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The Chromosomal Passenger Complex Is Required for Chromatin-Induced Microtubule Stabilization and Spindle Assembly

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Summary

In cells lacking centrosomes, such as those found in female meiosis, chromosomes must nucleate and stabilize microtubules in order to form a bipolar spindle. Here we report the identification of Dasra A and Dasra B, two new components of the vertebrate chromosomal passenger complex containing Incenp, Survivin, and the kinase Aurora B, and demonstrate that this complex is required for chromatin-induced microtubule stabilization and spindle formation. The failure of microtubule stabilization caused by depletion of the chromosomal passenger complex was rescued by codepletion of the microtubule-depolymerizing kinesin MCAK, whose activity is negatively regulated by Aurora B. By contrast, we present evidence that the Ran-GTP pathway of chromatin-induced microtubule nucleation does not require the chromosomal passenger complex, indicating that the mechanisms of microtubule assembly by these two pathways are distinct. We propose that the chromosomal passenger complex regulates local MCAK activity to permit spindle formation via stabilization of chromatin-associated microtubules.

Introduction

Eukaryotic cells utilize microtubules to drive chromosome movement during cell division. Bipolar spindle formation and chromosome alignment have classically been thought to occur by microtubule nucleation from centrosomes, followed by the capture of chromosomes via interaction of microtubule ends with kinetochores, the proteinaceous complexes assembled on centromeric chromatin during mitosis (reviewed in Biggins and Walczak [2003]). Nonetheless, centrosomes and kinetochores are not the only sites of microtubule nucleation and capture, respectively; bulk chromatin can also nucleate microtubules in a centrosome-independent manner (Heald et al., 1996). This pathway is crucial to the formation of bipolar spindles in cells lacking centrosomes, as found in female meiosis of several animal species (reviewed in Karsenti and Vernos [2001]; Rieder et al., 2001). Somatic mammalian cells also possess this pathway and can assemble bipolar spindles in the absence of centrosomes (Khodjakov et al., 2000). Yet, the mechanisms regulating this chromatin-induced spindle assembly process remain poorly understood.

To date, the best-characterized pathway of chromatin-induced microtubule assembly involves the small GTPase Ran (reviewed in Kahana and Cleveland [1999]). Metaphase chromosomes exist in a locally high concentration of Ran-GTP (Kalab et al., 2002), which is generated by chromosome bound RCC1, a Ran-GDP exchange factor (Bischoff and Ponstingl, 1991; Carazo-Salas et al., 1999). In *Xenopus* egg extracts, high concentrations of Ran-GTP are sufficient to induce microtubule aster formation, even in the absence of chromatin or centrosomes (Carazo-Salas et al., 1999; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999). Most recently, Ran-GTP has been proposed to promote microtubule stabilization through the activation of microtubule-associated proteins (MAPs), such as TPX2 and NuMA, in a mechanism involving their release from nuclear import factors (Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001). TPX2 has in turn been shown to activate Aurora A, which is required for the maturation of centrosomes (Tsai et al., 2003).

In addition to regulation by MAPs, microtubule stability is also controlled by microtubule-depolymerizing factors such as mitotic centromere-associated kinesin (MCAK, also known as XKCM1; Tournebize et al., 2000; Walczak et al., 1996). MCAK enzymatically induces catastrophe at both plus and minus ends of microtubules (Desai et al., 1999) and is required for global microtubule instability in *Xenopus* egg extracts (Tournebize et al., 2000). Very recently, it was demonstrated that the microtubule depolymerizing activity of MCAK can be suppressed through phosphorylation by the kinase Aurora B (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004). This was surprising, as Aurora has been proposed to increase the turnover of microtubules connecting kinetochores and centrosomes (Lampson et al., 2004; Tanaka et al., 2002). For this reason, it currently remains unclear what the physiological context and functional relevance of MCAK regulation by Aurora B might be.

Aurora B is one of the so-called "chromosomal passenger" proteins, which are enriched on the inner centromere during metaphase but are translocated to the spindle midzone at anaphase (reviewed in Adams et al. [2001]). Aurora B physically interacts with other chromosomal passenger proteins, Incenp and Survivin/BIR, to form the "chromosomal passenger complex" (Adams et al., 2000; Bolton et al., 2002; Losada et al., 2002). In *Xenopus* egg extracts, the majority of these three components form an 11S complex (Bolton et al., 2002; Losada et al., 2002). In nematodes, this complex also contains another essential protein, CSC-1, orthologs of which have not yet been identified in other eukaryotes (Romano et al., 2003). Several mitotic functions of the chromosomal passenger complex have been suggested, including the regulation of metaphase chromo-

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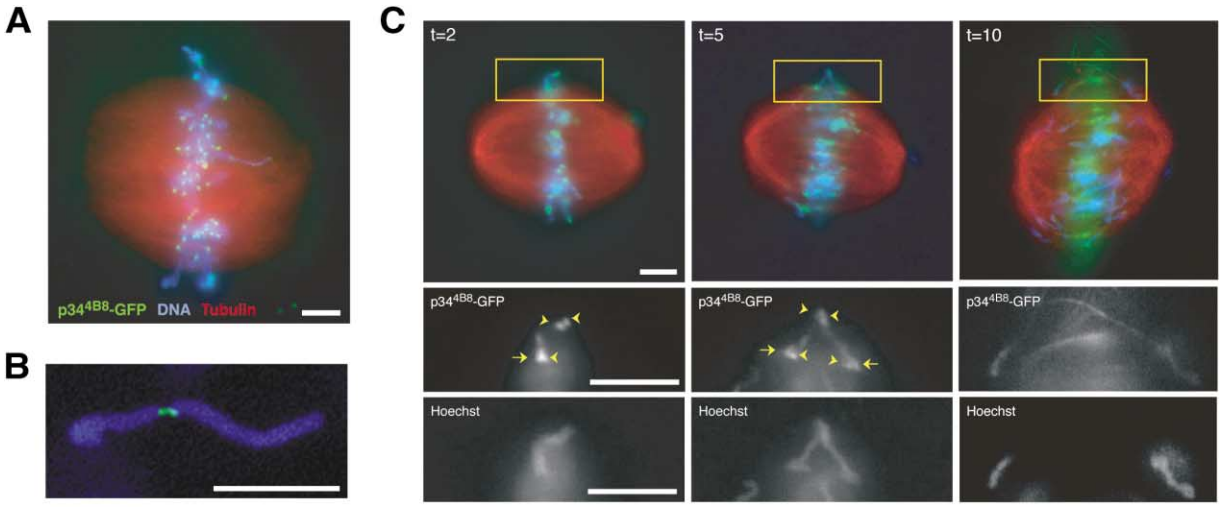


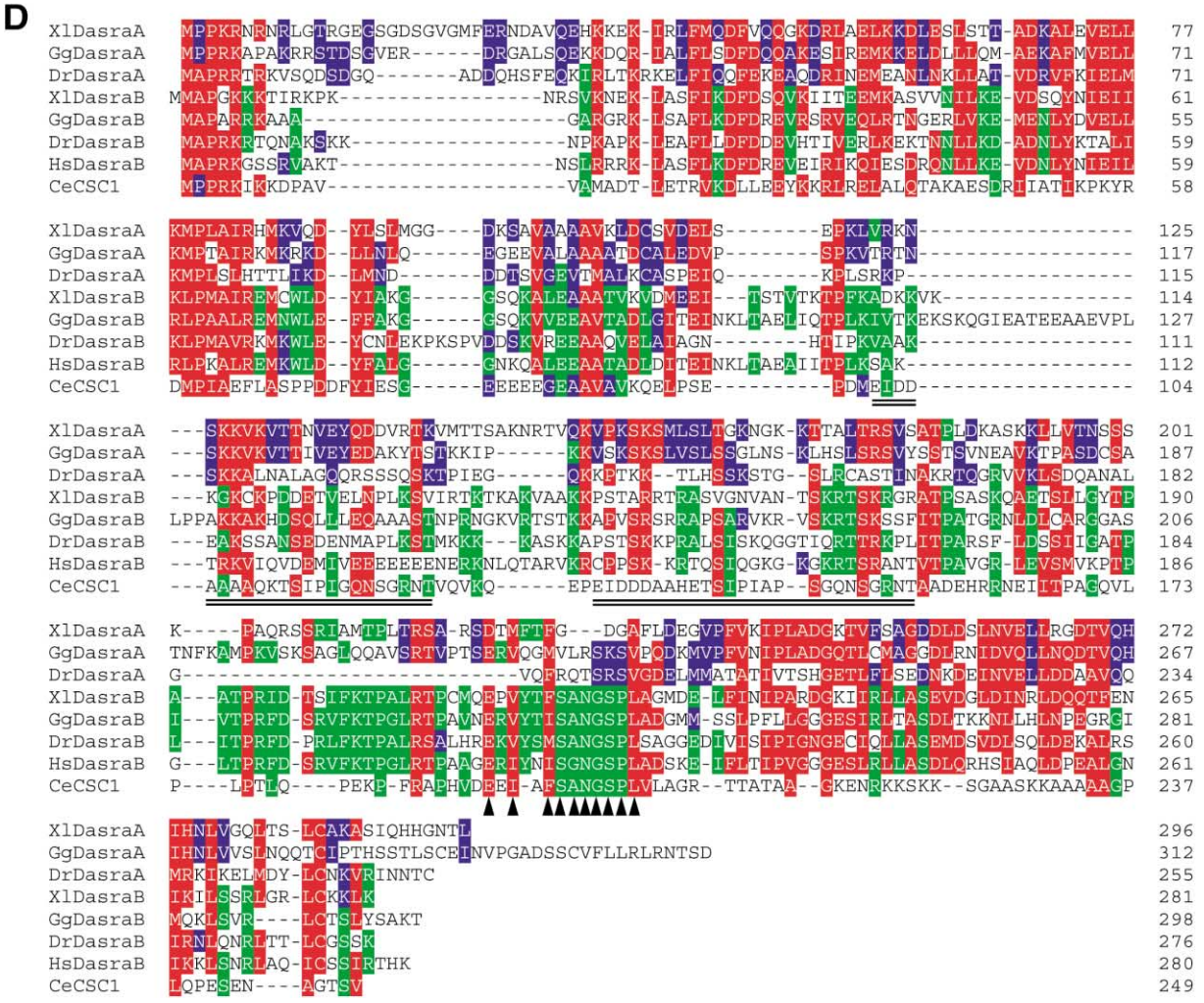


Figure 1. p34[4B8]-GFP Localizes to the Inner Centromere at Metaphase and to the Spindle Midzone at Anaphase

(A) Metaphase localization of p34[4B8]-GFP. mRNA encoding p34[4B8]-GFP (green) was added to CSF (meiotic metaphase II arrested) egg extract containing sperm nuclei, and the extract was cycled through interphase to metaphase. Rhodamine-tubulin (red) and DAPI (blue) were added to visualize microtubules and DNA, respectively. Scale bar, 5 μm.

