

Centrosomal Aki1 and cohesin function in separase-regulated centriole disengagement

Akito Nakamura,^{1,2} Hiroyuki Arai,² and Naoya Fujita¹

¹Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo 135-8550, Japan

²Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan

Sister chromatid separation at anaphase is triggered by cleavage of the cohesin subunit Scc1, which is mediated by separase. Centriole disengagement also requires separase. This dual role of separase permits concurrent control of these events for accurate metaphase to anaphase transition. Although the molecular mechanism underlying sister chromatid cohesion has been clarified, that of centriole cohesion is poorly understood. In this study, we show that Akt kinase-interacting protein 1 (Aki1) localizes to centrosomes and regulates centriole cohesion. Aki1 depletion causes formation of multipolar

spindles accompanied by centriole splitting, which is separase dependent. We also show that cohesin subunits localize to centrosomes and that centrosomal Scc1 is cleaved by separase coincidentally with chromatin Scc1, suggesting a role of Scc1 as a connector of centrioles as well as sister chromatids. Interestingly, Scc1 depletion strongly induces centriole splitting. Furthermore, Aki1 interacts with cohesin in centrosomes, and this interaction is required for centriole cohesion. We demonstrate that centrosome-associated Aki1 and cohesin play pivotal roles in preventing premature cleavage in centriole cohesion.

Introduction

The centrosome is the major microtubule-organizing center in most mammalian cells. It is composed of two centrioles and pericentriolar material (Bornens, 2002). A cell in the G1 phase of the cell cycle contains one centrosome. The centrosome is duplicated during the S phase. During duplication, new centrioles grow perpendicular to preexisting ones and connect to each other (centriole engagement). The duplicated centrosomes are separated and function as spindle poles in mitosis. The two centrioles are separated (centriole disengagement) during exit from mitosis, and this disengagement is required for centriole duplication in the next cell cycle. In this manner, the centrosome cycle is tightly regulated and coordinated with the cell cycle (Kuriyama and Borisy, 1981).

Separase, a well-known cysteine protease dissociating the cohesion between sister chromatids by cleaving Scc1 (a subunit of cohesin), has been recently found to be essential for centriole disengagement (Uhlmann et al., 1999; Waizenegger et al., 2000; Tsou and Stearns, 2006; Thein et al., 2007). Although these studies shed light on the mechanism of centriole disengagement, it remains to be seen which hypothetical proteins would connect

older with younger centrioles. In this context, it is interesting to consider that Scc1 might also function as a connector of a pair of centrioles that are cleaved by separase at anaphase onset.

We have previously shown that Akt kinase-interacting protein 1 (Aki1) functions as a scaffold protein to activate the phosphatidylinositol-3-OH kinase/3-phosphoinositide-dependent protein kinase 1–Akt pathway in EGF signaling (Nakamura et al., 2008). Furthermore, Aki1 has been shown to be five prime repressor elements under dual repression–binding protein-1, functioning as a transcriptional repressor of the serotonin-1A receptor gene (Ou et al., 2003). These studies suggest that Aki1 plays a distinct role depending on its localization. In this study, we focus on the role of Aki1 in the centrosome.

Results and discussion

Aki1 localizes to centrosomes

Aki1 was previously identified as a cytosolic and nuclear protein (Ou et al., 2003; Nakamura et al., 2008); however, its endogenous localization has not been precisely clarified. Immunofluorescence

Correspondence to Naoya Fujita: naoya.fujita@jcr.or.jp

Abbreviations used in this paper: Aki1, Akt kinase-interacting protein 1; CENP-E, centromere protein E; NuMA, nuclear mitotic apparatus; rAki1, RNAi refractory Aki1; SMC, structural maintenance of chromosomes; WT, wild type.

© 2009 Nakamura et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date [see <http://www.jcb.org/misc/terms.shtml>]. After six months it is available under a Creative Commons License [Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at <http://creativecommons.org/licenses/by-nc-sa/3.0/>].

