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The condensin complex is required for proper spindle assembly and chromosome segregation in *Xenopus* egg extracts

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Chromosome condensation is required for the physical resolution and segregation of sister chromatids during cell division, but the precise role of higher order chromatin structure in mitotic chromosome functions is unclear. Here, we address the role of the major condensation machinery, the condensin complex, in spindle assembly and function in *Xenopus laevis* egg extracts. Immunodepletion of condensin inhibited microtubule growth and organization around chromosomes, reducing the percentage of sperm nuclei capable of forming spindles, and causing dramatic defects in anaphase chromosome segregation. Although the motor CENP-E was recruited to kinetochores pulled poleward during anaphase, the disorganized chromosome mass was not resolved. Inhibition of condensin function during anaphase also inhibited chromosome segregation, indicating its continuous requirement. Spindle assembly around DNA-coated beads in the absence of kinetochores was also impaired upon condensin inhibition. These results support an important role for condensin in establishing chromosomal architecture necessary for proper spindle assembly and chromosome segregation.

Introduction

Chromosomes play an active role in their own segregation during cell division. Kinetochores assembled at the centromere of each sister chromatid attach to spindle microtubules and dictate chromosome alignment and segregation (Rieder and Salmon, 1998; Maney et al., 2000). Proteins associated with chromosome arms also contribute to chromosome movements, microtubule stabilization, and spindle organization (Andersen, 1999; Karsenti and Vernos, 2001). Fundamental to mitotic chromosome architecture is condensation, which in vertebrates reduces chromosome length ~100-fold relative to interphase, and is crucial to physically resolve entanglements and allow separation of the duplicated genome into two discrete sets (Heck, 1997). However, the contribution of the condensed state to mitotic chromosome functions is poorly understood.

Strongly implicated in mitotic chromosome condensation is the condensin complex, which was first purified from *Xenopus laevis* egg extracts. Depletion of this complex from clarified extracts severely compromised condensation of demembranated sperm nuclei into discrete chromosomes, and inhibition of condensin after condensation had occurred also caused defects, pointing to a role for the complex in both the establishment and maintenance of the condensed state (Hirano et al., 1997). The active condensin complex consists of five proteins including two members of the highly conserved structural maintenance of chromosomes (SMC)* ATPase superfamily that form a coiled-coil heterodimer (*Xenopus* chromosome–associated protein [XCAP]-C and XCAP-E; Hirano and Mitchison, 1994; Hirano et al., 1997). SMC proteins play multiple roles in chromosome organization and function, including sister cohesion, dosage compensation, and recombination-mediated repair (Strunnikov and Jessberger, 1999). Condensin also contains three non-SMC proteins (XCAP-H, -G, and -D2; Hirano et al., 1997; Uhlmann, 2001), which have been proposed to play targeting or regulatory roles in condensin function. In the presence of a type I topoisomerase, purified *Xenopus* condensin can reconfigure DNA structure in an ATP hydrolysis–dependent manner (Kimura and Hirano, 1997; Kimura et al., 1999). Electron spectroscopic imaging has revealed supercoiling of DNA by a single condensin complex, suggesting

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*Abbreviations used in this paper: CSF, cytostatic factor; FRET, fluorescence resonance energy transfer; GEF, guanine nucleotide exchange factor; SMC, structural maintenance of chromosomes; topoII, topoisomerase IIα; XCAP, *Xenopus* chromosome–associated protein.

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that it functions by generating positively supercoiled chromatin loops (Bazett-Jones et al., 2002).

Evidence supporting a role for condensin function in mitosis comes from several organisms. In *Drosophila melanogaster*, *Caenorhabditis elegans*, *Saccharomyces cerevisiae,* and *Schizosaccharomyces pombe*, loss of condensin subunit function results in chromosome segregation defects (Saka et al., 1994; Bhat et al., 1996; Sutani et al., 1999; Lavoie et al., 2000; Ouspenski et al., 2000; Steffensen et al., 2001; Bhalla et al., 2002; Hagstrom et al., 2002; Stear and Roth, 2002). The simplest explanation for these defects, consistent with the observations in *Xenopus* egg extracts (Hirano and Mitchison, 1994; Hirano et al., 1997), is that a gross failure in condensation prevents the chromosomes from being disentangled during anaphase. Mutation of condensin subunits in budding yeast increased the average distance between fluorescently labeled loci on a mitotic chromosome, supporting this model (Strunnikov et al., 1995; Lavoie et al., 2000; Ouspenski et al., 2000). However, chromosome condensation defects appeared more subtle upon loss of condensin function in *Drosophila* or *C. elegans*, resulting in a more diffuse prophase morphology, whereas metaphase chromosome compaction and longitudinal shortening of chromosomes were not dramatically affected (Steffensen et al., 2001; Hagstrom et al., 2002). In *C. elegans*, anaphase defects may be due at least in part to a failure in holocentric kinetochore organization. RNAi analysis revealed that condensin is required for the restricted orientation of kinetochores toward opposite spindle poles (Hagstrom et al., 2002), and mutation of one of the condensin subunits resulted in chromosome twisting and merotelic spindle attachments (Stear and Roth, 2002). Because holocentric kinetochores are thought to form as dispersed sequences coalesce, it has been proposed that these kinetochore defects arise from a failure in global chromosome condensation (Hagstrom et al., 2002). However, it is unclear whether the condensin complex also contributes to kinetochore organization in monocentric organisms.

Although the condensin complex has been shown to be required for the establishment and maintenance of chromosome condensation in clarified *Xenopus* extracts, its role in mitosis has not been studied using concentrated extracts that can support spindle assembly and function (Hirano et al., 1997). This system has the advantage of allowing independent examination of both kinetochore and chromatin activities during mitosis, because spindles can be formed both in the presence and absence of kinetochores. Upon incubation in *Xenopus* egg extracts, sperm chromosomes form functional kinetochores that mediate chromosome alignment and anaphase segregation in vitro (Murray et al., 1996; Desai et al., 1999), dependent on factors including the kinetochore kinesin-like protein CENP-E (Wood et al., 1997). In addition, plasmid DNA-coated beads drive bipolar spindle assembly in the absence of centrosomes and kinetochores, demonstrating a substantial role for mitotic chromatin in spindle assembly (Heald et al., 1996). The chromatin-dependent stabilization of microtubules is thought to be mediated primarily by RanGTP, which is generated by the chromatin-bound guanine nucleotide exchange factor (GEF) RCC1, causing localized release of cargoes from the transport factor importin β that promote spindle assembly specifically in the vicinity of chromosomes (for reviews see Clarke and Zhang, 2001; Dasso, 2001; Hetzer et al., 2002; Macara, 2002). In addition to RCC1, other chromatin-bound factors, such as chromosomal kinesin motors and Aurora and Polo kinases, play essential roles in chromosome alignment and segregation (Vernos et al., 1995; Antonio et al., 2000; Funabiki and Murray, 2000; Budde et al., 2001), but the relationship between mitotic chromosome architecture and the localization and function of these factors is not known.

