Aurora B controls the association of condensin I but not condensin II with mitotic chromosomes

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Accepted 1 February 2007
Journal of Cell Science 120, 1245-1255 Published by The Company of Biologists 2007
doi:10.1242/jcs.03425

Summary

The assembly of mitotic chromosomes is controlled by condensin complexes. In vertebrates, condensin I binds to chromatin in prometaphase, confers rigidity to chromosomes and enables the release of cohesin complexes from chromosome arms, whereas condensin II associates with chromosomes in prophase and promotes their condensation. Both complexes are essential for chromosome segregation in anaphase. Although the association of condensins with chromatin is important for the assembly and segregation of mitotic chromosomes, it is poorly understood how this process is controlled. Here we show that the mitotic kinase Aurora B regulates the association of condensin I, but not the interaction of condensin II with chromatin. Quantitative time-lapse imaging of cells expressing GFP-tagged condensin subunits revealed that Aurora B is required for efficient loading of condensin I onto chromosomes in prometaphase and for maintenance of the complex on chromosomes in later stages of mitosis. The three non-SMC subunits of condensin I are Aurora B substrates in vitro and their mitosis-specific phosphorylation depends on Aurora B in vivo. Our data indicate that Aurora B contributes to chromosome rigidity and segregation by promoting the binding of condensin I to chromatin. We have also addressed how Aurora B might mediate the dissociation of cohesin from chromosome arms.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/7/1245/DC1

Key words: Mitosis, Chromosome condensation, Aurora B, Condensin, Sgo1

Introduction

In mitosis and meiosis, the structure of eukaryotic chromosomes changes dramatically. In interphase, chromosomes occupy relatively large territories in which individual sister chromatids cannot be distinguished (Bolzer et al., 2005; Cremer et al., 2006). In prophase, the volume that is occupied by chromosomes becomes much smaller, chromosomes can thus be observed as individual elongated structures, and sister chromatids are partially resolved from each other in chromosome arm regions (Swedlow and Hirano, 2003). These morphological changes are believed to facilitate the attachments of chromosomes to the mitotic or meiotic spindle and the separation of chromosomes or sister chromatids in anaphase.

The morphological changes of chromosomes in early mitosis and meiosis are caused at least in part by changes in chromosomal protein composition and in the post-translational modification of chromosomal proteins. In mitotic *Xenopus* egg extracts, both phosphorylation of the linker histone H1 (Maresca et al., 2005) and association of the condensin complex with chromosomes contribute to chromatin condensation (Hirano et al., 1997; Hirano and Mitchison, 1994). However, in animal cells, assembly of metaphase chromosomes is not or only mildly affected if condensin subunits are deleted or inactivated (Bhat et al., 1996; Gassmann et al., 2004; Giet and Glover, 2001; Hagstrom et al., 2002; Hirota et al., 2004; Hudson et al., 2003; Kaitna et al., 2002; Saka et al., 1994; Savvidou et al., 2005; Steffensen et al., 2001). Chromosomes from such cells show increased sensitivity to hypotonic conditions (Hirota et al., 2004; Hudson et al., 2003; Ono et al., 2004) and higher elasticity in their centromeric regions (Gerlich et al., 2006), indicating that condensin is important to confer rigidity to mitotic chromosomes but is dispensable for chromosome condensation.

The canonical condensin complex is composed of two large ATPases of the structural maintenance of chromosomes (SMC) family, called Smc2-CAP-E and Smc4-CAP-C and of three non-SMC subunits (reviewed by Hagstrom and Meyer, 2003; Swedlow and Hirano, 2003). In human cells, the non-SMC subunits are called CAP-D2, CAP-G and kleisin-γ–CAP-H–hBARREN. Recently, a second condensin complex, called condensin II, has been identified in vertebrates (Ono et al., 2003; Schleiffer et al., 2003; Yeong et al., 2003). This complex contains the same Smc2 and Smc4 subunits that are present in the canonical condensin complex (which is now called condensin I) but in condensin II these subunits are bound to three different proteins, kleisin-β, CAP-D3 and CAP-G2. Whereas condensin I is cytoplasmic during interphase and prophase and can thus associate with chromosomes only after nuclear envelope breakdown (NEBD) in prometaphase, condensin II is nuclear, associates with chromosomes during prophase, and is required for normal kinetics of chromosome condensation (Gerlich et al., 2006; Hirota et al., 2004; Ono et

al., 2004; Yeong et al., 2003). Both condensin I and II are essential for proper chromosome segregation during anaphase (Gerlich et al., 2006; Hagstrom and Meyer, 2003).

While condensins associate with chromosomes in prophase and prometaphase, a related complex, called cohesin, dissociates from chromosome arms. Like condensins, cohesin is composed of two ATPases of the SMC family, called Smc1 and Smc3, that are bound to two non-SMC subunits, which in vertebrate cells are called Scc1/Rad21 and SA2 (Losada et al., 1998; Sumara et al., 2000). Cohesin is essential for the cohesion that holds sister chromatids together from S-phase until the subsequent anaphase (reviewed by Hagstrom and Meyer, 2003; Nasmyth and Haering, 2005). During prophase and prometaphase the bulk of cohesin is removed from chromosome arms by a pathway that depends in part on condensin I and on the mitotic kinases Aurora B and Plk1 (Gimenez-Abian et al., 2004; Hirota et al., 2004; Losada et al., 2002; Sumara et al., 2002). This cohesin dissociation process also requires phosphorylation of the cohesin SA2 subunit (Hauf et al., 2005), which is believed to be mediated by Plk1 (Losada et al., 2002; Sumara et al., 2002). By contrast, cohesin that is bound to centromeres is protected from this 'prophase pathway' by the protein Sgo1 (Kitajima et al., 2005; McGuinness et al., 2005; Salic et al., 2004; Tang et al., 2004). Centromeric cohesin complexes can therefore only be removed from chromosomes by proteolytic cleavage of the cohesin Scc1 subunit, which occurs at the onset of anaphase and is mediated by the protease separase (Hauf et al., 2001; Waizenegger et al., 2000). The selective release of cohesin from chromosome arms in prophase is required for the resolution of sister chromatid arms and causes the characteristic X or V shape of mitotic chromosomes (Gimenez-Abian et al., 2004; Losada et al., 2002).

In contrast to the regulation of cohesin, little is known about the regulation of condensins in vertebrates. It has been shown that phosphorylation of condensin I by Cdk1 stimulates the ability of the complex to hydrolyse ATP and to introduce positive supercoils into DNA, but these modifications did not increase the ability of condensin I to bind to DNA (Kimura et al., 1998). Genetic experiments in *Drosophila* (Giet and Glover, 2001), worms (Hagstrom et al., 2002; Kaitna et al., 2002) and fission yeast (Petersen and Hagan, 2003) have shown that orthologues of Aurora B kinase are required for the association of condensin with chromosomes, and in budding yeast Aurora B/Ipl1 kinase is required for chromosome condensation in anaphase (Lavoie et al., 2004). However, it is not known how the association of condensin I and II with chromosomes is regulated in vertebrate cells.

Here we show that in human cells Aurora B is required for efficient loading of condensin I onto chromosomes in prometaphase and for maintenance of the complex on chromosomes in later stages of mitosis, but surprisingly condensin II can associate with chromosomes normally in the absence of Aurora B activity. All three non-SMC subunits of condensin I are Aurora B substrates in vitro and their mitosis-specific phosphorylation depends on Aurora B in vivo. These observations indicate that Aurora B contributes to chromosome rigidity and segregation by promoting the binding of condensin I to chromatin. We have also addressed how Aurora B may mediate the dissociation of cohesin from chromosome arms.

