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Studies on premature centriole disengagement during a prolonged mitotic arrest

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ABSTRACT

Studies on premature centriole disengagement during a prolonged mitotic arrest

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Centrioles duplicate and segregate in a tight link to the cell cycle. During S phase, a new centriole grows orthogonally from preexisting centriole and then establishes engagement. At the end of mitosis, paired mother and daughter centrioles eventually disengaged from each other, which allow mother centriole to be able to duplicate in the following S phase. Disruption of the centriole cycle often results in aneuploidy and cancers. Here, I report that centrioles are prematurely disengaged when a cell is arrested at M phase for a period of time. Premature centriole disengagement still occurs in mitotic cells of which the separase activity is reduced by depletion of CDC20 or separase itself. However, centrioles remain engaged with treatment of BI2536, a PLK1 inhibitor, suggesting that the PLK1 activity is critical even for premature centriole disengagement in M-phase-arrested cells. I also observed that SAS6 is displaced in prematurely disengaged daughter centrioles, suggesting that SAS6 displacement precedes premature centriole disengagement. BI2536 inhibits the SAS6 displacement as well as premature centriole disengagement. Furthermore, the centriole disengagement rate was reduced in M-phase-arrested cells with the centriole-directed SAS6-PACT protein. Finally, I observed reduction of the centriole disengagement rate in M-phase-arrested cells with a higher dose of

nocodazole and taxol, and, therefore, whose cellular microtubules were completely disrupted. Furthermore, disengagement was rapidly rescued when each drug was washed out in CDC20-depleted cells. Based on the results, I propose that centrioles in M-phase-arrested cells are prematurely disengaged following two sequential steps: (i) removal of SAS6 from engaged centrioles with a help of PLK1 and (ii) a microtubule-mediated pushing force.

Key Words: Mitotic arrest, Centriole disengagement, SAS6
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INTRODUCTION

Centrosome consisting of centrioles and pericentriolar material (PCM) is a major microtubule organizing centers (MTOC) in mammalian cells and, therefore, is crucial for spindle pole assembly during mitosis. As a consequence, centrosome defect could result in genomic instability. Furthermore, it has been recognized long ago that there is a strong correlation between abnormal centrosome number and anueploid tumor cell, indicating that a precise regulation of centrosome number might be important for maintaining genome stability in actively proliferating cells (Nigg and Stearns, 2011).

Centrioles that acquire an ability to recruit PCM begin to function as centrosomes (Wang *et al*., 2011). Thus, answering how centriole number is controlled in normal cells resolves how cells stably maintain the centrosome number. Cells begin G1 phase with two centrosomes that consist of a single centriole surrounded by PCM. During S phase, like genomic replication, a new centriole (daughter) grows orthogonally from the lateral and proximal end of pre-existing centrioles (mother) only once per cell cycle. The juxtaposed form of paired mother and daughter centrioles ("engagement") at the core of each centrosome persists during S/G phase and, subsequent dissolution of engaged centriole pairs ("disengagement") occurs upon passage through M phase exit. In this context, Tsou *et al* demonstrate that Plk1 and Separase are required for centriole disengagement during M phase exit (Tsou *et al.*,2006, 2009). They proposed an attractive model that physical connections (i.e. engagement) between mother and daughter centriole create a block to further round of centriole assembly from the mother centriole. It is true that failure of disengagement ultimately suppressed the new centriole assembly in the following S phase. Moreover, when daughter centriole is intentionally destroyed by laser ablation, mother centrioles regain the ability to generate a single daughter centriole in same cell cycle, supporting that engagement prevents centriole reduplication and, as a

consequence, limits centrosome number (Loncarek *et al*., 2008). Thus, precocious centriole disengagement could result in centrosome amplification in actively cycling cells. Although such regulation of centriole disengagement play a fundamental role in maintaining centrosome number, the molecular mechanism underlying this event is not clear yet; for instance, what is a centriolar glue protein as substrate of Plk1 and Separase, and how do both enzymes coordinate to disengage paired centrioles?

Recent studies reported that while characterizing centrosomal proteins such as Aki1, Astrin, Sgo1, and Scc1, siRNA-mediated depletion of each protein exhibited premature centriole disengagements in mitotic cells, raising the possibility that these proteins are implicated in engagement (Thein *et al*., 2007; Wang *et al*., 2008; Nakamura *et al*., 2009). However, I noticed that downregulation of these proteins results in mitotic arrest as well as centriole disengagement. Thus, it is possible that mitotic arrest itself is involved in this event. Furthermore, two studies conducted in 1984 and 1992 showed that cells treated with colcemid or expressing non-degradable Cyclin B1 displayed centriole disengagement, suggesting that mitotic arrest is associated with centriole disengagement (Keryer *et al.*, 1984; Gallant and Nigg, 1992).

Here, I report that prolonged mitotic arrest induce premature centriole disengagement in human cells. This event does not depend on the activity of Separase, instead requires Plk1 activity even after prophase. I also noticed that during mitotic arrest SAS6 gradually disappeared from the daughter centrioles in a time-dependent and Plk1-dependent manner. I also observed that cells expressing SAS6-PACT suppressed premature centriole disengagement during mitotic arrest. Finally, I observed that premature centriole disengagement failed in M-phase-arrested cells with a higher dose of nocodazole and taxol. However, disengagement was rapidly rescued when the drug was washed out in CDC20-depleted cells.

MATERIALS AND METHODS

Cell culture, Cell synchronization and Drug treatments

HeLa and hTERT-RPE1 cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (Plasmocin; Invivogen, ANT-MPT). The PCNT-R2231A mutant and PCNT-WT stable cell lines were previously described (Lee and Rhee, 2012) and cultured with puromycin(0.5μg/ml) and hygromycin (200μg/ml) in DMEM supplemented with 10% FBS. Cells were synchronized at G1/S boundary by the double thymidine block. Briefly, 2mM thymidine was added to the culture medium for 17-20 hr, cells were incubated in fresh medium for 8 hr, and were incubated again in 2mM thymidine for 17 hr. For drug treatments, the following compounds and concentrations were used: STLC(5μM, 10μM), Nocodazole(50ng/ml, 200n/ml), Taxol (0.156μM, 5μM), BI2536(200nM), ZM447439(2μM).

Antibodies

The CEP135 (Kim *et al*., 2008) , Pericentrin (Lee and Rhee, 2011) antibodies were as described previously.

Antibodies for Centrin(Millipore, 20H5), Cdc20(Abcam, ab26483), Scc1(Abcam, ab992-ChIP Grade), Separase (Abcam, ab16170), Cyclin B1 (Santa Cruz, sc-245), phspho-histone H3 (Ser10) (Upstate, 06-570), GAPDH (Ambion, AM4300), SAS6(Santa Cruz, sc-81431), Alpha-tubulin (Abcam, ab18251) and FLAG (Sigma, F3165) were purchased.

Anti-mouse IgG-HRP (Sigma, A9044), anti-rabbit IgG-HRP (Calbiochem, DL03L) and Alexa 488, 555 (Invitrogen) were used as secondary antibodies for either Western blot or Immunocytochemistry analyses.

Immunofluorescence microscopy

Cells were fixed with methanol at -20℃ for 10 min, then blocked with 3% bovine serum albumin (w/v) in 0.3% PBST(Triton X-100) for 20 min. The cells were incubated with primary antibodies in the blocking solution for 1 h, and washed out with 0.3% PBST three times, and then treated with the secondary antibodies in the blocking solution for 30min. DNA was visualized with 4',6-diamidino-2-phenylinodole (DAPI). After staining for centrosomal antigens, 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.)

Lentiviral infection and shRNA sequences

To produce lentiviruses, *pLKO.1*(puro) , *pCMV-dR8.2 dvpr* and *pCMV-VSVG* were transfected into 293T cells. 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 hr and replaced with a normal culture medium. One day after the infection, puromycin(0.5μg/ml) was added for selection. *pLKO.1-puro*, *pCMV-dR8.2 dvpr* and *pCMV-VSVG* were gifted from Dr. Kyung S. Lee.

shRNA sequences used in this study are *shCTL* (GCA ATC GAA GCT CGG CTA CAT), shSeparase (GCA GGT TCT GTT CTT GCT TGA).

siRNA, DNA constructs and transfection

For Cdc20 depletion, Ambion Silencer Select siRNA against Cdc20 (s2748) were purchased. To deplete Scc1 and WAPL, siRNA sequence with 5′-AUACCUUCUUGCAGACUGUdTdT-3′ (Nakamura *et al.*, 2009, *J Cell Biol.*) and 5'-CGGACUACCCUUAGCACAATT-3' were used,

respectively. For control siRNA, scrambled siCTL (5'-GCAAUCGAAGCUCGGCUACTT-3') were used.

To express FLAG-SAS6-PACT, SAS6 was subcloned into pFLAG-CMV2-PACT and transfected into HeLa cells by Fugene HD(Promega).

Chromosome spread for Giemsa staining

It was previously described in (McGuiness *et al*. 2007. Plos Biol). Briefly, cells were harvested by trypsinization and pretreated with hypotonic buffer containing 40% medium and 60% D.W. for 5 min at room temperature. Cells were fixed with freshly made Carnoy's solution (75% methanol and 25% acetic acid), the fixative was changed three times, and the cells were then stored overnight at -20℃. For spreading, cells in Carnoy's solution were dropped onto glass slides and dried at room temperature. Slides were stained with 8% Giemsa (Merck, Darmstadt, Germany) at pH 6.8 for 30 min, washed briefly in D.W, air-dried, and mounted.

RESULTS

Prolonged mitotic arrest induces premature centriole disengagement

To test whether prolonged mitotic arrest *per se* causes centriole disengagement, RPE1 cells were arrested at S phase with double thymidine block and synchronously released. The cells were then treated with STLC (10μM), nocodazole (50ng/ml), taxol (0.156μM) which block prometaphase progression. I ensured that each drug was added eight hours after double thymidine block release to confirm no effect of each drug during interphase (Fig. 1a). At indicated time points, the cells were immunostained with the centrin antibody to determine centriole disengagement. The results showed that centrioles in M-phase-arrested cells are distinctly disengaged (Fig. 1b). In contrast to this, the centrioles in control cells are paired at spindle poles (Fig. 1b). The number of mitotic cells with disengaged centrioles increased in a time-dependent manner in STLC-treated cells (Fig. 1c). Nocodazole and taxol also induced centriole disengagement with reduced rates (Fig. 1c). I also treated other cell lines, such as HeLa and U2OS, with STLC and observed premature centriole disengagement in a time-dependent manner (Fig. 1d). These results suggest that mitotic arrest *per se* triggers premature centriole disengagement.

According to previous reports, centrin and C-Nap1 staining localization pattern (i.e. centrin and C-Nap1 foci ratios 2:1=engaged vs 1:1=disengaged) is often used to distinguish engaged from disengaged centrioles in interphase cells (Tsou *et al*., 2006, 2009). However, it is known that C-Nap1 disappears from centrosome mostly during mitosis (Fry *et al.*, 1998); therefore, it is unable to function as centriole disengagement marker. To resolve this problem, I have identified an additional disengagement marker for centrioles in mitosis. CEP135 known as a component of centriole assembly localizes to proximal ends of both mother and daughter centrioles and stably localizes at centrioles throughout cell cycle (Brito DA *et al.*, 2012).

