procedures with animals were approved by the Seoul National University Institutional Animal Care and Use Committees.

Immunoprecipitation and Immunoblot analyses

For immunoprecipitation, HeLa cells were lysed on ice for 20 min with a lysis buffer (150 mM NaCl, 0.1% triton X-100, 20 mM Tris-HCl at pH 7.5, 10 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 20 mM β-glycerophosphate, 5 mM $MgCl_2$, and 5% glycerol) containing a protease inhibitor cocktail (GenDEPOT, P3100) and centrifuged at 12,000 rpm for 20

min at 4°C. The supernatants were incubated with antibody against PCNT for 4-6 h at 4°C and then immunoprecipitated with Protein A bead (GE Healthcare Life Sciences, 17-0780-01) for 1 h at 4°C. The PCNT immunoprecipitants were eluted from Protein A bead by adding the SDS sample buffer (62.5 mM Tris-HCl at pH 6.8, 2% SDS, 10% Glycerol, 10 mM DTT and 0.01% Bromophenol blue) and boiled for 5 min. The samples were subjected to immunoblot analyses with indicated antibodies.

For immunoblot analyses, the cells were lysed on ice for 10 minutes with RIPA buffer (150 mM NaCl, 1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl at pH 8.0, 10 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, and 1 mM EGTA) containing a protease inhibitor cocktail (Sigma-Aldrich, P8340) and centrifuged with 12,000 rpm for 10 min at 4°C. The supernatants were mixed with 4×SDS sample buffer (250 mM Tris-HCl at pH 6.8, 8% SDS, 40% glycerol, and 0.04% bromophenol blue), and 10 mM DTT (Amresco, 0281-25G). Mixtures were boiled for 5 min.

To detect intact and cleaved PCNT, 15-20 μg of proteins were loaded in sodium dodecyl sulphate-polyacrylamide gel (3% stacking gel and 4% separating gel), electrophoresed, and transferred to Protran BA85 nitrocellulose membranes (GE Healthcare Life Sciences, 10401196). The membranes were blocked with blocking solution (5% nonfat milk in 0.1% Tween 20 in TBS or 5% bovine serum albumin in 0.1% Tween 20 in TBS) for 2 h, incubated with primary antibodies diluted in blocking solution for 16 h at 4°C, washed four times with TBST (0.1% Tween 20 in TBS), incubated with

secondary antibodies in blocking solution for 30 min, and washed again. To detect the signals of secondary antibodies, ECL reagent (ABfrontier, LF-QC0101) and X-ray films (Agfa, CP-BU NEW) were used. In the cases of other proteins, 5% stacking and 10% separating gels were used.

Immunstaining analysis

For immunocytochemistry, cells seeded on cover glass were fixed with cold methanol for 10 min and washed three times with cold PBS. After incubation of PBST (0.1% Triton X-100 in PBS) for 10 min, the cells were blocked with blocking solution (3% bovine serum albumin, and 0.3% triton X-100 in PBS) for 30 min, incubated with primary antibodies diluted in blocking solution for 1 h, washed three times with PBST, incubated with secondary antibodies in blocking solution for 30 min, washed twice with PBST, incubated with DAPI solution for 3 min, and washed twice with PBST. The cover glasses were mounted on a slide glass with ProLong Gold antifade reagent (Life technologies, P36930). Images were acquired from fluorescence microscopies equipped with digital cameras (Olympus IX51 equipped with QImaging QICAM Fast 1394 or Olympus IX81 equipped with ANDOR iXon^{EM}+) and processed in ImagePro 5.0 (Media Cybernetics) or MetaMorph 7.6 (Molecular Devices). Inset images were enlarged four times in Image J 1.49 (National Institutes of Health, USA) or Photoshop CS6 (Adobe) using option of bicubic interpolation. In the cases of quadruple staining, Image 5D (Fiji) which is a plug-in of Image J were used in order for pseudo-coloring.

To measure fluorescence intensities, I immunostained all cells at the same time with same diluent antibodies. All images were captured at same exposure time without stopping. Image J 1.49 was used to measure fluorescence intensities at centrosomes. In each measurement, background signals were subtracted from the sum of fluorescence signals at centrosome.

Statistical analysis

In order to statistical analysis, experiments were independently performed three times. To calculate P values, unpaired two-tailed t test or anova were performed in Prism 6 (GraphPad Software). All Measured fluorescent intensities were displayed with box-and-whisker plots in Prism 6 (Lines, median; vertical boxes; values from 25^{th} to 75^{th}; down error bars, 10^{th} value; up error bar, 90^{th} value; circles, outliers).

RESULTS

BI2536 blocks both PCM disassembly and centriole separation

In order to determine involvement of PLK1 in PCM disassembly during mitotic exit, I treated BI2536, a PLK1 inhibitor, to the M phase-arrested HeLa cells. The cells were forced to exit mitosis with ZM447439, an aurora kinase inhibitor (Ditchfield et al., 2003), and immunostained with antibodies against selected PCM proteins (Fig. 14a). The BI2536 treatment reduced centrosomal CEP192 and γ-tubulin to the basal levels even before mitotic exit (Fig. 14b,e,f). However, the centrosomal levels of PCNT and CEP215 remained relatively abundant in the BI2536-treated cells even after mitotic exit (Fig. 14b-d). These results imply that the PLK1 activity is required for removal of PCNT and CEP215 from the centrosome during mitotic exit.

Next, I determined effects of BI2536 on centriole separation during the mitotic exit. Centriole association was determined with 2:1 ratio of centrin-2 and the proximal centriole markers, such as C-NAP1 and CEP135 (Lee and Rhee, 2012; Tsou and Stearns, 2006b) (Fig. 14b). As expected, most of the daughter centrioles were separated from the mother centrioles once the cells were forced to exit mitosis (Fig. 14b,g,h). However, BI2536 blocked centriole separation in this condition (Fig. 14b,g,h). The distance between the associated centrioles was determined with the centrin-2 dots. Average distance between associated centrioles were less than 1 μm, and it did not significantly change after mitosis when BI2536 was treated (Fig. 14i). These results are consistent

with the previous report that the PLK1 activity is required for centriole separation during mitotic exit (Tsou et al., 2009).

PLK1 phosphorylation is necessary for PCNT cleavage

It is known that PCNT is specifically cleaved by separase and removed from the centrosome during mitotic exit (Lee and Rhee, 2012; Matsuo et al., 2012). In order to test whether PLK1 controls the separase-dependent cleavage of PCNT, I performed immunoblot analyses of PCNT in the BI2536-treated cells. The results showed that the specific cleavage of PCNT was suppressed in the BI2536-treated cells during mitotic exit (Fig. 15a). The specific PCNT cleavage was also suppressed in the PLK1-depleted cells (Fig. 15b). Finally, the cleavage of ectopic PCNT protein (FLAG-PCNT-Myc) was significantly reduced in the presence of BI2536 while the cleavage resistant FLAG-PCNT^{R2231A}-Myc mutant remained intact at all time (Fig. 15c). These results indicate that the PLK1 activity is necessary for the separase-dependent cleavage of PCNT during mitotic exit.

Kwanwoo Lee identified 22 specific phosphorylation sites of PLK1 within PCNT after a series of in vitro kinase assays (Lee and Rhee, 2011) (Fig. 15d and Fig. 16). Two of them at the N-terminal end (S1235 and S1241) have been known to be essential for centrosome maturation (Lee and Rhee, 2011) (Fig. 15d). To determine which phosphorylation sites are responsible for the separase-dependent cleavage of PCNT during mitotic exit, I performed a series

of PCNT cleavage assays with HeLa cells which expressed the phospho-resistant mutants of FLAG-PCNT-Myc in both transient and stable manners. All the phospho-resistant FLAG-PCNT-Myc proteins were localized at the centrosome (Fig. 17). The results showed that the 9 phosphorylation sites near the cleavage site (R2231) are responsible for the separase-dependent cleavage of PCNT during mitotic exit (Fig. 15e). All examined combinations of phospho-resistant mutants within the 9 sites were not as effective as FLAG-PCNT^{9A}-Myc was, although FLAG-PCNT^{S2259A}-Myc and FLAG-PCNT$^{S2259/2267A}$-Myc showed some resistance to the specific cleavage (Fig. 18). These results suggest that multiple phosphorylation of PCNT near the cleavage site is necessary for the separase-dependent cleavage during mitotic exit. To confirm that PCNT phosphorylation at the 9 residues is sufficient for separase-dependent cleavage of PCNT, I generated a HeLa cell line stably expressing FLAG-PCNT^{9D}-Myc in which the 9 phosphorylation sites were substituted to aspartic acid (Fig. 17b). The results showed that the phospho-mimetic PCNT mutant was efficiently cleaved during mitotic exit even in the presence of BI2536 (Fig. 15f). However, the specific cleavage of the phospho-mimetic PCNT mutant was significantly reduced in separase-depleted cells (Fig. 15g). These results collectively propose that the PLK1 phosphorylation at multiple sites of PCNT is essential for the separase-dependent cleavage of PCNT during mitotic exit.

Agircan and Schiebel recently developed the PCNT sensor protein for detection of separase-dependent cleavage of PCNT at the centrosome (Agircan

et al., 2014). The construct includes 2059-2398 residues of PCNT which contains 13 phosphorylation sites (Fig. 15d and Fig. 19a). I examined the importance of PLK1 phosphorylation on PCNT cleavage using the PCNT sensor protein (FLAG-mCherry-PCNT2059-2398-GFP-PACT). All PCNT sensor proteins were localized at the centrosome (Fig. 19b). As expected, the wild type PCNT sensor protein was efficiently cleaved during mitotic exit, while cleavage resistant PCNT sensor protein was not (Fig. 15h). In this condition, I observed a significant reduction of the specific cleavage of phospho-resistant PCNT sensor proteins (Fig. 15h). Furthermore, the phospho-mimetic PCNT sensor protein was cleaved even in the presence of BI2536 (Fig. 15i). The C-terminal fragments of the PCNT sensor proteins were not detected with the GFP antibody, implying that it is rapidly degraded by the N-end rule pathway (Fig. 15h,i). These results strongly support my proposal that multiple phosphorylation of PCNT near the cleavage site is a necessary step for separase-dependent cleavage of PCNT during mitotic exit.

PLK1 phosphorylates PCNT in vivo

In order to determine specific phosphorylation of PCNT in vivo, I generated a phospho-antibody specific to pS2259 of PCNT (pS2259PCNT; Fig. 20a). Immunoblot analyses with PCNT immunoprecipitants showed that pS2259PCNT-specific band was detected only in mitotic cells (Fig. 20b). Furthermore, pS2259PCNT specific signals at the centrosome were only restored in cells rescued with FLAG-PCNT-Myc, but not with phospho-

resistant FLAG-PCNT^{S2259A}-Myc (Fig. 20c,d). To more precisely observe specific phosphorylation of PCNT according to cell cycle, I synchronously released HeLa cells from double thymidine block and compared it with another phospho-PCNT antibody against pS1241 of PCNT (pS1241PCNT) which was previously characterized for centrosome maturation (Lee and Rhee, 2011) (Fig. 21a). Immunoblot analysis revealed that the specific cleavage bands of PCNT was detected at 10th hours with cyclin B1 degradation indicating that they reached to M phase at 10th hour and exited mitosis at 12th hour (Fig. 21b). Immunostaining analyses with the identical set of synchronous HeLa cells revealed that the centrosomal PCNT levels increased at 10th hour, and decreased afterwards (Fig. 21c,d). The centrosomal levels of both pS1241PCNT and pS2259PCNT also peaked at 10th hour but they were more dramatic than the PCNT protein (Fig. 21c,d). These results suggest that the centrosomal PCNT is simultaneously phosphorylated at both S1241 and S2259 during M phase.

I determined the phospho-PCNT levels at specific stages of mitosis. The immunostaining analyses revealed that the centrosomal signals of phospho-PCNT as well as PCNT were the highest at early mitosis and decreased at telophase (Fig. 21e,f). Immunoblot analyses with PCNT immunoprecipitants showed that the pS2259PCNT-specific band was only detected at early time points after the ZM447439 treatment (Fig. 21g). Furthermore, BI2536 significantly reduced the intensity of the pS2259PCNT-specific band in M phase-arrested cells (Fig. 21h). These results indicate that

PLK1 phosphorylation precedes the PCNT cleavage during mitotic exit.

PCNT phosphorylation is necessary for centriole separation

In order to examine if PLK1 phosphorylation of PCNT is necessary for centriole separation during mitotic exit, I prepared PCNT-rescued cells (Fig. 22a,b). In this study, I always carried out all knockdown-rescue experiments by depleting endogenous PCNT in cells which stably expressed siRNA-resistant FLAG-PCNT-Myc proteins (Fig. 22). To avoid unexpected effects of prolonged mitotic arrest and ZM443439, I synchronized the cell cycle at early G1 phase using double thymidine block release method (Fig. 23a). G1 phase cells were determined with two centrin-2 dots and centriole association was determined with 2:1 ratio of centrin-2 and C-NAP1. As expected, most of centrioles in the PCNT-depleted cells rescued with wild type FLAG-PCNT-Myc were separated, while those with cleavage-resistant FLAG-PCNT^{R2231A}-Myc remained associated after mitosis (Fig. 23b,c). In this condition, the proportion of cells with the associated centrioles increased, depending on the number of alanine substitutes within the ectopic PCNT of the rescued cells (Fig. 23b,c). I also observed that phospho-mimetic FLAG-PCNT^{9D}-Myc partially rescued centriole separation in BI2536-treated cells (Fig. 23d-f). These results imply that specific phosphorylation of PCNT is prerequisite for centriole separation during mitotic exit. The sum of specific phosphorylation near the cleavage site proportionally affects PCNT cleavage and eventually leads to centriole separation.

PCNT and CEP215 are essential for centriole association

PCNT and CEP215 are considered key components of the PCM (Kim and Rhee, 2014). It was recently reported that depletion of CEP215 from the centrosome is critical for centriole separation during mitotic exit (Pagan et al., 2015). In order to determine whether the PLK1 activity is still critical for separation of centrioles with limited PCM components, I treated BI2536 in the PCNT- and/or CEP215-depleted cells. Depletion of either PCNT or CEP215 did not affect one another in total cellular levels at M phase (Fig. 24a). However, the centrosomal levels of both proteins were significantly reduced in mitotic cells of any depletion of the proteins (Fig. 24b-d). This result reflects that CEP215 and PCNT are interdependent to localize at the mitotic spindle poles (Kim and Rhee, 2014). I determined centriole separation of PCNT- and/or CEP215-depleted cells in the presence of BI2536. The results showed that centrioles of the PCNT- and/or CEP215-depleted cells as well as control cells were separated during mitotic exit (Fig. 24e,f). BI2536 significantly inhibited centriole separation in the control cells, but not so much in the PCNT- and/or CEP215-depleted cells (Fig. 24e,f). No significant difference in the proportion of centriole separation was observed among the PCNT- and/or CEP215-depleted cells (Fig. 24e,f). These results support the notion that PLK1 phosphorylation of PCNT and eventual disintegration of PCM are essential for centriole separation. Furthermore, the results imply that another substrate of PLK1 is involved in centriole disengagement and separation.

I tested the hypothesis in which the PCNT cleavage leads to

disassembly of PCM components followed by centriole separation during mitotic exit. The hypothesis predicts that both PCNT and CEP215 should remain at the centrosome if PCNT were not cleaved during mitotic exit. To test the hypothesis, I determined the centrosomal levels of selected PCM proteins in cells rescued with the cleavage-resistant FLAG-PCNT^{R2231A}-Myc. As expected, all the tested PCM proteins in the FLAG-PCNT-Myc-rescued cells were reduced to basal level after mitotic exit (Fig. 25a-d). The centrosomal levels of CEP192 and γ-tubulin were also reduced in the FLAG-PCNT^{R2231A}-Myc-rescued cells during mitotic exit even with the centrioles associated (Fig. 25c,d). However, the centrosomal levels of PCNT and CEP215 remained relatively high in the FLAG-PCNT^{R2231A}-Myc-rescued cells with centriole associated even after mitosis (Fig. 25a,b). These results suggest that centrioles are associated as far as both PCNT and CEP215 remain at PCM even after mitotic exit.