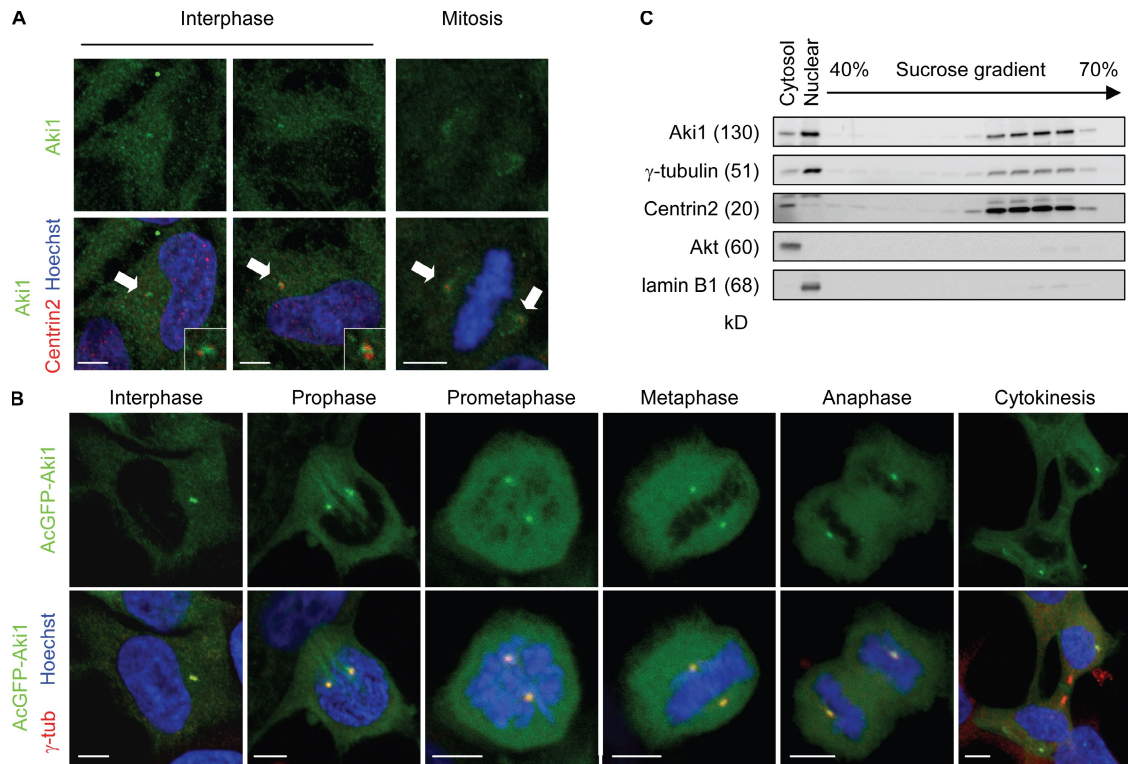


Figure 1. **Aki1 localizes to centrosomes.** (A) HeLa cells were costained for Aki1 (green) and centrin2 (red). Interphase or mitotic cells are shown. Arrows indicate the position of centrosomes, and insets show high magnification images of centrosomes. (B) HeLa cells stably expressing AcGFP-Aki1 (green) were counterstained for γ -tubulin (γ -tub; red). (C) Immunoblots were performed on cytoplasmic, nuclear, and sucrose gradient fractions (centrosome preparations) from HeLa cells with the indicated antibodies. Bars, 5 μ m.

analysis showed that Aki1 slightly localized in the vicinity of centrosomes (centrin2; a marker for individual centrioles) in interphase and mitosis (Fig. 1 A). To confirm this result, we established HeLa cells stably expressing AcGFP-Aki1. Like endogenous Aki1, AcGFP-Aki1 showed a centrosomal localization throughout the cell cycle (Fig. 1 B). To gain further evidence of the association of Aki1 with centrosomes, we purified centrosomes by sucrose gradient ultracentrifugation. Aki1 was detected in the fractions containing centrin2 and γ -tubulin, a centrosomal marker (Fig. 1 C). In contrast, Akt did not cofractionate with Aki1 in the centrosomal fractions (Fig. 1 C), suggesting that Aki1 does not function as a scaffold protein for Akt and PDK1 (Akt kinase) but plays a distinct role in centrosomes.

Absence of Aki1 results in formation of multipolar spindles

Considering its localization, we investigated whether Aki1 regulates the function of centrosomes. Aki1 depletion caused formation of multipolar spindles during mitosis, which had three or four γ -tubulin foci, and microtubules emanated from every focus without affecting expression of other proteins such as α -tubulin, γ -tubulin, and centrin2 (Fig. 2, A–C). Like γ -tubulin, pericentrin (a centrosome protein) localized to all spindle poles (Fig. 2 D). In quantitative terms, \sim 35% of mitotic cells had multipolar spindles in Aki1-depleted cells (Fig. 2 E). Furthermore, Aki1-depleted cells had only one or two γ -tubulin foci during interphase, and an increased number of centrosomes were observed only in mitosis (Fig. 2 F).

To exclude the possibility that Aki1 depletion affects spindle pole–focusing machinery, we examined the localization of nuclear mitotic apparatus (NuMA) and TPX2, which are spindle pole proteins (Merdes et al., 2000; Garrett et al., 2002). In Aki1-depleted cells, NuMA or TPX2 were present in the vicinity of every spindle pole as in control cells (Fig. S1, A and B).

Centrosome-associated Aki1 is required for formation of bipolar spindles

To clarify the region of Aki1 required for its centrosome localization, we established HeLa cell lines stably expressing deletion mutants of Aki1. We found that C-terminal–deleted Aki1, Δ C815-Aki1, did not localize to the centrosome (Fig. 2 G). Note that formation of multipolar spindles caused by Aki1 depletion was efficiently rescued by wild-type (WT) RNAi refractory Aki1 (rAki1) expression but not by Δ C815-rAki1 (Fig. 2 H). We also confirmed that Δ C815-Aki1 still retained previously defined functions such as a scaffold protein for PDK1 and Akt and a transcriptional repressor (unpublished data). We conclude that centrosomal localization of Aki1 is dependent on its C-terminal region and that centrosomal Aki1 is necessary for formation of bipolar spindles.

Aki1-depleted cells are arrested at the spindle checkpoint

We also found that Aki1 depletion caused an increase in the mitotic index and histone H3 phosphorylation (Fig. 3, A and B). To determine whether the spindle checkpoint is activated in