Although CEP135 is known to exist at both mother and newly formed centrioles, when using immunofluorescence microscopy, there is a single CEP135 dot at each centrosome rather than two dots due to resolution problem (Arquint *et al*., 2012). However, CEP135 begins to be seen as two dots in cells upon passage through telophase or early G1: a probable consequence of centriole disengagement during M phase exit (Arquint *et al*., 2012). I, therefore, thought that CEP135 could be used as centriole disengagement marker along with centrin instead of C-Nap1 particularly for centrioles of mitotic cells. Centrioles in M-phase-arrested cells with centrin and CEP135 staining foci ratios of either 2:1 or 1:1 indicated engaged or disengaged centrioles, respectively (Fig. 2). The same manners were used for centrioles in early G1 cells induced to exit mitosis with Cdk1 inhibitor RO3306 to distinguish disengaged centrioles. I found that there was no big difference in disengagement rates between centrin to CEP135 and centrin to C-Nap1 system (data not shown). Thus, this centrin to CEP135 foci ratio centriole disengagement marker system was used for all the following experiments.

I readdressed the issue of whether mitotic arrest induces premature centriole disengagement. In principle, prematurely disengaged centrioles in either microtubule disrupting drugs or STLC-treated cells could reflect a direct requirement for mitotic arrest or indirect negative effects of monopolar geometry and activation of spindle assembly checkpoint (SAC). To eliminate these possibilities, I performed siRNA-mediated CDC20 depletion to trigger mitotic arrest. CDC20 is a well known APC/C E3 ubiquitin ligase substrate, which is required for cyclinB1 and securin degradation during meta/anaphase transition. Thus, absence of CDC20 prevents CyclinB1 destruction and, as a consequence, tightly suppresses cells to exit mitosis, which does not activate SAC nor monopolar shape but rather results in the metaphase arrest (Huang *et al*., 2009). RPE1 cells were transfected with control and CDC20 siRNAs and cultured for 48 h. The cell lysates were subjected to immunoblot analysis with CDC20 and GAPDH antibodies to confirm effective depletion (Fig. 3a). The CDC20-depleted RPE1 cells were

arrested at S phase with thymidine and synchronously released. At indicated time points, the cells were coimmunostained with antibodies specific to centrin and CEP135 to determine centriole disengagement (Fig. 3b). As expected, cells without CDC20 exhibited premature centriole disengagement in time-dependent manner and, moreover, the graph of results was almost the same with that of control cells treated with STLC (Fig. 3c and d). Taken together, these results suggest that induction of premature centriole disengagement is independent of the pathway that lead to mitotic arrest; rather it is the length of time spent in mitosis that promotes engaged centrioles to disengage prior to M phase exit. Furthermore, I deduced that this event probably would not require the activity of Separase (a cystein protease responsible for triggering anaphase); depletion of CDC20 also prevents degradation of securin whose destruction is needed for Separase activation (Uhlmann *et al*., 1999; Zur and Brandeis., 2001). I next investigated the role of Separase in premature centriole disengagement in M-phase-arrested cells.

Separase is not required for the centriole disengagement in M-phase-arrested cells.

It has been demonstrated that Separase is important for centriole disengagement during M phase exit (Tsou *et al*., 2006, 2009). I wondered that whether Separase plays a critical role in premature centriole disengagement during prolonged mitotic arrest. Endogenous Separase in HeLa cells was depleted with lentiviral infection of shRNA targeting Separase (shSep). Immunoblot analysis was performed with antibodies specific to Separase and GAPDH. Both full-length Separase (220kda) and auto-cleaved C-terminal fragment (65kda) were effectively depleted using this method (Fig. 4a). Next, the Separase-depleted HeLa cells were arrested at S

phase with double thymidine block and synchronously released. Eight hours later, the cells were treated with nocodazole (200ng/ml) for 4 h and then released for 3 h to exit mitosis (Fig. 4b). At the indicated time points, the cells were coimmunostained with centrin and CEP135 antibodies to determine centriole disengagement in early G1 phase. The results showed that centrioles in Separase-deleted cells are paired. On the other hand, the centrioles in control cells were disengaged. (Fig. 4c) Consistent with previous studies, downregulation of Separase effectively blocked the dissolution of engaged centrioles during M phase exit (Fig. 4d).

Using this system, I tested whether Separase is required for premature centriole disengagement in M-phase-arrested cells. The Separase-depleted HeLa cells were arrested at S phase with a thymidine, synchronously released in the presence of STLC, and cultured further to indicated time points (Fig. 5a). The cells on each time point were coimmunostained with centrin and CEP135 antibodies to quantify the number of mitotic cells with disengaged centrioles. Parallel to the previous deduction in CDC20-depletion experiments, the inactivation of Separase did not perturb premature centriole disengagement in M-phase-arrested cells (Fig. 5b). These results suggest that Separase is not required for this event.

To further confirm this conclusion, I examined whether Separase-specific substrates, Scc1 and Pericentrin (PCNT), were cleaved during mitotic arrest, as their cleavage has recently been reported to play a role in centriole disengagement upon mitotic exit (Schckel L *et al*., 2011; Matsuo *et al*., 2012; Lee K and Rhee K, 2012). RPE1 cells were arrested at S phase with a thymidine block and synchronously released in the presence of STLC. Sixteen hours after the STLC treatment, ZM447439 (20μM), an aurora B inhibitor, was treated to induce mitotic exit (Fig. 6a). The cell lysates were subjected to immunoblot analysis with PCNT, Scc1, Separase, Cyclin b1, phospho Histone H3 (pHH3) and GAPDH antibodies. Bands corresponding to cleavage fragments of both substrates did not increase in time-dependent manner during mitotic arrest when compared to increase of premature centriole disengagement rate: moreover,

Separase's C-terminal fragment was absent at the same time (Fig. 6b). These results suggest that Separase is not activated during mitotic arrest. Scc1 specific cleaved bands were confirmed by immunoblot using Scc1 antibody with Scc1-depleted RPE1 cell lysates (Fig. 6c).

Next, PCNT-depleted HeLa cells were rescued with wild type or cleavage-resistant $PCNTB^{R2231A}$ and synchronously entered mitosis in the presence of STLC. The cells at indicated time points were coimmunostained with the centrin and CEP135 antibodies to quantify centriole disengagement. As expected, majority of centrioles in $PCNTB^{R2231A}$ cells were disengaged during mitotic arrest in time-dependent manner similar to control cells (Fig. 7). Taken together, I concluded that Separase is not required for premature centriole disengagement in mitotic arrested-cells.

Plk1 is critical for premature centriole disengagement in M-phase-arrested cells.

It has been suggested that Plk1 also plays a central role in centriole disengagement. In fact, centrioles that failed to disengage in synchrony with M exit in Separase knock-out cells were eventually disengaged in the following late S phase; however, the disengagement of centriole pairs was completely blocked when Plk1 was inactivated (Tsou *et al*., 2009). To further understand the role of Plk1 in premature centriole disengagement in M-phase-arrested cells, I used Plk1-specific small molecule inhibitor BI2536 (BI) to rapidly block Plk1 activity. RPE1 cells were arrested at S phase with a thymidine block and synchronously released in the presence of STLC and/or BI (200nM) (Fig. 8a). Indeed, combined treatments of STLC and BI before mitotic entry completely prevented premature disengagement during prolonged mitotic arrest (Fig. 8b). Parallel to these data, recent studies have proposed that it is during prophase

that when Plk1 plays a role in centriole disengagement (Wang *et al*., 2008; Tsou *et al*., 2009; Schckel *et al.*, 2011). This is similar to the prophase pathway responsible for removing cohesin from paired sister chromatid arms.

I next asked whether Plk1 acts for premature centriole disengagement even after the prophase. RPE1 cells were arrested at S phase with a thymidine block and synchronously released in the presence of STLC. Eight hours later, the cells were treated with DMSO or BI and cultured further to indicated time points (Fig. 9a). I ensured that, when BI was added, the majority of cells entered mitosis (Fig. 9b) and, moreover, most of the mitotic cells included sister chromatid pairs with open arms. However, sister chromatids in BI2536-treated cells and WAPL-depleted cells were closed (Fig. 9c). This indicates that the STLC-treated cells successfully passed through the prophase pathway before the BI treatment. The number of M phase cells with disengaged centrioles were counted. Interestingly, premature centriole disengagement completely failed in cells treated with STLC and then sequentially treated with BI timely after prophase (Fig. 9d). These data suggest that Plk1 is critical for premature centriole disengagement even after prophase in M-phase-arrested cells.

Prolonged mitotic arrest causes the loss of hSAS6 from centrioles in a Plk1-dependent manner.

SAS6 responsible for initiating assembly of new centriole localizes to the mother and daughter centrioles' interface. This is associated with engaged centriole pairs in cell cycle-dependent manner: i.e. SAS6 destructs in synchrony with M phase exit centriole disengagement (Strnad *et al*., 2007; Nigg and Stearn, 2011). In addition, dSAS6 (orthologue of SAS6 in *Drosophila*) has been suggested that it promotes centriole engagement in flies' spermatocytes (Stevens *et al.*, 2010). Although it was reported that expressing non-degradable SAS6 did not perturb

disengagement of M phase exit (Tsou *et al.*, 2009), previous evidence encouraged me to consider SAS6 as a candidate for the centriole engagement factor in human cell lines.

To test the involvement of SAS6 in this event, I first examined how SAS6 is regulated during mitotic arrest. RPE1 cells were arrested at S phase with a thymidine block and synchronously released in the presence of STLC. Eight hours later, Cells were treated with DMSO or BI and cultured further to indicated time points. The cells from each time point were coimmunostained with antibodies against SAS6, centrin and CEP135 (Fig. 10a). I observed that SAS6 was removed from centrioles in a time-dependent manner during mitotic arrest in DMSO-treated cells (Fig. 10b), which positively correlates with premature disengagement (Fig. 10c). Moreover, with close observation, I noticed that no SAS6 signal was observed in disengaged centrioles (Fig. 10b and c); however, part of centrioles that lack SAS6 dots exhibited a single CEP135 dot, indicating that they were not disengaged yet (Fig. 10b and d). It appears that SAS6 loss precedes premature centriole disengagement.

Recent paper shows that Plk1 inactivation can stabilize the SAS6 at centriole pairs that failed to disengage during M phase exit in the absence of Separase (Tsou *et al.*, 2009). I, therefore, tested whether Plk1 inactivation was able to suppress removal of SAS6 dots in M-phase-arrested cells. Interestingly, I observed that SAS6 remained at engaged centrioles in BI-treated mitotic cells (Fig. 10b and d), suggesting that SAS6 displacement as well as premature centriole disengagement are dependent on Plk1 activity.

I next investigated the cause of SAS6 removal from centrioles in M-phase-arrested-cells. There were two possibilities in terms of the SAS6 loss. First, SAS6 might be degraded during mitotic arrest. This is similar to previous interpretation that APC/C-Cdh1 dependent SAS6 destruction triggers SAS6 disappearance from centrioles. Second, it is possible that an unknown Plk1-dependent mechanism directly displaces SAS6 from centrioles during mitotic arrest. To distinguish between these two alternatives, RPE1 cells were arrested at S phase with a

thymidine block and synchronously released in the presence of STLC. Sixteen hours after the STLC treatment, ZM was treated to induce mitotic exit (Fig. 11a). Indeed, SAS6 cellular level did not change during mitotic arrest although it clearly disappeared in cells to undergo mitotic exit (Fig. 11b). These data suggest that the removal of SAS6 from centrioles during M phase arrest is probably a consequence of Plk1-dependent dislocation rather than its degradation.

Reduction of centriole disengagement rate in M-phase-arrested cells expressing centriole-directed SAS6-PACT protein.

To test whether SAS6 displacement is necessary for premature centriole disengagement, Flag-GFP, Flag-PACT, and Flag-SAS6-PACT was expressed in HeLa cells. The cells were arrested at S phase with a thymidine block and released in the presence of STLC (Fig. 12a). PACT derived from C-terminal of PCNT is known as centrosomal targeting domain, and, thus, SAS6-PACT was able to be stably localized to centriole during mitotic arrest (Fig.12b). The number of M phase cells with disengaged centrioles were counted. Unexpectedly, the cells expressing PACT showed a synergistic premature centriole disengagement rate when compared to control cells (Mock, Flag-GFP in Fig. 12c). However, the centriole disengagement rate was reduced in M-phase-arrested cells with the centriole-directed SAS6-PACT (Fig. 12c). These data suggest that SAS6 displacement seems to be required for premature centriole disengagement during M phase arrest.

