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Regulation of Sister Chromatid Cohesion between Chromosome Arms

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Summary

Sister chromatid separation in anaphase depends on the removal of cohesin complexes from chromosomes [1]. In vertebrates, the bulk of cohesin is already removed from chromosome arms during prophase and prometaphase [2, 3], whereas cohesin remains at centromeres until metaphase, when cohesin is cleaved by the protease separase [3, 4]. In unperturbed mitoses, arm cohesion nevertheless persists throughout metaphase and is principally sufficient to maintain sister chromatid cohesion [5]. How arm cohesion is maintained until metaphase is unknown. Here we show that small amounts of cohesin can be detected in the interchromatid region of metaphase chromosome arms. If prometaphase is prolonged by treatment of cells with microtubule poisons, these cohesin complexes dissociate from chromosome arms, and arm cohesion is dissolved. If cohesin dissociation in prometaphase-arrested cells is prevented by depletion of Plk1 or inhibition of Aurora B, arm cohesion is maintained. These observations imply that, in unperturbed mitoses, small amounts of cohesin maintain arm cohesion until metaphase. When cells lacking Plk1 and Aurora B activity enter anaphase, chromatids lose cohesin. This loss is prevented by proteasome inhibitors, implying that it depends on separase activation. Separase may therefore be able to cleave cohesin at centromeres and on chromosome arms.

Results and Discussion

Cohesin Persists in the Interchromatid Region of Chromosome Arms until Metaphase

Although cohesin begins to dissociate from chromosome arms in prophase [2, 3, 6–8], cohesion normally exists between both centromeric and arm regions of sister chromatids until metaphase. Cohesion is thought to be particularly strong at centromeres, but cohesion also persists until anaphase onset in acentric fragments. This observation indicates that centromeres are not required to maintain arm cohesion (reviewed by [5]). By severing centromeric cohesion with a laser microbeam, Rieder and Cole demonstrated that arm cohesion can even be sufficient to resist the pulling forces of the mitotic spindle until anaphase [5].

To address whether cohesion between chromosome arms is maintained by cohesin-independent mechanisms or by small amounts of cohesin, we reinvestigated cohesin localization on mitotic chromosomes in HeLa cells. Previous work had revealed the presence of cohesin on the arms of human prometaphase chromosomes [3, 7] and on chromosomes assembled in vitro in *Xenopus* egg extracts [9], but arm staining was not prominent on chromosomes of human cells in metaphase. In the present study, we utilized HeLa cells that stably express a myc-tagged version of the cohesin subunit Scc1, which is expressed at physiological levels and is incorporated into functional cohesin complexes [3, 4], and we examined the chromosomal localization of tagged cohesin by using a chromosome-spreading technique. Myc antibodies homogeneously stained a central thick rod in prophase chromosomes (Figure 1A). In prometaphase (Figure 1B) and metaphase cells (Figure 1C), Scc1-myc appeared to be concentrated at centromeres, but it is important to note that weak staining could also often be observed on chromosome arms (indicated by arrows in Figure 1C). Some metaphase figures only showed very little arm staining (data not shown). It is possible that these chromosomes were derived from cells that had already progressed to a later stage of metaphase. However, because not all cells of the cell line used in this experiment express detectable amounts of Scc1-myc [3], we suspect that the abundance of Scc1-myc and not the duration in metaphase determined how clearly cohesin could be detected on chromosome arms. As observed earlier [3], Scc1-myc could never be seen on sister chromatids in anaphase (Figure 1D). Colocalization experiments revealed that Scc1-myc was enriched inbetween the topoisomerase II (topo II) and condensin-containing axial structures of prometaphase and metaphase chromosomes, i.e., in the interchromatid region (Figure 1E and data not shown), consistent with a role of these cohesin complexes in maintaining cohesion between chromosome arms.