Next, I determined the centrosomal levels of the PCM proteins with associated or separated centrioles in the cells rescued with phospho-resistant FLAG-PCNT^{9A}-Myc (Fig. 25e,f). As expected, majority of the rescued cells had centrioles associated and only a small fraction of the cells had centrioles separated at the end of mitosis (Fig. 25e,f). The centrosomal levels of the ectopic PCNT proteins were higher in the centrosomes with associated centrioles than those with separated centrioles (Fig. 25e). The centrosomal levels of γ-tubulin, however, were reduced to basal levels, irrespective of the centriole status (Fig. 25f). These results support the hypothesis that the

centrosomal PCNT and CEP215 are essential for maintaining centriole association during mitosis. In consistent with this notion, I observed that PCNT was removed from the FLAG-PCNT^{9D}-Myc-rescued cells even in the presence of the PLK1 inhibitor (Fig. 23g).

Dual functions of PLK1 phosphorylation of PCNT

I previously reported that PLK1 phosphorylation of PCNT at S1235 and S1241 residues is an initial step for centrosome maturation at the onset of mitosis (Lee and Rhee, 2011). In this work, I revealed that PLK1 phosphorylation near the cleavage site of PCNT is critical for the PCNT cleavage. I examined whether these two events are independent or not. First, I observed that FLAG-PCNT^{AA}-Myc in which the serine residues for centrosome maturation (S1235 and S1241) were substituted to alanine was specifically cleaved during mitotic exit (Fig. 26a). Furthermore, I carried out knockdown-rescue experiments with stable cell lines expressing FLAG-PCNT^{AA}-Myc (Fig. 17c and Fig. 22e). The results showed that the centrioles were properly separated after mitosis when endogenous PCNT was rescued with FLAG-PCNT^{AA}-Myc (Fig. 26b,c). Second, I observed that both FLAG-PCNT^{9A}-Myc and FLAG-PCNT^{R2231A}-Myc were properly placed to the centrosome during mitotic entry (Fig. 26d,e). Furthermore, the centrosomal γ-tubulin levels were effectively rescued with both FLAG-PCNT^{9A}-Myc and FLAG-PCNT^{R2231A}-Myc, but not with FLAG-PCNT^{AA}-Myc (Fig. 26d,f). These results indicate that PLK1 phosphorylation of PCNT independently

governs two mitotic events: One is centrosome maturation at the onset of mitosis, and the other is the PCNT cleavage during mitotic exit.

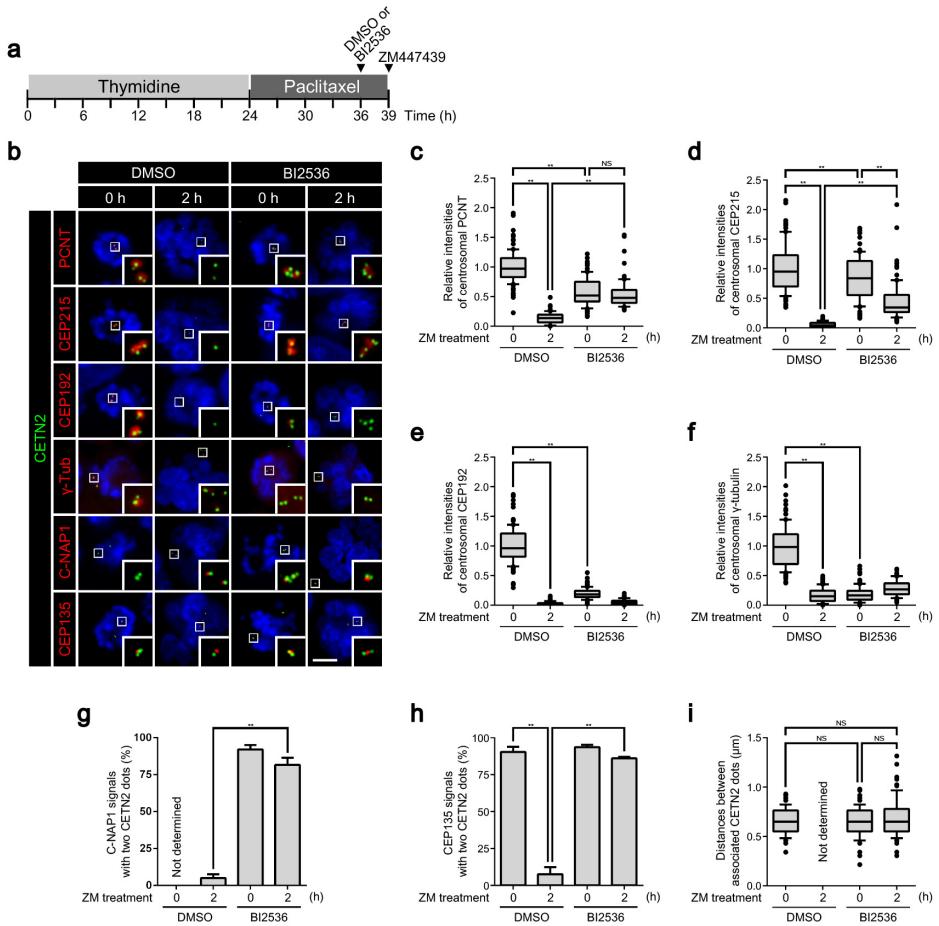

Figure 14. PLK1 regulation of PCM disassembly and centriole separation during mitotic exit (**a**) HeLa cells were arrested at M phase with sequential treatment of thymidine and paclitaxel. BI2536 was added for the last 3 h and then ZM447439 (ZM) for 2 h to induce mitotic exit. (**b**) The cells were fixed before and after the ZM447439 treatment and coimmunostained with the centrin-2 antibody (CETN2, green), along with the PCNT, CEP215, CEP192, γ-tubulin (γ-Tub), C-NAP1 and CEP135 antibodies (red). DNA was visualized with DAPI (blue). Scale bar, 10 μm. (**c-f**) Centrosomal intensities of the PCNT (**c**), CEP215 (**d**), CEP192 (**e**) and γ-tubulin (**f**) signals were shown with the box-and-whisker plot. n=90 per group in 3 independent experiments. (**g,h**) Centriole association was determined with 2:1 ratio of the centrin-2 and C-NAP1 (**g**) or CEP135 (**h**) dots. The C-NAP1 signals at prometaphase were undetectable in the absence of BI2536 (Mardin et al., 2010). n=300 per group in 3 independent experiments. Values are means with standard deviations. (**i**) Distance between associated centrioles in Fig. 1h was measured using the centrin-2 signals. Centriole was hardly associated in DMSO-treated G1 phase cells. n=90 per group in 3 independent experiments. The statistical significance was determined by anova in Prism 6 (NS, $p>0.05$; *, $p<0.05$; **, $p<0.01$).

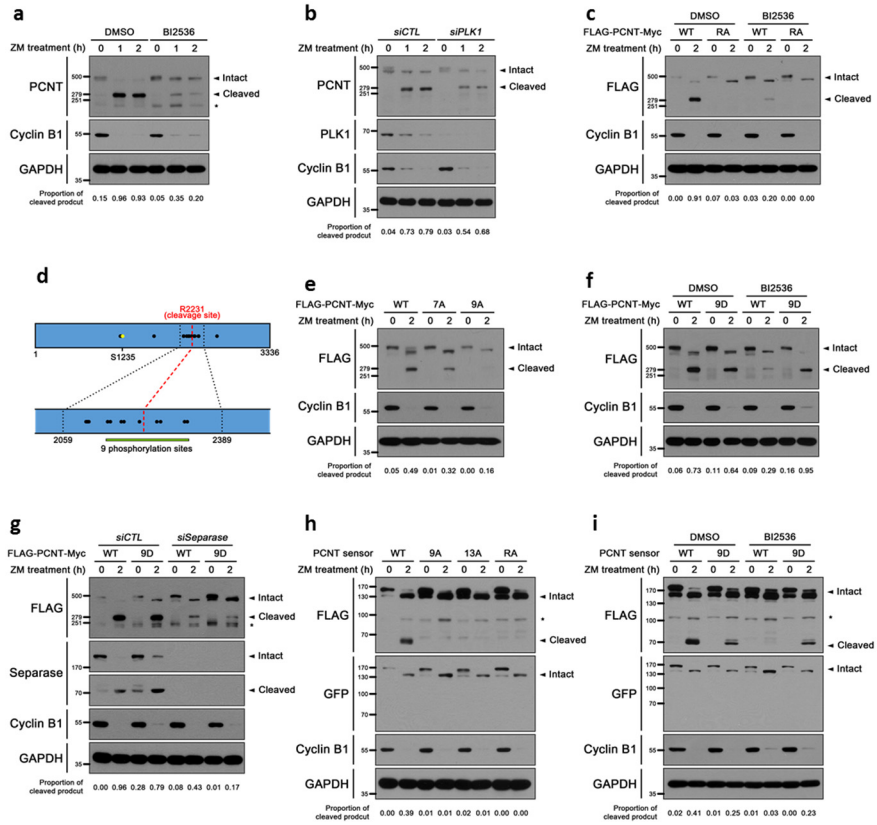

Figure 15. PLK1 regulation of the separase-dependent cleavage of PCNT during mitotic exit (**a**) HeLa cells were treated with BI2536 during mitotic exit and forced to mitotic exit with ZM447439 (ZM). Lysates were subjected to immunoblot analyses with antibodies specific to PCNT, cyclin B1, and GAPDH. (**b**) Immunoblot analysis was carried out to determine the PCNT cleavage in PLK1-depleted HeLa cells during the forced mitotic exit. (**c**) Specific cleavage of ectopic FLAG-PCNT-Myc (WT) and FLAG-PCNT^{R2231A}-Myc (RA) was determined in the presence of BI2536. (**d**) Summary of the PLK1 phosphorylation of PCNT. PLK1 phosphorylates 22 specific sites of PCNT (Fig. 13) and these sites are marked with circles. Among them, 9 phosphorylation sites (T2154, T2160, S2183, S2189, S2222, S2259, S2267, S2318, and T2324; green line) near the separase cleavage site (R2231; red) are critical for the PCNT cleavage. Two sites at the N-terminal end (S1235 and S1241; yellow circles) are essential for centrosome maturation. (**e**) Immunoblot analyses were carried out to determine specific cleavage of ectopic FLAG-PCNT^{7A}-Myc (7A) and FLAG-PCNT^{9A}-Myc (9A) during the forced mitotic exit. (**f,g**) Specific cleavage of ectopic FLAG-PCNT-Myc (WT) and FLAG-PCNT^{9D}-Myc (9D) was determined in the presence of BI2536 (**f**) and in the separase-depleted cells (**g**). (**h,i**) Immunoblot analysis was carried out to determine the specific cleavage of the FLAG-mCherry-PCNT$^{2059-2398}$-GFP-PACT fusion proteins with indicated phospho- (9A and 13A) and cleavage-resistant (RA) point mutations (**h**), and with phospho-mimetic mutation (9D) in the presence of BI2536 (**i**). The experiments were independently repeated at least twice. Asterisk, a non-specific band. (**a**,**b**,**c**,**e**) These results were adapted from [Jaeyoun Kim, master's thetis, Seoul National University, 2013].

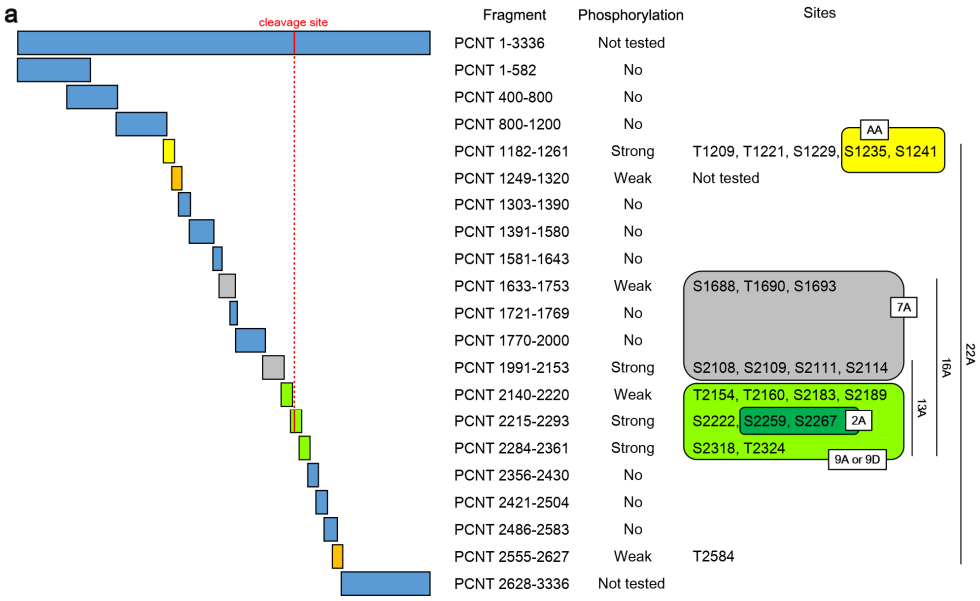

Figure 16. PLK1 phosphorylation of PCNT *in vitro* (**a**) Kwanwoo Lee performed *in vitro* kinase assays of PLK1 with PCNT fragments as substrates. The specific phosphorylation sites were confirmed with the PCNT fragments in which serine and threonine residues were substituted with alanines. In this way, He finally determined 22 residues for PLK1 phosphorylation. Results of T1209, T1221, S1229, S1235, and S1241 residues were previously described[10]. The phosphorylation sites of PCNT were grouped and colored with orange (T2584), yellow (AA; S1235A and S2141A), gray (7A; S1688A, T1690A, S1693, S2108, S2109, S2111, and S2114), green (9D or 9A; T2154A, T2160A, S2183A, S2189A, S2222A, S2259A, S2267A, S2318A, and T2324A), and dark green (2A; S2259A, S2267A).