Failure of centriole disengagement in M-phase-arrested cells with a higher dose of nocodazole and taxol.

I previously noticed that while different dose of STLC treatment did not have any effect on premature disengagement (data not shown), a dose-dependent premature centriole disengagement was observed in microtubule inhibitor-treated cells: premature centriole disengagement occurred in cells treated with a low dose of either nocodazole (50ng/ml) or taxol (0.156μM) in contrast to the ones treated with a high dose of drugs (nocodazole: 200ng/ml and taxol: 5μM) (Fig. 1c, Fig. 15c and Fig. 16c). It seemed that microtubule-mediated forces were associated with premature centriole disengagement.

I investigated that what extent to which microtubule was disrupted on different doses of nocodazole and taxol; because I assumed that microtubule-mediated forces are totally dependent on microtubule itself. Nocodazole or taxol-treated RPE1 cells were coimmunostained centrin and α-tubulin antibody. A low dose of nocodazole and taxol-treated cells still had remaining microtubules (Fig. 13), whereas, microtubules were totally abolished or severely aggregated in a high dose of nocodazole and taxol, respectively (Fig. 13). These data imply that microtubule-mediated force cannot act in a higher dose of nocodazole and taxol condition.

On the other hand, reduction of centriole disengagement in high dose of each drug could be due to failure in SAS6 displacement as seen in Fig. 12c rather than disruption of microtubule-mediated force. To exclude this possibility, RPE1 cells were treated with high dose of nocodazole, taxol and/or with BI2536. Eighteen hours later, the cells were coimmunostained with antibodies specific to SAS6 and CEP135 (Fig. 14a). The number of mitotic cells with centriolar SAS6 dot signals was counted. I noticed that SAS6 dots were absent from engaged centrioles in majority of cells under high dose of nocodazole or taxol treatment when compared to control cells treated along with BI (Fig. 14b). These data suggest that the block of centriole disengagement in a high dose of each drug is not because of failure in hSAS6 displacement.

Rescue of centriole disengagement when nocodazole or taxol is washed out in CDC20-depleted cells.

I hypothesized that microtubule-dependent forces could play a part in premature centriole disengagement in M-phase-arrested cells. To address this hypothesis, CDC20-depleted RPE1 cells were arrested at S phase with a thymidine block and then released. Eight hours later, the cells were treated with a high dose of nocodazole or taxol for 12 h, shaken off for mitotic cell collection. The cells were washed with PBS well, reseeded to a poly-L-lysine-coated coverslip, and then cultured further to indicated time points (Fig. 15a).

Premature centriole disengagement rate was significantly reduced in cells treated with both CDC20 targeting siRNA and high dose of nocodazole or taxol when compared to mitotic arrested-cells induced by CDC20 depletion. However, over 40% of all cells released from either nocodazole or taxol block rescued centriole disengagement one or two hours later (Fig. 15c and 16c). By carefully observing DAPI staining, I could ensure that the cells processed in this manner did not exit mitosis because of CDC20 depletion (Fig. 15b, Fig. 16a). Moreover, I noticed that DAPI and α-tubulin in drugs washed-out cells returned back to the condition similar to that of CDC20 depleted mitotic cells, suggesting that microtubule-mediated forces were rescued (Fig. 15b, 16b). Taken together, these data suggest that microtubule-dependent forces might be required to disengage centriole pairs where SAS6 has already been removed during prolonged mitotic arrest.

Fig. 1. Premature centriole disengagement in M-phase-arrested cells. (a) RPE1 cells were arrested at S phase with double thymidine block and synchronously released. Eight hours later, the cells were treated with STLC (10μM), nocodazole (50ng/ml) and taxol (0.156μM), and cultured further to indicated time points. (b) The cells were immunostained with the centrin antibody (green). DNA was stained with DAPI (blue). Centrioles in the DMSO-treated control cells remained engaged. On the other hand, those in the M-phase-arrested cells became disengaged. Scale bar: 10μm (c) The number of M phase cells with disengaged centrioles were counted. Experiments were repeated three times, n>150 for each time point within each experiment. Values are means and standard errors. (d) Asynchronous HeLa, U2OS, and RPE1 cells were treated with STLC (5μM) and immunostained with the centrin antibody at indicated time points. The number of mitotic cells with disengaged centrioles were counted. Experiments were repeated twice, n>100 for each time point within each experiment. Values are means and standard deviation.

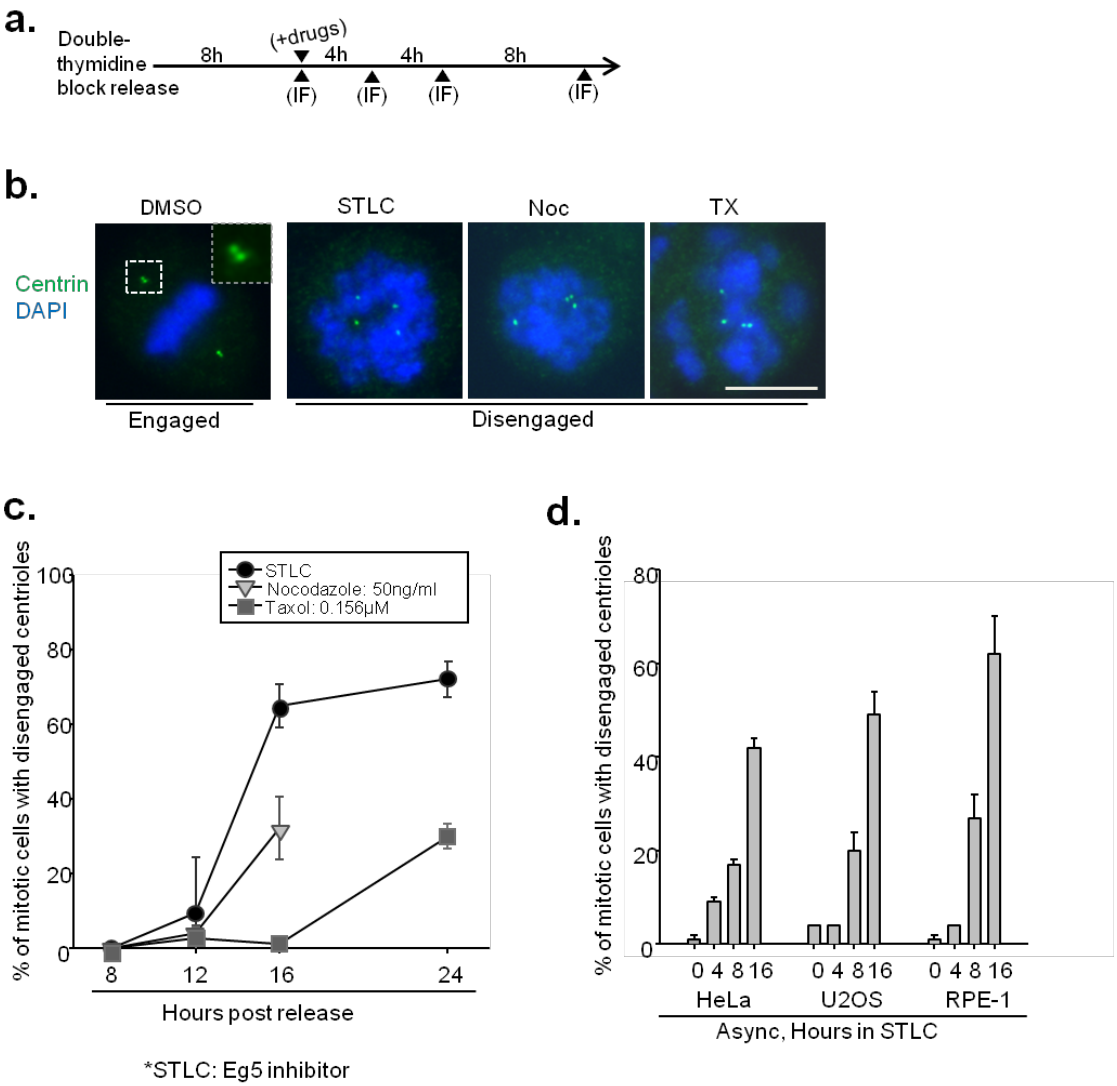

a.
Double-
thymidine
block release
8h
(+drugs)
4h
4h
8h
(IF)
(IF)
(IF)
(IF)
b.
DMSO
STLC
Noc
TX
Centrin
DAPI
Engaged
Disengaged
c.
STLC
Nocodazole: 50ng/ml
Taxol: 0.156μM
% of mitotic cells with disengaged centrioles
100
80
60
40
20
0
8
12
16
24
Hours post release
*STLC: Eg5 inhibitor
d.
% of mitotic cells with disengaged centrioles
80
60
40
20
0
0 4 8 16
0 4 8 16
0 4 8 16
HeLa
U2OS
RPE-1
Async, Hours in STLC

Fig. 2. Centrin and CEP135 number ratios differentially mark the engaged centrioles versus disengaged centrioles. Centriole disengagement can be determined with immunostaining pattern of centrin (green) and CEP135 (red). Engaged centrioles are stained with a single dot of CEP135 along with two dots of centrin. Disengaged centrioles are stained with two pairs of CEP135 and centrin signals.

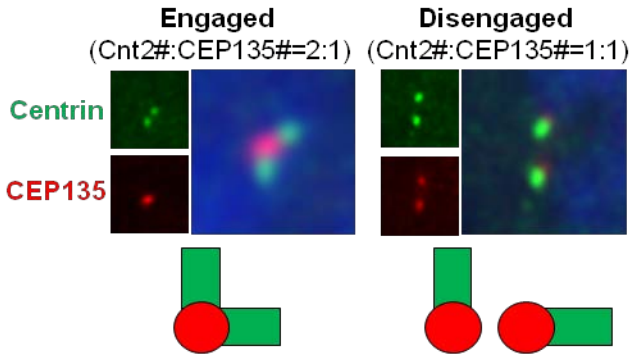
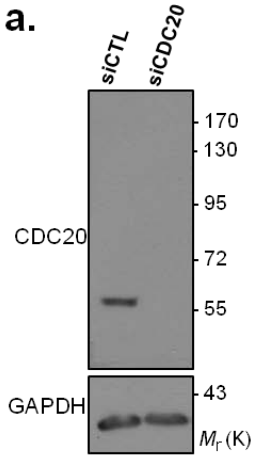
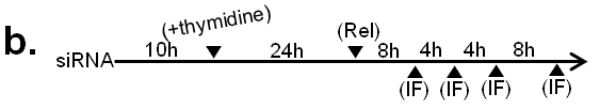
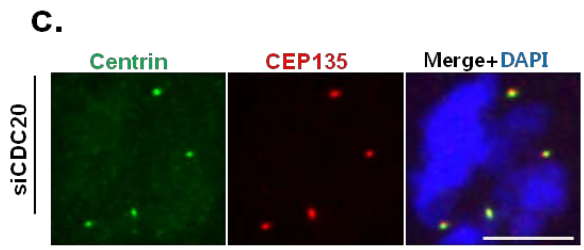