Here, we address the role of condensin during spindle assembly and anaphase chromosome segregation in *Xenopus* egg extracts. We find that condensin activity establishes a chromosomal architecture that promotes proper microtubule organization during spindle assembly and is required

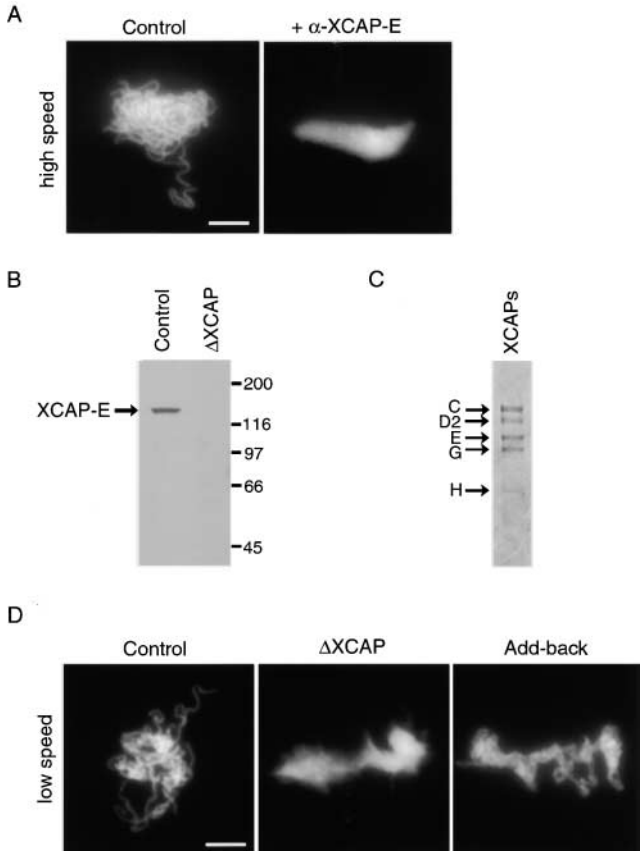

Figure 1. **Condensin depletion from crude *Xenopus* egg extracts causes defects in chromosome condensation.** (A) Chromosomes assembled in clarified extract to which buffer (Control) or 0.26 mg/ml XCAP-E antibody (+ α-XCAP-E) was added. *Xenopus* sperm nuclei were incubated in CSF extract for 90 min, and fixed and stained with Hoechst 33258. Bar, 10 μm. (B) Western blot analysis of crude *Xenopus* egg extracts depleted using IgG (Control) or XCAP-E and XCAP-G antibodies (ΔXCAP), and probed with affinity-purified XCAP-E antibody, which recognized a single band of 140 kD in control extract. (C) Silver-stained 8% gel of *Xenopus* condensin complex purified from *Xenopus* egg extract using XCAP-G antibody–coupled beads and eluted using XCAP-G peptide. All five subunits of the complex are present, though XCAP-H appears lighter and more diffuse. (D) Chromosomes assembled in control and condensin-depleted crude extracts. *Xenopus* sperm nuclei were added to extracts, which were then cycled through interphase and back into mitosis. The reactions were fixed in metaphase, 60 min after initiation of mitosis. Bar, 10 μm.

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continuously to allow chromosome resolution and segregation during anaphase.

Results

Depletion of the condensin complex from crude extracts causes chromosome condensation defects

To address the role of chromosome architecture in mitosis, we first established conditions to deplete the condensin complex from *Xenopus* egg extracts. We generated peptide antibodies against two components of the *Xenopus* condensin complex, XCAP-E and XCAP-G, using sequences identical to those described previously (Hirano et al., 1997; Kimura and Hirano, 2000). Consistent with previous studies, immunodepletion of condensin using these antibodies or addition of anti–XCAP-E to diluted, high speed *Xenopus* egg extracts resulted in defective sperm chromosome condensation (Fig. 1 A and not depicted; Hirano and Mitchison, 1994; Hirano et al., 1997). However, these clarified extracts are arrested in metaphase and, therefore, chromosome condensation in this system does not fully mimic in vivo condensation. Therefore, we tested the ability of our antibodies to deplete the condensin complex from crude (10,000 × g) extracts capable of transiting through the cell cycle. After two rounds of depletion using a mixture of both antibodies, >98% of XCAP-E was removed from the extract, as judged by Western blot (Fig. 1 B). A similar reduction in XCAP-G was observed (unpublished data), indicating that the entire 13S condensin complex was depleted. Elution of the complex from the antibody beads with peptide yielded highly purified condensin (Fig. 1 C). To test the effects on chromosome morphology, sperm nuclei were added to control and condensin-depleted crude extracts, which were cycled through interphase to allow DNA replication, and then back into mitosis. In control extracts, individual condensed chromosomes were clearly visible (Fig. 1 D). However, chromosomes assembled in the absence of the condensin complex were aberrant. The chromatin formed a diffuse mass and individual chromosomes were not apparent, suggesting that both chromosome condensation and resolution were impaired. Similar defects were observed if XCAP-E antibodies were added to the extract at the start of mitosis (not depicted), and the aberrant chromosomes were similar to those seen upon condensin inhibition in the high speed extract (Fig. 1 A). Importantly, just as for the high speed reactions, chromosome condensation and resolution could be rescued upon addition of purified condensin complex to the depleted extract (Fig. 1 D). Therefore, condensin immunodepletion can be achieved in crude extracts and results in mitotic chromosome defects morphologically similar to those described in clarified ex-

