# Essential roles for cohesin in kinetochore and spindle function in *Xenopus* egg extracts

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## Summary

**To facilitate their accurate distribution by the mitotic spindle, sister chromatids are tethered during DNA replication, attached by their kinetochores and bi-oriented on the spindle, and then simultaneously released at the metaphase to anaphase transition, allowing for their segregation to opposite spindle poles. The highly conserved cohesin complex is fundamental to this process, yet its role in mitosis is not fully understood. We show that depletion of cohesin from *Xenopus* egg extracts impairs sister chromatid cohesion and kinetochore-microtubule interactions, causing defective spindle attachments and chromosome alignment during metaphase and mis-segregation during anaphase. In the absence of cohesin, sister kinetochore pairing and centromeric localization of chromosomal passenger proteins INCENP and aurora B were lost upon bipolar spindle attachment. However, kinetochores remained paired with normal passenger localization if bipolar spindle formation was prevented by inhibiting the kinesin-5 motor (Eg5). These observations indicate that cohesin is not required to establish sister association, but is necessary to maintain cohesion in the presence of bipolar spindle forces. Co-depletion of cohesin together with another major SMC complex, condensin, revealed cumulative effects on spindle assembly and chromosome architecture. These data underscore the essential requirement for cohesin in sister chromatid cohesion, kinetochore and spindle function.**

Key words: Cohesin, Mitosis, Chromosome, Spindle, Anaphase, Chromosomal passenger

## Introduction

Genetic information must be faithfully transmitted during each repetition of the cell cycle and requires the coordination of multiple complex events. When a new copy of the genome is created during DNA replication, sister chromatids remain paired so that following condensation and attachment to the spindle, they can orient towards opposite poles and correctly segregate during anaphase (Gadde and Heald, 2004). Chromatid attachment to the spindle occurs at the kinetochore, a specialized structure located on each sister, where cohesion must be robust to resist their premature separation by spindle forces. Furthermore, checkpoint mechanisms operating at the kinetochore ensure that proper attachment of chromosomes to the spindle is established before sister chromatid cohesion is dissolved and poleward transport occurs (Cleveland et al., 2003).

The tethering of sister chromatids is thought to be physically mediated by the cohesin complex, a conserved set of four proteins including a heterodimeric pair of the structural maintenance of chromosomes (SMC) family members Smc1 and Smc3, and two additional regulatory proteins, Scc1/Rad21 and Scc3/SA (Hirano, 2002; Lee and Orr-Weaver, 2001; Losada et al., 1998). In yeast, proteolytic cleavage of Scc1 at the metaphase-anaphase transition abolishes cohesion, whereas in vertebrate systems there is also an earlier step of cohesin dissociation from chromosome arms during prophase, dependent on the activity of polo and aurora B kinases (Haering and Nasmyth, 2003). Inhibition of individual cohesin subunits by depletion, RNAi or gene disruption impairs sister cohesion in higher eukaryotes, such as *Xenopus* and *C. elegans*, as well as in chicken and *Drosophila* cells (Losada et al., 1998; Sonoda et al., 2001; Vass et al., 2003; Wang et al., 2003). In addition, loss of the Scc1/Rad 21 subunits in chicken or *Drosophila* cells caused mislocalization of the inner centromere protein (INCENP) (Sonoda et al., 2001; Vass et al., 2003). INCENP is a member of the chromosomal passenger complex also containing aurora B kinase, survivin and borealin (also known as dasra) that has been implicated in multiple mitotic functions including chromosome condensation, kinetochore attachment and function, spindle assembly and cytokinesis (Vagnarelli et al., 2004). However, the functional relationship between cohesin and the passenger proteins is poorly understood.

Conserved complexes containing SMC family members also mediate other crucial chromosome functions, such as chromosome condensation (Hirano, 2005; Jessberger, 2002). We have previously characterized the role of the SMC2-SMC4 (XCAP-E–XCAP-G)-containing condensin complex in *Xenopus* egg extracts, and shown that condensin depletion compromises compaction and resolution of mitotic chromosomes, impairing both spindle assembly and anaphase and distorting chromosome architecture (Wignall et al., 2003). We wished to similarly examine the impact of the cohesin complex on mitotic processes. Although previous work in *Xenopus* has demonstrated a requirement for this complex in the establishment of sister chromatid cohesion in egg extract (Losada et al., 1998), its roles in the context of a mitotic spindle have not been characterized.

Here, we show that cohesin depletion from in vitro *Xenopus* spindle assembly reactions results in aberrant kinetochore-microtubule interactions contributing to chromosome alignment defects during metaphase, and mis-segregation during anaphase. Without cohesin, sister kinetochore pairing and centromeric localization of chromosomal passenger proteins INCENP and aurora B was lost upon bipolar spindle attachment, but not if spindles were monopolar, indicating that cohesin is not required to maintain sister association in the absence of bi-directional pulling forces. Co-depletion of cohesin together with the other major SMC complex, condensin, caused additive defects in spindle assembly and altered the outcome of cohesin depletion on chromosome architecture. These data provide mechanistic insight into the essential requirement for cohesin in sister chromatid cohesion, kinetochore and spindle function.

## Results

### Immunodepletion of cohesin from extracts impairs sister chromatid cohesion

To analyze the role of the cohesin complex in *Xenopus* egg extracts, we generated affinity purified antibodies against XSmc1 and XRad21 for immunodepletion experiments, using peptides identical to those previously described (Losada et al., 1998). After two rounds of depletion using both antibodies, western blot analysis revealed that >98% of XSmc1 and XRad21 were removed from metaphase, cytostatic factor (CSF)-arrested egg extract compared with the mock IgG-depleted control, whereas levels of condensin subunits XCAP-G or XCAP-E were not altered (Fig. 1A and data not shown).

We tested the ability of cohesin-depleted CSF extract to support sister chromatid cohesion by adding sperm nuclei and inducing cell cycle progression through interphase by adding $Ca^{2+}$, thereby releasing the metaphase arrest and allowing DNA replication. The return to a metaphase-arrested state was subsequently induced by addition of fresh, cohesin-depleted CSF extract. To evaluate chromosome condensation and morphology, reactions were spun onto coverslips under conditions that preserved chromosome architecture but not spindle microtubules, and were subjected to immunofluorescence with an antibody against the XCAP-G subunit of the condensin complex. Kinetochores were visualized with an antibody against the spindle-checkpoint protein BubR1. Proper chromatid cohesion was observed in the mock-depleted extracts in both chromosome clusters and isolated chromosomes, with double BubR1 spots indicative of paired sister kinetochores (Fig. 1B). The clustering of sperm chromosomes made precise quantification of pairing difficult. However, in the absence of cohesin, we often observed single unpaired chromatids as well as paired chromatids whose kinetochores were further apart than those in the controls, demonstrating that sister cohesion was impaired, comparable to previously published results in extracts (Losada et al., 1998). Also in line with previous studies, we did not observe any defects in chromosome condensation in the absence of cohesin (Losada et al., 1998) and kinetochores formed – as judged by the punctate localization of BubR1 (Fig. 1B).