Fig. 3. Premature centriole disengagement in CDC20-depleted cells. (a) RPE1 cells were transfected with control and CDC20 siRNAs and cultured for 48 h. The cell lysates were subjected to immunoblot analysis with CDC20 and GAPDH antibodies. (b) The CDC20-depleted RPE1 cells were arrested at S phase with thymidine for 24 h and released. (c) At indicated time points the cells were coimmunostained with antibodies specific to centrin (green) and CEP135 (red). DNA was stained with DAPI (blue). Scale bar, 10μm. (d) The number of M phase cells with disengaged centrioles were counted in CDC20-depleted cells. STLC-treated cells were used as a control. Experiments were repeated three times, n>150 for each time point within each experiment. Values are means and standard errors.


a.
siCTL
siCDC20
170
130
95
CDC20
72
55
GAPDH
43
Mr (K)


b.
siRNA
(+thymidine)
10h
24h
(Rel)
8h
4h
4h
8h
(IF)
(IF)
(IF)
(IF)


c.
Centrin
CEP135
Merge+DAPI
siCDC20

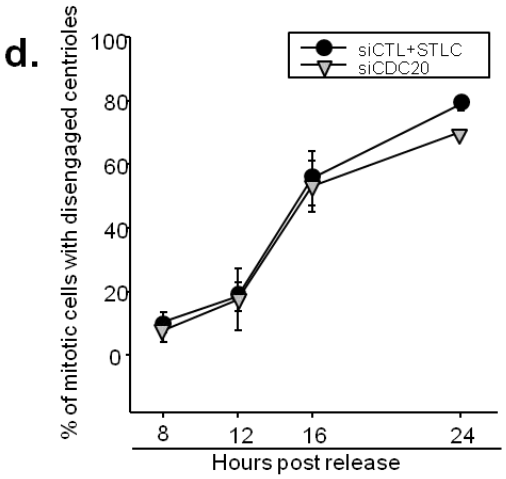
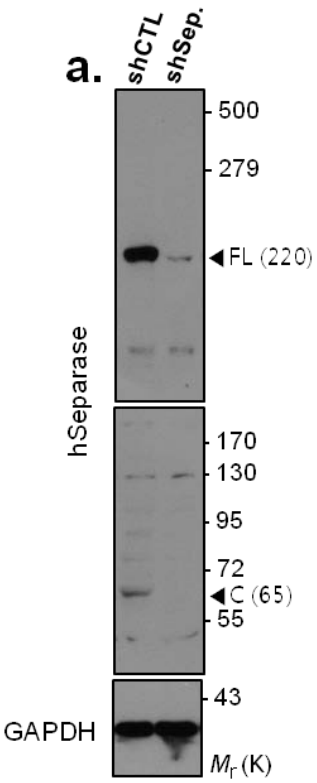
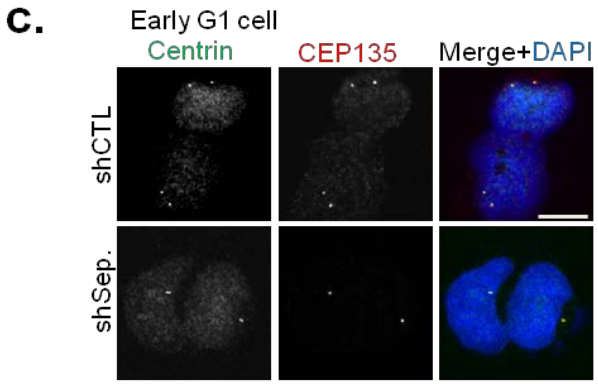

d.
% of mitotic cells with disengaged centrioles
100
80
60
40
20
0
siCTL+STLC
siCDC20
8
12
16
24
Hours post release

Fig. 4. Failure in centriole disengagement during M phase exit in Separase-depleted cells. (a) Endogenous Separase in HeLa cells was depleted with lentiviral infection of shRNA targeting Separase (shSep). Immunoblot analysis was performed with antibodies specific to Separase and GAPDH. The C-terminal fragment of Separase was also detected. (b) The Separase-depleted HeLa cells were arrested at S phase with double thymidine block and synchronously released. Eight hours later, the cells were treated with nocodazole (200ng/ml) for 4 h and then released for 3 h. (c) The cells were coimmunostained with centrin (green) and CEP135 (red) antibodies. DNA was stained with DAPI (blue). Scale bar, 10μm. (d) The number of disengaged centrioles were counted. Experiments were repeated three times, n>360 for each time point within each experiment. Values are means and standard errors.


a.
shCTL
shSep.
500
279
FL (220)
hSeparase
170
130
95
72
C (65)
55
43
GAPDH
Mr (K)


b.
Double-
thymidine
block release
8h
(Noc)
4h
(Rel)
3h
(IF/M)
(IF/G1)


c.
Early G1 cell
Centrin
CEP135
Merge+DAPI
shCTL
shSep.

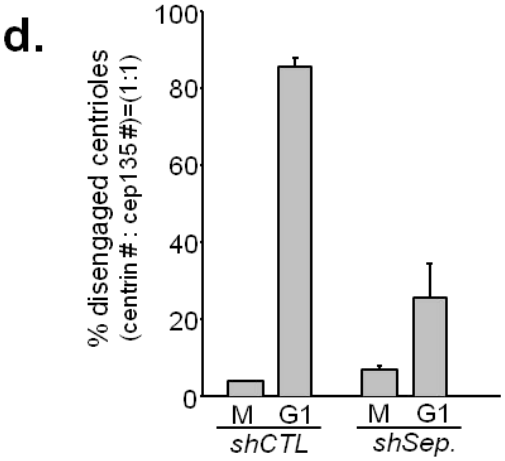

d.
% disengaged centrioles
(centrin # : cep135 #)=(1:1)
100
80
60
40
20
0
M
G1
M
G1
shCTL
shSep.

Fig. 5. Premature centriole disengagement at M-phase-arrested cells is independent of the Separase activity. (a) Separase-depleted HeLa cells were arrested at S phase with a thymidine block, synchronously released in the presence of STLC, and cultured further to indicated time points. (b) The number of mitotic cells with disengaged centrioles were counted at each time point. Experiments were repeated three times, n>150 for each time point within each experiment. Values are means and standard errors.

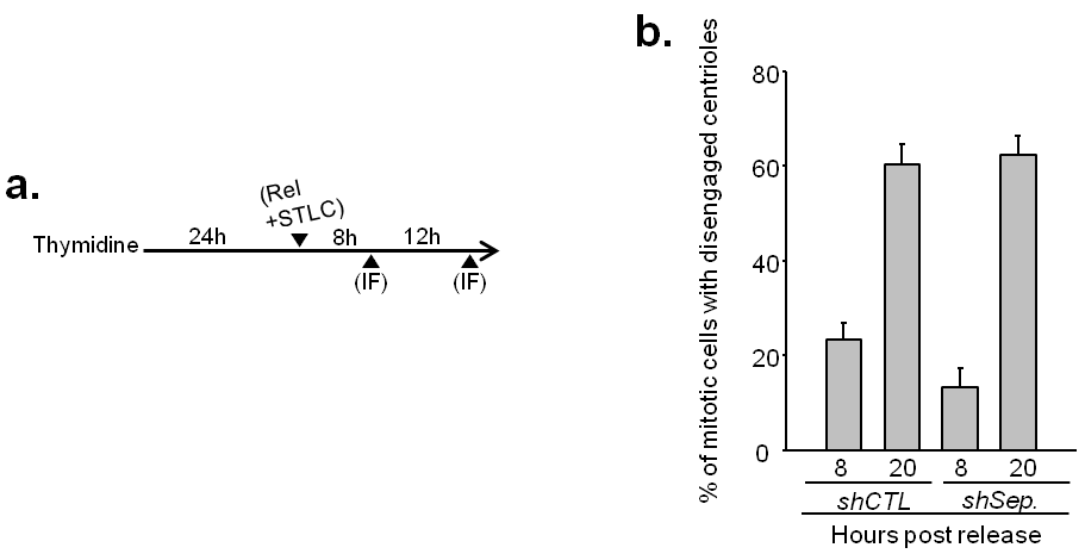
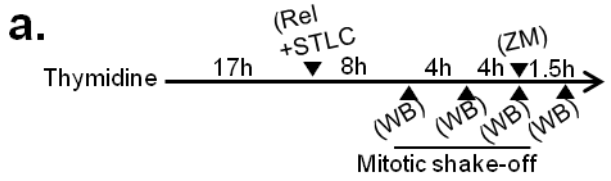


Fig. 6. SCC1 cleavage is not accompanied in M-phase-arrested cells. (a) RPE1 cells were arrested at S phase with a thymidine block and synchronously released in the presence of STLC. Sixteen hours after the STLC treatment, ZM447439 (20μM), an aurora B inhibitor, was treated to induce mitotic exit. (b) The cell lysates were subjected to immunoblot analysis with the PCNT, SCC1, separase, cyclin B1, phospho-Histone H3 (pHH3) and GAPDH antibodies. (c) Lysates from control or SCC1 siRNA-transfected RPE1 cells were subjected to immunoblot analysis with the SCC1 and GAPDH antibodies. Arrows indicate non-specific bands.


a.
(Rel
+STLC
(ZM)
Thymidine
17h
8h
4h
4h
1.5h
(WB)
(WB)
(WB)
(WB)
Mitotic shake-off

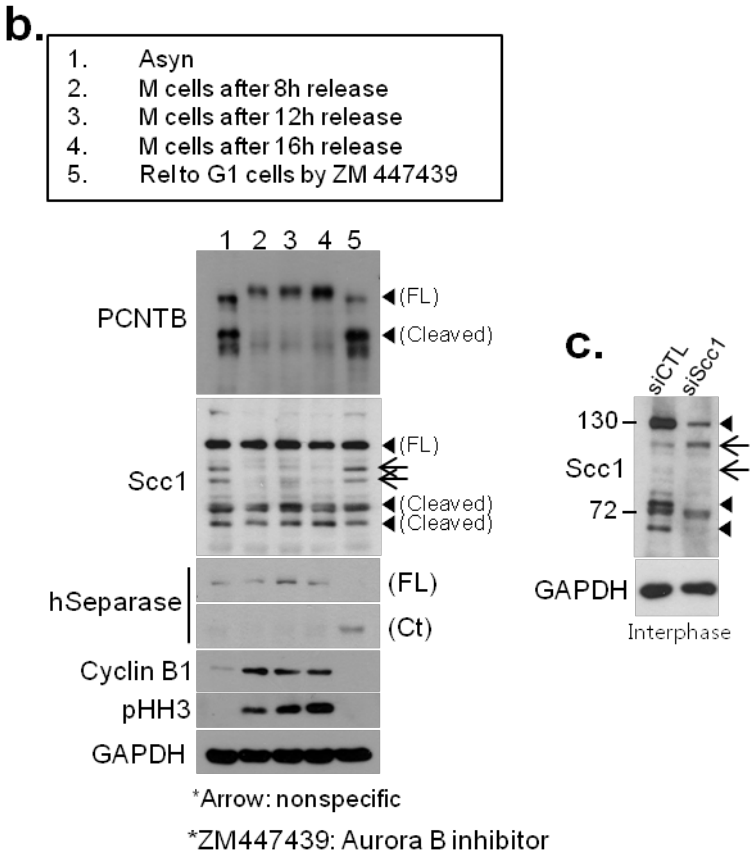

b.
1. Asyn
2. M cells after 8h release
3. M cells after 12h release
4. M cells after 16h release
5. Rel to G1 cells by ZM 447439
1 2 3 4 5
PCNTB
(FL)
(Cleaved)
Scc1
(FL)
(Cleaved)
(Cleaved)
hSeparase
(FL)
(Ct)
Cyclin B1
pHH3
GAPDH
*Arrow: nonspecific
*ZM447439: Aurora B inhibitor
c.
siCTL
siScc1
130
Scc1
72
GAPDH
Interphase

Fig. 7. PCNT cleavage is not accompanied in M-phase-arrested cells.