(B) An individual chromosome (blue) from egg extract expressing p34[4B8]-GFP (green). Scale bar, 5 μm.

(C) Time-lapse microscopy of p34[4B8]-GFP-containing spindles after induction of anaphase. The boxed regions of merged images (p34[4B8]-GFP, green; DAPI, blue; rhodamine-tubulin, red) are shown at increased magnification in monochrome. Arrows indicate centromeres, which lead chromosome movement during anaphase, and arrowheads telomeres. Scale bars, 5 μm.

some alignment, sister chromatid resolution, spindle checkpoint signaling, and cytokinesis (reviewed in Carmena and Earnshaw [2003]). Despite these multiple roles, there has been no strong evidence that this complex is involved in spindle assembly.

To better understand the role of chromosomes in organizing mitotic processes, we initiated an expression screen using *Xenopus* egg extracts to identify new metaphase chromosome binding proteins. This approach led us to discover two novel components of the vertebrate chromosomal passenger complex, Dasra A and Dasra B, the latter of which shows limited similarity to CSC-1. To our surprise, depletion of the chromosomal passenger complex from *Xenopus* egg extracts, using antibodies against either Dasra A or Incenp, blocked bipolar spindle assembly. This block was caused by failure to stabilize microtubules around chromatin, and we found that this microtubule instability depended on MCAK. The chromosomal passenger complex was not required for Ran-induced microtubule nucleation, suggesting that the complex stabilizes microtubules by a mechanism distinct from that of the Ran-GTP pathway. Our findings demonstrate an unexpected role for the chromosomal passenger complex in chromatin-induced microtubule stabilization.

Results

Identification of a New Vertebrate Chromosomal Passenger Protein

We applied an expression screening strategy to identify metaphase chromosome binding proteins using *Xenopus* egg extracts (Funabiki and Murray, 2000; see Supplemental Figure S1A at http://www.cell.com/cgi/content/full/118/2/187/DC1). After initial biochemical identification, the localization patterns of putative chromosome binding proteins were further examined by GFP tagging. Among these, the localization of a protein temporarily named p34^{4B8} drew our special attention. The p34^{4B8}-GFP protein was found throughout metaphase chromosomes, with enrichment at the primary constriction (Figures 1A and 1B); this localization onto chromosome arms and centromeres was also observed by immunofluorescence (see below). The p34^{4B8}-GFP protein was also localized to the subtelomeric regions of some if not all chromosomes (Figure 1C). During anaphase, p34^{4B8}-GFP disappeared from centromeres but was relocalized to the spindle midzone (Figure 1C). This dynamic localization pattern mimicked that of the chromosomal passenger proteins, including Incenp, Aurora B, and Survivin (reviewed in Carmena and Earnshaw [2003]).

xDasra A Is a Component of the Chromosomal Passenger Complex

To test if p34^{4B8} physically interacts with the known chromosomal passenger proteins, we raised antibodies against a C-terminal p34^{4B8} peptide. This antibody specifically recognized a 34 kDa protein in *Xenopus* egg extracts by Western blot (Figure 2A), consistent with the size of p34^{4B8} protein translated in vitro (see Supplemental Figure S1B on the *Cell* web site). The antibody precipitated p34^{4B8} from egg extract (Figures 2C and 2F) and coprecipitated xIncenp, xAurora B, and xSurvivin (Figure 2C). More than 90% of the p34^{4B8} protein present in egg extract was depleted using this antibody, leading to ~70% codepletion of xIncenp, xAurora B, and xSurvivin (Figure 2C, Supplemental Figure S2A, and data not shown). Conversely, depletion of greater than 90% of xIncenp using anti-xIncenp antibodies led to ~90% codepletion of p34^{4B8} (Figure 2B and Supplemental Figure S2A). This depletion was specific to components of the chromosomal passenger complex, as no significant codepletion of other proteins, such as MCAK, XMAP215, Aurora A, or ICIS, was detected (Supplemental Figure S3 and data not shown). Further supporting the conclusion that xIncenp, xAurora B, xSurvivin, and p34^{4B8} form a physical complex, these proteins cofractionated after sucrose density gradient centrifugation (Figure 2D). Moreover, the immunolocalization patterns of p34^{4B8} and xIncenp were identical in metaphase and anaphase (Supplemental Figure S2B). Thus, we conclude that the majority of xIncenp, xAurora B, xSurvivin, and p34^{4B8} form a complex in *Xenopus* egg extracts. We renamed the p34^{4B8} protein xDasra A in reference to the Hindu mythological deity *Dasra*, who acts as a harbinger of the dawn (*Aurora* of Roman mythology).

xDasra A Can Associate with Chromosomes Lacking Centromeres

Immunolocalization of xDasra A and xIncenp on metaphase chromosomes revealed that both are localized not only to centromeres but also to chromosome arms (Supplemental Figure S2C; S.C.S. and H.F., unpublished data). To determine whether xDasra A can be targeted to chromosomes lacking centromeres, we analyzed its localization on plasmid-coated chromatin beads in egg extracts. Anti-Dasra A antibodies clearly stained such chromatin beads (Figure 2E), demonstrating that Dasra A can be efficiently recruited to noncentromeric chromatin.

Identification of Dasra B Proteins

The *Xenopus laevis* Dasra A cDNA encodes a protein of 296 amino acids, which is 23% identical to human CDCA8 (Figure 1D). CDCA8 is a protein of unknown function, whose cell cycle expression profile is strongly correlated with those of known cell cycle regulators such as cyclins (Walker, 2001). We were able to identify *Xenopus laevis* EST sequences encoding a protein 42% identical to CDCA8. We named this *Xenopus* protein xDasra B, as it displays weak (26% identity) but significant homology to xDasra A (Figure 1D) and interacts with the chromosomal passenger complex (see below). Although we were able to identify both Dasra A and Dasra B

(D) Sequence alignment of *Xenopus laevis* Dasra A, *Gallus gallus* Dasra A, *Xenopus laevis* Dasra B, *Gallus gallus* Dasra B, *Homo sapiens* Dasra B/CDCA8, and *Caenorhabditis elegans* CSC-1. Identical or conserved amino acids are boxed. Identity or similarity between Dasra A sequences is shown in blue, between Dasra B sequences in green, and between all sequences in red. Double underlines indicate the direct repeat region of CSC-1, and arrowheads indicate the region of high homology between Dasra B sequences and CSC-1.