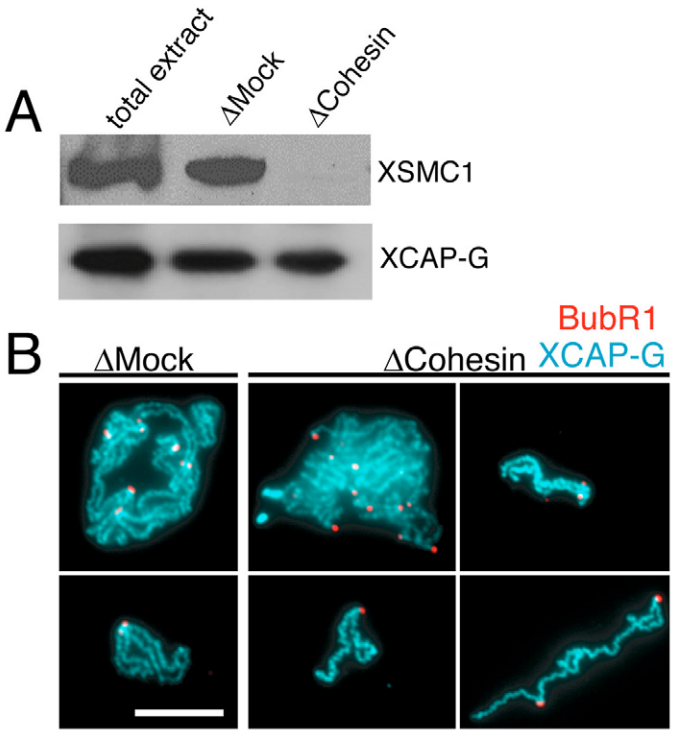


**Fig. 1.** Sister chromatid cohesion defects in *Xenopus* egg extracts depleted of cohesin. (A) Upper panel. Western blot of untreated total (total extract) and IgG-depleted (ΔMock) *Xenopus* egg extract, and *Xenopus* egg extract depleted by antibodies against XSmc1 and XRad21 (ΔCohesin). The blot was probed with an affinity-purified XSmc1 antibody that recognizes a single band of 155 kDa (present in total extract and ΔMock), which is absent in cohesin-depleted extract (ΔCohesin). Lower panel. The same blot probed with an affinity-purified antibody against the XCAP-G condensin subunit, recognizing a single band of 130 kDa (present in control, IgG- and cohesin-depleted extracts. (B) Immunofluorescence images of sperm chromosomes assembled in mock- and cohesin-depleted extracts that had been cycled through interphase to allow DNA replication, then back into mitosis. Chromosomes were spun down onto coverslips 60 minutes after induction of mitosis and processed for immunofluorescence using an antibody that recognizes a subunit of the condensin complex, XCAP-G (blue) and the kinetochore protein BubR1 (red). Bar, 10 μm.

### Cohesin is essential for metaphase chromosome alignment and anaphase segregation

To elucidate the role of cohesin in the context of spindle function, we examined chromosome alignment during metaphase and segregation during anaphase, monitoring kinetochore behavior using antibodies against Ndc80, a component of the outer kinetochore plate (McCleland et al., 2003). Immunofluorescence microscopy showed that spindles in mock-depleted extracts displayed tightly paired kinetochores aligned along the metaphase plate (Fig. 2A, left panel). By contrast, sister kinetochores were often unpaired in spindles lacking cohesin, and chromatids were observed throughout the central spindle, at the poles, or occasionally outside of the spindle (Fig. 2A, middle and right panels). We quantified more than 300 spindle structures for each condition in three independent experiments and observed an approximately fourfold increase in the number of chromosomes that had completely strayed from the metaphase plate upon cohesin immunodepletion (Fig. 2B). Chromosomes found within the central spindle were disorganized and never