The Complete Dissociation of Cohesin from Chromosome Arms Correlates with the Loss of Arm Cohesion

Although arm cohesion is normally maintained until anaphase onset, it has been known since the 1930s that arm cohesion is lost if prometaphase is delayed by treatment with microtubule poisons such as colcemid or nocodazole (colcemid mitosis/c-mitosis; [10–13]; reviewed by [14]). These compounds activate the spindle checkpoint, a surveillance mechanism that delays anaphase until all chromosomes have been attached to both spindle

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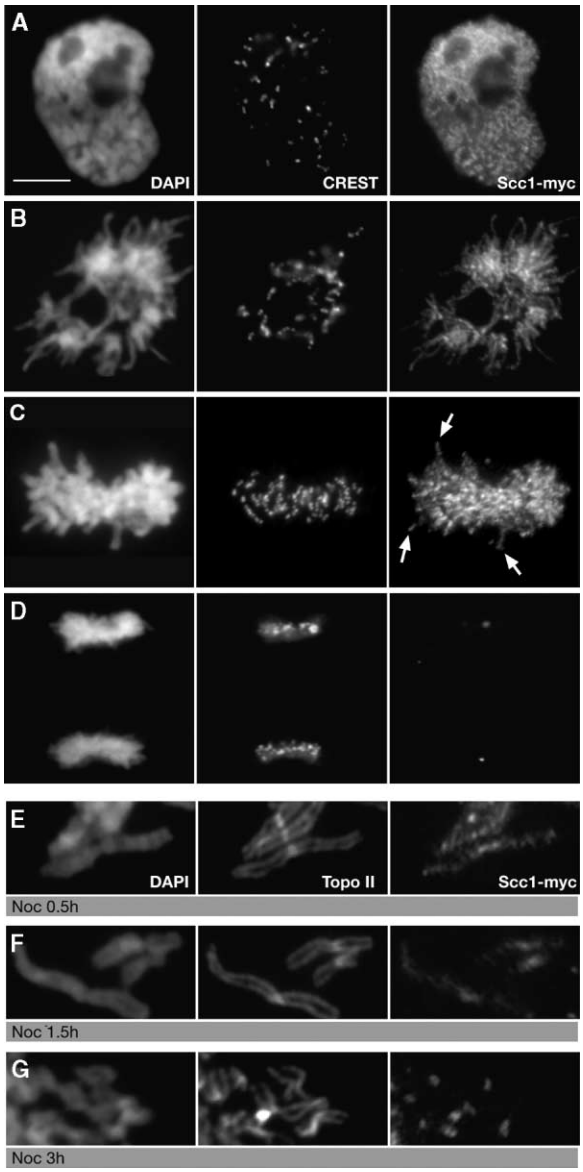

Figure 1. Cohesin Can Be Detected on Arms of Metaphase Chromosomes as Long as Arm Cohesion Is Maintained.

(A–D) Analysis of Scc1-myc localization in fixed mitotic cells by immunofluorescence microscopy. Specimens were prepared from synchronized HeLa cells expressing Scc1-myc and costained with CREST sera that recognize kinetochores (middle panels) and antibodies to the myc epitope (right panels). DNA was counterstained with DAPI (left panels). The images shown are from cells in prophase (A), prometaphase (B), metaphase (C), and anaphase (D). Scc1-myc can be seen on chromosome arms in metaphase (C) but not in anaphase (D). Note that some Scc1-myc is also detected at spindle poles (D), consistent with an earlier report [20]. The scale bar represents 10 μm.

(E, F) Scc1-myc localization after nocodazole treatment. Mitotic cells were collected by shake-off after 8 hr from the release from double thymidine block, and nocodazole was added for 0.5 hr (E), 1.5 hr (F), or 3 hr (G), and Scc1-myc localization was analyzed. Cells were costained with antibodies to Topo II (middle panels) and the myc epitope (right panels). DNA was counterstained with DAPI (left panels).

poles. Under these conditions, chromosome arms open, whereas centromeric cohesion is maintained and thereby results in the typical X or V shape (depending on the position of the centromere) that is seen in chromosome spreads from cells treated with spindle poisons.

To test if arm cohesion is mediated by residual amounts of cohesin, we analyzed Scc1-myc staining in nocodazole-treated cells. Staining with Giemsa, DAPI, or topo II antibodies revealed that chromosome arms opened between 1.5 and 3 hr of nocodazole treatment (Figures 1E–1G, 2G, 2H, and data not shown). Scc1-myc could be seen in the interchromatid region of chromosomes in which arm cohesion was still present (Figures 1E and 1F). In contrast, Scc1-myc was exclusively seen at centromeres but never in arm regions in chromosomes whose arms had opened (Figure 1G). These observations are consistent with the notion that arm cohesion is maintained by residual amounts of cohesin.

