Cell Cycle 11:13, 2476-2485; July 1, 2012; © 2012 Landes Bioscience

Separase-dependent cleavage of pericentrin B is necessary and sufficient for centriole disengagement during mitosis

Kwanwoo Lee and Kunsoo Rhee*

Department of Biological Sciences; Seoul National University; Seoul, Korea

Keywords: pericentrin, separase, centrosome, centriole disengagement, mitosis

Centriole disengagement is considered an essential step for licensing a new round of centriole duplication in the next cell cycle. Separase is critical for centriole disengagement. Here, we showed that pericentrin B (PCNTB) is specifically cleaved by separase at the exit of mitosis. The cleavage-resistant PCNTB mutant blocks the centriole disengagement and duplication. We also observed that an artificial cleavage of PCNTB during M phase induced premature disengagement of centrioles. Based on these results, we concluded that the separase-dependent cleavage of PCNTB is necessary and sufficient for centriole disengagement during mitosis.

Introduction

A centriole pair is surrounded by pericentriolar matrix (PCM) within the centrosome.[1] An accurate control of centriole number is critical for genomic stability, since abnormality in centriole number might cause defects in bipolar spindle formation during mitosis. In fact, amplified centrioles are detected in many cancer cells.[2]

The centriole is duplicated once per cell cycle during S phase.[1] The duplicated procentriole is engaged with the mother centriole until M phase. The centrioles are disengaged at the end of mitosis, and this is considered an essential step for licensing a new round of centriole duplication in the next cell cycle. First, centriole duplication is inhibited in cells whose centrioles are not disengaged even at G_1 phase.[3] Second, a laser ablation of the engaged procentriole induces reduplication of the mother centriole.[4] Third, premature centriole disengagement is observed in cells whose cell cycle is arrested at S or G_2 phase, and centriole reduplication is accompanied.[5] These observations indicate that centriole disengagement is a precondition for centriole duplication.

Centriole disengagement is regulated by the protease activity of separase.[3,6] When endogenous separase is depleted or replaced with an inactive form, centriole disengagement is inhibited at G_1 phase. Nonetheless, the centrioles in separase-depleted cells are eventually disengaged at a later stage of the cell cycle. Separase is known to cleave Scc1, a cohesin component for sister chromatid separation during mitosis.[7] It was reported that cohesin is an essential factor for centriole engagement, and it is cleaved by separase during mitosis.[8] However, there is a contradictory report in which the cleavage of Scc1 is not required for centriole disengagement.[3]

Pericentrin (PCNT) is a centrosomal protein that functions as a scaffold for recruiting a number of centrosomal proteins.[9-12] Mutations in the *PCNT* gene are associated with several human disorders, including primordial dwarfism.[13-16] It is known that PCNT plays an important role in spindle pole formation along with other PCM proteins, such as CEP215 and CEP192.[9,11,12] We previously reported that PLK1-mediated phosphorylation of PCNT initiates centrosome maturation by organizing the spindle pole-specific PCM lattice.[9]

In this paper, we showed that PCNTB is specifically cleaved by separase. Our results indicate that PCNTB is a separase substrate that is responsible for centriole disengagement during the mitotic exit.

Results

PCNTB is specifically cleaved by separase during mitotic exit. Immunoblot analyses with the pericentrin antibody revealed specific bands corresponding to A and B isoforms of pericentrin and an additional band 340 kDa in size.[9] This additional band is likely a cleaved fragment of pericentrin B (PCNTB), because it disappears with the siRNAs specific to PCNTB.[9] We determined the PCNTB protein levels in synchronized cell populations. The results showed that the full-length PCNTB levels significantly decreased, and the fragmented PCNTB levels simultaneously increased at 12 h after the double thymidine block and release (**Fig. 1A**). These results suggest that PCNTB is fragmented during the mitotic exit. We examined PCNTB degradation in detail with HeLa cells synchronized with nocodazole. The full-length PCNTB levels were significantly reduced within 1 h after the nocodazole release, and the fragmented PCNTB levels were

*Correspondence to: Kunsoo Rhee; Email: rheek@snu.ac.kr
Submitted: 05/23/12; Accepted: 05/24/12
http://dx.doi.org/10.4161/cc.20878