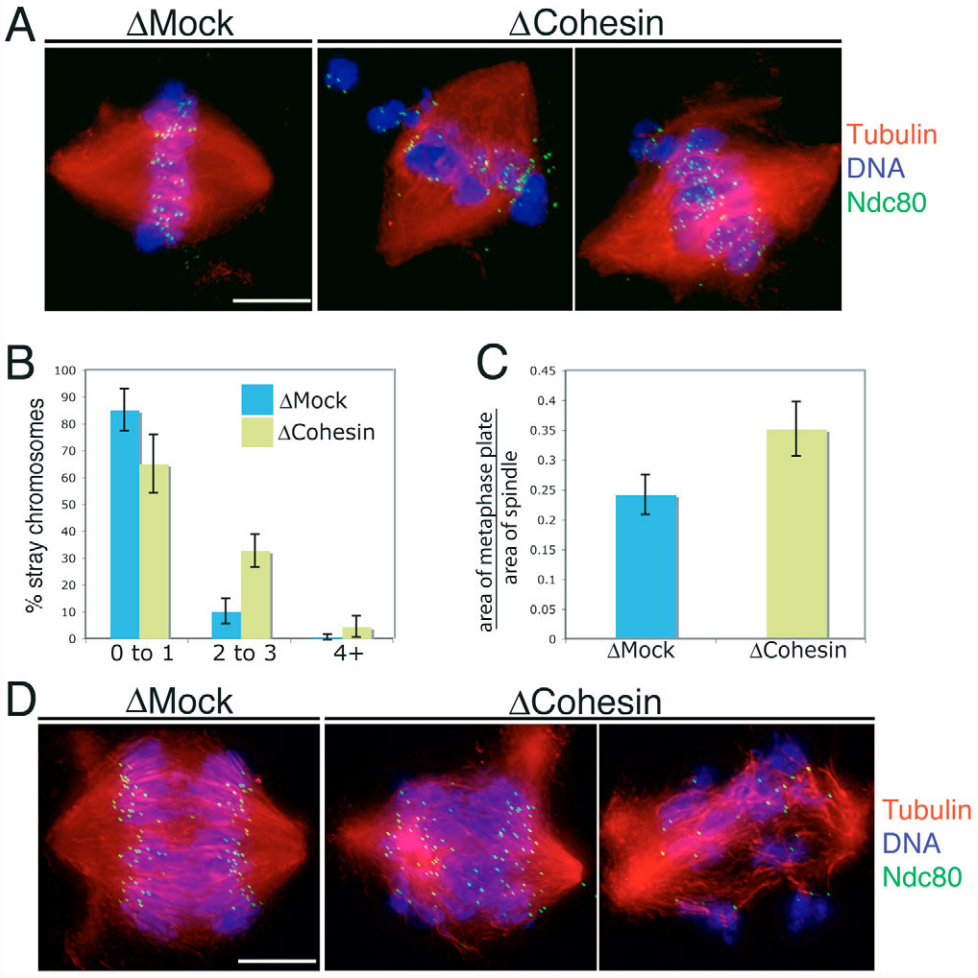


**Fig. 2.** Defects in metaphase chromosome alignment and anaphase segregation in the absence of cohesin.(A) Fluorescence images showing spindles assembled around *Xenopus* sperm nuclei in mock- and cohesin-depleted extracts, in the presence of Rhodamine-labeled tubulin (red). After 60 minutes in metaphase, reactions were spun down onto coverslips and processed for immunofluorescence with an antibody against the kinetochore protein Ndc80 (green). DNA was stained with Hoechst-33258 dye (blue), and microtubules are red. Bar, 10 μm. (B) Quantification of chromosome misalignment. The number of chromosomes that had strayed completely away from the metaphase plate in each spindle were counted. Bars represent averages for three independent experiments and a total of $n$=328 and $n$=317 spindles in mock- and cohesin-depleted extracts, respectively. Error bars are standard deviations (± s.d.). (C) Quantification of metaphase plate compaction relative to spindle size in mock- and cohesin-depleted spindles. Bars represent the area of the metaphase plate divided by the area of the spindle, to account for differences in spindle size. At least ten spindles were measured in each of three independent experiments, using Metamorph software (Molecular Devices). Error bars are standard deviations. (D) Fluorescence images showing microtubules (red), kinetochore component Ndc80 (green) and DNA (blue) in spindles undergoing anaphase in mock- and cohesin-depleted extracts. Reactions were spun onto coverslips 20 minutes post anaphase induction and processed for immunofluorescence. Bar, 10 μm.

aligned as tightly as controls. We quantified this defect by calculating the area of the metaphase plate and dividing by the area of the spindle. This chromosome congression measure revealed a 32% expansion in area of the metaphase plate in the absence of cohesin (Fig. 2C). Therefore, the cohesin complex is required for chromosome organization and their proper alignment at the metaphase plate.

The observed defects in chromosome organization during metaphase indicated not only a deficiency in sister chromatid cohesion, but also faulty spindle attachments. To evaluate the effects on chromosome segregation, we assembled spindles around duplicated chromosomes in mock- and cohesin-depleted extracts and added $Ca^{2+}$, activating the anaphase promoting complex (APC) and releasing the metaphase arrest. In controls, chromosomes moved poleward in a synchronized manner. By contrast, most cohesin-depleted spindles did not segregate the chromosomes, which often failed clear the spindle midzone (Fig. 2D). When anaphase movements occurred, many chromosomes appeared to be lagging compared with mock-depleted reactions, and the chromosomes were not distributed evenly to opposite spindle poles (Fig. 3). These results indicate a role for the cohesin complex in chromosome attachment to and movement within the spindle.

### Cohesin is required for correct kinetochore-microtubule attachments that are not rescued by sister catenation

To elucidate the basis of the chromosome alignment and segregation defects observed in the absence of cohesin, we treated spindles with nocodazole, which preferentially depolymerizes non-kinetochore microtubules, to allow better visualization of kinetochore fibers. In a representative experiment, in which a high percentage of attachments was unequivocally determined (four spindles per condition, 71 chromosomes total), 90% of chromosomes in mock-depleted controls possessed bi-oriented (amphitelic) kinetochore attachments, whereas the remainder appeared associated with only one pole through a single kinetochore attachment (mono-oriented) (Fig. 4A and Table 1). By contrast, cohesin-depleted chromosomes displayed a variety of maloriented kinetochores, with 53% showing a complete loss of pairing between sisters. Interestingly, 34% of chromosomes possessed sister kinetochores attached to the same pole (syntelic), and their pairing was maintained. Merotelic attachments were also

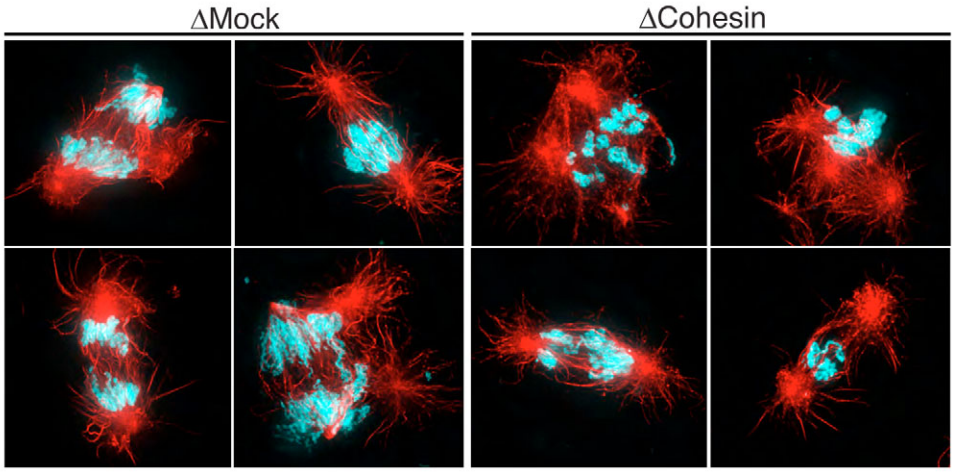


**Fig. 3.** Examples of anaphase chromosome segregation defects observed in the absence of cohesin; mock- and cohesin-depleted extracts containing spindles fixed 15 minutes after anaphase induction. Note that, chromosomes clearly segregated in three out of four control spindles, whereas cohesin-depletion causes segregation failure and/or unequal distribution of chromatids. Microtubules are red and chromosomes are blue-green.

observed in the absence of cohesin (6%), in which an individual kinetochore was attached to both poles. Thus, kinetochore attachment defects caused by cohesin depletion probably contribute to the chromosome alignment and segregation defects observed.

In addition to cohesin, sister chromatid catenation that occurs during DNA replication may also contribute to their association. These linkages must be resolved by topoisomerase II (topoII) to allow sister separation at the metaphase-to-anaphase transition in yeast, *Xenopus* egg extracts and mammalian cells (Downes et al., 1991; Holm et al., 1989; Shamu and Murray, 1992; Uemura et al., 1987). It has previously been observed that addition of topoII inhibitors rescued metaphase chromosome alignment defects caused by Scc1 depletion in chicken cells (Vagnarelli et al., 2004). We tested the effects of increased chromosome catenation in egg extracts depleted of cohesin by adding the topoII inhibitors etoposide or ICRF-193 (data not shown) to cycled reactions following DNA replication. The presence of a topoII inhibitor was able to partially rescue metaphase chromosome alignment defects, decreasing the number of stray chromosomes observed and reducing the area of the metaphase plate (Fig. 4B and data not shown). However, when kinetochores were visualized by immunofluorescence microscopy, unpaired sister chromatids did not appear aligned or bi-oriented, but accumulated at the periphery of the chromosome mass. Therefore, our results are consistent with a role for cohesin in establishing proper microtubule-kinetochore interactions in addition to sister chromatid cohesion.