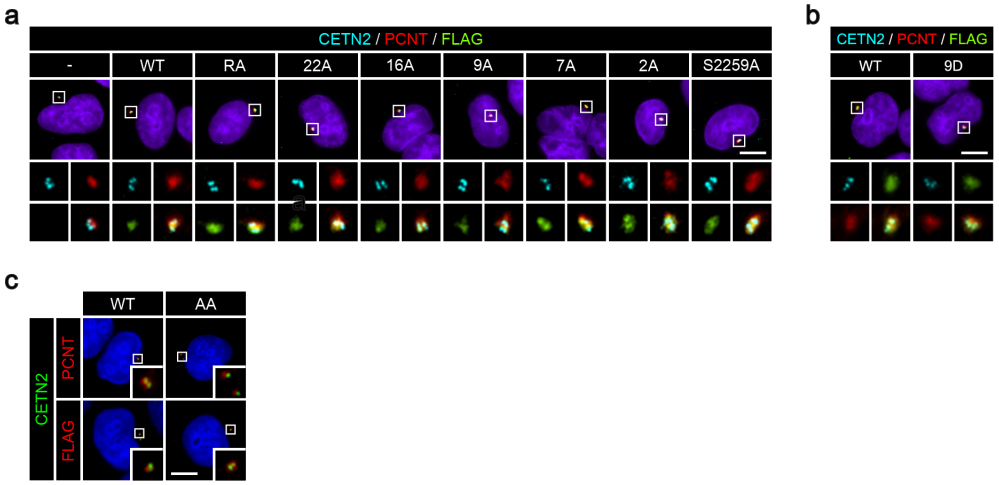

Figure 17. Centrosomal localization of the ectopic FLAG-PCNT-Myc proteins (**a**,**b**,**c**) The HeLa cells stably expressing FLAG-PCNT-Myc mutants were coimmunostained with antibodies specific to centrin-2 (CETN2; cyan in **a**,**b** and green in **c**), PCNT (red), and FLAG (green in **a**,**b** and red in **c**). DNA was visualized with DAPI (violet in **a**,**b** and blue in **c**). Scale bars, 10 μm

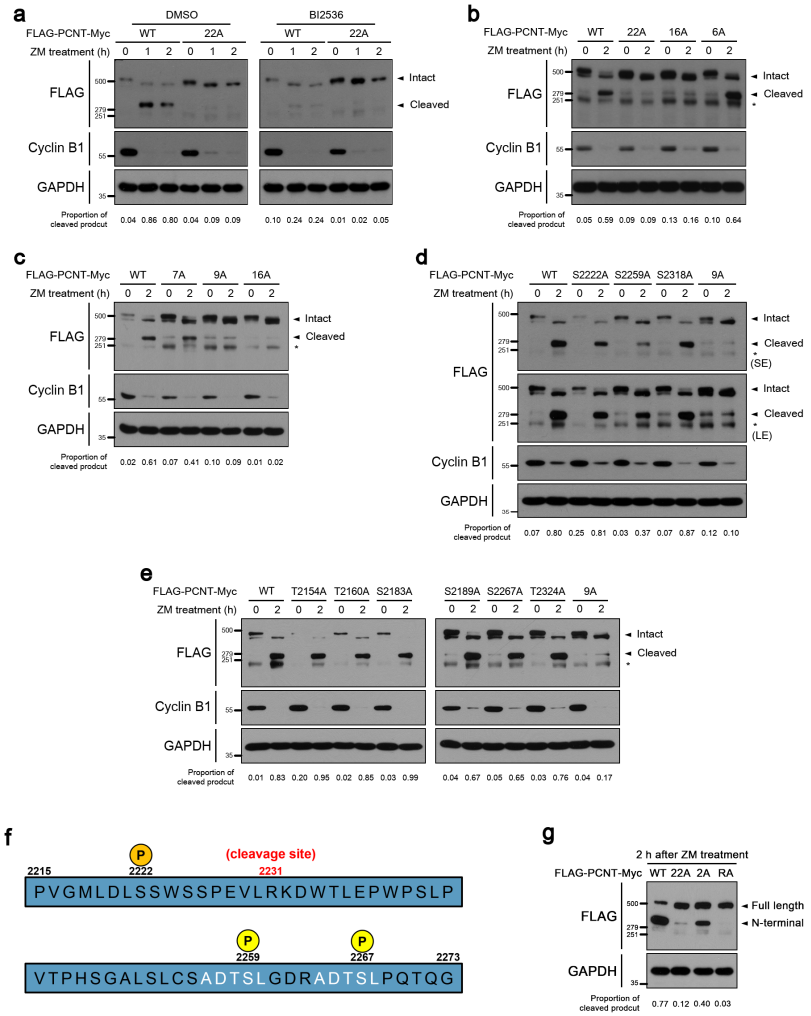

Figure 18. Multiple phosphorylation sites for PCNT cleavage (**a**) The HeLa cells stably expressing FLAG-PCNT-Myc and FLAG-PCNT^{22A}-Myc were arrested at M phase and treated with BI2536 for 3 h. After the ZM447439 (ZM) treatment, the cells were harvested at indicated time points and subjected to immunoblot analyses with antibodies specific to FLAG, cyclin B1 and GAPDH. (**b**-**e**) The HeLa cells were transiently transfected with indicated FLAG-PCNT-Myc mutants and subjected to PCNT cleavage assays. (**f**) The amino acid sequences near the cleavage site (R2231) of PCNT. S2222, S2259, and S2267 are specifically phosphorylated by PLK1 *in vitro* (yellow). The ADT(pS)L sequence (white) is repeated twice. (**g**) Cleavage bands of ectopic PCNT mutants in the stable cell lines were determined during mitotic exit. All experiments were independently repeated at least twice. (**a**-**f**) These results were adapted from [Jaeyoun Kim, master's thetis, Seoul National University, 2013]

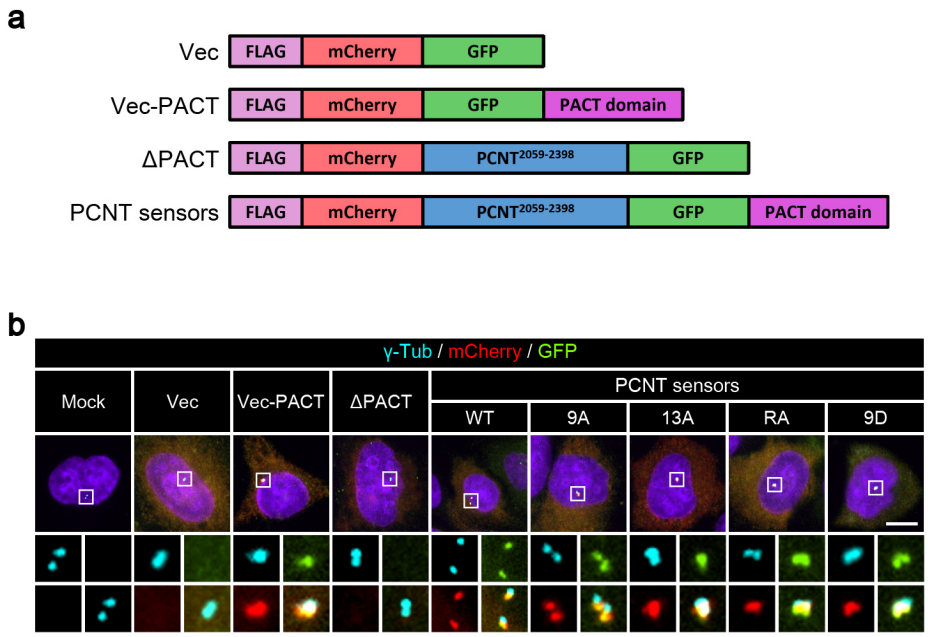

Figure 19. Centrosomal localization of the PCNT sensor proteins (**a**) Constructs of PCNT sensors (FLAG-mCherry-PCNT$^{2059\text{-}2398}$-GFP-PACT). mCherry (red) and GFP (green) were tagged at N- and C-terminus of PCNT$^{2059\text{-}2398}$ fragment (blue), respectively. PACT domain (violet) were attached at the end of GFP. FLAG (pink) was additionally tagged at N-terminus of mCherry. Vec, Vector. (**b**) HeLa cells transiently transfected with the PCNT sensor proteins were coimmunostained with antibodies specific to γ-tubulin (γ-Tub, cyan), mCherry (red) and GFP (green). DNA were visualized with DAPI (violet). Scale bar, 10 μm.

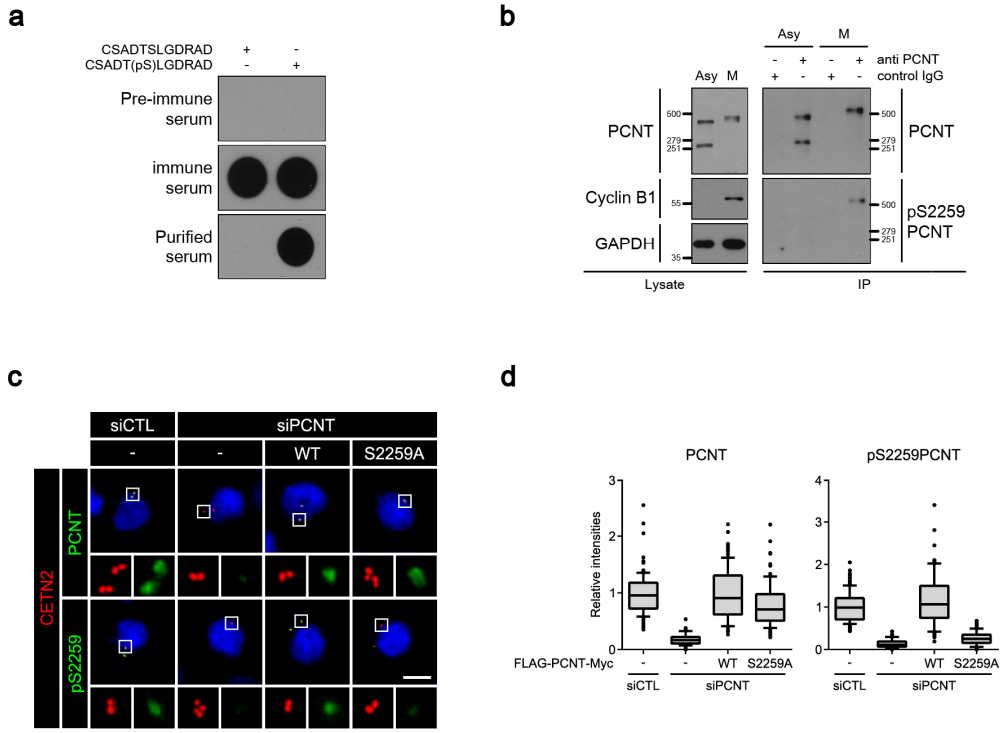

Figure 20. Characterization of the pS2259PCNT antibody (**a**) The pS2259PCNT antiserum was affinity-purified, and subjected to dot blot analyses with the synthetic peptides with or without pS2259. (**b**) Asynchronous and mitotic arrested lysates were immunoprecipitated with control IgG or the PCNT antibody and followed by immunoblot analysis with antibodies against PCNT and pS2259PCNT. (**c**) Endogenous PCNT was depleted in the cells stably expressing FLAG-PCNT-Myc (WT) or FLAG-PCNT^{S2259A}-Myc (S2259A). The cells were coimmunostained with the centrin-2 antibody (CETN2, red), along with the PCNT (red) and pS2259PCNT (red) antibodies. DNA was visualized with DAPI (blue). Scale bar, 10 μm (**d**) The centrosomal signals of PCNT and pS2259PCNT were determined and analyzed with the box-and-whisker plot. n=90 per group in 3 independent experiments.

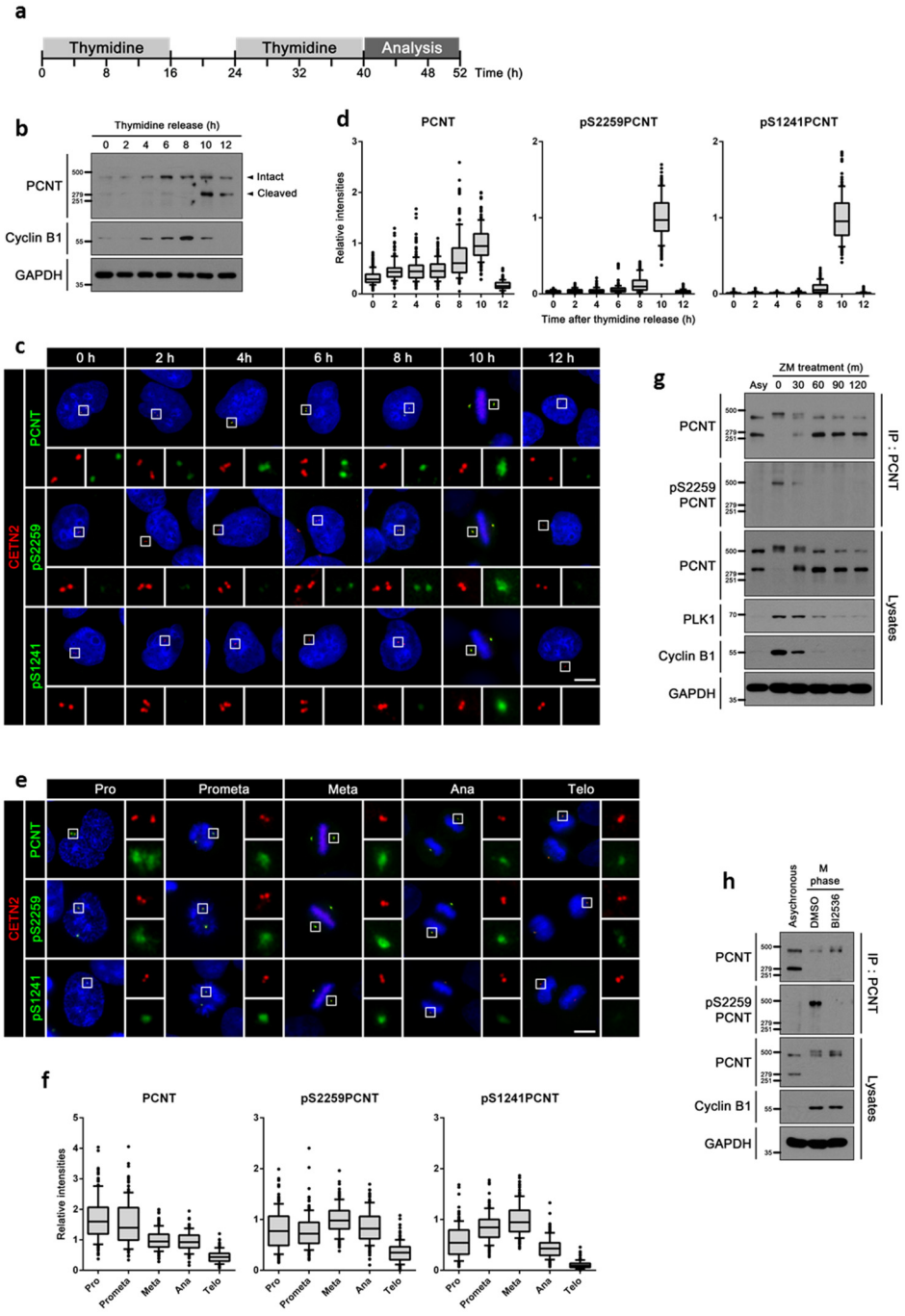

Figure 21. PLK1 phosphorylation of PCNT in vivo

Figure 21. PLK1 phosphorylation of PCNT in vivo (Cont'd) (**a**) HeLa cells were synchronously released from G1/S phase with the double thymidine block method and harvested every two hours for the immunoblot and immunostaining analyses. (**b**) The cells were subjected to immunoblot analyses with the PCNT, cyclin B1, and GAPDH antibodies. (**c**) Coimmunostaining analysis was performed with the centrin-2 antibody (CETN2, red) along with the PCNT and phospho-PCNT (pS2259PCNT and pS1241PCNT) antibodies (green). Scale bar, 10 μm. (**d**) Centrosomal intensities of PCNT and phospho-PCNT were densitometrically determined and shown with the box-and-whisker plot. n=153 per group in 3 independent experiments. (**e**) Coimmunostaining analysis of mitotic HeLa cells was performed with indicated antibodies. The mitotic stages were determined with DAPI staining patterns. Scale bar, 10 μm. (**f**) Centrosomal intensities of PCNT and phospho-PCNT were determined in cells at specific mitotic stages. n=153 per group in 3 independent experiments. (**g**) M phase-arrested HeLa cells were forced to exit mitosis with ZM447439 (ZM). At indicated time points, the cells were subjected to immunoprecipitation followed by immunoblot analyses with antibodies specific to PCNT, pS2259PCNT, PLK1, cyclin B1, and GAPDH. (**h**) The M phase-arrested HeLa cells were treated with BI2536 for 3 h and subjected to immunoprecipitation followed by immunoblot analyses with indicated antibodies.

