

Distinct functions of condensin I and II in mitotic chromosome assembly

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Summary

Condensin is a protein complex associated with mitotic chromosomes that has been implicated in chromosome condensation. In vertebrates, two types of condensin complexes have recently been identified, called condensin I and II. Here, we show that in mammalian cells condensin II associates with chromatin in prophase, in contrast to condensin I which is cytoplasmic and can thus interact with chromosomes only after nuclear envelope breakdown. RNA interference experiments in conjunction with imaging of live and fixed cells revealed that condensin II is required for chromosome condensation in early prophase, whereas condensin I appears to be dispensable at this stage. By contrast, condensin I is required for the complete dissociation of cohesin from chromosome arms, for chromosome shortening and for normal timing of

progression through prometaphase and metaphase, whereas normal condensin II levels are dispensable for these processes. After depletion of both condensin complexes, the onset of chromosome condensation is delayed until the end of prophase, but is then initiated rapidly before nuclear envelope breakdown. These results reveal that condensin II and I associate with chromosomes sequentially and have distinct functions in mitotic chromosome assembly.

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Introduction

During mitosis, chromatin is structurally reorganized into condensed chromosomes that become first visible as long threads in prophase. In prometaphase and metaphase, chromosomes are shortened and the sister chromatids, which each contain one copy of the replicated DNA, are partially resolved from each other, resulting in the formation of 'chromosome arms'. By contrast, chromatids remain tightly connected at centromeres, where kinetochores assemble and are then captured by spindle microtubules. In anaphase, cohesion between sister chromatids is lost, chromatids are segregated towards opposite spindle poles and chromatin is subsequently decondensed during telophase.

Both chromosome condensation and cohesion depend on large protein complexes that contain two ATPases of the Smc family, a Kleisin that is thought to bridge the ATPase domains of the Smc proteins, and one or two additional subunits (reviewed in Haering and Nasmyth, 2003; Hagstrom and Meyer, 2003; Hirano, 2002; Jessberger, 2002; Schleiffer et al., 2003). One type of complex, called cohesin, associates with chromatin in telophase, remains bound throughout interphase, and is required for sister chromatid cohesion from S phase until anaphase onset. Before anaphase, cohesion is dissolved by stepwise removal of cohesin from chromosomes. In prophase and prometaphase the bulk of cohesin is removed from chromosome arms, whereas cohesin persists at centromeres

and in small amounts on chromosome arms until metaphase when its Kleisin subunit Sccl is cleaved by the protease separase (reviewed in Nasmyth and Schleiffer, 2004).

A different Smc-Kleisin complex, called condensin, becomes specifically enriched in axial structures of sister chromatids during mitosis (Hirano and Mitchison, 1994; Maeshima and Laemmli, 2003; Saitoh et al., 1994). This observation and the finding that depletion of condensin from *Xenopus leavis* egg extracts prevents chromosome assembly (Hirano et al., 1997) implies that condensin is required for chromosome condensation. Consistent with this hypothesis, it has been observed that worm and chicken cells, which lack functional condensin, have chromosome-condensation defects during prophase (Hagstrom et al., 2002; Kaitna et al., 2002; Hudson et al., 2003). However, condensin mutants largely show normal degrees of chromosome condensation at later stages of mitosis, and the major defect observed in these mutants is abnormal anaphase (Saka et al., 1994; Bhat et al., 1996; Giet and Glover, 2001; Steffensen et al., 2001; Hagstrom et al., 2002; Kaitna et al., 2002; Hudson et al., 2003). Condensin is also essential for cell division in budding yeast, where only a small degree of mitotic chromosome condensation is observed (Guacci et al., 1994; Lavoie et al., 2004), and where condensin function is required for the organization and segregation of ribosomal DNA and other repetitive DNA sequences (D'Amours et al., 2004; Freeman et

al., 2000; Machin et al., 2004; Sullivan et al., 2004; Wang et al., 2004).

Condensin is composed of five proteins, Smc2, Smc4, CAP-D2/Eg7, CAP-G and Kleisin- γ /CAP-H (reviewed in Gassmann et al., 2004; Hagstrom and Meyer, 2003; Swedlow and Hirano, 2003), but recent work has shown that Smc2 and Smc4 are also subunits of a separate complex, called condensin II (Ono et al., 2003; Schleiffer et al., 2003; Yeong et al., 2003). In addition to Smc2 and Smc4, condensin II contains CAP-D3/hHCP-6, CAP-G2 and Kleisin- β /CAP-H2, proteins that are distantly related to the non-Smc subunits of the originally identified complex, now called condensin I. Condensin I and II are enriched at different sites along chromatid axes, and their depletion causes different morphological changes when chromosomes are treated with hypotonic buffers (Ono et al., 2003), but their relative contribution to chromosome structure and function is poorly understood. Here, we report that condensin I and II differ in their intracellular location, the timing of their association with mitotic chromosomes, and that these complexes have distinct functions in chromosome condensation, dissociation of cohesin from chromosomes, chromosome shortening and mitotic progression.

Materials and Methods

Antibodies

Polyclonal rabbit antibodies raised against the following peptides were used: Smc2 (Pep639; CAKSKAKPPKGAHVEV), Smc4 (Pep638, KSVAVNPKEIASKGLC). Antibodies against CAP-D3 and Kleisin- γ were prepared as described (Yeong et al., 2003); CAP-D2 antibodies were a generous gift from E. Watrin, UMR 6061-CNRS, Rennes. Monoclonal mouse antibodies specific for the following proteins were used: GFP (7.1 and 13.1, Roche), histone H3-phospho-Ser10 (6G3, Cell Signaling Technologies), myc (4A6, Upstate), and topoisomerase II α (Ki-S1, Boehringer Mannheim). Human CREST serum was kindly provided by A. Kromminga, IPM, Hamburg.

RNA interference

The sequences of the small interfering RNAs (siRNAs) were as follows: CAP-D2 5'-CCAU AUGCUCAGUGCUACATT-3', CAP-D3 5'-CAUGGAUCUAUGGAGAGUATT-3', Smc2 5'-UGCUAUCACUGGCUUAAAUTT-3'. Synthetic sense and antisense oligonucleotides were obtained from VBC Genomics. Annealing of siRNA oligonucleotides was performed according to the instructions of Dharmacon. For control transfections, annealing reactions were carried out with the control siRNA 5'-UUCUCCGAACGUGUC-ACGUTT-3', or dH₂O instead of siRNA oligonucleotides. Transfections were carried out by incubating 120–200 nM duplex siRNA with oligofectamine (Invitrogen) in medium containing 0.3% (v/v) fetal calf serum (FCS). After 4 hours more FCS was added to a final concentration of 20% (v/v).

Cells

HeLa, human retinal pigment epithelium (RPE) and normal rat kidney (NRK) cells were cultured in a complete medium of DME:F12 (Sigma) supplemented with 10% FBS, 0.2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. To generate NRK cells stably expressing EGFP- and Flag-tagged Kleisin- γ , cells were transfected with pEGFP-C1-Flag-Kleisin- γ using FuGene6 reagent (Roche), and cells were selected in a complete medium containing G418 (800 μ g/ml; Gibco-BRL), and were screened by fluorescence microscopy for expression of GFP-Kleisin- γ . HeLa cells stably

expressing GFP-H2B were established as previously described, except that cells were selected with 2.0 μ g/ml Blasticidin-S (Kanda et al., 1998).