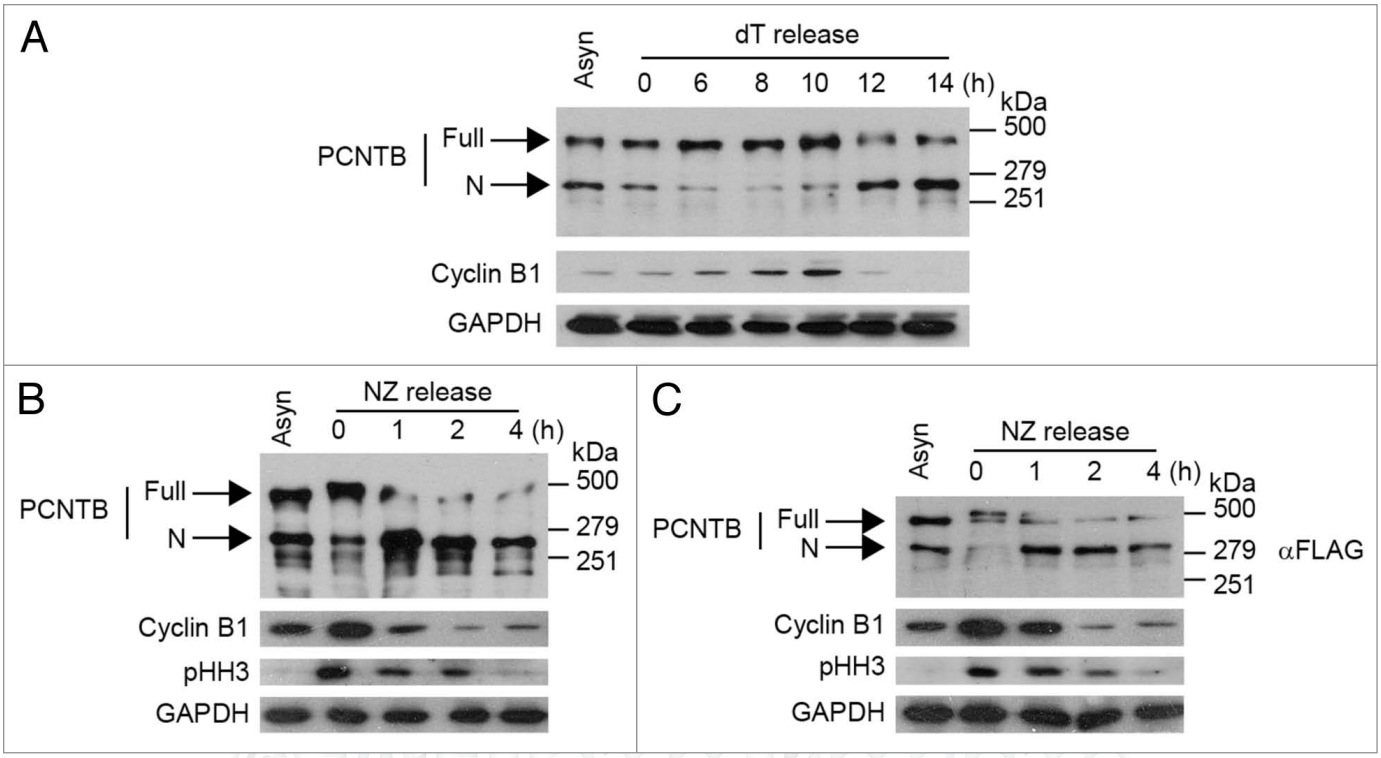

Figure 1. PCNTB is cleaved during mitotic exit. (A) HeLa cells were accumulated at G_1/S phase with a double thymidine block and synchronously released. The cells were harvested at indicated time points and subjected to immunoblot analysis with antibodies specific for PCNT, cyclin B1 and GAPDH. Asynchronous cells were used as a control. (B) HeLa cells were accumulated at M phase with a nocodazole treatment for 16 h. The mitotic cells were shaken off for a synchronous release. The cells were harvested at indicated time points and subjected to immunoblot analysis with antibodies specific for PCNT, cyclin B1, phospho-histone H3 (pHH3) and GAPDH. Asynchronous cells were used as a control. (C) A stable cell line expressing FLAG-GFP-PCNTB-Myc was accumulated at M phase with a nocodazole treatment for 16 h. The mitotic cells were shaken off for a synchronous release. At indicated time points, the cells were harvested and subjected to immunoblot analysis with antibodies specific for FLAG, cyclin B1, phospho-histone H3 (pHH3) and GAPDH. Asynchronous cells were used as a control.

simultaneously induced (**Fig. 1B**). Identical results were observed with FLAG-GFP-PCNTB-Myc, which was stably expressed in HeLa cells (**Fig. 1C**). Based on these results, we concluded that PCNTB is specifically cleaved during the mitotic exit.

We tried to identify a protease that is responsible for PCNTB cleavage. Previous papers reported that separase localizes at the centrosome and functions for procentriole disengagement from the mother centriole during mitotic exit.[3,17] In fact, we observed reduction of the cleaved PCNTB levels in the separase-depleted cells (**Fig. 2A**). Immunoblot analysis of PCNTB was performed in the separase-depleted cells, which synchronously entered mitosis with the thymidine-nocodazole block and release. The results showed that the amount of the fragmented PCNTB was significantly reduced in separase-depleted mitotic cells, but those of the full-length PCNTB remained constant (**Fig. 2B**). It is well known that separase becomes active after securin is degraded by anaphase-promoting complex during mitosis.[7,18] Therefore, we determined the PCNTB protein levels in cells expressing nondegradable securin. The results showed that the fragmented PCNTB is significantly reduced during mitosis in cells expressing the nondegradable securin (**Fig. 2C**). Based on these results, we concluded that separase is essential for the PCNTB cleavage during mitotic exit.

A consensus sequence for separase cleavage (S/T-x-E-x-x-R↓) was well defined.[19,20] Among the six potential separase cleavage sites, the one near R2231 was the most promising, based on the predicted size of the fragmented PCNTB. Furthermore, R2231 of PCNTB is conserved among selected vertebrates (**Fig. 2D**). The molecular size of FLAG-GFP-PCNTB$^{1-2231}$ is identical to that of the fragmented form of the wild type FLAG-GFP-PCNTB-Myc (**Fig. 2E**). Finally, we determined specific cleavage at R2231 with a cleavage-resistant mutant of PCNTB. The results showed that the PCNTB^{R2231A} mutant was not cleaved during the mitotic exit, indicating that separase specifically cleaves next to R2231 of PCNTB during the mitotic exit (**Fig. 2F**).

The C-terminal fragment of PCNTB is rapidly degraded after cleavage. Once PCNTB was cleaved during mitosis, the N-terminal fragment of PCNTB was readily detected in immunoblot analysis, but the C-terminal fragment was not. We hypothesized that the C-terminal fragment is degraded rapidly after cleavage. The C-terminal fragment of PCNTB was able to be detected with immunoprecipitation followed by immunoblotting with the PCNTB antibody generated from GST-PCNTB$^{2628-2969}$ (**Fig. 3A**). When the cells were forced to exit mitosis with ZM447439, an Aurora B inhibitor, the C-terminal fragment disappeared as soon as it is formed (**Fig. 3A**). The

