Contribution of hCAP-D2, a non-SMC subunit of Condensin I, to chromosome and chromosomal protein dynamics during mitosis.

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Running Title: Contribution of hCAP-D2 to mitotic chromosome dynamics

Material and Methods: 926 words

Introduction, Results and Discussion: 3883 words
Abstract

Condensins are heteropentameric complexes that were first identified as structural components of mitotic chromosomes. They are composed of two SMC and three non-SMC subunits. Condensins play a role in the resolution and segregation of sister chromatids during mitosis, as well as in some aspects of mitotic chromosome assembly. Two distinct Condensin complexes, Condensin I and Condensin II, exist, that differ only by their non-SMC subunits. Here we used an RNA interference approach to deplete hCAP-D2, a non-SMC subunit of Condensin I in HeLa Cells. We found that the association of hCAP-H, another non-SMC subunit of Condensin I, with mitotic chromosomes, depends on the presence of hCAP-D2. Moreover, chromatid axes, as defined by Topoisomerase II and hCAP-E localisation, are disorganised in the absence of hCAP-D2, and resolution and segregation of sister chromatids are impaired. In addition, hCAP-D2 depletion affects chromosome alignment in metaphase and delays entry into anaphase. This suggests that Condensin I is involved in the correct attachment between chromosome kinetochores and microtubules of the mitotic spindle. These results are discussed relatively to the effects of depleting both Condensin complexes.
Introduction

During mitosis, the two copies of replicated genomic DNA are segregated into each daughter cell. Due to the size of cells and to physical constraints linked to the segregation process, the replicated genome is converted into condensed chromosomes at the beginning of mitosis (32, 58). Among the factors involved in chromosome assembly process, literature has focused on a pentameric complex termed Condensin (28). This complex was isolated from mitotic chromosomes assembled in Xenopus egg extracts (34) and is composed of two SMC (structural maintenance of chromosomes; for review, see (30)) subunits (CAP-E/SMC2 and CAP-C/SMC4) and three non-SMC subunits (CAP-D2, CAP-G and CAP-H). Homologues of each Condensin subunit have been identified in all eukaryotes studied so far and were shown to be required for proper chromosome segregation (For review, see (28, 42)). In vitro studies showed that Condensin has a DNA-stimulated ATPase activity and is able to introduce constrained positive supercoils into DNA in the presence of ATP (39, 40).

The first functional studies of Condensin were made in mitotic extracts prepared from Xenopus eggs. When incubated in these extracts, sperm nuclei are converted into condensed chromosomes and this process was found to be totally blocked upon immunodepletion of Condensin (33). In living higher eukaryotes, mutating or inactivating individual Condensin subunits have more discrete effects on chromosome condensation. In Caenorhabditis and Drosophila embryos lacking the Condensin subunit SMC4, mitotic chromosomes are formed but adopt a dumpy morphology, indicating that lateral, rather than longitudinal compaction is altered (27, 55).

It was recently revealed that two distinct complexes, Condensins I and II exist in metazoans. Condensin I is predominant in Xenopus eggs whereas both Condensins are present at similar levels in human HeLa cells (8, 48). Each of these complexes can be independently inactivated by repressing the expression of non-SMC subunits specific for either complexes (48). Inactivating either Condensin I or II has distinct, subtle effects on chromosome architecture, whereas inactivating both Condensin complexes severely impairs assembly of mitotic chromosomes (48).

In mitosis, cells have also to remove topological links that exist between sister chromatids (resolution) and eventually between different chromosomes (individualisation). Most of the links generated between replicated fibres are removed during replication but sister chromatids remain largely catenated upon entry into mitosis. Resolution of these remaining links, which is necessary for proper segregation of sister chromatids in anaphase, is accounted for by Topoisomerase II (TopoII) activity (13, 18). As a consequence, TopoII mutation or inactivation leads to severe...
segregation defects (16, 35, 59). Since TopoII activity is in equilibrium between catenation and decatenation, a mechanism must exist on chromosomes, that would be able to orientate this activity toward decatenation. According to a currently accepted model, this mechanism would involve a step-by-step coupling between resolution and chromosome compaction activities (31, 32). Several observations suggest that such a coupling could involve a functional interaction between Condensins and TopoII. First, Condensins and TopoII are both structural components of mitotic chromosomes (19, 22) and both participate in the assembly of condensed chromosomes (1, 33). Furthermore, Condensin (9, 27, 51, 55-57) and TopoII (16, 35, 59) mutants exhibit similar segregation defects. Finally, Ycs4, the yeast homologue of CAP-D2, was shown to be indirectly involved in recruiting TopoII onto chromosomes (9). To date, the respective contributions of Condensins I and II to the localisation and activity of TopoII are not known.