Results

Aurora B is required for the association of EGFP-kleisin-γ with chromosomes in prometaphase

To inactivate Aurora B, we first treated HeLa cells with Hesperadin, a compound that inhibits Aurora B (Hauf et al., 2003) by inserting into its ATP binding pocket (Sessa et al., 2005). At a concentration of 100 nM, Hesperadin prevents phosphorylation of histone H3 on Ser10 (Hauf et al., 2003), a modification that depends on Aurora B activity (Giet and Glover, 2001; Hauf et al., 2003; Hsu et al., 2000). At this concentration, the related kinase Aurora A is still active, as judged by its phosphorylation on Thr288 (see supplementary material Fig. S1) which depends on Aurora A activity (Eyers et al., 2003). We therefore used 100 nM Hesperadin for all experiments. To monitor the behavior of condensin complexes in real time, we performed quantitative video microscopy imaging on HeLa cells that stably express EGFP-tagged versions of either the condensin-I-specific subunit kleisin-γ or the condensin-II-specific subunit kleisin-β (Gerlich et al., 2006; Hirota et al., 2004).

These cells were synchronised with respect to their cell cycle state by double-thymidine treatment and were filmed during entry into mitosis. Chromosomes were visualised by applying the dye Hoechst 33342, and the chromosomal Hoechst signal was used as a reference to quantify changes in EGFP signal intensity on chromosomes. Addition of Hesperadin prior to entry into mitosis did not detectably change the association of EGFP-kleisin-β with chromatin in prophase (Fig. 1A,B), indicating that Aurora B activity is not required for association of condensin II with chromosomes.

Unlike condensin II, which is nuclear throughout interphase, the cytoplasmic condensin I complex only gains access to chromosomes at nuclear envelope breakdown (NEBD) and normally binds to them at the onset of prometaphase (Hirano, 2004; Ono et al., 2004). As shown before (Gerlich et al., 2006; Hirota et al., 2004) EGFP-kleisin-γ clearly reflected this behavior and accumulated on chromosomes rapidly after NEBD (Fig. 1C,D). However, when cells entered mitosis in the presence of Hesperadin, the association of EGFP-kleisin-γ with chromosomes was strongly reduced (Fig. 1C,D). Both the rate of association and the final levels that were reached until end of the experiment were greatly decreased (10 minutes after NEBD to 33±11% of the control value).

To test whether these effects were caused by inactivation of Aurora B, as opposed to being due to inhibition of some other Hesperadin-sensitive kinase, we used RNA interference to deplete Aurora B. Immunofluorescence (data not shown) and immunoblotting experiments (see supplementary material Fig. S2F) showed that Aurora B was depleted efficiently. Also when these cells were analysed, the rate of EGFP-kleisin-γ binding to chromosomes and the final levels achieved were substantially reduced (10 minutes after NEBD, 36±13%), whereas the chromosome association of EGFP-kleisin-β was not detectably affected (Fig. 1A,B and data not shown). These data show that Aurora B is required for the rapid and efficient association of EGFP-kleisin-γ with chromosomes at the onset of prometaphase.

Video microscopy of Hesperadin-treated and Aurora-B-depleted cells for longer periods of times showed that EGFP-kleisin-γ did also not accumulate to higher levels on mitotic chromosomes in later stages of mitosis (see supplementary

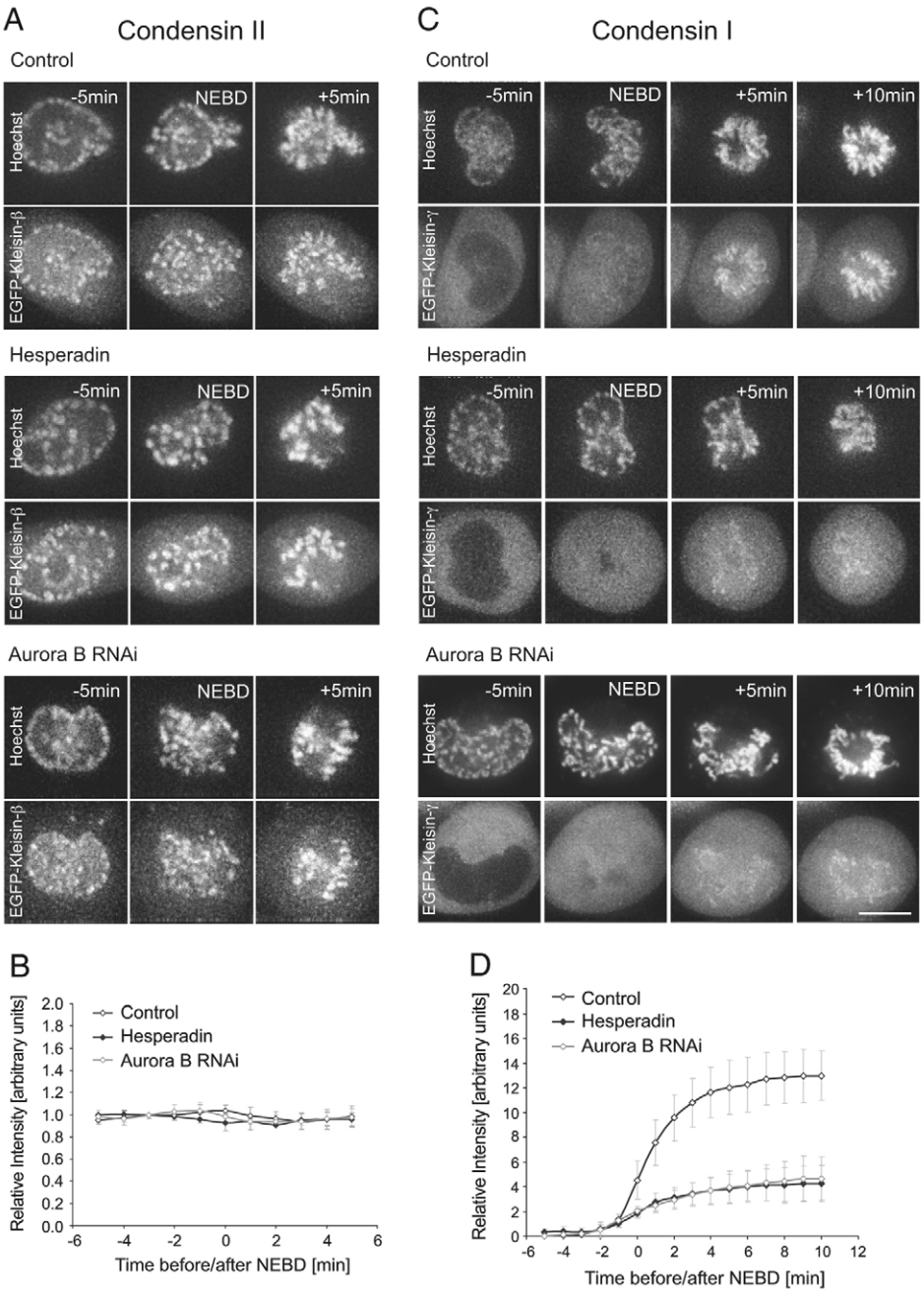

Fig. 1. Aurora B activity is required for loading condensin I in prometaphase. (A) HeLa cells stably expressing EGFP-tagged kleisin-β (condensin II) were enriched in S-phase by a double-thymidine arrest and synchronously released into mitosis. The levels of EGFP-kleisin-β associating with chromatin were monitored over time as cells entered mitosis. Chromosomes were visualised by adding the DNA-intercalating dye Hoechst 33342 (0.2 μg/ml). Aurora B activity was compromised either by adding Hesperadin (100 nM) 1 hour before the bulk of the cells entered mitosis or by depleting the protein with siRNAs specific to Aurora B for 30 hours. Movies were taken on an Olympus DeltaVision microscope (63× objective, three slices every 1 minute, 1×1 binning). Projected images are shown. (B) Quantification of chromatin-bound EGFP-kleisin-β in control, Hesperadin-treated and Aurora B siRNA-transfected cells over time. Intensities were quantified using the Hoechst signal as a reference. The average cytoplasmic intensity was subtracted as background intensity. Averages of seven cells (n=7) for each condition are shown. Time point 0 (t=0) corresponds to nuclear envelope breakdown (NEBD). (C,D) Same experiment as in A and B using a HeLa cell line stably expressing kleisin-γ tagged with EGFP (condensin I). Curves represented in D are averages from seven cells (n=7) for each condition.

