The complete removal of cohesin from chromosome arms depends on separase

Masato Nakajima[1,2], Kazuki Kumada[3], Katsuyoshi Hatakeyama[2], Tetsuo Noda[3], Jan-Michael Peters[4] and Toru Hirota[1,*]

[1]Department of Experimental Pathology, Cancer Institute of the Japanese Foundation for Cancer Research (JFCR), Tokyo, Japan
[2]Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
[3]Department of Cell Biology, Cancer Institute of the Japanese Foundation for Cancer Research (JFCR), Tokyo, Japan
[4]Research Institute of Molecular Pathology (IMP), Vienna, Austria
*Author for correspondence (e-mail: thirota@jfcr.or.jp)

Accepted 13 September 2007
Journal of Cell Science 120, 4188-4196 Published by The Company of Biologists 2007
doi:10.1242/jcs.011528

Summary

Cohesin needs to be removed from chromosomes to allow sister chromatid separation in mitosis. In vertebrates, two pathways contribute to this process. The prophase pathway, which requires phosphorylation of the cohesin subunit SA2 and a cohesin-binding protein, called Wapl, removes the bulk of cohesin from the chromosome arms in early mitosis and allows the resolution of the chromosome arms. At anaphase onset, the protease separase removes centromere-enriched cohesin by proteolytic cleavage of another cohesin subunit, Scc1 (Rad21, Mcd1), which allows the separation of sister chromatids. When anaphase onset is delayed by the spindle-assembly checkpoint, the complete removal of cohesin from chromosome arms but not from centromeres generates typical X- or V-shaped chromosomes. Here, we found that cohesion between chromosome arms is preserved if mitosis is arrested with the proteasome inhibitor MG132. This arm cohesion depends on cohesin complexes that are protected by the shugoshin protein Sgo1, which appears to be distributed on chromosome arms as well as on centromeres in early mitosis. In cells lacking separase or expressing non-cleavable Scc1, arm cohesion was not efficiently removed during nocodazole arrest. Our observations suggest that a fraction of arm cohesin is protected by Sgo1, which prevents cohesin from being removed by the prophase pathway, and that separase is partly activated in nocodazole-arrested cells and removes the arm cohesin protected by Sgo1.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/23/4188/DC1

Key words: Anaphase-promoting complex/cyclosome, Centromere, Shugoshin, Spindle-assembly checkpoint

Introduction

Sister chromatid cohesion is essential for bi-orientation of sister kinetochores during mitosis and hence for propagation of the genome during cell proliferation. It is mediated by a multi-subunit complex called cohesin (Nasmyth and Haering, 2005). Cohesin is dismantled in at least two phases using different machineries (Losada et al., 1998; Waizenegger et al., 2000). During the first phase, which starts in prophase, the bulk of cohesin is removed from chromosome arms via a process that requires the Wapl (Wapal) protein and involves the polo-like kinase Plk1 and Aurora B but does not require proteolytic cleavage of the cohesin subunit Scc1 (Rad21, Mcd1) (Losada et al., 2002; Sumara et al., 2002; Gimenez-Abian et al., 2004; Hauf et al., 2005; Gandhi et al., 2006; Kueng et al., 2006). The second and more important phase only occurs when all chromosomes have bi-oriented, at which point a thiol protease called separase is activated by a ubiquitin protein ligase called the anaphase-promoting complex/cyclosome (APC/C), which cleaves Scc1, removes all remaining cohesin on chromosomes and triggers sister chromatid disjunction (Uhlmann et al., 2000; Hauf et al., 2001).

When cells are arrested in mitosis for extended periods of time using spindle poisons such as nocodazole or colcemid, which inhibit APC/C activation, all cohesion along chromosome arms is eventually lost, whereas cohesion at the centromere persists, thereby creating X- or V-shaped chromosomes (Rieder and Palazzo, 1992). Although the X or V configuration are most people's image of the chromosome, they are in fact an artefact rarely seen in nature, except in cells during the second meiotic division. It has been assumed that the loss of cohesin/cohesion along chromosome arms in nocodazole- or colcemid-treated cells is due entirely to the prophase pathway, which is inhibited from removing cohesin at centromeres by a protein called Sgo1 (Kitajima et al., 2004; Salic et al., 2004; Tang et al., 2004; McGuinness et al., 2005).

The prophase and the separase pathways are not completely differentiated, as was initially predicted, because compensatory functions of these two pathways have been observed. When the prophase pathway is impaired and an excess amount of cohesin remains on the arms, the separase pathway appears to be able to remove all cohesin from chromosomes at the onset of anaphase (Gimenez-Abian et al., 2004; Hauf et al., 2005; Kueng et al., 2006). Conversely, in the absence of Sgo1, cohesins on arms and at centromeres is removed solely by the prophase pathway (Salic et al., 2004; McGuinness et al., 2005; Kitajima et al., 2005). These observations imply that both pathways are in principle competent to remove cohesin from anywhere on chromosomes.

We have previously shown that not all cohesin is removed from chromosome arms and that sister chromatids remain associated along the arms until anaphase onset by the prophase pathway during undisturbed mitoses (Gimenez-Abian et al., 2004). However, it was unknown whether metaphase is too short to allow removal of all cohesin from arms by the prophase pathway, or whether the prophase pathway alone cannot remove all the arm cohesin before anaphase.

Results

To further address how the dissociation of cohesin on chromosome arms is regulated, we reinvestigated sister chromatid cohesion in cells that were arrested in mitosis under different conditions. Mitotic HeLa cells collected from a synchronized culture were treated either with the spindle poison nocodazole or with the proteasome inhibitor MG132 (Fig. 1A). Nocodazole and MG132 treatment both cause a