In Caenorhabditis elegans, Condensins were shown to be involved in the proper orientation of centromeres toward the spindle poles. In the absence of functional Condensins, this orientation is altered but the attachment of kinetochores to spindle microtubules remains robust (27). This is unlikely due to the fact that nematode chromosomes are holocentric (i.e. they have several centromeres all along their length) since early reports are in favour of a role of Condensins in the bipolar attachment of chromosomes in yeast as well (41, 49). It is interesting to note that passenger proteins (for review, see (2)) are involved in both the recruitment of Condensins onto chromosomes (23, 27, 44) and the control of bipolar attachment of chromosomes to spindle microtubules (3, 11, 38), possibly by transmitting microtubule tension or attachment defects to the spindle checkpoint (12, 17, 29, 50). An opened possibility is that such a role of passenger proteins in controlling the bipolar attachment of chromosomes could be somehow related to Condensin functions. Condensins are able to introduce positive coils into DNA (7, 39, 40) and this activity could for instance participate in the correct assembly and orientation of centromeric structures (27).

In the present study, we used siRNAs specific for hCAP-D2 mRNA to specifically inactivate Condensin I and to study the contribution of this complex to chromosome dynamics in mitotic HeLa cells.

Materials and Methods

Cell culture
HeLa cells (ECACC 93021013) were grown in Dulbecco’s modified Eagle medium (Gibco-Invitrogen) supplemented with 10% fetal calf serum and penicillin-streptomycin at 37°C under a 5% CO₂ atmosphere.

RNA interference
siRNAs were provided by Dharmacon Research Inc. and used according to the providers instructions. hCAP-D2 siRNA sequence (AACCAUAUGCUCAGUGCUACA) starts at position 761 (702 from the initiation codon) in hCAP-D2/CNAP1 mRNA (accession NM_014865). An unrelated dsRNA (AACCAGCAGGACGACCUCUAA) was used for control experiments. siRNA transfections were made with Oligofectamine (Invitrogen) in OPTIMEM medium (Invitrogen) according to the manufacturer instructions.

Antibodies
Rabbit anti-hCAP-D2 were described in (54). Rabbit anti-hCAP-E/SMC2, and anti hCAP-H polyclonal sera were provided by J.M. Peters (62) and U.K. Laemmli (43), respectively. Mouse monoclonal antibodies against Topoisomerase II α (clone Ki-S1) and AuroraB (clone AIM-1) were from Chemicon International and BD Transduction Laboratories, respectively. Antibodies were used at 1:2,000 for Western Blot and at 1:400 for immunofluorescence experiments.

Immunofluorescence staining
Cells were grown on glass coverslips, rinsed in PBS, fixed in methanol for 6 minutes at -20°C, permeabilised for 3 minutes in PBS containing 0.1% Triton and blocked in PBS containing 1% BSA. When indicated, soluble antigens were extracted by incubation in 0.1 % Triton for 2 minutes followed by 3 minutes in PBS at room temperature prior to fixation in methanol. Cells were successively incubated for two hours at room temperature in primary antibodies (1:400 in PBS containing 1% BSA) and for one hour at room temperature in secondary antibodies (Rhodamine-coupled anti-mouse and FITC-coupled anti-rabbit from goat (Sigma) at 1:400 in PBS containing 1% BSA). After each step, cells were washed three times for 5 minutes in PBS at room temperature. Cells were counterstained in DAPI (0.5 μg/ml in PBS) and observed under a fluorescence microscope. When indicated, image stacks were acquired with a Leica DMRXA2 microscope driven by the Metavue driver. Z-steps of 0.3 μm were submited to
deconvolution (nearest neighbor method) by using Metamorph software. Presented pictures result from projections of three 0.3\( \mu \)m optical sections.

Flow cytometry analysis

Cells were recovered by standard trypsin treatment, rinsed six times in PBS and fixed in ethanol for 12 hours at -20°C. Cells were rinsed in PBS and treated with RNase A (0.1 mg/ml in PBS) for one hour at 37°C. DNA was stained by propidium iodide (0.1 mg/ml in PBS) and cells were analysed by FACS (Becton Dickinson).