material Fig. S2). In anaphase, however, chromosomal signals increased slightly by about 20% (compared with levels in metaphase). A similar increase has previously been observed in control cells (Gerlich et al., 2006). Aurora B inactivation therefore reduces both the rate of EGFP-kleisin-γ binding to chromosomes and the maximal levels that are reached during mitosis, but an increase in chromosome association of EGFP-kleisin-γ in anaphase does not depend on Aurora B. The behavior of the condensin II subunit EGFP-kleisin-β was unaffected by the addition of Hesperadin also at later stages of mitosis (see supplementary material Fig. S3).

Aurora B is required for the association of endogenous condensin I subunits with chromosomes

Previous experiments have indicated that EGFP-kleisin-β and EGFP-kleisin-γ are incorporated into functional condensin complexes (Gerlich et al., 2006; Hirota et al., 2004). We nevertheless wanted to exclude that the differential effects of Aurora B inactivation on EGFP-kleisin-β and EGFP-kleisin-γ are an artefact of the ectopic expression of these tagged proteins. We therefore analysed the effects of Aurora B inactivation on endogenous condensin subunits. For this purpose, we treated logarithmically proliferating HeLa cells with Hesperadin, arrested them with Nocodazole in mitosis, fixed them and analysed the specimens by immunofluorescence microscopy with condensin antibodies. Chromosome staining of Smc2, which is shared by condensin I and condensin II, showed a moderate but significant reduction in fluorescence intensity (64±9% of the control value) upon Hesperadin treatment. The signals of the condensin-I-specific

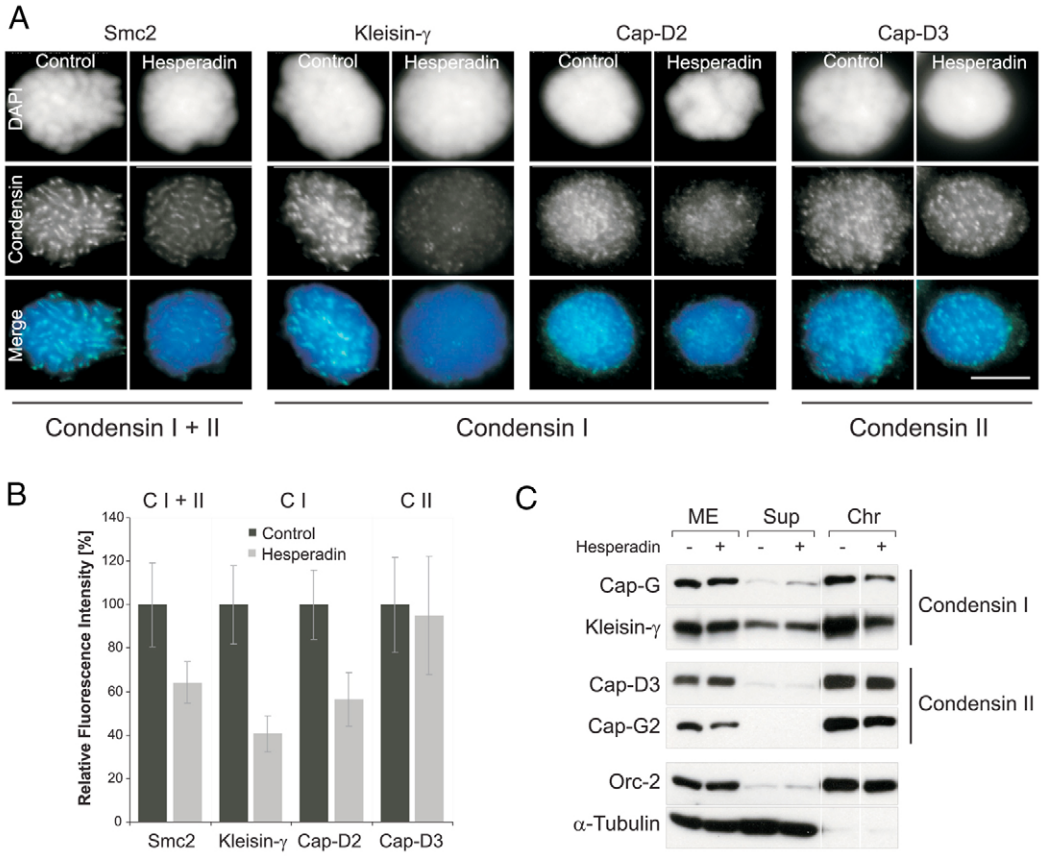

Fig. 2. Condensin I binding to chromatin is dependent on Aurora B activity. (A) Nocodazole-arrested human HeLa cells were either treated with 100 nM Hesperadin or DMSO for 1 hour, fixed and stained for the condensin subunits Smc2 (shared between condensin I and II), kleisin-γ, Cap-D2 (both condensin I specific) and Cap-D3 (condensin II specific). Chromosomes were counterstained with DAPI (1 μg/ml). Bar, 10 μm. (B) Average fluorescence intensities of condensin on chromatin were quantified using the DAPI channel as a reference and to determine the area occupied by chromatin. The background intensity was calculated by measuring the fluorescence intensity outside this area and subtracted from the condensin signal. The presented graphs are averaged from at least 30 different cells for each condition (n>30) and normalised to the control value ± s.d. (C) Biochemical fractionation of Nocodazole-arrested HeLa cells which had either been treated with 100 nM Hesperadin for 1 hour or left untreated before harvesting. The levels of condensin-I- (Cap-G and kleisin-γ) and condensin-II-specific subunits (Cap-D3 and Cap-G2) were detected by western blotting. Total mitotic extracts (ME), cytoplasm (Cyt) and chromatin (Chr) fractions are shown.

subunits kleisin-γ (41±8%) and Cap-D2 (57±12%) were also diminished, whereas the levels of the condensin-II-specific subunit Cap-D3 on mitotic chromosomes were not significantly changed (95±27%; Fig. 2A,B). Similar results were obtained when mitotic cells were used that had not been treated with Nocodazole (data not shown), indicating that the observed differences were not due to prolonged mitotic arrest.

To confirm these observations we prepared chromosomal and cytoplasmic fractions from Nocodazole-arrested HeLa cells and asked if the previous treatment of these cells with Hesperadin changed the relative distribution of condensin I and condensin II. Immunoblotting experiments revealed a reduction in the amounts of kleisin-γ, Cap-G and Cap-D2 in the chromosome pellet that was obtained from Hesperadin treated cells (Fig. 2C and data not shown). Probing the same membrane with antibodies against the condensin-II-specific subunits Cap-G2 and Cap-D3 revealed no detectable differences. In whole-cell lysates, condensin levels were unaffected by the addition of Hesperadin implying that the reduction of condensin I in chromosome pellets was not due to lower protein levels in the extract. Taken together, these data indicate that also the levels of endogenous condensin I on mitotic chromosomes, but not the levels of condensin II, are dependent on the activity of Aurora B.