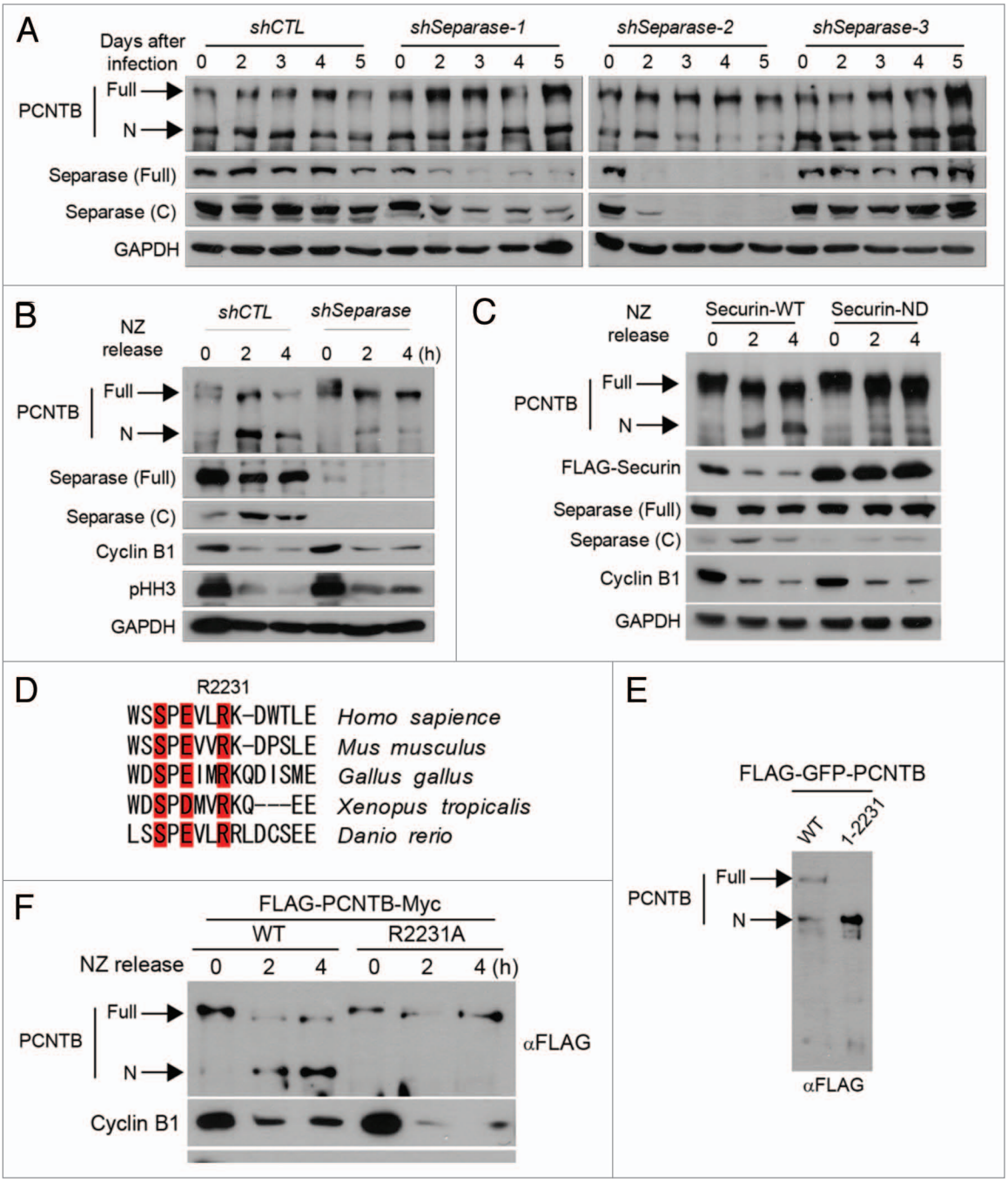

Figure 2. Determination of the specific separase cleavage site within PCNTB. (A) HeLa cells were infected with the lentiviruses with 3 different shRNAs (*shSeparase*) for separase and a control shRNA (*shCTL*). The infected cells were harvested at indicated days and subjected to immunoblot analysis with antibodies specific for PCNT, separase and GAPDH. (B) HeLa cells were infected with the lentiviruses expressing *shCTL* or *shSeparase-2*. Forty-eight hours later, the cells were accumulated at M phase with a thymidine-nocodazole block and synchronously released. The cells were harvested at indicated time points and subjected to immunoblot analysis with antibodies specific for PCNT, separase, cyclin B1, phospho-histone H3 (pHH3) and GAPDH. (C) HeLa cells were transfected with expression vectors for the wild type (WT) and nondegradable (ND) forms of securin. Forty-eight hours later, the cells were treated with doxycycline, accumulated at M phase with a thymidine-nocodazole block and synchronously released. The cells were harvested at indicated time points and subjected to immunoblot analysis with antibodies specific for PCNT, cyclin B1, FLAG, separase and GAPDH. (D) Sequence alignment near the separase cleavage site of PCNTB among representative vertebrates. (E) The transiently expressing FLAG-GFP-PCNTB$^{1-2231}$ protein was compared with the stably expressing FLAG-GFP-PCNTB-Myc protein. The immunoblot was performed with the FLAG antibody. (F) Stable cell lines expressing the wild-type or R2231A point mutant of FLAG-PCNTB-Myc were accumulated at M phase with a thymidine-nocodazole block and synchronously released. The cells were harvested at indicated time points and subjected immunoblot analysis with antibodies specific for PCNT, cyclin B1 and GAPDH.

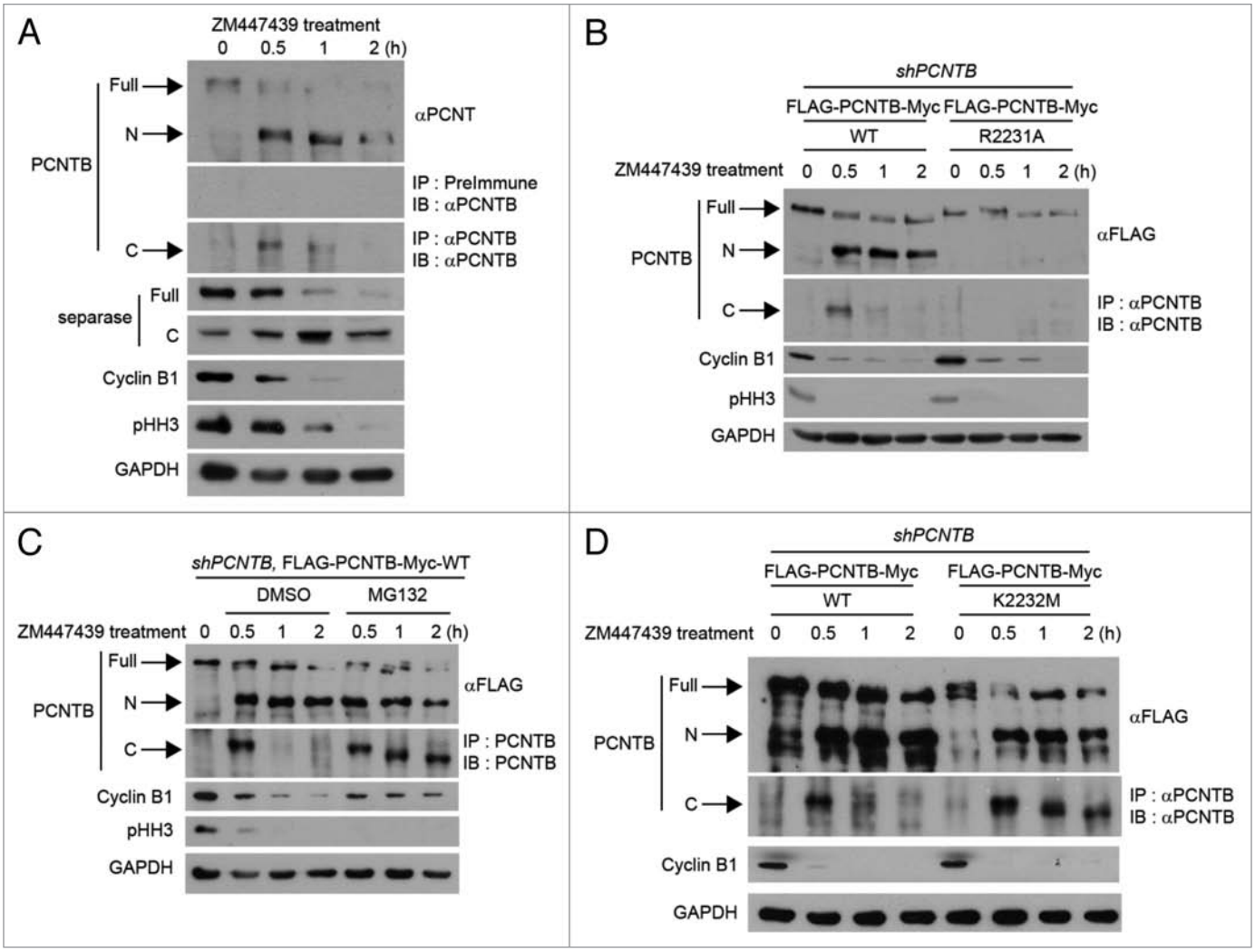

Figure 3. The C-terminal fragment of PCNTB is immediately degraded after cleavage. (A) HeLa cells were accumulated at metaphase with a thymidine-taxol block and then treated with ZM447439 to force exiting mitosis. The cells were harvested at indicated time points and subjected to immunoblot analysis with antibodies specific for PCNT, separase, cyclin B1, phospho-histone H3 and GAPDH. For detection of the C-terminal fragment of PCNTB, the cell lysates were immunoprecipitated and subsequently immunoblotted with the PCNTB antibody. (B) The same scheme of experiments was performed with the cells in which endogenous PCNTB is depleted and rescued with the wild type (WT) or cleavage-resistant mutant (R2231A) of FLAG-PCNTB. (C) The same scheme of experiments was performed except MG132 was added 0.5 h after the ZM447439 treatment. (D) The same scheme of experiments was performed with the cells in which endogenous PCNTB is depleted and rescued with the wild type (WT) or K2232M point mutant of FLAG-PCNT-Myc.

endogenous PCNTB was depleted with shRNA and rescued with the wild type or cleavage-resistant PCNTB (**Figs. S1 and S3B**). The C-terminal fragment was detected only in the cells rescued with the wild type PCNTB, confirming that the short-lived C-terminal fragment is formed by the separase-directed cleavage during mitotic exit (**Fig. 3B**).