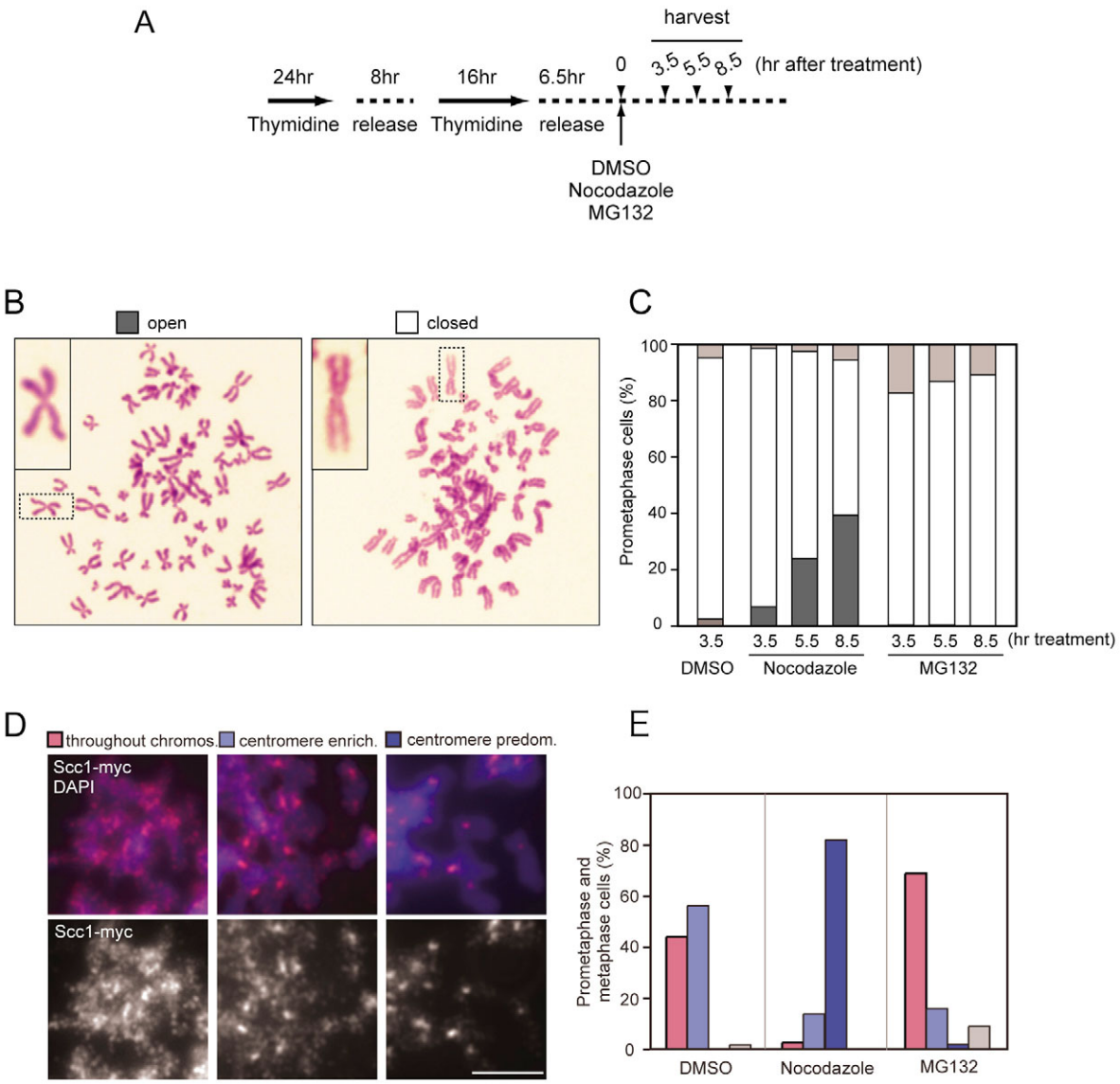

Fig. 1. Preservation of chromosome arm cohesion/cohesin in MG132-arrested cells. (A) Schematic overview of the experimental procedure. Synchronized HeLa cells were released from early S phase and treated with 100 ng/ml nocodazole, 25 μM MG132 or the solvent DMSO at G2/M transition. Mitotic cells were collected at the indicated times (hours) after the treatment and chromosome morphology was analyzed by Giemsa staining. (B) Representative pictures of chromosomes in the absence (left panel; 'open') or presence (right panel; 'closed') of arm cohesion. Insets show magnified images of the single chromosomes in the boxed region. (C) Four-hundred prometaphase/metaphase cells were scored for arm status at each time-point; the results are summarized in the histogram. The dark-grey and white populations represents cells with open and closed arms, respectively, as exemplified in B. An unclassified population is shown in light grey. (D) HeLa cells that had been induced to express Scc1-Myc for 2 days were treated with nocodazole or MG132 for 2 hours to enrich mitotic cells, which were then collected and further incubated in a new dish for 2 hours in the presence of the respective drugs. For a control experiment, cells were treated with an equivalent amount of DMSO for 4 hours. Mitotic cells were shaken off, cytospun, fixed and stained with Myc antibodies. Representative Myc-staining patterns including 'throughout chromosomes', 'centromere enrichment' and 'centromere predominant' are shown. (E) One-hundred prometaphase/metaphase cells, indicated by condensin-I-specific subunit CAP-G staining (not shown), were classified into three categories based on Myc-staining pattern as exemplified and colour-coded in D. An unclassified population is shown in grey. Bar, 10 μm.

mitotic arrest before anaphase onset, but they perturb mitotic progression through different mechanisms. Nocodazole disrupts microtubules and activates the spindle-assembly checkpoint. Upon activation, Mad2 (Mad2l1) inhibits the APC/C by interacting with the activator of this complex, Cdc20 (reviewed in Musacchio and Salmon, 2007). MG132 also blocks protein degradation, but in this case by direct inhibition of the proteasome, regardless of the spindle checkpoint. When we analyzed Giemsa-stained chromosomes that had been isolated from nocodazole-arrested cells, cohesion on chromosome arms had been lost, resulting in characteristic 'X-shaped' chromosomes. Unexpectedly, however, such chromosomes with open arms were hardly seen in MG132-arrested cells. Instead, cohesion between chromosome arms was preserved for at least 8.5 hours in MG132-treated cells (Fig. 1B,C).

Cohesion between chromosome arms in MG132-treated cells could be maintained by cohesin complexes that hold sister chromatids, or by a cohesin-independent mechanism. To address this, we first localized cohesin by immunofluorescence microscopy of HeLa cells that express Myc-epitope-tagged Scc1. The Myc staining of fixed cells and of spread chromosomes, which reflects the behaviour of endogenous cohesin complexes (Waizenegger et al., 2000; Hauf et al., 2001), revealed that a small amount of Scc1-Myc remains on chromosomes along their entire length in MG132-arrested cells, whereas the signal was found almost exclusively at centromeres in nocodazole-arrested cells (Fig. 1D,E). We then studied whether preservation of cohesion depends on this residual cohesin, the association of which with mitotic chromosomes might be protected by Sgo1. Depletion of Sgo1 by RNAi was performed during a double thymidine block cell synchronization regimen (McGuinness et al., 2005), and we examined whether sister chromatids remain paired during prolonged mitosis in the presence of MG132 (Fig. 2A,B). Most of the control-treated cells showed persistent sister chromatid cohesion, but, in Sgo1-depleted cells, many sister chromatids dissociated from each other and became unpaired over time, indicating that cohesion along the chromosome length in MG132-arrested cells is maintained by cohesin complexes that are protected by Sgo1 (Fig. 2C,D).

The finding that Sgo1 protects cohesin from the prophase pathway not only at centromeres but also on chromosome arms prompted us to examine whether Sgo1 localizes along the arms. We first addressed this in a HeLa cell line that stably express EGFP-tagged Sgo1. Both in fixed cells (Fig. 3A) and in live cells (supplementary material Fig. S1), the enrichment of EGFP-Sgo1 at centromeres was seen from prophase to metaphase, as was previously characterized (Kitajima et al., 2004; Salic et al., 2004; Tang et al., 2004; McGuinness et al., 2005). We noticed that a small amount of EGFP-Sgo1 was distributed on chromosome arms, homogenously throughout the chromosome length, during the early phases of mitosis. Notably, the EGFP-Sgo1 signal on arms became undetectable when cells were treated with nocodazole, supporting the idea that the arm distribution of EGFP-Sgo1 is not merely due to a