PCNT-depleted HeLa cells were rescued with wild type or cleavage-resistant PCNTB^{R2231A} and synchronously entered mitosis in the presence of STLC. The cells at indicated time points were coimmunostained with the centrin and CEP135 antibodies. The number of M phase cells with disengaged centrioles was counted. Experiments were repeated three times, n>150 for each time point within each experiment. Values are means and standard errors.

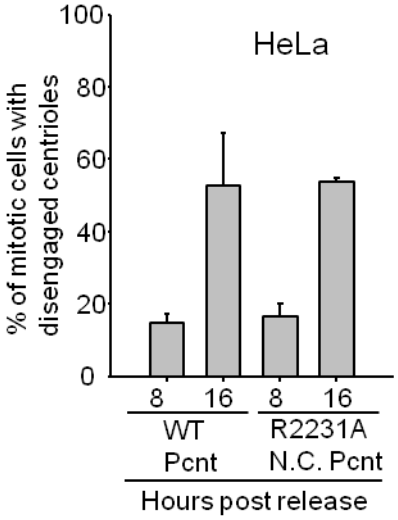
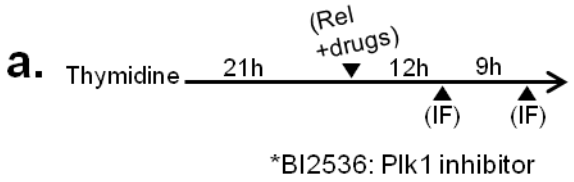


Fig. 8. The Plk1 activity is required for centriole disengagement at M-phase-arrested cells. (a) RPE1 cells were arrested at S phase with a thymidine block and synchronously released in the presence of STLC and/or BI2536 (BI, 200nM), a PLK1 inhibitor. (b) At indicated time points, the number of mitotic cells with disengaged centrioles was counted. Experiments were repeated three times, n>150 for each time point within each experiment. Values are means and standard errors.



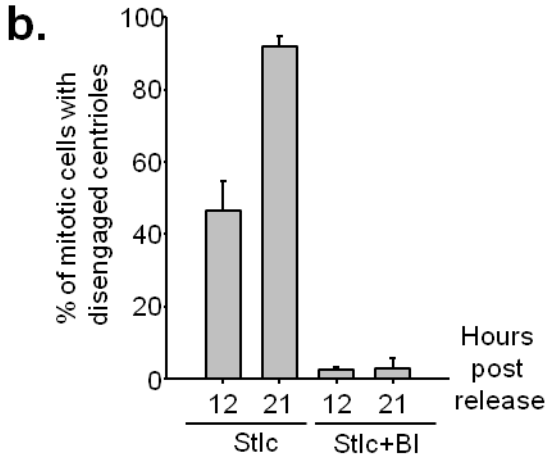
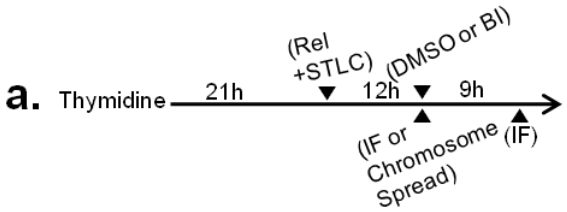
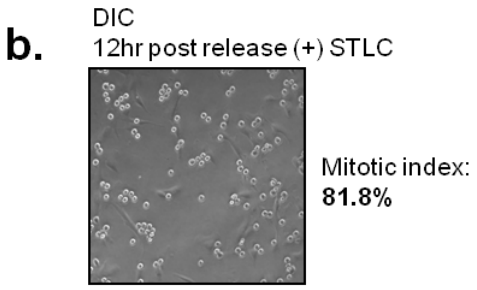
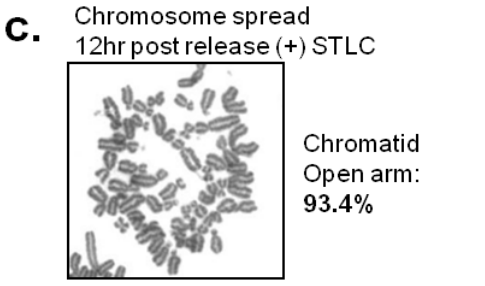
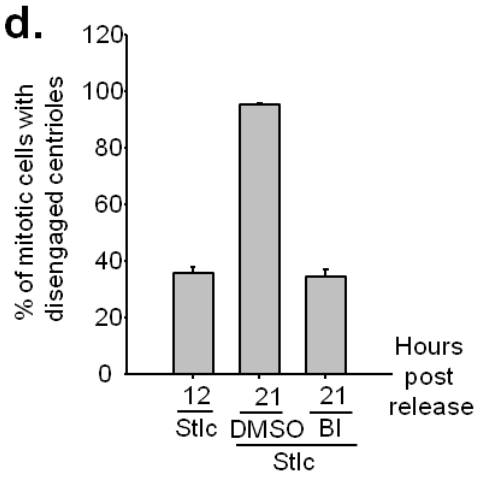


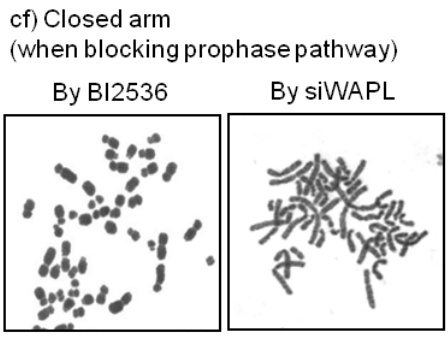
Fig. 9. BI2536 effectively blocks centriole disengagement in M-phase-arrested cells even after prophase. (a) RPE1 cells were arrested at S phase with a thymidine block and synchronously released in the presence of STLC. Eight hours later, the cells were treated with DMSO or BI and cultured further to indicated time points. (b) Phase-contrast microscope image of the cells which cultured for 12 h after release from the thymidine block. About 82% of the cells entered mitosis. (c) Chromosome spreads were prepared and stained with Giemsa. Most of the mitotic cells included sister chromatid pairs with open arms. However, sister chromatids in BI2536-treated cells and WAPL-depleted cells were closed. (d) The number of M phase cells with disengaged centrioles were counted. Experiments were repeated three times, n>150 for each time point within each experiment. Values are means and standard errors.











*PLK1 & WAPL:
Regulator of
Prophase pathway

Fig. 10. Loss of SAS6 from centrioles in a Plk1-dependent manner correlates strictly with premature centriole disengagement in mitotic arrest cells. (a) RPE1 cells were arrested at S phase with a thymidine block and synchronously released in the presence of STLC. Eight hours later, Cells were treated with DMSO or BI and cultured further to indicated time points. (b) The cells were coimmunostained with the SAS6 (green) and CEP135 (red) antibodies. DNA was stained with DAPI (blue). Scale bar, 10μm. (c) The number of M phase cells with disengaged centrioles was counted at the indicated time points. No SAS6 signal was observed in disengaged centrioles. Experiments were repeated twice, n>100 for each time point within each experiment. Values are means and standard errors. (d) The number of mitotic cells without SAS6 signal at the centrioles was counted. The diagonal pattern in histograms indicates the portion of cells with disengaged centrioles. Experiments were repeated twice, n>100 for each time point within each experiment. Values are means and standard errors.

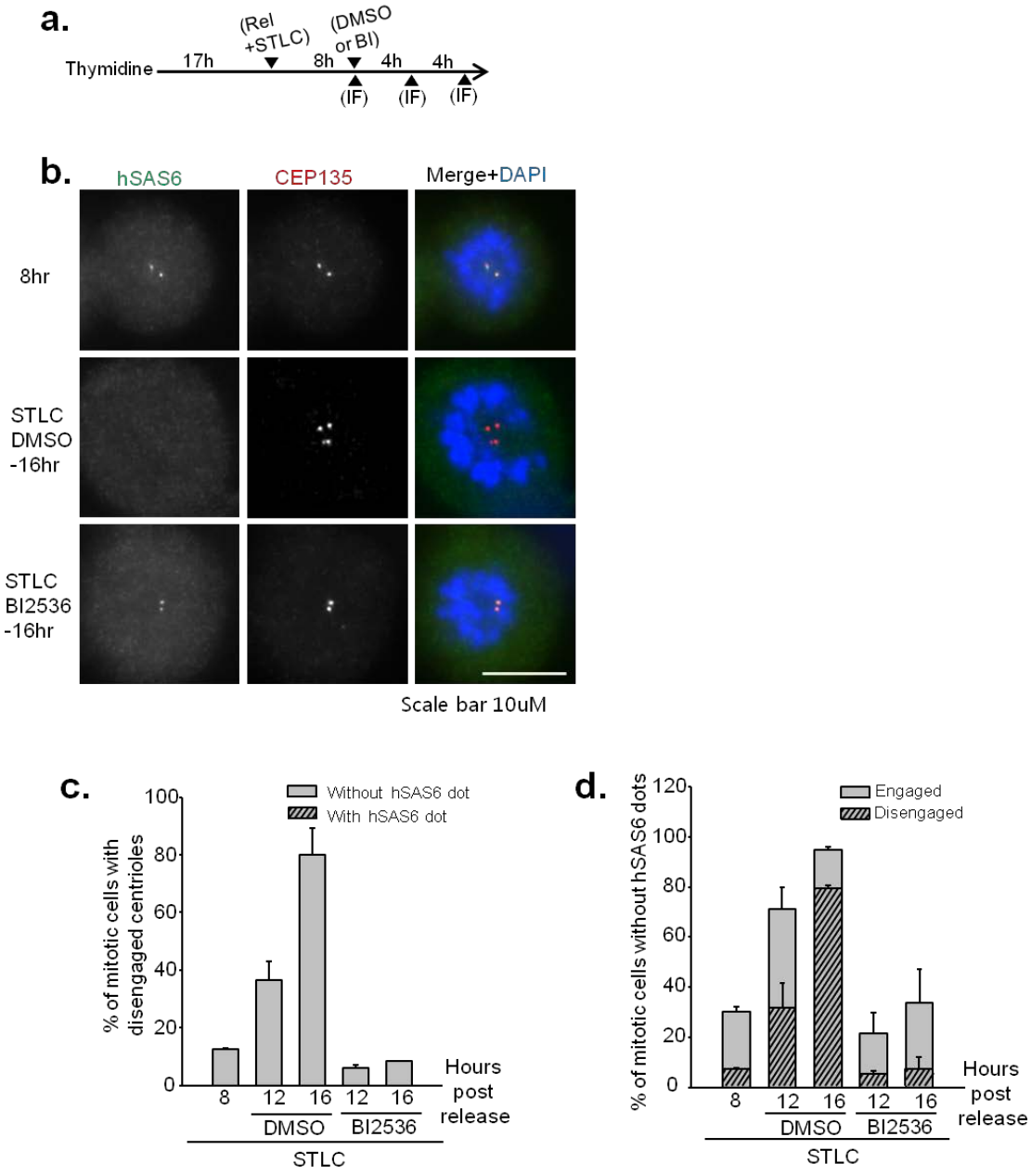

a.
Thymidine
17h
(Rel +STLC)
8h
(DMSO or BI)
4h
4h
(IF)
(IF)
(IF)
b.
hSAS6
CEP135
Merge+DAPI
8hr
STLC DMSO -16hr
STLC BI2536 -16hr
Scale bar 10uM
c.
Without hSAS6 dot
With hSAS6 dot
% of mitotic cells with disengaged centrioles
100
80
60
40
20
0
8
12
16
12
16
DMSO
BI2536
STLC
Hours post release
d.
Engaged
Disengaged
% of mitotic cells without hSAS6 dots
120
100
80
60
40
20
0
8
12
16
12
16
DMSO
BI2536
STLC
Hours post release

Fig. 11. Cellular SAS6 levels during the M phase arrest. (a) RPE1 cells were processed as same manner in Fig. 6a. (b) The cell lysates were subjected to immunoblot analysis with antibodies specific to SAS6, cyclinB1, pHH3 and GAPDH.