Chromosome spreading

Non confluent cells (about 10^6) were treated with Nocodazole (5 \( \mu \)g in 10 ml of culture medium) for 6 hours. Cells were recovered by mitotic shake off and sedimented by centrifugation (450 g for 3 minutes). The cell pellet was resuspended in 2 ml of culture medium and mixed with 3 ml of tap water. After a 5 minute incubation at room temperature, the cell suspension was mixed with 7 ml Carnoy fixative (25% acetic acid and 75% ethanol). Cells were sedimented by centrifugation and washed in 10 ml Carnoy fixative. This step was repeated three times before recovering the cells in 1 ml Carnoy fixative. Samples (80 \( \mu \)l of cell suspension were dropped from a 10 cm height onto a microscope slide and air dried. Slides were immersed for 6 minutes in a freshly prepared Giemsa solution (5% in PBS) and rinsed briefly in tap water. Liquid excess was removed by applying a paper towel to the slide edges. Once air dried, slides were mounted in Entellan and spread chromosomes were observed under a light microscope. Alternatively, spread chromosomes were stained in SYBR-green (Molecular Probes), rinsed in PBS and mounted in Vectashield (Vector Laboratories).

Chromosome preparation

Non confluent cells (about 10^6) were treated with Nocodazole (0.5 \( \mu \)g/ml in culture medium) for 15 hours. Cells were sedimented by centrifugation (450 g for 10 minutes). The cell pellet was resuspended in 10 ml of ice-cold culture medium and kept on ice for 30 minutes. Cells were sedimented, resuspended in 10 ml of ice-cold 75 mM KCl and kept on ice for 20 minutes. After a second centrifugation, cells were resuspended in 1 ml of ice-cold aqueous disruption buffer (ADB: 10 mM Tris-HCl pH 7.4, 120 mM KCl, 20 mM NaCl, 0.1% Triton X-100, 0.1 mM PMSF, 2 mM CaCl2). Cells were kept on ice for 5 minutes and broken by passing 10 times through a hypodermic needle.
Interphase nuclei and debris were removed by centrifugation (400 g for 3 minutes at 4°C) and supernatant ("total"
fraction) was centrifuged again for 15 minutes at 3,000 g. The chromosome pellet was resuspended in 0.2 ml of
nuclear isolation buffer (NIB: 10 mM Hepes pH 7.5, 2 mM MgCl₂, 250 mM sucrose, 25 mM KCl, 1 mM DTT, 1
mM PMSF, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin). The chromosome suspension was centrifuged through a 0.5
ml sucrose cushion (750 mM in NIB, giving a final sucrose concentration of 1 M) for 20 minutes at 10,000 g. After
having removed the supernatant and sucrose cushion, the chromosome pellet was solubilised in SDS sample buffer
and analysed by polyacrylamide gel electrophoresis and western blotting.

Western Blot Analysis
Electrophoresis in SDS-polyacrylamide gels and electrotransfer onto nitrocellulose membranes were performed
following standard procedures. Membranes were blocked in TBST (25 mM Tris-HCl, 150 mM NaCl, 0.05% Tween
20 (Sigma)) containing 5% skimmed milk powder for 2 h at 4 °C. Membranes were incubated at 4 °C for 1 h with
crude sera diluted 1,000-fold in TBST containing 2.5% skimmed milk. Immunocomplexes were revealed with
alkaline phosphatase-coupled anti-rabbit antibodies (Jackson Immunoresearch Laboratories, Inc.) using a
chemifluorescence assay (Amersham Biosciences) according to the manufacturer’s instructions. Signals were
analysed using ImageQuant (Amersham Biosciences).

Results

Localisation of hCAP-D2 during the cell cycle.
In order to test antibodies used in this study and to address hCAP-D2 behaviour during the cell cycle, asynchronous
HeLa cells were fixed in methanol and analysed by immunofluorescence using a mixture of anti-Aurora B and anti-
hCAP-D2 antibodies (Fig. 1A). Aurora-B was reported to be undetectable in G1/S HeLa cells and to accumulate into
nucleus in G2 cells (15). In G1/S (Aurora-B negative) cells, hCAP-D2 signal was almost uniformly distributed
between nucleus and cytoplasm. Nuclear hCAP-D2 signal strongly diminished in G2 (Aurora-B positive) cells (Fig.
1A). As a consequence, hCAP-D2 appeared to be mainly cytoplasmic, although nuclear signal remained above the
background. To investigate further hCAP-D2 localisation, cells were treated with Triton prior to fixation in order to
extract soluble antigens (Fig. 1B). When G1/S cells were examined under these conditions, hCAP-D2 signal strongly
decreased in both nucleus and cytoplasm as compared with non extracted cells, indicating that a major part of hCAP-D2 is soluble in these cells. Interestingly, nuclear hCAP-D2 signals were similar in Triton-extracted G1/S and G2 cells, indicating that most of hCAP-D2 present in G1/S nuclei is soluble. As suggested previously (53), this soluble nuclear fraction of hCAP-D2 in G1/S cells could result from condensin complexes that has dissociated from chromosomes at the end of the preceding mitosis. These observations additionally suggest that a minor part of the hCAP-D2 is associated to an insoluble nuclear component throughout interphase.