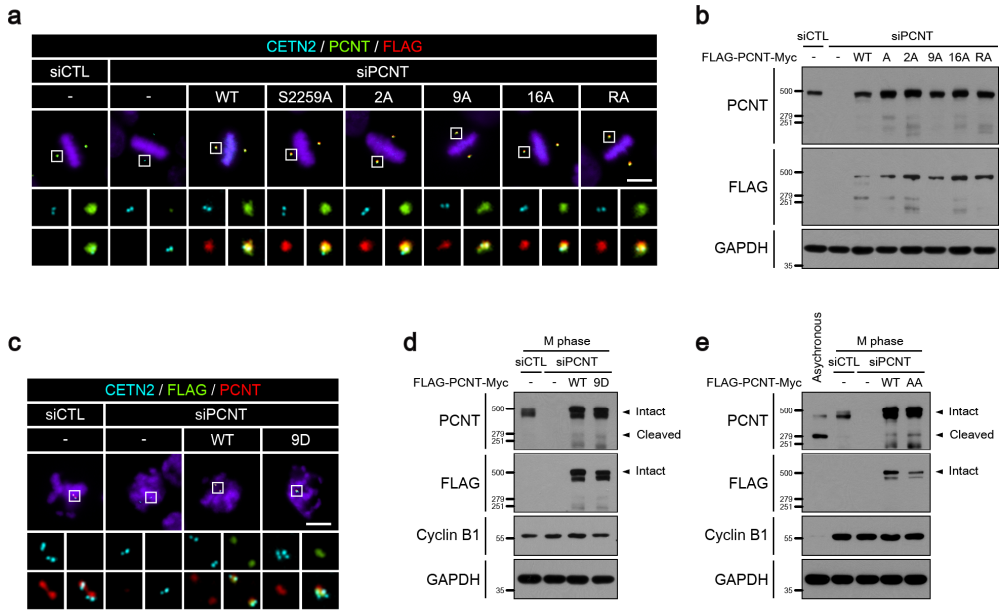

Figure 22. Generation of the FLAG-PCNT-Myc-rescued cells (**a**,**c**) Endogenous PCNT was depleted in HeLa cells in which the FLAG-PCNT-Myc mutant proteins were stably expressed. Mitotic cells were coimmunostained with antibodies against centrin-2 (CETN2, cyan), PCNT (green), and FLAG (red). DNA was visualized with DAPI (violet). Scale bars, 10 μm. (**b**) The PCNT rescued cells were arrested in G1/S phase with the double thymidine block method and subjected to immunoblot analyses with antibodies specific to PCNT, FLAG, and GAPDH. 'A' of lane 4 means FLAG-PCNT^{S2259A}-Myc. (**d**,**e**) The HeLa cells rescued with FLAG-PCNT^{9D}-Myc (9D) (**d**) and FLAG-PCNT$^{S2259/2267A}$-Myc (AA) (**e**) were arrested at M phase and subjected to immunoblot analysis with antibodies specific to PCNT, FLAG, cyclin B1, and GAPDH.

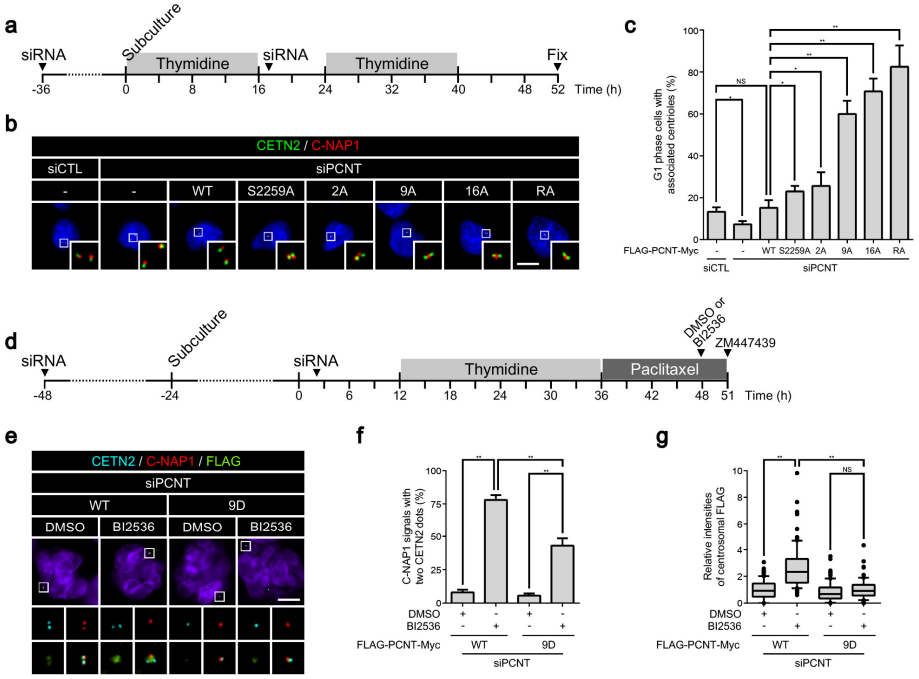

Figure 23. PLK1 phosphorylation of PCNT for centriole separation during mitotic exit (**a**) Endogenous PCNT was depleted in the stable cell lines expressing FLAG-PCNT-Myc proteins. As results, endogenous PCNT was rescued with the ectopic PCNT proteins (Fig. 17a,b). The cell cycle was synchronized with the double thymidine block and release. (**b**) The PCNT-rescued cells were subjected to coimmunostaining analyses with antibodies specific to C-NAP1 (red) and centrin-2 (CETN2, green). Scale bar, 10 μm. (**c**) Centriole association in G1 phase cells was determined with the 2:1 ratio of centrin-2 and C-NAP1. Cells with only two centrin-2 dots were determined in G1 phase. Values are means with standard deviations. n=300 per group in 3 independent experiments. (**d**) The cells rescued with FLAG-PCNT-Myc (WT) or FLAG-PCNT^{9D}-Myc (9D) were arrested at M phase, treated with BI2536 to block the PLK1 activity, and with ZM447439 (ZM) to force mitotic exit. (**e**) The HeLa cells rescued with FLAG-PCNT^{9D}-Myc (9D) were subjected to coimmunostaining analyses with antibodies specific to centrin-2 (cyan), C-NAP1 (red) and FLAG (green). Scale bar, 10 μm. (**f**) Centriole association was determined with the 2:1 ratio of centrin-2 and C-NAP1. Values are means with standard deviations. n=300 per group in 3 independent experiments. (**g**) The centorsomal levels of ectopic FLAG-PCNT-Myc were statistically analyzed and shown with the box-and-whisker plot. n=90 per group in 3 independent experiments. The statistical significance was determined by anova in Prism 6 (NS, $p>0.05$; *, $p<0.05$; **, $p<0.01$).

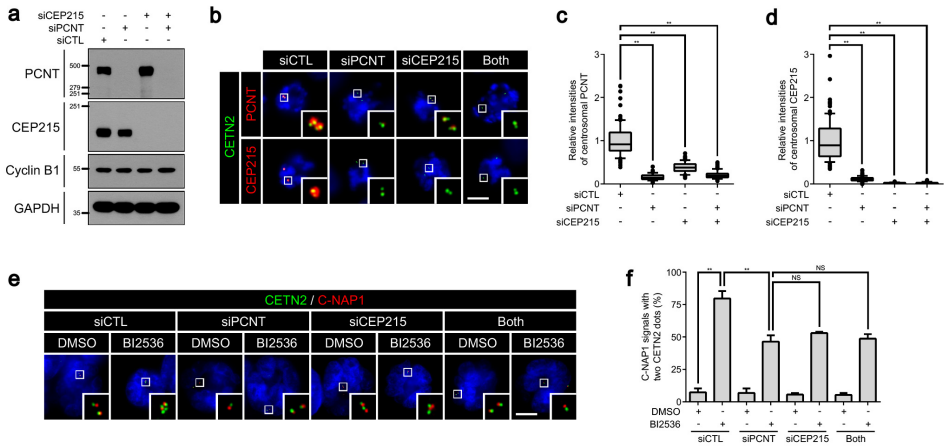

Figure 24. Centriole separation in the PCNT- and/or CEP215-depleted cells (**a**-**d**) Depletion of PCNT and CEP215 was confirmed with immunoblot (**a**) and coimmunostaining (**b**) with the PCNT and CEP215 antibodies. Scale bar, 10 μm. (**c**,**d**) Centrosomal intensities of PCNT and CEP215 in mitotic cells were determined and shown with the box-and-whisker plot. n=90 per group in 3 independent experiments. (**e**) The PCNT- and/or CEP215-depleted cells were arrested at M phase, treated with BI2536 for 3 hours, forced to exit mitosis with ZM447439 (ZM) for 2 hours, and coimmunostained with antibodies specific to centrin-2 (CETN2, green) and C-NAP1 (red). Scale bar, 10 μm. (**f**) Centriole association was determined with the 2:1 ratio of centrin-2 and C-NAP1. Values are means with standard deviations. n=300 per group in 3 independent experiments. The statistical significance was determined by anova in Prism 6 (NS, $p>0.05$; *, $p<0.05$; **, $p<0.01$).

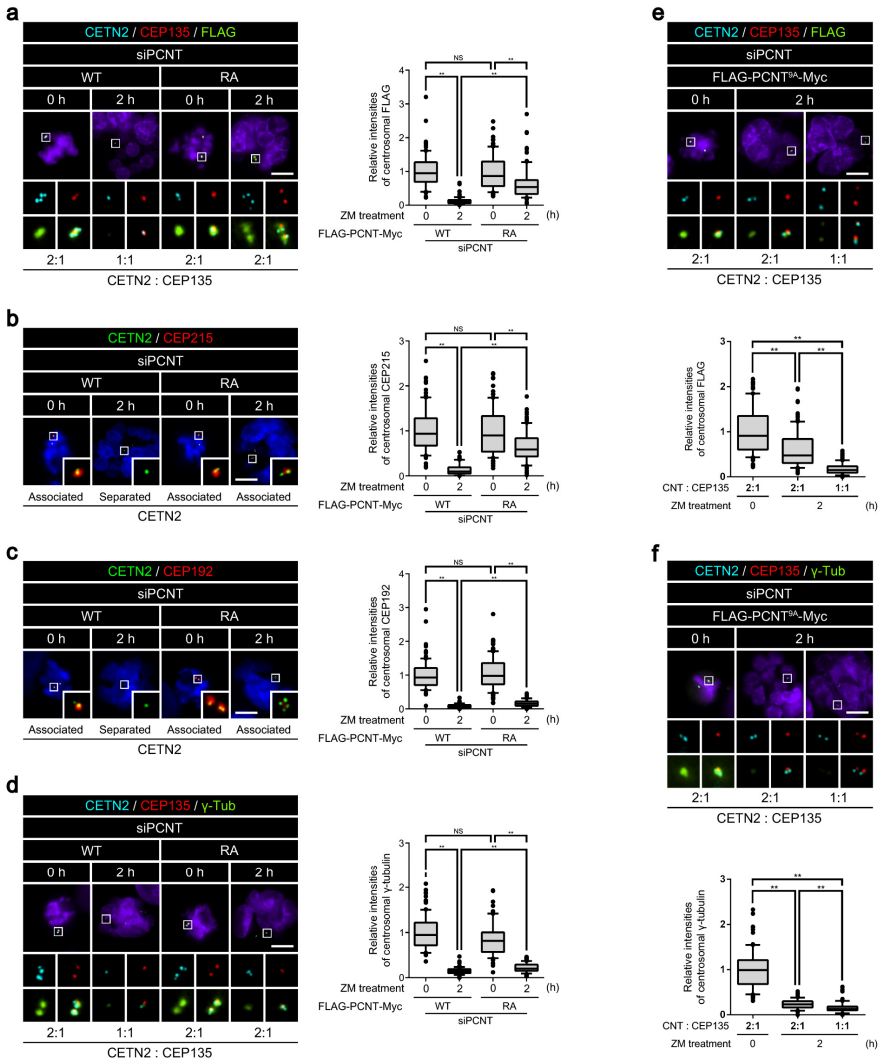

Figure 25. Importance of PCM for maintaining centriole association (**a-f**) The M phase-arrested cells rescued with FLAG-PCNT-Myc (WT) or FLAG-PCNT^{R2231A}-Myc (RA) (**a-d**) and FLAG-PCNT9A-Myc (9A) (**e,f**) were forced to exit mitosis with ZM447439 (ZM) and coimmunostained with indicated antibodies. Scale bars, 10 μm. Centrosomal intensities of the indicated proteins were determined before and after the ZM447439 treatment and shown with the box-and-whisker plot. Centriole association was determined with the 2:1 ratio of centrin-2 and CEP135 dots in **a**, and **d-f**. The centriole separation in **b** and **c** was determined, based on the proximity of centriole pairs (< 1μm). n=90 per group in 3 independent experiments. The statistical significance was determined by anova in Prism 6 (NS, p>0.05; *, p<0.05; **, p<0.01).

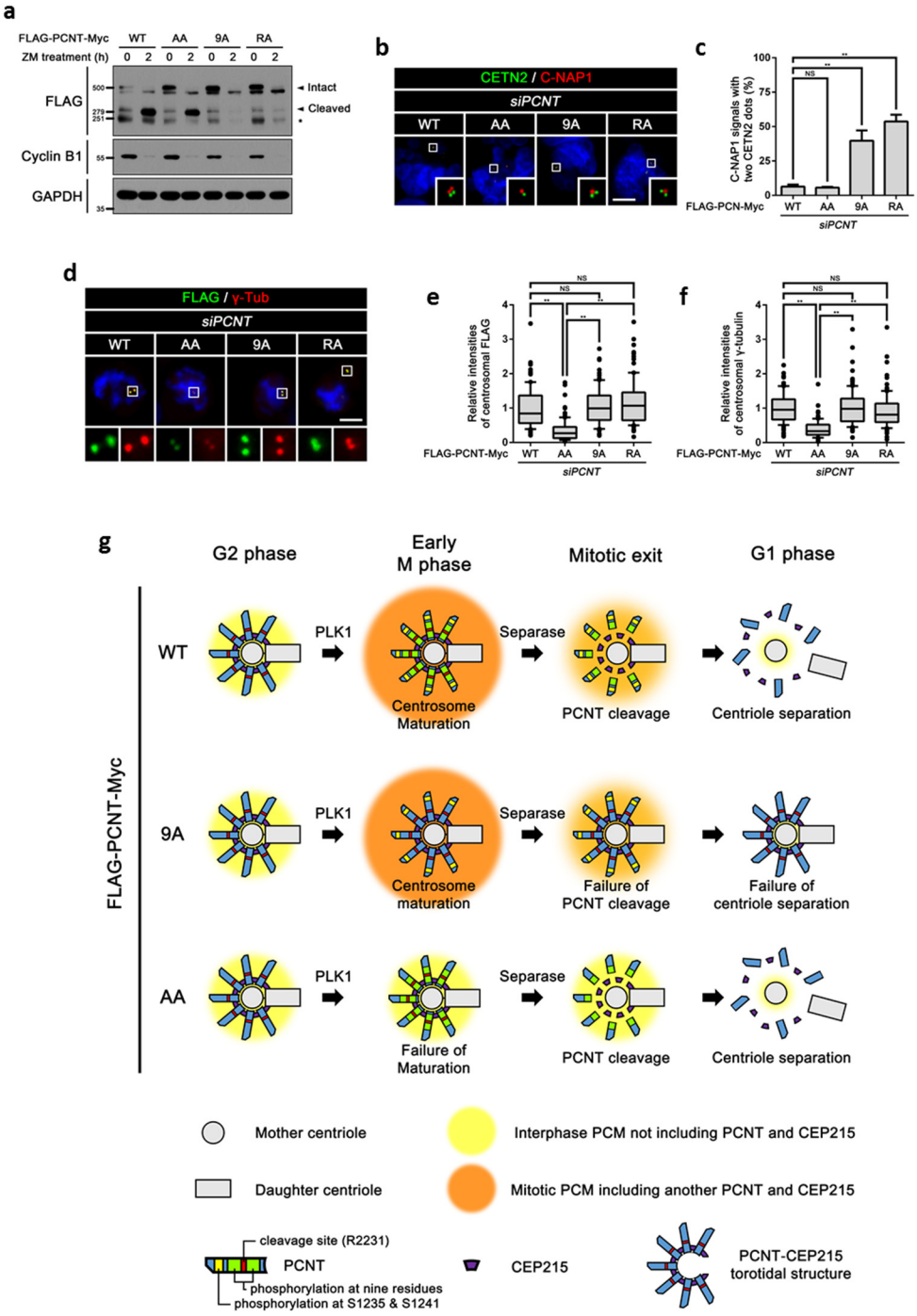

Figure 26. PLK1 control for dual functions of PCNT

Figure 26. PLK1 control for dual functions of PCNT (Cont'd) (**a**) HeLa cells were transiently transfected with the wild type and mutant FLAG-PCNT-Myc (WT, AA, 9A and RA), arrested at M phase and forced to exit mitosis with ZM447439 (ZM). Immunoblot analyses were performed to determine specific cleavage of FLAG-PCNT-Myc during mitotic exit. Asterisk, a non-specific band. (**b**) The PCNT-depleted cells were rescued with the wild type and mutant FLAG-PCNT-Myc, and subjected to coimmunostaining analyses with antibodies specific to centrin-2 (CETN2, green) and C-NAP1 (red). Scale bar, 10 μm. (**c**) Centriole association was determined with the 2:1 ratio of centrin-2 and C-NAP1 dots. Values are means with standard deviations. n=300 per group in 3 independent experiments. (**d**) The PCNT-rescued cells were coimmunostained with antibodies against FLAG (green), and γ-tubulin (red). Scale bar, 10 μm. (**e**,**f**) Centrosomal intensities of ectopic FLAG-PCNT-Myc (**e**) and γ-tubulin (**f**) were determined. n=90 per group in 3 independent experiments. The statistical significance was determined by anova in Prism 6 (NS, $p>0.05$; *, $p<0.05$; **, $p<0.01$). (**g**) Model. PLK1 phosphorylation of PCNT induces centrosome maturation in early M phase and PCNT cleavage during mitotic exit. As a result, centrioles are separated from each other with little PCM surrounded. The centrosomes in $PCNT^{9A}$-rescued cells were properly matured in early M phase but PCNT was not cleaved by separase during mitotic exit. As a result, centrioles remained associated within the PCM scaffold. The centrosomes in $PCNT^{AA}$-rescued cells were not matured in early M phase but PCNT was cleaved by separase during mitotic exit. As a result, centrioles are separated from each other with little PCM surrounded.