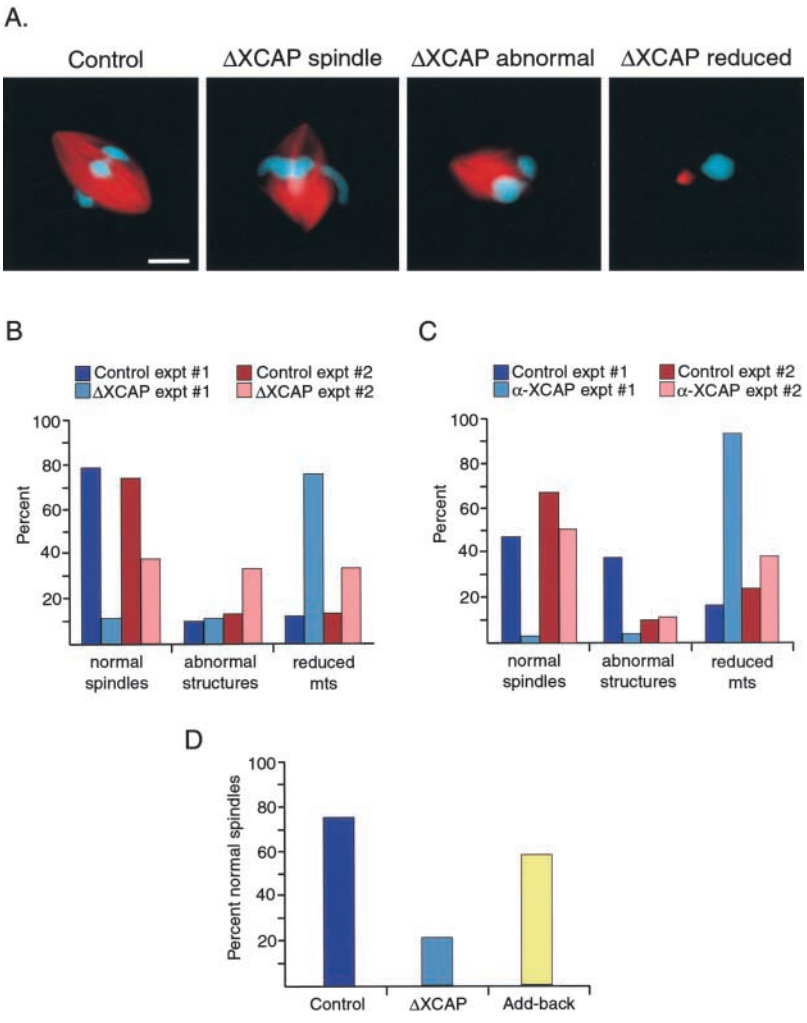

Figure 2. **Condensin inhibition reduces the fidelity of sperm spindle assembly.** (A) Mitotic structures formed in extracts depleted using IgG (Control) or condensin antibodies (ΔXCAP) that were fixed 60 min after the induction of mitosis. The three ΔXCAP images show structures representative of the three categories quantified in B and C: normal spindles, abnormal structures, and reduced microtubules. In all images, microtubules appear red and chromosomes blue. Bar, 10 μm. (B) Quantification of the effect of condensin depletion on spindle assembly 60 min after the start of mitosis. Two experiments (red and blue bars) showing the percent sperm nuclei in each category depicted in A. For each condition, >100 structures were counted. (C) Quantification of the effect of XCAP-E antibody addition on spindle assembly. Two separate experiments are shown. To each spindle reaction, either buffer (Control) or 0.26 mg/ml α-XCAP-E antibody (α-XCAP) was added at the start of mitosis. Samples were fixed at 60 min and the microtubule structures were assigned to the categories depicted in A. (D) Spindle assembly reactions in extracts depleted with IgG (Control) or XCAP antibodies (ΔXCAP), and in XCAP-depleted extract to which purified *Xenopus* condensin complex was added (Add-back). Samples were fixed at 60 min after the start of mitosis and the microtubule structures were examined. Percent normal spindles is shown.

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tracts, suggesting that the condensin complex is the major condensation machinery under both conditions.

Condensin depletion reduces the fidelity of spindle assembly

Next, we tested whether spindles could form around aberrant sperm chromosomes lacking the condensin complex. Rhodamine-labeled tubulin was added to the reactions, which were monitored at 15-min intervals after entry into mitosis. In control extracts, most sperm nuclei directed the formation of bipolar spindles by 60 min. However, depletion of the condensin complex significantly reduced the percentage of normal structures formed. The condensin-depleted spindles were morphologically similar to control spindles, though many were smaller, suggesting that microtubule polymerization was less robust (Fig. 2 A). To quantify the severity of the spindle defects, reactions were fixed at 60 min. Each sperm nucleus on the slide was examined, and the microtubule array surrounding it was classified as a normal spindle, an abnormal structure, or a structure containing a reduced number of microtubules. Representative structures are shown in Fig. 2 A. After condensin depletion, a reduction in the percentage of normal spindles was always observed (Fig. 2 B). However, the extent of inhibition varied depending on the experiment. In seven independent experiments, the percent reduction varied from 32 to 93%, with an average of 64%. The levels of depletion in all experiments were indistinguishable by Western blot, suggesting that the variability did not arise from altered efficiency of condensin depletion, but rather resulted from differences among extract preparations. In support of this hypothesis, we observed similar variability among extracts when we inhibited condensin function by adding XCAP-E antibodies (Fig. 2 C). Effects of antibody addition were neutralized by preincubation with peptide (unpublished data). Furthermore, as with the condensation defects, spindle assembly phenotypes could be substantially rescued upon addition of purified condensin to the depleted reactions (Fig. 2 D). Therefore, we conclude that although condensin function is not required for formation of bipolar microtubule arrays around sperm chromosomes in *Xenopus* egg extracts, it significantly enhances the fidelity of spindle assembly.

Chromosomes lacking condensin fail to resolve and segregate during anaphase

To determine whether the spindles formed in the absence of the condensin complex were functional, we induced anaphase and evaluated the effects on chromosome segregation.

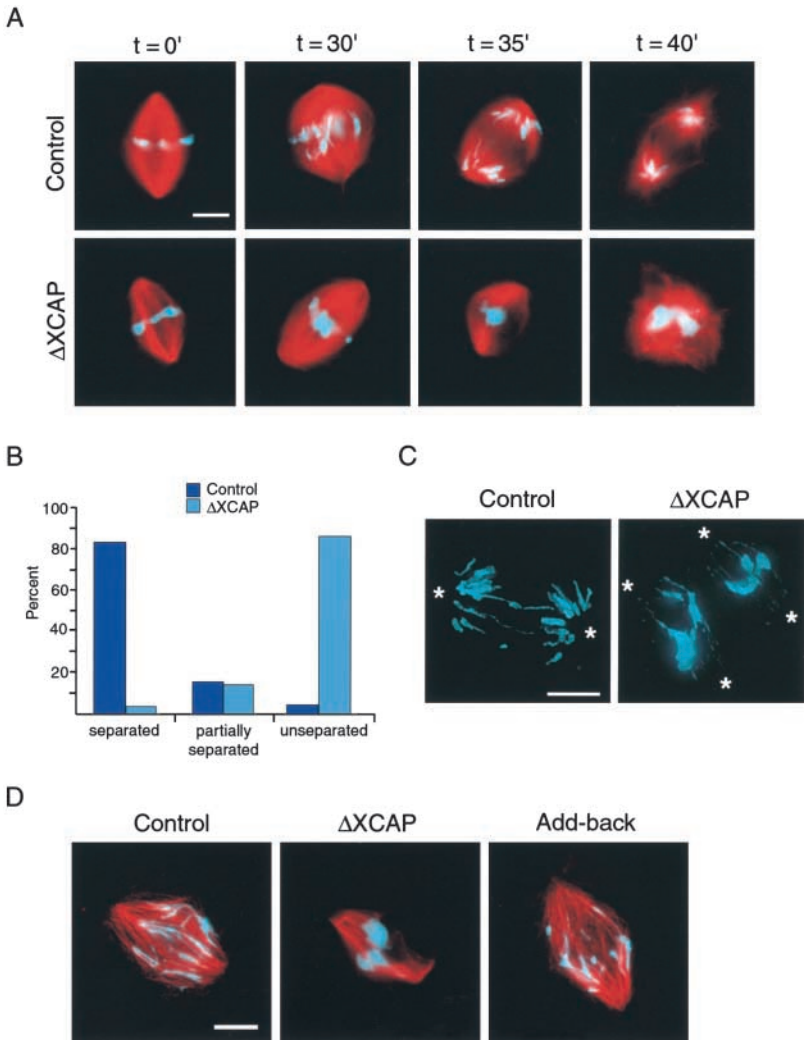

Figure 3. **Depletion of the condensin complex from *Xenopus* egg extracts causes defects in anaphase chromosome segregation.** (A) Chromosome segregation in extracts depleted with IgG (Control) or condensin antibodies (ΔXCAP). Once metaphase spindles were assembled (t = 0′), anaphase was induced and samples were fixed at 30, 35, and 40 min. Bar, 10 μm. (B) Quantification of the effect of condensin depletion on chromosome segregation. One representative experiment is shown. Samples were fixed 40 min after anaphase initiation, and the chromosomes in each spindle were classified as completely separated, partially separated, or unseparated. 188 and 162 structures were counted for the control and condensin depletion (ΔXCAP), respectively. Percent sperm nuclei in each category is depicted. (C) Deconvolution microscopy of chromosomes during anaphase in control and condensin-depleted (ΔXCAP) extracts. The samples were fixed 35 min after anaphase induction. The poles of each spindle are marked with asterisks (*). Two side-by-side spindles are shown in the condensin-depleted extract. Bar, 10 μm. (D) Chromosome segregation in extracts depleted with IgG (Control) or XCAP antibodies (ΔXCAP), and in the XCAP-depleted extract to which purified *Xenopus* condensin complex was added (Add-back). The structures were fixed 30 min after anaphase initiation. Bar, 10 μm.