In mitosis, as previously described, hCAP-D2 was found to decorate chromosome arms in both metaphase and anaphase cells and Aurora-B labelling was found on centromeric regions of chromosomes in metaphase and on the spindle midzone in anaphase. These mitotic stainings were not affected by Triton treatment prior to fixation (compare Fig. 1A and Fig. 1B).

**Depletion of hCAP-D2 by dsRNA interference in HeLa cells.**

In order to deplete hCAP-D2, HeLa cells were treated with either siRNA specific for hCAP-D2 mRNA or control dsRNA (see Materials and Methods). Protein extracts were prepared at different times after treatment and protein levels were monitored by immunoblotting with anti-hCAP-D2 antibodies (Fig. 2A). 24 hours after transfection, hCAP-D2 level was decreased up to 80% in siRNA treated cells as compared with control cells and no further decrease was observed between 24 and 72 hours. As monitored in the same experiment, the expression of hCAP-H was not affected. It is to be noted that in experiments where residual hCAP-D2 was undetectable, a decrease in hCAP-H levels was sometimes observed (data not shown). This suggests that in the absence of hCAP-D2, hCAP-H does not associate with SMC subunits anymore, and might become less stable. However, in the different experiments presented here, we only used conditions were hCAP-D2 depletion did not affect hCAP-H level. In some experiments, repression of hCAP-D2 was more progressive, reaching a maximal level of depletion at 72 hours. The expression and localisation of hCAP-D2 were further analysed by immunofluorescence 72 hours after dsRNA treatment (Fig. 2B). In control cells, consistently with previous studies, hCAP-D2 was found to decorate mitotic chromosomes. This chromosomal hCAP-D2 signal was either strongly diminished or absent in most siRNA treated cells. However, it has to be noted that this staining remained observable in about 10% of treated cells, probably due to the fact that some of these cells escaped transfection with ds RNA (not shown), which is consistent with the quantified depletion of hCAP-D2.
Effect of hCAP-D2 depletion on Condensin subunits and TopoII chromosome association.

Since hCAP-D2 was proposed to play a role in the recruitment of Condensin onto chromosomes (5), localisations of two other Condensin subunits, hCAP-E (one of the two SMC subunits) and hCAP-H (one of the three non-SMC subunits) were analysed by immunofluorescence in dsRNA-treated cells (Fig. 3A). In control cells, antibodies specific for hCAP-E and hCAP-H stained chromosome arms, giving pictures very similar to that obtained with anti-hCAP-D2 antibodies. TopoII signal was also observed on chromosomes, consistently with previous studies (20, 43). It is to be noted that a strong TopoII signal is present at the spindle poles, consistently with previous studies describing the presence of this protein in centrosomes (6). Whereas both TopoII and hCAP-E remained present on chromosomes in hCAP-D2 depleted cells, chromosomal localisation of hCAP-H was strongly diminished (Fig. 3A).

To further analyse the effect of hCAP-D2 depletion on the chromosomal recruitment of Condensin subunits, chromosomes were purified from mitotic cells and their protein contents analysed by immunobloting (Fig. 3C). In siRNA treated cells, hCAP-D2 levels were found to decrease in both the total and chromosome-associated fractions. These fractions were also probed with anti-TopoII and anti-Condensin antibodies. In siRNA treated cells, the levels of TopoII and hCAP-E remained constant in both total and chromosomal fractions as compared with fractions obtained from control cells. In contrast, although total hCAP-H levels were not affected, this Condensin subunit dissociated from mitotic chromosomes in hCAP-D2 depleted cells.

TopoII and hCAP-E localisation in hCAP-D2 depleted cells.

Since several reports are in favour of Condensin being involved in the dynamic recruitment of TopoII onto chromosomes (9, 36), we analysed in more detail the localisation of this enzyme in hCAP-D2 siRNA-treated cells. To this purpose, immunofluorescence pictures of mitotic cells were taken at higher resolution and treated by deconvolution (Fig. 4A). In control cells, both hCAP-D2 and TopoII antibodies decorated the axial region of chromosomes, giving an interspersed staining pattern consistent with previous studies (43). In cells treated with hCAP-D2 siRNAs, immunofluorescence signals of TopoII were more diffuse, suggesting either that its localisation was no longer confined to chromosome axes or that axes themselves were disorganised. To discriminate between these hypotheses, we compared immunofluorescence signals obtained by double staining with anti-TopoII and anti-hCAP-E antibodies (Fig. 4B). In control cells, hCAP-E was found to form patches aligned along the chromosome.
axes. Such a patchy pattern could be reminiscent to the previously described organisation of Condensins as a spiral structure along chromosome axes (48). TopoII was also found along chromosome axes, although not strictly colocalised with hCAP-E. In hCAP-D2 depleted cells, hCAP-E signals, although remaining punctuate, failed to form aligned patches, suggesting that Condensin-defined chromosome axes were somehow perturbed. TopoII signals also failed to form aligned structures and became more diffuse than in control cells. Therefore, spatial organisation of both TopoII and Condensins are altered in hCAP-D2 depleted cells.