When a protein is cleaved by a protease at a specific site, the stability of the cleaved C-terminal fragment depends on the new N-terminal residue, which is known as the N-end rule.[21] Since PCNTB in a mitotic cell is cleaved next to arginine 2231, the C-terminal fragment of PCNTB should start from lysine 2232, which may be easily ubiquitinated and degraded following the N-end rule (**Fig. 3C**). In support of our prediction, the C-terminal fragment of PCNTB was stabilized with MG132 (**Fig. 3C**). We generated a PCNTB point mutant, in which lysine at 2232 was substituted to methionine, and tested the stability of the C-terminal fragment. The results showed that the C-terminal fragment with M2232 becomes stabilized after the forced mitotic exit (**Fig. 3D**). This indicates that the C-terminal fragment of PCNTB is rapidly degraded by the N-end rule pathway.

The PCNTB cleavage is essential for timely centriole disengagement and duplication. It is known that the separase activity is essential for centriole disengagement.[3,6] Therefore, we examined whether the separase cleavage of PCNTB is required for centriole disengagement or not. The centriole disengagement was determined in the PCNTB-depleted cells rescued with the cleavage-resistant PCNTB mutant. Skp2 was used to distinguish G_1 phase cells from S and G_2 phase cells, since it is expressed

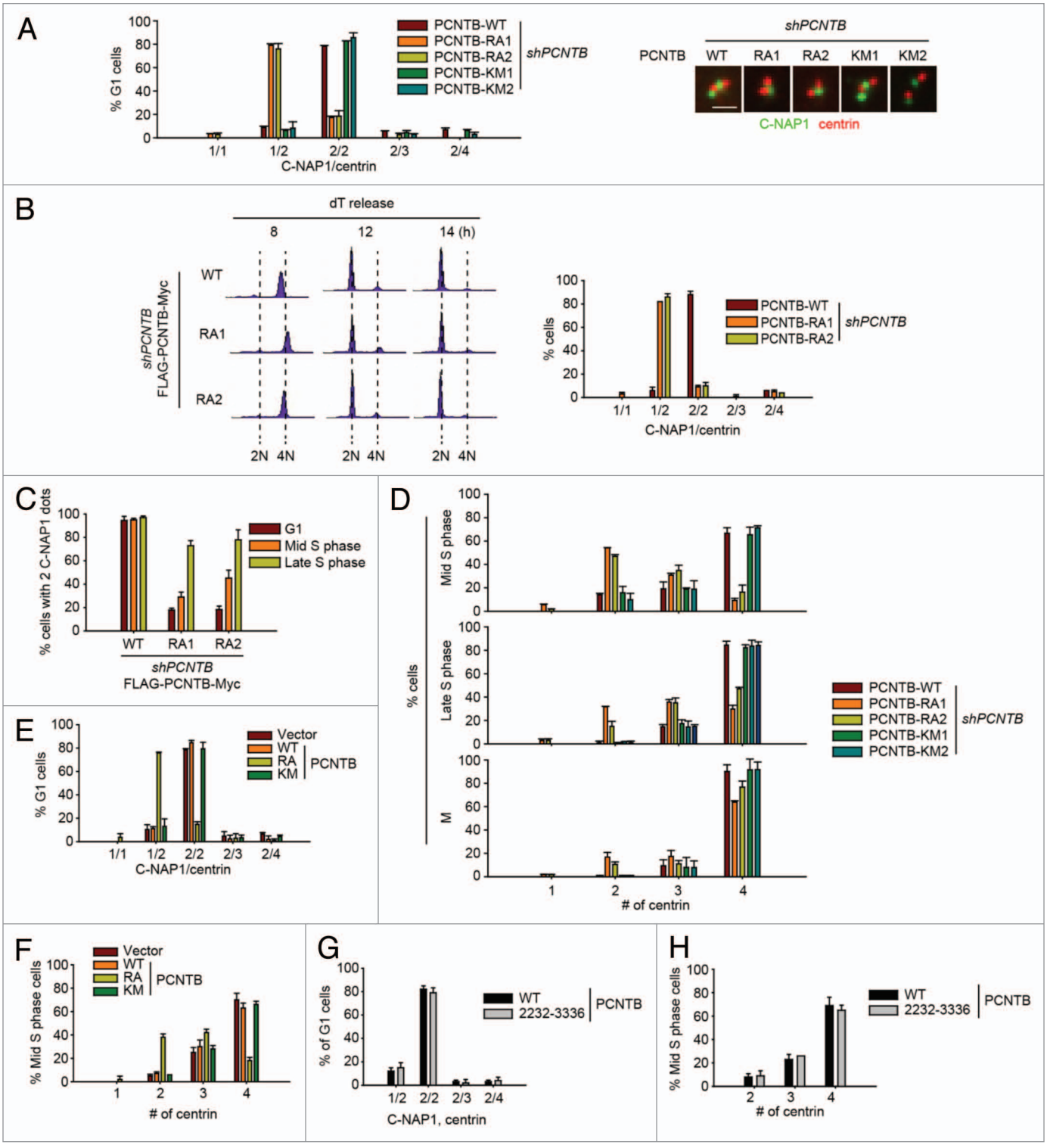

Figure 4. For figure legend, see page 6.

from S phase and degraded by the anaphase-promoting complex during mitosis.[22] In accordance, centrioles in most of the Skp2-negative cells were not duplicated yet (**Fig. S2**). We determined centriole disengagement in the Skp2-negative cells by counting the number of centrin and C-NAP1 signals. The results showed that centriole disengagement was inhibited in most of the cells with the cleavage-resistant PCNTB^{R2231A} mutant but not in cells with the wild type or PCNTB^{K2232M} mutant (**Fig. 4A**). We also observed the centriole disengagement in a synchronized population. The FACS analysis revealed that most of the cells are at

Figure 4. PCNTB cleavage is essential for centriole disengagement and duplication. (A) The PCNTB-depleted cells were stably rescued with the ectopic PCNTB (wild-type, R3321A or K2232M) and immunostained with antibodies specific for centrin (red), C-NAP1 (green) and Skp2. The numbers of centrin and C-NAP1 dots were counted at the Skp2-negative, G_1 phase cells. The scale bar indicates 1 μm. (B) The HeLa cells stably rescued with wild-type or R2231A PCNTB were synchronized with the double thymidine block and release, and harvested at indicated time points. FACS analysis was performed to determine the cell cycle stage. Twelve hours after the release, the numbers of centrin and C-NAP1 dots were counted to determine centriole disengagement. (C) The HeLa cells rescued with the wild type or R2231A mutant of FLAG-PCNTB-Myc were coimmunostained with the antibodies specific for C-NAP1, PCNA and Skp2. Cell cycle stages of the individual cells were determined based on the staining patterns of Skp2 or PCNA. The number of C-NAP1 dots was counted to determine centriole disengagement. (D) HeLa cells rescued with FLAG-PCNTB, FLAG-PCNTB^{R2231A} and FLAG-PCNTB^{K2232M} were coimmunostained with the antibodies specific for C-NAP1 and centrin. Cell cycle stages of the individual cells were determined based on the staining patterns of Skp2 or PCNA. (E) HeLa cells expressing FLAG-PCNTB-Myc, FLAG-PCNTB^{R2231A}-Myc and FLAG-PCNTB^{K2232M}-Myc were coimmunostained with antibodies specific for centrin, C-NAP1 and Skp2 to determine centriole disengagement at G_1 phase. (F) The same set cells were coimmunostained with antibodies specific for centrin and PCNA to determine centriole duplication. (G) HeLa cells expressing FLAG-PCNTB-Myc and FLAG-PCNTB$^{2232–3336}$ were coimmunostained with antibodies specific for centrin, C-NAP1 and Skp2 to determine centriole disengagement at G_1 phase. (H) HeLa cells expressing FLAG-PCNTB-Myc and FLAG-PCNTB$^{2232–3336}$ were coimmunostained with antibodies specific for centrin and PCNA to determine centriole duplication at mid S phase. All experiments in this figure were repeated twice with more than 100 cells per experimental group. The values are means and standard deviations.