Cell extracts and fractionations

HeLa cell extracts were prepared as previously described (Waizenegger et al., 2000). Proteins were resolved by SDS-PAGE, and transferred to Immobilon-P membrane (Millipore). Blocking and antibody-incubations were in 4% (w/v) low-fat milk in TBS-T [150 mM NaCl, 20 mM Tris pH 8.0, 0.05% (v/v) Tween 20], and washes were in TBS-T. Blots were developed by enhanced chemiluminescence (ECL). Crude chromatin-enriched fractions were obtained by centrifugation of total cell extracts at 1300 g for 20 minutes using a table top centrifuge. For fractionation, 100,000 g supernatant fractions of cell extracts from NRK cells expressing EGFP-Flag-Kleisin- γ were prepared using IP buffer (Waizenegger et al., 2000) and centrifuged through a 5–20% (w/v) sucrose density gradient for 18 hours at 150,000 g at 4°C, using a SW40 rotor (Beckman).

Immunofluorescence microscopy and chromosome spreading

Cells grown on poly-L-lysine-coated coverglass were fixed with 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 20 minutes at room temperature, or with ice cold acetone-methanol (1:1) solution for 2 minutes. In the latter case, cells were washed for 2 minutes at room temperature with PBS containing decreasing amounts of methanol [95%, 90%, 80%, 70%, 50% (v/v)]. Fixed cells were permeabilized with 0.2% (v/v) Triton X-100-PBS, and incubated with 3% (w/v) BSA-PBS for 1 hour. Pre-extraction was carried out for 2 minutes in 0.1% Triton X-100-PBS and followed by a 3-minute incubation in PBS before fixation. For Sec1-myc staining, mitotic cells were spun onto glass slides for 5 minutes at 1500 rpm using a cytospin centrifuge (Shandon), pre-extracted and fixed with 4% (w/v) paraformaldehyde. Cells were incubated with the 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 (all 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 μ g/ml 4',6-diamidino-2-phenylindole (DAPI), cells were mounted in Vectorshield mounting medium (Vector Laboratories). Images were captured on a Zeiss Axioplan 2 microscope equipped with epifluorescence and a Photometrics Cool Snap HQ CCD camera driven by MetaMorph software (Universal Imaging Corp.). Fluorescence intensities of chromosome area, defined by DAPI staining, were quantified with Image-J software.

For chromosome spreading, mitotic cells were collected by shake-off, incubated either in isotonic buffer (PBS) or hypotonic buffer (cell culture medium:tap at 2:3) for 5 minutes and 30 seconds, then fixed and washed with Carnoy solution (methanol:acetic acid at 3:1). The cells were stored overnight at -20°C. The next day, fixed cells were dropped onto slides, dried and stained with 5% (v/v) Giemsa (Merck) at pH 6.8 for 7 minutes, washed briefly in tap water, dried and mounted with Entellan (Merck). To stain heterochromatin, chromosome spreads were treated with 0.2 mg/ml distamycin dissolved in McIlvaine's 0.1 M citric acid-0.2 M phosphate buffer (pH 7.0) for 15 minutes, followed by DAPI staining.

Video microscopy

Cells were grown in Labtek chambered cover glasses (Nunc) and transfected with siRNA oligonucleotides using oligofectamine. Medium was changed to CO₂-independent medium without Phenol Red 56–70 hours after transfection and chambers were sealed with

silicon grease. For some experiments, DNA was stained in living cells with 0.2 µg/ml Hoechst 33342. Three-dimensional image stacks of live cells were captured over time with an in house developed software macro (Rabut and Ellenberg, 2004) on a Zeiss LSM510 confocal microscope. The macro is available under <http://www.embl-heidelberg.de/ExternalInfo/ellenberg/homepage/macros.html>.

Results and Discussion

Condensin II and I associate with chromosomes in prophase and prometaphase, respectively

Based on immunofluorescence (IF) microscopy experiments, condensin I has been reported to be located in the nucleus of interphase cells (Cabello et al., 2001; Uzbekov et al., 2003); however, the complex has also been found to be cytoplasmic (Ball et al., 2002; Cabello et al., 2001; Maeshima and Laemmli, 2003; Schmiesing et al., 2000; Steffensen et al., 2001; Uzbekov et al., 2003). The latter finding was surprising considering the genetic evidence that condensin subunits are required for chromosome condensation in prophase (Hagstrom et al., 2002; Kaitna et al., 2002; Hudson et al., 2003), i.e. before nuclear envelope breakdown (NEBD). We therefore reinvestigated the intracellular distribution of condensin I. In NRK cells, we generated a line that stably expresses an EGFP-Flag-tagged version of Kleisin-γ, at levels below those of endogenous Kleisin-γ (Fig. 1A). Immunoprecipitation (IP) and sucrose-density-gradient centrifugation experiments showed that EGFP-Flag-Kleisin-γ is quantitatively incorporated into condensin I complexes (Fig. 1A,B); subcellular fractionation experiments suggested that these EGFP-tagged complexes specifically associate with chromosomes in mitosis, similarly to endogenous condensin I (Fig. 1C). In fluorescence microscopy experiments, we observed that EGFP-Flag-Kleisin-γ was excluded from the nucleus in interphase cells, whereas in mitotic cells it localized to chromatid axes (Fig. 1E). To determine the exact timing of chromosome association of condensin I, we performed time-lapse video microscopy experiments. We found that EGFP-Flag-Kleisin-γ could be detected only in the cytoplasm during interphase and prophase, but immediately after NEBD, EGFP-Flag-Kleisin-γ became enriched in axial structures of sister chromatids and remained associated with these axes until late anaphase (Fig. 1D; see supplementary material, Movie 1). IF experiments using antibodies against CAP-D2 confirmed these observations in fixed cells (Fig. 1F). In agreement with earlier IF data (Maeshima and Laemmli, 2003), our results therefore indicate that condensin I is cytoplasmic until NEBD and is thus presumably not directly involved in chromosome condensation in prophase.

To analyze whether condensin II has a role in chromosome condensation during prophase, we first analyzed the intracellular distribution of CAP-D3 by IF microscopy. In contrast to condensin I subunits, CAP-D3 was enriched in nuclei during interphase (Fig. 1F) (Yeong et al., 2003) and prophase (Fig. 1F). When soluble proteins were extracted from cells before fixation and immunostaining, CAP-D3 was still seen on prophase chromatin, which indicates condensin II stably associates with chromosomes already in prophase (data not shown). From prometaphase until anaphase, CAP-D3 was enriched in chromatid axes, similar to CAP-D2. These results indicate that condensin II is nuclear in interphase and associates with chromosomes in prophase.

Smc2, a shared subunit of both complexes, has previously been reported to be located on chromosomes only after NEBD (Maeshima and Laemmli, 2003). However, in our experiments Smc2 antibodies stained both the cytoplasm and nuclei of interphase cells and, in pre-extracted cells, Smc2 staining on chromosomes was clearly seen in prophase nuclei (Fig. 1G). These data are consistent with the notion that in prophase, condensin I and II are located in the cytoplasm and nucleus, respectively.

Condensin I and II associate with chromosomes independently of each other

To examine specific functions of condensin I and II we established conditions that allow the depletion of CAP-D2, CAP-D3 or Smc2 by RNA interference (RNAi). Immunoblot (Fig. 2A) and IF analyses (see below) revealed that abundance of these proteins was greatly decreased between 48 and 72 hours after the transfection with specific siRNAs.