Effect of hCAP-D2 depletion on mitotic chromosomes.

Condensins and TopoII were reported to play a role in both compaction and resolution of mitotic chromosomes. To examine chromosome structure in more detail, nocodazole-treated mitotic cells were spread and chromosomes stained with Giemsa (Fig. 5A). In control cells, chromosomes were individualised and had a characteristic rod shaped structure and well separated sister chromatids. In hCAP-D2 depleted cells, chromosomes were poorly individualised and sister chromatids often failed to be fully separated. When stained with SYBR-Green (Fig. 5B), chromosomes from hCAP-D2 depleted cells appeared more entangled in each other and sister chromatids were much less distinguishable than in chromosomes spread from control cells. In addition, whereas chromatids from control cells had a well defined structure typical of super-condensed chromosomes (43), chromosomes from hCAP-D2 depleted cells adopted a diffuse and fluffy appearance, reminiscent to what was observed by depleting hCAP-G, another non-SMC subunit of condensin I (48). Therefore hCAP-D2 depletion alters both the structure of chromosomes and the resolution of sister chromatids.

Mitotic effects of hCAP-D2 depletion.

The effects of hCAP-D2 depletion on both resolution and individualisation of mitotic chromosomes were likely to have consequences on the course of mitosis. To address this issue, mitotic cells were observed at different stages in both control and hCAP-D2-siRNA series. When scored manually, the mitotic index (about 6%) was not significantly different in control and hCAP-D2 depleted cells (data not shown). The numbers of cells in different mitotic stages were scored in both series. The relative populations in each stage were constant in control cells at 48, 72 and 96 hours (Fig. 6A, left panel). In contrast, the proportion of mitotic cells in prometaphase or metaphase increased between 48 h and 96 h after treatment with hCAP-D2 siRNAs. Conversely, the proportion of cells in anaphase or
telophase progressively decreased in course of time in these treated cells (Fig. 6A, right panel). This suggests that entry into anaphase was delayed in hCAP-D2 depleted cells. To estimate the possible consequences of such a delay on their DNA content, control and depleted cells were analysed by flow cytometry (Fig. 6B). FACS profiles confirmed that the mitotic index was not significantly different between control and siRNA treated cells. Despite the increased metaphase to anaphase ratio observed above, no polyploid cells were found to accumulate up to 96 h after siRNA treatment. Rather, cells with a DNA content lower than 2n were found to accumulate in hCAP-D2 depleted cells. These cells with low DNA contents could correspond to apoptotic cells although it cannot be formally excluded that aneuploid cells also exist in this population.

The above observations indicate that entry into anaphase is impaired in the absence of hCAP-D2. This impairment could be due to a persistence of topological links between sister chromatids that in turn would mechanically block segregation in early anaphase. On the other hand, entry into anaphase could be delayed by the persistence of metaphase defects. In order to explore these possibilities, cells in anaphase or metaphase were observed in more detail. First, we observed hCAP-D2 depleted cells that entered anaphase. Some of these cells showed segregation defects resulting in the persistence of chromatin bridges between the two sets of chromosomes (Fig. 7A). Such defects were also observed in control cells and represented 10% of all anaphases, whatever the observation time was. In hCAP-D2 depleted cells analysed 48h and 72h after siRNA treatment, about 30% of all anaphases were abnormal and this score reached 45% at 96h (Fig. 7B). hCAP-D2 depletion therefore resulted in an increase in segregation defects.

According to previous studies, a delayed entry into anaphase could result from improper attachment and/or alignment of chromosomes in metaphase, which activates the spindle checkpoint (for review, see (64)). This would suggest that, on the contrary to what is observed in SMC2 or SMC4 depleted cells (14, 36) hCAP-D2 depletion does not affect spindle checkpoint activation. Since spindle checkpoint control depends on passenger proteins, localisation of these proteins should not be disrupted in hCAP-D2 depleted cells. To address this issue, control and hCAP-D2 depleted cells were stained with anti-Aurora B and anti-survivin antibodies. Aurora B and survivin are passenger proteins that localise on centromeres between prophase and metaphase. Upon entry into anaphase, they are released from centromeres to reach the midzone of mitotic spindles. In both series, Aurora B (Fig. 8B) and survivin (not shown) localised properly on mitotic chromosomes. In addition, when observed by fluorescence microscopy, hCAP-D2 depleted cells exhibited misaligned chromosomes in metaphase (Fig. 8A, b, c, d) as compared with control cells.
Aurora B being used as a centromeric marker that is specific for prometaphase and metaphase cells (Fig. 8B). In control cells, Aurora B staining showed discrete dots in prometaphase (Fig. 8B, a") that aligned on the metaphase plate (Fig. 8B, b"). Aurora B localisation was not altered in prometaphase from hCAP-D2 depleted cells (Fig. 8B, c"). However, Aurora B labelled centromeres appeared to be stretched in metaphase (Fig. 8B, d"-e") and some of these seemed to escape from the metaphase plate (Fig. 8B, e") to reach spindle regions closer to the poles. This indicates that the alignment of mitotic chromosomes on the metaphase plate is impaired in hCAP-D2 depleted cells.