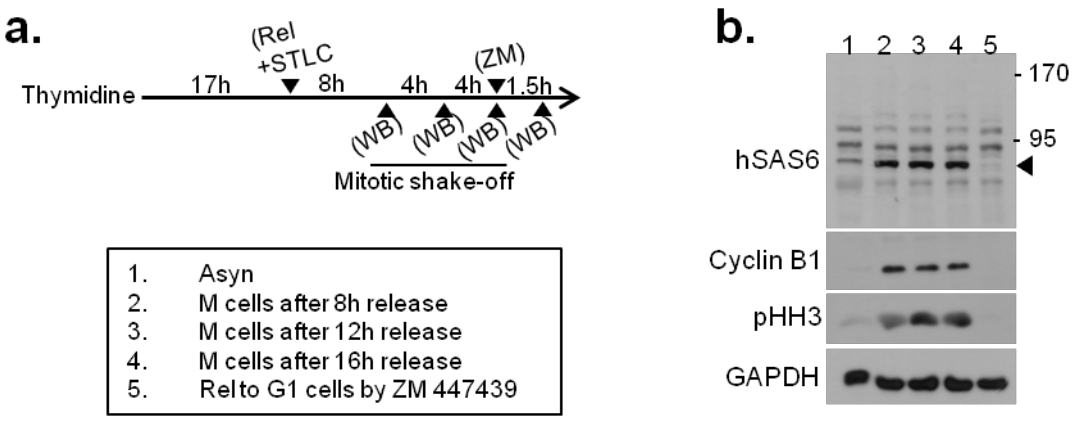


Fig. 12. Effect of SAS6-PACT on premature centriole disengagement in M-phase-arrested cells. (a) The Flag-GFP-, Flag-PACT- or Flag-SAS6-PACT-expressing HeLa cells were arrested at S phase with a thymidine block and synchronously released in the presence of STLC. (b) The cells were coimmunostained with the Flag (green) and CEP135 (red) antibodies. DNA is stained with DAPI (blue). Scale bar, 10μm. (c) The number of mitotic cells with disengaged centrioles was counted. Note that the disengagement was determined when CEP135 foci exhibited more than three dots and less than four dots in Flag-PACT- or Flag-SAS6-PACT-expressing cells: their intense Flag signals colocalized with CEP135 foci. Experiments were repeated three times, n>180 for each time point within each experiment. Values are means and standard errors.

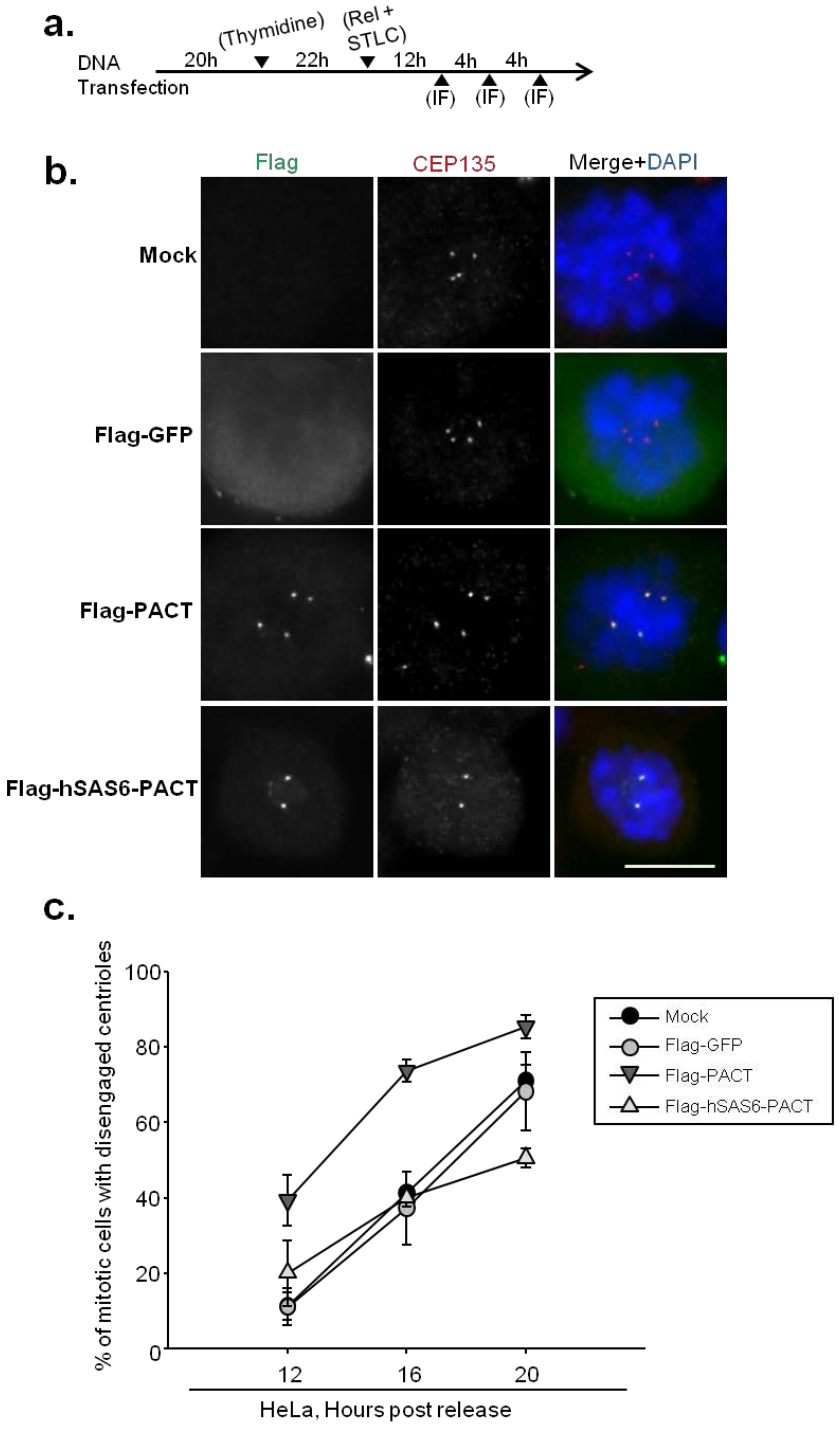

a.
DNA
Transfection
20h
(Thymidine)
22h
(Rel +
STLC)
12h
4h
4h
(IF)
(IF)
(IF)
b.
Flag
CEP135
Merge+DAPI
Mock
Flag-GFP
Flag-PACT
Flag-hSAS6-PACT
c.
% of mitotic cells with disengaged centrioles
100
80
60
40
20
0
12
16
20
HeLa, Hours post release
Mock
Flag-GFP
Flag-PACT
Flag-hSAS6-PACT

Fig. 13. Microtubule patterns in cells treated with nocodazole and taxol.

RPE1 cells were arrested at S phase with a thymidine block and released in the presence of different doses of nocodazole (50ng/ml or 200ng/ml) or taxol (0.156μM or 5μM). Twenty-four hours later, the cells were coimmunostained with antibodies against centrin (green) and α-tubulin (red). DNA is stained with DAPI (blue). Scale bar, 10μm.

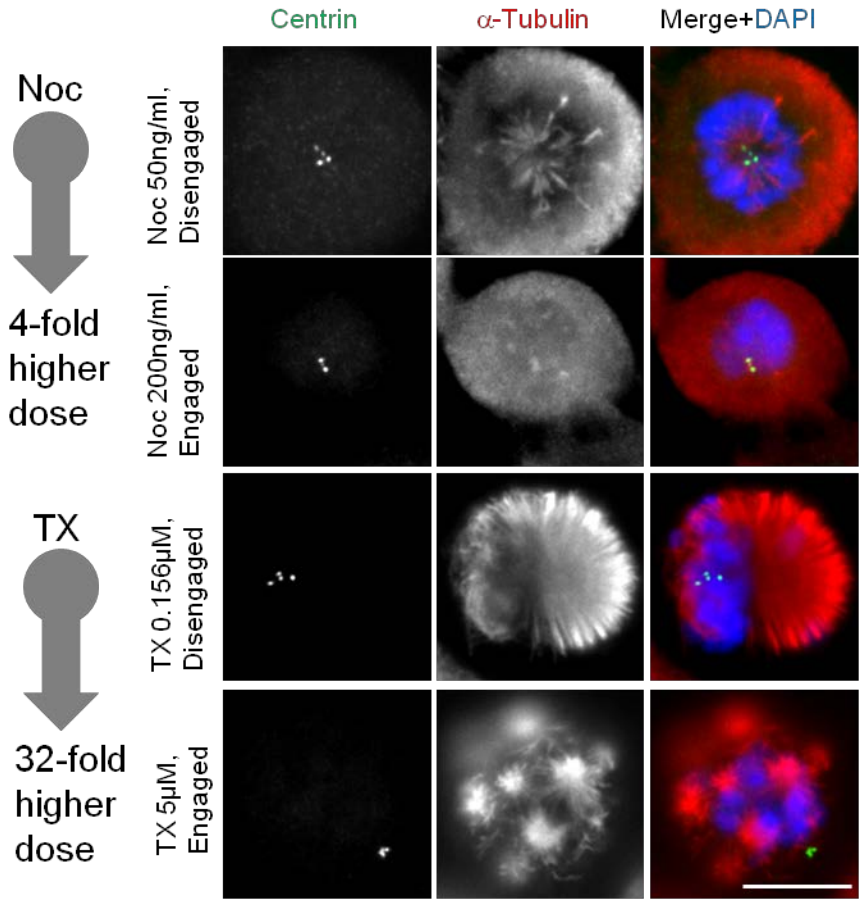


Fig. 14. Absence of SAS6 dot signal at engaged centrioles in high dose of nocodazole or taxol treated cells. (a) RPE1 cells were treated with nocodazole (200ng/ml), taxol (5μM) and/or along with BI2536 (200nM). Eighteen hours later, the cells were coimmunostained with antibodies specific to SAS6 (green) and CEP135 (red). DNA is stained with DAPI (blue). Scale bar, 10μm. (b) The number of mitotic cells with centriolar SAS6 dot signals was counted. Experiments were repeated twice with more than 120 cells per experimental groups. Values are means and standard errors.