Enrichment of condensin I at centromere-proximal regions depends on Aurora B

Our data so far indicated that Aurora B inactivation reduced the association of condensin I with mitotic chromosomes but did not entirely prevent it, perhaps because Aurora B activity was not completely abolished in our experiments. To determine whether condensin I loading was equally reduced in different chromosomal regions, we prepared chromosome spreads from mitotic HeLa cells under hypotonic conditions and analysed them by immunofluorescence microscopy with kleisin-γ and Cap-D2 antibodies. Under these conditions, chromosomes from control cells showed axial condensin I staining on chromosome arms and a slightly increased staining intensity at centromeres (Fig. 3A), consistent with data obtained in living cells that express GFP-tagged condensin I subunits (Gerlich et

al., 2006). However, chromosomes from Hesperadin-treated cells showed a different staining pattern. On these chromosomes, the staining intensities were reduced both in arm and centromere regions, but the reduction in signal intensity was much stronger at the centromere and in centromere-proximal regions (Fig. 3A-C and data not shown). Interestingly, Aurora B itself is enriched in these parts of the chromosome (reviewed by Andrews et al., 2003). It has previously been reported that depletion of Aurora B also reduced enrichment of condensin II at centromeres (Ono et al., 2004), but such an effect could not be observed in our experiments (see supplementary material Fig. S4).

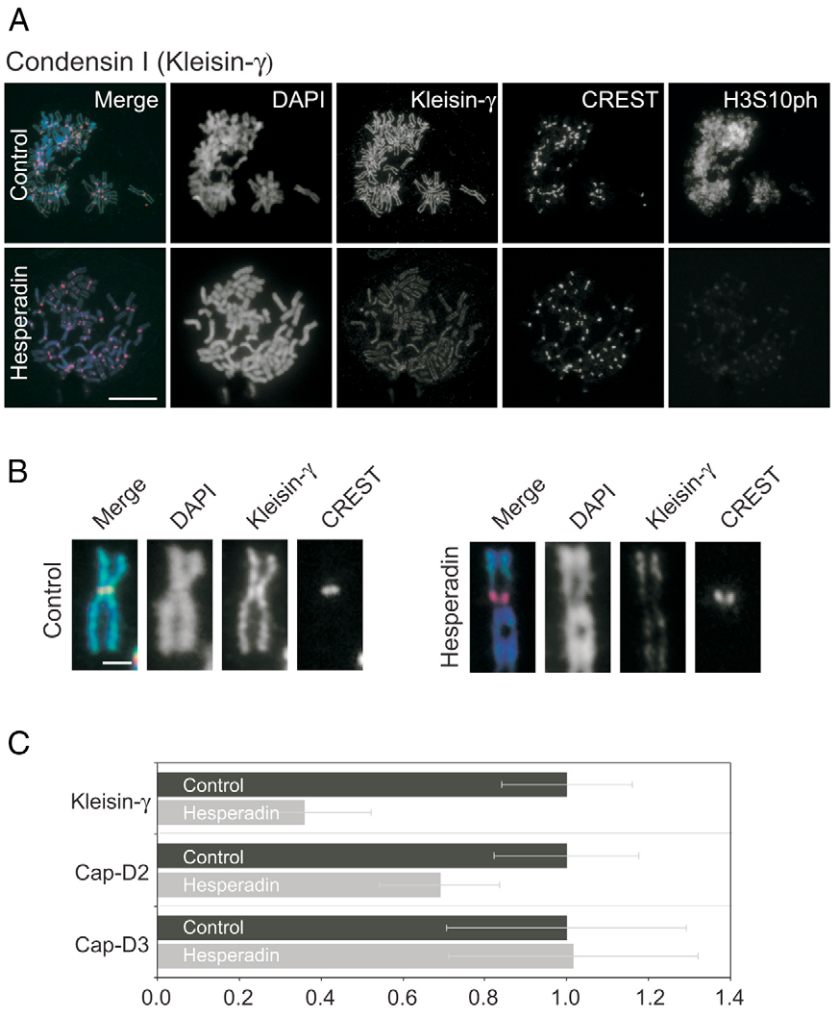

Fig. 3. Aurora B regulates centromeric enrichment of condensin I. (A) Mitotic HeLa cells were spread on glass slides under hypotonic conditions, fixed and stained with antibodies against condensin I (kleisin-γ). CREST autoimmune serum was used to detect centromeric regions. Inactivation of Aurora B kinase activity by Hesperadin treatment (100 nM) was monitored by staining for phosphorylated Histone H3 Serine 10 (H3S10ph). Chromosomes were counterstained with DAPI (1 μg/ml). Bar, 10 μm. (B) A magnified chromosome of the same experiment is shown stained with DAPI, antibodies against kleisin-γ and CREST autoimmune serum. Bar, 2 μm. (C) Quantification of condensin I (kleisin-γ and Cap-D2) and condensin II (Cap-D3) at centromeric regions was done using the CREST signal as a reference and to determine the location of centromeres during image segmentation. Mean ± s.d. from more than 100 different centromeres of at least ten different cells for each condition are shown. The presented graphs are normalised to the control value.

Aurora B activity is required for maintenance of condensin I on chromosomes

Our data so far showed that Aurora B is needed for the rapid association of condensin I with chromosomes after NEBD. To address whether Aurora B activity is also required to maintain normal levels of condensin on chromosomes we arrested EGFP-kleisin-γ and EGFP-kleisin-β cells in prometaphase with Nocodazole and monitored EGFP signals on chromosomes. In the absence of Hesperadin, the EGFP signals remained constant and at a high level over a period of 90 minutes, after which time we added Hesperadin to the cells (Fig. 4A). Approximately 10 minutes later, the fluorescence signal of EGFP-kleisin-γ began to decrease until a constant level of 41±8% of the initial value (t=0 minutes; addition of Hesperadin) was reached after about 2 hours (Fig. 4A,B). The decrease of EGFP-kleisin-γ on chromosomes did not stem from continuous bleaching because low levels of light were used in this experiment and the fluorescence intensity stayed constant over a period of 1.5 hours before inhibition of Hesperadin. Furthermore, the intensities of the condensin II subunit EGFP-kleisin-β did not decrease significantly under the same conditions.

We performed the same experiment using cells arrested in metaphase by the proteasome inhibitor MG132 and obtained very similar results (data not shown), ruling out the fact that the behavior of condensins was artificially affected by Nocodazole. Aurora B is therefore not only required for loading but also for maintenance of normal condensin I levels on mitotic chromosomes.

The non-SMC subunits of condensins are phosphorylated by Aurora B in vitro

Aurora B could facilitate the association of condensin I with chromosomes either by modifying chromosome structure, by regulating unknown loading or unloading factors, or by directly phosphorylating condensin I subunits. As a first step towards testing the latter possibility we investigated whether recombinant Aurora B (bound to a fragment of its activating subunit INCENP) (Sessa et al., 2005) could phosphorylate purified condensin complexes in vitro. We immunoprecipitated condensins from the same cell lines that were analysed by video microscopy. For this purpose we used antibodies to a Flag tag that is located between EGFP and kleisin in the EGFP-kleisin-γ and EGFP-kleisin-β proteins, respectively. The purified proteins were incubated with Aurora B in the presence of [γ-^{32}P]ATP, separated by gel electrophoresis, stained with silver and subsequently analysed by phosphorimaging. The identity of all protein bands was confirmed by mass spectrometry (performed on parallel samples that had not been incubated with Aurora B and [γ-^{32}P]ATP).