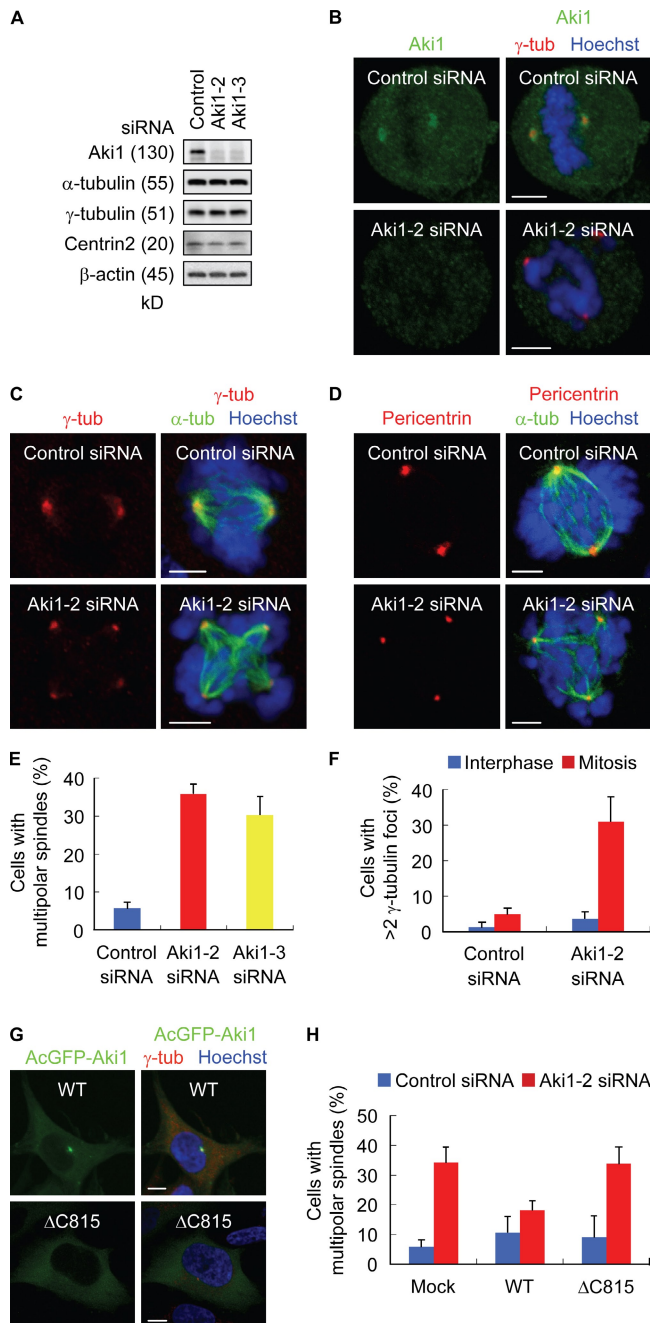


Figure 2. **Centrosomal Aki1 is essential for formation of bipolar spindles.** (A) HeLa cells were transfected with control, Aki1-2, or Aki1-3 siRNA. Cell lysates were immunoblotted with the indicated antibodies. (B–D) HeLa cells were transfected with control or Aki1-2 siRNA and costained for Aki1 (green) and γ -tubulin (γ -tub; red), or for α -tubulin (α -tub; green) and γ -tubulin or pericentrin (red; C and D). (E) Percentage of multipolar cells of mitotic cells in control, Aki1-2, or Aki1-3 siRNA-treated cells. (F) Percentage of multipolar cells in control or Aki1-2 siRNA-treated cells in interphase or mitosis. (G) HeLa cells stably expressing AcGFP-WT or Δ C815-Aki1 (green) were counterstained for γ -tubulin (red). (H) Percentage of multipolar cells of mitotic cells in control or Aki1-2 siRNA-treated cells expressing AcGFP-mock, AcGFP-WT-rAki1, or AcGFP- Δ C815-rAki1. Error bars represent SD of triplicate experiments (150–200 total cells were scored per condition). Bars, 5 μ m.

Aki1-depleted cells, we examined localization of the spindle checkpoint proteins BubR1 and centromere protein E (CENP-E; Abrieu et al., 2000; Chen, 2002). Immunofluorescence analysis

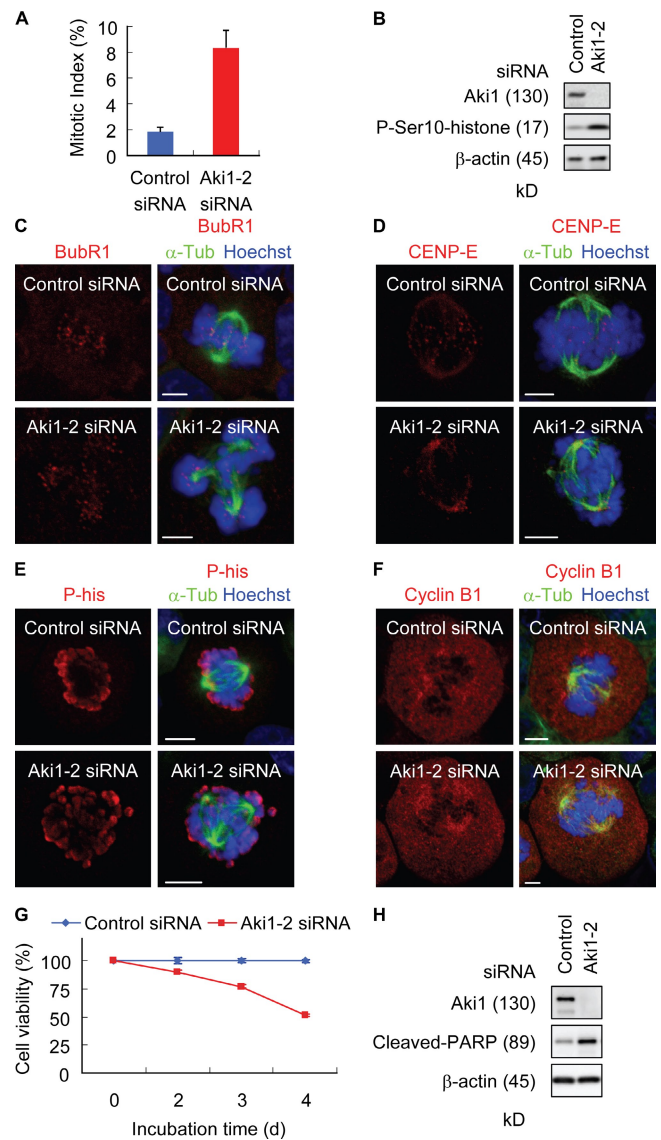


Figure 3. **Depletion of Aki1 induces spindle checkpoint arrest.** (A) Percentage of mitotic cells in control or Aki1-2 siRNA-treated cells. Error bars represent SD of triplicate experiments (2,000–2,500 total cells were scored per condition). (B) HeLa cells were transfected with control or Aki1-2 siRNA. Cell lysates were immunoblotted with the indicated antibodies. (C–F) HeLa cells were transfected with control or Aki1-2 siRNA and costained for α -tubulin (α -tub; green) and BubR1, CENPE, phospho-Ser¹⁰-histone (P-his), or cyclin B1 (red). (G) HeLa cells were transfected with control or Aki1-2 siRNA. Cell viability was measured using the cell viability assay. (H) HeLa cells were transfected with control or Aki1-2 siRNA. Cell lysates were immunoblotted with the indicated antibodies. Error bars represent SD of triplicate experiments. Bars, 5 μ m.

showed that Aki1-depleted cells displayed BubR1 and CENP-E staining intensity at misaligned chromosomes, indicating activation of the spindle checkpoint (Fig. 3, C and D). Because histone H3 is dephosphorylated and cyclin B1 is degraded at anaphase onset (Gurley et al., 1978; Rieder and Maiato, 2004), they can be useful markers for preanaphase arrest. Aki1-depleted cells were strongly stained for phospho-Ser¹⁰-histone H3 and cyclin B1 as control cells at metaphase (Fig. 3, E and F). These results indicate that Aki1 depletion causes activation of the spindle checkpoint and preanaphase mitotic arrest.