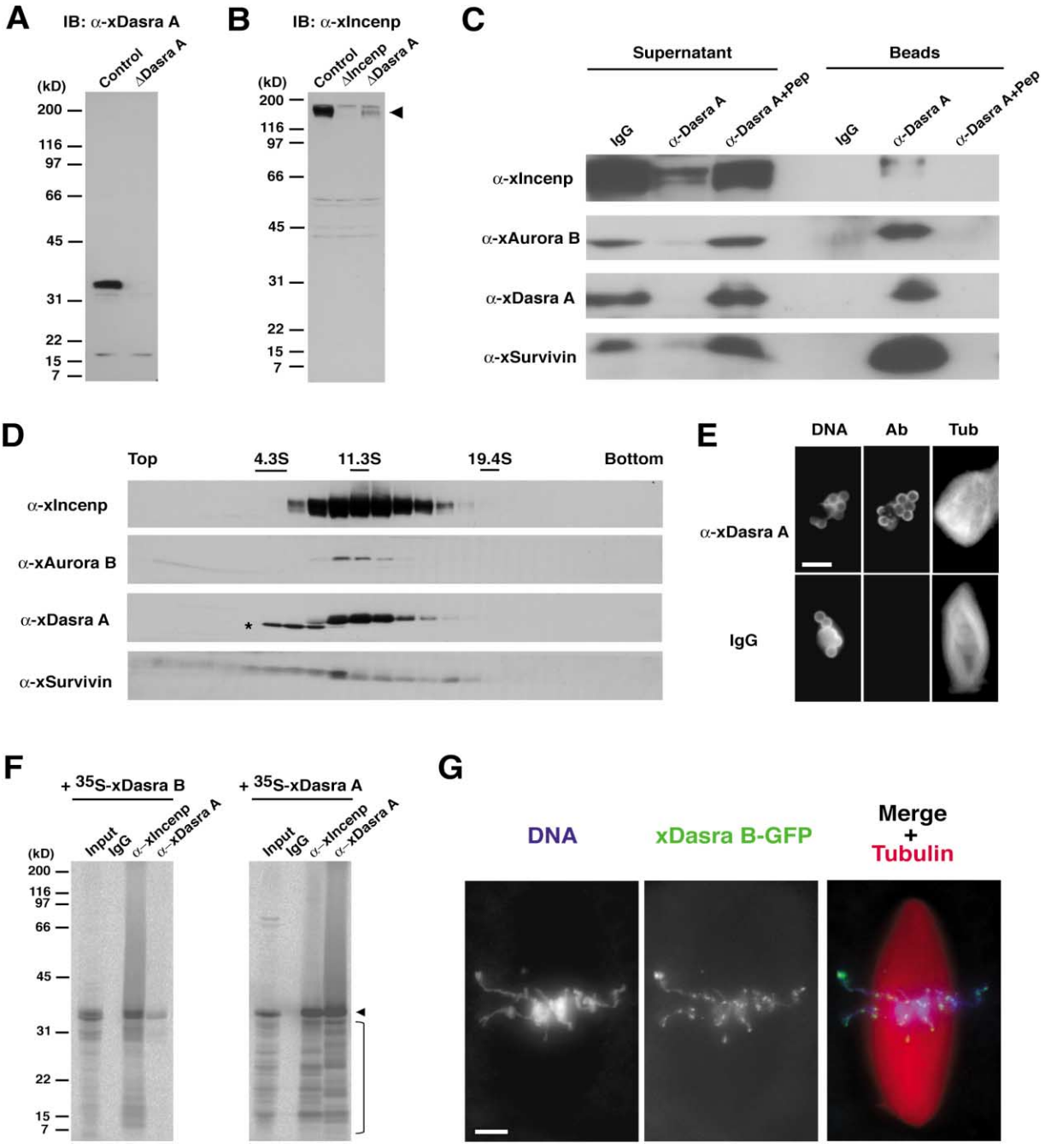


Figure 2. Dasra A and Dasra B Are Components of the Chromosomal Passenger Complex

p34^{4B8} is hereafter referred to as xDasra A (see text for details). (A) Specificity of anti-xDasra A antibodies and immunodepletion of xDasra A from *Xenopus* egg extract. Metaphase egg extracts were depleted either with control IgG beads or anti-xDasra A beads. A Western blot of total protein from each is shown probed with anti-xDasra A antibodies. (B) Immunodepletion of xIncenp from egg extract and codepletion of xIncenp with xDasra A. Metaphase egg extracts were depleted with either control IgG beads, anti-xIncenp beads, or anti-xDasra A beads. A Western blot of total protein from each is shown probed with anti-xIncenp antibodies. Arrowhead indicates the position of xIncenp. The minor crossreacting species are not depleted by either anti-xIncenp or anti-xDasra A beads. (C) xDasra A physically interacts with the chromosomal passenger complex. High-speed supernatants of metaphase *Xenopus* egg extracts were depleted with either control IgG beads, anti-xDasra A beads, or anti-xDasra A beads in the presence of xDasra A peptide competitor. A Western blot of total protein from the supernatant (left) or bead bound (right) fractions was prepared and probed with the indicated antibodies. (D) Cofractionation of xDasra A with the chromosomal passenger complex after sucrose density gradient centrifugation. High-speed supernatant of metaphase egg extract was separated over a 5%–40% continuous sucrose gradient, and fractions were collected and analyzed by Western blot using the indicated antibodies. Asterisk indicates a minor lower molecular weight form of unknown significance, which cannot be depleted using anti-xDasra A beads. (E) Localization of Dasra A to noncentromeric chromatin. Chromatin beads were assembled in metaphase egg extract containing rhodamine-tubulin and were stained with either anti-xDasra A antibodies (top) or control IgG antibodies (bottom). DNA was stained with Hoechst 33258. Scale bar, 10 μm. (F) xDasra B coprecipitates with xIncenp and xDasra A in egg extract. ^{35}S-labeled xDasra B (left) or xDasra A (right) protein was incubated

sequences in *Xenopus*, chicken (*Gallus gallus*), and zebrafish (*Danio rerio*), only Dasra B sequences could be found in mammalian sequence databases. Homology search analyses failed to identify Dasra homologs in yeasts or nematodes. However, we recognized limited regions showing sequence identity between Dasra B and *C. elegans* CSC-1. This similarity is most prominent in a C-terminal region (marked with arrowheads in Figure 1D), which is highly conserved among Dasra B sequences, but not among Dasra A sequences.

To determine whether Dasra B interacts with the chromosomal passenger complex, ^{35}S-labeled *Xenopus laevis* Dasra B (xDasra B) protein was incubated with egg extracts, and the complex was immunopurified using either anti-xIncenp or anti-xDasra A antibodies. A much greater amount of labeled xDasra B was copurified using anti-xIncenp antibodies than with anti-xDasra A antibodies (Figure 2F), suggesting that, while xDasra B can interact with xDasra A, the majority of xDasra B is associated with xIncenp lacking xDasra A. It is thus possible that two types of the chromosomal passenger complex exist, one containing Dasra A and the other containing Dasra B. To verify its localization, mRNA encoding GFP-tagged xDasra B (xDasra B-GFP) was translated in egg extracts. xDasra B-GFP was localized to metaphase chromosomes with centromeric enrichment, similar to the pattern observed for xDasra A-GFP (Figure 2G).

Human Dasra B Is Required for Proper Metaphase Chromosome Alignment in Mammalian Cells

To examine the function of human Dasra B (hDasra B)/CDCA8, we raised an antibody against a C-terminal hDasra B peptide. Immunofluorescence on metaphase HeLa cells with the anti-hDasra B antibody revealed punctate dots on chromosomes, which were coincident with anti-Aurora B staining (Figures 3A and 3B). Although this antibody did not work for immunoblotting (S.C.S., unpublished data), it coimmunoprecipitated Aurora B and Survivin, suggesting that hDasra B interacts with the chromosomal passenger complex (Figure 3C).

To examine whether hDasra B is required for proper function of the chromosomal passenger complex, HeLa cells were treated with an siRNA oligonucleotide targeting the hDasra B mRNA. In hDasra B siRNA-treated cells arrested in metaphase with the proteasome inhibitor MG132, 41% of mitotic cells demonstrated severe chromosome misalignment (Figures 3B and 3E; quantified in Figure 3F, left); in such cells, anti-hDasra B, anti-Aurora B, and anti-Survivin antibodies all failed to stain chromosomes by immunofluorescence (Figure 3B and data not shown). hDasra B siRNA-treated cells also accumulated multiple interphase nuclei (Figure 3F, right). These phenotypes are reminiscent of those seen following loss of Aurora B function (Ditchfield et al., 2003; Hauf et al., 2003). Furthermore, Survivin protein levels were reduced by 60%–70% after treatment with hDasra B siRNAs, whereas Aurora B and Incenp levels were unchanged (Figure 3D; S.C.S., E. Knatko, and J. Swedlow, unpublished data), indicating that hDasra B is required to maintain normal levels of Survivin protein.

The Chromosomal Passenger Complex Is Required for Bipolar Spindle Formation in Frog Egg Extracts

To investigate the function of the Dasra A-containing chromosomal passenger complex, we monitored spindle assembly on replicated sperm chromosomes in *Xenopus* egg extracts that had been immunodepleted using anti-xDasra A antibodies. Seventy minutes after inducing M phase entry at 15.5°C, bipolar metaphase spindles were observed in mock-depleted control extracts (Figure 4A). To our surprise, however, bipolar spindle formation was severely inhibited after xDasra A depletion, and 79% of mitotic figures were either monopolar spindles or asters (Figures 4A and 4B).

To verify the phenotype caused by Dasra A immunodepletion, we asked whether depletion of the chromosomal passenger complex using anti-xIncenp antibodies caused a similar effect. In xIncenp-depleted extracts, an even more severe spindle formation defect was observed (Figures 4A and 4C); chromosomes were associated with either very small astral microtubules (42%) or no detectable microtubules at all (57%). The severity of the spindle formation phenotype correlated with the amount of residual xIncenp and xAurora B; while approximately 30% of xIncenp and xAurora B remained undepleted using anti-xDasra A antibodies, less than 10% of xIncenp and xAurora B remained undepleted using anti-xIncenp antibodies (Figure 2B, Supplemental Figure S2A, and data not shown).

We did not expect to perturb spindle assembly by depleting the chromosomal passenger complex, since such defects had not been reported previously for loss of function of any known components of the complex, either in somatic tissue culture cells or in the first embryonic cell division of *C. elegans*. We observed spindle assembly defects in egg extracts using two antibodies recognizing different components of the complex, however, suggesting that the phenotype is specific. For our further studies on the role of the chromosomal passenger complex in microtubule assembly, we focused on the defects in extracts depleted with anti-xIncenp antibodies, due to the more complete immunodepletion of the complex achieved and the stronger phenotype observed by this method. We will refer to these as ΔIncenp extracts.

The Chromosomal Passenger Complex Is Required for Chromatin-Induced Spindle Assembly

Microtubules nucleate from both centrosomes and chromosomes in *Xenopus* egg extracts. To determine

for 30 min with CSF extract containing cycloheximide. The extract was then treated with either control IgG beads, anti-xIncenp beads, or anti-xDasra A beads, and the bead bound fraction of each was analyzed by SDS-PAGE, followed by autoradiography. Arrowhead indicates the positions of xDasra B and xDasra A. Bracket indicates low molecular weight products from in vitro translation. (G) Metaphase localization of xDasra B-GFP. mRNA encoding xDasra B-GFP (green) was added to CSF extract containing sperm nuclei, and the extract was cycled through interphase to metaphase. Rhodamine-tubulin (red) and DAPI (blue) were added to visualize microtubules and DNA, respectively. Scale bar, 5 μm.