Anaphase was triggered in extracts by the addition of calcium or its downstream target, CamKII (Morin et al., 1994), and chromosome segregation was monitored by fixation of samples at 5-min intervals. In control extracts, chromosomes began moving to the poles and were completely segregated by 40 min (Fig. 3 A). In contrast, the chromosomes in condensin-depleted extracts remained a disorganized mass in the center of the spindle and were not moved to the poles. Nevertheless, spindle poles separated, and by 40 min the spindles in condensin-depleted extracts began to disassemble, leaving an unsegregated mass of DNA. This effect was quantified in a representative experiment by counting the number of morphologically normal spindles with segregated chromosomes at 40 min, revealing 82% segregation in control extracts, compared with 5% in the absence of condensin (Fig. 3 B).

To examine the chromosome segregation defect at a higher resolution, we performed deconvolution microscopy on spindles in anaphase. Individual chromosomes resolved from one another and moved poleward in control spindles (Fig. 3 C). In contrast, although the DNA in condensin-depleted extracts appeared stretched toward the poles during anaphase, it remained as one mass and individual chromosomes were not apparent. Importantly, anaphase chromosome segregation defects could largely be rescued if purified condensin was added back to the depleted extracts (Fig. 3 D). Therefore, the condensin complex is specifically required to condense and resolve chromosome arms in order to ensure proper chromosome segregation during anaphase.

Condensin function is required throughout anaphase

The anaphase defects observed are consistent with studies in other organisms showing segregation problems after condensin subunit inhibition (Saka et al., 1994; Bhat et al., 1996; Sutani et al., 1999; Lavoie et al., 2000; Ouspenski et al., 2000; Steffensen et al., 2001; Bhalla et al., 2002; Hagstrom et al., 2002; Stear and Roth, 2002). However, these

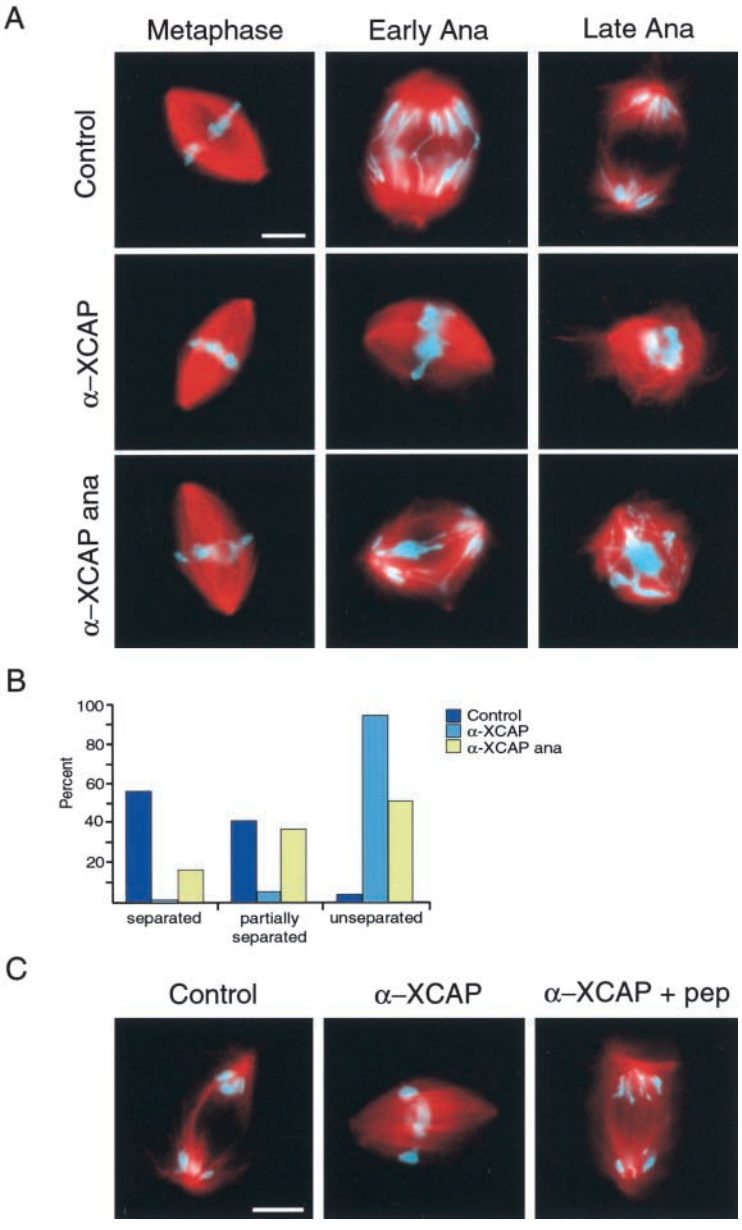

Figure 4. **Inhibition of the condensin complex either before mitosis or after anaphase initiation causes chromosome segregation defects.** (A) Segregation of sperm chromosomes in *Xenopus* egg extracts in the presence of buffer or 0.26 mg/ml XCAP-E antibody, added either as the extract was entering mitosis (α-XCAP) or immediately after anaphase induction (α-XCAP ana). Samples were fixed at metaphase (Metaphase), and early and late anaphase (Early Ana and Late Ana). Bar, 10 μm. (B) Quantification of the effect of condensin antibody addition on chromosome segregation. Spindles were fixed 40 min after anaphase induction, and the chromosomes were classified as completely separated, partially separated, or unseparated. For each reaction, >100 structures were counted. (C) Chromosome segregation in the presence of IgG (Control), XCAP-E antibody preincubated with buffer (α-XCAP), or XCAP-E antibody preincubated with antigen (α-XCAP + pep). Samples were fixed 35 min after anaphase induction. Bar, 10 μm. Videos 1–6 are available at http://www.jcb.org/cgi/content/full/jcb.200303185/DC1.

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studies could not distinguish whether condensin function is required early in mitosis for chromosome condensation and resolution, or continuously throughout metaphase and anaphase. This question can be easily addressed in the in vitro extract system, by adding antibodies to spindle reactions at different time points and monitoring subsequent effects on anaphase chromosome segregation. Addition of XCAP-E antibody at the transition to mitosis caused severe chromosome segregation defects (Fig. 4 A), similar to those seen in condensin-depleted extracts (Fig. 3 A). Preincubation of the condensin antibody with the peptide antigen completely neutralized its effects (Fig. 4 C).

To test whether condensin function is also required once chromosomes have properly condensed and aligned on the metaphase plate, spindles were assembled and XCAP-E antibody was added immediately after anaphase induction. Although chromosomes moved toward the poles under these conditions, a higher frequency of lagging and mis-segregated chromosomes was observed compared with control spindles (Fig. 4, A and B). We also monitored chromosome segregation using time-lapse fluorescence video microscopy in control extracts and in extracts to which XCAP-E antibody had been added. Consistent with analysis of fixed samples, we observed chromosome segregation defects when condensin antibody was added either at the start of metaphase or at the start of anaphase (Videos 4–6 available at http://www.jcb.org/cgi/content/full/jcb.200303185/DC1; unpublished data). These results support observations that the condensin complex is necessary to establish and maintain proper chromosome condensation (Hirano et al., 1997). Thus, condensin is involved both in establishing a chromosome architecture capable of being segregated, and also in actively maintaining that structure throughout anaphase.