To understand the fate of Aki1-depleted cells, we established HeLa cells stably expressing AcGFP–histone H2B. Most Aki1-depleted cells with multipolar spindles (33/35) remained arrested for a long time (4–22 h) and finally underwent apoptotic death (Video 1). In support of this result, cells undergoing prolonged mitotic arrest may eventually undergo caspase-dependent apoptosis (Varetti and Musacchio, 2008). In accordance with this idea, Aki1 depletion resulted in reduction in the number of viable cells and increased the amount of cleaved poly(ADP-ribose) polymerase fragment, which is generated by caspase-mediated cleavage (Fig. 3, G and H).

Aki1 depletion causes centriole splitting

Formation of multipolar spindles harboring extracentrosomal foci could result from various types of mechanism (Keryer et al., 1984; Fukasawa, 2007). Determining the number of centrioles at each spindle pole is useful in understanding the mechanism by which multipolar spindles are formed. Immunofluorescence analysis showed that Aki1 depletion caused centriole splitting (Fig. 4 A). Although ~80% of spindle poles contained a pair of centrioles in control cells, ~60% of spindle poles contained only one centriole in Aki1-depleted cells (Fig. 4 B). Aki1 depletion also led to a modest increase in poles with no centrioles (Fig. 4 B). Presumably, spindle poles with no centrioles may generate contingently when centrioles are split. Consistent with our result, Thein et al. (2007) previously reported that astrin depletion led to centriole splitting with an increase in poles containing no centrioles.

Centriole splitting was recently shown to occur as a result of premature separase activation (Thein et al., 2007). Interestingly, separase depletion significantly suppressed formation of multipolar spindles induced by Aki1 depletion (Fig. 4, C and D). We investigated whether separase was prematurely activated by Aki1 depletion. It was reported that active separase undergoes self-cleavage, resulting in C-terminal fragment production (Waizenegger et al., 2002). Indeed, the C-terminal fragment was detected in cells released from mitotic arrest in which separase was activated (Fig. 4 E). We found that Aki1 depletion did not promote fragment production (Fig. 4 E). We also noted that Aki1 in mitotic cells had reduced mobility in immunoblot analysis (Fig. 4 E). Phosphatase treatment suggested that the shift is attributable to mitotic phosphorylation of Aki1 (unpublished data), but the role of this phosphorylation remains unclear. Collectively, centriole splitting in Aki1-depleted cells depends on separase, whereas premature separase activation is not induced.

Sccl is essential for centriole cohesion

Cohesin is a chromosome-associated multisubunit protein complex consisting of structural maintenance of chromosomes protein 1 (SMC1), SMC3, Scc1, and either SA1 or SA2 (Losada et al., 1998; Waizenegger et al., 2000). Intriguingly, cohesin is also associated with the centrosome (Guan et al., 2008; Kong et al., 2009). Considering that separase regulates both sister chromatid separation and centriole disengagement, an analogous separase–cohesin system may be involved in centriole cohesion.

To verify the centrosomal localization of cohesin, we purified centrosomes by sucrose gradient ultracentrifugation.

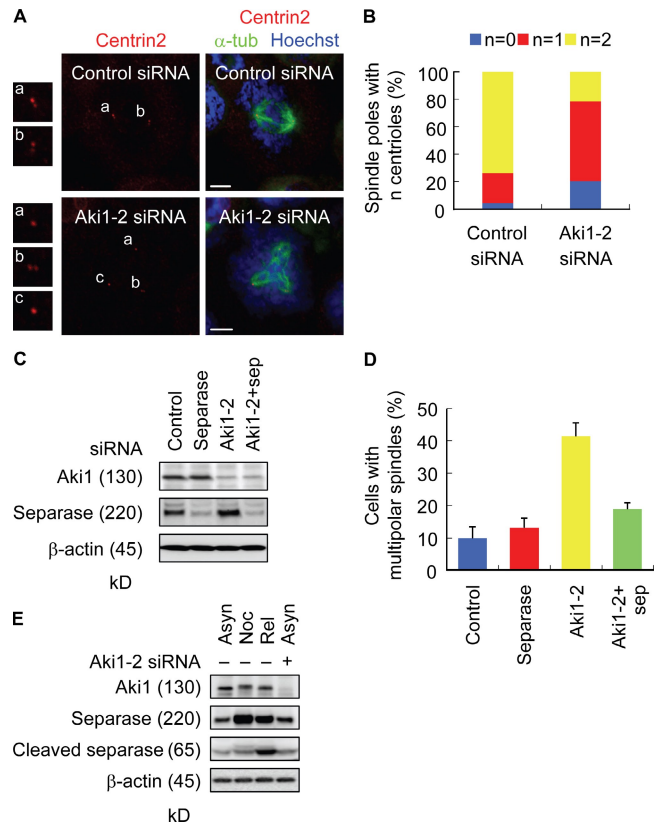


Figure 4. Formation of multipolar spindles induced by Aki1 depletion is dependent on separase. (A) HeLa cells were transfected with control or Aki1-2 siRNA and costained for α -tubulin (α -tub; green) and centrin2 (red). Centrosomes are shown enlarged on the left. (B) Quantitative analysis of centriole number at each spindle pole in control or Aki1-2 siRNA-treated cells (mean of three experiments; 100 total cells were scored per condition). (C) HeLa cells were transfected with the indicated siRNA. Cell lysates were immunoblotted with the indicated antibodies. (D) Percentage of multipolar cells of mitotic cells in indicated siRNA-treated cells. Error bars represent SD of triplicate experiments (150–200 total cells were scored per condition). (E) HeLa cells were transfected with control or Aki1-2 siRNA and harvested asynchronously (Asyn) or 0 or 2 h after release (Rel) from nocodazole (Noc) arrest. Cell lysates were immunoblotted with the indicated antibodies. Bars, 5 μ m.