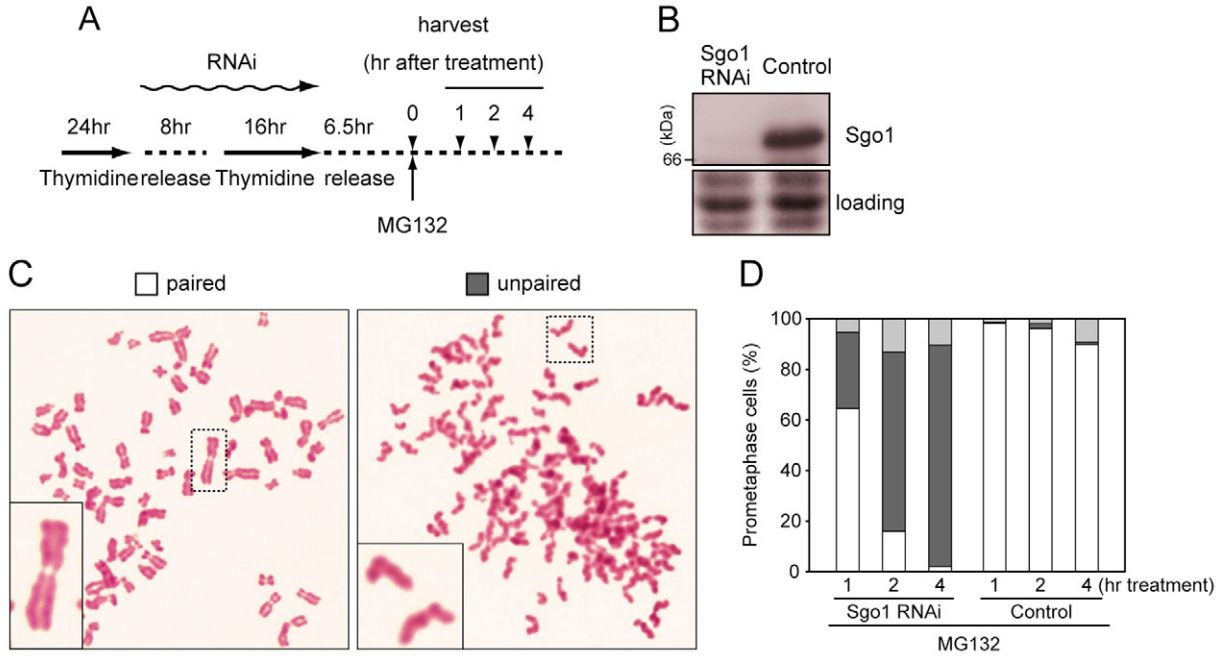

Fig. 2. Persistence of sister chromatid cohesion on arms depends on a cohesin complex protected by Sgo1. (A) Schematic overview of synchronization and RNAi treatment. HeLa cells were transfected with a mock or siRNA to *Sgo1* during synchronization by the double-thymidine-block protocol and 25 μM MG132 was added at G2/M transition. Mitotic cells were collected at the indicated times (hours) after MG132 treatment and analyzed by Giemsa staining of chromosome spreads. (B) Reduction of Sgo1 in the collected mitotic cells was assessed by immunoblot analysis. (C) Representative chromosomes with paired (left) or unpaired (right) sister chromatids are shown. Insets show magnified images of the single chromosome in the boxed regions. (D) Quantification results from 200 cells for each time-point are summarized in the histogram. The dark-grey and white population represents cells containing unpaired and paired chromatids, respectively. An unclassified population is shown in light grey.

non-specific binding to chromatin or to an excess amount of EGFP-Sgo1. To further verify the localization of Sgo1 on chromosome arms, we immunostained cells with Sgo1-specific antibodies (Fig. 3B). Similarly to the EGFP-Sgo1 distribution pattern, reactivity of Sgo1 antibodies were seen on chromosome arms as well as at centromeres, and both of these stainings disappeared when Sgo1 was depleted by RNAi (Fig. 3B).

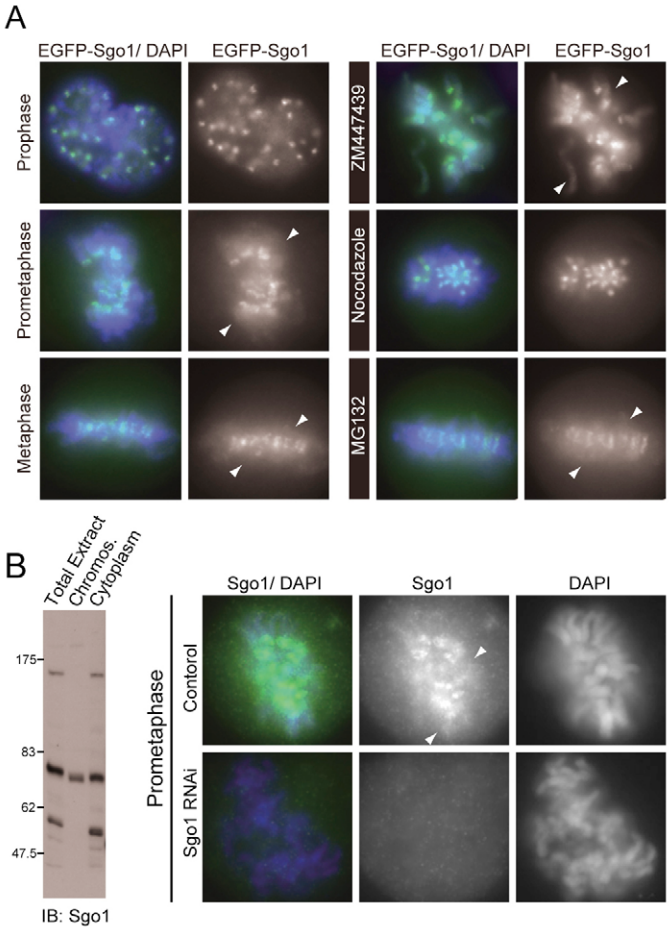

Fig. 3. Localization of Sgo1 on chromosome arms. (A) HeLa cells expressing EGFP-tagged Sgo1 were synchronized with thymidine, and, at 9.5 hours after the release, cells were fixed and DNA was counterstained with DAPI. The left panels are representative pictures of prophase, prometaphase and metaphase cells from unperturbed mitosis. The right panels represent cells treated either with 3 μM of an Aurora B inhibitor, ZM447439, 100 ng/ml nocodazole or 25 μM MG132 for 1 hour before fixation. Note that some EGFP signals are discernible on chromosome arms (arrowheads) that are not clearly detectable in nocodazole-treated cells. (B) Total extract of nocodazole-arrested HeLa cells (lane 1) was fractionated into a chromosome-enriched fraction (lane 2) and a cytoplasmic fraction (lane 3). Each fraction was analyzed by immunoblotting with antibodies to Sgo1. Note that these antibodies specifically react with Sgo1 in the chromosome-enriched fraction (left). (Right panels) HeLa cells were transfected with siRNA to *Sgo1* during thymidine treatment for 21 hours. At 9 hours after the release, cells were fixed with 4% paraformaldehyde and stained with antibodies to Sgo1. DNA was counterstained with DAPI. Note that signals on chromosome arms (arrowheads) were abolished by Sgo1 depletion (right).

These cytological observations suggest that a small amount of Sgo1 distributes on chromosome arms, in addition to its enrichment at centromeres. It has been shown that higher levels of Sgo1 remain on chromosome arms in metaphase when the activity of Aurora B (Lipp et al., 2007) or Bub1 (Kitajima et al., 2006) is depleted. Taken together with our documentation of Sgo1 on chromosome arms, a tempting possibility is that Sgo1 is initially distributed throughout the chromosome length, and is thereafter removed from arms and enriched at centromeres by a mechanism mediated by these kinases during early mitotic phases.

How Sgo1 protects cohesin from being dissociated has been explained by the binding of Sgo1 to phosphatase PP2A (Ppp2r1a), which prevents SA2 (Stag2; a mammalian homologue of the yeast Scc3) phosphorylation and thereby confers resistance to the prophase pathway (Riedel et al., 2006; Kitajima et al., 2006; Tang et al., 2006). If a subset of cohesin on chromosome arms is in fact protected by Sgo1, one interesting possibility is that activity of separase is required for the removal of cohesin from chromosome arms and the dissociation of arm cohesion. To directly test this hypothesis, we used fibroblasts derived from mouse embryos in which both separase alleles can be conditionally knocked out by the induction of Cre recombinase (Kumada et al., 2006). Cells were depleted of separase or were mock treated by infecting adenovirus encoding Cre or β-galactosidase, respectively, for 2 days. Mitotic cells were collected and incubated in the presence of nocodazole or MG132, and chromosomes were analyzed by Giemsa staining (Fig. 4A). As in HeLa cells, arm cohesion was lost in chromosomes from an increasing number of the control cells over time during nocodazole treatment, whereas arm cohesion was preserved in MG132-treated cells. Under these conditions, we noticed that dissociation of arm cohesion was less efficient when separase-depleted cells were incubated with nocodazole (Fig. 4B,C).

Formation of diplochromosomes, in which two chromosomes were connected together at centromeres (four chromatids), is a characteristic feature of cells depleted of separase activity (Wirth et al., 2006; Kumada et al., 2006; Jager et al., 2001). Thus, it would be reasonable to find that arm cohesion was preserved more frequently in these diplochromosomes after nocodazole treatment (Fig. 4C), but the diplo-state of chromosomes per se might prevent arms from opening for geometrical reasons. To eliminate such a possibility, we synchronized the cells after adenovirus infection and increased the number of the first mitosis after separase deprivation (Fig. 4D). In this experimental setting, similar results were obtained as in the initial experiments, namely that separase is required for an efficient dissociation of arm cohesion (Fig. 4E).