Many proteins target properly to chromosomes in condensin-depleted extracts, but kinetochore morphology is disrupted

To begin investigating the origin of the spindle assembly defects observed in the absence of condensin, we examined both chromosome arms and kinetochores to determine whether the localization of known proteins required for spindle assembly and anaphase was affected. Immunofluorescence microscopy of metaphase spindles did not reveal significant changes in the localization of the chromokinesins Xklp1 and Xkid, the RanGEF RCC1, or the mitotic kinases Aurora B and Plx1 (unpublished data). To determine whether the chromosomal Ran-GTP gradient was disrupted despite recruitment of the RanGEF RCC1, we examined the local generation of Ran-GTP using a fluorescence resonance energy transfer (FRET) probe. The probe binds RanGTP, but not RanGDP, exhibiting a loss of FRET in the vicinity of mitotic chromatin (Kalab et al., 2002). In high or low speed extracts to which condensin antibody was added, we still observed a significant RanGTP gradient surrounding sperm nuclei (Fig. 5 A and not depicted). These results indicate that the defects in spindle assembly caused by condensin inhibition do not arise from mislocalization of the chromokinesins, chromatin-associated kinases, or loss of RCC1 activity. With the exception of RCC1, however, we do not know whether the activities of these chromatin factors are affected.

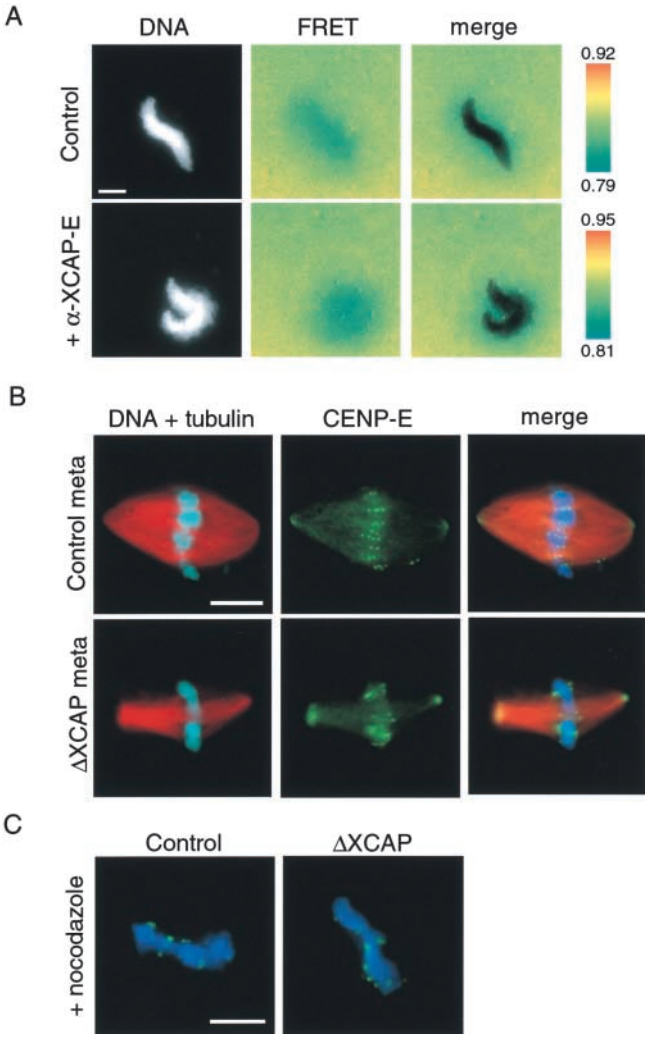

Figure 5. **Depletion of the condensin complex does not affect the Ran-GTP gradient, but disrupts kinetochore morphology.** (A) RanGTP was visualized by addition of a probe that shows a loss of FRET when bound to RanGTP. Fluorescence images show sperm nuclei, the FRET ratio signal (I_{FRET}/I_{CFP}), and an overlay. Ratios range from low FRET (blue) to high FRET (red). Gradients appeared indistinguishable surrounding chromosomes in clarified extracts treated with buffer or α-XCAP-E. Bar, 10 μm. (B) Immunofluorescence images of CENP-E (green) in spindles formed in extracts depleted using IgG (Control) or condensin antibodies (ΔXCAP), revealing aberrant kinetochore morphology in the absence of condensin. Samples were fixed 60 min after the induction of mitosis. Bar, 10 μm. (C) Addition of 1 μM nocodazole to metaphase reactions increased the CENP-E signal and partially alleviated its distortion in ΔXCAP extracts. Bar, 10 μm.

To evaluate whether kinetochore formation was affected by condensin depletion, we performed immunofluorescence microscopy using antibodies against the outer kinetochore-associated kinesin-like protein CENP-E (Wood et al., 1997). In control metaphase extracts, CENP-E localized in a punctate pattern corresponding to kinetochores (Wood et al., 1997; Fig. 5 A). In contrast, after condensin depletion, CENP-E was localized in irregular lines. Many of these lines were oriented along the axis of the spindle, suggesting that

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the altered morphology was due to microtubules exerting force on the kinetochores and stretching the underlying chromatin. In support of this hypothesis, addition of the microtubule depolymerizing drug nocodazole to condensin-depleted extracts caused the CENP-E staining to resume a more normal, punctate distribution (Fig. 5 C). Therefore, we propose that depletion of the condensin complex causes kinetochores to lose structural integrity so that when spindle microtubules move chromosomes, kinetochores become stretched and their morphology is disrupted.

Depletion of the condensin complex reduces the fidelity of DNA bead spindle assembly

As condensin inhibition did not lead to mislocalization of several chromatin-associated proteins with known mitotic functions, but did affect kinetochore morphology, we next sought to determine if the reduction in the fidelity of spindle assembly after condensin depletion was caused solely by defects in kinetochore formation. Therefore, we analyzed the effects of condensin inhibition on the assembly of spindles lacking kinetochores by using DNA-coated beads (Fig. 6). Plasmid DNA beads function as "artificial chromosomes," recruiting the condensin complex and other mitotic chromatin proteins when incubated in *Xenopus* egg extracts (Heald et al., 1996; Kimura and Hirano, 2000; unpublished data). In this system, microtubules polymerize around the chromatin beads and are sorted into a bipolar array by microtubule-based motors (Walczak et al., 1998). Depletion of the condensin complex reduced the fidelity of DNA bead spindle assembly, resulting in a higher percentage of structures with abnormal or reduced microtubule polymerization (Fig. 6 A). As with the effects on spindle assembly around sperm nuclei, the severity was variable among extracts, with the percent inhibition averaging 33% in five independent experiments. Quantification of two representative experiments is shown in Fig. 6 B. These data indicate that disrupting chromatin condensation is sufficient to cause a reduction in the fidelity of spindle assembly in extracts, even in the absence of kinetochores. However, the defects observed in bead spindle assembly were less severe than with sperm nuclei, when comparing either immunodepletion or antibody addition (Fig. 2, B and C, and Fig. 6, B and C). This observation suggests that kinetochore defects may increase the severity of sperm spindle assembly inhibition. Alternatively, the compact nature and short DNA sequences characteristic of the plasmid DNA-coated beads may render them less sensitive to defects in chromatin condensation. In conclusion, the activity of mitotic chromatin to induce spindle assembly is enhanced in the presence of condensin, indicating that chromosome condensation contributes to mitotic chromatin function. As condensin inhibition did not alter microtubule polymerization induced by isolated centrosomes (unpublished data), we propose that the contribution of condensin to spindle assembly is indirect, by promoting the localization and/or activity of other factors.