Plk1 and Aurora B Are Required for the Complete Dissociation of Cohesin from Chromosome Arms

To directly test the above hypothesis, we wanted to analyze if the loss of arm cohesion in prometaphase-arrested cells depends on the removal of cohesin complexes. To achieve this, we first had to identify conditions that inhibit cohesin dissociation in prometaphase. In in vitro assays with *Xenopus* egg extracts, the separase-independent dissociation of cohesin from chromosomes depends on the Polo-like kinase Plx1 [15] and on Aurora B [16], but it is unknown if these kinases are required for cohesin dissociation in living cells.

To address this question, we first inhibited expression of Plk1 by using RNA interference (RNAi), which arrests cells in a prometaphase-like state (I.S. and J.-M.P., unpublished data), and then analyzed the behavior of cohesin. In immunoblot experiments, Scc1 could be detected in chromosome fractions from Plk1 RNAi cells, whereas most Scc1 had been lost from chromosomes in mitotic cells obtained by nocodazole arrest (Figure 2A). Immunofluorescence microscopy of Plk1-depleted cells revealed Scc1-myc staining along the entire length of prometaphase chromosomes, with equal intensity between arm and centromere regions (Figure $2B_b$). The intensity of Scc1-myc signals on chromosome arms was much higher in Plk1 RNAi cells than Scc1-myc staining was on the arms of control prometaphase chromosomes (compare Figures $2B_a$ and $2B_b$).

To confirm these results in living cells, we inhibited Plk1 expression in a rat kidney (NRK) cell line that stably expresses a form of the cohesin subunit SA1 that is N-terminally tagged with enhanced green fluorescent protein (EGFP). The levels of EGFP-SA1 in this cell line are below the levels of endogenous SA1, and EGFP-SA1 is incorporated into cohesin complexes in which chromatin association and dissociation behavior is indistinguishable from the behavior of endogenous cohesin (D.G., F. Dupeux, J.-M.P. and J.E., unpublished data). In mitosis, enrichment of EGFP-SA1 molecules on chromosomes was difficult to observe at high resolution due to a high soluble pool in the cytoplasm. Therefore, we reduced cytoplasmic fluorescence by photobleaching a small region in the cellular periphery (Figure 2C).

Then, EGFP-SA1 was typically seen enriched in patches, which presumably represent centromeric regions on prometaphase and metaphase chromosomes (indicated by arrows in the middle panel of Figure 2C). In contrast, in Plk1 RNAi cells, EGFP-SA1 was distributed at high levels nearly homogeneously along the entire length of the chromosomes (Figures 2C and 2D). These data indicate that Plk1 is not only required for cohesin dissociation in *Xenopus* extracts in vitro, but also in vertebrate cells in vivo.

To address if Aurora B is also required for cohesin dissociation in human cells, we treated cells with Hesperadin, a small molecule that inhibits the activity of Aurora B [17]. Immunofluorescence microscopy revealed that Scc1-myc was present in higher amounts on chromosome arms of Hesperadin-treated prometaphase cells than on control prometaphase cells (Figure $2B_c$), indicating that Aurora B is also required for cohesin dissociation in human cells. In these experiments, we also noticed that Aurora B and Plk1 are not only required for cohesin dissociation from chromosome arms but also for formation of the primary constriction at the centromeres (See Figures S1 and S2 in the Supplemental Data available with this article online). The possible implications of these findings are discussed in the Supplemental Data.