G_1 phase when they are cultured for 12 h after the double thymidine block and release (**Fig. 4B**). The results confirmed that centriole disengagement occurred at most of the cells rescued with the wild type PCNTB but not with the cleavage-resistant PCNTB^{R2231A} mutant (**Fig. 4B**). It is known that centrioles in the separase-deficient cells are eventually disengaged during S phase with an unknown mechanism.[3] We tested whether centrioles in the PCNTB^{R2231A}-expressing cells are disengaged during S phase or not. We subdivided S phase into four stages based on the staining patterns of proliferating cell nuclear antigen (**Fig. S3**).[23] The results showed that the PCNTB^{R2231A}-expressing cells were gradually disengaged during the S phase progression (**Fig. 4C**).

Centriole disengagement is considered a precondition for centrosome duplication, so that centriole duplication as well as disengagement is blocked in the separase-deficient cells.[3] Centriole duplication was eventually observed at a late cell cycle stage, when centrioles are disengaged.[3] We determined centriole duplication in the PCNTB-depleted cells rescued with the cleavage-resistant PCNTB^{R2231A}. The results show that centriole duplication was inhibited in the PCNTB^{R2231A}-expressing cells (**Fig. 4D**). However, centriole duplication slowly occurred when the PCNTB^{R2231A}-expressing cells progress to M phase (**Fig. 4D**). These results indicate that the PCNTB cleavage is not required for a delayed centriole disengagement and duplication, which occurs during S phase.

The centriole disengagement was determined in the PCNTB-expressing cells without depletion of endogenous PCNTB using *pRTS*, an episomal-inducible vector.[24] The results showed that the centriole disengagement and duplication was inhibited in the PCNTB^{R2231A}-expressing cells, but not in wild type or PCNTB^{K2232M}-expressing cells (**Fig. 4E and F**). These results indicate that the separase cleavage of PCNTB is a necessary event for centriole disengagement duplication. We also observed that overexpression of FLAG-PCNTB$^{2232–3336}$ does not affect the centriole disengagement and duplication (**Fig. 4G and H**). These results suggest that the linkage rather than presence of the C-terminal fragment of PCNTB is critical for centriole disengagement and duplication.

PCNTB cleavage is sufficient for centriole disengagement during mitosis. In order to test whether the PCNTB cleavage is sufficient for centriole disengagement and duplication, we established a stable cell line in which PCNTB is cleaved only with the ectopic TEV protease (PCNTB^{R2231A}-TEV). The centriole disengagement and duplication was inhibited in this cell, because PCNTB^{R2231A}-TEV is not cleaved during the mitotic exit (data not shown). The levels of the transiently expressed TEV protease were very low (**Fig. S4**). Therefore, we indirectly confirmed TEV protease expression with the GFP protein which was coexpressed by the bidirectional promoter in the *pRTS* vector (**Fig. 5A**). The immunoblot analysis confirmed that PCNTB^{R2231A}-TEV is specifically cleaved with transient expression of the TEV protease (**Fig. 5A**). As expected, the TEV protease induction resumed centriole disengagement at G_1 phase and centriole duplication at S phase in PCNTB^{R2231A}-TEV-expressing cells (**Fig. 5B and C**).

Next, we tested whether the cleavage of PCNTB is sufficient for centriole disengagement and reduplication in mitotic cells. Centriole disengagement was determined by CEP135 instead of C-NAP1, which is not detectable in mitotic cells.[25] CEP135 is localized at proximal end of centriole, so that it is detected as a single dot in the engaged centrioles like C-NAP1.[26] PCNTB^{R2231A}-TEV was artificially cleaved in S phase-arrested cells, and the cells were allowed to proceed to prometaphase. The results showed that artificial cleavage of PCNTB^{R2231A}-TEV prematurely induced centriole disengagement (**Fig. 5D and F**). At the same time, the centrioles were overduplicated (**Fig. 5E and F**). On the other hand, neither premature centriole disengagement nor duplication occurred when PCNTB^{R2231A}-TEV was artificially cleaved by TEV protease in S phase-arrested cells (**Fig. 5G**). These results indicate that PCNTB cleavage is sufficient for centriole disengagement in cells at M phase but not in those at S phase.

Discussion

We observed that PCNTB is specifically cleaved by separase at the end of mitosis. PCNTB cleavage is significantly reduced in separase-deficient cells. Furthermore, the cleavage-resistant PCNTB mutant blocks the centriole disengagement and duplication. We also observed that an artificial cleavage of PCNTB during M phase induced premature disengagement of centrioles. Based on these results, we concluded that PCNTB is a substrate