Depletion of the condensin complex likely has direct effects on chromosome segregation

In contrast to the spindle assembly defects observed in the absence of condensin, the inhibition of chromosome segregation in anaphase appeared to be the direct result of a failure in chromosome compaction and resolution. To address this issue, we first examined CENP-E kinetochore localization after anaphase induction to determine whether kineto-

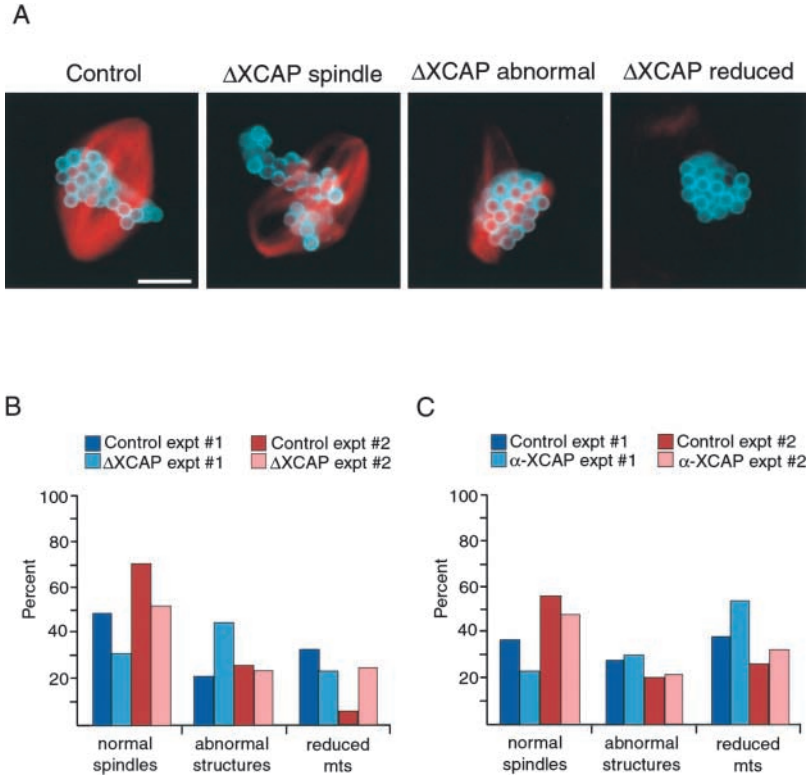

Figure 6. **Condensin inhibition reduces the fidelity of DNA bead spindle assembly.** (A) Microtubule structures formed around DNA beads in extracts depleted using IgG (Control) or condensin antibodies (ΔXCAP) that were fixed in metaphase 90 min after initiation of mitosis. The three ΔXCAP images show structures representative of the three categories quantified in B and C: normal spindles, abnormal structures, and reduced microtubules. Bar, 10 μm. (B) Quantification of the effect of condensin depletion on bead spindle assembly in two separate experiments (red and blue bars). Samples were fixed 90 min after the start of mitosis. Each cluster of 10 or more DNA beads was examined and the microtubule array surrounding it was assigned to the categories depicted in A. For each reaction, >100 structures were counted. Percent of bead clusters in each category are depicted. (C) Quantification of the effect of XCAP-E antibody addition on bead spindle assembly in two separate experiments. To each spindle assembly reaction, either buffer (Control) or 0.26 mg/ml α-XCAP-E antibody (α-XCAP) was added at the start of mitosis. Samples were fixed at 90 min, and the microtubule structures were grouped into the categories depicted in A.

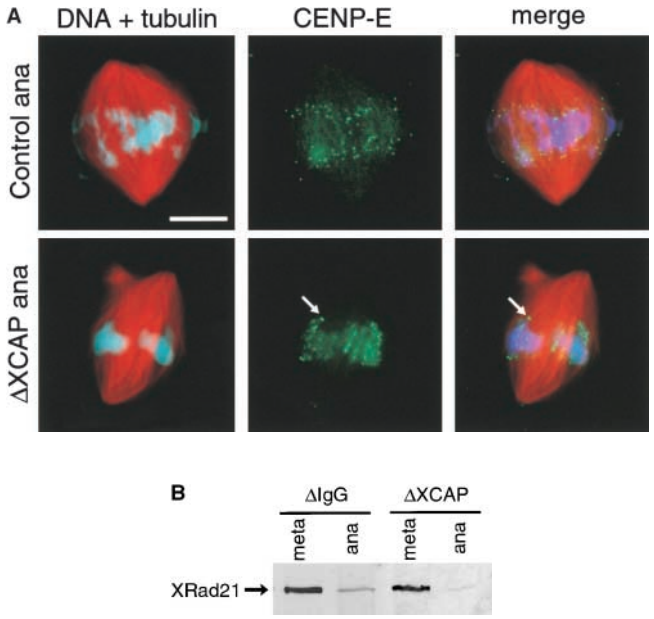

Figure 7. **Condensin depletion does not affect CENP-E localization in anaphase or XRad21 degradation.** (A) CENP-E staining (green) in spindles formed in extracts depleted using IgG (Control) or condensin antibodies (ΔXCAP). Samples were fixed 15 min after anaphase initiation. Bar, 10 μm. CENP-E is localized to regions of DNA that is stretched toward the spindle poles (arrow), suggesting that the kinetochores are still functional. (B) Analysis of XRad21 degradation in anaphase. Chromosomes were purified from extracts depleted with IgG (control) or condensin antibodies (ΔXCAP) in either metaphase or anaphase. Proteins were separated on an 8% gel and blotted using affinity-purified XRad21 antibodies, revealing a similar reduction of XRad21 in the presence and absence of condensin. The blot was also probed with antibodies against topoII as a loading control (not depicted).

chore–microtubule attachments were capable of generating poleward forces in the absence of condensin function. In the control extracts, punctate CENP-E staining was observed on the ends of sister chromatids migrating toward the poles, illustrating kinetochore movements that normally drag chromosomes poleward (Fig. 7 A). After condensin depletion, we observed trails of chromatin extending toward the poles, although the chromosomes were unable to separate (Fig. 3 C and Fig. 7 A). Although CENP-E staining was aberrant in these structures, it was clearly visible at the ends of the stretched segments of DNA (Fig. 7 A, arrow). This localization suggests that in the absence of condensin function, CENP-E is able to target to discrete sites on chromosomes that mediate microtubule attachment and force generation.

Although structural disorganization and loss of sister chromatid resolution may be sufficient to explain the segregation defects in the absence of condensin, other factors also participate in the regulation of chromosome segregation at the metaphase to anaphase transition that could be affected by defects in chromosome architecture. Loss of cohesion between sister chromatids must occur, which is due to specific proteolysis of a cohesin subunit, termed XRad21 in *Xenopus* (Losada et al., 1998; Uhlmann et al., 1999). In addition, the chromokinesin Xkid must be degraded to allow poleward movement of chromosome arms (Antonio et al., 2000; Funabiki and Murray, 2000). Finally, topoisomerase IIα (topoII) activity is required for sister segregation (Shamu and Murray, 1992). Therefore, we compared the behavior of these proteins in control and condensin-depleted extracts. As reported in clarified extracts, we found no effect of condensin depletion on the localization of topoII (Hirano et al., 1997; unpublished data). To test whether the activity of topoII was nevertheless affected, we assayed the decatenation of kinetoplast DNA added to control and condensin-depleted extracts (Shamu and Murray, 1992). The efficiency of kinetoplast decatenation was not altered after condensin depletion or XCAP-E antibody addition (unpublished data), suggesting that bulk topoII activity was not affected by condensin inhibition. To examine the loss of XRad21 and Xkid in anaphase, sperm nuclei were isolated from extracts arrested in metaphase or induced to enter anaphase as described previously (Funabiki and Murray, 2000). XRad21 (Fig. 7 B) and Xkid (unpublished data) were similarly diminished from chromosomes in both mock- and condensin-depleted extracts upon anaphase induction. Thus, condensin inhibition does not appear to impair proteins functioning at the metaphase–anaphase transition. Together with our observation that condensin is required even after anaphase induction (Fig. 4), these results indicate a direct and continuous requirement for condensin function to allow chromosome resolution and segregation during anaphase.