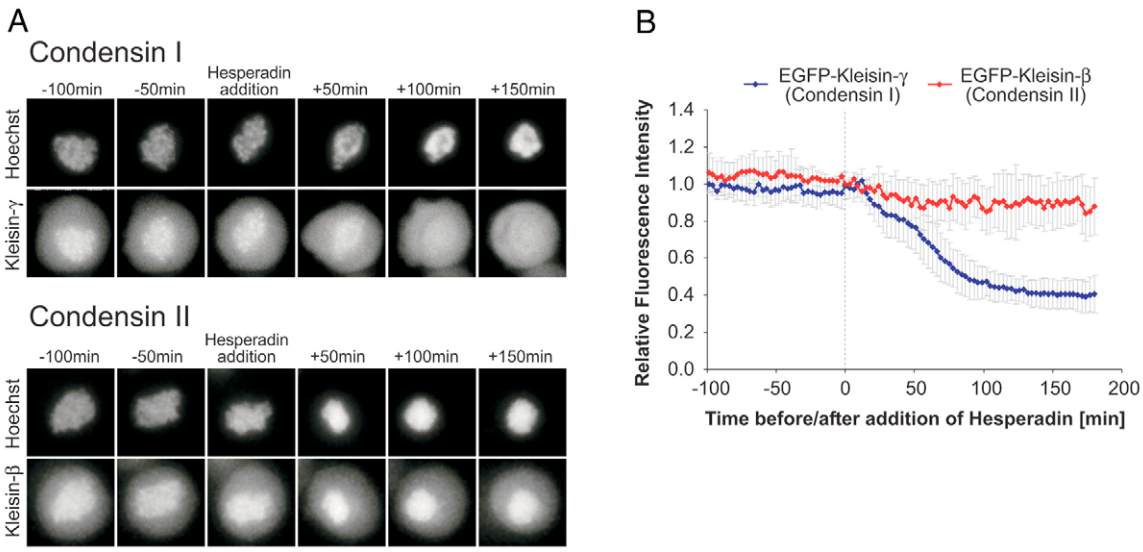

Fig. 4. Maintenance of condensin I is dependent on Aurora B activity. (A) Nocodazole-arrested HeLa cells either expressing EGFP-kleisin-γ (condensin I) or EGFP-kleisin-β (condensin II). At time point 0 (t=0), 100 nM Hesperadin was added to the cells and the levels of condensin followed over time. Hoechst 33342 (0.2 μg/ml) was used to stain chromatin. (B) EGFP-kleisin-γ (condensin I) (red, n=25) and EGFP-kleisin-β (condensin II) (blue, n=19) signals were quantified as in Fig. 1B and Fig. 1D over time. Note that the increase in EGFP-kleisin-β (condensin II) visible in A upon Hesperadin treatment is masked by the normalisation with Hoechst, which likewise increases. Movies were taken on an Olympus DeltaVision microscope [40× objective, five slices every 2 minutes, 1×1 binning (EGFP-kleisin-γ) and 2×2 binning (EGFP-kleisin-β)]. Projected images are shown.

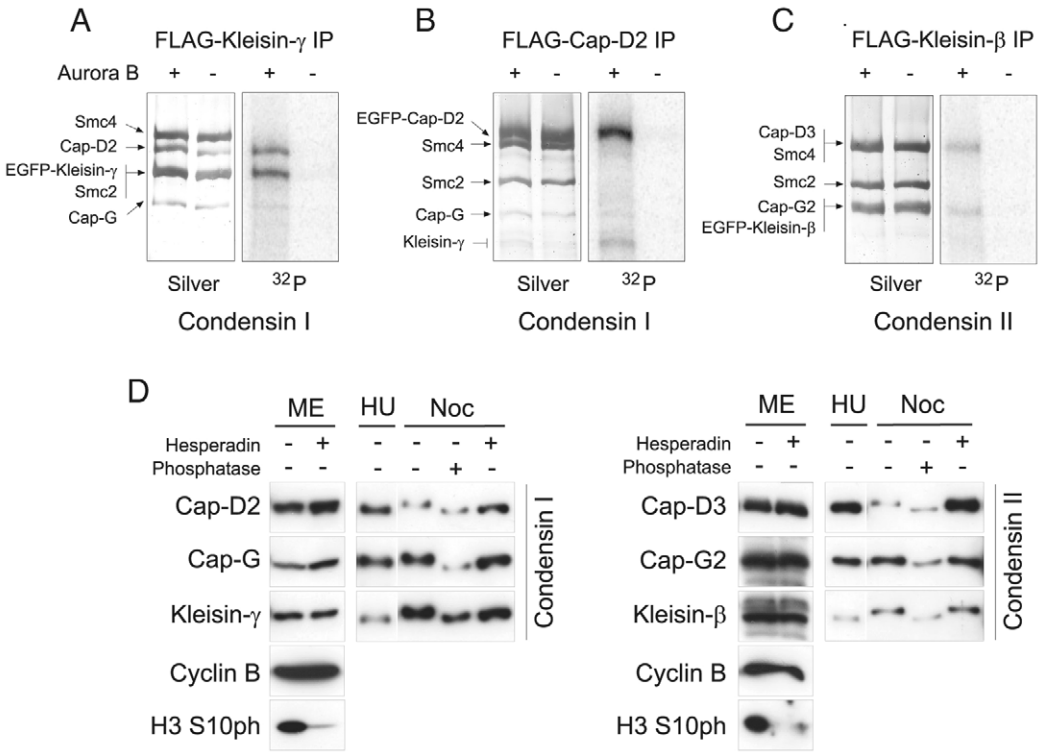

Fig. 5. Aurora B phosphorylates condensin I regulatory subunits in vitro and in vivo. (A-C) Condensin was purified by immunoprecipitation from EGFP-FLAG-kleisin-γ (A), EGFP-FLAG-Cap-D2 (B) (both condensin I) and EGFP-FLAG-kleisin-β (C) (condensin II) cell lines. Immunoprecipitates were incubated either with or without recombinant Aurora B and [γ-^{32}P]ATP in vitro, resolved on a gel, silver stained (Silver) and exposed (^{32}P). The identity of the bands was determined by mass spectrometry. (D) Condensin I (EGFP-FLAG-kleisin-γ, first panel) and condensin II (EGFP-FLAG-kleisin-β, second panel) were immunoprecipitated either from interphase (hydroxyurea arrested; HU) or mitotic cells (Nocodazole arrested; Noc) and analysed for electrophoretic mobility shifts that are sensitive to pre-treatment of the HeLa cells with Hesperadin (100 nM for 1 hour) or to treatment of the immunoprecipitates with λ-phosphatase. Total mitotic extracts (ME) were probed with antibodies against H3S10ph to monitor Aurora B activity and against cyclin B to verify that cells were in mitosis.

In condensin I immunoprecipitates, two prominent ^{32}P bands were observed which corresponded to Cap-D2 and EGFP-kleisin-γ/Smc2 (the latter two were not separated by electrophoresis). In addition, a faint band was detected at the position of Cap-G (Fig. 5A). To investigate whether ^{32}P had been incorporated into EGFP-kleisin-γ or Smc2 we immunoprecipitated condensin I from a different stable HeLa cell line in which an EGFP-Flag tag was fused to Cap-D2 but not to kleisin-γ (Gerlich et al., 2006). As predicted, kleisin-γ migrated much faster than EGFP-kleisin-γ and could therefore be separated from Smc2 (Fig. 5B and supplementary material Fig. S5A). Under these conditions, the ^{32}P signal co-migrated with kleisin-γ but not with Smc2 (see supplementary material Fig. S5A). In all cases, the ^{32}P bands were absent when Aurora B was omitted from the reaction mixtures (Fig. 5A,B) and were strongly reduced by the addition of Hesperadin (see supplementary material Fig. S5), indicating that the observed phosphorylation reactions had been catalyzed by Aurora B and not by a contaminating enzyme that could have been present in the immunoprecipitates. In vitro, Aurora B is therefore able to phosphorylate all three non-SMC subunits of condensin I.