Requirement of separase for cohesin dissociation specifically from chromosome arms was first noticed in yeast meiosis I, in which cohesin removal from the distal part to the chiasmata is the crucial step for the separation of chromosome homologues in anaphase I (Buonomo et al., 2000). Experiments in mice have demonstrated that the function of separase in meiosis I is conserved throughout higher eukaryotes (Gorr et al., 2006; Kudo et al., 2006). In this respect, our observations suggest that separase-mediated cohesin dissociation on chromosome arms might be a universal machinery not only among species but also between mitosis and meiosis.

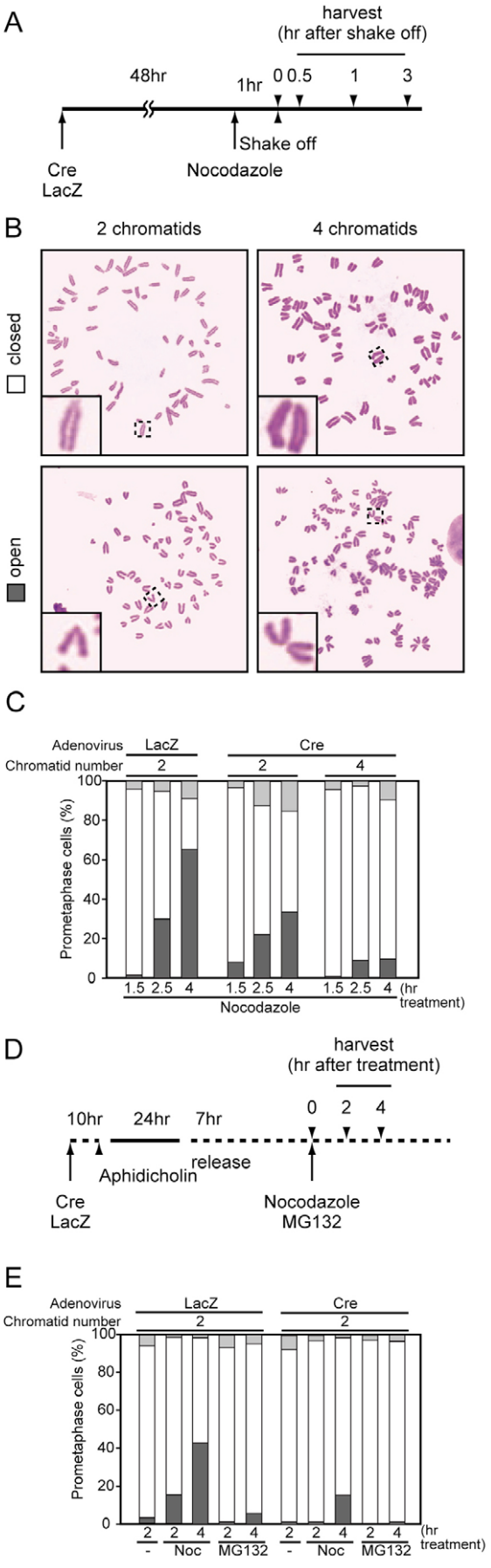

Fig. 4. Separase is required for efficient dissociation of arm cohesion. (A) Experimental design using asynchronous cells. Immortalized fibroblasts derived from separase conditional-knockout mouse embryos were infected with adenovirus encoding Cre recombinase or β-galactosidase (*lacZ*) at MOI 200. After 2 days, mitotic cells were collected following 1 hour of treatment with 100 ng/ml nocodazole (or with 25 μM MG132, as a control; data not shown) and incubated in the presence of nocodazole (or MG132) for another 0.5, 1 or 3 hours, when cells were fixed and chromosome spreads were stained with Giemsa. (B) Representative chromosome configurations with a normal number of chromatids (two chromatids; left panels) and diplochromosomes (four chromatids; right panels) are shown. Note that chromosomes appear V-shaped when arm cohesion is dissolved, because mouse chromosomes are acrocentric. Insets show magnification images of the chromosomes in the boxed regions. (C) Approximately 200 cells were assessed for arm cohesion and summarized. Note that diplochromosomes (four chromatids) appeared only in Cre-introduced separase-depleted cells, which were scored separately from chromosomes with two chromatids. (D) Experiment designed to enrich the first mitosis after separase depletion. At 10 hours after adenovirus infection, cells were treated with 1 μM aphidicholin for 24 hours to arrest cells at early S phase. At 7 hours after the release from aphidicholin, when many cells were in G2 phase, either 100 ng/ml nocodazole (Noc) or 25 μM MG132 were added and cells were incubated for 2-4 hours. Mitotic cells were collected and analyzed by chromosome spreading. (E) Quantification data of arm cohesion from the experiment in D. Approximately 200 cells with two chromatids were assessed for each time-point.

The finding that separase is required to dissociate arm cohesion implies that cohesin is removed by proteolytic cleavage of the cohesin subunit Scc1. To test this, we used HeLa cells that can inducibly express a non-cleavable mutant of Scc1 by the addition of doxycycline (Hauf et al., 2001) and studied whether arm cohesion was preserved. In this experiment, we needed to take into account that cohesin used for linking sister chromatids is incorporated in S phase (Haering et al., 2004) and that sister chromatids often fail to disjoin in mitosis if non-cleavable Scc1 is incorporated (Hauf et al., 2001). Therefore a reasonable amount of non-cleavable Scc1 needed to be expressed at the beginning of S phase and those cells needed to be analyzed in the following mitosis. Because we found that exogenous Scc1 appeared 24 hours after induction (Fig. 5A), cells were incubated for a following 16 hours, by which time we expect that the majority of mitotic cells had incorporated a significant amount of exogenous Scc1 (Fig. 5B). Many chromosomes from mitotic cells that expressed non-cleavable Scc1 maintained arm cohesion, whereas cohesion was dissolved and arms opened widely in chromosomes from cells that expressed wild-type Scc1, or from cells that did not induce either wild-type or mutant Scc1 (Fig. 5C,D).

To further examine whether this persistence of arm cohesion was due to the inefficient removal of cohesin complex, spread chromosomes were immunostained with Myc antibodies to examine the distribution of exogenously expressed Scc1. The majority of wild-type Scc1-Myc-expressing cells showed Myc staining almost exclusively at centromeres after 4 hours of nocodazole treatment, as previously described (Waizenegger et al., 2000; Hauf et al., 2001; Gimenez-Abian et al., 2004). By contrast, we found that Myc signals were distributed along the entire chromosome in many of the non-cleavable Scc1-Myc-

expressing cells, and the centromeric enrichment was less discernible (Fig. 5E,F).

Because cohesin distribution throughout the chromosome length is normally seen in prophase (Gimenez-Abian et al., 2004), it was formally possible that prophase was prolonged, resulting in chromosomes with homogenous Myc staining being frequently seen in non-cleavable Scc1 cells. Therefore, chromosomes were co-stained with antibodies to the condensin-I-specific subunit CAP-G (Ncapg), because condensin I is known to associate with chromosomes only after prometaphase (Ono et al., 2004; Hirota et al., 2004). The experiments clearly revealed association of condensin I with mitotic chromosomes, confirming that the chromosomes we analyzed were isolated from cells in prometaphase or metaphase. Notably, the staining pattern of non-cleavable Scc1 was reminiscent to that of wild-type Scc1 when cells were treated with MG132 (Fig. 1E), further supporting the idea that MG132 treatment suppressed Scc1 cleavage.

An alternative possible interpretation for why arm cohesin persists in MG132-arrested mitosis is that the proteasome-mediated protein-degradation machinery is involved in the prophase pathway; e.g. in the activation of the mitotic kinases Plk1 or Aurora B, or in Wapl function. However, MG132-arrested cells do not show the phenotypes that are characteristic for cells in which Plk1 or Aurora B have been inhibited (Lenart et al., 2007; Sumara et al., 2004; Gimenez-Abian et al., 2004; Hauf et al., 2003; Ono et al., 2004). MG132 treatment did not disrupt the formation of the metaphase plate or of spindle poles (data not shown), did not disrupt the primary constriction of chromosomes and did not prevent the association of condensin I with mitotic chromosomes (data not shown).