**Discussion**

Condensin I (initially referred as Condensin) has been shown to play important roles in the assembly and segregation of mitotic chromosomes. Recently, a second distinct complex, termed Condensin II, that differs from Condensin I by its non-SMC subunits, has been characterised (48). Here, we studied the effect of depleting hCAP-D2, a non-SMC subunit of Condensin I on the chromosome composition and dynamics in mitosis. First, we observed that depleting hCAP-D2 up to 80% with specific siRNAs led to a strong decrease of hCAP-D2 levels on chromosome axes. Interestingly, hCAP-D2 depletion provoked hCAP-H (the Barren homologue) to be released from chromosomes to a great extent. A two-hybrid screen performed in our lab identified CAP-H as a direct partner of CAP-D2 (unpublished data) and this interaction was confirmed by a direct two-hybrid assay (21). It is therefore conceivable that depleting hCAP-D2 weakens the association of hCAP-H to the Condensin I core complex. hCAP-D2 was shown to contain several HEAT motifs (46) and a role of hCAP-D2 in the assembly of Condensin I subunits would be consistent with a more general role of HEAT proteins in stabilising macromolecular complexes (4, 24). hCAP-H is a member of the Kleisin protein family which comprises CAP-H/Barren, CAP-H2/KLE2 and Scc1 in Condensin I, Condensin II and Cohesin, respectively (52, 62). Condensin and Cohesin SMC heterodimers were shown to exhibit a V shape that would be able to embrace or trap two segments of chromatin. Accordingly, Cohesin would ensure sister chromatid cohesion by embracing the two sister chromatids (25, 26) and Condensins would structure mitotic chromosomes by embracing two looped adjacent segments of chromatin (63). In this model, Kleisins would act as a gate that would stably close the V-shaped SMC-containing complexes (25). Based on this model, releasing hCAP-H would directly dissociate the Condensin I core complex from chromosomes. This is consistent with data suggesting that both CAP-
D2 and CAP-H are involved in the recruitment of Condensin I onto chromosomes (5, 21). However, in conditions where both hCAP-D2 and hCAP-H were absent from chromosomes, levels of bound hCAP-E were not affected. One explanation would be that residual levels of hCAP-E are accounted for by the intact Condensin II complex. However, HeLa cells were shown to contain equal quantities of Condensin I and Condensin II (48) and we did not observe any decrease in the level of chromosome-associated hCAP-E, under conditions where hCAP-H was clearly diminished in purified chromosomal fractions. Furthermore, we obtained very similar results in Xenopus egg extracts (60), an experimental model where Condensin I is five fold more abundant than Condensin II (48). These observations raise the possibility that in a situation where hCAP-D2 is not completely depleted, the remaining Condensin I would contribute to recruit and/or stabilise Condensin core complexes (consisting of the two SMC subunits) by protein-protein interactions. This would be consistent with in vitro studies showing that 8S Condensin core complexes can be processively recruited onto DNA by an aggregation process (63).

Depletion of hCAP-D2 also affected both the individualisation of mitotic chromosomes and the resolution of sister chromatids. This strongly suggests that the action of TopoII is somehow perturbed in the absence of functional Condensin I. We observed that chromatid axes, as defined by hCAP-E localisation, are altered in the absence of hCAP-D2. Possibly as a consequence, TopoII, although remaining on the chromosomes, also fails to localise along well defined axes in depleted cells. Such a mislocalisation of TopoII has also been observed in Drosophila (14) and Chicken (36) cells lacking SMC4. It is very unlikely that the observed resolution and individualisation defects come from TopoII inactivation in hCAP-D2 depleted cells. Rather, we propose that its activity only fails to be orientated toward the decatenation (removal of DNA topological links between sisters) due to its failure to adopt a correct axial localisation. An assay able to discriminate between catenating and decatenating activities of TopoII in living cells would be of great interest to address this issue.