To interpret possible phenotypes caused by these depletions, we first analyzed whether the binding of condensin I to chromosomes depends on the presence of condensin II or vice versa. When nocodazole-arrested mitotic cells were analyzed by immunoblotting, CAP-D2 was detected in chromosome-enriched fractions from CAP-D3-depleted cells, and CAP-D3 was detected in chromosome fractions from CAP-D2-depleted cells (Fig. 2B), suggesting that condensin I and II can associate with chromosomes independently of each other. This notion was confirmed by IF microscopy (Fig. 2C). These experiments also showed that condensin I becomes normally enriched in axial structures on chromatids in condensin-II-depleted cells and vice versa. However, the axial CAP-D3 staining was more intense in CAP-D2-depleted cells than in control cells, indicating that the loss of condensin I could result in the loading of higher than normal amounts of condensin II (Fig. 2C and see supplementary material, Fig. S1). It is therefore possible that loss of condensin I function can be partially compensated for by condensin II.

Depletion of Smc2 reduced the levels of CAP-D2 and CAP-D3 on chromosomes, suggesting that these non-Smc subunits alone do not directly associate with chromosomes (Fig. 2B,C). Consistent with earlier observations (Coelho et al., 2003; Hudson et al., 2003), the amount of topoisomerase II (Topo II) in chromosome fractions was not affected by the depletion of condensin I or II, or both (Fig. 2B), although immunofluorescence microscopy revealed that Topo II was not enriched in axial structures under these conditions (data not shown).

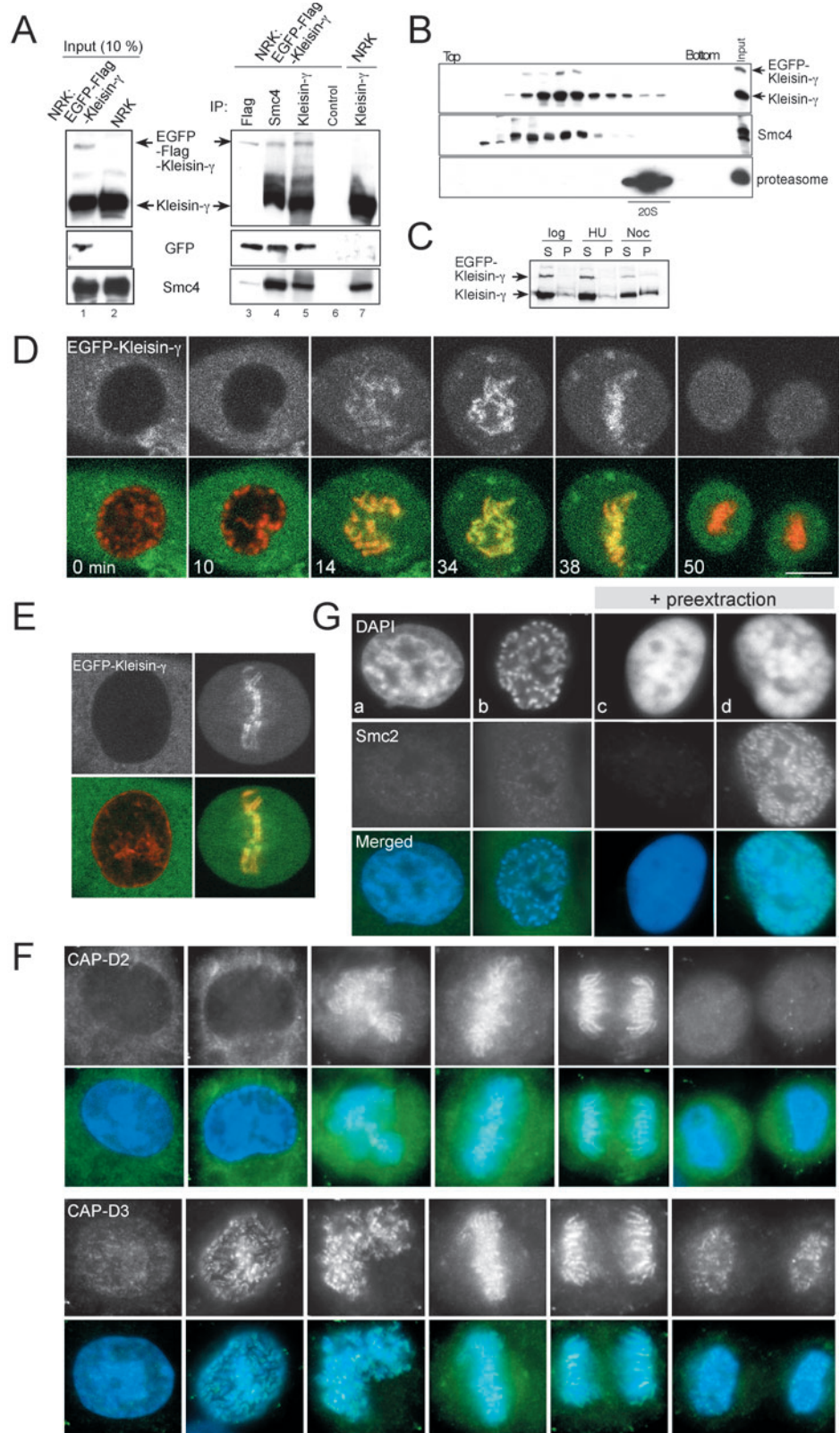
Condensin II is required for normal chromosome condensation in early prophase

The different intracellular distribution of condensin I and II suggested a role in chromosome condensation in prophase for condensin II, but not condensin I. To test this hypothesis, we analyzed chromosome condensation by IF and time-lapse confocal microscopy in cells in which CAP-D2, CAP-D3 or Smc2 had been depleted. In IF experiments, prophase cells were identified by the presence of histone H3-phospho-Ser10 staining and an intact nuclear envelope (Fig. 3A). In control experiments, DAPI staining showed that chromatin was

strongly condensed in 50% of prophase cells, while moderate and low degrees of condensation in 30% and 20% of these cells, respectively, were observed. When condensin I was

inactivated by CAP-D2 depletion, chromosome condensation was not detectably affected. By contrast, inactivation of condensin II by CAP-D3 depletion caused a strong reduction

Fig. 1. Localization of condensin I and II during the cell cycle. (A-E) Analysis of NRK cells stably expressing EGFP-Flag-Kleisin- γ . (A) Incorporation of ectopically expressed EGFP-Flag-Kleisin- γ into condensin I complexes. Extracts of NRK cells expressing EGFP-Flag-Kleisin- γ (lane 1) or parental NRK cells (lane 2) were analyzed by SDS-PAGE and immunoblotting using antibodies to Kleisin- γ (upper panel), to GFP (middle panel) and to Smc4 (lower panel). The same extracts were also subjected to immunoprecipitations with antibodies to Flag-epitope (lane 3), Smc4 (lane 4), Kleisin- γ (lanes 5 and 7), or control IgG (lane 6) and analyzed by immunoblotting, as indicated. (B) Extracts of EGFP-Flag-Kleisin- γ -expressing NRK cells were fractionated by 5-20% sucrose-density-gradient centrifugation. Fractions were analyzed by immunoblotting using antibodies against Kleisin- γ , Smc4 or a subunit of the proteasome. (C) Extracts of exponentially proliferating (log), hydroxyurea treated (HU) or nocodazole treated (Noc) NRK cells expressing EGFP-Flag-Kleisin- γ were separated by centrifugation into pellet (P) and supernatant (S). Endogenous and exogenous Kleisin- γ proteins were detected by immunoblotting using Kleisin- γ antibodies. EGFP-Flag-Kleisin- γ was expressed at approximately 10% of endogenous protein levels, estimated by quantifying the intensity of the bands. (D, E) Localization of EGFP-Kleisin- γ in live interphase and metaphase NRK cells. DNA was stained with Hoechst 33342 (red in overlay). (D) Mitotic time-lapse imaging of EGFP-Kleisin- γ -expressing cells stained with Hoechst 33342 (red). Time from start of filming is indicated in minutes. Bar, 10 μ m. (F) Localization of CAP-D2 and CAP-D3 by IF microscopy. Logarithmically proliferating HeLa cells were fixed with acetone-methanol solution, incubated with CAP-D2 and CAP-D3 antibodies and stained with Alexa 488 (upper panels). Merged signals of Alexa 488 (green) and DAPI (blue) are shown in the lower panels. (Left to right) Representative cells in interphase, prophase, prometaphase, metaphase, anaphase and telophase. (G) Smc2 is located on chromosomes in prophase. HeLa cells were fixed with 4% paraformaldehyde either after pre-extraction (c,d) or without pre-extraction (a, b), and stained with Smc2 antibodies (green). DNA was stained with DAPI (blue). Representative cells in interphase (a, c) and in prophase (b, d). Notice, staining of Smc2 on axial chromosome structures can be seen in prophase after pre-extraction, although condensed chromosomes are not seen as clearly after pre-extraction treatment.