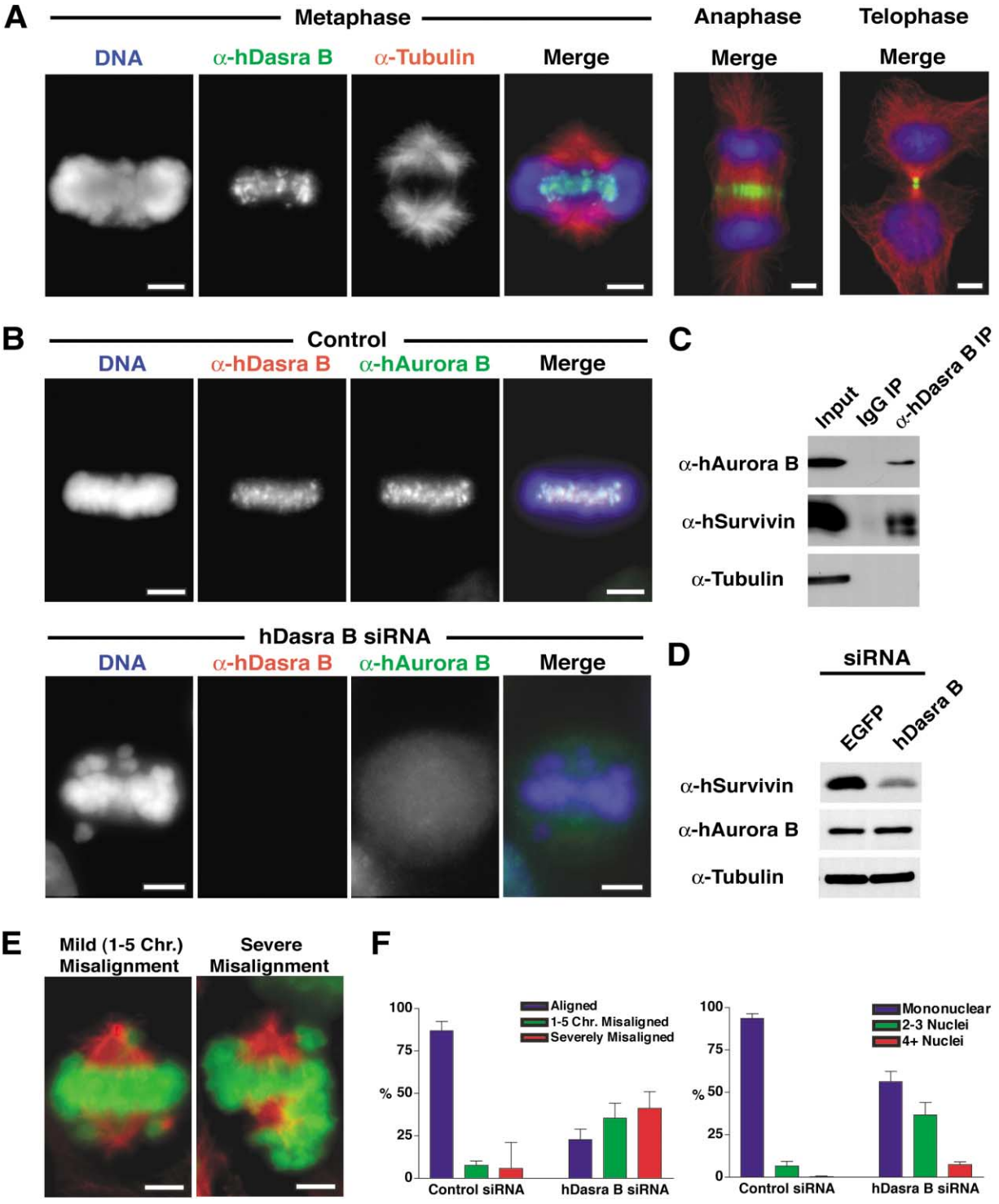


Figure 3. Human Dasra B (hDasra B) Is Associated with the Chromosomal Passenger Complex and Is Required for Proper Metaphase Chromosome Alignment

(A) Localization of hDasra B during mitosis in human cells. Asynchronous HeLa cells were stained with anti-hDasra B antibodies (green), anti-α-tubulin (red), and Hoechst 33258 (blue). Scale bars, 5 μm.

(B) Loss of hDasra B expression causes Aurora B mislocalization and metaphase chromosome misalignment. HeLa cells were treated with either a control EGFP siRNA oligo (top row) or an hDasra B-specific siRNA oligo for 30 hr, arrested with the proteasome inhibitor MG132 for 2 hr, and analyzed by immunofluorescence using anti-hDasra B (red) and anti-hAurora B (green) antibodies. DNA was stained with Hoechst 33258 (blue). Scale bars, 5 μm.

(C) Physical interaction between hDasra B, hAurora B, and hSurvivin. HeLa cells were synchronized in M phase by thymidine-nocodazole arrest, and complexes were immunoprecipitated from lysates using either control IgG or anti-hDasra B antibodies. Western blots of the eluted fractions were performed using the indicated antibodies.

if the chromosomal passenger complex is required for microtubule nucleation from centrosomes and/or chromosomes, we first monitored astral microtubule formation from centrosomes associated with sperm nuclei. Because nucleation of microtubules from these centrosomes well precedes reorganization of the sperm chromatin, it is possible to distinguish between centrosome- and chromosome-mediated microtubule nucleation. Robust microtubule asters formed from centrosomes in both control extracts and ΔIncenp extracts within 10 min after incubation at 15.5°C, demonstrating that centrosomes can nucleate microtubules in the absence of the chromosomal passenger complex (Figure 5, left). Twenty minutes after incubation, the centrosomes had migrated away from the majority of sperm chromosomes in control and ΔIncenp extract, although they still maintained an interaction with a part of the chromosomal mass (Figure 5, middle). At 40–60 min after incubation, however, while half-spindles or bipolar spindles had formed around 96% of chromosomes in control extracts, 83% of sperm chromosomes in ΔIncenp extracts were associated with few or no detectable microtubules (Figure 5, right). This result indicates that chromosomes can neither nucleate nor stabilize microtubules in the absence of the chromosomal passenger complex.

To directly test this hypothesis, we used DNA-coupled beads as the substrate for spindle assembly, since it has been shown that the chromatin assembled onto DNA beads is sufficient to promote microtubule assembly and bipolar spindle formation (Heald et al., 1996). We monitored microtubule assembly using rhodamine-labeled tubulin and live microscopy. In mock-depleted extracts, microtubules nucleated and stabilized around chromatin beads after approximately 10 min of incubation, and bipolar spindles began to form after 20 min (Figures 6A and 6C). In ΔIncenp extracts, however, no detectable microtubule assembly was observed over the time course of 60 min (Figures 6A and 6C and data not shown). We therefore conclude that the chromosomal passenger complex is required for chromatin-induced spindle formation.

Aurora B and Incenp can act together to phosphorylate MCAK and reduce its microtubule depolymerizing activity in vitro (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004). To examine whether the lack of microtubule assembly around chromatin in ΔIncenp extracts depends on MCAK function, we coimmunodepleted MCAK and the chromosomal passenger complex from egg extracts. In ΔIncenpΔMCAK extracts, microtubules were clearly stabilized around chromatin beads (Figures 6B and 6C). This rescue could be reverted by adding back recombinant MCAK protein to endogenous levels (Figures 6B and 6C and Supplemental Figure S3). Similar rescue of microtubule assembly by MCAK depletion from ΔIncenp extract was observed when replicated sperm chromosomes were used rather than chromatin beads (R.O. and H.F., unpublished data).

To determine whether chromatin still served as the site of microtubule nucleation in the absence of MCAK, we monitored microtubule assembly in ΔIncenpΔMCAK extracts by live microscopy. As shown in Figure 6A (bottom row), microtubules nucleated and stabilized around chromatin beads in ΔIncenpΔMCAK extract. These data demonstrate that chromatin still maintains the ability to *nucleate* microtubules in the absence of the chromosomal passenger complex but that under these conditions such microtubules are extremely unstable due to MCAK activity.

The Chromosomal Passenger Complex Is Not Essential for Ran-GTP-Dependent Microtubule Nucleation

What mechanism underlies the chromatin-induced microtubule nucleation observed in ΔIncenpΔMCAK extracts? To test whether the Ran-GTP pathway contributes to the nucleation of microtubules under these conditions, we performed live imaging of microtubule nucleation around chromatin beads in ΔIncenpΔMCAK extract after adding a dominant-negative mutant of Ran, RanT24N, which prevents production of Ran-GTP by blocking its exchange factor, RCC1 (Carazo-Salas et al., 2001). Whereas 100% of bead aggregates in control ΔIncenpΔMCAK extract exhibited robust microtubule nucleation, this chromatin-induced microtubule growth was severely inhibited by the addition of RanT24N protein, in which 98% of aggregates demonstrated no microtubule nucleation (Figure 7A). This finding demonstrates that the Ran-GTP pathway can act to nucleate microtubules from chromatin in the absence of the chromosomal passenger complex. Consistent with this finding, both RCC1 and Ran were capable of associating with chromosomes in ΔIncenp extracts (Supplemental Figure S4).

We found that RanT24N also prevented chromatin-induced microtubule nucleation in extract lacking only MCAK. Whereas 100% of chromatin bead aggregates demonstrated microtubule nucleation in ΔMCAK extract, this nucleation was suppressed in 90% of all aggregates in the presence of RanT24N (Figure 7A). Thus, while the instability of chromatin-associated microtubules in ΔIncenp extract requires MCAK, the lack of chromatin-induced microtubule nucleation caused by RanT24N does not depend on MCAK activity.