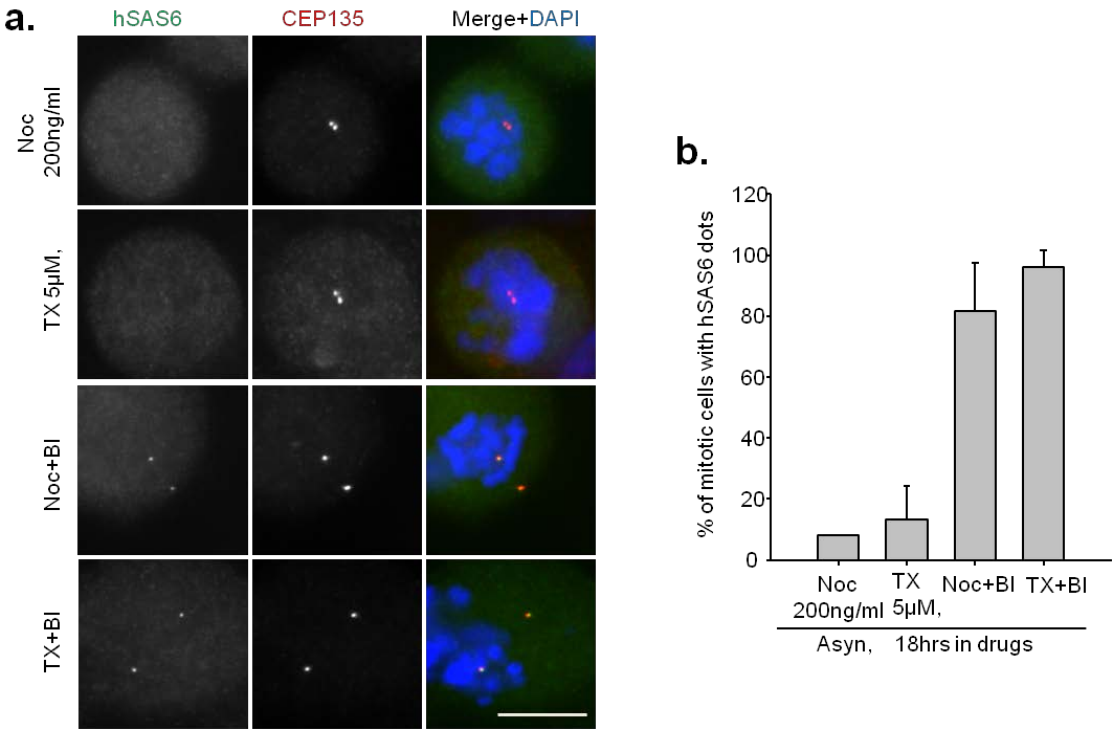
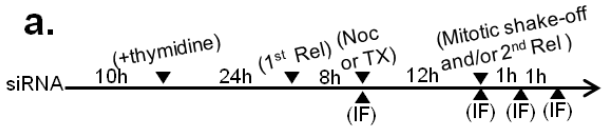
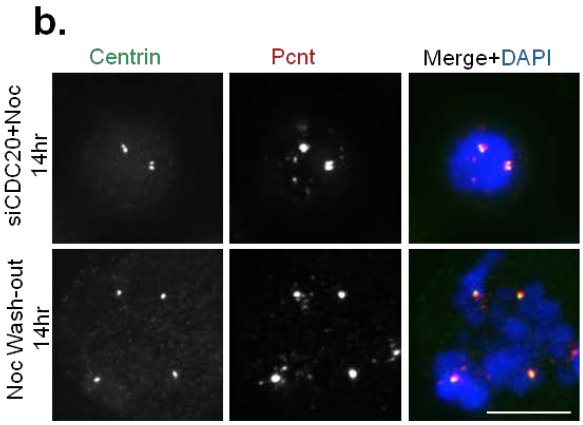


Fig. 15. Effects of nocodazole concentration on premature centriole disengagement in M-phase-arrested cells. (a) CDC20-depleted RPE1 cells were arrested at S phase with a thymidine block and then released. Eight hours later, the cells were treated with nocodazole (200ng/ml) for 12 h, shaken off for mitotic cell collection, washed with PBS well, seeded to a poly-L-lysine-coated coverslip, and then cultured further to indicated time points. (b) The cells at 14 hours after nocodazole treatment were coimmunostained with the centrin (green) and PCNT (red) antibodies. DNA is stained with DAPI (blue). Scale bar, 10μm. (c) At indicated time points, the number of mitotic cells with disengaged centrioles were counted using centrin and CEP135 dots. Experiments were repeated twice, n>100 for each time point within each experiment. Values are means and standard errors.


a.
siRNA
10h
(+thymidine)
24h
(1st Rel)
8h
(Noc or TX)
12h
(Mitotic shake-off and/or 2nd Rel)
1h
1h
(IF)
(IF)
(IF)
(IF)


b.
Centrin
Pcnt
Merge+DAPI
siCDC20+Noc 14hr
Noc Wash-out 14hr

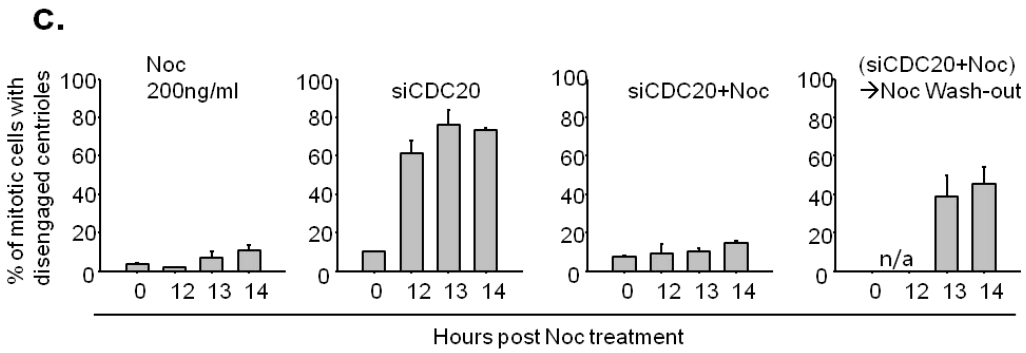

c.
% of mitotic cells with disengaged centrioles
Noc 200ng/ml
siCDC20
siCDC20+Noc
(siCDC20+Noc) →Noc Wash-out
n/a
0
12
13
14
Hours post Noc treatment

Fig. 16. Effects of taxol concentration on premature centriole disengagement in M-phase-arrested cells. (a) RPE1 cells were processed as same manner in Fig. 15a, only except using taxol (5μM). The cells at 14 hours after taxol treatment were coimmunostained with the centrin (green) and CEP135 (red) antibodies. DNA is stained with DAPI (blue). Scale bar, 10μm. (b) The cells at 14 hours after taxol treatment were coimmunostained with the centrin (green) and α-tubulin (red) antibodies. DNA is stained with DAPI (blue). Scale bar: 10μm (c) The number of mitotic cells with disengaged centrioles were counted. Experiments were repeated twice, n>100 for each time point within each experiment. Values are means and standard errors.

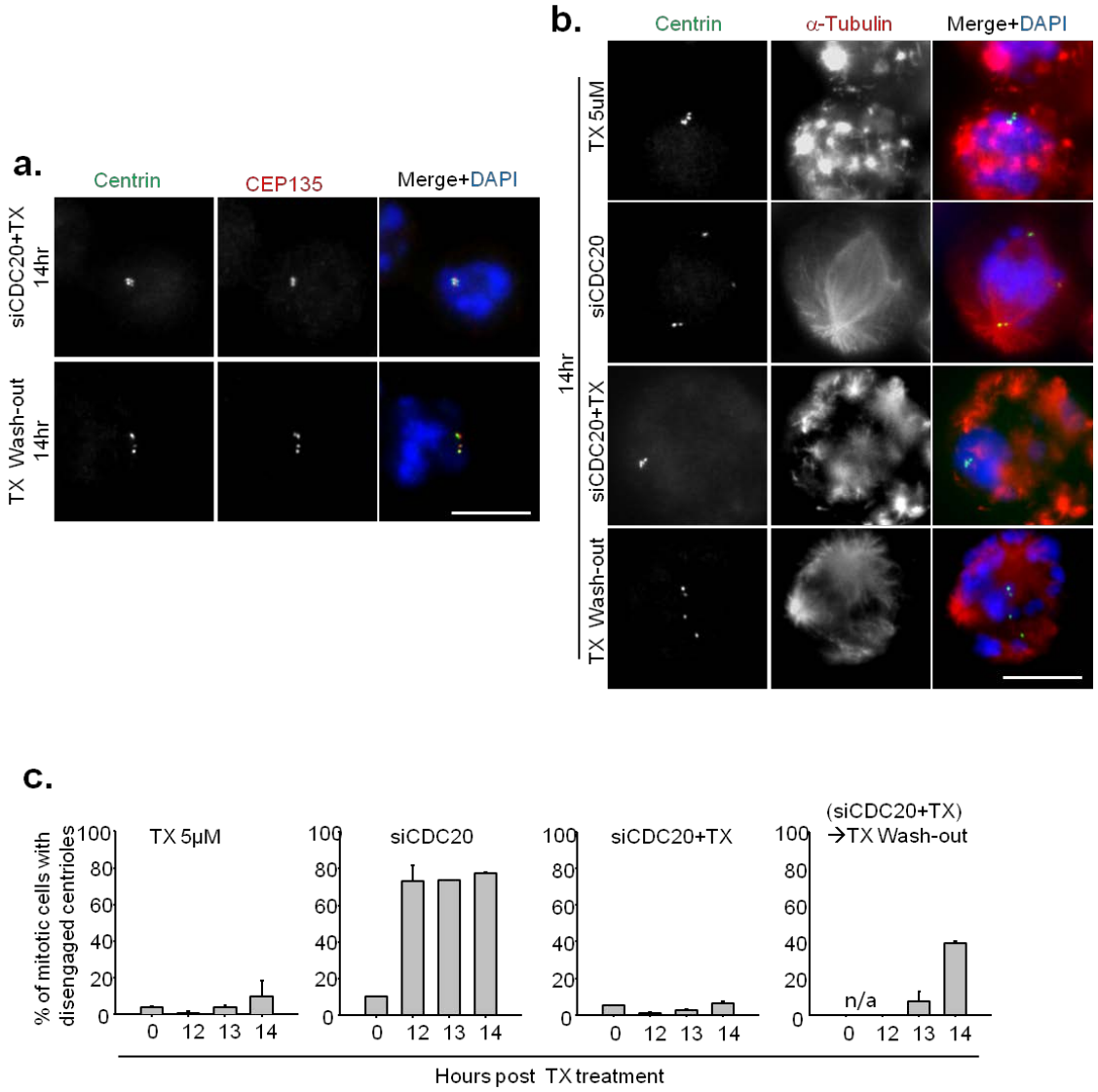
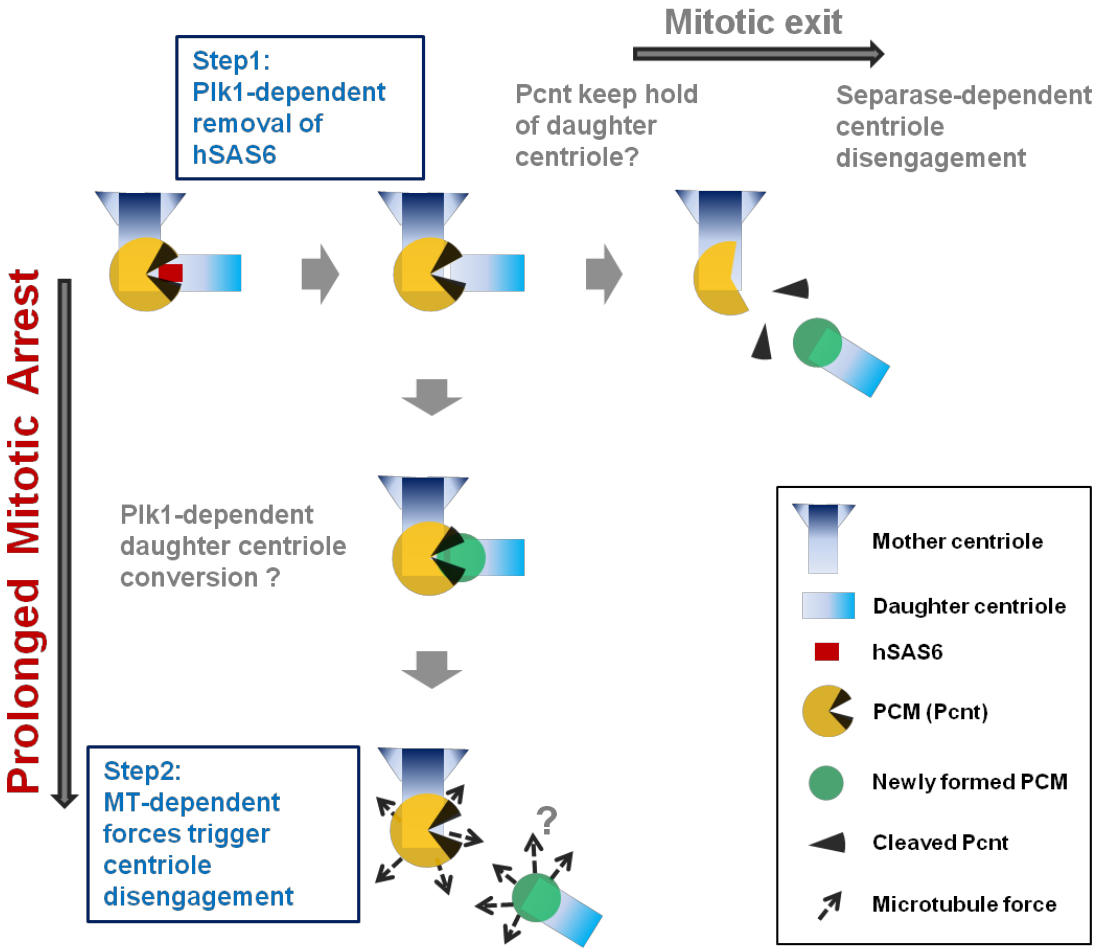

a.
Centrin
CEP135
Merge+DAPI
siCDC20+TX 14hr
TX Wash-out 14hr
b.
Centrin
α-Tubulin
Merge+DAPI
14hr
TX 5uM
siCDC20
siCDC20+TX
TX Wash-out
c.
% of mitotic cells with disengaged centrioles
TX 5μM
siCDC20
siCDC20+TX
(siCDC20+TX) →TX Wash-out
n/a
0 12 13 14
Hours post TX treatment