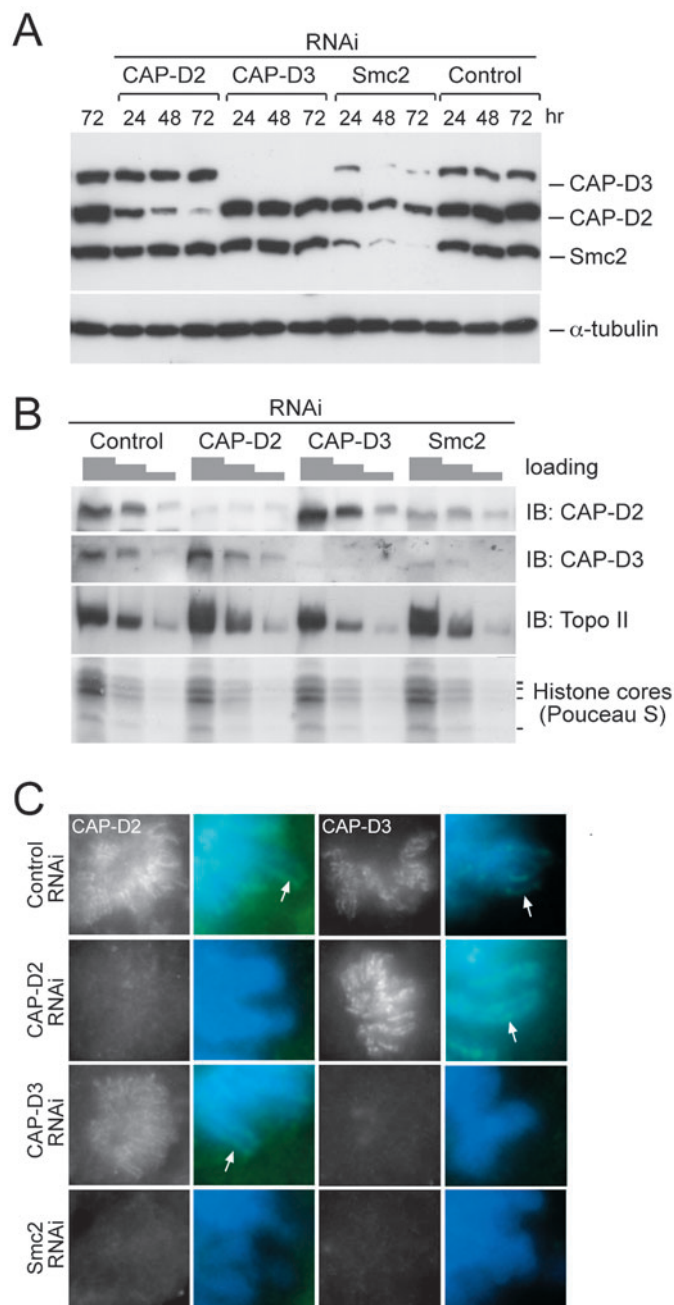


in chromosome condensation in prophase. Co-staining of these cells with anti-CAP-D3 antibodies revealed that there was a clear inverse correlation between the amount of CAP-D3 and the degree of chromosome condensation (Fig. 3A and data not shown). Similar results were obtained when prophase cells were identified by the presence of cyclin B in the nucleus (data not shown).

To follow the progression of chromosome condensation *in vivo* and to confirm the effects described above, we established a HeLa cell line that stably expresses EGFP-tagged histone H2B (H2B-EGFP) (Kanda et al., 1998) as a marker for chromatin. Using automated confocal microscopy over long periods of time, we observed initiation of chromatin condensation approximately 15-20 minutes before NEBD in control cells (Fig. 3B, $n=34$). Upon CAP-D3 depletion,

initiation of condensation was significantly delayed by 5 to 10 minutes in 71% of the observed cells (Fig. 3B, $n=24$), whereas no significant delays were seen in CAP-D2 depleted cells (Fig. 3B, $n=11$). In Smc2-depleted cells chromosome condensation was delayed at similar rates to CAP-D3-depleted cells (Fig. 3B, 76%, $n=17$).