Cytosolic MAPK/extracellular signal-regulated kinase kinase and nuclear envelope protein lamin B1 were not detected in centrosomal fractions, confirming that there were little or no cytosolic and nuclear contaminations. Under the condition, all cohesin subunits were detected in centrosomal fractions, especially SMC1 and SMC3 (Fig. 5 A). Furthermore, immunofluorescence analysis showed that SMC1 localized to centrosomes in interphase and mitosis (Fig. S2 A) as previously reported (Guan et al., 2008; Kong et al., 2009). Next, we examined whether centrosomal Scc1 is cleaved by separase. The C-terminal cleaved product of Scc1 (~95 kD) was detected in chromatin fraction but not in supernatant fraction in cells released from mitotic arrest (Fig. S3; Waizenegger et al., 2000). We detected the cleaved fragment of Scc1 and the self-cleavage product of separase in centrosomal fraction in cells released from mitotic arrest (Fig. 5 B, fractions 9–12), suggesting that Scc1 is cleaved not only in the chromatin but also in the centrosome at anaphase onset. Furthermore, we investigated whether cohesin mediates centriole cohesion. Losada et al. (2005) showed that depletion

of Scc1 causes aberrant sister chromatid cohesion and formation of multipolar spindles. We also observed the extensive formation of multipolar spindles and spindle checkpoint arrest in Scc1-depleted cells and found that the number of spindle poles was mostly three or four, which is the same number as Aki1-depleted cells (Fig. S2, B–G). Importantly, we demonstrated that Scc1 depletion induced centriole splitting (Fig. 5 C). Meanwhile, metaphase chromosome spreads revealed that Scc1 depletion brought about loss of sister chromatid cohesion as reported previously (Losada et al., 2005); however, Aki1 depletion did not affect the assembly of metaphase chromosomes (Fig. S2 H). To show whether the disengagement phenotype is directly induced by depletion of Aki1 or Scc1 or indirectly caused by mitotic arrest, we established HeLa cells stably expressing AcGFP- γ -tubulin. Live cell imaging analysis revealed that multi- γ -tubulin foci occurred early in mitosis in Aki1- and Scc1-depleted cells (Videos 2 and 3), indicating that the Aki1 or Scc1 depletion itself triggers centriole splitting.

Aki1 contributes to mitotic centrosomal localization of Scc1

We also noted that the peak of cohesin expression in sucrose gradient ultracentrifugation coincided with that of Aki1 expression but not with that of NuMA or pericentrin expression (Fig. 5 A). From this result, we assumed that Aki1 associates with cohesin in the centrosome. Centrosomal extracts purified from G1/S-arrested cells, mitotically arrested cells, or mitotically arrested and released cells were used to test this hypothesis. We initially observed that Scc1 and SA-2 (but not SMC proteins) levels in centrosomal extracts were significantly increased in mitotic cells, although their levels in nuclear/chromatin extracts do not seem to be affected by the cell cycle phase (Fig. 5 D). Intriguingly, immunoprecipitation analysis using centrosomal extracts revealed that Scc1 and SA-2 were coprecipitated with Aki1 especially in mitotic cells, whereas SMC proteins relatively associated with Aki1 irrespective of the cell cycle phase (Fig. 5 D). Consistent with the disagreement of the peak shown in Fig. 5 A, Aki1 did not associate with NuMA or pericentrin (Fig. 5 D). Furthermore, cytoplasmic protein Akt and nuclear protein lamin B1 were not coprecipitated with Aki1, indicating that we do not just pull down intact centrosomes or cellular fragments where Aki1 is localized.

We next tried to identify the regions in Aki1 that are critical for complex formation with cohesin. Endogenous SMC1 formed a complex with WT-Aki1 but not with Δ N415- and Δ N765-Aki1 (Fig. 5 E), suggesting that the N-terminal region of Aki1 is associated with binding to SMC1. Importantly, formation of multipolar spindles caused by Aki1 depletion was efficiently rescued by WT-rAki1 but not by Δ N415-rAki1 expression (Fig. 5 F), although Δ N415-rAki1 showed centrosomal localization (not depicted). We also confirmed that Δ N415-Aki1 still acts as a scaffold protein and a transcriptional repressor as well as WT-Aki1 (unpublished data). These data indicate that formation of a complex of Aki1 with SMC1 (cohesin) is necessary for centriole cohesion. To obtain more direct evidence that Aki1 regulates centrosome-associated cohesin, we performed centrosome purification from control or Aki1 siRNA-treated cells. Interestingly, the Scc1 level in centrosome fraction was decreased in Aki1 siRNA-treated

cells harvested by mitotic shake off compared with mitotically arrested control cells, whereas Scc1 levels in other (cytoplasmic and nuclear) fractions from these cells were almost identical (Fig. 5 G). We also noticed that the cleaved fragment of centrosomal Scc1 was not detected in Aki1 siRNA-treated cells (unpublished data). These results suggest that, in Aki1-depleted mitotic cells, the loss of recruited Scc1 in spindle poles leads to premature cleavage and centriole splitting by separase, and the decrease in the amount of the cleaved fragment that was unable to detect. Thus, Aki1 would regulate mitotic centrosomal localization of Scc1 to prevent premature cleavage in centriole cohesion.

The timing of centriole disengagement needs to be tightly regulated because defects in this process would lead to centrosome abnormalities. Although multiple systems have been elucidated to regulate separase enzymatic activity (Ciosk et al., 1998; Stemmann et al., 2001; Thein et al., 2007), the mechanism of centriole engagement and its players are poorly understood. Our results suggest a new model. Aki1 may associate with Scc1 during mitosis to recruit Scc1 to centrosomes, which mediates centriole cohesion to avoid premature centriole splitting.

Materials and methods

Cell culture conditions

HeLa and 293T cells were cultured in DME supplemented with 10% FBS. For G1/S phase synchronization, cells were incubated with 2 mM thymidine for 20 h. For mitotic phase synchronization, cells were incubated with 100 ng/ml nocodazole for 16 h. Mitotic cells were harvested by shake off. For release from mitotic arrest (to obtain cells in which separase is active), mitotic cells were isolated by shake off and released from mitotic arrest by washing three times with PBS and plating into fresh medium for 2 h.

Plasmid construction

Human WT *Aki1*, deletion mutant *Aki1*, and γ -tubulin and histone H2B cDNAs were generated by PCR with an IMAGE clone (clone 6585236; Invitrogen), a Mammalian Gene Collection clone (clone 3345973; Thermo Fisher Scientific), or a pBOS-H2BGFP vector (BD) as the template, respectively. The cDNAs were cloned into pRetroQ-AcGFP1 N1 (Takara Bio Inc.) or pFlag-CMV2 (Sigma-Aldrich). *rAki1* cDNAs were generated by mutating GATCTGGAT of the Aki1-2 siRNA target sequence to GACCTCGAC without changing the amino acid sequence.