We also showed that hCAP-D2 depletion had two major effects on the progression of mitosis: segregation defects in anaphase and failure of chromosomes to align on the metaphase plate. The effects on chromosome segregation are consistent with many studies that highlighted a role of Condensins in this process (10, 27, 41, 49, 51, 55, 56, 61). Such an impaired segregation is probably a direct consequence of either an altered activity (9, 14) and/or a mislocalisation (14, 36) (and this study) of TopoII.

We also observed a misalignment of chromosomes onto the metaphase plate in the absence of hCAP-D2.

Centromeric regions are stretched in metaphase cells and some centromeres even escape from the metaphase plate in
hCAP-D2 depleted cells. Similar alignment defects and kinetochore stretching were recently observed by inactivating either Condensin I or II (47). Previous studies suggested that misaligned chromosomes could result from bipolar attachment defects due to an altered assembly and/or orientation of kinetochores in the absence of functional Condensin (27, 41, 49). Interestingly, alignment defects were also reported in cells whose passenger proteins had been depleted or inactivated (3, 23, 38). Passenger proteins such as Aurora-B (23, 27, 38) and Bir1p/Survivin (44) have been shown to be involved in the recruitment of Condensins. Condensins could therefore mediate, at least partially, the role of passenger proteins in the attachment of kinetochores to spindle microtubules (11, 29, 45). Accordingly, depletion of hCAP-G, another non-SMC subunit of Condensin I, was recently shown to alter the geometry of kinetochores (47). One could propose that Condensins contribute to assemble and orientate kinetochores through their supercoiling activity. Alternatively, they could act as an intermediate platform to recruit and stabilise factors involved in the bipolar attachment of chromosomes to the spindle.

Finally, we observed that hCAP-D2 depletion led to a delayed entry into anaphase. This delay is likely to be due to the activation of the spindle checkpoint that responds to an impaired bipolar attachment of chromosomes to the spindle and/or a lack of tension at the kinetochores (64). In contrast to what we observed for CAP-D2, depleting SMC4/CAP-C, which is common to Condensins I and II, also leads to alignment defects (47) but fails to delay entry into anaphase (14), implying that the spindle checkpoint is overridden under these conditions. This is reminiscent of recent observations that inactivating Aurora B impairs not only chromosome alignment but also the activation of the spindle checkpoint, due to a role of this protein in recruiting spindle checkpoint factors (17, 29, 37). Given that the recruitment of Condensins onto chromosomes depends on Aurora B, it can be proposed that Condensins act as an intermediate for Aurora B functions in both chromosome attachment and spindle checkpoint activation. Inactivating both Condensins would therefore impair chromosome attachment as well as activation of the spindle checkpoint. Inactivating Condensin I only would impair the correct attachment of chromosomes to microtubules but not the activation of checkpoint. However, we cannot formally exclude that a complete depletion of hCAP-D2 would had inactivate the spindle checkpoint as well.

Taken together with recent studies (47), these observations suggest that Condensins I and II have distinct contributions to the composition, structure or function of centromeric regions.

Acknowledgements
We thank U.K. Laemmli, T. Hirano and J.M. Peters for providing some of the antibodies used in this study. We are also grateful to C. Kraft, S. Küng and H.B. Osborne for critical reading of the manuscript. Microscopy analyses were performed thanks to the IFR97 microscopy service with the assistance of its manager Stephanie Dutertre.

The author’s research is supported by grants from the Association pour la Recherche contre le Cancer, Contract number 5711. CNRS UMR 6061 is a component of the Federative Research Institute IFR97, Functional Genomics and Health. E.W. was a recipient of a fellowship from the french Fondation pour la Recherche Médicale.

References


Legends to figures

Figure 1: Detection of Aurora-B and hCAP-D2 in interphase and mitotic cells.
Asynchronous HeLa cells were treated (B) or not (A) by Triton prior to fixation in methanol (see Material and Methods) and immunostained with a mixture of anti Aurora-B (red) and anti-hCAP-D2 (green) antibodies. Cells were counterstained with DAPI (blue) and observed by fluorescence microscopy. Cells in G1/S, G2, metaphase (Meta) and anaphase (Ana) are presented.

Figure 2: Depletion of hCAP-D2 by siRNAs.
HeLa cells were treated with control or hCAP-D2 siRNAs as described in Material and methods. A) Proteins were extracted at the indicated times after siRNA transfection, separated on a 8% polyacrylamide gel and analysed by western blotting with anti-hCAP-D2 and anti-hCAP-H antibodies. B) Cells were fixed 72 h after siRNA treatment, immunostained with anti-hCAP-D2 antibodies and counterstained with DAPI. Pictures of prometaphase and anaphase cells are shown for each series.