## Sister cohesion is essential for inner centromere localization of INCENP and Aurora B

Whereas localization of several outer kinetochore proteins, including Ndc80, BubR1 and CENP-E, appeared normal in the absence of cohesin (Fig. 1, Fig. 2A and data not shown), we were curious about the fate of inner centromere factors and

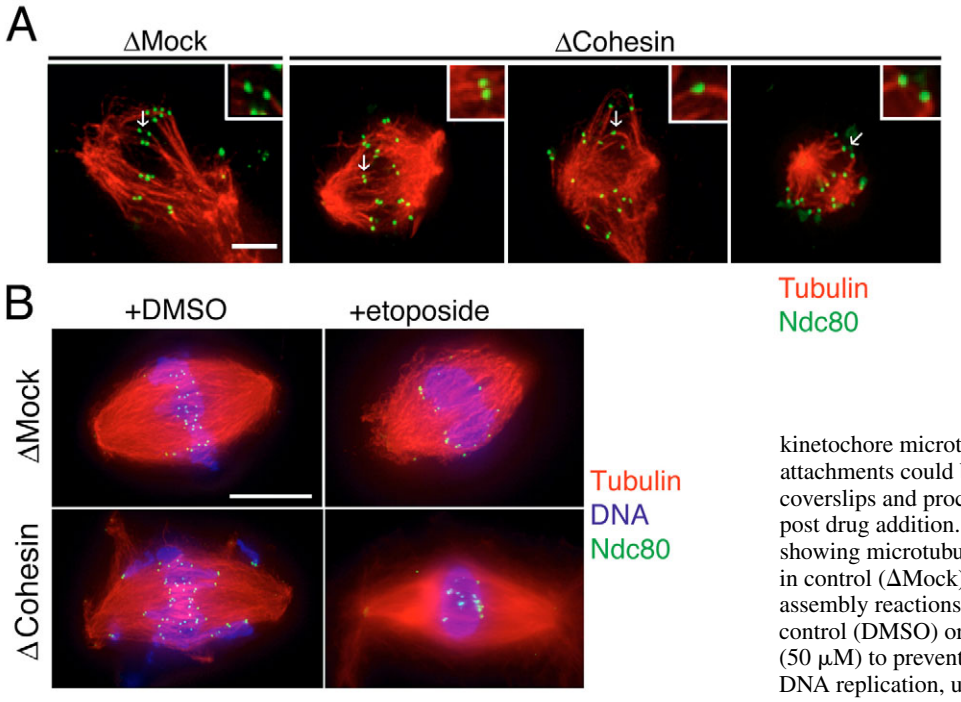


**Fig. 4.** Kinetochore-spindle-attachment defects in the absence of cohesin are not rescued by topologically linking sister chromatids. (A) Fluorescence images showing microtubules (red) and the kinetochore component Ndc80 (green) in spindles formed in mock- or cohesin-depleted extracts that had been treated with 0.3 μM nocodazole to preferentially depolymerize non-kinetochore microtubules so that kinetochore-microtubule attachments could be visualized. Reactions were spun onto coverslips and processed for immunofluorescence 20 minutes post drug addition. Bar, 5 μm. (B) Fluorescence images showing microtubules (red), Ndc80 (green) and DNA (blue) in control (ΔMock) and cohesin (ΔCohesin) depleted spindle assembly reactions that had been incubated with a solvent control (DMSO) or the topoismerase II inhibitor etoposide (50 μM) to prevent DNA decatenation. Drug was added after DNA replication, upon induction of mitosis. Bar, 10 μm.

**Table 1. Quantification of kinetochore attachments in control and cohesin-depleted spindles**

| | Bipolar | Mono-oriented | Syntelic | Merotelic | Lost sister pairs | Unable to determine |
|---|---|---|---|---|---|---|
| Control | | | | | | |
| 1 | 9 | 0 | 0 | 0 | 0 | 1 |
| 2 | 8 | 1 | 0 | 0 | 0 | 1 |
| 3 | 11 | 2 | 0 | 0 | 0 | 3 |
| 4 | 7 | 1 | 0 | 0 | 0 | 3 |
| Total | 35 | 4 | 0 | 0 | 0 | 8 |
| Cohesin-depleted | | | | | | |
| 1 | 0 | 0 | 3 | 0 | 3 | 3 |
| 2 | 0 | 1 | 2 | 0 | 5 | 0 |
| 3 | 0 | 0 | 6 | 1 | 4 | 0 |
| 4 | 0 | 1 | 0 | 1 | 5 | 2 |
| Total | 0 | 2 | 11 | 2 | 17 | 5 |

Images of four different nocodazole-treated spindles were analyzed under each condition and stained with antibodies against Ndc80. A variable number of kinetochores were visible in each image.

Categories: bipolar, sister kinetochores attached to opposite spindle poles; mono-oriented, one sister kinetochore attached to one pole, the other appeared unattached; syntelic, both sister kinetochores attached to the same pole; merotelic, a single kinetochore attached to both poles; lost sister pairs, sister kinetochores were dissociated; unable to determine, kinetochores were visible but their linkages could not be unambiguously determined.

whether their alteration may contribute to the defective spindle-kinetochore interactions observed. Previous studies reported that loss of the Scc1/Rad21 subunits in chicken or *Drosophila* cells caused mislocalization of the inner centromere protein INCENP (Sonoda et al., 2001; Vass et al., 2003).

On control *Xenopus* chromosomes, INCENP and aurora B localized between sister kinetochores that were labelled with BubR1 or Ndc80 antibodies (Fig. 5A, upper panels and Fig. 5B left panel). By contrast, the centromeric localization of these chromosomal passengers in cohesin-depleted reactions was often distorted, although faint chromosome-arm staining was not noticeably diminished. Fragmentation of the inner centromere staining appeared to occur when chromosomes were attached to both poles and stretched across the metaphase plate, whereas more normal-sized foci were present between sister chromatids near the poles that had presumably failed to make bipolar attachments (syntelic or mono-oriented) (Fig. 5A lower panels). Examination of isolated chromosomes at higher magnification revealed residual INCENP staining adjacent to the kinetochore of unpaired chromatids, as well as inner centromere localization in cases where sisters appeared only partially separated (Fig. 5B, right panels). Therefore, the fragmentation of INCENP and aurora B staining probably reflects distortion of the inner centromere upon spindle attachments that cause loss of sister cohesion. Consistent with this view, topoII inhibition, which topologically linked sisters and partially rescued metaphase plate formation – but not kinetochore pairing (Fig. 4B), did not restore correct localization of the passenger protein complex (data not shown).

A striking behavior of the passenger proteins is their dramatic relocalization from the inner centromere during metaphase to the spindle midzone during anaphase that is required for completion of cytokinesis (Vagnarelli and Earnshaw, 2004). We analyzed aurora B and INCENP distribution during anaphase in mock- and cohesin-depleted extracts, and found no obvious defect in their ability to relocalize during late anaphase, despite obvious chromosome segregation defects (Fig. 5C). These results are consistent with those observed in Drad21/Scc1-depleted *Drosophila* cells (Vass et al., 2003), and demonstrate that proper recruitment of INCENP and aurora B to the inner centromere is not a prerequisite for their spindle localization at anaphase, which may occur from chromatin-associated or cytoplasmic pools.