When purified condensin II was analysed in the same assay, we also detected two distinct Aurora-B-dependent ^{32}P bands (Fig. 5C). By using different gel conditions, we could determine the lower signal to contain both phosphorylated kleisin-β and Cap-G2. The upper one co-migrated with Smc4 and Cap-D3, but we were unable to determine which of these proteins had incorporated ^{32}P (see supplementary material Fig. S5B).

The mitosis-specific phosphorylation of Cap-D2, Cap-G and kleisin-γ depends on Aurora B in vivo

Previous work has shown that the three non-SMC subunits of condensin I are hyperphosphorylated in mitotic *Xenopus* egg extracts (Kimura et al., 1998) and in human cells (Takemoto et al., 2004). In *Xenopus* extracts, these phosphorylation reactions have been attributed to Cdk1 (Kimura et al., 1998), but the kinases responsible for condensin I phosphorylation in human cells have not been identified. We therefore analysed whether the mitotic phosphorylation of these proteins depends on Aurora B in vivo.

We immunoprecipitated condensin I and condensin II from EGFP-kleisin-γ or EGFP-kleisin-β cells, respectively, which had either been arrested in mitosis with Nocodazole or in S phase with hydroxyurea. Immunoblot experiments revealed that the electrophoretic mobility of the non-SMC subunits in both condensin I and condensin II was retarded in mitotic samples, with the exception of Cap-G2, for which a mobility shift was difficult to detect. Pretreatment of the samples with protein phosphatase abolished the mobility shifts, indicating that they were caused by phosphorylation (Fig. 5D). Importantly, the mitosis specific mobility shifts of the condensin I subunits, but not those of condensin II subunits, were also abolished when complexes were isolated from cells that had been treated with Hesperadin (Fig. 5D). This effect was not due to cells exiting mitosis because Hesperadin treatment did not cause a reduction in the levels of cyclin B, which is degraded when cells exit mitosis (Fig. 5D). Therefore, Aurora B is required for the phosphorylation-induced mobility shift of Cap-D2, Cap-G and kleisin-γ in vivo, whereas Aurora B is dispensable for the mobility shifts of the condensin II subunits CAP-D3 and kleisin-β.

How does Aurora B contribute to cohesin dissociation from chromosome arms?

It has previously been shown that both Aurora B (Gimenez-Abian et al., 2004; Losada et al., 2002) and condensin I (Hirota et al., 2004) are required for the complete dissociation of cohesin from chromosome arms. Our observation that Aurora B is required for the normal association of condensin I with chromosomes therefore raises the interesting question whether Aurora B contributes to cohesin dissociation indirectly by promoting the chromatin binding of condensin I. Unfortunately we have not been able to test this possibility so far because we have not yet found conditions under which normal amounts of condensin I can associate with chromosomes in the absence of Aurora B activity.

However, we observed that Aurora B is also required for the proper chromosomal localisation of Sgo1, the protein that protects cohesin at centromeres from precocious dissociation (Fig. 6) (Kueng et al., 2006). As reported previously (Kitajima et al., 2004; McGuinness et al., 2005; Salic et al., 2004; Tang et al., 2004), Sgo1 was almost exclusively detected at centromeres when control cells were fixed and analysed by immunofluorescence microscopy (Fig. 6A). By contrast, when cells were analysed in which Aurora B had either been inactivated by Hesperadin treatment or been depleted by RNAi, Sgo1 could also be detected on chromosome arms (Fig. 6A). To confirm this observation we generated a cell line that stably expresses an EGFP-tagged version of Sgo1. To avoid potential overexpression artefacts we introduced a bacterial artificial chromosome (BAC) into HeLa cells, so that the *SGO1* gene was expressed under the control of its own regulatory sequence elements. Also in these cells, Sgo1 signals were seen in distinct chromosomal patches that represent centromeres, but Sgo1 accumulated on chromosome arms within 10 to 20 minutes after inactivation of Aurora B by Hesperadin (Fig. 6D). Aurora B activity is therefore required to confine the localisation of Sgo1 to centromeres in human cells. Recently, similar observations have been published by Resnick et al. who observed that the Aurora B subunit INCENP is required for centromeric localisation of the Sgo1 orthologue MEI-332 during meiosis in *Drosophila* (Resnick et al., 2006) and by Dai et al. who found that Sgo1 becomes visible on chromosome arms after Aurora B depletion in human cells (Dai et al., 2006).

We next tested whether Aurora B inactivation causes a defect in cohesin dissociation from chromosome arms by recruiting Sgo1 to these sites. We depleted Sgo1 by RNAi and simultaneously inactivated Aurora B either by RNAi or by treatment of cells with Hesperadin. Cells were then fixed and analysed by immunofluorescence microscopy with antibodies to Sgo1, kinetochores (CREST autoimmune sera) and cohesin (detected by stable expression of an epitope-tagged cohesin subunit, Scc1-Myc). If only Sgo1 was depleted, the majority of cells contained individual sister chromatids on which neither Sgo1 nor Scc1-Myc could be detected, as reported previously (McGuinness et al., 2005). However, when both Sgo1 and Aurora B were inactivated, sister chromatid cohesion remained intact and Scc1-Myc could clearly be detected on chromosomes. The cohesin dissociation defect that is caused by inactivation of Aurora B does therefore not appear to

depend on Sgo1. This finding implies that Aurora B inactivation must cause at least one other effect besides the relocalisation of Sgo1 to chromosome arms that also prevents the dissociation of cohesin from chromosomes. This effect could be the defect in condensin I binding to chromosomes.

It is important to note that the results of our Sgo1-Aurora-B double inactivation experiments differ from published results that showed that Sgo1 depletion led to precocious sister chromatid separation even after depletion of Aurora B (McGuinness et al., 2005; Dai et al., 2006). By contrast, the effect of Sgo1 depletion on cohesion was reproducibly reverted in our experiments by inactivation of Aurora B (Fig. 6). We presently do not know the reason for this discrepancy, but it is possible that Sgo1 and Aurora B were inactivated to different degrees in the published and our experiments.