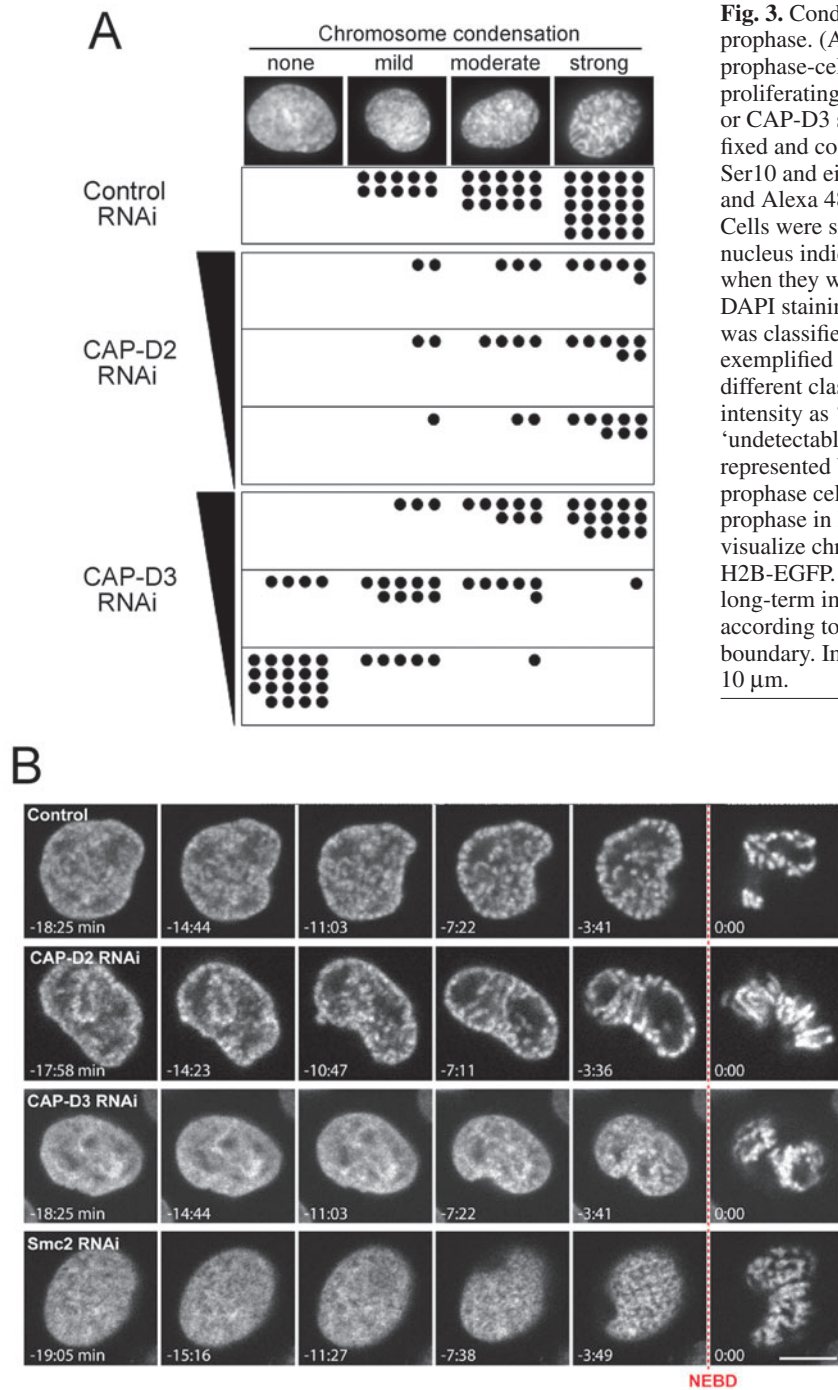
These observations indicate that condensin II, but not condensin I, is required for the initiation of chromosome condensation in prophase. This is consistent with the finding that Smc2 is required for chromosome condensation during prophase in chicken DT40 cells (Hudson et al., 2003), and with observations made in worms, whose single type of condensin is required for chromosome condensation in prophase (Hagstrom et al., 2002; Kaitna et al., 2002) and whose subunits are more closely related to vertebrate condensin II than to condensin I (Schleiffer et al., 2003). During preparation of this manuscript, Ono et al. have also reported that the CAP-G2 and Kleisin- β subunits of human condensin II are found in prophase nuclei, and that depletion of CAP-G2 leads to an increase in the frequency of prophase cells in which chromatin is poorly condensed (Ono et al., 2004).



Normal levels of condensin I and condensin II are dispensable for chromosome condensation in prometaphase and metaphase

It is interesting that in condensin-II-depleted cells chromosome condensation was delayed but still initiated just before NEBD (Fig. 3B), i.e. before condensin I can be detected on chromosomes (Fig. 1D,F). Similar observations were made in Smc2-depleted cells, arguing further that condensin I is not responsible for condensation at the end of prophase (Fig. 3B). We cannot rule out the possibility that these cells contain residual amounts of condensin II sufficient to initiate condensation before NEBD. However, the observation that two independent depletion protocols (targeting either CAP-D3 or Smc2) resulted in the initiation of chromosome condensation shortly before NEBD raises the more interesting possibility that the process of NEBD itself could be causally related to chromosome condensation at this stage, for example by

Fig. 2. Condensin I and II associate with chromosomes independently of each other. (A) Depletion of condensin subunits by RNAi in HeLa cells. Logarithmically proliferating cells were transfected with CAP-D2, CAP-D3, Smc2 siRNAs or with control reagents, and total cell extracts were analyzed by immunoblotting at the indicated times after the transfection. Because the targeted proteins were depleted between 48-72 hours after transfection, subsequent analyses were carried out in this time window. (B) HeLa cells were transfected with control, CAP-D2, CAP-D3 or Smc2 siRNAs for 48 hours. Following treatment with 50 ng/ml nocodazole for 12 hours, mitotic cells were collected and chromosome enriched fractions were prepared and analyzed by immunoblotting as indicated. Three serial dilutions were loaded for each sample. Ponceau S staining of core histones is shown as a loading control in the bottom panel. IB, immunoblot. (C) IF microscopy of condensin I and condensin II in CAP-D2- or CAP-D3-depleted cells. Forty-eight hours after the transfection of indicated siRNAs, cells were fixed and stained with antibodies against CAP-D2 or CAP-D3 (gray in the first column, green in the second), and DNA was counterstained with DAPI (blue). Representative prometaphase cells are shown. Arrows indicate chromosome axes where CAP-D2 and CAP-D3 are enriched.



increasing the permeability of nuclear pore complexes that could allow access of cytoplasmic molecules to chromatin (Lenart et al., 2003).

Significantly, we also noticed that, during prometaphase and metaphase, obvious defects in chromosome condensation were not seen in either live (Fig. 4A) or fixed cells (see Fig. 5 for an example) in which CAP-D2, CAP-D3 or Smc2 had been depleted, except that chromosome arms appeared slightly more fuzzy in the absence of Smc2 (Fig. 4A). To analyze the effect of condensin I and II depletion on chromosome structure in more detail, we analyzed mitotic cells by chromosome spreading and Giemsa staining. When the spreading procedure

Fig. 3. Condensin II is required for chromosome condensation in prophase. (A) Analysis of chromosome condensation in fixed prophase-cells depleted of condensin I or II. Logarithmically proliferating immortalized RPE cells were transfected with CAP-D2 or CAP-D3 siRNAs. Sixty hours after the transfection cells were fixed and co-incubated with antibodies against histone H3-phospho-Ser10 and either CAP-D2 or CAP-D3, and probed with Texas Red and Alexa 488, respectively. DNA was counterstained with DAPI. Cells were scored as being in prophase when the shape of their nucleus indicated that they possessed an intact nuclear envelope, and when they were positive for histone H3-phospho-Ser10. Based on DAPI staining, the extent of chromosome condensation in these cells was classified into 'none', 'mild', 'moderate' or 'strong' as exemplified in the top panels. Cells were also grouped into three different classes according to their CAP-D2 or CAP-D3 staining intensity as 'no reduction', 'reduced but still detectable' or 'undetectable' (these different signal intensities are graphically represented by black triangles on the left). Each dot represents one prophase cell. (B) Analysis of chromosome condensation during prophase in live HeLa cells depleted of condensin I or II. To visualize chromatin, we analyzed HeLa cells that stably express H2B-EGFP. Prophase image-sequences were extracted from long-term imaging experiments and aligned on the time axis according to NEBD, as determined from the loss of a defined nuclear boundary. Imaging was 56-70 hours after siRNA transfection. Bar, 10 μ m.

was carried out using isotonic buffer solutions, chromosomes were condensed regardless of whether condensin I and II had been depleted or not (Fig. 4B, left panels). When the specimens were pretreated with hypotonic buffer, chromosomes from CAP-D2- or CAP-D3-depleted cells also appeared normally condensed, but chromosomes from Smc2-depleted cells or from cells in which both CAP-D2 and CAP-D3 had been depleted acquired an amorphous appearance (Fig. 4B, middle panels). These data indicate that, as suggested by Earnshaw and colleagues based on Smc2 depletion experiments (Hudson et al., 2003; Gassmann et al., 2004), normal levels of condensin complexes are not essential for mitotic chromosome condensation but that these complexes confer rigidity to chromosomes, making them more resistant to hypotonic conditions. Furthermore, our results imply that condensin I and II are at least partly redundant in their ability to confer rigidity to chromosomes as only the depletion of the

shared subunit Smc2 leads to a phenotype under hypotonic conditions.