To further examine whether the chromosomal passenger complex acts through a Ran-GTP-independent

(D) Loss of hDasra B expression leads to decreased hSurvivin protein levels. HeLa cells were treated with either a control or a hDasra B-specific siRNA oligo, as described in (B). After 30 hr, cells were lysed and analyzed by Western blot using anti-hSurvivin, anti-hAurora B, and anti-α-tubulin antibodies.

(E) Chromosome misalignment caused by loss of hDasra B. HeLa cells were treated with either a control or an hDasra B-specific siRNA oligo and analyzed using anti-α-tubulin antibodies (red) and Hoechst 33258 (green). A cell was scored as "severely misaligned" if the chromosomal mass either was not organized into a metaphase plate or was extended beyond the spindle or metaphase plate. Scale bars, 5 μm.

(F) Quantitation of metaphase chromosome misalignment (left) and interphase multinuclearity (right). Values shown are the mean plus standard deviation from three independent experiments, with at least 100 cells counted per experiment. Chromosome misalignment was evaluated by scoring the percentage of mitotic cells with bipolar spindles having the indicated phenotypes, as determined by Hoechst 33258 staining.

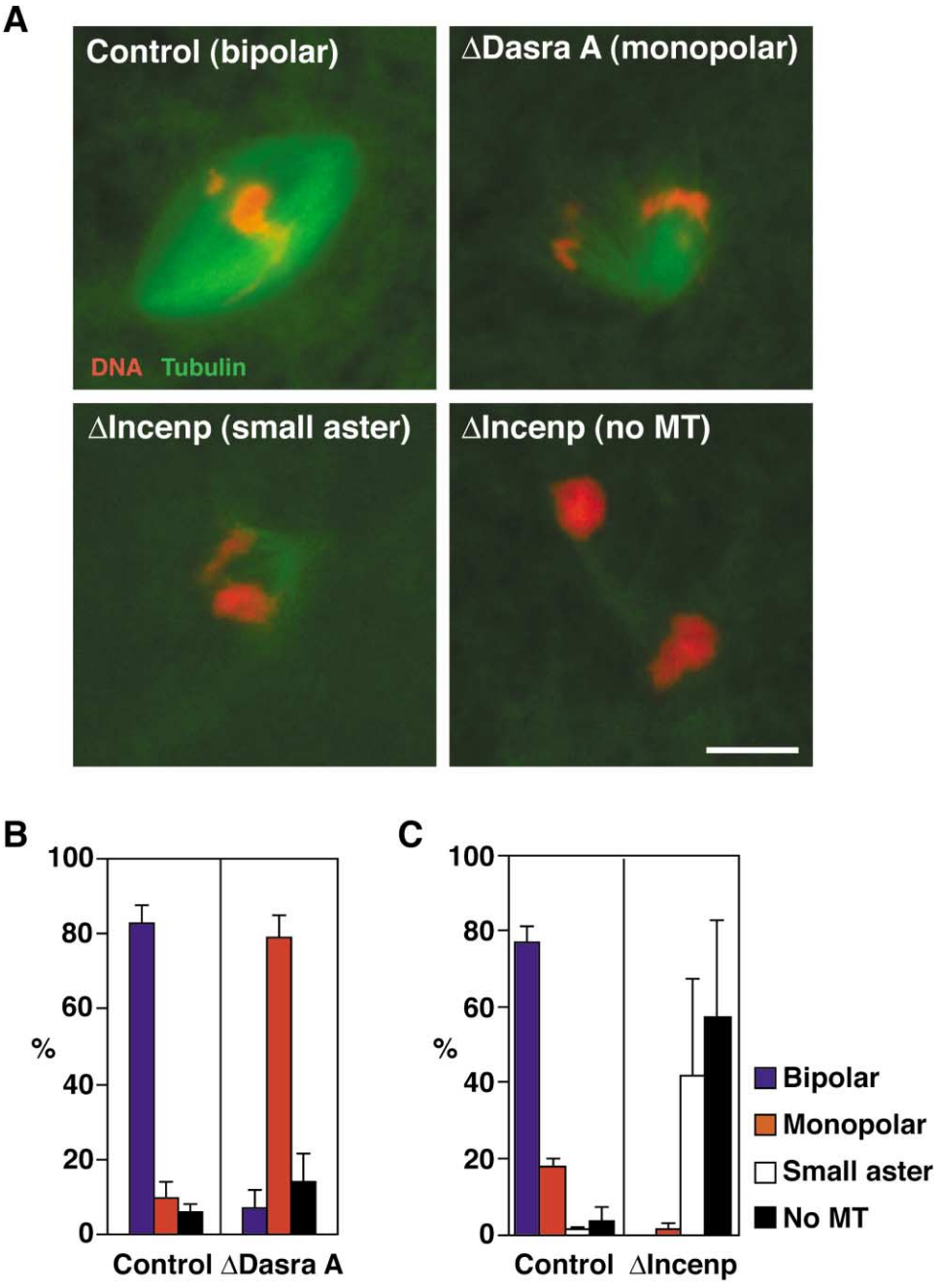


Figure 4. xDasra A and xIncenp Depletion Induce Defects in Spindle Formation in Egg Extracts

(A) Spindles assembled on replicated sperm chromosomes (containing centrosomes) in mock-depleted extract, ΔDasra A extract (depleted with anti-xDasra A antibodies), or ΔIncenp extract (depleted with anti-xIncenp antibodies). Chromosomes were visualized with Hoechst 33258 (red) and microtubules with rhodamine-labeled tubulin (green). Scale bar, 10 μm.

(B) Quantitation of spindle structures assembled on replicated chromosomes in control or ΔDasra A extracts. Spindles were scored 70 min after entry into M phase at 15.5°C. Values shown are the mean plus standard deviation from four independent experiments. Spindle classification was as indicated in (A).

(C) Quantitation of spindle structures assembled on replicated chromosomes in control or ΔIncenp extracts. Spindles were scored at 70 min after entry into M phase at 15.5°C. Values shown are the mean plus standard deviation from three independent experiments. Spindle classification was as indicated in (A).

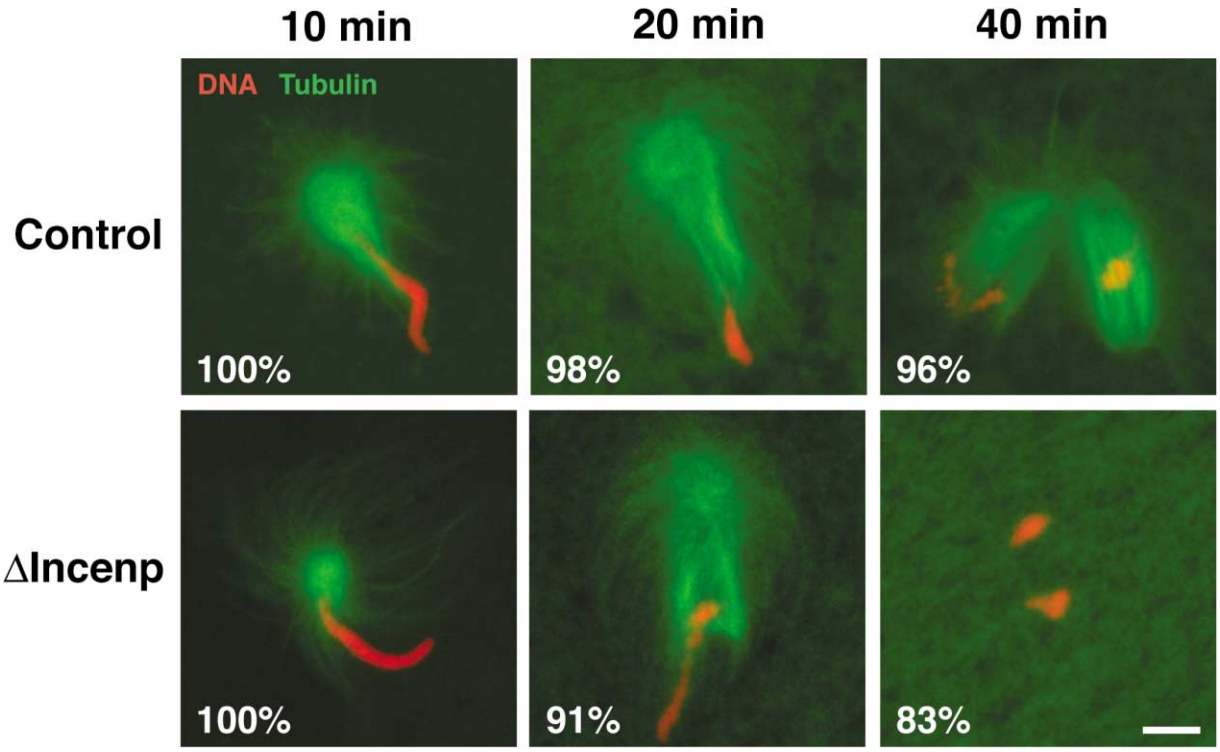


Figure 5. Microtubule Nucleation from Sperm Centrosomes and Chromosomes in ΔIncenp Extract

Microtubule nucleation was visualized from centrosomes associated with demembranated sperm nuclei in control or ΔIncenp extracts. Sperm nuclei were incubated in extracts at 15.5°C for the time indicated and were visualized with Hoechst 33258 (red) in the presence of rhodamine-labeled tubulin (green). For each time point, quantitation is given of the indicated microtubule morphology. At least 200 sperm nuclei were scored at each time point. Scale bar, 10 μm.

pathway, we added hydrolysis-deficient mutant Ran protein (Coutavas et al., 1993) to metaphase extracts; this is known to induce aster formation in a manner dependent on the presence of microtubule binding proteins such as TPX2, XMAP215, and γTuRC (Gruss et al., 2001; Wilde and Zheng, 1999). Addition of this mutant Ran protein induced aster formation in both mock-depleted and ΔIncenp egg extracts (Figure 7B). We could not detect any quantitative or qualitative difference in aster structures between mock-depleted extracts and those lacking the Aurora B complex (Figure 7B). This demonstrates that the chromosomal passenger complex is not required for Ran-GTP-induced aster formation. In addition, this argues against the possibility that the anti-xIncenp antibody either specifically or non-specifically codepletes other microtubule stabilizing proteins working downstream of Ran-GTP.