Discussion

The reorganisation of interphase chromatin into condensed

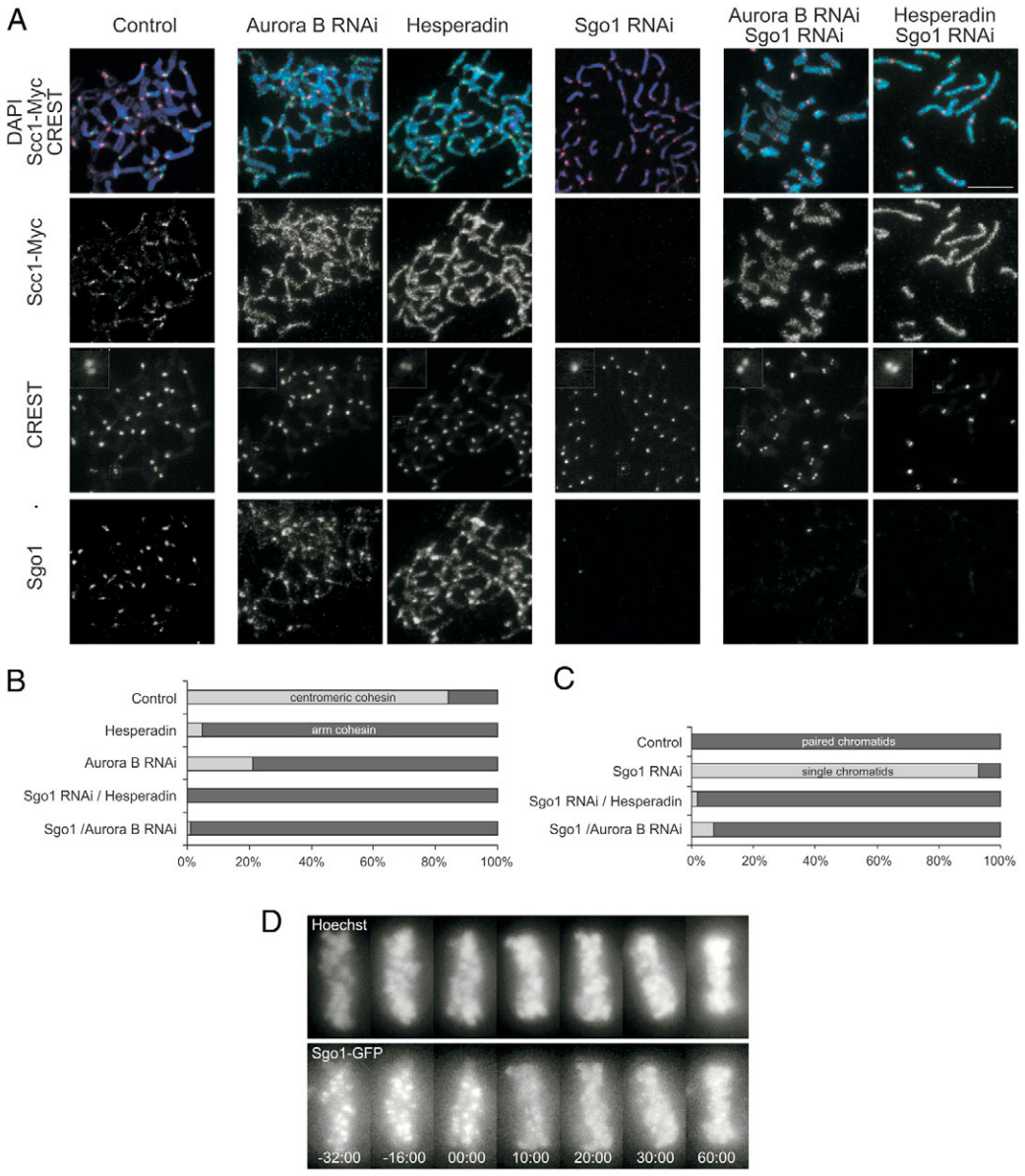

Fig. 6. The persistence of cohesin on chromosome arms in Aurora B depleted cells does not depend on Sgo1. (A) In order to visualise cohesin in mitosis, expression of Scc1-9xMyc was induced by applying Doxycyclin (1 μg/ml for 48 hours). Cells were spread onto glass slides using hypotonic conditions, fixed and stained with antibodies against Myc, Sgo1 and the autoimmune serum CREST to detect centromeres. Sgo1 and Aurora B were depleted by siRNA transfection. 100 μm Hesperadin was used to inhibit Aurora B activity. Bar, 10 μm. (B) Number of cells (%) showing only centromeric cohesin versus cells which display cohesin also on chromosome arms. (C) Number of cells (%) showing paired versus single sister centromeres. (D) Images from a movie of HeLa cells stably expressing Sgo1-GFP. Centromeric enrichment of Sgo1-GFP is lost when Hesperadin (100 nM) is added to the medium (t=0). Chromosomes were visualised with Hoechst 33342 (0.2 μg/ml). Movies were taken on an Olympus DeltaVision microscope (63× objective, five slices every 2 minutes, 2×2 binning). Projected images at times indicated (in minutes) are shown.

rigid chromosomes is one of the most dramatic morphological changes that occurs in mitotic and meiotic cells of higher eukaryotes. Condensin complexes play an important part in this process because their association with chromatin from prophase until telophase contributes to chromosome condensation, confers rigidity to chromosomes and is essential for proper chromosome segregation in anaphase (reviewed by Hirano, 2005). Despite the importance of this process, surprisingly little is known about the mechanisms that promote the association of condensin complexes with chromosomes in vertebrate cells.

Our work shows that the activity of the mitosis-specific kinase Aurora B is required for the rapid and complete association of condensin I with chromosomes in prometaphase and also for maintenance of normal condensin I levels on chromosomes at later stages of mitosis. Together with previous genetic studies in yeast (Petersen and Hagan, 2003; Lavoie et al., 2004) and invertebrates (Giet and Glover, 2001; Hagstrom et al., 2002; Kaitna et al., 2002) these findings indicate that members of the Aurora B kinase family are universal regulators of condensin complexes in eukaryotes.

The roles of Aurora B in chromosome segregation

Aurora B has a number of functions in chromosome assembly and segregation, such as the correction of erroneous kinetochore-microtubule attachments (Hauf et al., 2003; Tanaka et al., 2002), the stabilisation of correct kinetochore-microtubule attachments (Sampath et al., 2004) and the dissociation of cohesin complexes and heterochromatin proteins from specific chromosomal regions (Fischle et al., 2005; Gimenez-Abian et al., 2004; Hirota et al., 2005; Losada et al., 2002). Our work implies that Aurora B in addition contributes to chromosome segregation by facilitating the association of condensin I with chromosomes. In support of this notion, we have observed that chromosomes from cells lacking Aurora B activity tend to become swollen and fuzzy when they are exposed to hypotonic conditions (T.H. and J.J.L., unpublished observations), a property that is characteristic of chromosomes lacking condensin I (Hirota et al., 2004; Ono et al., 2003). Our experiments have not revealed detectable defects in axial chromosome compaction when Aurora B was inactivated (Hauf et al., 2003) (this study). However, this observation is not inconsistent with a role for Aurora B in controlling the behavior of condensin I because quantitative depletion of the condensin I complex in vertebrate cells does also not cause visible structural defects under isotonic conditions (Gerlich et al., 2006; Hirota et al., 2004).

How is condensin II regulated?

Previous studies have shown that condensin I and condensin II are regulated differently because the latter complex is nuclear in interphase and associates with chromatin in prophase, whereas the former is cytoplasmic and can thus associate with chromosomes only after NEBD in prometaphase (Gerlich et al., 2006; Hirota et al., 2004; Maeshima and Laemmli, 2003; Ono et al., 2004; Yeong et al., 2003). This difference in intracellular distribution is principally sufficient to explain the temporal difference in condensin I and condensin II association with chromosomes. It was thus surprising to find that the abilities of these otherwise closely related complexes to bind to chromosomes differ in their requirements for Aurora B.

Condensin I has recently also been found to differ from condensin II in another property, namely its average residence time on mitotic chromosomes, which is much shorter in the case of condensin I than in the case of condensin II (Gerlich et al., 2006). These observations raise the question of whether we failed to see an effect of Aurora B inactivation on condensin II simply because our experiments did not last for long enough to reveal such an effect. This remains a possibility for those experiments where Aurora B was only inactivated once condensin II had already bound to chromosomes. However, our finding that condensin II loading was also not affected when cells entered mitosis in the absence of Aurora B argues strongly for the possibility that this kinase is not essential for the association of condensin II with chromosomes.

Our data do not exclude the possibility that condensin II is also regulated by phosphorylation, either by kinases other than Aurora B or by enzymes that function redundantly with Aurora B. Our observation that the CAP-D3 and kleisin-β subunits of condensin II are specifically phosphorylated in mitosis indeed suggests that the localisation of condensin II may also be controlled by phosphorylation.

How does Aurora B promote the association of condensin I with chromosomes?