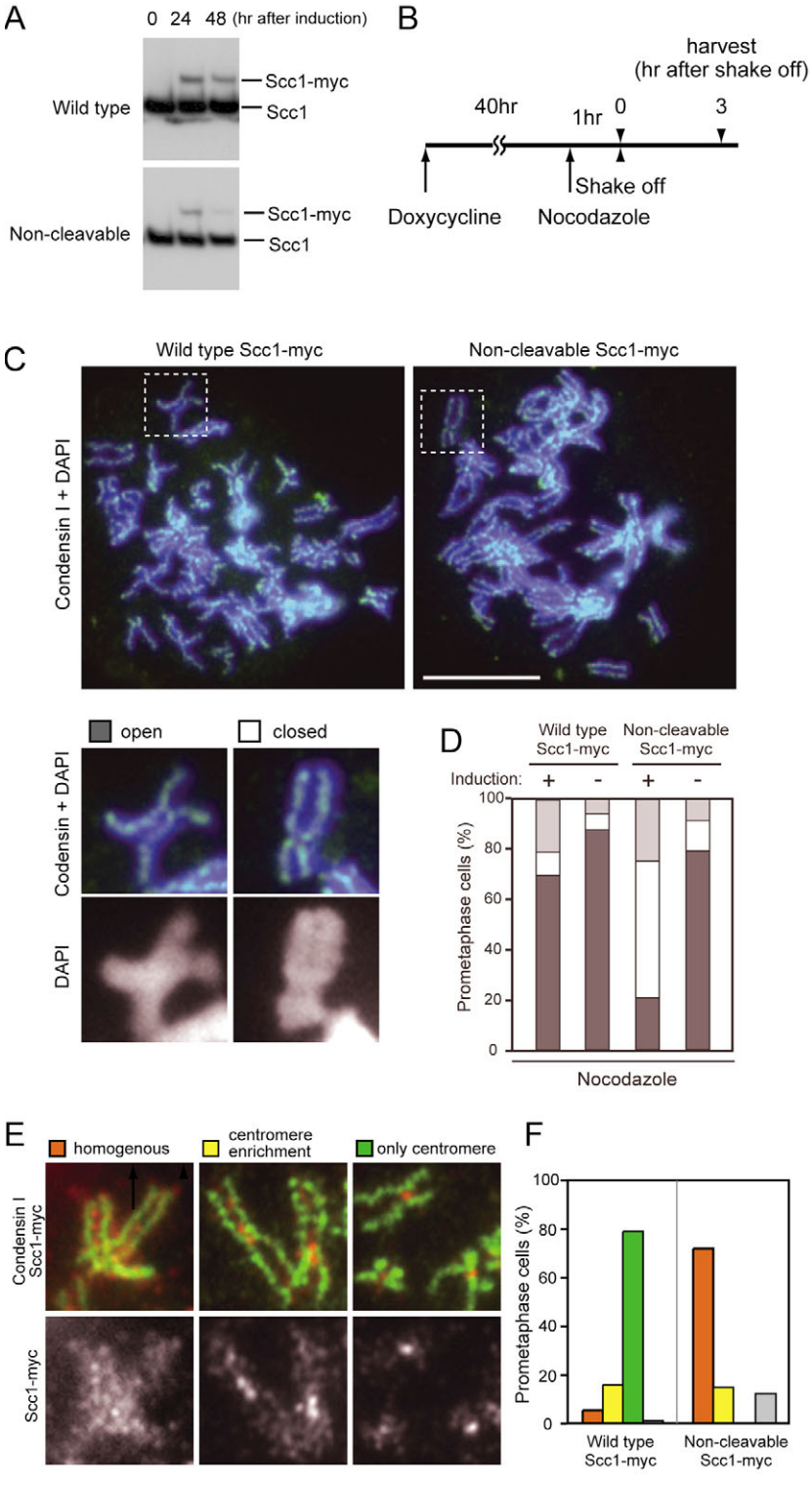

Fig. 5. Scc1 cleavage is required for the complete removal of cohesin from chromosome arms. (A) Induction of Scc1-Myc. Both wild-type and non-cleavable Scc1-Myc could be detected 24 hours after doxycycline addition. Scc1 antibodies recognize both endogenous and exogenous Scc1, as indicated. (B) Experimental protocol. HeLa cells that can inducibly express either wild-type or non-cleavable Scc1-Myc were induced of their expression by the addition of doxycycline for 40 hours. Mitotic cells enriched by 1 hour of treatment with 100 ng/ml nocodazole were collected and further incubated for 3 hours in the presence of nocodazole. (C) Representative images of wild-type and non-cleavable Scc1-Myc-expressing cells (top two panels). After a hypotonic treatment, cells were cytospun onto glass slides and immunostained with antibodies to the Myc epitope (not shown, but red in E) and the condensin I subunit CAP-G (green), and DNA was counterstained with DAPI (blue). Chromosomes in the boxed regions are magnified and shown as an example for arm-open or -closed chromosomes (bottom panels). (D) Quantification results from ~100 prometaphase cells for each experiment are summarized in the histogram. The cell population for the open and closed arms is shown in dark grey and white, respectively, and an unclassified population is in light grey. (E) The Scc1-Myc-positive prometaphase and metaphase cells were further classified accordingly to the distribution pattern of Scc1-Myc on chromosomes, as colour-coded. (F) One-hundred prometaphase/metaphase cells were classified into three categories based on Myc-staining pattern, as exemplified in E. An unclassified population is shown in grey. Bar, 10 μm.

MG132 treatment did not detectably affect mitotic progression (Fig. 6D) and cells could reach to a metaphase-like state, which is not the case for Wapl-depleted cells (Gandhi et al., 2006; Kueng et al., 2006). It is therefore unlikely that MG132 inhibits the prophase pathway for cohesin dissociation.

Our results are consistent with a counterintuitive notion that, during nocodazole-induced prometaphase arrest, progressive removal of cohesin on chromosome arms involves activation of separase and the cleavage-dependent mechanism. If this process involved the APC/C, depletion of the APC/C activator Cdc20 should prevent the arm opening in nocodazole-arrested cells. Two siRNAs targeting different parts of the *Cdc20* mRNA achieved efficient depletions of the protein beyond immunoblot detection levels (Fig. 6A,B). In a condition in which arm cohesion was lost in chromosomes from more than 80% of mock-transfected prometaphase cells, the chromosome arms were still widely opened in ~60% of cells after Cdc20 depletion using two different siRNAs (Fig. 6C). This observation suggests that dissociation of chromosome arms during nocodazole arrest is largely mediated by a mechanism that does not involve APC-Cdc20, which could be reasonable because, in such a situation, the spindle-assembly checkpoint must rigorously inhibit the action of APC-Cdc20.