DISCUSSION

In this report, I revealed that PCNT should be phosphorylated by PLK1 in order to be a suitable substrate of separase. It is known that the separase-dependent cleavage of PCNT is an inevitable event for centriole separation during mitotic exit (Lee and Rhee, 2012; Matsuo et al., 2012). Therefore, I propose that PLK1 phosphorylation is a priming step for PCNT cleavage and eventually for centriole separation during mitotic exit (Fig. 26g). My results are consistent with the previous reports in which the PLK1 activity is required for centriole separation during mitotic exit (Tsou et al., 2009).

It is well known that separase is activated at the anaphase onset when securin and cyclin B1 are degraded by the APC complex (Ciosk et al., 1998; Goff et al., 2015). However, a residual amount of the separase activity is also detected prior to the anaphase and even in interphase cells (Agircan et al., 2015; Nagao, 2004). The PLK1-dependent phosphorylation would preclude premature centriole separation, which might result in multipolar spindle formation and mitotic catastrophe. Therefore, PLK1 phosphorylation of PCNT provides an additional layer of regulatory mechanism to ensure the timeliness and fidelity of centriole separation during mitotic exit. It was recently reported that PP2A forms a complex with inactive separase (Hellmuth et al., 2014). It is possible that PCNT may be a substrate of the separase-bound PP2A. If this is the case, centriole separation mechanisms would be additionally fine-tuned

with a counterbalance between PLK1 and PP2A for the separase-dependent cleavage of PCNT. My results are consistent with the proposal that PLK1 and APC/C activities cooperatively function for centriole separation in the short time-window of the cells finishing mitosis (Hatano and Sluder, 2012).

PCNT and CEP215 are major PCM components in the mitotic spindle poles as well as in the interphase centrosomes (Kim and Rhee, 2014). Once PCNT is cleaved by separase, the C-terminal fragment of PCNT is immediately degraded following the N-end rule pathway (Lee and Rhee, 2012). Since both the CEP215-interacting and the centrosome targeting domains reside in the C-terminal fragment of PCNT, the separase-dependent cleavage of PCNT results in disassembly of itself from centrosome and subsequently centriole separation during mitotic exit (Fig. 26g). Depletions of either one or both of PCNT and CEP215 probably limit the assembly of PCM scaffold which plays critical roles in maintaining centriole association (Fig. 24b-d). Consistent with this notion, CEP215 depletion results in centriole separation in the cells even with a non-cleavable PCNT mutant (Pagan et al., 2015).

Two different models for the mother and daughter centriole association have been proposed. First, the orthogonal configuration of engaged centrioles is maintained by a glue protein that may form a linker between the mother and daughter centriole (Schockel et al., 2011). Second, the mother and daughter centrioles are trapped within PCM so that centrioles remain associated (Cabral et al., 2013; Lee and Rhee, 2012; Matsuo et al.,

2012; Pagan et al., 2015). These two models are not mutually exclusive, so that it is possible that daughter centriole is disengaged but trapped within the PCM until the PCM scaffold is disassembled (Pagan et al., 2015; Sluder, 2013). In this study, I revealed that PLK1 is critical for centriole separation by regulating PCM disassembly during mitotic exit. However, PLK1 may be also involved in the centriole disengagement to break orthogonal arrangement prior to the centriole separation. In fact, PLK1 depletion prevented centriole disengagement and reduplication in S phase-arrested U2OS cells (Hatano and Sluder, 2012; Liu and Erikson, 2002). Centriole disengagement and reduplication in G2-arrested cells also depend upon PLK1 activity (Loncarek et al., 2010). Finally, when PLK1 activity is inhibited in late G2 phase, but not in late M phase, centriole disengagement is completely blocked in the separase-null cells (Tsou et al., 2009). These results suggest that PLK1 regulates both centriole disengagement and centriole separation events. I identified that PCNT is a key substrate of PLK1 for centriole separation. However, it remains to be identified what is a PLK1 substrate for centriole disengagement.

It is known that PLK1 phosphorylation of PCNT at S1231 and S1241 residues are required for centrosome maturation (Lee and Rhee, 2011). Here, I report that another set of PCNT residues should be phosphorylated by PLK1 for centriole separation. Therefore, two different functions of PCNT are induced by PLK1 phosphorylation at distinct sites of PCNT (Fig. 26g). It is well known that PLK1 carries out multiple mitotic functions by

phosphorylating diverse substrates. My results provide an example that PLK1 can carry out multiple functions by phosphorylating specific sets of residues within a single protein species.

CHAPTER 2

Phenotypic analyses of PCNT-deleted cells

- PCNT has an active role in maintaining centriole engagement -

ABSTRACT

Duplication and segregation of centrioles occur in tight link to cell cycle. A daughter centriole is assembled next to the mother centriole during S phase, and remained in an engaged state until cells exit M phase. However, it is largely unknown how centriole engagement is maintained until the end of M phase. Pericentrin (PCNT) is a PCM protein which is important for maturation process of centrosome to become spindle poles in cells entering M phase. Here, I generated *PCNT* knockout cell lines and analyzed the phenotypes in relation to PCM assembly and centriole association. Deletion of PCNT hardly affected interphase centrosomes but conferred defects in centrosome maturation in cells entering M phase. The centrioles in *PCNT*–deleted cells were prematurely separated in early phase of mitosis and frequently amplified in M phase-arrested cells. Abnormal multi-nuclear cells repeatedly appeared in *PCNT*-deleted cells at interphase. My results confirmed that PCNT is critical for centriole association during M phase.

INTRODUCTION

The centrosome is a very small organelle which is composed of centrioles and pericentriolar materials (PCM) (Bettencourt-Dias and Glover, 2007). Centriole duplication begins at S phase when daughter centriole starts to grow next to mother centriole with perpendicular angle (Nigg, 2007). Daughter centriole is engaged with mother centriole until mitosis while limiting additional round of centriole duplication (Nigg, 2007). Therefore, centriole disengagement is considered a licensing step for centriole duplication. Misregulation of centriole disengagement can cause chromosomal instabilities, which is one of hallmarks of cancer (Holland and Cleveland, 2009; Vitre and Cleveland, 2012).

PCNT is a versatile PCM component related to mitotic PCM assembly and centriole separation (Lee and Rhee, 2011; Lee and Rhee, 2012). Interestingly, both events are governed by PLK1 (Lee and Rhee, 2011, Kim et al., 2015). At G2/M transition, PLK1 phosphorylates PCNT at S1235 and S1241 residues, which is a switch leading to mitotic PCM assembly (Lee and Rhee, 2011). During mitotic exit, PLK1 phosphorylates PCNT at 9 residues, which is a priming step for separase-dependent PCNT cleavage (Kim et al., 2015).

Studies with super-resolution microscopy revealed that PCM is highly organized with a toroidal structure and PCNT stands like a pillar (Fu and Glover, 2012; Lawo et al., 2012; Sonnen et al., 2012). This observation leads

to think PCNT is a scaffold protein for PCM toroid. In this chapter, I observed that PCM proteins are normally recruited to interphase centrosome, but not mitosis in PCNT-deleted cells. Furthermore, centrioles separation and overduplication were observed during mitosis in the absence of PCNT. Premature centriole separation was rescued with DD-Flag-PCNT$^{1235/1251A}$-Myc mutant. These results imply that PCNT is not scaffold protein for interphase PCM assembly, but it is important for maintaining centriole engagement during mitosis.

MATERIALS AND METHODS

Plasmids, siRNA, and cell culture

All PCNT (NCBI reference sequence: NM_006031.5) constructs were subcloned into pcDNA^{TM}5/FRT/TO vector (Invitrogen) and tagged with 3×FLAG-DD and Myc at 5'- and 3'- ends, respectively. In order to generate siRNA-resistant PCNT construct, I induced silent mutations of PCNT with the following primers: 5'-GCA GCA GAA CTC AAG GAG-3' and 5'-TGG ACC TCT TCG AAT GAG-3'.

The HeLa cells were cultured in DMEM (Welgene, LM 001-05) supplemented with 10% FBS (Welgene, S101-01) and antibiotics (Invivogen, ANT-MPT) at 37°C and 5% CO_2. To generate stable cell lines, I seeded 2.4×10^5 HeLa cells on 60 mm dish and 2.5 μg of plasmid DNA was transfected in the next day. The plasmids were transfected into the HeLa cells using Fugene HD (Promega, E2311). One day after the transfection, the cells were transferred to a 100 mm dish and treated with 0.2 mg/ml hygromicin B for 2-3 weeks. Monoclonal cell lines were established with the dilution cloning method.

To synchronize cell cycle, I used 2 mM thymidine (Sigma, T9250), 5 μM paclitaxel (Sigma, T7402), and 2 μM ZM447439 (Cayman chemical company, 13601). For inhibition of PLK1 activity during mitosis, I treated the cells with 100 nM BI2536 (Selleck chemicals, S1109) for 3 h after the thymidine-paclitaxel. To induce expression of ectopic proteins, 10 ng/ml

Doxycycline and 50 nM shield1 was treated.

Generation of knockout cell lines with CRIPSR/CAS9 system

Knockout cell lines were generated as described previously (Ran et al., 2013). In brief, guide RNA sequence of target was subcloned into pSpCAS9(BB)-2A-Puro vector and transfected to HeLa cells. One day after transfection, 1 mg/ml puromycin was treated for three day and monoclones were obtained. To determine in-del, the genomic DNA near the guide RNA target site were amplified by PCR and sequences were analyzed.

Antibodies

The CEP135 (ICC 1:5000; Jeong et al., 2007), CEP215 (ICC 1:5000, IB 1:5000; Lee and Rhee, 2010), and PCNT (ICC 1:20000, IB 1:5000; Kim and Rhee, 2011) antibodies were previously described. Antibodies specific to centrin-2 (Merck Millipore, 04-1624; ICC 1:1000), CEP192 (Bethyl Laboratories, A302-324A; ICC 1:20000), GAPDH (Life technologies, AM4300; IB 1:20000), FLAG (Sigma-Aldrich, F3165; ICC 1:2000, IB 1:10000), and γ-tubulin (Santa Cruz Biotechnology, sc-7396; ICC 1:200) were purchased. The secondary antibodies conjugated with fluorescent dye (Alexa-488, Alexa-594, and Alexa-647, Life Technologies; 1:1000) and for with HRP (Sigma-Aldrich or Millipore, 1:10000) were purchased.

Immunoblot analysis

The cells were lysed on ice for 10 minutes with RIPA buffer (150 mM NaCl, 1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl at pH 8.0, 10 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, and 1 mM EGTA) containing a protease inhibitor cocktail (Sigma-Aldrich, P8340) and centrifuged with 12,000 rpm for 10 min at 4°C. The supernatants were mixed with 4×SDS sample buffer (250 mM Tris-HCl at pH 6.8, 8% SDS, 40% glycerol, and 0.04% bromophenol blue), and 10 mM DTT (Amresco, 0281-25G). Mixtures were boiled for 5 min.

To detect intact and cleaved PCNT, 15-20 μg of proteins were loaded in sodium dodecyl sulphate-polyacrylamide gel (3% stacking gel and 4% separating gel), electrophoresed, and transferred to Protran BA85 nitrocellulose membranes (GE Healthcare Life Sciences, 10401196). The membranes were blocked with blocking solution (5% nonfat milk in 0.1% Tween 20 in TBS or 5% bovine serum albumin in 0.1% Tween 20 in TBS) for 2 h, incubated with primary antibodies diluted in blocking solution for 16 h at 4°C, washed four times with TBST (0.1% Tween 20 in TBS), incubated with secondary antibodies in blocking solution for 30 min, and washed again. To detect the signals of secondary antibodies, ECL reagent (ABfrontier, LF-QC0101) and X-ray films (Agfa, CP-BU NEW) were used. In the cases of other proteins, 5% stacking and 10% separating gels were used.

Immunstaining analysis

For immunocytochemistry, cells seeded on cover glass were fixed with cold methanol for 10 min and washed three times with cold PBS. After incubation of PBST (0.1% Triton X-100 in PBS) for 10 min, the cells were blocked with blocking solution (3% bovine serum albumin, and 0.3% triton X-100 in PBS) for 30 min, incubated with primary antibodies diluted in blocking solution for 1 h, washed three times with PBST, incubated with secondary antibodies in blocking solution for 30 min, washed twice with PBST, incubated with DAPI solution for 3 min, and washed twice with PBST. The cover glasses were mounted on a slide glass with ProLong Gold antifade reagent (Life technologies, P36930). Images were acquired from fluorescence microscopies equipped with digital cameras (Olympus IX51 equipped with QImaging QICAM Fast 1394 or Olympus IX81 equipped with ANDOR iXon^{EM}+) and processed in ImagePro 5.0 (Media Cybernetics) or MetaMorph 7.6 (Molecular Devices). Inset images were enlarged four times in Image J 1.49 (National Institutes of Health, USA) or Photoshop CS6 (Adobe) using option of bicubic interpolation. In the cases of quadruple staining, Image 5D (Fiji) which is a plug-in of Image J were used in order for pseudo-coloring.

To measure fluorescence intensities, I immunostained all cells at the same time with same diluent antibodies. All images were captured at same exposure time without stopping. Image J 1.49 was used to measure fluorescence intensities at centrosomes. In each measurement, background signals were subtracted from the sum of fluorescence signals at centrosome.

Statistical analysis

In order to statistical analysis, experiments were independently performed three times. To calculate P values, unpaired two-tailed t test or anova were performed in Prism 6 (GraphPad Software). All Measured fluorescent intensities were displayed with box-and-whisker plots in Prism 6 (Lines, median; vertical boxes; values from 25^{th} to 75^{th}; down error bars, 10^{th} value; up error bar, 90^{th} value; circles, outliers).