Our observations that Aurora B is able to phosphorylate the non-SMC subunits of condensin I and is required for their mitosis-specific phosphorylation raise the interesting possibility that Aurora B facilitates the binding of condensin I to chromosomes directly by phosphorylating these subunits. Obviously this hypothesis will have to be tested through the identification and mutation of Aurora-B-dependent phospho-sites on condensin I, and it does not exclude the possibility that the phosphorylation of other Aurora B substrates also contributes to condensin I loading. However, it is interesting to note that phosphorylation has already been shown to regulate the association between chromosomes and cohesin, although in this case in a negative manner (Hauf et al., 2005). It will therefore be interesting to test whether the chromosome association of other SMC-kleisin complexes is also regulated by phosphorylation of their non-SMC subunits.

Does Aurora B cooperate with Cdk1 in the regulation of condensin I?

We observed that Aurora B can specifically phosphorylate the non-SMC subunits of condensin I and II, but not Smc2 and Smc4. Interestingly, the same set of subunits is also phosphorylated by Cdk1 in *Xenopus* egg extracts (Kimura et al., 1998). In vitro, these modifications did not increase the ability of condensin I to bind to naked DNA but increased the ATPase activity of condensin I and its ability to induce positive supercoils into DNA in the presence of topoisomerase I (Kimura and Hirano, 1997; Kimura et al., 1999). The latter activity has been proposed to contribute to chromosome condensation (Kimura and Hirano, 1997). It is therefore possible that Cdk1 and Aurora B cooperate in the regulation of condensin I, Cdk1 by controlling the enzymatic properties of condensin, and Aurora B by controlling its chromosome association. A detailed analysis of the phospho-sites that are created by these kinases and their functional consequences will be needed to test this idea further.

Materials and Methods

Immunofluorescence microscopy

Immunofluorescence staining and chromosome spreads were performed as described in Hirota et al. (Hirota et al., 2005). Primary antibodies against the following proteins were used: Smc2, kleisin-γ, kleisin-β (see Yeong et al., 2003), Cap-G (see Gerlich et al., 2006), Cap-G2 (rabbit autoimmune serum raised against the following peptide: CLEEDSIERFLYESSSRTL), Cap-D2 (generous gift from E. Watrin, IMP Vienna), Cap-D3 (gift from E. Ogris, University of Vienna), CREST (A. Kromminga, IPM Hamburg), H3S10ph (clone 6G3), Aurora A T288ph (Cell Signalling Technologies), Myc (clone 4A6, Upstate). Images were taken on a Zeiss Axioplan 2 microscope as described (Hirota et al., 2004). Quantifications were done using ImageJ software.

Live-cell imaging

HeLa cells expressing EGFP-kleisin-γ, EGFP-kleisin-β and EGFP-Cap-D2 are described elsewhere (Gerlich et al., 2006; Hirota et al., 2004). Cell synchronisation and siRNA transfections were done as described (Hirota et al., 2004). Cells were grown on LabTek chambered cover glasses (Nunc). Immediately before transferring the cells to the microscope the medium was changed to prewarmed CO_2-independent medium without Phenol Red (Invitrogen) and chambers sealed with silicon grease. To stain chromatin, Hoechst 33342 (0.2 μg/ml) was added to the medium 30 minutes before the start of filming. Image series were taken on an Olympus DeltaVision microscope using the conditions indicated in the corresponding figure legend.

The levels of chromatin-bound condensin were quantified essentially as described (Gerlich et al., 2006). In brief, image segmentation into chromosome and cytoplasm was done using the Hoechst channel as a mask. Average cytoplasmic intensities of condensin were substracted from those on chromatin and normalised to the Hoechst signal. In summary, [bound $EGFP_{chromatin}$]=([$EGFP_{chromatin}$]–[$EGFP_{cytoplasm}$])/([$Hoechst_{chromatin}$]–[$Hoechst_{cytoplasm}$]). Quantifications were done using ImageJ software.

Biochemistry and in vitro phosphorylation

Biochemical fractionation of mitotic cells was performed as described in (Hirota et al., 2005). Immunoprecipitations were done using pre-bound anti-FLAG IgG Sepharose beads (Sigma). Typically, 20 μl beads were saturated with pre-cleared cell lysate containing 5 mg protein. Binding was done at 4°C on a rotator for 1 hour. Beads were washed three times each with IP buffer [20 mM Tris-HCl (pH 7.5), 5% glycerol] containing either 0.1% NP-40 and 100 mM NaCl (low stringency) or 0.2% NP-40 and 400 mM NaCl (high stringency). For in vitro phosphorylation we used recombinant Aurora B bound to a fragment of its activator INCENP (Sessa et al., 2005). Phosphorylation was carried out on beads in a buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA, 150 mM NaCl. The reaction was done at 30°C for 30 minutes followed by washing in TBST buffer (0.04% Tween-20). Bound condensin complexes were elution from the beads using 100 mM glycine (pH 2).

When looking for shifts in electrophoretic mobility, extraction and immunoprecipitation was done in the presence of phosphatase inhibitors (50 mM β-glycerophosphate, 50 mM NaF, 1 mM sodium vanadate, 1 μM okadaic acid). Dephosphorylation of purified condensin was performed on beads using λ-phosphatase in a buffer containing 50 mM Tris-HCl pH 7.5, 0.1 mM Na_2EDTA, 5 mM DTT, 2 mM $MnCl_2$ for 30 minutes at 30°C.

BAC recombination and stable HeLa cell transfection

The BAC RP24-185F17, harboring mouse *SGO1* (*mSGO1*), was obtained from the BACPAC Resources Center (http://bacpac.chori.org). The EGFP-IRES-Neo cassette was PCR amplified using primers that carry 50 nucleotides of homology to the C-terminus of Sgo1. Recombination of the BAC was performed as described (Zhang et al., 2000). Briefly, two electroporation steps with the *E. coli* strain containing the BAC vector were performed: first, a plasmid carrying two recombinases was introduced; second, the PCR fragment containing the homology arms. Precise incorporation of the EGFP-IRES-Neo cassette in-frame in front of the stop codon was verified by sequencing.

Supercoiled BAC DNA was purified with the Large-construct kit (Qiagen) and subsequently transfected into HeLa cells using Effectene (Qiagen). HeLa cells were seeded 16 hours before transfection into 6-cm dishes with a density of 200,000 cells per well in 5 ml medium (DMEM, 10% FBS, 2 nM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin). 24 hours later, cells were transferred to 10-cm dishes and cultivated in selection medium containing 500 μg/ml geneticin (G418, Invitrogen).

Finally, the pool of HeLa cells stably expressing mSgo1-GFP, was single-cell-sorted into 96-well plates using a FACSAria™ Cell Sorter (BD Biosciences). Stable HeLa clones, that had survived further G418 selection, were analysed at the protein and mRNA level to ensure equal expression of endogenous Sgo1 and EGFP-fusion protein.

We would like to thank F. Sessa and A. Musacchio for recombinant Aurora B/INCENP, D. Gerlich, B. Koch and J. Ellenberg for EGFP-tagged condensin cell lines, N. Kraut for Hesperadin, Y. Watanabe for antibodies, K. Mechtler for mass spectrometry and A. Hyman and L. Pelletier for generous support. We are grateful to S. Kueng for helpful discussions and sharing unpublished data. T.H. acknowledges a fellowship from the Japanese Society for the Promotion of Science (JSPS). Research in the laboratory of J.-M.P. is supported by Boehringer Ingelheim, the 6th Framework Programme of the European Union via the Integrated Project MitoCheck and the European Research Foundation and the Austrian Science Fund (FWF) via the EuroDYNA programme.

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