An obvious following question is how can separase gain its activity when the spindle-assembly checkpoint is functioning and is inhibiting APC/C-Cdc20? Interestingly, we realized that

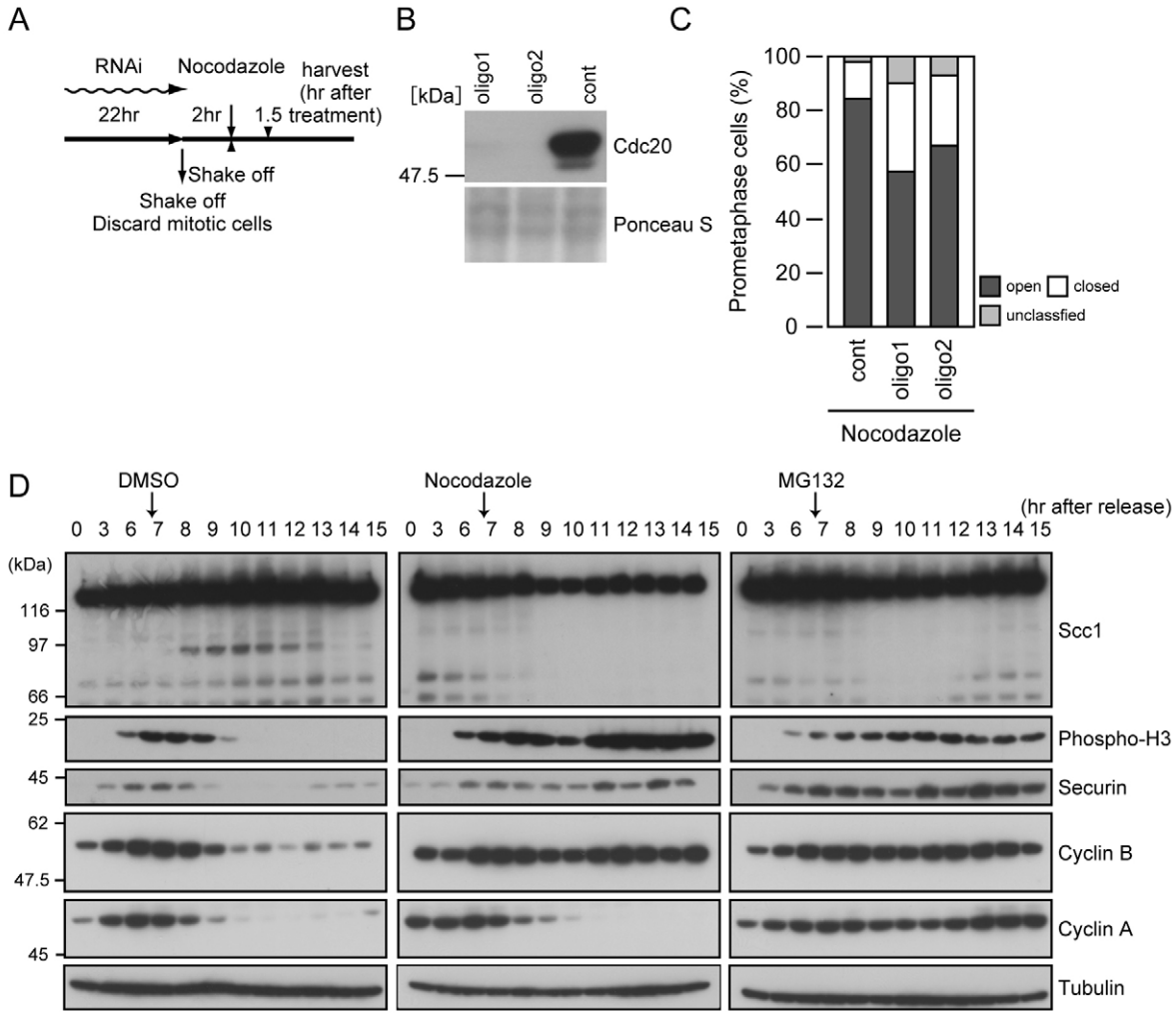

Fig. 6. Decrease in securin levels in nocodazole-arrested cells. (A) Schematic overview of the experimental settings. Asynchronously grown HeLa cells were transfected with *Cdc20* siRNA for 22 hours. To obtain a synchronous culture of mitotic cells, we first shook-off mitotic cells to remove cells that had already spent some time in mitosis, and then collected cells that entered and stayed in mitosis within the next 2 hours. Subsequently, cells were transferred to a growing medium containing 100 ng/ml nocodazole, incubated for another 1.5 hours, and analyzed by chromosome spreading and Giemsa staining. (B) Depletion of Cdc20 by RNAi. Total cell extracts were prepared from mitotic cells that were used in the experiment. (C) One-hundred prometaphase/metaphase cells were classified based on chromosome-arm status. The dark-grey and white populations represent cells with open and closed arms, respectively. An unclassified population is shown in light grey. (D) HeLa cells were synchronized at early S phase by the double-thymidine-block regimen and, at 6.5 hours after the release, cells were treated either with 100 ng/ml nocodazole or 25 μM MG132, or with the solvent DMSO. Total cell extracts were analyzed by immunoblotting with the indicated antibodies. In nocodazole-arrest experiments, we noticed a slight reduction in the protein levels of securin. Note that phospho-H3 first appears at 6 hours after the release in both nocodazole- and MG132-treated cells, as in DMSO-treated cells, suggesting that the timing of mitotic entry is not largely affected by these treatments.

securin levels always showed a slight decrease during nocodazole arrest, whereas it remained stable in MG132-arrested cells (Fig. 6D). This partial degradation of securin might be causally related to partial activation of separase during spindle-assembly checkpoint arrest. At present, we do not know why nocodazole treatment caused degradation of securin, but the easiest interpretation is that securin becomes unstable when the spindle microtubules are depolymerized. This idea could be reasonable, because a fraction of securin and separase has been found to associate with spindle microtubules (Funabiki et al., 1996; Kumada et al., 1998; Hagting et al., 2002).

Discussion

Stepwise removal of cohesin is an important feature of mitotic chromosome assembly. Our finding that separase activity is required for the complete removal of cohesin from chromosome arms is not mutually exclusive with the pre-existing model, which proposes that the prophase pathway promotes cohesin dissociation by SA2 phosphorylation and Wapl association (Waizenegger et al., 2000; Hauf et al., 2001; Losada et al., 2002; Sumara et al., 2002; Gimenez-Abian et al., 2004; Hauf et al., 2005; Gandhi et al., 2006; Kueng et al., 2006). Chromosome arms might comprise a mixture of cohesin complexes that require different mechanisms for their dissociation; one might require phosphorylation and another one proteolytic cleavage (Fig. 7). To what extent does cohesin on arms require separase to be removed? If all of the arm cohesins have to be cleaved, one would expect that the Myc signal intensities of non-cleavable Scc1-Myc or of wild-type Scc1-Myc in MG132-treated cells to be maintained at a level as high as in early prophase cells. But, in both cases, the intensity was reduced remarkably (data not shown), suggesting that the majority of cohesin can be removed by the prophase pathway. Taking this into account, it is not too surprising that we have so far been unable to detect cleaved products of Scc1 during nocodazole-induced cohesin dissociation in immunoblot analysis (Waizenegger et al., 2000) (Fig. 6D).

Unlike budding yeast mitosis, in which the majority of cohesin is removed from chromosomes by separase, it seems that, in vertebrates, chromosome arm cohesin is a mixture of at least two fractions, the dissociation of which relies on either phosphorylation or proteolysis. What defines these different properties of cohesin? What determines their distribution on chromosomes? An implication from our observation is that a fraction of cohesin on chromosome arms is normally protected by Sgo1, and these particular cohesin complexes can then only be removed by separase (Fig. 7). In this model, it is important to find out whether phosphorylation of Scc1, likewise to SA2 (Riedel et al., 2006; Kitajima et al., 2006; Tang et al., 2006), is also prevented by Sgo1, because phosphorylation of Scc1 and its counterpart in meiosis, Rec8, enhances its cleavability by separase (Alexandru et al., 2001; Lee et al., 2003; Clyne et al., 2003; Hornig and Uhlmann, 2004; Hauf et al., 2005).

In mammalian cells, separase has so far been believed to be essential only for the cleavage of cohesin complexes that are located at centromeres, where these complexes are protected from the prophase pathway by Sgo1. By contrast, our work indicates that the separase pathway is also required to remove a subset of cohesin complexes from the chromosome arms. Our data further indicate that these cohesin complexes cannot be removed by the prophase pathway because they are protected by small amounts of Sgo1. The complete loss of cohesion between chromosome arms that is observed in cells arrested by the spindle-assembly checkpoint therefore depends on low amounts of separase activity. Whether low amounts of separase are also activated in the early stages of an unperturbed mitosis remains to be tested in the future. It will also be important to understand why separase is unable to remove all cohesin from centromeres in checkpoint-arrested cells. One possibility is that there are simply so many cohesin complexes at centromeres that separase activity during early mitosis cannot cleave them all. Another, more interesting, possibility is that cohesin at centromeres is not only protected from the prophase pathway but also somehow from separase. Understanding how the mitotic kinases and separase collaborate in regulating cohesin at centromeres and on chromosome arms awaits further investigation.