Inhibition of Cohesin Dissociation from Chromosome Arms Prevents Loss of Arm Cohesion in Prometaphase-Arrested Cells

The finding that Plk1 and Aurora B are required for cohesin dissociation from chromosome arms in prometaphase allowed us to directly test if the loss of arm cohesion depends on the removal of these complexes. First, we treated Plk1 RNAi cells with nocodazole for different periods of time and measured the presence of cohesin on chromosome arms and the maintenance of arm cohesion. The latter was analyzed by chromosome spreading and Giemsa staining, which visualizes particularly clearly if arm cohesion is present (Figure 2E) or absent (Figure 2F). The levels of Scc1-myc on chromosome arms remained high over a 4 hr time course (Figure $2B_e$) and arm cohesion was maintained in the chromosomes of almost all cells (Figures 2G and S3). In contrast, Scc1-myc dissociated from chromosome arms in nocodazole treated control cells (Figure $2B_d$), and after 4 hr arm cohesion had been lost in 80% of these cells (Figure 2G). Scc1-myc was also maintained on chromosome arms when cells were treated with nocodazole and Hesperadin (Figure $2B_f$), and 70% of these cells maintained arm cohesion during the course of the experiment (Figure 2H). These data indicate that the loss of arm cohesion in nocodazole treated cells depends on the complete dissociation of cohesin from chromosome arms. Cohesion between chromosome arms therefore appears to be mediated by residual amounts of cohesin complexes.

In an Unperturbed Mitosis, Residual Amounts of Cohesin Appear to Be Removed from Chromosome Arms by Separase

Our observations imply that cohesin can be completely removed from chromosome arms in a prolonged prometaphase arrest, resulting in opening of chromosome arms, but that unperturbed cells normally arrive in metaphase with some cohesin complexes on their arms. To address if cohesin on chromosome arms can be cleaved by separase at the metaphase-anaphase transition, we inactivated the early mitotic cohesin dissociation pathway by simultaneously depleting Plk1 and inhibiting Aurora B and then analyzed the behavior of cohesin. Cells lacking Plk1 are unable to enter anaphase due to activation of the spindle checkpoint, but inhibition of Aurora B overrides the checkpoint arrest and allows rapid exit from mitosis without chromosome segregation and cytokinesis (I.S. and J.-M.P., unpublished data). Immunofluorescence microscopy revealed that Hesperadin treatment of Plk1 RNAi cells caused a strong decrease in the number of mitotic cells that contain Scc1-myc on chromosomes, whereas the number of mitotic cells without Scc1-myc on chromosome increased (Figures 3A and 3C). (Note that one third of mitotic cells did not contain Scc1-myc on chromosomes at the beginning of the experiment, because the Scc1-myc transgene is only detectably expressed in two thirds of the cells.) At the same time, the frequency of cells with decondensed chromosomes increased, indicating that many cells exited mitosis. Cells which had just begun to exit mitosis contained no or little Scc1-myc on their chromosomes, whereas cells which had completed mitotic exit and had formed reconstitution nuclei showed Scc1-myc staining on their chromatin (Figure 3A). These observations imply that cohesin is transiently removed from chromosomes when cells begin to exit mitosis, but that cohesin rapidly rebinds to chromatin after mitotic exit. In unperturbed cells, cohesin also reassociates with chromosomes during telophase and G1 [2, 3, 6]. In contrast, the frequency of mitotic cells that contained Scc1-myc on chromosomes remained constant in Plk1 RNAi cells that were not treated with Hesperadin (Figure 3B).

The observation that cohesin is lost from chromosomes during mitotic exit even in the absence of Plk1 and Aurora B activity suggests that this process may require separase activation. Separase activation depends on ubiquitination of the separase inhibitor securin by the anaphase-promoting complex/cyclosome (APC) and subsequent degradation of securin by the proteasome. Once activated, separase cleaves itself and Scc1 [3, 18]. Degradation of the APC/C substrate cyclin B and cleavage of separase and Scc1 could be observed in Plk1 depleted cells after treatment with Hesperadin (I.S. and J.-M.P., unpublished data), consistent with the possibility that cohesin is removed from chromosomes by separase. To further test this possibility, we analyzed the behavior of cohesin in Plk1 RNAi cells that were treated with both Hesperadin and the proteasome inhibitor MG132, which should prevent separase activation. Under these conditions the number of cells containing Scc1-myc positive chromosomes remained almost constant during the course of the experiment (Figure 3D), supporting the notion that separase is able to remove cohesin from both chromosome arms and centromeres.