Discussion

Dasra Proteins Are Components of the Vertebrate Chromosomal Passenger Complex

Our studies on the functions of the chromosomal passenger complex were initiated by our identification of Dasra A and Dasra B, two new components of this complex in vertebrates. We suggest that vertebrate Dasra B may represent a functional homolog of nematode CSC-1 on the basis of their slight sequence similarity and because both physically associate with other members of the chromosomal passenger complex. Depletion of hDasra B and CSC-1 by siRNA in HeLa cells and *C. elegans*, respectively, led to decreased Survivin protein levels and to failure in targeting Aurora B to chromosomes (Romano et al., 2003). Thus, Dasra B and CSC-1 may act to regulate the stability of Survivin, which, together with Incenp, serves to target Aurora B to chromosomal arms and centromeres. Nonetheless, since Dasra A shows little or no sequence similarity to CSC-1, it is possible that the function of Dasra A may be distinct from that of CSC-1 and Dasra B.

Whereas Dasra B is well conserved among vertebrates, Dasra A homologs can be found in zebrafish, frogs, and chicken but not in mammals. Although we cannot rule out the possibility that a mammalian ortholog of Dasra A exists, it is also possible that Dasra A plays a function that is dispensable in mammals. One of the characteristics distinguishing Dasra A-containing vertebrates from mammals is the rapid cell divisions that occur during early embryonic development in the former (Beddington et al., 2002). Thus, a hypothetical role of Dasra A, distinct from Dasra B, might be to aid in rapid spindle formation and chromosome segregation during early embryonic cell cycles. Further studies are needed to determine the precise molecular functions of the Dasra proteins.

The chromosomal passenger complex is a large (~11S) entity, whose constituent proteins exist in unknown stoichiometries. Addition of a recombinant

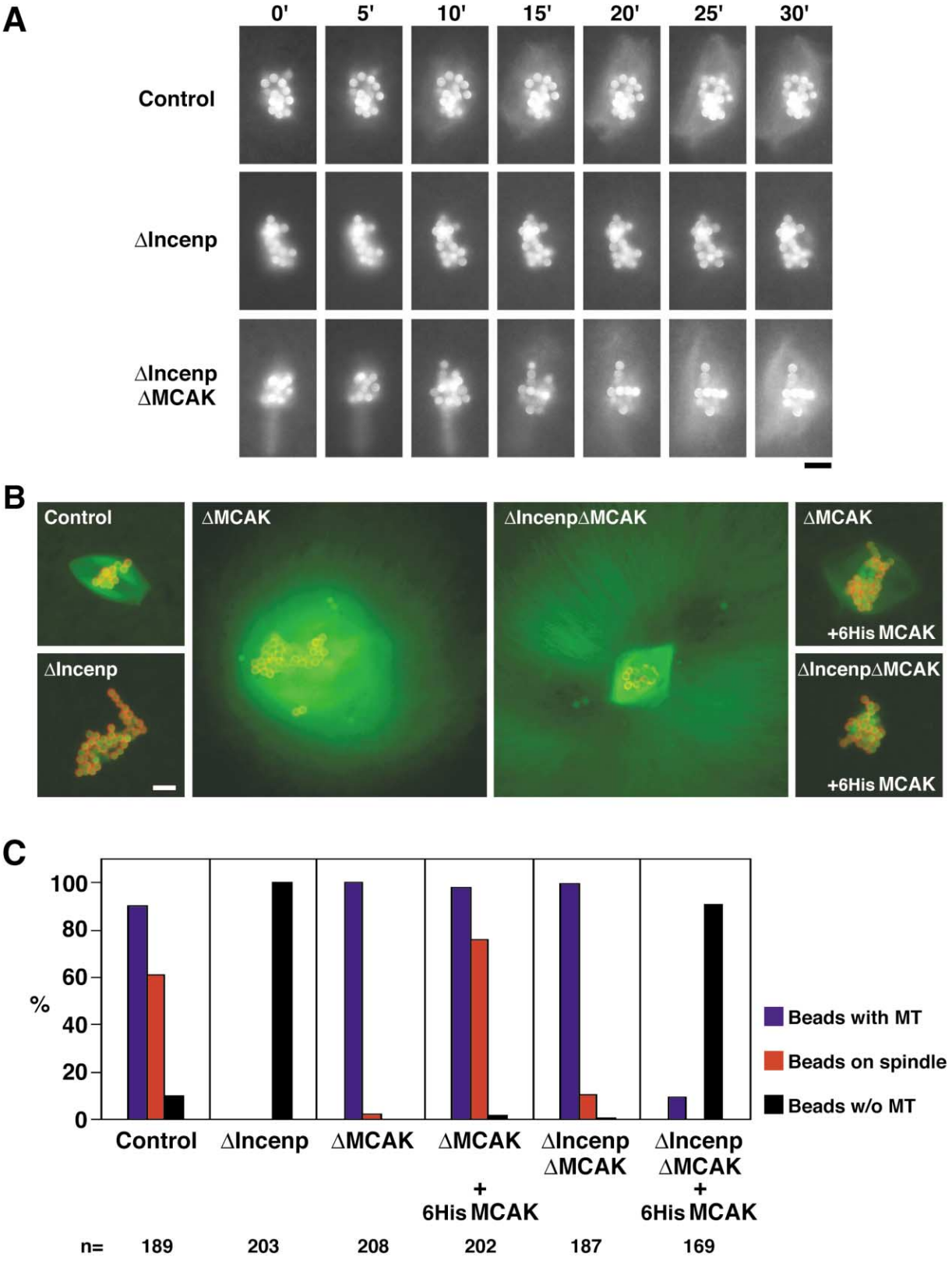


Figure 6. Microtubule Nucleation from Chromatin Beads in ΔIncenp Extract

(A) Spindle formation was monitored by time-lapse video microscopy immediately after adding control, ΔIncenp, or ΔIncenpΔMCAK extracts to DNA-coated beads at 20°C. Microtubules were visualized with rhodamine-tubulin, and chromatin beads were simultaneously observed by their autofluorescence. Time is in minutes after the beginning of image acquisition. The first images were taken 4 min after placing the ice-cold extracts on slides at 20°C. Scale bar, 10 μm.

xIncenp/xAurora B complex (which can phosphorylate and suppress MCAK in vitro [Ohi et al., 2004]), together with recombinant xSurvivin, xDasra A, and xDasra B to ΔIncenp or ΔDasra A extracts, did not rescue their microtubule stabilization and spindle assembly defects (R.O. and H.F, unpublished data), indicating that there are yet more proteins associated with this complex. Identification of these missing factors is crucial to understand how the chromosomal passenger complex is spatially and temporally regulated.

Chromatin-Induced Microtubule Stabilization via the Chromosomal Passenger Complex

The discovery of the Dasra proteins led us to investigate the role of the chromosomal passenger complex in spindle assembly. Spindle-associated microtubules can nucleate from both chromosomes and centrosomes, but these two pathways can be distinguished by examining spindle formation around chromatin beads (lacking centrosomes) and sperm nuclei (containing centrosomes). We found that removing the chromosomal passenger complex from *Xenopus* egg extracts (ΔIncenp extracts) completely abolished the capacity of chromatin to drive microtubule assembly, demonstrating that the chromosomal passenger complex is required for the stabilization of chromatin-nucleated microtubules. By contrast, centrosomes associated with sperm nuclei efficiently nucleated microtubules in ΔIncenp extracts. This centrosome-induced microtubule nucleation might be regulated by proteins such as Aurora A and Ran-GTP, which are known to localize to the centrosome (reviewed in Carmena and Earnshaw [2003]; Keryer et al., 2003).