Figure 3: Effect of hCAP-D2 depletion on Condensin subunit and TopoII chromosome association.
Cells were treated with control or hCAP-D2 siRNAs and analysed 72 h later. A) Treated cells were fixed and double-labelled with anti-TopoII antibodies and antibodies against either hCAP-D2, hCAP-H or hCAP-E as indicated. Pictures of mitotic cells are shown. B) Total proteins were analysed as in figure 2 with anti-hCAP-D2 and anti-hCAP-H antibodies. C) 60 h after siRNA treatment, cells were incubated in nocodazole-containing culture medium for an additional 12 hours. Mitotic cells were recovered by shake off and lysed (total extract). Chromosomes were isolated by centrifugation through a sucrose cushion. Total and chromosomal proteins were separated by polyacrylamide gel electrophoresis and analysed by western blotting with anti-TopoII, anti-hCAP-D2, anti-hCAP-E and anti-hCAP-H antibodies.

Figure 4: Effect of hCAP-D2 depletion on the localisation of TopoII and hCAP-E in prometaphase.
Cells treated with control or h-CAP-D2 siRNAs were fixed and immunostained after 72h. A) Prometaphase cells were double-labelled with anti-TopoII (red) and anti-hCAP-D2 (green) antibodies and counterstained with DAPI. B) Prometaphase cells were double-labelled with anti-hCAP-E (green) and anti-TopoII (red) antibodies. Pictures were
taken with a 63X objective and treated by deconvolution. Inserts correspond to enlarged areas taken from corresponding pictures.

**Figure 5:** Effect of hCAP-D2 depletion on the shape, resolution and individualisation of mitotic chromosomes.

Cells were treated with siRNAs. After a 66 hour recovery, they were blocked in mitosis by a 6-hour nocodazole treatment. Mitotic cells were recovered by shake off and chromosomes were spread after hypotonic treatment as described in materials and methods. Spread chromosomes were stained with Giemsa (A) or SYBR-green (B).

**Figure 6:** Effect of hCAP-D2 depletion on the progression of mitosis.

A) Cells were treated with siRNAs, fixed at the indicated times and stained with DAPI. Cells in prophase (black bars), prometaphase/metaphase (open bars) or anaphase/telophase (gray bars) were scored and expressed as a percentage of total mitotic cells. B) Cells were recovered by trypsin treatment 72 h or 96 h after siRNA treatment. Suspended cells were fixed in ethanol and treated with RNase A. After DNA staining with propidium iodide, cells were analysed by flow cytometry. Graphs indicate cell counts as a function of their DNA content. Note that cells with a DNA content lower than 2n accumulate in the absence of hCAP-D2.

**Figure 7:** Chromosome segregation is impaired in the absence of hCAP-D2.

Cells were treated with siRNAs, fixed at different times and stained with DAPI. A) Examples of normal (a) and abnormal (b-d) anaphase pictures obtained in hCAP-D2 depleted cells. B) Normal and abnormal anaphase pictures were scored at the indicated times in control (open bars) and hCAP-D2 depleted cells (black bars). Results are expressed as a percentage of total anaphase/telophase cells.

**Figure 8:** Effect of hCAP-D2 depletion on the metaphase plate.

Cells were treated with control or hCAP-D2 siRNAs and analysed by immunofluorescence after a 72 h recovery. A) Example of normal (a) and abnormal metaphase pictures obtained in hCAP-D2 depleted cells (b-d). Cells were immunostained with anti-tubulin antibodies (red) and counterstained with DAPI (blue). B) Cells treated with control (left panel) or hCAP-D2 siRNAs (right panel) were double-stained with anti-Aurora B and anti-hCAP-D2 antibodies and counterstained with DAPI. Cells in prometaphase (a-a”, c-c”) or metaphase (b-b”, d-d”, e-e”) are shown.
**Fig. 3**

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Fig. 4

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![Image A](image1)

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![Image B](image2)
Fig. 6

(A) Bar graphs showing the percentage of cells in different phases of the cell cycle (prophase, metaphase, anaphase) at different time points (48, 72, 96 hours) for Control siRNA and hCAP-D2 siRNA. The percentages are as follows:

- Control siRNA:
  - 48 hours: 10% prophase, 50% metaphase, 40% anaphase
  - 72 hours: 11% prophase, 55% metaphase, 34% anaphase
  - 96 hours: 11% prophase, 54% metaphase, 35% anaphase

- hCAP-D2 siRNA:
  - 48 hours: 15% prophase, 46% metaphase, 29% anaphase
  - 72 hours: 9% prophase, 83% metaphase, 29% anaphase
  - 96 hours: 10% prophase, 70% metaphase, 20% anaphase

(B) Flow cytometry histograms for 2n and 4n cell counts at 72 and 96 hours for Control siRNA and hCAP-D2 siRNA.
Fig. 8

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siRNA

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