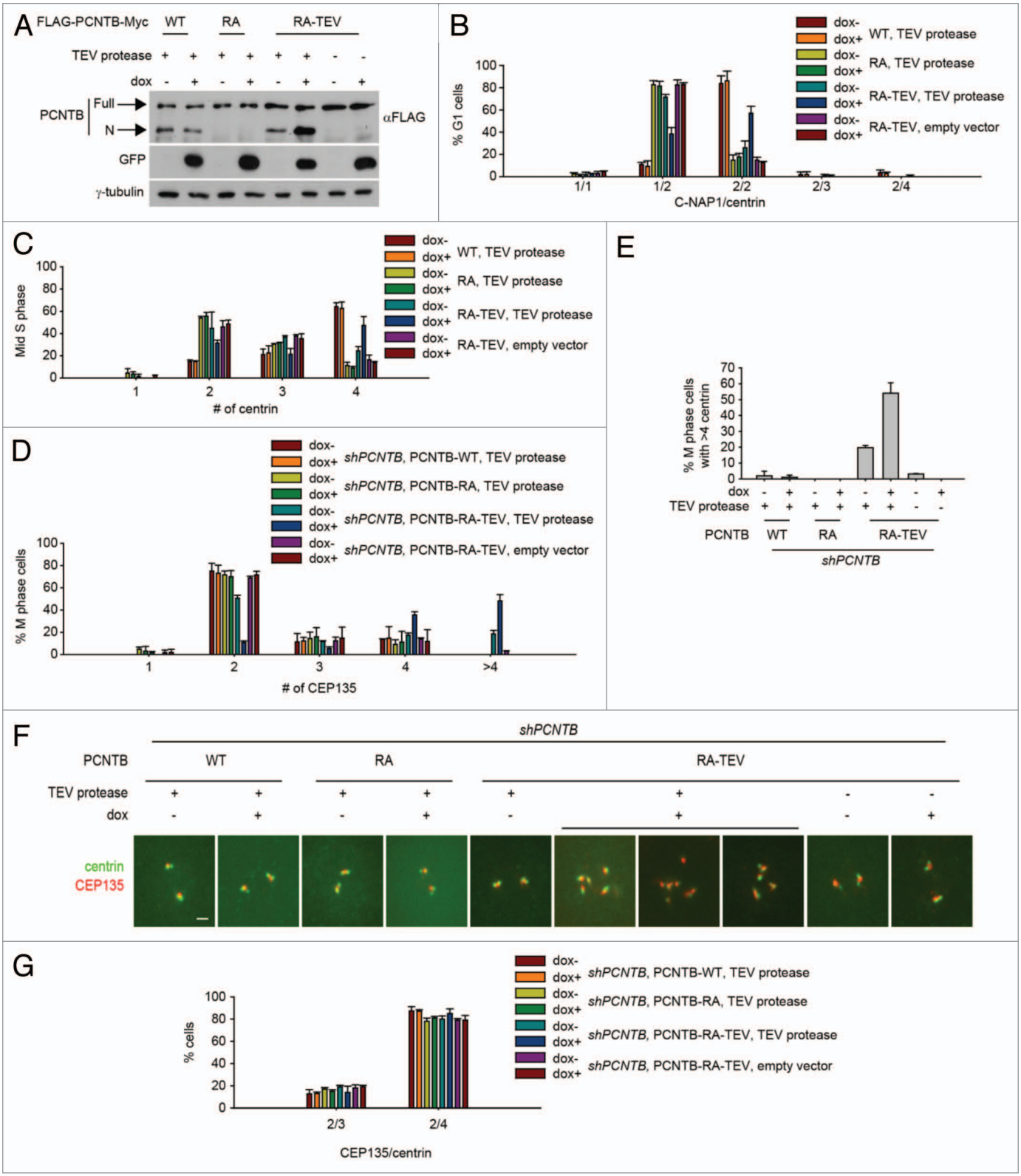

Figure 5. For figure legend, see page 8.

of separase, which is responsible for centriole disengagement. Furthermore, PCNTB cleavage is sufficient for centriole disengagement during M phase.

It was previously reported that Scc1, a cohesin component, is a target of separase for centriole disengagement as well as for sister chromatid separation during mitosis.[8] In fact, cohesin

Figure 5 (See previous page). A TEV protease-directed cleavage of PCNTB induced the premature centriole disengagement and duplication during mitosis. (A) The inducible *pFLAG-TEV protease* expression vector was transfected into stable lines expressing the wild-type, R2231A and R2231A-TEV PCNTB. TEV protease expression was induced by doxycycline for 6 h and the cells were subjected to immunoblot analysis with antibodies specific for FLAG, GFP and γ-tubulin. (B) The centriole disengagement at G_1 phase was determined by coimmunostaining the same set of cells with antibodies specific for C-NAP1, centrin and Skp2. In this experiment, TEV protease expression was induced for 48 h. (C) The centriole duplication at S phase was determined by coimmunostaining the same set of cells with antibodies specific for C-NAP1, centrin and PCNA. (D and E) The inducible *pFLAG-TEV protease* expression vector was transfected into the wild-type, R2231A and R2231A-TEV PCNTB-rescued cells. The cells were accumulated S phase with the thymidine treatment for 30 h and the TEV expression was induced by doxycycline 20 h after the thymidine treatment. The cells were released from the thymidine block, accumulated at M phase with STLC for 8 h and then immunostained with antibodies specific for centrin and CEP135. (F) The coimmunostaining pattern with the centrin (green) and CEP135 (red) antibodies. The scale bar indicates 1 μm. (G) The same set of cells were fixed before thymidine release and coimmunostained with the antibodies specific for centrin and CEP135 to determine centriole disengagement. More than 100 cells per experimental group were analyzed in two independent experiments. The values are means and standard deviations.

components were detected at the centrosome.[8] A non-cleavage form of Scc1 blocked the centriole disengagement. Furthermore, when endogenous Scc1 is replaced by artificially cleavable Scc1, the corresponding site-specific protease triggers centriole disengagement.[8] We currently do not have an explanation how cohesin and PCNTB cooperate for maintaining centriole engagement. Both Scc1 and PCNTB may be involved in maintaining topological linkage between centrioles and simultaneously cleaved by separase during mitosis.[8]

Centrioles in the cells with the cleavage-resistant PCNTB may not be disengaged at G_1 phase but eventually are disengaged later at S phase. Delayed centriole disengagement was observed in separase-depleted cells.[3] The centriole disengagement at later stage of cell cycle may be independent of the separase activity. PCNTB cleavage is important only in separase-dependent disengagement during M phase. In accord, artificial cleavage of PCNTB did not induce centriole disengagement during the prolonged S phase (**Fig. 5G**).

It remains to be determined how PCNTB cleavage induces centriole disengagement. We believe that the absence of the C-terminal fragment of PCNTB is a critical event for centriole disengagement. The C-terminal fragment of PCNTB includes functional domains for centriole localization and for interaction with CEP215.[10,27,28] Once PCNTB loses those functional domains, the centriole would be disengaged. In fact, it is known that CEP215 is involved in centriole engagement.[29] The C-terminal fragment of PCNTB should be linked to the N-terminal end for its function, because the centriole disengagement still occurs in cells with the ectopic C-terminal fragment (**Fig. 4E–H**). It is likely that the N-terminal fragment of PCNTB also includes a functional domain with unknown centrosomal component. PCNTB might link the N-terminal domain with the C-terminal domain, and this is critical for maintenance of centriole engagement.

While preparing this manuscript, we learned that Matsuo et al. (2012) reported that PCNT is a substrate of separase.[30] They also concluded that PCNTB cleavage is involved in the licensing centriole duplication. Our observation is basically identical to their report. However, we additionally report that C-terminal fragment of PCNTB is degraded by N-end rule pathway rapidly after PCNTB cleavage. Furthermore, PCNTB cleavage is sufficient for centriole disengagement in M phase. Future work should focus on how the PCNTB cleavage induces centriole disengagement.

Materials and Methods

Cell culture and stable cell line establishment. HeLa and 293T cells were cultured in DMEM supplemented with 10% FBS and antibiotics. FLAG-PCNTB-Myc was subcloned into *p3x FLAG-CMV10* (Sigma) or *pLVX-IRES-Puro* (Clonetech) and transfected into HeLa cells by Fugene HD (Roche). The cells were subcultured into 96-well dish and treated with neomycin (500 μg/ml) or puromycin (0.5 μg/ml). Two or three weeks later, drug-resistant cells were harvested, cloned and expanded for future experiments.

Lentiviral infection. For lentivirus production, *pLKO.1* (puro or hygro), *pCMV-dR8.2 dvpr* and *pCMV-VSVG* were transfected into 293T cells with polyethylenimine (Sigma, 408727). Two days later, the media were harvested and centrifuged for 15 min at 4,000 g. For lentivirus infection, the harvested media were added into the same volume of normal culture media with polybrene (9 μg/ml), cultured for 18 h and replaced with a normal culture medium. Puromycin (0.5 μg/ml) or hygromycin (200 μg/ml) was treated after 1 day after infection for selection. *pLKO.1-hygro* was obtained from Dr. Robert A. Weinberg via Addgene Inc., (Addgene plasmid 24150). *pLKO.1-puro*, *pCMV-dR8.2 dvpr* and *pCMV-VSVG* were gifted by Dr. Kyung S. Lee.