RESULTS

Generation of *PCNT*;*TP53* double knockout cell lines

To investigate roles of PCNT in centriole separation and PCM assembly, I generated *PCNT* knockout cell lines using CRISPR/CAS9 system. It is known that the p53 pathway is frequently activated by loss of centrosomal genes, resulting in cell cycle arrest and eventually cell death (Cuella-Martin et al., 2016; Fong et al., 2016; Lambrus et al., 2016; Meitinger et al., 2016). Therefore, I eliminated the *TP53* gene prior to deletion of the *PCNT* gene in the Flp-IN T-REx HeLa cells (Spitzer et al., 2013). Immunoblot analysis revealed that both PCNT and p53 were undetectable in my double knockout cell line (Fig. 27a). The sequencing analyses revealed frameshift mutations in both the *TP53* and *PCNT* genes (Fig. 27b,c). Therefore, I used the *PCNT*;*TP53* double knockout cell line for my thesis experiments.

Interphase centrosomes in the *PCNT*-deleted cells

To investigate importance of PCNT in the PCM structure of interphase centrosomes, I determined the centrosomal levels of selected PCM proteins in a *TP53*;*PCNT* double knockout cell line (Fig. 28a). The wild type and *TP53*-deleted HeLa cells were used as controls. The results showed that the centrosomal signals of PCNT were hardly detectable in the *PCNT*-deleted cells (Fig. 28a,b). However, centorosomal levels of the other examined PCM

proteins, such as CEP215, CEP192 and γ-tubulin, were unaffected in the absence of PCNT (Fig. 28a,c-e). The number of centrioles per cell was more or less the same among the wild type and knockout cells (Fig. 28a,f). Mitotic index was also unaffected in the double knockout cells (Fig. 29a,b). However, the number of multinucleated cells increased in the double knockout cells (Fig. 29c,d). These results suggest that structural and functional roles of PCNT are minimal in interphase centrosome. On the contrary, PCNT may be critical in mitotic centrosome, since *PCNT* deletion augmented proportion of multinuclear cells.

Spindle poles in the *PCNT*-deleted mitotic cells

When cells enter mitosis, centrosomes mature to become spindle poles with a robust microtubule organizing activity. PCM enlargement is accompanied during the centrosome maturation (Feng et al., 2017). To investigate importance of PCNT in PCM structure of M-phase cells, I determined the centrosomal levels of selected PCM proteins in a *TP53*;*PCNT* double knockout cell line (Fig. 30a). After the release from a thymidine block, the cells were arrested at prometaphase with treatment of STLC, an EG5 inhibitor, for 10 h (Seo et al., 2015). The wild type and *TP53*-deleted HeLa cells were used as controls. The results showed that the centrosomal signals of PCNT were hardly detectable in the *PCNT*-deleted cells (Fig. 30a,b). At the same time, significant reduction of other PCM proteins, such as CEP215, CEP192 and γ-tubulin, was accompanied (Fig. 30a,c-e). These results indicate

that PCNT is essential for mitotic PCM assembly.

Daughter centrioles are formed next to the mother centrioles in S phase and remain engaged until mitotic exit. Therefore, most of the mother and daughter centrioles are associated when cells are arrested at prometaphase with the STLC treatment (Seo et al., 2015; Figure 27f,g). However, I observed that centrioles were separated in the *PCNT*-deleted cells with STLC treatment (Fig. 30f,g). At the same time, about 30% of the *PCNT*-deleted cells included amplified centrioles (Fig. 30f,h). Similar results were observed when the *PCNT*-deleted cells were treated with the other cell cycle blockers, such as nocodazole and paclitaxel (Fig. 31a-c). These results indicate that PCNT plays a key role for centriole association in prometaphase-arrested cells. Centriole association is likely to be linked to inhibition of unscheduled amplification of centrioles during the prometaphase arrest.

It is known that a prolonged treatment of STLC results in premature centriole separation, probably due to stress-induced PCM dispersal (Seo et al., 2015). I compared premature centriole separation in the *TP53* knockout and *TP53*;*PCNT* double knockout cell lines. After the thymidine release, the cells were treated with STLC for up to 20 h (Fig. 32a). The results showed that centriole separation in control cells increased in 15 h and reached maximal in 20 h after the STLC treatment (Fig. 32b,c). On the other hand, centriole separation in *PCNT*-deleted cells reached maximal in 10 h, about when the cells entered M phase (Fig. 32b,c). Centriole amplification also increased significantly in 10 h after the STLC treatment and reduced afterwards (Fig.

32b,d). These results support the notion that PCNT is essential for centriole engagement when cells enter M phase. As far as centrioles are associated, they may not be amplified (Novak et al., 2014). It is not clear why centriole overduplication was reduced in prometaphase-arrested cells with a prolonged treatment of STLC. One possibility may be that disintegration of PCM structure does not provide a favorable environment for centriole amplification.

In the previous works, I investigated spindle pole morphology of the *PCNT*-deleted cells whose cell cycle was arrested at prometaphase with STLC. Next, I wanted to confirm roles of PCNT in the centrosomal structure and functions of the *PCNT*-deleted cells undergoing mitosis. I began to determine proportion of mitotic phases of the *PCNT*-deleted cells. The *PCNT*-deleted cells were enriched at M phase with double thymidine block and release, and their mitotic phases were determined by morphology of condensed chromosomes and spindles (Fig. 33a). The results showed that the *PCNT*-deleted cells were staged more at prometaphase and less at metaphase than the wild type cells, suggesting that mitotic progression was slightly delayed in the *PCNT*–deleted cells (Fig. 33b).

I determined the centrosomal levels of selected PCM proteins in *PCNT*-deleted cells at prometaphase (Fig. 33c). The results showed that the centrosomal signals of PCNT were hardly detectable in the *PCNT*-deleted cells (Fig. 33c,d). At the same time, significant reduction of other PCM proteins, such as CEP215, CEP192 and γ-tubulin, was observed (Fig. 33c,e-g). These results are very similar to the previous results with STLC-arrested

cells (Fig. 30a-e). Therefore, I conclude that PCNT should be crucial for PCM assembly during mitotic spindle pole formation, even if it may not be essential for toroidal structure of centrosomes in interphase cells.

Finally, I determined centriole separation in PCNT-deleted cells during mitosis. Centriole separation was determined with 1:1 ratio of the centrin-2 and CEP135 signals at the spindle poles of prometaphase cells (Fig. 33c). As expected, most centrioles were associated in control cells (Fig. 33h). However, many centrioles in the spindle poles of PCNT-deleted cells were separated (Fig. 33h). At the same time, centriole amplification was frequently observed in the PCNT-deleted mitotic cells (Fig. 33i). Taken together, I conclude that PCNT is essential not only for PCM assembly but also for centriole association in cells entering mitosis.

Active role of PCNT for centriole association

In order to investigate the role of PCNT in centriole association in depth, I generated rescue cell lines in which ectopic DD-Flag-PCNT-Myc protein was induced by doxycycline and actively degraded by removal of shield1 (Banaszynski et al., 2006). The immunoblot analysis revealed that DD-Flag-PCNT-Myc was hardly detected in the stable lines but was induced by doxycycline and shield1 to a comparable level with endogenous PCNT (Fig. 34a). I determined the centrosomal levels of PCNT and CEP215 in the rescued cells at prometaphase with the STLC treatment (Fig. 34b). As expected, the

DD-Flag-PCNT-Myc fusion protein was detected at the centrosome as much as the endogenous PCNT protein (Fig. 34c). At the same time, the centrosomal levels of CEP215 was also rescued in the DD-Flag-PCNT-Myc-expressing cells (Fig. 34d). I also observed that centriole association was rescued with DD-Flag-PCNT-Myc (Fig. 35a,b). However, centriole amplification was not effectively reduced in the DD-Flag-PCNT-Myc-expressing cells (Fig. 35a,c). Collectively, I concluded that the DD-Flag-PCNT-Myc fusion protein can rescue most of the *PCNT* deletion phenotypes except centriole amplification in M phase.

A previous work in my laboratory revealed that PLK1 phosphorylation at S1235 and S1241 of PCNT is a critical event for centrosome maturation (Lee and Rhee, 2011). When endogenous PCNT was depleted with siRNA transfection and rescued with the phospho-resistant mutant of PCNT, the cells are arrested at prometaphase with monopolar spindle (Lee and Rhee, 2011). I repeated the same experiments using the *TP53*;*PCNT* double knockout cell line and DD-Flag-PCNT-Myc fusion protein (Fig. 36). As expected, expressions of the wild type (DD-Flag-PCNT-Myc, $PCNT^{WT}$) and phospho-resistant mutant (DD-Flag-$PCNT^{S1235/1241A}$-Myc, $PCNT^{AA}$) forms of ectopic PCNT were similarly induced by doxycycline and shield1 in the *PCNT*-deleted cells (Fig. 36a). The ectopic PCNT proteins were absent at the centrosomes of the stable cell lines but were detected after the induction (Fig. 36b,c). The centrosomal levels of the phospho-resistant $PCNT^{AA}$ mutant were somewhat lower than the $PCNT^{WT}$ in the prometaphase-

arrested cells (Fig. 36b,c). The centrosomal levels of endogenous CEP215 was also minimal in the stable line before induction, but significantly enhanced with ectopic PCNT fusion proteins (Fig. 36b,d). However, the phospho-resistant $PCNT^{AA}$ mutant was not as good as the $PCNT^{WT}$, supporting the notion that specific phosphorylation of PCNT is essential for PCM recruitment during centrosome maturation (Fig. 36b,d) (Lee and Rhee, 2011).

I also determined centriole separation in the PCNT rescued cells (Fig. 37a). As shown previously, most of centrioles were prematurely separated at prometaphase when the ectopic PCNT fusion protein was not expressed in the rescue cells (Fig. 37a,b). Ectopic expression of $PCNT^{WT}$ significantly inhibited premature separation of centrioles at prometaphase (Fig. 37a,b). Ectopic expression of the phospho-resistant $PCNT^{AA}$ mutant also significantly inhibited centriole separation (Fig. 37a,b). The unscheduled centriole amplification was also inhibited by both the wild type and phospho-resistant mutant PCNT proteins (Fig. 37a,c). These results suggest that PCNT has an active role for maintaining centrioles engaged. This function may be independent of its role in centrosome maturation.

PLK1 plays key roles in multiple mitotic events, including centrosome maturation. PLK1 phosphorylation of PCNT is the critical event for centrosome maturation during mitotic entry (Lee and Rhee, 2011). I used BI2536, a PLK1 inhibitor, to test whether PLK1 is also essential for maintaining centriole association during mitotic entry (Fig. 38a). The results showed that centriole separation was significantly inhibited by BI2536 in

control cells, but not in *PCNT*-deleted cells (Fig. 38a,b). In accord, premature centriole amplification was observed only in the *PCNT*-deleted cells irrespective of BI2536 (Fig. 38a,c). These results suggest that PLK1 is involved in centriole engagement as far as PCNT is present in the centrosome. In the absence of PCNT, centrioles are prematurely separated even if PLK1 is inhibited.

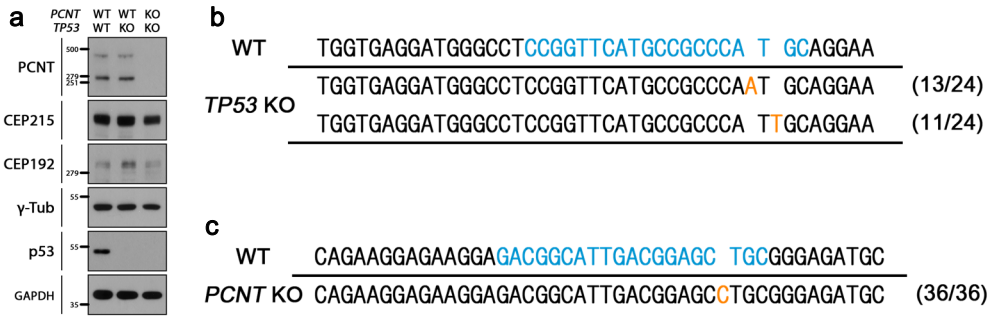

Figure 27. Generation of a *PCNT* knockout cell line in the *TP53*-deletion background
(**a**) The *PCNT* genes are deleted in the *TP53*-deleted HeLa cells with the CRIPSR/CAS9 method. Immunoblot analysis was carried out with antibodies specific to pericentrin (PCNT), CEP215, CEP192, γ-tubulin (γ-Tub), p53 and GAPDH. (**b**,**c**) In-del types of the *TP53* and *PCNT* genes of the double knockout HeLa cells. Target sequences of CRISPR/CAS9 and in-dels are marked in blue and orange letters, respectively.

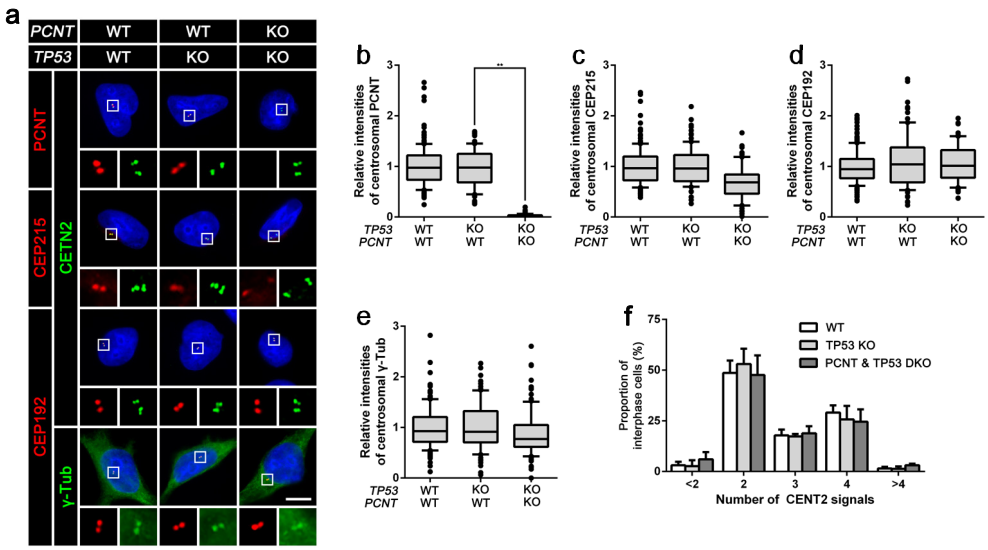

Figure 28. Interphase centrosomes in the *PCNT*-deleted cells (**a**) The *PCNT*-deleted Hela cells were immunostained with antibodies specific to centrin-2 (CETN2), pericentrin (PCNT), CEP215, CEP192 and γ-tubulin (γ-Tub). DNA was visualized with DAPI (blue). Scale bar, 10 μm. (**b**-**e**) Centrosomal intensities of the PCNT (**b**), CEP215 (**c**), CEP192 (**d**) and γ-Tub (**e**) signals were measured. n=90 per group in three independent experiments. (**f**) The number of centrin-2 signals per cell was counted. Values are means with s.d.'s. n≥300 per group in three independent experiments. (*, P<0.05; **, P<0.01)

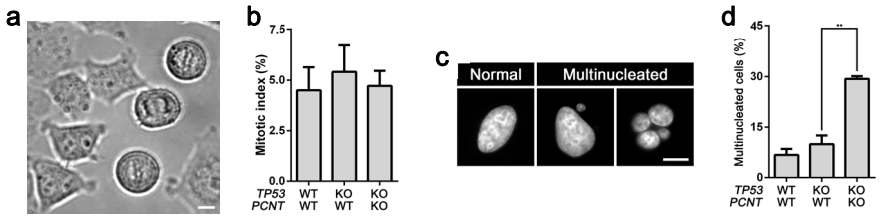

Figure 29. Multinucleated cells in *PCNT*-deleted cells (**a**) Mitotic cells were determined by cell morphology. Round cells with condensed chromosomes were judged as mitotic cells. (**b**) Mitotic indexes were calculated based on the proportion of mitotic cells. (**c**) Multinucleated cells were determined with DAPI staining patterns. (**d**) The number of multinucleated cells were counted. Scale bar, 10 μm. Values are means with s.d.'s. n≥300 per group in three independent experiments. (*, P<0.05; **, P<0.01)