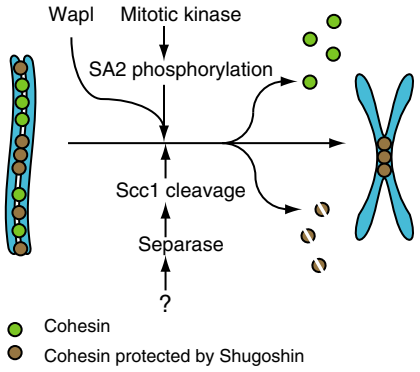

Fig. 7. A model illustrating how dissociation of cohesin from chromosome arms is regulated.

Materials and Methods

Cell culture and chromosome preparations

HeLa cells were cultured in DME medium supplemented with 10% FCS, 0.2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For synchronization, HeLa Kyoto cells were grown in the presence of 1 mM thymidine (Sigma) for 24 hours, washed twice with PBS and released in a fresh medium for 8 hours. Thymidine was added again to a final concentration of 1 mM to block cells at G1/S. After another 16-hour incubation, cells were released, and, at the G2/M transition, shortly before an increase of mitotic index, either 100 ng/ml nocodazole or 25 μM MG132, or the solvent DMSO, was added and samples were harvested over time. Immortalized mouse embryonic fibroblasts were generated by the 3T3 protocol (Kumada et al., 2006). Synchronization of fibroblasts were performed by 1 μM Aphidicholin treatment. Chromosome spreading and Giemsa staining were performed as described previously (Gimenez-Abian et al., 2004).

Antibodies

The following monoclonal mouse antibodies were used: Myc (4A6, Upstate Biotechnology), securin (DCS-280, MBL), cyclin A (BF683, Cell Signaling Technologies), cyclin B (clone 18, BD Biosciences), Scc1 (53A303, Upstate Biotechnology), tubulin (B-5-1-2, Sigma-Aldrich Co.). The following polyclonal rabbit antibodies were used: phospho-histone H3 Ser10 (Cell Signaling Technologies). Polyclonal antibodies to CAP-G were raised against two synthetic peptides FRLAQQPHQNQAKL and YKREPAVERVIEF corresponding to amino acids 14-27 and 59-71, respectively.

RNA interference

The targeted sequences were as follows: Sgo1, 5′-CCCAAUAGUGAUGA-CAGCUCCAGAA-3′; Cdc20 oligo1, 5′-AAACCGUUCAGGUUCAAAGCCC-AGG-3′ and oligo2, 5′-AGUAGUUGCCCUCUUUGAUCCAGGC-3′ (Stealth, Invitrogen). Transfection of siRNA oligonucleotides was performed to HeLa cells during the cell synchronization regimen. Transfections were carried out by incubating 50 nM duplex siRNA with RNAi MAX (Invitrogen) in the antibiotics-free growth medium. For control transfections, the same annealing reaction was set up using H_2O instead of siRNA oligos.

Immunofluorescence microscopy

Scc1-Myc-expressing cells were spun onto glass slides with a cytospin centrifuge (Shandon) for 5 minutes at 1500 rpm, pre-extracted and fixed with 4% (w/v) paraformaldehyde. Cells were incubated with primary antibodies overnight at room temperature, followed by incubation with secondary antibodies for 45 minutes. The secondary antibodies used in this study were: goat anti-rabbit IgG Alexa-Fluor-488 and -568, goat anti-mouse Alexa-Fluor-488 and -568, and goat anti-human IgG Alexa-Fluor-568 (Molecular Probes). For antibody dilutions, 0.01% (v/v) Triton X-100 in PBS with 1% BSA (w/v) was used. After a 5-minute incubation with 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI), cells were mounted with the Fluorescent Mounting Medium (Dako Cytomation). Images were captured on a Zeiss Imager M1 microscope equipped with epifluorescence and a Photometrics Cool Snap HQ CCD camera driven by MetaMorph software (Universal Imaging).

Western blot analysis

Cells were lysed in a buffer consisting of 20 mM Tris (pH 7.4), 100 mM NaCl, 20 mM beta-glycerophosphate, 5 mM $MgCl_2$, 1 mM NaF, 0.1% Triton X-100, 10% glycerol, 1 mM DTT, supplemented with a cocktail of protease inhibitors (Complete Mini EDTA-free, Roche Diagnostics). Total-protein concentration was measured and controlled by the Bradford method (Protein Assay system, Bio-Rad Laboratories), and the cell extracts were resolved by SDS-PAGE and transferred to a PVDF membrane (Immobilon-P, Millipore). Blocking and antibody incubations were in 5% non-fat dry milk or 4% Block Ace solution (Snow Brand Milk Products). The horseradish peroxidase-labelled secondary antibodies (Amersham) were developed by chemiluminescence using luminol and coumaric acid (Sigma).

We are grateful to G. Wutz (IMP, Vienna) for communicating unpublished results; to T. Kitajima, Y. Tanno, K. Tanaka and Y. Watanabe (University of Tokyo) for Sgo1 antibodies, EGFP-Sgo1 cells and comments on the manuscript; to M. Hosojima (Niigata University) for providing CREST sera; and to K. Takagaki, T. Ohishi and Y. Hirayama (JFCR, Tokyo) for discussions and support. Research in the laboratory of J.-M.P. is supported by Boehringer Ingelheim, Wiener Wirtschaftsfoerderungsfonds (WWFF) and EMBO. Research in the laboratories of T.H. and T.N. are supported by grants from the Japan Society for the Promotion of Science (JSPS), and from the Ministry of Education, Culture, Sports and Technology of Japan.

References

Alexandru, G., Uhlmann, F., Mechtler, K., Poupart, M. A. and Nasmyth, K. (2001). Phosphorylation of the cohesin subunit Scc1 by Polo/Cdc5 kinase regulates sister chromatid separation in yeast. *Cell* **105**, 459-472.

Buonomo, S. B., Clyne, R. K., Fuchs, J., Loidl, J., Uhlmann, F. and Nasmyth, K. (2000). Disjunction of homologous chromosomes in meiosis I depends on proteolytic cleavage of the meiotic cohesin Rec8 by separin. *Cell* **103**, 387-398.

Clyne, R. K., Katis, V. L., Jessop, L., Benjamin, K. R., Herskowitz, I., Lichten, M. and Nasmyth, K. (2003). Polo-like kinase Cdc5 promotes chiasmata formation and cosegregation of sister centromeres at meiosis I. *Nat. Cell Biol.* **5**, 480-485.

Funabiki, H., Yamano, H., Kumada, K., Nagao, K., Hunt, T. and Yanagida, M. (1996). Cut2 proteolysis required for sister-chromatid seperation in fission yeast. *Nature* **381**, 438-441.

Gandhi, R., Gillespie, P. J. and Hirano, T. (2006). Human Wapl is a cohesin-binding protein that promotes sister-chromatid resolution in mitotic prophase. *Curr. Biol.* **16**, 2406-2417.

Gimenez-Abian, J. F., Sumara, I., Hirota, T., Hauf, S., Gerlich, D., de la Torre, C., Ellenberg, J. and Peters, J. M. (2004). Regulation of sister chromatid cohesion between chromosome arms. *Curr. Biol.* **14**, 1187-1193.

Gorr, I. H., Reis, A., Boos, D., Wuhr, M., Madgwick, S., Jones, K. T. and Stemmann, O. (2006). Essential CDK1-inhibitory role for separase during meiosis I in vertebrate oocytes. *Nat. Cell Biol.* **8**, 1035-1037.

Haering, C. H., Schoffnegger, D., Nishino, T., Helmhart, W., Nasmyth, K. and Lowe, J. (2004). Structure and stability of cohesin's Smc1-kleisin interaction. *Mol. Cell* **15**, 951-964.