### Cohesin is required to maintain, but not to establish, the inner centromere and sister cohesion

Our observations that sister chromatids lacking bipolar attachments maintained paired kinetochores (Figs 2, 4) and inner centromere localization of chromosomal passengers (Fig. 5A) suggested that residual cohesion links sister chromatids in the absence of the cohesin complex, but that this linkage is not stable enough to resist spindle forces. To address this issue more directly, we took advantage of monastrol, a small-molecule inhibitor of the kinesin-5 motor (Eg5), which causes the formation of monopolar spindles with microtubule minus-ends focused at the center of the astral structure and the chromosomes attached around the periphery (Kapoor et al., 2000; Mayer et al., 1999). Because sister kinetochores are attached to a single pole, they are by definition syntelic, and biorientation never occurs. When monastrol was added at the beginning of mitosis, mock- and cohesin-depleted microtubule arrays looked similar, with paired sister kinetochores attached at the aster periphery (Fig. 6A). The average distance between paired kinetochores was slightly greater in the absence of cohesin at 1.41±0.17 μm compared to 0.80±0.14 μm in the mock-depleted control (90 sisters measured for each condition in three independent experiments). However, INCENP- and aurora-B-staining appeared normal in the absence of cohesin, indicating that in the absence of bipolar forces, cohesin is not required to link sister kinetochores and maintain the inner centromere (Fig. 6B and data not shown). To test whether sister pairing could be rescued upon dissipation of spindle forces, monastrol was added after spindle formation, leading to their collapse into monoaster structures. Interestingly, paired sister kinetochores were apparent in cohesin-depleted reactions, but often stretched even further apart compared with controls (Fig. 6C), and INCENP and aurora B localization was often fragmented (Fig. 6D and data not shown). Therefore, we hypothesize that other factors also contribute to the association of sister chromatids, but that cohesin is essential to maintain this pairing in the presence of bipolar spindle forces.

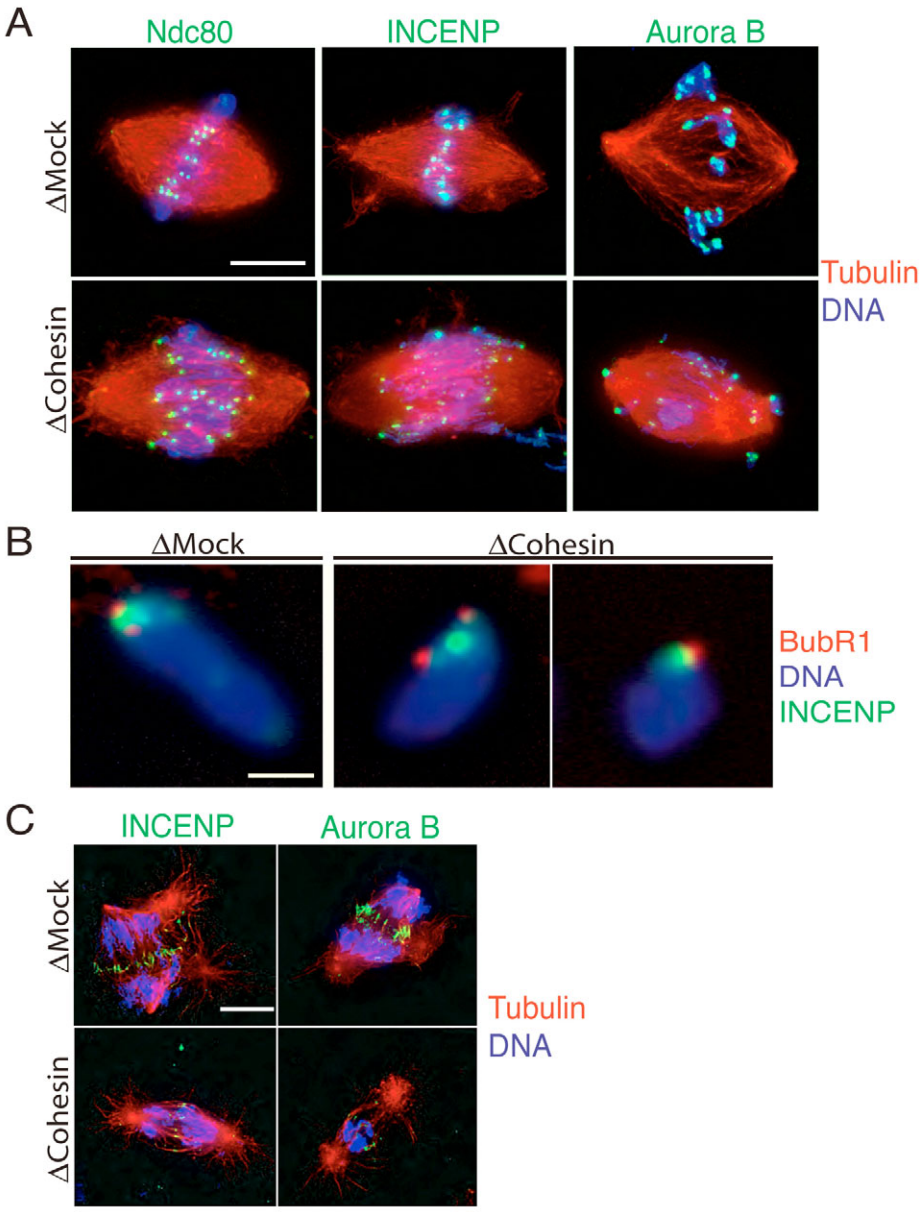


**Fig. 5.** Centromeric localization of INCENP and aurora B is fragmented in cohesin-depleted spindles.
(A) Fluorescence images showing DNA (blue), tubulin (red) and Ndc80, INCENP or aurora B (green) in spindles formed in cycled extracts in the presence (ΔMock) or absence (ΔCohesin) of cohesin. Reactions were spun onto coverslips 60 minutes into metaphase. Bar, 10 μm. (B) Fluorescence images of replicated, isolated sperm chromosomes assembled in mock- and cohesin-depleted extracts, spun onto coverslips 60 minutes after induction of mitosis and processed for immunofluorescence with antibodies to INCENP (green) and the kinetochore protein BubR1 (red). DNA was counterstained with Hoechst-33258 dye (blue). Bar, 2.5 μm. (C) Bipolar spindles assembled in the presence of Rhodamine-labeled tubulin (red) in control (ΔMock) or cohesin (ΔCohesin) depleted extracts were induced to enter anaphase, fixed after 20 minutes and spun onto coverslips for immunofluorescence using antibodies against INCENP and aurora B (green). DNA was counterstained with Hoechst-33258 dye (blue) and microtubules are shown in red. Bar, 10 μm.

### Co-depletion of condensin and cohesin amplifies spindle-assembly defects and alters chromosome morphology

We have previously shown that depletion of the conserved chromosome condensation factor condensin from egg extracts caused chromosome condensation, spindle assembly, anaphase and kinetochore morphology defects (Wignall et al., 2003). To investigate the mitotic consequences of removing both of these SMC-containing *Xenopus* complexes, we simultaneously depleted them from extracts and evaluated spindle assembly and chromosome morphology. Western blot analysis indicated that both condensin and cohesin subunits could be removed (Fig. 7A).