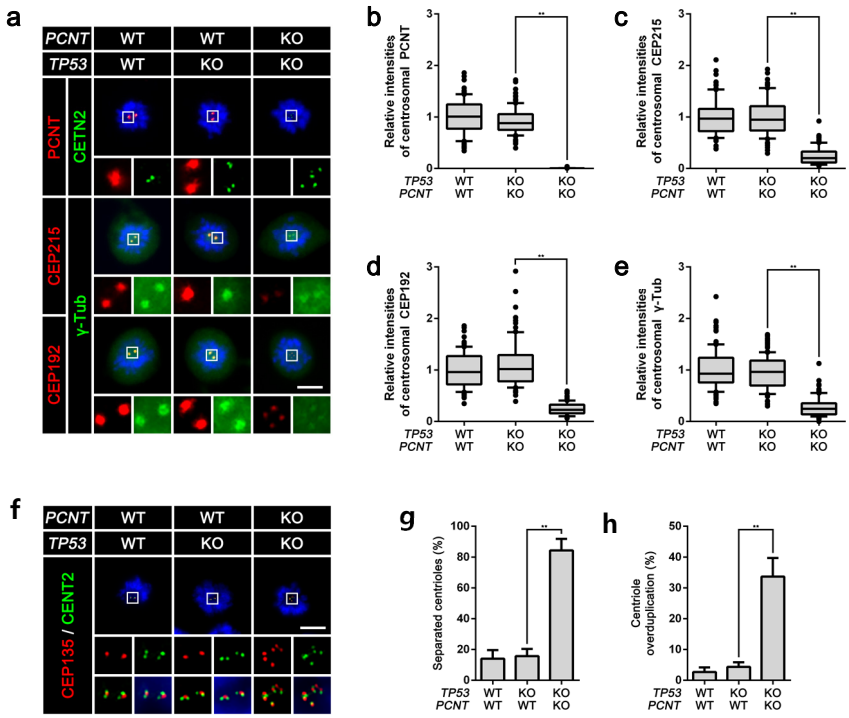

Figure 30. Centrosomes of the *PCNT*-deleted cells during M phase arrest (**a,f**) The *PCNT*-deleted cells were arrested at M phase with STLC and immunostained with antibodies specific to centrin-2 (CETN2, green), pericentrin (PCNT, red), CEP215 (red), CEP192 (red), CEP135 (red) and γ-tubulin (γ-Tub, red). DNA were visualized with DAPI (blue). Scale bar, 10 μm. (**b**-**e**) Centrosomal intensities of the PCNT (**b**), CEP215 (**c**), CEP192 (**d**), and γ-Tub (**e**) signals were measured. n=90 per group in three independent experiments. (**g**) Centriole separation in STLC-arrested cells was determined with 1:1 ratio of the centrin-2 and CEP135 signals. (**h**) Centriole overduplication was determined when cells had five or more CETN2 signals. Values are means with s.d.'s. n≥300 group in three independent experiments. (*, P<0.05; **, P<0.01)

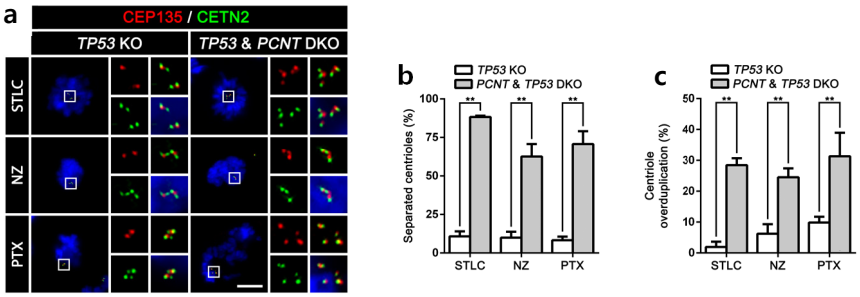

Figure 31. Centrosomes of the *PCNT*-deleted cells in several conditions of M phase arrest (**a**) The cells were arrested at M phase with STLC, nocodazole (NZ) and paclitaxel (PTX) for 10 h, and immunostained with antibodies specific to CETN2 (green) and CEP135 (red). DNA was visualized with DAPI (blue). Scale bar, 10 μm. (**b**) Centriole separation in STLC-arrested cells was determined with 1:1 ratio of the centrin-2 and CEP135 signals. (**c**) Centriole overduplication was determined when cells had five or more CETN2 signals. Values are means with s.d.'s. n≥300 group in three independent experiments. (*, P<0.05; **, P<0.01)

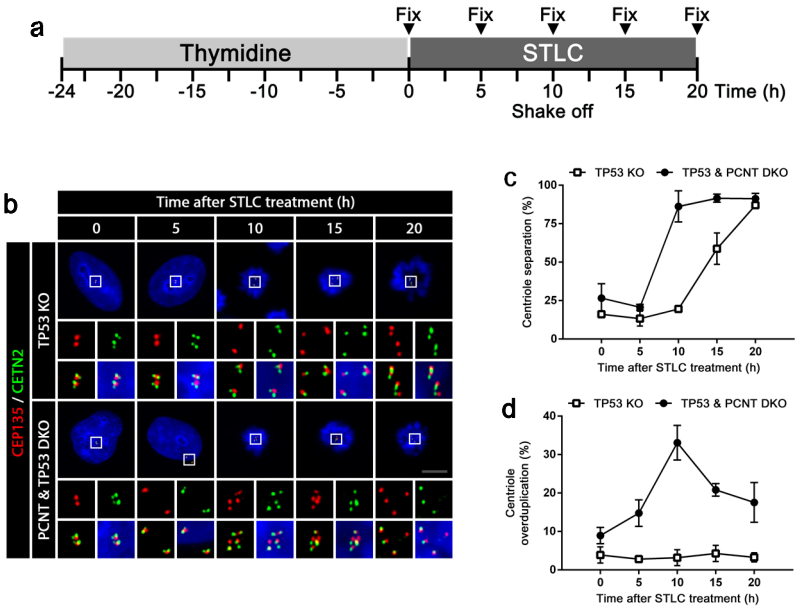

Figure 32. Centriole separation of the *PCNT*-deleted cells during prolonged M-phase-arrest (**a**) The *PCNT*-deleted cells were arrested at M phase with STLC treatment after thymidine release. Cells were collected every 5 h after thymidine release. (**b**) Cells were coimmunostained with antibodies specific to CEP135 (red), and centrin-2 (CETN2). DNA was visualized with DAPI (blue). Scale bar, 10 μm. (**c**) Centriole separation was determined with 1:1 ratio of the centrin-2 and CEP135 signals. (**d**) Cells with five or more centrin-2 signals were counted. Values are means with s.d.'s. n≥300 per group in three independent experiments. (*, P<0.05; **, P<0.01)

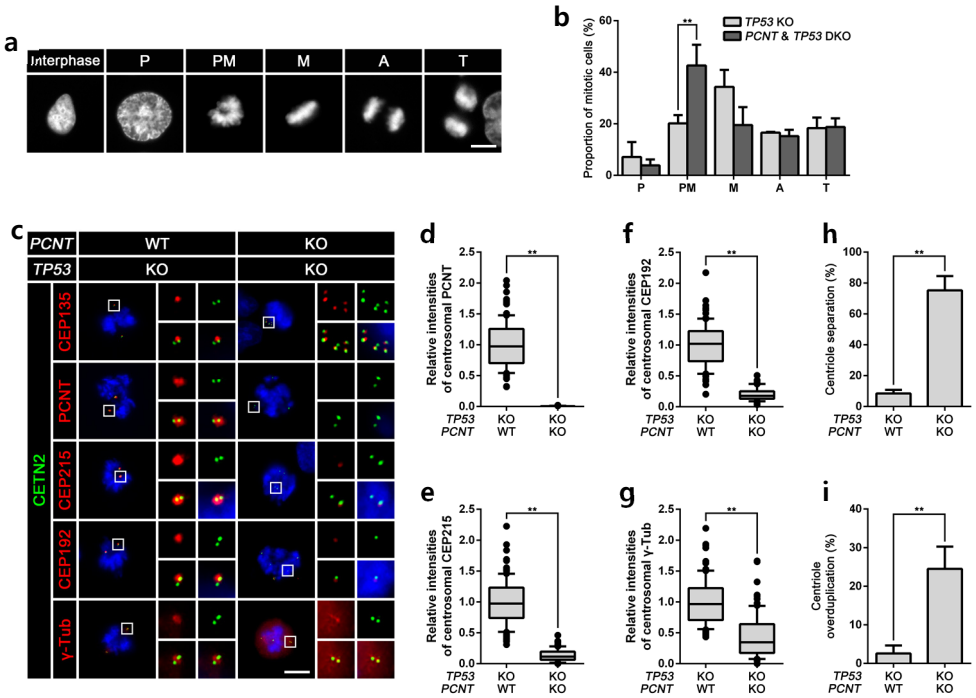

Figure 33. Mitotic centrosomes in the *PCNT*-deleted cells (**a**) The *PCNT*-deleted cells were enriched at M phase with double thymidine block and release. Mitotic stages were determined with DAPI staining patterns. (P, prophase; PM, prometaphase; M, metaphase; A, anaphase; T, telophase) (**b**) Proportion of the cells at prophase, prometaphase, metaphase, anaphase, and telophase were determined. (**c**) The cells enriched at M phase were immunostained with antibodies specific to centrin-2 (CENT2, green), CEP135 (red), pericentrin (PCNT, red), CEP215 (red), CEP192 (red), and γ-tubulin (γ-Tub, red). DNA was visualized with DAPI (blue). Scale bar, 10 μm. (**d**-**f**) Centrosomal intensities of the PCNT (**d**), CEP215 (**e**), CEP192 (**f**), and γ-Tub (**g**) signals were measured. n=90 per group in three independent experiments. (**h**) Centriole separation was determined with 1:1 ratio of the centrin-2 and CEP135 signals. (**i**) Cells with five or more centrin-2 signals were counted. Values are means with s.d.'s. n≥300 per group in three independent experiments. (*, P<0.05; **, P<0.01)

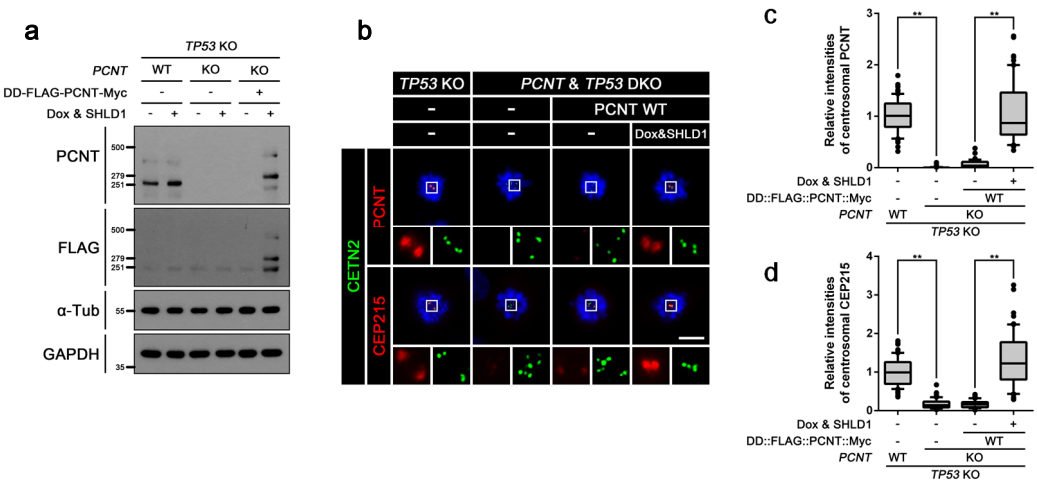

Figure 34. Rescue of the *PCNT*-deleted cell with ectopic DD-Flag-PCNT-Myc (**a**,**b**) Expression of ectpoic DD-Flag-PCNT-Myc was induced with doxycycline (Dox, 10 ng/ml) and shield1 (SHLD1, 50nM) in the *PCNT*-deleted HeLa cells. Immunblot (**a**) and co-immunostaining (**b**) analyses were performed with the antibodies specific to pericentrin (PCNT), Flag, CEP215, centrin-2 (CENT2), α-tubulin (α-Tub) and GAPDH. DNA was visualized with DAPI (blue). Scale bar, 10 μm. (**c**,**d**) Centrosomal intensities of the PCNT (**c**), and CEP215 (**d**) signals were measured. n=90 per group in three independent experiments. (*, P<0.05; **, P<0.01)

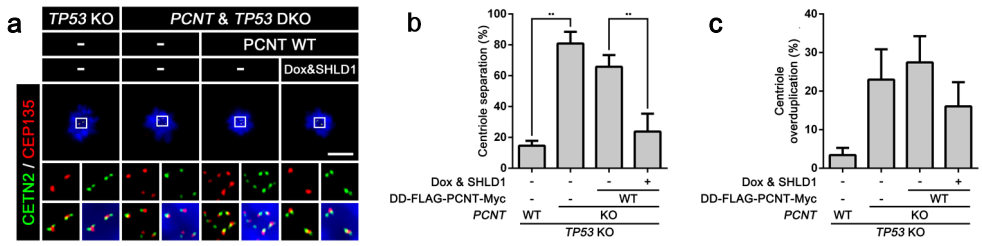

Figure 35. Importance of PCNT for centriole association in M-phase-arrested HeLa cells (**a**) Expression of DD-Flag-PCNT-Myc was induced in the *PCNT*-deleted cells. The cells were treated with STLC for M phase arrest and immunostained with antibodies specific to centrin-2 (CENT2, green) and CEP135 (red). DNA was visualized with DAPI (blue). Scale bar, 10 μm. (**b**) Centriole separation was determined with 1:1 ratio of the centrin-2 and CEP135 signals. (**c**) Cells with five or more centrin-2 signals were counted. Values are means with s.d.'s. n≥300 per group in three independent experiments. (*, P<0.05; **, P<0.01)

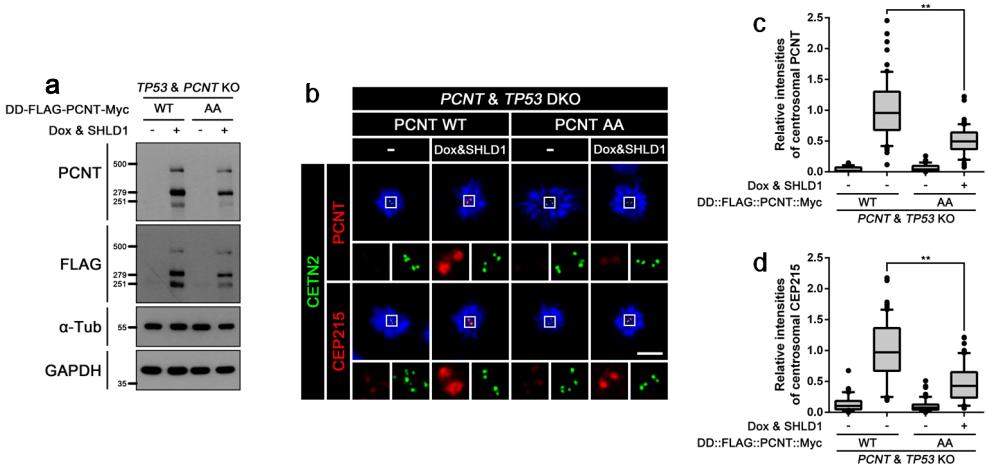

Figure 36. Ectopic expression of DD-Flag-PCNT-Myc mutants in the *TP53* and *PCNT* double knockout cell line (**a**,**b**) Expression of DD-Flag-PCNT-Myc (WT) and DD-Flag-PCNT$^{S1235/1241A}$-Myc (AA) was confirmed with immunblot (**a**) and coimmunostaining (**b**) analyses with antibodies specific to pericentrin (PCNT), Flag, CEP215, centrin-2 (CENT2), α-tubulin (α-Tub) and GAPDH. Doxycycline (Dox, 10 ng/ml) and shield1 (SHLD1, 50nM) were treated to induce expression of ectopic PCNT proteins. DNA was visualized with DAPI (blue). Scale bar, 10 μm. (**c**,**d**) Centrosomal intensities of the PCNT (**c**), and CEP215 (**d**) signals were measured. n=90 per group in three independent experiments. (*, P<0.05; **, P<0.01)