Cell cycle synchronization. The double thymidine block was performed by treating HeLa cells with thymidine (2 mM) for 19 h, a release for 8 h with thymidine again for 17 h. For a thymidine-nocodazole block, HeLa cells were incubated with thymidine for 19 h, washed out, then incubated with nocodazole (50 ng/ml) for 12 h. The mitotic cells were shaken off, washed out with PBS and replated. For forced mitotic exit by ZM447439, the cells were treated with thymidine for 19 h, washed out and treated with paclitaxel (5 μM) for 12 h. The mitotic cells were shaken off and treated with ZM447439 (2 μM).

shRNA sequence. *shRNA* sequences used in this study are *shCTL* (GCA ATC GAA GCT CGG CTA CAT), *shSeparase-1* (GGT TTA TGA CTT TGC CCA AGG), *shSeparase-2* (GCA GGT TCT GTT CTT GCT TGA), *shSeparase-3* (AAG CTT GTG ATG CCA TCC TGA) and *shPCNTB* (GGA CGT CAT CCA ATG AGA AAG).

Antibodies. The CEP215, PCNT, C-NAP1 and CEP135 antibodies were as described previously.[31-34] The PCNTB antibody was generated from rabbit with GST-PCNTB$^{2628–2969}$. Antibodies

for FLAG (Sigma, F3165), Aurora A (Cell signaling, 3092), CyclinB1 (Santa Cruz, sc-245), phospho-histone H3 (Ser10) (Upstate, 06-570), PCNA (Dakocytomation, PC10), GAPDH (Ambion, AM4300), centrin (Millipore, 20H5), γ-tubulin (Sigma, GTU-88), separase (Abcam, ab16170), Skp2 (Cell signaling, 4358), GFP (Santa Cruz, sc-9996) were purchased. Anti-mouse IgG-HRP (Sigma, A9044), anti-rabbit IgG-HRP (Calbiochem, DL03L) and Alexa 488, 555 and 647 (Invitrogen) were used as secondary antibodies for immunoblot and immunostaining analyses.

Immunofluorescence, microscopy. Immunofluorescence and microscopy were previously described.[32] Cells were fixed with cold methanol for 10 min or 4% PFA for 15 min. The cells were blocked, permeabilized by 3% BSA in 0.3% PBST, incubated with primary antibodies in the blocking solution for 1 h, washed out with 0.3% PBST three times and treated with the secondary antibodies in the blocking solution for 30 min and with DAPI for 3 min. Secondary antibodies and DAPI were washed out with 0.3% PBST, and the cells were mounted onto a slide glass. The cells were observed with a fluorescence microscope (Olympus IX51) with a 60x/1.25 oil Iris (UFlanFl) objective lens. The images were analyzed with a CCD camera (Qicam fast 1394, Qimaging) and ImagePro 5.0 (Media Cybernetics, Inc.).

DNA constructs and transfection. FLAG-GFP-PCNTB-Myc was previously described.[9] FLAG-PCNTB-Myc was subcloned into *pLVX-IRES-Puro* (Clonetech) and used for generating stable cell lines. FLAG-PCNTB-Myc was also subcloned into *pRTS-puro*.[24] Securin-WT and ND (EN10-11AA, R61A) were subcloned into *pRTS-puro*. TEV protease was subcloned into *pRTS-Neo*.[24] *pRTS-puro* and *pRTS-Neo* is obtained from Dr. Georg Bornkamm. The human securin cDNA was obtained from Dr. Mark W. Kirschner.[35] The TEV protease cDNA was obtained from Dr. David Waugh via Addgene Inc., (Addgene plasmid 8827).[36]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgements

We thank Drs. Robert A. Weinberg, Kyung S. Lee, Georg Bornkamm, Mark W. Kirschner and David Waugh for providing us with their valuable materials. This study was supported by grants from the BioImaging Research Center at GIST; the Basic Research Program [grant number 3344-20100052]; the Science Research Center Program [grant number R11-2005-009-03005-0] of the Ministry of Education, Science and Technology; and the second stage of the Brain Korea 21 Project in 2007 (to K.L.).-

Supplemental Material

Supplemental material may be found here:
www.landesbioscience.com/journals/cc/article/20878