Discussion

Condensation is required for successful chromosome segregation for several reasons. First, if interphase chromosomes exceed the length of the cell, compaction is essential for the spindle to separate the genome into two discrete sets. Second, condensation is required for the resolution of tangled sister chromatids, and is thought to provide mechanical strength to withstand spindle forces. Third, the condensed chromosome architecture may aid in the structural organization and orientation of kinetochores. Here, we have examined the role of the condensin complex in mitotic chromosome functions in crude *Xenopus* egg extracts capable of reconstituting spindle assembly in vitro. As in the clarified extracts examined previously (Hirano et al., 1997), we find that chromosome condensation and resolution are compromised when condensin is depleted. These defects have a significant impact on the ability of the mitotic spindle to form, and dramatically inhibit chromosome segregation when anaphase is induced.

Condensin and the function of mitotic chromatin

Mitotic chromatin is a major driving force for spindle assembly in *Xenopus* egg extracts. Chromatin-associated factors, such as RCC1, chromokinesins, and the kinase Plx1, are essential for proper spindle assembly (Vernos et al., 1995; Ohba et al., 1999; Antonio et al., 2000; Funabiki and Murray, 2000; Budde et al., 2001). Chromatin itself is sufficient, as illustrated by the ability of DNA-coated beads to induce spindle assembly in the absence of centrosomes and kinetochores (Heald et al., 1996). The inhibition of microtubule polymerization and spindle assembly in the absence

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of condensin function is, therefore, likely due to impairment of chromatin function due to defects in its higher-order organization. However, our examination of several known chromatin proteins by immunofluorescence, and the generation of a RanGTP gradient in the vicinity of chromatin did not reveal significant changes upon condensin depletion (unpublished data; Fig. 5 A), though we cannot rule out subtle defects in the RanGTP gradient undetectable in our assay. In the future, biochemical comparison of chromatin proteins recruited to sperm nuclei or DNA beads in the presence or absence of condensin may reveal proteins with altered association, phosphorylation state, or activity.

We observed variation in the effects of condensin inhibition on spindle assembly from experiment to experiment, which we believe is due to extract variability because depletion efficiencies were identical by Western blot. Spindle phenotype variability likely results from the fact that multiple and partly redundant mechanisms promote spindle assembly in vitro, and that these activities are not equally preserved among extract preparations from different batches of eggs. Although spindle assembly defects due to loss of condensin function have not been well elucidated in other organisms, budding yeast condensin mutants have been reported to have defects in microtubule organization (Lavoie et al., 2000; Ouspenski et al., 2000). More extensive examination of condensin function in other cell types will be important to distinguish the generality of the role of the complex in spindle formation.

Despite the spindle phenotype variability, we consistently found that spindle assembly around sperm chromosomes was more dramatically affected than that induced by DNA beads, although both recruit a similar complement of chromatin proteins (Kimura and Hirano, 2000; Budde et al., 2001). One possible explanation is that due to their compact physical nature, the plasmid DNA beads generate a higher local concentration of chromatin factors than the decondensed sperm chromosomes, making them less susceptible to disorganization by lack of condensin function. Alternatively, kinetochores may contribute to spindle formation around sperm nuclei, and, therefore, the disruption of kinetochore morphology seen in condensin-depleted extracts might contribute to the reduced fidelity of spindle assembly.

Condensin and kinetochores

Kinetochores form upon depletion of condensin, as indicated by the recruitment of the outer kinetochore kinesin CENP-E to discrete foci (Wood et al., 1997). However, CENP-E distribution becomes stretched and distorted upon spindle assembly, suggesting that in the absence of proper condensation, the kinetochore and underlying chromatin cannot resist deformation upon microtubule attachment and chromosome movement. During anaphase, despite the general failure of sperm chromosome segregation in the absence of condensin function, CENP-E foci were stretched toward the spindle poles. This observation suggests that despite their distorted morphology, the kinetochores are attached to microtubules and acted on by anaphase forces. In the future, fluorescence video microscopy analysis of kinetochore markers will be extremely informative to elucidate how chromosome structure impacts kinetochore attachment and movement within the spindle.

An active role for condensin in anaphase

We found that condensin function is required throughout anaphase, as its inhibition after anaphase induction caused chromosome segregation defects. This analysis was possible because addition of condensin antibodies inhibited its function to the same extent as immunodepletion, and the in vitro system allowed us to inhibit condensin at any time during the cell cycle. Although it is possible that condensin plays an enzymatic role in decatenation, it is more likely required to actively maintain chromosome organization, preventing decondensation and sister entanglements. Another possibility was that condensin inhibition interfered with the function of topoII, which is required for anaphase chromosome segregation (Shamu and Murray, 1992). Consistent with results in clarified extracts, condensin depletion did not alter topoII localization to chromosomes in crude extracts (Hirano et al., 1997) or inhibit the decatenation of kinetoplast DNA (unpublished data). Therefore, it is unlikely that the condensin complex modulates the function of topoII directly, though it may be required to generate a chromosome architecture that is capable of being efficiently decatenated. Other chromosomal factors whose proper regulation is required for sister separation include the chromokinesin Xkid and the cohesin subunit XRad21. Both of these factors appear to be degraded normally in the absence of condensin, supporting work in budding yeast that showed Scc1 is properly removed from chromosomes in a condensin mutant (Bhalla et al., 2002). Altogether, these data support a direct role for the condensin complex in anaphase chromosome resolution.

Does chromosome condensation occur in the absence of condensin?

In contrast to the dramatic effects on chromosome morphology seen in *Xenopus* (Hirano et al., 1997), studies in other systems reported more moderate defects in chromosome compaction upon loss of condensin function. In mitotic neuroblasts of a *Drosophila* mutant lacking an SMC condensin component, longitudinal shortening of chromosomes appeared normal, but chromatid resolution was strikingly disrupted during anaphase (Steffensen et al., 2001). Similar effects were observed in *C. elegans* embryos, as RNAi against condensin subunits did not cause a dramatic decrease in chromosome compaction during metaphase, but inhibited anaphase segregation (Hagstrom et al., 2002). Anaphase defects in mutants can also be attributed to a chromosome twisting phenotype that disrupts kinetochore orientation (Stear and Roth, 2002). Our studies show that the differences between the phenotypes of condensin inhibition in *Xenopus* and other systems are not attributable to an artifact of the high speed extract system. We have shown that condensin depletion from low speed extracts causes severe defects in condensation, which are rescued by addition of purified condensin. It is possible that variability between the level of condensin inactivation in different systems causes variable phenotypes. For example, there may be a phenotypic difference between depletion of the entire condensin

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complex, compared with mutation of one of its subunits. Alternatively, it is possible that there are differences in the chromosome condensation pathways in different organisms.

In summary, our studies show that the condensin complex is required throughout mitosis to promote spindle formation and ensure proper chromosome segregation in *Xenopus* egg extracts. The emerging model is that depending on the organism, other factors may contribute to chromatin compaction during mitosis, but that condensin function is required to generate a chromosome structure that allows sister chromatid resolution, kinetochore orientation, and prevents chromosome stretching and distortion in response to spindle forces.

Materials and methods

Preparation of *Xenopus* egg extracts, DNA, and antibodies

Crude cytostatic factor (CSF) extracts were prepared from *X. laevis* eggs arrested in metaphase of meiosis II as described previously (Murray, 1991; Desai et al., 1999), except that the crushing spin was performed at 10,200 rpm in a rotor (model HB-6, Sorvall) for 15 min. To prepare clarified extracts, CSF extract was spun an additional 90 min at 55,000 rpm in a rotor (model TLS-55; Beckman Coulter). Sperm nuclei and DNA beads were prepared as described previously (Murray, 1991; Heald et al., 1996). Sperm nuclei were a gift from G.O. Nads (University of California, Berkeley).