Compared with individual depletions, co-depletion of condensin and cohesin resulted in an additive effect on spindle assembly, decreasing the percentage of normal spindles formed to approximately 35%, compared with 79%, 66% and 48% in control, cohesin and condensin depletions, respectively (>400 microtubule structures counted for two independent experiments, Fig. 7B). The abnormal spindles observed were often small and monopolar, or bipolar with dramatically reduced microtubules (Wignall et al., 2003) (data not shown). Interestingly, within the normal spindles that formed, co-depletion of condensin and cohesin appeared to largely rescue problems in chromosome alignment observed in the absence of cohesin (Fig. 7C, ΔCohesin vs ΔCohesin + ΔCondensin), similar to the effects of co-inhibiting topoII (Fig. 4B). These observations are consistent with the requirement for both topoII and condensin for chromosome resolution, without which improperly paired or attached chromosomes are not free to dissociate and move away from the spindle midzone to cause misalignment. Co-depletion of condensin and cohesin also rescued a defect observed upon condensin depletion alone, which was the stretching and distortion of sites on chromosomes corresponding to kinetochores (Fig. 7C), perhaps because centromeric cohesion simply gave way,

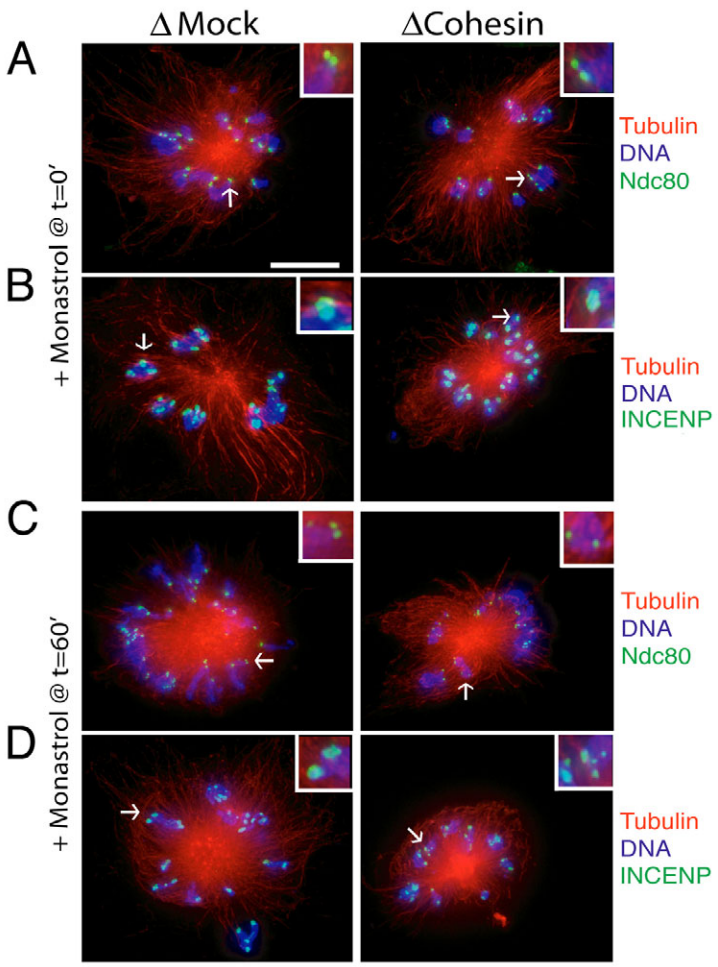


**Fig. 6.** Inhibition of bipolar spindle attachments can rescue inner centromeric cohesion and protein passenger localization in the absence of cohesin. (A-D) Fluorescence images of spindle reactions treated with the Eg5 inhibitor monastrol (125 μM) either prior to spindle assembly for 30 minutes (A,B), or for 30 minutes following spindle assembly (C,D) in mock- and cohesin-depleted extracts. DNA is blue, microtubules are red and Ndc80, INCENP or aurora B are green. Bar, 10 μm.

allowing kinetochores to separate rather than distend. These results highlight the distinct contributions that condensin and cohesin make to mitotic chromosome architecture and dynamics.

## Discussion

We have characterized the role of the cohesin complex during mitosis using *Xenopus* egg extracts. Our results support previous studies in other systems demonstrating the essential function of cohesin in sister chromatid cohesion, metaphase alignment and anaphase segregation. We have elucidated the potential basis of these defects by examining kinetochore-microtubule interactions in greater detail in the presence of low doses of nocodazole, revealing aberrant syntelic and merotelic attachments and improperly targeted inner centromere proteins INCENP and aurora B. Whereas increasing topological linkage of sisters by topoII inhibition rescued metaphase plate compaction, it did not restore kinetochore cohesion or bi-orientation, indicating an essential role for cohesin for proper spindle-kinetochore interactions. Importantly, we have also shown that sister kinetochore association and the inner centromere persist in the absence of cohesin when bipolar attachment of chromosomes is blocked by monastrol treatment. Finally, the effects of co-depletion of both condensin and cohesin highlight that, despite their different roles in chromosome organization, both of these chromosomal complexes contribute to spindle assembly.

### Cohesin and chromosome passenger proteins

Our results are consistent with previous studies examining the effects of cohesin inhibition on proteins of the inner centromere, particularly those belonging to the INCENP–aurora-B–survivin chromosomal passenger protein complex that relocalizes to the spindle mid-zone during anaphase. Mutation of the Rad21/Scc1 cohesin subunit in fission yeast resulted in the mistargeting of the survivin and aurora kinase homologues (Morishita et al., 2001), and INCENP recruitment was altered when a cohesin subunit was knocked down in either vertebrate or *Drosophila* cells (Sonoda et al., 2001; Vass et al., 2003). However, aurora B localization in the absence of cohesin had not been characterized in higher eukaryotes.

In *Xenopus*, we observed abnormal staining of both INCENP and aurora B at cohesin-depleted centromeres (Fig. 5A,B). Western blot analysis showed that levels of aurora B and INCENP were not altered by cohesin depletion. We also could not detect a biochemical interaction between cohesin and chromosomal passengers (data not shown). We therefore propose that cohesin is indirectly responsible for passenger recruitment by physically maintaining a functional inner centromere in the presence of spindle forces. Intriguingly, defects at the inner centromere could contribute to the improper kinetochore-microtubule attachments observed in the absence of cohesin, because aurora B is involved in an error-correction mechanism that regulates activity of the microtubule-destabilizing kinesin-13, MCAK at the centromere, which is thought to sever faulty kinetochore connections (Lampson et al., 2004; Lan et al., 2004). It will be interesting to investigate whether the unstable recruitment of passenger proteins contributes to the kinetochore defects observed upon cohesin depletion.