References

1. Bettencourt-Dias M, Glover DM. Centrosome biogenesis and function: centrosomics brings new understanding. Nat Rev Mol Cell Biol 2007; 8:451-63; PMID:17505520; http://dx.doi.org/10.1038/nrm2180.
2. Nigg EA, Stearns T. The centrosome cycle: Centriole biogenesis, duplication and inherent asymmetries. Nat Cell Biol 2011; 13:1154-60; PMID:21968988; http://dx.doi.org/10.1038/ncb2345.
3. Tsou MF, Wang WJ, George KA, Uryu K, Stearns T, Jallepalli PV. Polo kinase and separase regulate the mitotic licensing of centriole duplication in human cells. Dev Cell 2009; 17:344-54; PMID:19758559; http://dx.doi.org/10.1016/j.devcel.2009.07.015.
4. Loncarek J, Hergert P, Magidson V, Khodjakov A. Control of daughter centriole formation by the pericentriolar material. Nat Cell Biol 2008; 10:322-8; PMID:18297061; http://dx.doi.org/10.1038/ncb1694.
5. Loncarek J, Hergert P, Khodjakov A. Centriole reduplication during prolonged interphase requires procentriole maturation governed by Plk1. Curr Biol 2010; 20:1277-82; PMID:20656208; http://dx.doi.org/10.1016/j.cub.2010.05.050.
6. Tsou MF, Stearns T. Mechanism limiting centrosome duplication to once per cell cycle. Nature 2006; 442:947-51; PMID:16862117; http://dx.doi.org/10.1038/nature04985.
7. Uhlmann F, Lottspeich F, Nasmyth K. Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. Nature 1999; 400:37-42; PMID:10403247; http://dx.doi.org/10.1038/21831.
8. Schöckel L, Möckel M, Mayer B, Boos D, Stemmann O. Cleavage of cohesin rings coordinates the separation of centrioles and chromatids. Nat Cell Biol 2011; 13:966-72; PMID:21743463; http://dx.doi.org/10.1038/ncb2280.
9. Lee K, Rhee K. PLK1 phosphorylation of pericentrin initiates centrosome maturation at the onset of mitosis. J Cell Biol 2011; 195:1093-101; PMID:22184200; http://dx.doi.org/10.1083/jcb.201106093.
10. Buchman JJ, Tseng HC, Zhou Y, Frank CL, Xie Z, Tsai LH. Cdk5rap2 interacts with pericentrin to maintain the neural progenitor pool in the developing neocortex. Neuron 2010; 66:386-402; PMID:20471352; http://dx.doi.org/10.1016/j.neuron.2010.03.036.
11. Haren L, Stearns T, Lüders J. Plk1-dependent recruitment of gamma-tubulin complexes to mitotic centrosomes involves multiple PCM components. PLoS One 2009; 4:5976; PMID:19543530; http://dx.doi.org/10.1371/journal.pone.0005976.
12. Zimmerman WC, Sillibourne J, Rosa J, Doxsey SJ. Mitosis-specific anchoring of gamma tubulin complexes by pericentrin controls spindle organization and mitotic entry. Mol Biol Cell 2004; 15:3642-57; PMID:15146056; http://dx.doi.org/10.1091/mbc.E03-11-0796.
13. Griffith E, Walker S, Martin CA, Vagnarelli P, Stiff T, Vernay B, et al. Mutations in pericentrin cause Seckel syndrome with defective ATR-dependent DNA damage signaling. Nat Genet 2008; 40:232-6; PMID:18157127; http://dx.doi.org/10.1038/ng.2007.80.
14. Rauch A, Thiel CT, Schindler D, Wick U, Crow YJ, Ekici AB, et al. Mutations in the pericentrin (PCNT) gene cause primordial dwarfism. Science 2008; 319:816-9; PMID:18174396; http://dx.doi.org/10.1126/science.1151174.
15. Anitha A, Nakamura K, Yamada K, Iwayama Y, Toyota T, Takei N, et al. Association studies and gene expression analyses of the DISC1-interacting molecules, pericentrin 2 (PCNT2) and DISC1-binding zinc finger protein (DBZ), with schizophrenia and with bipolar disorder. Am J Med Genet B Neuropsychiatr Genet 2009; 150:967-76; PMID:19191256; http://dx.doi.org/10.1002/ajmg.b.30926.
16. Delaval B, Doxsey SJ. Pericentrin in cellular function and disease. J Cell Biol 2010; 188:181-90; PMID:19951897; http://dx.doi.org/10.1083/jcb.200908114.
17. Chestukhin A, Pfeffer C, Milligan S, DeCaprio JA, Pellman D. Processing, localization and requirement of human separase for normal anaphase progression. Proc Natl Acad Sci USA 2003; 100:4574-9; PMID:12672959; http://dx.doi.org/10.1073/pnas.0730733100.
18. Zur A, Brandeis M. Securin degradation is mediated by fzy and fzr, and is required for complete chromatid separation but not for cytokinesis. EMBO J 2001; 20:792-801; PMID:11179223; http://dx.doi.org/10.1093/emboj/20.4.792.
19. Hauf S, Waizenegger IC, Peters JM. Cohesin cleavage by separase required for anaphase and cytokinesis in human cells. Science 2001; 293:1320-3; PMID:11509732; http://dx.doi.org/10.1126/science.1061376.
20. Sullivan M, Lehane C, Uhlmann F. Orchestrating anaphase and mitotic exit: separase cleavage and localization of Slk19. Nat Cell Biol 2001; 3:771-7; PMID:11533655; http://dx.doi.org/10.1038/ncb0901-771.
21. Sriram SM, Kim BY, Kwon YT. The N-end rule pathway: emerging functions and molecular principles of substrate recognition. Nat Rev Mol Cell Biol 2011; 12:735-47; PMID:22016057; http://dx.doi.org/10.1038/nrm3217.
22. Bashir T, Dorrello NV, Amador V, Guardavaccaro D, Pagano M. Control of the SCF(Skp2-Cks1) ubiquitin ligase by the APC/C(Cdh1) ubiquitin ligase. Nature 2004; 428:190-3; PMID:15014502; http://dx.doi.org/10.1038/nature02330.
23. Celis JE, Celis A. Cell cycle-dependent variations in the distribution of the nuclear protein cyclin proliferating cell nuclear antigen in cultured cells: subdivision of S phase. Proc Natl Acad Sci USA 1985; 82:3262-6; PMID:2860667; http://dx.doi.org/10.1073/pnas.82.10.3262.

24. Bornkamm GW, Berens C, Kuklik-Roos C, Bechet JM, Laux G, Bachl J, et al. Stringent doxycycline-dependent control of gene activities using an episomal one-vector system. Nucleic Acids Res 2005; 33:137; PMID:16147984; http://dx.doi.org/10.1093/nar/gni137.
25. Fry AM, Mayor T, Meraldi P, Stierhof YD, Tanaka K, Nigg EA. C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated protein kinase Nek2. J Cell Biol 1998; 141:1563-74; PMID:9647649; http://dx.doi.org/10.1083/jcb.141.7.1563.
26. Kleylein-Sohn J, Westendorf J, Le Clech M, Habedanck R, Stierhof YD, Nigg EA. Plk4-induced centriole biogenesis in human cells. Dev Cell 2007; 13:190-202; PMID:17681131; http://dx.doi.org/10.1016/j.devcel.2007.07.002.
27. Gillingham AK, Munro S. The PACT domain, a conserved centrosomal targeting motif in the coiled-coil proteins AKAP450 and pericentrin. EMBO Rep 2000; 1:524-9; PMID:11263498.
28. Fong KW, Hau SY, Kho YS, Jia Y, He L, Qi RZ. Interaction of CDK5RAP2 with EB1 to track growing microtubule tips and to regulate microtubule dynamics. Mol Biol Cell 2009; 20:3660-70; PMID:19553473; http://dx.doi.org/10.1091/mbc.E09-01-0009.
29. Barrera JA, Kao LR, Hammer RE, Seemann J, Fuchs JL, Megraw TL. CDK5RAP2 regulates centriole engagement and cohesion in mice. Dev Cell 2010; 18:913-26; PMID:20627074; http://dx.doi.org/10.1016/j.devcel.2010.05.017.
30. Matsuo K, Ohsumi K, Iwabuchi M, Kawamata T, Ono Y, Takahashi M. Kendrin is a novel substrate for separase involved in the licensing of centriole duplication. Curr Biol 2012; 22:915-21; PMID:22542101; http://dx.doi.org/10.1016/j.cub.2012.03.048.
31. Lee S, Rhee K. CEP215 is involved in the dynein-dependent accumulation of pericentriolar matrix proteins for spindle pole formation. Cell Cycle 2010; 9:774-83; PMID:20139723; http://dx.doi.org/10.4161/cc.9.4.10667.
32. Kim K, Rhee K. The pericentriolar satellite protein CEP90 is crucial for integrity of the mitotic spindle pole. J Cell Sci 2011; 124:338-47; PMID:21224392; http://dx.doi.org/10.1242/jcs.078329.
33. Jeong Y, Lee J, Kim K, Yoo JC, Rhee K. Characterization of NIP2/centrobin, a novel substrate of Nek2, and its potential role in microtubule stabilization. J Cell Sci 2007; 120:2106-16; PMID:17535851; http://dx.doi.org/10.1242/jcs.03458.
34. Kim K, Lee S, Chang J, Rhee K. A novel function of CEP135 as a platform protein of C-NAP1 for its centriolar localization. Exp Cell Res 2008; 314:3692-700; PMID:18851962; http://dx.doi.org/10.1016/j.yexcr.2008.09.016.
35. Zou H, Stemman O, Anderson JS, Mann M, Kirschner MW. Anaphase specific auto-cleavage of separase. FEBS Lett 2002; 528:246-50; PMID:12297314; http://dx.doi.org/10.1016/S0014-5793(02)03238-6.
36. Kapust RB, Tözsér J, Fox JD, Anderson DE, Cherry S, Copeland TD, et al. Tobacco etch virus protease: mechanism of autolysis and rational design of stable mutants with wild-type catalytic proficiency. Protein Eng 2001; 14:993-1000; PMID:11809930; http://dx.doi.org/10.1093/protein/14.12.993.