Antibodies

The following antibodies were used in this study: rabbit Aki1, SA-1, SMC1 (Bethyl Laboratories, Inc.), mouse Aki1 (Abnova), Akt (Cell Signaling Technology), rabbit α -tubulin, CENPE, pericentrin, separase (clone XJ11-1B12; Abcam), mouse α -tubulin (clone B-5-1-2), α -tubulin-FITC, mouse γ -tubulin (clone GTU-88; Sigma-Aldrich), β -actin (clone C-2), centrin2, cyclin B1 (Santa Cruz Biotechnology, Inc.), rabbit γ -tubulin (BioLegend), lamin B, NuMA, SMC1 (EMD), MAPK/extracellular signal-regulated kinase, phospho-Ser¹⁰-histone H3, Scc1, SMC3 (Millipore), SA-2, TPX-2 (Novus Biologicals), and BubR1 (provided by T. Hirota, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan).

Microscopy

Cells grown on glass coverslips were fixed in 4% PFA for 15 min or -20°C methanol for 5 min. After fixation, cells were permeabilized in 0.3% Triton X-100 for 10 min. In situ cell extraction was performed to remove the majority of cytoplasmic SMC1 to visualize SMC1 associated with the centrosome (Kong et al., 2009). After incubation for 1 h with 3% BSA, cells were labeled by overnight incubation at 4°C with primary antibodies followed by 30-min incubation with secondary antibodies conjugated to Alexa Fluor 488 or 568 (Invitrogen). DNA was stained with Hoechst 33342 (Invitrogen). Mitotic chromosome spreads were prepared as described previously (Ono et al., 2003). In brief, cells were treated with 100 ng/ml colcemid for 3 h before harvest. Mitotic cells were collected by shake off, treated with 75 mM KCl at 37°C for 30 min, and centrifuged onto coverslips at 1,300 rpm for 10 min using a cytocentrifuge (Cytospin 4; Shandon).

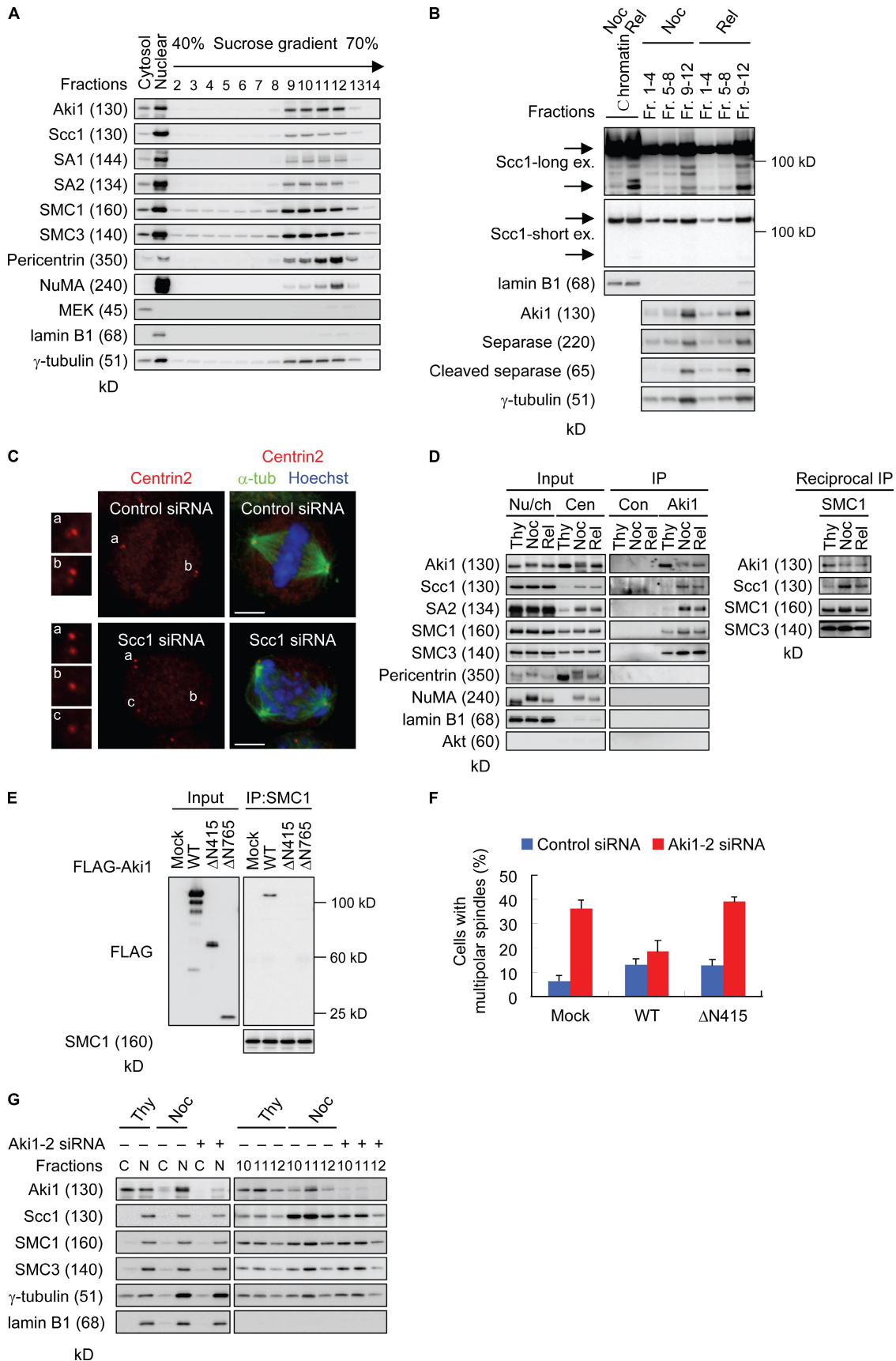


Figure 5. **Formation of a centrosomal Aki1-cohesin complex is required for centriole cohesion.** (A) Immunoblots were performed on cytoplasmic, nuclear, and sucrose gradient fractions (centrosome preparations) from HeLa cells with the indicated antibodies. (B) HeLa cells were harvested 0 or 2 h after release from nocodazole arrest, and centrosomes were purified. Extracts obtained from indicated sucrose gradient fractions and chromatin fractions were

For high resolution images and time-lapse microscopy, cells were viewed under a confocal microscope (FV1000-IX81; Olympus) equipped with a 60x/1.42 NA Plan Apo N oil immersion objective (Olympus) and photomultipliers. Z-series images were acquired and deconvolved, and z planes were projected onto a single view using FV10-ASW software (version 1.7; Olympus). Images were saved as tif files and processed using Photoshop (CS; Adobe).