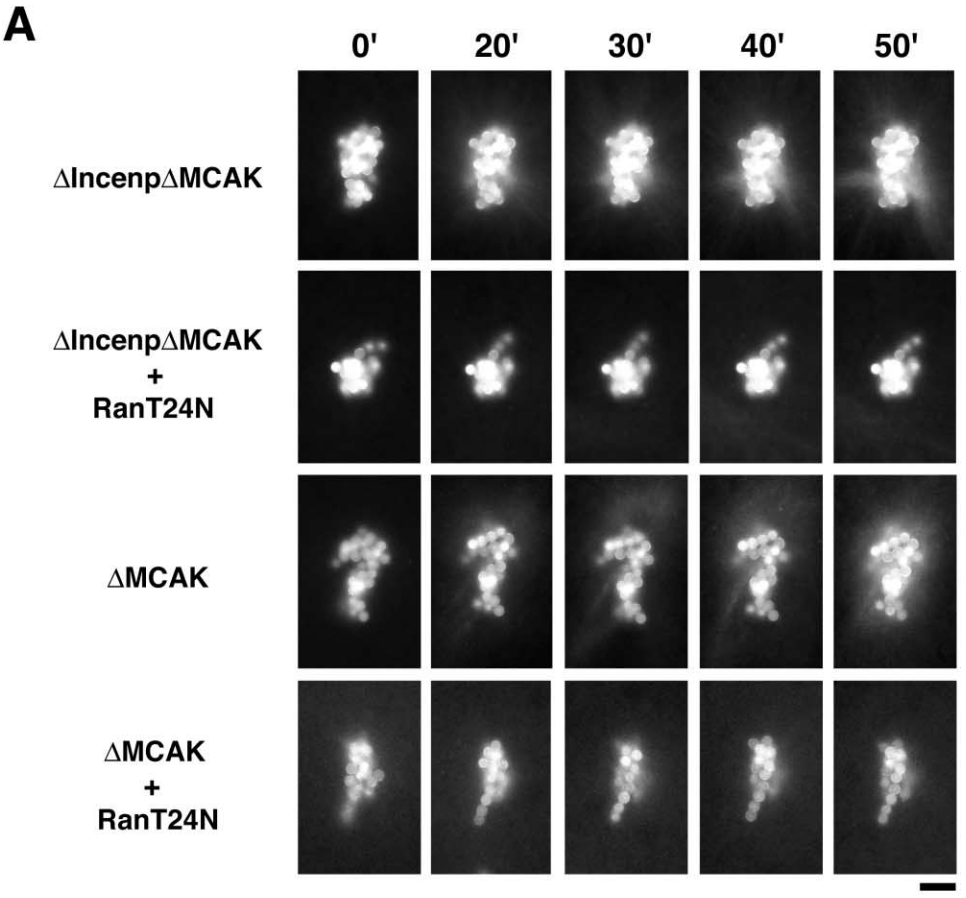
The lack of chromatin-induced microtubule assembly in ΔIncenp extracts was rescued by depletion of the microtubule depolymerase MCAK. Under these conditions, chromatin-associated microtubules became hyperstabilized, forming giant halo-like structures similar to those observed in extracts depleted of MCAK alone. In contrast, simultaneous depletion of MCAK and XMAP215, a protein which plays crucial roles in stabilizing microtubules (Tournebize et al., 2000), does not lead to such halo-like microtubule structures (M. Shirasu-Hiza, personal communication). This suggests that the chromosomal passenger complex and XMAP215 use distinct mechanisms to stabilize microtubules and that the rescue of microtubule nucleation in ΔIncenpΔMCAK extracts reflects a unique functional relationship between Aurora B and MCAK. Indeed, whereas XMAP215 can directly stabilize microtubules by increasing microtubule growth rate and suppressing the microtubule catastrophe rate in the absence of MCAK (Kinoshita et al., 2001), Aurora B was found to phosphorylate and suppress the microtubule-destabilization activity of MCAK in vitro (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004). Although this does not rule out the possibility that Aurora B also regulates MCAK activity through other mechanisms or that it controls other regulators of microtubule stability, the simplest model to explain our results is that phosphorylation and inactivation of MCAK represent one of the major pathways by which Aurora B supports microtubule stabilization around chromosomes (Figure 8).

It has been suggested that MCAK phosphorylation is required for the conversion of monopolar to bipolar spindles; reconstitution of MCAK-depleted extract with the alanine mutant MCAK-4A, which is resistant to inhibition by Aurora B, does not support bipolar spindle formation around sperm chromosomes (Ohi et al., 2004). This is an effect reminiscent of the monopoplar spindles observed after microtubule assembly around sperm chromosomes in ΔDasra A extract (Figure 4A) and indicates that the chromosomal passenger complex likely facilitates the conversion of monopolar to bipolar spindles on sperm chromosomes via MCAK regulation. It remains unclear, however, whether this conversion is promoted by regulation of MCAK located on chromosome arms or at centromeres. Since MCAK is known to interact with its activator, ICIS, at centromeres (Ohi et al., 2003), it is possible that the differential regulation of MCAK at centromeres and on chromosome arms is crucial for spindle bipolarity.

Consistent with the role of the chromosomal passenger complex in stabilizing microtubules near chromosome arms, the complex is localized to noncentromeric regions of chromosomes in *Xenopus* egg extracts (Figure 2E). Incenp initially shows such a distribution during prophase-prometaphase in somatic cells, but, as metaphase advances, it becomes progressively confined to chromosomal regions closely associated with spindle microtubules (Cooke et al., 1987; Earnshaw and Cooke, 1991). In such somatic cells, chromosome arms are often excluded from the center of metaphase spindles (Figure 3). This is in striking contrast to the situation in *Xenopus* egg extracts, in which the majority of the chromosome mass is inside the metaphase spindle (Figure 1A). This difference in the amount of chromosomal area associated with spindle microtubules may explain why depletion of the chromosomal passenger complex in somatic cells does not cause a dramatic spindle assembly phenotype. While chromatin-induced microtubule stabilization is essential in *Xenopus* egg extracts (and thus in female meiosis), it may be less important in somatic mitotic cells. Indeed, female meiotic cells must nucleate microtubules from chromosomes without centrosomes, whereas mitotic cells lacking chromosomes can still form spindles (Faruki et al., 2002). Finally, it is also possible that the lack of a spindle assembly

(B) Microtubules assembled on chromatin beads in control extract, ΔIncenp extract, ΔMCAK extract, ΔIncenpΔMCAK extract, ΔMCAK extract supplemented with 10 μg/ml 6His-MCAK, and ΔIncenpΔMCAK extract supplemented with 10 μg/ml 6His-MCAK. DNA-coated beads were incubated in extracts at 15.5°C for 60 min and were visualized with Hoechst 33258 (red) in the presence of rhodamine-labeled tubulin (green). Scale bar, 10 μm.

(C) Quantitation of microtubule structures assembled on chromatin beads as described in (B). Structures associated with bead aggregates consisting of more than six beads were scored. Classification, "Beads with MT," chromatin beads are associated with any visible microtubule fibers (including those beads associated with bipolar spindles); "Beads on spindle," chromatin beads are associated with a bipolar spindle; and "Beads w/o MT," chromatin beads are not associated with any visible microtubule fibers.



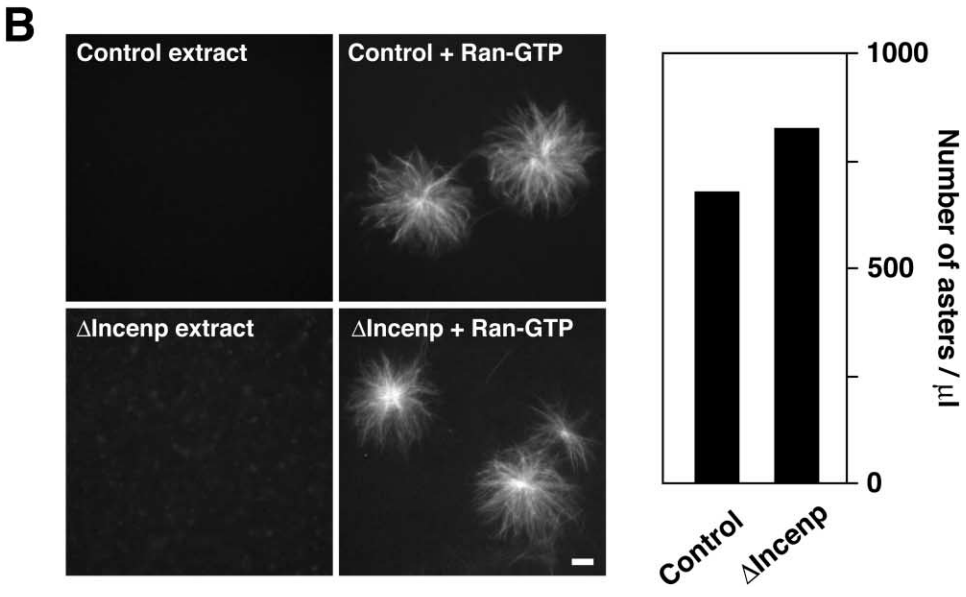


Figure 7. Ran-GTP Nucleates Microtubules in ΔIncenp Extract

(A) Chromatin-induced microtubule nucleation in ΔIncenpΔMCAK extract depends on the Ran-GTP pathway. Spindle formation was monitored by time-lapse video microscopy immediately after placing ΔIncenpΔMCAK or ΔMCAK extracts containing DNA-coated beads and rhodamine-labeled tubulin at 20°C, with or without 7 μM RanT24N protein (a gift of E. Arias and J. Walter). Time is in minutes after the beginning of image acquisition. The first images were taken 4 min after placing the ice-cold extracts on slides at 20°C. Scale bar, 10 μm.

(B) Metaphase control or ΔIncenp extracts were incubated with or without 25 μM GTPase-defective Ran-G19V/Q69L (loaded with GTP; a gift of E. Coutavas and J. Gaetz) for 20 min at 15.5°C. Microtubules were visualized with rhodamine-labeled tubulin. Scale bar, 10 μm. (Right) Quantitation of asters induced by GTP-loaded Ran-G19V/Q69L. The number of asters in whole slides was counted after 1 μl of each extract was mounted on a slide with Fix solution.