A Refined Model of the Differential Regulation of Arm Versus Centromere Cohesion

It is widely accepted that cohesin is essential in vertebrate cells for maintaining cohesion at centromeres until

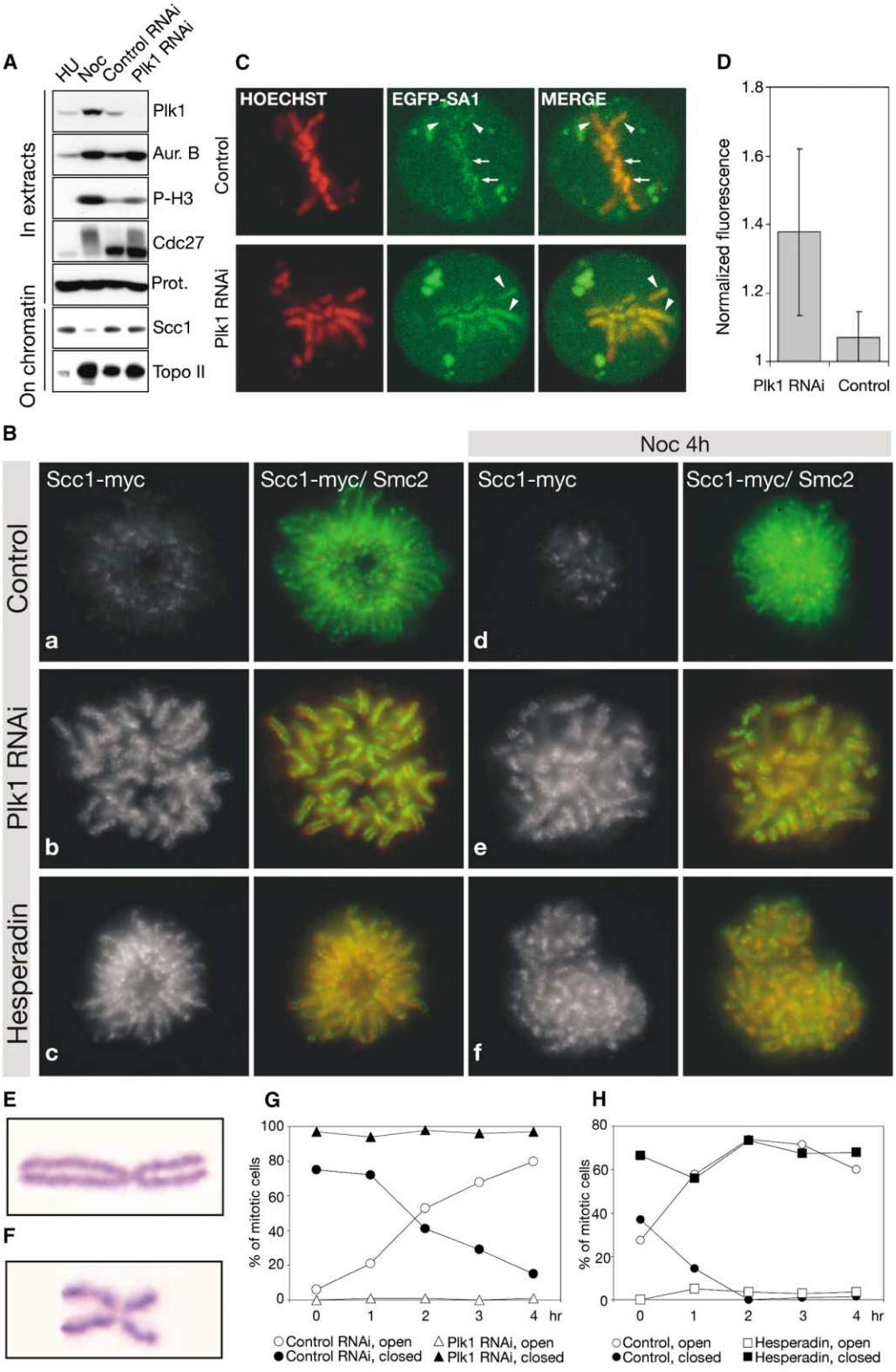

Figure 2. Loss of Plk1 Expression or Inhibition of Aurora B Reduces Dissociation of Cohesin from Chromosome Arms in Prometaphase