Synthetic peptides corresponding to the COOH-terminal regions of XCAP-E, XCAP-G, and XRad21 were identical to those described previously (Hirano et al., 1997; Losada et al., 1998; Kimura and Hirano, 2000). Peptide synthesis and antibody production were performed by Zymed Laboratories. Antibodies were affinity-purified as described previously (Sawin et al., 1992). XCAP-G serum was a gift from N. Cozzarelli and V. Holmes (University of California, Berkeley). CENP-E antibody was a gift from S. Gadde (University of California, Berkeley). Plx1 and Aurora B antibodies were a gift from P. Budde (University of California, Berkeley).

In vitro spindle assembly and anaphase

CSF extracts were supplemented with demembranated sperm (final 500 nuclei/μl) or with DNA beads on ice, and transferred to 20°C. Extracts were driven into interphase by adding 0.1 extract volume of $CaCl_2$ buffer (4 mM $CaCl_2$, 10 mM Hepes, pH 7.7, 150 mM sucrose, 100 mM KCl, and 1 mM $MgCl_2$). To cycle into mitosis, an equal volume of CSF extract was added with X-rhodamine–labeled tubulin (50 μg/ml final; Hyman et al., 1991). To fix, 1-μl samples were spotted onto slides, overlaid with 5 μl of spindle fix (48% glycerol, 11% formaldehyde in MMR [5 mM Hepes, pH 7.8, 2 mM KCl, 1 mM $MgSO_4$, 100 mM NaCl, 2 mM $CaCl_2$, and 0.1 mM EGTA], and 5 μg/ml Hoechst 33258) and squashed with a coverslip (Wignall and Heald, 2001). To induce anaphase, 0.1 volume of $CaCl_2$ buffer was added. Alternatively, an activated version of CamKII expressed in reticulocyte lysate was added as described previously (Morin et al., 1994). CamKII plasmid was a gift from M. Doree (CNRS, Montpellier, France). Clarified extract condensation reactions were set up as in Hirano et al. (1997).

Antibody addition, immunodepletion, and rescue

For antibody addition, α-XCAP-E was added to extract to 0.26 mg/ml (final) at the start of interphase, upon entry into mitosis, or after anaphase induction. As a control, equivalent volumes of CSF-XB or rabbit IgG were added. To block with antigen, 20 μg α-XCAP-E was incubated with a fivefold molar excess of XCAP-E peptide or buffer overnight. Reactions were dialyzed into XB (Desai et al., 1999) to remove the peptide and added to extract to 0.2 mg/ml.

Extracts were depleted of condensin in two rounds. For each round, 15 μg α-XCAP-E and 7 μg α-XCAP-G (ΔXCAP) or 23 μg rabbit IgG (control) were added to 55 μl of protein A–Dynabeads (Dynal), the volume was brought to 200 μl with TBS + 0.1% Triton X-100, and rotated at 4°C overnight. After coupling, the beads were washed with CSF-XB + 10 μg leupeptin, pepstatin, and chymostatin. (Desai et al., 1999). Beads were used to deplete a 70-μl CSF extract in two 1-h rounds. During each round, the extract was kept on ice and mixed frequently.

Xenopus 13S condensin was purified from mitotic clarified extracts as described previously (Hirano et al., 1997; Kimura and Hirano, 2000). 100 μg α-XCAP-G was coupled to 200 μl protein A–agarose beads (GIBCO BRL). Beads were incubated with a 2-ml high speed extract for 4 h, poured into a column, and washed with 80 column vol XBE2-gly (containing 20 mM β-glycerophosphate), 10-column vol XBE2-gly + 0.4 M KCl, and 10-column vol XBE2-gly. Condensin was eluted with XBE2-gly containing 0.8 mg/ml XCAP-G peptide, concentrated to 1–2 mg/ml using Microcon-30 concentrators (Amicon), and added to depleted extracts at 1:10.

Fixation and staining of spindles and chromosomes

To perform immunofluorescence on spindles, reactions were spun onto coverslips as described previously (Desai et al., 1999) with minor modifications. In brief, 10-μl reactions were added to 1 ml of dilution buffer (1× BRB80, 30% glycerol, and 0.5% Triton X-100) and fixed by the addition of 1 ml of dilution buffer + 5% formaldehyde. After 10 min, samples were spun onto coverslips through a 4-ml cushion (BRB80 + 40% glycerol) at 5,500 rpm for 20 min using an HB-4 rotor. To preserve chromosome structure, 10-μl reactions were fixed for 10 min with 100 μl XBE2 + 2% formaldehyde (Losada et al., 2000) and spun through a 5-ml cushion (XBE2 + 30% glycerol). Coverslips were fixed after in −20° methanol for 5 min, blocked with 3% BSA in PBS, and processed for immunofluorescence using FITC-conjugated secondary antibodies. DNA was stained with 1 μg/ml Hoechst 33258 in PBS.

The RanGTP gradient was visualized using a RanGTP-binding FRET probe YFP-RBD-CFP as described previously (Kalab et al., 2002), except the probe (final 4 μM) was preincubated with extract on ice for 10 min before starting the condensation or spindle reactions. Probe was a gift from P. Kalab (University of California, Berkeley).

Chromosome purification

Chromosomes were purified as in Funabiki and Murray (2000) with minor changes. In brief, 120 μl extract containing sperm nuclei (2500/μl) and biotin dUTP (25 μM) was cycled into interphase as described in previous paragraphs. Addition of 60 μl fresh extract and cyclin B Δ90 (final 24 μg/μl) cycled the extract into mitosis. Control or $CaCl_2$ buffer was added to form metaphase or anaphase chromosomes, respectively. Purification was performed as described previously (Funabiki and Murray, 2000).

Microscopy and data analysis

Images and time-lapse movies were collected using a fluorescence microscope (model E600; Nikon) equipped with a CCD camera (model Orca II; Hamamatsu), a shutter controller (model Lambda 10-2; Sutter Instrument Co.), and Metamorph software (Universal Imaging Corp.). Images were imported into Adobe Photoshop® software for processing. For quantification of spindle phenotypes, microtubule structures were visually categorized. At least 75 structures were evaluated for each condition, and at least five independent experiments were performed. Deconvolution microscopy was performed using a DeltaVision (Applied Precision, Inc.) imaging station: an inverted microscope (model IX70; Olympus) with 40×, 1.3 NA or 100×, 1.35 NA oil immersion lenses, and a cooled CCD (model Photometrics; Roper Scientific). All images were taken with a Z-step size of 0.2 μm, saved as three-dimensional stacks, and subjected to constrained iterative deconvolution (Chen et al., 1996).

Online supplemental material

All videos were acquired using the microscope set-up described in the previous section. Frames were collected every 30 s, and are displayed at a rate of 10 frames/s.

Videos 1–3 show chromosome segregation in extract to which buffer was added at the start of mitosis. Microtubules are shown in red and chromosomes in green. The microtubules and chromatin are shown together in Video 1. Video 2 shows the DNA alone, and Video 3 shows the microtubules.

Videos 4–6 show anaphase in extract to which 0.26 mg/ml XCAP-E antibody was added at the start of mitosis. The microtubules and DNA are shown together in Video 4, and separately in Video 5 (DNA) and Video 6 (microtubules). Videos 1–6 are available at http://www.jcb.org/cgi/content/full/jcb.200303185/DC1.

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