The passenger protein complex also has been implicated in spindle assembly, because its depletion from *Xenopus* egg extracts impaired chromatin-induced microtubule polymerization (Sampath et al., 2004). We did not observe dramatic spindle assembly defects in the absence of cohesin. However, the chromosome-arm-associated pool of these proteins did not appear diminished, and is thought to be the source of activity contributing to chromatin-mediated microtubule stabilization and spindle assembly (Sampath et al., 2004). Furthermore, relocalization of the passengers to the spindle during anaphase was not disrupted by cohesin depletion (Fig. 5C) (Vass et al., 2003). Therefore, different mechanisms function to recruit the passenger proteins to different sites during mitosis, and it is primarily the centromeric pool that is disrupted in egg extracts upon cohesin depletion.

### Cohesin is required to maintain, but not to establish, the inner centromere and sister cohesion

By carefully examining spindles and kinetochores, we observed that not all sister chromatids lost association upon cohesin depletion (Fig. 1B, Fig. 2A, Fig. 4A, Fig. 5). Interestingly, we found that sisters with syntelic or mono-oriented spindle attachments in bipolar spindles maintained their pairing and inner centromere, whereas those attached to opposite poles appeared to separate. Likewise, treatment of cohesin-depleted extracts with the Eg5 inhibitor monastrol (which prevents bipolar spindle assembly) also preserved sister association and the inner centromere localization of INCENP and aurora B. These observations suggest that cohesin is dispensable for the initial tethering of sisters during DNA replication, but is required to maintain their association upon bipolar spindle assembly. Although cohesin was depleted to nearly undetectable levels in our experiments, it is also possible that residual complex mediates the remaining aberrant cohesion. Distortion of centromeres in the absence of cohesin could be due to the effects of spindle forces on incorrectly linked sister kinetochores. Alternatively, effects could be indirect, caused by a failure to recruit essential centromere-stabilizing factors in the absence of cohesin. Because some degree of sister pairing was rescued upon spindle collapse in the absence of cohesin, our results also indicate that loss of cohesion upon spindle attachment is partially reversible. Thus, cohesin is essential for tight and robust sister chromatid cohesion, but other factors may also contribute to their physical linkage. Catenation is one possible source of cohesion that may not effectively resist spindle forces. Other protein factors have been identified that contribute to cohesion, including sororin and SGO1, but these factors are not known to play direct roles in sister linkage and, more probably, function upstream of cohesin (Rankin et al., 2005; Salic et al., 2004; Watanabe and Kitajima, 2005). Intriguingly, other factors in addition to cohesin have been shown to contribute to cohesion and resolution of telomeres and rDNA (Dynek and Smith, 2004; Sullivan et al., 2004), and our results are consistent with the model that at least two distinct activities contribute to centromeric cohesion in *Xenopus* egg extracts.

### Interplay between cohesin and condensin

Consistent with previous studies, we found no role for cohesin in condensin loading or chromosome condensation (Fig. 1) (Losada et al., 1998). Although defects in spindle assembly upon cohesin depletion were minor, additive effects were observed upon co-depletion of condensin (Fig. 7B). Because we did not see a significant decrease in the fidelity of spindle formation around chromatin-coated beads in the absence of either complex (data not shown) (Wignall et al., 2003), we propose that the spindle assembly defects result from impaired kinetochore function, rather than effects on chromatin-mediated microtubule stabilization. More extensive analysis of spindle- and kinetochore-fiber dynamics in the absence of these

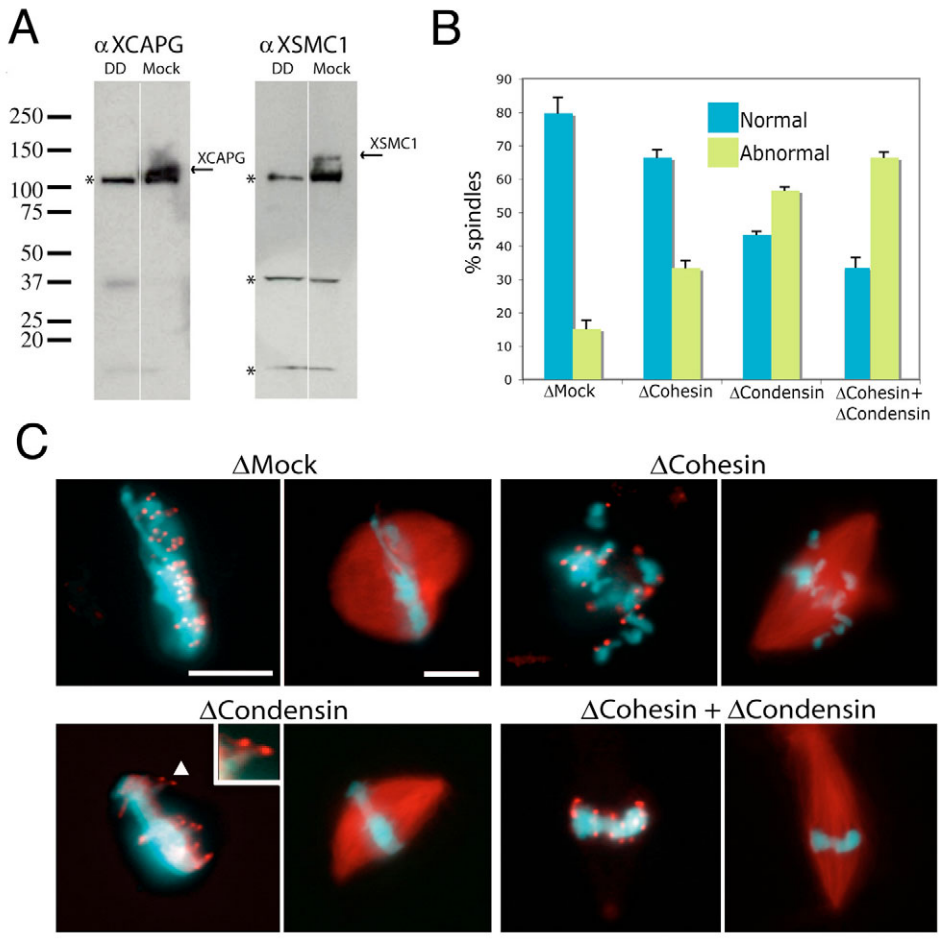


**Fig. 7.** Effects of co-depleting both condensin and cohesin from *Xenopus* egg extracts. (A) Western blot of mock- and double-depleted (DD) extracts showing that a cohesin subunit (SMC1), and a condensin subunit (XCAP-G) have been depleted. Background bands are marked with asterisks. (B) Spindle assembly defects are additive. Spindles assembled in extracts depleted using control (ΔMock), cohesin (ΔCohesin), condensin (ΔCondensin) or both (ΔCohesin+ΔCondensin) antibodies were evaluated for spindle assembly defects. Bars represent the average of two experiments, with more than 400 microtubule structures evaluated under each condition. Error bars represent standard deviation (± s.d.). (C) Chromosome morphology in single- and double-depleted extracts. Right set of panels show the position of chromosomes stained with Hoechst-33258 dye (blue) within metaphase spindles (microtubules are red). Left set of panels show higher magnification images of the same chromosomes (blue) stained with antibodies to kinetochore checkpoint protein BubR1 (red). Note misaligned chromosomes in the absence of cohesin. Arrowhead shows chromosome distortion apparent in the absence of condensin, probably due to pulling at kinetochores by spindle microtubules. Both the alignment and distortion defects are rescued in the double depletion. Bars, 10 μm.

chromosomal factors could shed light on the basis of these defects.