(A) HeLa cells arrested in S-phase by thymidine treatment were released for 8 hr and then incubated with transfection mixtures either lacking (lane 3) or containing Plk1 siRNA (lane 4). Together with the transfection mixture, thymidine was added a second time to arrest cells at the onset of S-phase. Eighteen hours after release from the second arrest, cells were collected, cytoplasmic fractions ("In extracts") and chromatin fractions ("On chromatin") were isolated and analyzed by SDS-PAGE, and immunoblotting was performed with antibodies against the indicated proteins. In parallel, cells were released from the second thymidine arrest into hydroxyurea (lane 1) or into nocodazole (lane 2) and analyzed 18 hr later as above. (B) Immunofluorescence analysis of cohesin dissociation. Plk1 expression was inhibited in synchronized HeLa cells

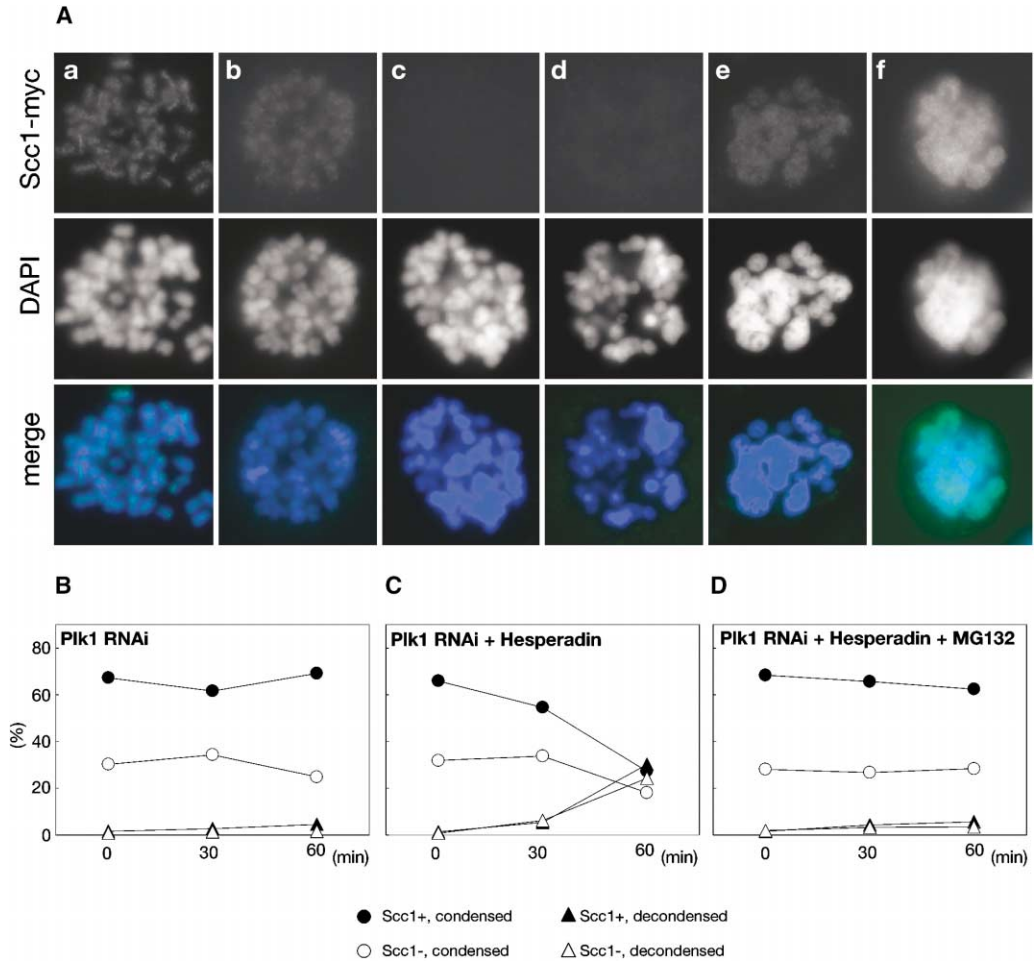

Figure 3. Cohesin Can Be Removed from Chromosomes in the Absence of Plk1 and Aurora B Activity at Anaphase Onset.