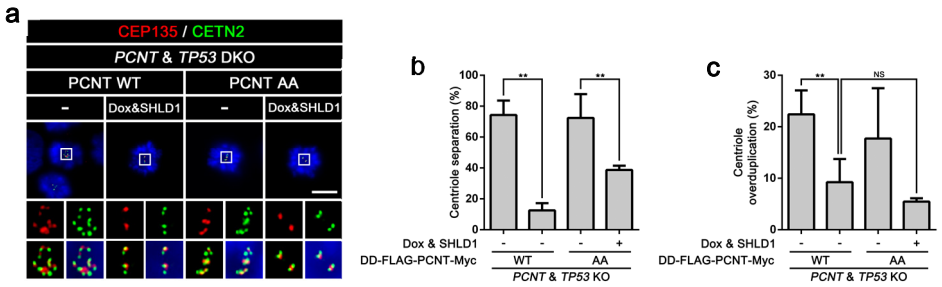

Figure 37. Centriole association in DD-Flag-PCNT$^{S1235/1241A}$-Myc-expressing HeLa cells (**a**) Expressions of DD-Flag-PCNT-Myc (WT) and DD-Flag-PCNT$^{S1235/1241A}$-Myc (AA) were induced in *PCNT*–deleted HeLa cells. The cells were treated with STLC for M phase arrest and coimmunostained with antibodies specific to centrin-2 (CENT2, green), and CEP135 (red). Scale bar, 10 μm. (**b**) Centriole separation was determined with 1:1 ratio of the centrin-2 and CEP135 signals. (**c**) Cells with five or more centrin-2 signals were counted. Values are means with s.d.'s. n≥300 per group in three independent experiments. (*, P<0.05; **, P<0.01)

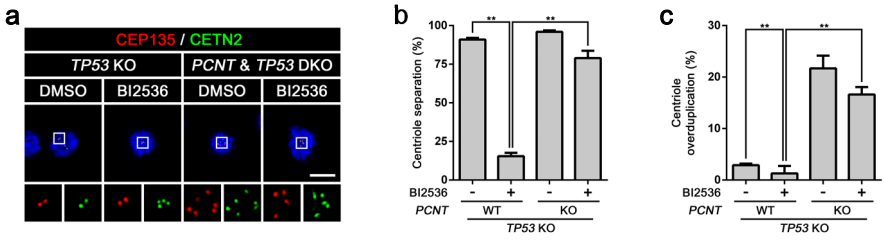

Figure 38. Centriole association of the PNCT-deleted cells in the presence of BI2536 (**a**) The cells were treated with STLC and BI2536 (100 nM) for 20 h after thymidine release. The prolonged M-phase-arrested cells coimmunostained with antibodies specific to centrin-2 (CENT2, green), and CEP135 (red). DNA was visualized with DAPI (blue). Scale bar, 10 μm. (**b**) Centriole separation was determined with 1:1 ratio of the centrin-2 and CEP135 signals. (**c**) Cells with five or more centrin-2 signals were counted. Values are means with s.d.'s. n≥300 per group in three independent experiments. (*, P<0.05; **, P<0.01)

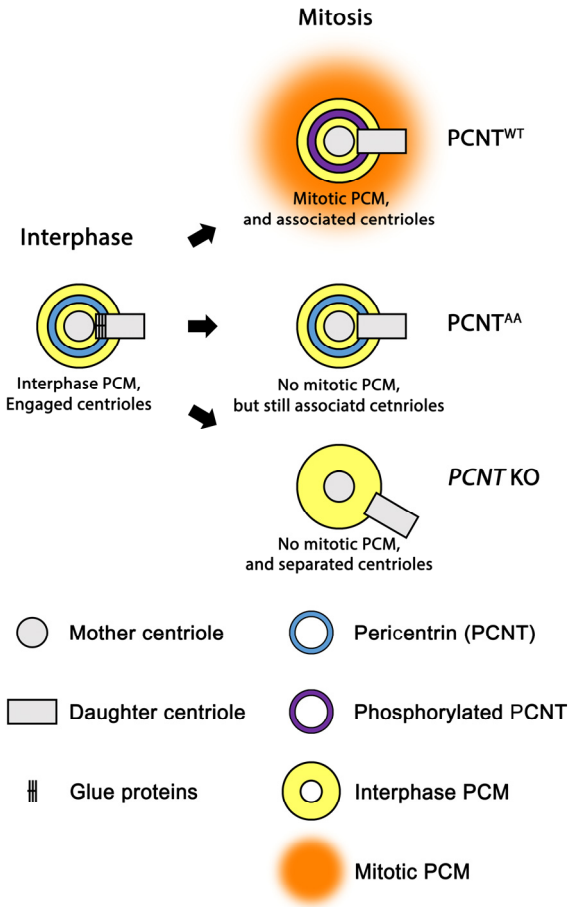

Figure 39. Model At the onset of mitosis, daughter centriole is released from mother centriole as unknown glue protein is removed. However, centrioles are still associated by PCNT. In the absence of PCNT, centrioles were prematurely separated in mitosis. $PCNT^{1235/1241A}$ mutant rescued centriole separation, but not mitotic PCM assembly. It suggests that PCNT in toroid structure is critical for centrioles association in mitosis.

DISCUSSION

I generated *PCNT* knockout cell lines and analyzed the phenotypes in relation to PCM assembly and centriole association. Deletion of PCNT hardly affected interphase centrosomes but conferred defects in centrosome maturation in cells entering M phase. The centrioles in *PCNT*–deleted cells were prematurely separated in early phase of mitosis and frequently amplified in M phase-arrested cells. Abnormal multi-nuclear cells repeatedly appeared in *PCNT*-deleted cells at interphase. My results confirmed that PCNT is critical for centriole association during M phase.

It is surprising that deletion of *PCNT* hardly affected centrosomes in interphase cells. Studies with super-resolution microscopes suggested that PCM in interphase centrosome forms a toroidal structure in which specific PCM proteins concentrically surround mother centrioles (Fu and Glover, 2012; Lawo et al., 2012; Sonnen et al., 2012). According to the model, PCNT stands perpendicularly within the toroid structure of PCM, suggesting a pillar-like role of PCNT for maintaining the toroidal structure of PCM in interphase centrosomes (Lawo et al., 2012). However, my results revealed that the centrosomal levels of selected PCM proteins were hardly affected in *PCNT*-deleted cells. The centriole numbers per cells in *PCNT*-deleted interphase cells are identical to those in wild type cells (Fig. 28f). These results suggest that PCNT may not be so critical for PCM assembly and centriole duplication in interphase centrosomes. It remains to be investigated whether the toroid structure of PCM is maintained without PCNT or not.

Deletion of *PCNT* may not affect PCM of interphase centrosomes, it conferred significant reduction of PCM of mitotic spindle poles (Fig. 28, 30). As a result, the centrosomes in *PCNT*-deleted cells failed to mature during mitotic entry. A delay of mitosis at prometaphase may be another consequence of the centrosome maturation defects in *PCNT*-deleted cells (Fig. 33b). These results are consistent with the previous report with *PCNT*-depleted cells with siRNA transfection (Lee and Rhee, 2011). In fact, my laboratory revealed that PLK1 phosphorylation of PCNT is an essential for recruitment of PCM proteins during centrosome maturation (Lee and Rhee, 2011). Consistent with the notion, defects in centrosomal maturation in *PCNT*-deleted cells were rescued with wild type DD-Flag-PCNT-Myc, but not with DD-Flag-PCNT^{AA}-Myc, a phosphor-resistant mutant. Therefore, I confirmed that PCNT plays key roles in centrosomal maturation to spindle pole formation during mitotic entry. It remains to be investigated how PCNT recruits additional PCM proteins during the maturation process. It was proposed that specific phosphorylation enhances PCNT interactions with other PCM proteins during mitotic spindle formation (Lee and Rhee, 2011).

The most interesting phenotype of the *PCNT*-deleted cells may be premature separation of centrioles during mitotic entry. I observed that most of the mitotic centrioles were already disengaged and separated in *PCNT*-deleted cells. Centrioles are frequently amplified if the *PCNT*-deleted cells were arrested at prometaphase. These results suggest that PCNT is essential for engagement of mother and daughter centrioles during M phase. However,

PCNT may not be critical for centriole engagement during interphase, since centrioles in interphase cells remained associated in the absence of PCNT (Fig. 28). It is possible that the mother and daughter centrioles in interphase centrosomes are linked by a glue protein whose identity has not been known yet. The glue protein may be dissociated by separase as soon as the cells enter mitosis. Consistent with this view, centriole disengagement was separase-dependent in G2-arrested cells (Seo et al., 2015). It may be PCNT which plays a key role for holding the mother and daughter centriole together during mitosis.

What are the outcomes of the premature centriole separation in PCNT-deleted cells? First, centrioles can be amplified even in M phase, because centrioles are liberated from inhibitory environment exerted from centriole association (Tsou and Stearns, 2006a, b). As results, multiple spindle poles may be formed during mitosis (Holland and Cleaveland, 2009). Activation of spindle checkpoint should be block cell cycle progression at metaphase for a while (Nigg, 2001). Some of the checkpoint-activated cells would follow apoptosis after prolonged arrest at M phase (Uetake and Sluder, 2010). Others may proceed mitosis after centrosome clustering, but eventually forms multinuclear cells (Holland and Cleveland, 2009). In fact, I observed a significant increase of multinuclear cells in *PCNT*-deleted cells at interphase.

In this chapter, I investigated PCNT functions for PCM formation and centriole association (Fig. 39). I believe that PCNT also had an important function in PCM disintegration during mitotic exit. I plan to investigate this

topic with the PCNT-deleted cells with a cleavage-resistant DD-Flag-PCNT^{RA}-Myc mutant. My study would be useful for elucidation of pleiotropic defects of the congenital patients with mutation in the *PCNT* gene (Delaval and Doxsey, 2010; Rauch et al., 2008; Chen et al., 2014).

PERSPECTIVE

The assembly and segregation of centrioles are tightly linked to the cell cycle. Daughter centrioles are assembled next to the mother centrioles at S phase and remained associated until mitotic exit. Premature separation of centrioles may induce multipolar spindles and frequently lead to aneuploidy. My long term goal was to elucidate how centriole separation is tightly regulated during mitosis.

A working hypothesis of mine states that centriole association is maintained by PCM. Previous works in my laboratory revealed that a specific cleavage of PCNT is a critical event of centriole separation. In this dissertation, I determined importance of PCNT in centriole association during mitosis. I also refined a regulatory mechanism of the PCNT cleavage during mitosis. Based on my results, I propose a model for centriole separation (Fig. 40). Centriole separation occurs in two steps. In the first step, the daughter centriole is disengaged from the mother centriole as soon as cells enter mitosis. The disengaged centrioles remain associated within a spindle pole during mitosis. My works revealed that PCNT is essential for centriole association during mitosis. At the end of mitosis, PCNT is cleaved by separase and PCM is disintegrated, resulting in centriole separation (Fig. 40).

The proposed model needs to be extensively tested in the future. First, it needs to be elucidated how PCM is disintegrated by the PCNT cleavage during mitotic exit. It would be important to elucidate how a released centriole

become competent to be a template for a new centriole. Finally, it is important to identify a glue protein which links a daughter centriole to a mother centriole.

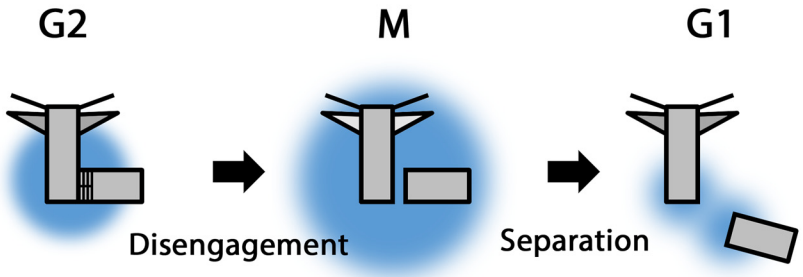

Figure 40. Two steps of centriole detachment In interphase, centrioles are engaged by PCNT (blue) and a glue protein to be identified. When cells enter mitosis, the glue protein is removed from the centrosome. As a result, daughter centriole is released from mother centriole. However, centrioles are still associated because PCNT holds mother and daughter centrioles. During mitosis exit, PCNT is cleaved by separase and removed from the centrosome. Therefore, centrioles are separated and eventually disengaged after mitosis.

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ABSTRACT IN KOREA
국문 초록

중심립 복제 및 분리 과정은 세포주기와 밀접하게 연관되어 있다. 세포가 S기에 진입하면, 딸 중심립은 모체 중심립에 직각으로 결속되어 형성된다. 결속된 모체 중심립은 복제 능력이 잃어버리기 때문에, 중심립 분리 과정이 중심체 복제과정에서 면허 기전으로 여겨진다. 하지만 중심립이 어떻게 결속되어 있고, 어떻게 분리되는지에 대해서는 아직 많은 연구가 이루어지지 않았다.

Pericentrin (PCNT)는 중심체 성숙 및 방추극 형성에 중요한 역할을 담당하는 중심구 단백질이다. 또한 PCNT는 세포분열 후기에 일어나는 중심립 분리 과정에 관여한다. 이 과정에서 PCNT는 separase에 의해 절단된다. 이 절단은 중심립 분리 과정에 필수적인 사건으로 여겨진다. 이 연구의 목적은 세포분열기동안 중심립 결속 및 분리과정에서 PCNT의 생물학적 기능을 이해하는데 있다.

제 1 장에서 본인은 PCNT가 separase에 의해 절단되기 위해서는 PLK1에 의해 인산화가 이루어 져야함을 말하고자 한다. 인산화 내성 PCNT 돌연변이형은 separase에 의해 절단되지 않으며, 이는 결국 중심립 분리 현상을 억제시킨다. 인산화 모방 PCNT 돌연변이형은 BI2536이 처리된 상황에서도 중심립 분리 현상을 회복시킨다. 이 결과들을 바탕으로 본인은 PLK1에 의한 PCNT 인산화가 separase에 의한 PCNT 절단 과정과 중심립 분리 현상의 선행 과정이라고 제안한다. PLK1에 의한 PCNT의 인산화는 세포분열 후기에 일어나는 중심립 분리 과정을 정교하게 조절하기 위한 추가적인 기전으로 여겨진다.

제 2 장에서 본인은 *PCNT* 유전자가 제거된 세포주를 이용하여,

중심구와 중심립 분리간의 상관관계를 분석하였다. *PCNT* 유전자가 제거되었을 경우, 세포분열기에 중심체 성숙 과정이 이루어지지 않고, 중심립 분리와 과복제가 빈번하게 관찰된다. 반면, 간기동안에는 *PCNT* 유전자가 제거되더라도 중심체에서의 이상은 관찰되지 않는다. 이 결과는 PCNT이 세포분열기 동안 중심립 결속 및 중심구 형성에 중요함을 의미한다.