Fig. 17. Model for premature centriole disengagement during prolonged mitotic arrest. During prolonged mitotic arrest, SAS6 disappears from centriole pairs in Plk1-dependent manner and subsequently microtubule-mediated forces trigger premature centriole disengagement. This event is independent of the Separase activity.



DISCUSSION

Here I report that prolonged mitotic arrest triggers premature centriole disengagement, which is independent of the pathway that leads mitotic arrest. Downregulation of Separase activity does not perturb premature centriole disengagement in M-phase-arrested cells. Parallel to these data, Separase specific substrates such as Scc1 and PCNT, are not cleaved during M phase arrest. However, Plk1 inactivation by BI treatment effectively blocks premature centriole disengagement. I also observed that prolonged mitotic arrest induce loss of SAS6 from centriole pairs in a Plk1-dependent manner. BI inhibits the SAS6 displacement as well as premature centriole disengagement in M-phase-arrested cells. Importantly, the expression of SAS6-PACT resulted in reduction of premature centriole disengagement during prolonged mitotic arrest. Finally, I report that microtubule-dependent forces seem to be associated with premature centriole disengagement in M-phase-arrested cells.

It remains to be determined how Plk1 regulates SAS6 localization as well as whether SAS6 is a direct substrate of the Plk1 during mitosis. It would be interesting to test whether expressing SAS6 displacement mutant can block centriole disengagement. In contrast to SAS6, dSAS6 actually does not disappear from centrioles during cell cycle (Mennella *et al*., 2012). However, dSAS6 mutant flies displayed disengaged centrioles (Rodrigues-Martins *et al*., 2007). Further studies in this area will reveal the fundamental role of SAS6 and dSAS6 in centriole engagement. Nevertheless, possibility that the mechanism responsible for centriole disengagement might have changed evolutionally between varying species of vertebrates and invertebrates still remains to be investigated.

It is unclear how PACT (PCNT C-terminal domain) expression synergistically induced premature centriole disengagement in M-phase-arrested cells, but it appears that the ectopic PACT expression impaired the role of PCNT played in centriole engagement. Using high

resolution fluorescence microscopy, recent papers observed the formation of gap 150-200nm in length around PCM coinciding with the position of daughter centrioles (Mennella *et al*., 2012; Lawo *et al*., 2012; Lüders J., 2012). According to their structural model of human centrosome, it seems that horseshoe shaped PCM around the centriole pairs are tethered to the proximal end of daughter centrioles: especially N terminal of PCNT could be the fundamental domain for this (Fig. 16). It would be interesting to explore how either C-terminal domain of PCNT, PACT, or N-terminal domain is involved in centriole engagement.

When cells treated with high dose of microtubule inhibitors, it is possible that physically disengaged centrioles only appear engaged because they are closely clustered: i.e. exhibiting single CEP135 foci. However, I don't believe that is true. Important fact is that disengaged centrioles in STLC-treated cells did not return to appear as a single CEP135 dot when high dose of nocodazole or taxol was sequentially treated after premature disengagement (data not shown).

Currently, I do not have all the information to understand how microtubule-mediated force contributes to premature centriole disengagement in M-phase arrested-cells at molecular level. However, I occasionally observed that each disengaged daughter centriole has its own PCM, indicating the centriole to MTOC conversion occurred during mitotic arrest. It has been proposed that the conversion requires sequential steps composed of primary Plk1-dependent modification during mitosis and the passage through mitotic exit (Wang *et al*., 2011). Furthermore, a recent study showed that ectopically expressed Plk1 can lead to centriole reduplication, and this seems to be related to premature conversion (Loncarek *et al*., 2010; Wang *et al*., 2011). Based on these collective data, I think daughter centrioles that are exposed to Plk1 during mitotic arrest may acquire the ability to nucleate its own PCM (conversion) and, thus, this is probably able to form microtubule-dependent pushing force to its engaged mother centriole, resulting in subsequent premature disengagement (Fig. 16). In future, it would be of

great interest to investigate the relationship between conversion and Separase-independent premature centriole disengagement.

I believe that premature centriole disengagement promoting system described in this study has several benefits compared to centriole reduplication during interphase arrest (Loncarek *et al*., 2010) in the aspect of studying mechanism for the centriole disengagement. First, the disengagement of this mitotic arrest system does not require Separase-activity in contrast to that of interphase arrest cells (Prosser *et al*., 2012). Second, it takes relatively shorter time to trigger disengagement than interphase arrest. Last but not least, further progress in this field will explore the question of whether cancer cells with abnormal centrosome number exert such Separase-independent centriole disengagement mechanism.

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국문초록

중심립은 세포 주기에 맞추어 복제되고 나눠진다. 염색체 복제 기간 동안 새로운 중심립이 기존에 존재하던 중심립으로부터 단 하나가 수직 형태로 돋아 나온다. 이는 단단히 고정된 형태로 분열기가 끝날 때까지 직교형태를 유지하는 데 이를 engagement라 부르고, 분열기에서 간기로 빠져나갈 때 이 둘이 떨어지는 것을 disengagement라 부른다. 그리고 이 떨어짐으로 인해 기존에 존재하던 중심립은 다시 새로운 중심립을 복제할 수 있다. 본 논문에서 저자는 이 중심립 분리 현상이 분열기에서 간기로 빠져나가는 시점이 아닌, 분열기 정체 동안에도 이것이 일어날 수 있음을 관찰하였다. 이 현상은 Separase를 직접 또는 CDC20 단백질을 감소시켜 Separase activity를 억제시킨 분열기 정체 세포에서도 일어났다. 그러나 Plk1 인산화효소 특이적 저해제 BI2536이 처리된 세포의 중심립은 계속 중심립 쌍의 형태를 유지함으로써, Plk1이 이 현상에 중요함을 알 수 있었다. 저자는 분열기 정체 동안 연결고리로 의심되는 SAS6 단백질이 중심립 쌍으로부터 점차 사라지고, 이는 Plk1에 의해 조절됨을 확인함으로써 SAS6의 위치 조절과 특발성 중심립 분리 현상이 상관관계가 있음을 보였다. 또한 SAS6-PACT를 발현하여 분열기 정체 동안의 중심립 분리 현상이 감소하는 것도 확인했다. 끝으로 이 현상은 높은 농도의 미세소관 억제제, 즉 Nocodazole 또는 Taxol이 처리되었을 때는 억제되었다. 그러나 이것은 CDC20이 억제된 세포에서 높은 농도의 두 drugs이 제거되었을 때 빠르게 회복되었다. 따라서, 본 연구는 분열기 정체동안 일어나는 중심립 분리 현상이 두 가지 단계에 의해 조절됨을 제안한다. 1) Plk1의 도움으로 SAS6 단백질이 중심립

쌍으로부터 사라진다. 2) 세포질 내 미세소관의 힘이 중심립 쌍을 떨어뜨리는 데 작용한다.

주요어: 분열기 정체, 중심립 분리현상, SAS6
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모였다가 때가 되어 흩어지는 일.
삼라만상에 깃든 물리법칙이 비단 사람의 생이라고 비켜가지 않음을 새삼 곱씹는다. 한 때 장 씨와 이 씨, 둘이서 부산에 신혼 집을 차렸다가 세월을 두르는 사이 단란한 다섯 식구가 되었다. 그러나 또 한 번 세월의 고개를 넘고보니 다시 1979 년 그때 신혼집처럼 두 사람만 그곳에 남았다. 안방 이부자리에 늙음이라는 섭리를 대신 품으시고는.
그 부부가 자랑스러워 하는 막내 아들 하나 있다. 여기에 그 녀석 이야기 잠깐 하고자 한다. 얘는 딴 건 죄다 젬뱅인데 연필 쥐고 책상에 앉아있는 건 곧잘 해 사실 아직도 그걸 하고 있다. 채 서른 해도 겪지 않은 고놈의 짧은 생도 세상의 그것이라, 자리를 풀었다 어떤 연유로 다시 자리틀고 일어나는 일이 이 참에 있었다고 한다. 석사학위 이 년 동안 이백칠호 같은 공간에서 서로의 눈을 마주보고 혹은 목소리를 나눠 들으며 교감했던 그것들이 소중했고, 감사했음을 전하고 싶었음에 글공간을 빌려 달라고 내게 부탁했다. 직접 말하는 건 쑥스러워하는 꼰대 같은 놈이라 이해해달라며.
선생님과 선배 후배 동기들이 있었는데...어떤 날은 이들 중 누군가로부터 삶을 대하는 자세를 경험했다. 학문에 빛을 비추는 일이 어떤 의미가 있는지를 설명해준 이도 있었으며, 과학의 논리와 실험방법, 실험자체에 대한 논의, 실험재료 등을 함께 공유해 준 이들도 있었단다. 그 아이의 학위논문을 특별히 신경 써준 이도 있었다. 하지만 무엇보다, 바다 위 작은 파도들과 같은 일상의 단조로움과 빈번함에서 그냥 곁에 있다는 이유만으로 가까이 와 한 마디씩 말을 건네줬던, 혹은 과자를 건네며 작음 관심을 줬던 아무날의 아무개들 모두에게 고마워해야 함이 마땅하겠다고. 끝으로 종종 행동이나 말투가 거만하고, 자기 잘난 맛에

거들먹거리며 참견을 일삼고, 늘 한 발 물러나 뻣뻣하게 있던 자신의 겸손하지 못했던 점들을 이 글을 빌려 심심한 사과를 하고 싶어했다.

Sequence of scrambled si*Komaunsaramdle*

5’-가나다라아메더워박빈이영자장창한류용법수항학혁지거조임거남우정수준규선장이준호성경미희최명진은유문건해윤성보란원조고차우김훈좋임님활장현최미옥원분은선김훈기야백연이랑다화경혁박손교재연신환조선관웅현원종를경종돈울히신매란진조이용민후을재원인강쏘엽최이니사이김정숙할보효께존정철을팀영우스박천승김지시태맙헌고진병우카해싶박선승유정가만김규연화조그한럴닉레열최미중정령양성배습재원모강구신장아대관리턴균덕경현신행익션비캐창현야봉스님경장미렬호해차타파하-3’

형광펜 + 동그라미 몇 번= (_____)