Plk1 expression was inhibited in synchronized HeLa cells expressing Scc1-myc. Cells were collected by shake-off at 13 hr after the release from the second thymidine block. To override the prometaphase arrest induced by Plk1 depletion, the cells were incubated with Hesperadin, in the absence (C) or presence (D) of MG132 for indicated times, and Scc1-myc distribution was analyzed by immunofluorescence microscopy (A). Control samples were obtained at the identical time points without Hesperadin treatment (B).Cells were classified based on the Scc1-myc staining and chromosome condensation. (A) shows representative images of cells with strong (a) or weak (b) myc staining with condensed chromosomes, cells negative for myc staining with condensed (c) or decondensed (d) chromosomes, and cells with weak (e) or strong (f) myc staining with decondensed chromosomes. For each experiment, 200–300 cells were assessed for myc staining and classified as above. Frequency of category (a) plus (b) [Scc1+, condensed], (c) [Scc1-, condensed], (d) [Scc1-, decondensed], and (e) plus (f) [Scc1+, decondensed] were plotted over time (B-D).

expressing Scc1-myc as described in (A). For Aurora B inhibition, Hesperadin was added 7 hr after the release from the second thymidime block at the final concentration of 100 nM [4]. Cells were collected 11.5 hr after the release with (panels d–f) or without (panels a–c) nocodaozle treatment for 4 hr, and subjected to immunofluorescence analysis with antibodies to the myc epitope (first panels of each experiment) and antibodies against Smc2, a subunit of condensin I and II. Merged pictures of Scc1-myc (red) and Smc2 (green) are shown in the right-hand panels. (C) Still images from video microscopy movies of living NRK cells that stably express the cohesin subunit SA1 tagged at its N terminus with EGFP (green). Cells had either been left untreated (top) or were transfected with Plk1 siRNA (bottom). DNA was visualized by addition of 0.2 μg/ml Hoechst 33342, 30 min before the onset of imaging (red). The cytoplasmic background fluorescence from soluble EGFP-SA1 was removed by repeated photobleaching at regions outside of chromatin. The arrowheads indicate chromosome arms that contain lower EGFP-SA1 signals in control cells than in Plk1 RNAi cells. Arrows in the top panel indicate EGFP-SA1-enriched sites, which might represent centromeres. Cytoplasmic fluorescent aggregates are induced by the transfection procedure. (D) EGFP-SA1 fluorescence was measured on chromosome arms of control NRK cells or Plk1 RNAi cells as in (C), except that these cells were not photobleached. Fluorescence intensity was determined as the mean of three intensity measurements on chromosome arm regions in each cell and normalized to mean cytoplasmic fluorescence. For Plk1 RNAi and control cells each, $n = 10$ cells. A Student's t test demonstrated that the difference between control and Plk1 RNAi cells was significant ($p < 0.01$). (E–H) Loss of arm cohesion in Plk1-depleted or Aurora-B-inhibited cells was analyzed by Giemsa staining of chromosome spreads, which unambiguously reveals if arm cohesion is present or absent (see examples in [E] and [F], respectively). Plk1 expression was inhibited in synchronized HeLa cells as in (A), and the frequency of spreads with chromosomes having arms that had opened or remained closed was plotted over time (G). For Aurora B inhibition, logarithmically proliferating HeLa cells were treated for 30 min with Nocodazole in either the absence or presence of Hesperadin, mitotic cells were collected by shake-off and cultured in nocodazole, and either in the absence or presence of Hesperadin for up to 4 hr (H). The difference in the kinetics of arm opening in control cells in (G) and (H) may be due to the different experimental settings.

anaphase onset, but so far, it has remained unclear if cohesion between chromosome arms is also mediated by cohesin binding or by alternative mechanisms. Several observations indicate that cohesin maintains arm cohesion in a normal mitosis. First, small amounts of cohesin can be detected in the interchromatid region of chromosome arms in prometaphase [3, 7] and metaphase (Figure 1C). Second, the loss of cohesion between chromosome arms in prometaphase-arrested cells correlates with the loss of cohesin from chromosome arms (Figure 1G). Third, inhibition of cohesin dissociation in prometaphase-arrested cells by either Plk1 depletion or inhibition of Aurora B prevents the opening of chromosome arms (Figures 2G and 2H).