Condensin I is required for the complete dissociation of cohesin from chromosome arms

During prophase and prometaphase the bulk of cohesin complexes dissociates from chromosome arms, resulting in partial resolution of sister chromatids from each other (Waizenegger et al., 2000; Losada et al., 2002). When cells are arrested in prometaphase by spindle poisons such as nocodazole, this cohesin dissociation process can go to

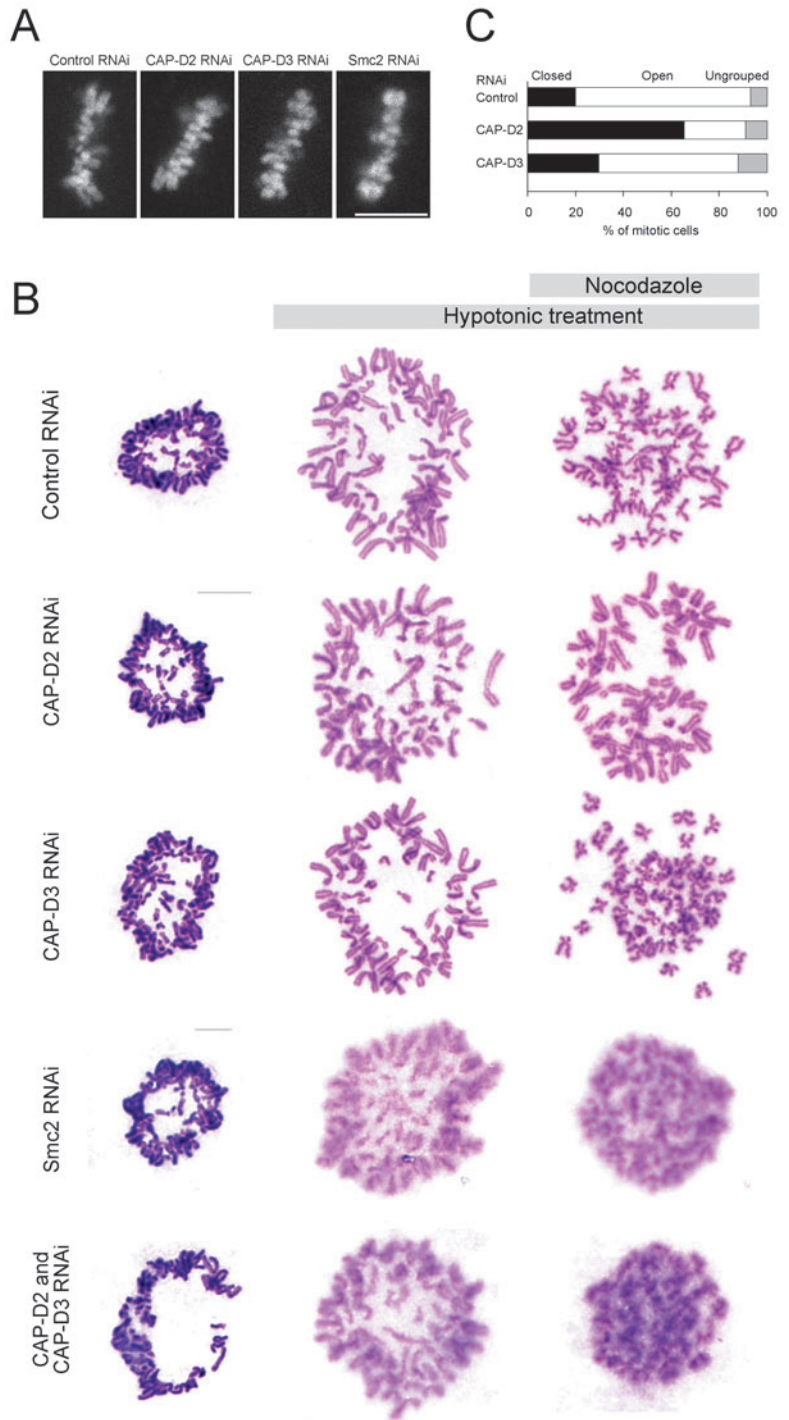
Fig. 4. Analysis of chromosome morphology in cells depleted of condensins. (A) Metaphase plates in live HeLa cells stained with Hoechst 33342 after transfection with the indicated siRNAs. Single confocal sections of 3D-stacks at 1.6 μm slice-thickness of representative examples ($n=10$ for each condition) are shown. (B) Chromosome spreads from condensin-depleted cells. HeLa cells were transfected with control, CAP-D2, CAP-D3 or Smc2 siRNAs for 48 hours. Mitotic cells were collected without treatment (left and middle panels) or after 4 hours of nocodazole treatment (right panel), and incubated with either isotonic buffer (left panels) or with hypotonic buffer (middle and right panels), and fixed with Carnoy solution. Chromosome spreads made on slide-glass were stained with Giemsa. (C) Quantification of the number of spread cells whose chromosome arms had opened or remained closed after 4 hours of nocodazole treatment. 200 cells were assessed for each experiment.

completion in the region of chromosome arms (but not at centromeres), resulting in the complete loss of arm cohesion and the formation of 'X-shaped' chromosomes (Gimenez-Abian et al., 2004). When we analyzed Giemsa-stained chromosomes that had been isolated from nocodazole-arrested cells, we noticed that arm cohesion was lost in chromosomes from most CAP-D3-depleted cells, but that arm cohesion was maintained in chromosomes from many CAP-D2-depleted cells (Fig. 4B, right panels, and C). Under these conditions, the average arm-to-arm distance between sister chromatids was $1.30 \pm 0.33 \mu\text{m}$ (mean \pm s.d.) in control cells, $1.19 \pm 0.35 \mu\text{m}$ in CAP-D3 RNAi cells but only $0.97 \pm 0.17 \mu\text{m}$ in CAP-D2 RNAi cells (Fig. 5A).

This observation prompted us to examine whether condensin I is required for complete dissociation of cohesin from chromosome arms. To test this, we performed condensin RNAi experiments in HeLa cells that stably express a myc-tagged version of the cohesin subunit Scc1 (Waizenegger et al., 2000). In control and CAP-D3-depleted cells that were arrested in mitosis Scc1-myc was seen only at centromeres but in CAP-D2-depleted cells, significant amounts of Scc1-myc were present on chromosome arms (Fig. 5B,C). Colocalization of Scc1-myc and Smc2 revealed that cohesin is enriched in-between chromatid axes following CAP-D2-depletion, consistent with the notion that these cohesin complexes maintain cohesion between chromosome arms (Fig. 5B, bottom panel).

These results indicate that condensin I but not condensin II is required for the complete dissociation of cohesin from chromosome arms. In theory, condensin I could have a direct role in this process by displacing the structurally related cohesin complexes from chromatin. However, because condensin I and cohesin are enriched in distinct domains of chromosomes (Fig. 5B) it is more plausible that condensin I changes chromosome structure in a way that is required for cohesin dissociation.

It has previously been reported that condensin I is not required for the dissociation of the bulk of cohesin from chromosomes in *Xenopus* egg extracts (Losada et al., 1998). However, in these extracts it is difficult to detect the small



amounts of cohesin that persist on mitotic chromosomes. It is therefore possible that condensin I loading is essential only for the dissociation of those cohesin complexes that remain on chromosome arms after prophase, and that this effect cannot easily be detected in *Xenopus* egg extracts.