Transfection, immunoprecipitation, and Western blot analysis

Cells were transfected with appropriate plasmids or siRNA using Lipofectamine 2000 or RNAiMAX (Invitrogen) according to the manufacturer's instructions. Negative control and stealth *Aki1* siRNAs were purchased from Invitrogen. The coding strands of *Aki1* siRNAs were 5'-CCCTGGC-GATCTGGATGCTTTGTT-3' (*Aki1-2*) and 5'-GCGTGGCTAAGAGCTTT-GATGCTGT-3' (*Aki1-3*). Coding strands of *separate* and *Sccl* siRNAs were described previously (Losada et al., 2005; Thein et al., 2007). Cell lysis, immunoprecipitation, and immunoblotting were performed as described previously (Nakamura et al., 2008).

Centrosome purification

Centrosomes were isolated from HeLa cells by discontinuous gradient ultracentrifugation as described previously (Moudjou and Bornens, 1994). In brief, cell pellet was washed with TBS and 0.1x TBS/8% sucrose. Cells were resuspended with 0.1x TBS/8% sucrose and mixed with 0.5% NP-40 lysis buffer. The suspension was shaken slowly for 30 min at 4°C and spun at 2,500 g for 10 min. The supernatant was added with 1 mM Hepes and 1 mg/ml DNase to make final concentrations of 10 mM and 1 µg/ml, respectively. After incubation for 30 min at 4°C, the mixture was gently underlaid with 60% sucrose solution and spun at 10,000 g for 30 min. The obtained centrosomal suspension was vortexed, loaded onto a discontinuous sucrose gradient (70, 50, and 40% sucrose solutions from the bottom), and spun at 120,000 g for 1 h. Fractions were collected from the top, diluted with Pipes buffer (10 mM Pipes), and spun at 20,400 g for 15 min. The supernatants were removed and centrosomes were resuspended with SDS sample buffer. Simultaneously, we prepared lysates from different steps of the purification: cytoplasmic and nuclear/chromatin fractions were supernatant and pellet at the first centrifugation after cell lyses, respectively. For immunoprecipitating centrosomal proteins, the centrosomal pellets sedimented from sucrose gradient fractions (fractions 9–12) were reconstituted in 1% NP-40 lysis buffer.

Cell viability assay

To assess cell viability, cells were incubated with 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide for 2.5 h, and formazan products were solubilized with DMSO. The optical density was measured at 525 nm with a reference at 650 nm using a microplate spectrophotometer (Benchmark Plus; Bio-Rad Laboratories).

Online supplemental material

Fig. S1 shows that *Aki1* depletion induces formation of multipolar spindles without affecting the spindle-focusing machinery. Fig. S2 shows that *Sccl* depletion causes similar and extensive phenotypes compared with *Aki1* depletion. Fig. S3 shows that *Sccl* is cleaved and its C-terminal fragment is produced in chromatin fraction. Video 1 shows *Aki1*-depleted cells expressing AcGFP-histone H2B (green) progressing through mitosis. Videos 2 and 3 show *Aki1* or *Sccl*-depleted cells expressing AcGFP-γ-tubulin (green) progressing into mitosis. The merged AcGFP/differential interference contrast images are shown.

We thank Dr. Toru Hirota and Dr. Kentaro Takagaki for technical advice and for providing reagents, Dr. Akira Nakanishi for help with centrosome purification, and Dr. Oji Suzuki for technical advice.

This study was supported in part by special grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (grant 20015046 to N. Fujita). N. Fujita was also supported by the Novartis Foundation (Japan) for the Promotion of Science and the Vehicle Racing Commemorative Foundation. A. Nakamura was supported by Research Fellowships for Young Scientists from the Japan Society for the Promotion of Science.