Hagting, A., Den Elzen, N., Vodermaier, H. C., Waizenegger, I. C., Peters, J. M. and Pines, J. (2002). Human securin proteolysis is controlled by the spindle checkpoint and reveals when the APC/C switches from activation by Cdc20 to Cdh1. *J. Cell Biol.* **157**, 1125-1137.

Hauf, S., Waizenegger, I. C. and Peters, J. M. (2001). Cohesin cleavage by separase required for anaphase and cytokinesis in human cells. *Science* **293**, 1320-1323.

Hauf, S., Cole, R. W., LaTerra, S., Zimmer, C., Schnapp, G., Walter, R., Heckel, A., van Meel, J., Rieder, C. L. and Peters, J. M. (2003). The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *J. Cell Biol.* **161**, 281-294.

Hauf, S., Roitinger, E., Koch, B., Dittrich, C. M., Mechtler, K. and Peters, J. M. (2005). Dissociation of cohesin from chromosome arms and loss of arm cohesion during early mitosis depends on phosphorylation of SA2. *PLoS Biol.* **3**, e69.

Hirota, T., Gerlich, D., Koch, B., Ellenberg, J. and Peters, J. M. (2004). Distinct functions of condensin I and II in mitotic chromosome assembly. *J. Cell Sci.* **117**, 6435-6445.

Hornig, N. C. and Uhlmann, F. (2004). Preferential cleavage of chromatin-bound cohesin after targeted phosphorylation by Polo-like kinase. *EMBO J.* **23**, 3144-3153.

Jager, H., Herzig, A., Lehner, C. F. and Heidmann, S. (2001). Drosophila separase is required for sister chromatid separation and binds to PIM and THR. *Genes Dev.* **15**, 2572-2584.

Kitajima, T. S., Kawashima, S. A. and Watanabe, Y. (2004). The conserved kinetochore protein shugoshin protects centromeric cohesion during meiosis. *Nature* **427**, 510-517.

Kitajima, T. S., Hauf, S., Ohsugi, M., Yamamoto, T. and Watanabe, Y. (2005). Human Bub1 defines the persistent cohesion site along the mitotic chromosome by affecting Shugoshin localization. *Curr. Biol.* **15**, 353-359.

Kitajima, T. S., Sakuno, T., Ishiguro, K., Iemura, S., Natsume, T., Kawashima, S. A. and Watanabe, Y. (2006). Shugoshin collaborates with protein phosphatase 2A to protect cohesin. *Nature* **441**, 46-52.

Kudo, N. R., Wassmann, K., Anger, M., Schuh, M., Wirth, K. G., Xu, H., Helmhart, W., Kudo, H., McKay, M., Maro, B. et al. (2006). Resolution of chiasmata in oocytes requires separase-mediated proteolysis. *Cell* **126**, 135-146.

Kueng, S., Hegemann, B., Peters, B. H., Lipp, J. J., Schleiffer, A., Mechtler, K. and Peters, J. M. (2006). Wapl controls the dynamic association of cohesin with chromatin. *Cell* **127**, 955-967.

Kumada, K., Nakamura, T., Nagao, K., Funabiki, H., Nakagawa, T. and Yanagida, M. (1998). Cut1 is loaded onto the spindle by binding to Cut2 and promotes anaphase spindle movement upon Cut2 proteolysis. *Curr. Biol.* **8**, 633-641.

Kumada, K., Yao, R., Kawaguchi, T., Karasawa, M., Hoshikawa, Y., Ichikawa, K., Sugitani, Y., Imoto, I., Inazawa, J., Sugawara, M. et al. (2006). The selective continued linkage of centromeres from mitosis to interphase in the absence of mammalian separase. *J. Cell Biol.* **172**, 835-846.

Lee, J., Iwai, T., Yokota, T. and Yamashita, M. (2003). Temporally and spatially selective loss of Rec8 protein from meiotic chromosomes during mammalian meiosis. *J. Cell Sci.* **116**, 2781-2790.

Lenart, P., Petronczki, M., Steegmaier, M., Di Fiore, B., Lipp, J. J., Hoffmann, M., Rettig, W. J., Kraut, N. and Peters, J. M. (2007). The small-molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. *Curr. Biol.* **17**, 304-315.

Lipp, J. J., Hirota, T., Poser, I. and Peters, J. M. (2007). Aurora B controls the association of condensin I but not condensin II with mitotic chromosomes. *J. Cell Sci.* **120**, 1245-1255.

Losada, A., Hirano, M. and Hirano, T. (1998). Identification of Xenopus SMC protein complexes required for sister chromatid cohesion. *Genes Dev.* **12**, 1986-1997.

Losada, A., Hirano, M. and Hirano, T. (2002). Cohesin release is required for sister chromatid resolution, but not for condensin-mediated compaction, at the onset of mitosis. *Genes Dev.* **16**, 3004-3016.

McGuinness, B. E., Hirota, T., Kudo, N. R., Peters, J. M. and Nasmyth, K. (2005). Shugoshin prevents dissociation of cohesin from centromeres during mitosis in vertebrate cells. *PLoS Biol.* **3**, e86.

Musacchio, A. and Salmon, E. D. (2007). The spindle-assembly checkpoint in space and time. *Nat. Rev. Mol. Cell Biol.* **8**, 379-393.

Nasmyth, K. and Haering, C. H. (2005). The structure and function of SMC and kleisin complexes. *Annu. Rev. Biochem.* **74**, 595-648.

Ono, T., Fang, Y., Spector, D. L. and Hirano, T. (2004). Spatial and temporal regulation of Condensins I and II in mitotic chromosome assembly in human cells. *Mol. Biol. Cell* **15**, 3296-3308.

Riedel, C. G., Katis, V. L., Katou, Y., Mori, S., Itoh, T., Helmhart, W., Galova, M., Petronczki, M., Gregan, J., Cetin, B. et al. (2006). Protein phosphatase 2A protects centromeric sister chromatid cohesion during meiosis I. *Nature* **441**, 53-61.

Rieder, C. L. and Palazzo, R. E. (1992). Colcemid and the mitotic cycle. *J. Cell Sci.* **102**, 387-392.

Salic, A., Waters, J. C. and Mitchison, T. J. (2004). Vertebrate shugoshin links sister centromere cohesion and kinetochore microtubule stability in mitosis. *Cell* **118**, 567-578.

Sumara, I., Vorlaufer, E., Stukenberg, P. T., Kelm, O., Redemann, N., Nigg, E. A. and Peters, J. M. (2002). The dissociation of cohesin from chromosomes in prophase is regulated by Polo-like kinase. *Mol. Cell* **9**, 515-525.

Sumara, I., Gimenez-Abian, J. F., Gerlich, D., Hirota, T., Kraft, C., de la Torre, C., Ellenberg, J. and Peters, J. M. (2004). Roles of polo-like kinase 1 in the assembly of functional mitotic spindles. *Curr. Biol.* **14**, 1712-1722.

Tang, Z., Sun, Y., Harley, S. E., Zou, H. and Yu, H. (2004). Human Bub1 protects centromeric sister-chromatid cohesion through Shugoshin during mitosis. *Proc. Natl. Acad. Sci. USA* **101**, 18012-18017.

Tang, Z., Shu, H., Qi, W., Mahmood, N. A., Mumby, M. C. and Yu, H. (2006). PP2A is required for centromeric localization of Sgo1 and proper chromosome segregation. *Dev. Cell* **10**, 575-585.

Uhlmann, F., Wernic, D., Poupart, M. A., Koonin, E. V. and Nasmyth, K. (2000). Cleavage of cohesin by the CD clan protease separin triggers anaphase in yeast. *Cell* **103**, 375-386.

Waizenegger, I. C., Hauf, S., Meinke, A. and Peters, J. M. (2000). Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase. *Cell* **103**, 399-410.

Wirth, K. G., Wutz, G., Kudo, N. R., Desdouets, C., Zetterberg, A., Taghybeeglu, S., Seznec, J., Ducos, G. M., Ricci, R., Firnberg, N. et al. (2006). Separase: a universal trigger for sister chromatid disjunction but not chromosome cycle progression. *J. Cell Biol.* **172**, 847-860.