Condensin I is required for longitudinal compaction of chromosomes in prolonged prometaphase

Chromosome condensation normally continues throughout prometaphase. If prometaphase is delayed by treatment with

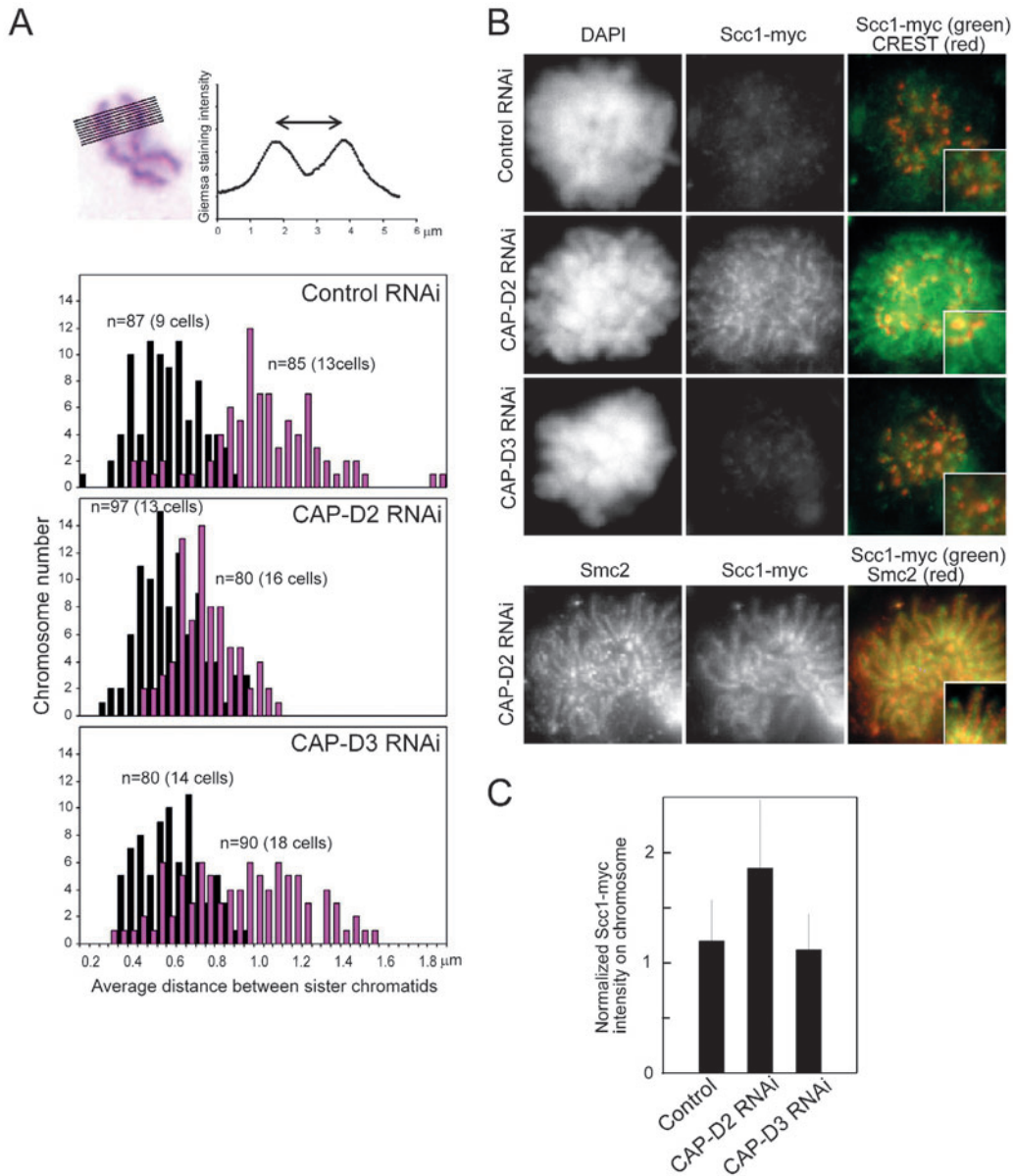


Fig. 5. Cohesin dissociation from chromosome arms in prometaphase requires condensin I. (A) Quantitative analysis of sister chromatid cohesion in condensin RNAi cells. Chromosome spreads from HeLa cells transfected with either CAP-D2 or CAP-D3 siRNAs were prepared as described in Fig. 4B, using hypotonic conditions. For each chromosome, 20 line scans were randomly selected on chromosome arms excluding regions where chromatids converge into centromeres (exemplified in the upper left panel). The signal intensities over the defined lines were averaged, and the distance between the peak intensities were scored (upper right panel). The resulting distance values were plotted against chromosome number in histograms (bottom panel). Data are from cells that were not treated (black bars) or treated with nocodazole for 4 hours (purple bars). (B) Analysis of cohesin in condensin depleted cells. HeLa cells, stably expressing Scc1-myc, were transfected with control or condensin siRNAs and processed for IF microscopy following a 50 ng/ml nocodazole treatment for 4 hours. Cells were incubated with anti-myc antibodies (green) and either antibodies against kinetochores (CREST sera, red) or Smc2 antibodies (red, bottom panel). DNA was counterstained with DAPI. (C) Quantitative analysis of Scc1-myc signal-intensity on chromosomes. Chromosome regions were defined by DAPI staining, and the ratio of fluorescence intensities of myc signals to CREST signals was calculated. A Student's *t*-test demonstrated that the difference between CAP-D2 RNAi cells and control or CAP-D3 RNAi cells was significant ($P < 0.01$).

spindle poisons, chromosomes can condense to abnormally high degrees (Fig. 4B; compare middle and right columns). We noticed that this hypercondensation was reduced in nocodazole-arrested cells from which CAP-D2 had been depleted (Fig. 4B). To quantify this result, we identified chromosome number 1 by staining its characteristically large pericentromeric heterochromatin cluster by using a special

DAPI-treatment protocol (Fig. 6A). We found that prolongation of prometaphase by nocodazole treatment for 4 hours resulted in shortening of chromosome 1 by an average of 37% in control cells, by 40% in CAP-D3-depleted cells, but only by 15% in CAP-D2-depleted cells (Fig. 6B; $P < 0.01$, Student's *t*-test). These results show that condensin I contributes to longitudinal chromosome shortening in a

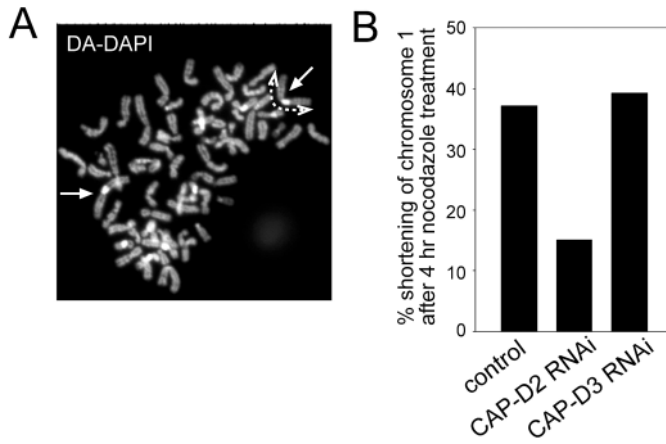


Fig. 6. Condensin I is required for longitudinal chromosome compaction in prolonged prometaphase. (A) Identification of chromosome 1. Spread chromosomes were stained for heterochromatin with distamycin (DA) followed by DAPI staining. Arrows indicate chromosome 1 with characteristic pericentromeric heterochromatin. (B) Quantitative analysis of chromosome length after nocodazole treatment in cells depleted of condensin I or II. Forty-eight hours after transfection with CAP-D2 or CAP-D3 siRNAs, cells were either incubated with or without nocodazole for 4 hours before being processed for chromosome spreading and DA-DAPI staining. The telomere-to-centromere-to-telomere distance of chromosome 1 was measured in 30 cells for each experiment (exemplified by a dashed line in A), and the average length by which chromosomes shortened during the 4-hour nocodazole treatment was calculated.