Submitted: 4 June 2009

Accepted: 27 October 2009

References

- Abrieu, A., J.A. Kahana, K.W. Wood, and D.W. Cleveland. 2000. CENP-E as an essential component of the mitotic checkpoint in vitro. *Cell*. 102:817–826. doi:10.1016/S0092-8674(00)00070-2
- Bornens, M. 2002. Centrosome composition and microtubule anchoring mechanisms. *Curr. Opin. Cell Biol.* 14:25–34. doi:10.1016/S0955-0674(01)00290-3
- Chen, R.H. 2002. BubR1 is essential for kinetochore localization of other spindle checkpoint proteins and its phosphorylation requires Mad1. *J. Cell Biol.* 158:487–496. doi:10.1083/jcb.200204048
- Ciosk, R., W. Zachariae, C. Michaelis, A. Shevchenko, M. Mann, and K. Nasmyth. 1998. An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell*. 93:1067–1076. doi:10.1016/S0092-8674(00)81211-8
- Fukasawa, K. 2007. Oncogenes and tumour suppressors take on centrosomes. *Nat. Rev. Cancer.* 7:911–924. doi:10.1038/nrc2249
- Garrett, S., K. Auer, D.A. Compton, and T.M. Kapoor. 2002. hTPX2 is required for normal spindle morphology and centrosome integrity during vertebrate cell division. *Curr. Biol.* 12:2055–2059. doi:10.1016/S0960-9872(02)01277-0
- Guan, J., E. Ekwurtzel, U. Kvist, and L. Yuan. 2008. Cohesin protein SMC1 is a centrosomal protein. *Biochem. Biophys. Res. Commun.* 372:761–764. doi:10.1016/j.bbrc.2008.05.120
- Gurley, L.R., J.A. D'Anna, S.S. Barham, L.L. Deaven, and R.A. Tobey. 1978. Histone phosphorylation and chromatin structure during mitosis in Chinese hamster cells. *Eur. J. Biochem.* 84:1–15. doi:10.1111/j.1432-1033.1978.tb12135.x
- Keryer, G., H. Ris, and G.G. Borisy. 1984. Centriole distribution during tripolar mitosis in Chinese hamster ovary cells. *J. Cell Biol.* 98:2222–2229. doi:10.1083/jcb.98.6.2222
- Kong, X., A.R. Ball Jr., E. Sonoda, J. Feng, S. Takeda, T. Fukagawa, T.J. Yen, and K. Yokomori. 2009. Cohesin associates with spindle poles in a mitosis-specific manner and functions in spindle assembly in vertebrate cells. *Mol. Biol. Cell.* 20:1289–1301. doi:10.1091/mbc.E08-04-0419
- Kuriyama, R., and G.G. Borisy. 1981. Centriole cycle in Chinese hamster ovary cells as determined by whole-mount electron microscopy. *J. Cell Biol.* 91:814–821. doi:10.1083/jcb.91.3.814
- Losada, A., M. Hirano, and T. Hirano. 1998. Identification of *Xenopus* SMC protein complexes required for sister chromatid cohesion. *Genes Dev.* 12:1986–1997. doi:10.1101/gad.12.13.1986
- Losada, A., T. Yokochi, and T. Hirano. 2005. Functional contribution of Pds5 to cohesin-mediated cohesion in human cells and *Xenopus* egg extracts. *J. Cell Sci.* 118:2133–2141. doi:10.1242/jcs.02355
- Merdes, A., R. Heald, K. Samejima, W.C. Earnshaw, and D.W. Cleveland. 2000. Formation of spindle poles by dynein/dynactin-dependent transport of NuMA. *J. Cell Biol.* 149:851–862. doi:10.1083/jcb.149.4.851
- Moudjou, M., and M. Bornens. 1994. Isolation of centrosomes from cultured animal cells. In *Cell Biology: A Laboratory Handbook*. J.E. Celis, editor. Academic Press, San Diego. 595–604.
- Nakamura, A., M. Naito, T. Tsuruo, and N. Fujita. 2008. Freud-1/Aki1, a novel PDK1-interacting protein, functions as a scaffold to activate the PDK1/

immunoblotted with the indicated antibodies. Arrows indicate the full length (top) and C terminus (bottom) of *Sccl*. The top *Sccl* blot shows a longer exposure of the bottom blot. (C) HeLa cells were transfected with control or *Sccl* siRNA and costained for α-tubulin (α-tub; green) and centrin2 (red). Centrosomes are shown enlarged on the left. (D) Centrosomes were purified from thymidine (Thy)-arrested cells, nocodazole (Noc)-arrested cells, or cells released (Rel) from a nocodazole arrest for 2 h. Centrosomal proteins were immunoprecipitated with control rabbit IgG, anti-Aki1, or anti-SMC1 (for reciprocal immunoprecipitation [IP]) antibody. Immunoprecipitated proteins, nuclear/chromatin (Nu/ch) lysates, and centrosomal (Cen) lysates were immunoblotted with the indicated antibodies. (E) 293T cells were transfected with pFlag-CMV-2 vector encoding none (mock), WT-Aki1, or Aki1 mutants as indicated. Immunoprecipitated proteins and cell lysates were immunoblotted with antibodies to SMC1 or Flag. (F) Percentage of multipolar cells of mitotic cells in control or Aki1-2 siRNA-treated cells expressing AcGFP-mock, AcGFP-WT-rAki1, or AcGFP-ΔN415-rAki1. Error bars represent SD of triplicate experiments (150–200 total cells were scored per condition). (G) Centrosomes were purified from thymidine-arrested control cells, nocodazole-arrested control cells, or Aki1-depleted cells harvested by mitotic shake off. Cytoplasmic (C), nuclear (N), and centrosomal lysates (fractions 10–12) were immunoblotted with the indicated antibodies. Bars, 5 µm.

Akt pathway in epidermal growth factor signaling. *Mol. Cell. Biol.* 28:5996–6009. doi:10.1128/MCB.00114-08

- Ono, T., A. Losada, M. Hirano, M.P. Myers, A.F. Neuwald, and T. Hirano. 2003. Differential contributions of condensin I and condensin II to mitotic chromosome architecture in vertebrate cells. *Cell.* 115:109–121. doi:10.1016/S0092-8674(03)00724-4
- Ou, X.M., S. Lemonde, H. Jafar-Nejad, C.D. Bown, A. Goto, A. Rogaeva, and P.R. Albert. 2003. Freud-1: A neuronal calcium-regulated repressor of the 5-HT1A receptor gene. *J. Neurosci.* 23:7415–7425.
- Rieder, C.L., and H. Maiato. 2004. Stuck in division or passing through: what happens when cells cannot satisfy the spindle assembly checkpoint. *Dev. Cell.* 7:637–651. doi:10.1016/j.devcel.2004.09.002
- Stemmann, O., H. Zou, S.A. Gerber, S.P. Gygi, and M.W. Kirschner. 2001. Dual inhibition of sister chromatid separation at metaphase. *Cell.* 107:715–726. doi:10.1016/S0092-8674(01)00603-1
- Thein, K.H., J. Kleylein-Sohn, E.A. Nigg, and U. Gruneberg. 2007. Astrin is required for the maintenance of sister chromatid cohesion and centrosome integrity. *J. Cell Biol.* 178:345–354. doi:10.1083/jcb.200701163
- Tsou, M.F., and T. Stearns. 2006. Mechanism limiting centrosome duplication to once per cell cycle. *Nature.* 442:947–951. doi:10.1038/nature04985
- Uhlmann, F., F. Lottspeich, and K. Nasmyth. 1999. Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. *Nature.* 400:37–42. doi:10.1038/21831
- Varetti, G., and A. Musacchio. 2008. The spindle assembly checkpoint. *Curr. Biol.* 18:R591–R595. doi:10.1016/j.cub.2008.06.012
- Waizenegger, I.C., S. Hauf, A. Meinke, and J.M. Peters. 2000. Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase. *Cell.* 103:399–410. doi:10.1016/S0092-8674(00)00132-X
- Waizenegger, I., J.F. Giménez-Abián, D. Wernic, and J.M. Peters. 2002. Regulation of human separase by securin binding and autocleavage. *Curr. Biol.* 12:1368–1378. doi:10.1016/S0960-9822(02)01073-4