Although cohesin can be completely removed from chromosome arms by Plk1 and Aurora B-dependent mechanisms in prometaphase-arrested cells, our results imply that those cohesin complexes that still reside on chromosome arms in metaphase are removed from chromosomes at anaphase onset by separase mediated Scc1 cleavage. First, the cleavage-independent removal of cohesin in prometaphase-arrested cells takes a few hours until it results in loss of arm cohesion (Figures 1E–1G, 2G and 2H), whereas loss of cohesion in anaphase usually occurs earlier in an unperturbed mitosis; for example, in Ptk1 cells, anaphase is initiated 23 min after completion of prometaphase [19]. Second, cohesin disappears from chromatids in anaphase, even in cells that lack Plk1 and Aurora B activity, i.e., even when the cleavage-independent cohesin dissociation pathway has been inactivated (Figure 3). Under these conditions, the removal of cohesin from chromosomes requires proteasome activity, implying that APC/C and separase activation are necessary.

In human cells, separase cleaves only a minor fraction of cohesin at the onset of anaphase [3]. So far, this fraction was thought to represent cohesin at centromeres, and it was therefore conceivable that specific properties of centromeres are required for cohesin cleavage. However, our data suggest that separase can also cleave cohesin on chromosome arms, implying that separase's substrate specificity may be determined by some feature common to all cohesin complexes bound to chromosomes.

Our findings suggest that the differential regulation of arm versus centromere cohesion, a phenomenon that has been recognized more than sixty years ago (reviewed by [14]), is a reflection of the differential regulation of cohesin complexes (schematically summarized in Figure 4). We propose that cohesin on chromosome arms can be removed by either kinase- or separase-dependent mechanisms, explaining why chromosome arms can open in prolonged prometaphase arrests but normally do not open until anaphase. In contrast, cohesin at centromeres can be removed from chromosomes only by separase, explaining why centromeres remain connected for many hours in prometaphase-arrested cells and normally separate only in anaphase.

Supplemental Data

Supplemental Data including Supplemental Results and Discussion, Experimental Procedures, and three additional figures are available at http://www.current-biology.com/cgi/content/full/14/13/1187/DC1/.

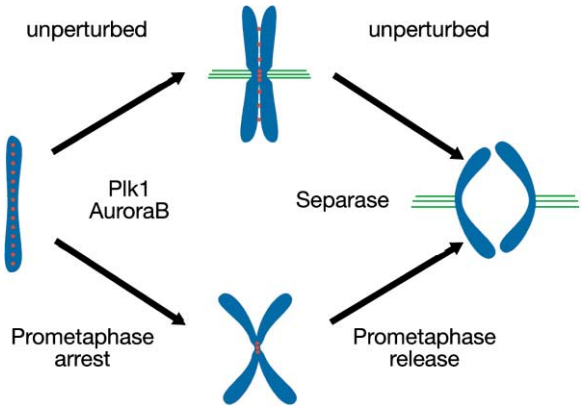

Figure 4. A Model that Illustrates the Differential Regulation of Cohesion between Sister Chromatid Arms and Centromeres

In unperturbed mitoses, Plk1- and Aurora-B-dependent mechanisms remove the bulk of cohesin (red dots) from chromosome arms, but cohesin persists at centromeres and in small amounts on chromosome arms. In normal metaphase cells, cohesion is therefore maintained between sister chromatid centromeres and arms. If prometaphase is delayed by spindle poisons, Plk1- and Aurora-B-dependent mechanisms can completely remove cohesin from chromosome arms, whereas cohesin at centromeres remains protected. Under these conditions, cohesion is therefore lost between sister chromatid arms but maintained at centromeres. When cells reach metaphase, either normally or after washout of spindle poisons, separase is activated and cleaves those cohesin complexes that are still associated with chromosomes.

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