Similar to treatment of cohesin-depleted extracts with etoposide, co-depletion of the condensin complex eliminated chromosome mis-alignment that occurs in the absence of cohesin. Presumably, this is a result of incorrect condensation and resolution of sister chromatids, rendering them unable to separate from the amalgam of chromatin at the metaphase plate. Morphologically, co-depletion of the cohesin complex appeared to rescue stretching and distortion of kinetochores observed in condensin-depleted spindles (Fig. 7C) that is probably due to spindle microtubule attachment, because this distortion was reversible upon nocodazole treatment (Wignall et al., 2003). We believe that cohesin depletion rescues because sister chromatid cohesion fails upon bipolar attachment in the absence of cohesin and rather than becoming distorted, the centromere is more elastic and sister kinetochores separate. These results highlight the dynamic and complementary roles played by condensin and cohesin in generating the functional mitotic chromosome architecture essential for proper segregation.

## Materials and Methods

### *Xenopus* egg extracts

Cytostatic factor (CSF)-arrested extracts were prepared from freshly laid, unfertilized *Xenopus* eggs arrested in metaphase of meiosis II as previously described (Desai et al., 1999; Murray, 1991). A crushing centrifugation at 10,200 rpm (15 minutes at 16°C, Sorvall HB-6 rotor) marked the only departure from this protocol.

### Antibodies

C-terminal peptides of XSMC1, XRad21, XCAP-E and XCAP-G, identical to those described (Losada et al., 1998) were synthesized and used for antisera production by Synpep Corporation, and antibody-affinity purification (Sawin et al., 1992). Antibodies against aurora B were a gift from P. P. Budde (University of California, Berkeley); against Ndc80 and INCENP a gift from P.T. Stukenberg (University of Virginia); and against BubR1 a gift from D. Cleveland (University of California, San Diego).

### Immunodepletion and western blotting

For immunodepletion experiments from *Xenopus* egg extracts, antibodies were first coupled to Protein A dynabeads (Dynal) overnight at 4°C as previously described (Wignall et al., 2003). Antibodies and beads were combined in the following proportions: cohesin: 7.5 μg α-XSMC1, 7.5 μg α-XRad21 and 37.5 μl beads; mock-depletion control: 15 μg random rabbit IgG (Sigma) and 37 μl beads; condensin: 9 μg α-XCAP-E, 9 μg of α-XCAP-G and 30 μl beads; condensin-cohesin co-depletions: same amount of condensin antibody, together with 11.5 μg of α-XSMC and 11.5 μg of α-XRad21 plus 50 μl beads. Coupling reactions were prepared in duplicates for two successive rounds of depletion. Antibody-coupled Protein A dynabeads were incubated with 50 μl of fresh *Xenopus* egg extract for 1 hour at 4° per round. Depletion efficiency was assayed by SDS-PAGE and western blot of 1 μl extract samples using standard procedures and ECL™ reagents (Amersham Biosciences).

### In vitro spindle assembly and anaphase

To assemble and visualize spindles, sperm nuclei without their membrane (final concentration 500 nuclei/μl) and carboxy-X-Rhodamine-labeled tubulin (final concentration 75 μg/ml) (Hyman et al., 1991) were added to immunodepleted CSF extracts on ice. To initiate DNA replication and chromosome decondensation, extracts were driven into interphase by the addition of one-tenth extract volume $CaCl_2$ buffer (10 mM Hepes pH 7.7, 150 mM sucrose, 100 mM KCl, 1 mM $MgCl_2$ and 4 mM $CaCl_2$) and incubated for 60 minutes at 20°C in a water bath. To initiate assembly of mitotic spindles, an equal volume of fresh immunodepleted CSF-arrested extract was added to each tube and incubated for an additional 60 minutes to form metaphase-arrested spindles. In reactions containing nocodozole or etoposide (Sigma), the drugs were added post interphase together with fresh depleted CSF extract to a final concentration of 0.2 μM or 10 μM, respectively. When indicated, 5 mM of monastrol was diluted 1:40 in extracts either before or after spindles were formed. To induce anaphase, an additional one-tenth volume of $CaCl_2$ buffer was added and reactions were incubated for 30-40 minutes at 20°C.

In vitro reactions were monitored by dispensing 1.2 μl of extract onto a slide, overlaying with 5 μl of spindle fix [48% glycerol, 11% formaldehyde and 25 μg/ml Hoechst-33258 in MMR (2 mM $CaCl_2$, and 0.1 mM EGTA, 5 mM Hepes pH 7.8, 2 mM KCl, 1 mM $MgSO_4$, 100 mM NaCl)] and gentle squashing with a coverslip (Wignall and Heald, 2001).

### Immunofluorescence

Metaphase and anaphase spindles were spun onto coverslips as described (Desai et al., 1999) with the changes described by Wignall and Heald (Wignall and Heald, 2001). Metaphase chromosomes were preserved by diluting extract samples 100-fold with XBE2 buffer in 2% formaldehyde [XBE2: 5 mM Hepes pH 7.7, 50 mM KCl, 0.05 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM EGTA, 25 mM sucrose (Losada et al., 1998)], incubating at room temperature for 15 minutes and spinning onto coverslips through 6 ml of a 30% glycerol/XBE2 cushion. Coverslips were post-fixed in methanol for 5 minutes at –20°C and blocked overnight at 4°C in PBS/3% BSA. Primary antibodies were detected by incubation with Rhodamine-, FITC- or Alexa Fluor 488-labeled secondary antibodies (Molecular Probes™). DNA was visualized by a 1-minute incubation of coverslips with Hoechst 33258 (1 μg/ml) diluted in PBS before mounting onto slides with Vectashield® mounting medium.

### Microscopy

Deconvolution microscopy was performed using an Olympus IX70 wide-field inverted fluorescence microscope with 60× 1.4 NA, or 100× 1.35 NA oil immersion lenses. Images were obtained with a cooled Photometrics CCD CH350 camera, captured using DeltaVision image acquisition software (Applied Precision, Issaquah, WA) with a Z-step of 0.15-0.20 μm and deconvolved using a conservative algorithm (Chen et al., 1996). Additional images were taken with a CCD camera (Hamamatsu) mounted on an E600 fluorescence microscope (Nikon) using Metamorph software (Universal Imaging Corp.) All images were imported and processed using Adobe Photoshop®.

### Phenotype quantification

To evaluate defects in spindle assembly, microtubule structures were examined by fluorescence microscopy and counted as either normal (bipolar) or abnormal (monopolar, multipolar or abnormal microtubule nucleation) as previously described (Wignall et al., 2003). Chromosome alignment deficiencies were assessed by counting the number of chromosomes located outside of the metaphase plate. To measure metaphase compaction defects, we used Metamorph software (Molecular Devices) to calculate the area of the metaphase plate and divided that number by the area of the spindle.

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