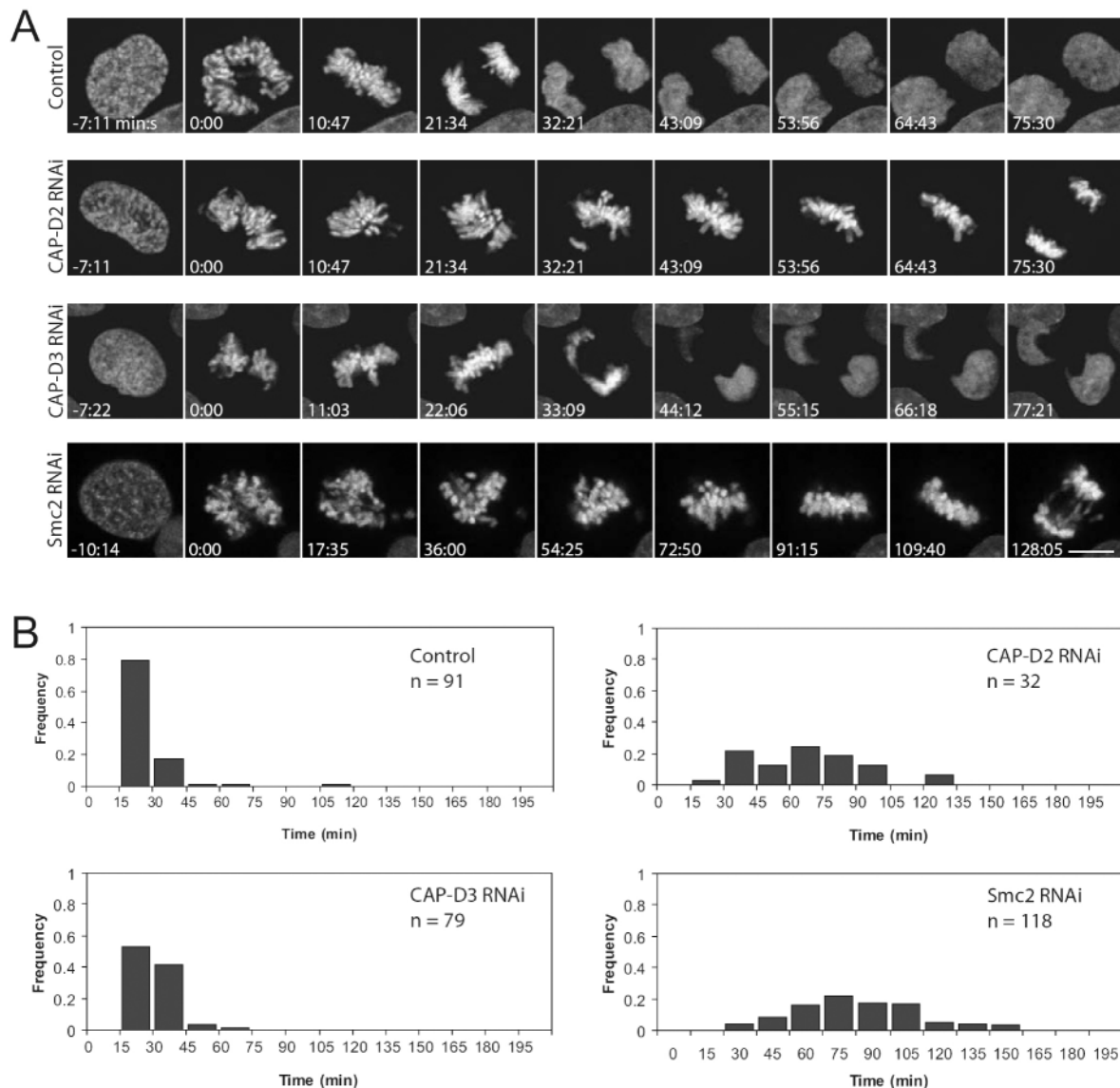


Fig. 7. Mitotic progression in condensin-depleted cells. Mitotic progression was followed in long-term time-lapse experiments of HeLa cells expressing H2B-EGFP. (A) Representative image sequences of control cells and condensin-depleted cells. (B) The time interval between NEBD and onset of segregation was measured based on the observed chromatin pattern.

prolonged prometaphase arrest. In the absence of nocodazole treatment, we found that the length of chromosome 1 in CAP-D2-depleted cells was similar to that in control cells (Fig. 6B). Because CAP-D2-depleted cells progress through prometaphase much more slowly than control cells (see below), this finding is consistent with the possibility that condensin I is also required for chromosome shortening in an unperturbed mitosis.

Condensin I is required for normal timing of progression from prometaphase to anaphase

To analyze mitotic progression in cells depleted of condensin I or II in real time, we used time-lapse imaging in HeLa H2B-EGFP cells to measure the time from NEBD to the onset of anaphase (Fig. 7, see also supplementary material, Movies 2-5). Control cells required 27 ± 12 minutes (mean \pm s.d., $n=91$) after NEBD to initiate anaphase, and similar time periods were observed in CAP-D3-depleted cells (32 ± 9 minutes, $n=79$). By contrast, CAP-D2-depleted cells were significantly ($P < 0.001$, Student's *t*-test) delayed in prometaphase to anaphase progression and required 68 ± 26 minutes ($n=32$); similar observations were made in Smc2-depleted cells. These analyses suggest that condensin I is important for normal timing of mitotic progression, and that its function cannot be replaced by condensin II. We presently do not know why condensin-I-depleted cells progress more slowly through mitosis. It is possible that this phenomenon is caused by defects in cohesin dissociation. However, we have not yet observed similarly strong delays in mitotic progression in cells in which cohesin dissociation from chromosomes was perturbed by other means (Silke Hauf, B.K. and J.-M.P., unpublished). We therefore consider it more likely that condensin I is required to generate physical properties of chromosomes, such as their rigidity at the centromere, which is needed to allow efficient bi-orientation of chromosomes on the mitotic spindle. In the future, it will be important to test this possibility by measuring the physical properties of condensin-depleted chromosomes and by analyzing how such chromosomes align on metaphase plates.

Conclusions

Our results show that the two condensin complexes coexisting in mammalian cells differ greatly in their intracellular distribution, the timing of their chromosome association and in their mitotic functions, despite the fact that both complexes share two subunits and associate with axial structures of sister chromatids. Condensin II is nuclear throughout interphase and mitotic prophase and is required for chromosome condensation in early prophase. By contrast, condensin I is cytoplasmic in interphase, associates therefore with chromosomes only at the onset of prometaphase, and is needed for normal timing of prometaphase and metaphase progression, and the complete removal of cohesin from chromosome arms. Our analyses revealed defects in condensin-II-depleted cells only during prophase but not at later stages of mitosis. In condensin-I-depleted cells prometaphase defects were seen, although condensin II seemed to associate normally with chromosomes, suggesting that condensin I confers properties to chromosomes that cannot be conferred by condensin II. Finally, we observed

that normal levels of condensin I and II are not required for chromosome condensation at the end of prophase, during prometaphase and metaphase, and that even condensin-I and -II depleted cells segregated their chromosomes after more than a 2-hour delay. These data further support the hypothesis that condensin is not solely responsible for chromosome condensation.

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