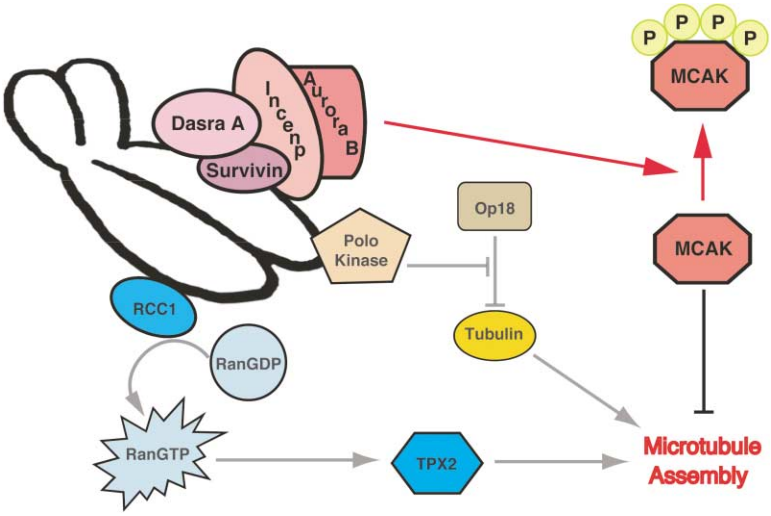


Figure 8. A Model for Chromatin-Induced Microtubule Assembly

defect in somatic cells is due to insufficient hDasra B knockdown, which may, however, be sufficient to cause chromosome misalignment.

Pathways Promoting Chromatin-Induced Microtubule Assembly

In addition to the chromosomal passenger complex-dependent pathway described here, at least two other mechanisms contribute to the nucleation of microtubules around chromatin: the RCC1/Ran-GTP system and Polo-like kinase activity (reviewed in Karsenti and Vernos [2001]). Three lines of evidence argue that the chromosomal passenger complex can stabilize microtubules through a Ran-independent mechanism. First, both Ran and RCC1 are properly loaded onto metaphase chromosomes in the absence of the chromosomal passenger complex. Second, Ran-GTP is able to induce microtubule aster formation in ΔIncenp extracts, demonstrating that the complex is not required downstream of Ran-GTP. Finally, microtubule nucleation in ΔIncenp-ΔMCAK extracts can be inhibited by the addition of dominant-negative Ran and is therefore Ran dependent but Aurora B independent. Though Aurora B, like Cdc2 (Li and Zheng, 2004), may also serve in part to activate the Ran pathway, this latter finding strongly suggests that Aurora B is not essential per se for the generation of chromosomal Ran-GTP. Thus, we support a model in which the microtubule-stabilizing effect of Aurora B is achieved through the regulation of other factors, including but not limited to MCAK.

While MCAK is the major microtubule-depolymerizing factor in egg extract (Tournebize et al., 2000), we demonstrated that, in the presence of dominant-negative Ran, depletion of MCAK is not sufficient to support chromatin-induced microtubule nucleation. Without the Ran-dependent activation of MAPs, such as TPX2 and NuMA, depletion of MCAK may not be sufficient to stabilize microtubules. Alternatively, Ran-GTP might promote the production of "seeds" for microtubule nucleation around chromatin in a process independent of the regulation of microtubule dynamic instability by molecules such as MCAK. This latter model may fit well with the fact that RanT24N does not change the various parameters of microtubule dynamics in *Xenopus* egg extracts (Carazo-Salas et al., 2001).

A chromosomal Polo-like kinase (Plx1 in *Xenopus*) is also required for chromatin-induced microtubule assembly in *Xenopus* egg extracts (Budde et al., 2001). Budde and colleagues proposed that the effect of Plx1 is through phosphorylation of Op18/Stathmin, a protein known to contribute to destabilization of microtubules in M phase, possibly by sequestering tubulin heterodimers. Microtubule assembly may be promoted around chromosomes by increasing the local concentration of tubulin, which can be accomplished by releasing tubulin dimers from Op18 upon phosphorylation by chromosomal Plx1 (Budde et al., 2001).

Taken together, we propose that chromatin-induced microtubule stabilization requires at least three pathways (Figure 8). First, the effective concentration of tubulin heterodimers around chromosomes must be increased by Plx1-mediated release of tubulin from Op18. Second, Ran-GTP must be generated in order to activate microtubule-associated proteins, which serve to nucleate and/or stabilize microtubules. Third, the chromosomal passenger complex must act near chromosomes to inhibit MCAK-dependent microtubule depolymerization. Working in concert, these mechanisms could initiate the earliest steps of bipolar spindle formation around chromatin.

Experimental Procedures

See Supplemental Data available on the *Cell* website for detailed descriptions and additional methods.

Frog Egg Extracts

Meiotic metaphase II-arrested (CSF) *Xenopus laevis* egg extracts were used (Murray, 1991).

Chromosome Binding Screen

An expression screening method described previously (Funabiki and Murray, 2000) was followed with modifications, utilizing a full-length, normalized *Xenopus laevis* egg cDNA library (Carninci et al., 2000) built in a modified pCS2 expression vector.

To purify chromosomes, extracts containing biotinylated chromosomes were mixed with 540 μl of Dilution Buffer 2 (DB2; 10 mM K-HEPES [pH 7.7], 50 mM β-glycerophosphate, 50 mM NaF, 20 mM

EGTA, 2 mM EDTA, 0.5 mM spermine, 1 mM PMSF, 200 mM sucrose, 10 μg/ml LPC [leupeptin, pepstatin, chymostatin]), layered over 60SDB2 (DB2 with 60% w/v sucrose), and spun for 30 min in a refrigerated microcentrifuge. This crude chromosome preparation was incubated with 15 μl Dynabeads M-280 streptavidin (Dynal) for 1 hr at 4°C, and the beads were recovered using a magnetic particle separator. Copurifying labeled proteins were resolved by SDS-PAGE, followed by exposure to a PhosphorImager screen (Fujifilm BAS-2500).

Live Imaging of GFP Fusion Proteins

pSCS012 and pSCS021, encoding xDasra A-GFP and xDasra B-GFP, respectively, were in vitro transcribed using the mMessage mMachine SP6 kit (Ambion). pAFS210, encoding GFP only, was used as a negative control (a gift of A. Straight). For each spindle assembly reaction, 1 μl of mRNA was added to 30 μl of *Xenopus* CSF extract containing sperm nuclei (400/μl), and the extract was cycled through interphase to metaphase, when 0.05 μg/ml DAPI and ~100 nM rhodamine tubulin were added. To induce anaphase, 0.5 mM calcium chloride was added, and 3.5 μl of extract was squashed under a coverslip and sealed with Valap (Desai et al., 1999). Chromosomes, GFP-fusion protein, and microtubules were imaged by time-lapse microscopy using a Carl Zeiss Axioplan 2 microscope equipped with a Photometrics CoolSnap HQ cooled CCD camera and controlled by MetaMorph software (Universal Imaging).

Generation of Peptide Antibodies

Methods previously described (Field et al., 1998) were followed. Peptides corresponding to the C termini of xDasra A (CAKASI QHHGNTL), xIncenp (CSNRHHLAVGYGLKY), and hDasra B (SNRLA QICSSIRTHK) were synthesized and conjugated to hemocyanin (Sigma), and polyclonal antibodies were raised in rabbits (Cocalico Biologicals). Antibodies were affinity purified using SulfoLink Coupling Gel (Pierce) according to the manufacturer's directions.

Immunodepletion from *Xenopus* Egg Extracts

For each 50 μl immunodepletion reaction, 50 μl of Protein A-Dynabeads (Dynal) were conjugated to either 5 μg affinity-purified anti-xDasra A, anti-xIncenp, or control rabbit IgG or to 12.5 μg anti-MCAK (Ohi et al., 2004). Extracts were depleted in 50 μl aliquots in 0.6 ml microcentrifuge tubes for 2 hr on ice.

All incubations for spindle assembly using immunodepleted egg extracts were carried out at exactly 15.5°C, and manipulations were carried out at 20°C. For spindle assembly on replicated chromosomes, 40 μl of immunodepleted egg extract containing sperm nuclei (400/μl) and 0.3 mM calcium chloride were incubated for 120 min at 15.5°C to prepare depleted interphase extracts. Metaphase-depleted spindles were prepared by adding 9 μl of this interphase extract to 27 μl of fresh immunodepleted extract, followed by incubation at 15.5°C for 70 min. To score depletion phenotypes, 1 μl of extract was placed on a slide, 3 μl of Fix (Murray, 1991) was added, and an 18 × 18 mm coverslip was placed on top.

For spindle assembly on chromatin beads, 2 μl of plasmid-coated beads (Heald et al., 1998) was added to 66 μl of depleted extract containing cycloheximide and rhodamine-tubulin and was incubated for 2 hr at 15.5°C after adding 0.3 mM calcium chloride. To induce M phase, 24 μg/ml cyclin BΔ90 and 33 μl of depleted extract were added, and the extract was incubated for 30 min at 15.5°C. The beads were then retrieved and resuspended in 100 μl of fresh depleted extract. To monitor spindle assembly, 20 μl of the bead suspension was incubated for 60 min at 15.5°C, or 2.5 μl was used for live imaging at 20°C.

Immunofluorescence Microscopy

Spindles assembled in *Xenopus* egg extract were processed for immunofluorescence as described (Desai et al., 1999; Funabiki and Murray, 2000). Affinity-purified anti-xDasra A and anti-xIncenp antibodies were used at 1 μg/ml in AbDil (TBS/0.1% Triton X-100 + 2% BSA) for 1 hr and were visualized with FITC- or X-rhodamine-conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch).

For immunofluorescence on mammalian cells, HeLa cells grown on coverslips were fixed with methanol, permeabilized in TBS + 0.5% Triton X-100, and blocked in AbDil. Affinity-purified anti-hDasra B, anti-AIM-1 (BD Biosciences), and anti-hSurvivin (R & D Systems) were used at 1 μg/ml and anti-α-tubulin (DM1, Sigma) at 1:1000, all after dilution in AbDil. Each image is a maximum projection derived from 0.1 μm serial sections through the cell.

Cell Culture and siRNA Treatment

HeLa cells were passaged in DMEM/10% FBS with penicillin and streptomycin at 37°C/5% CO_2. siRNAs were used at 200 nM and were transfected using Oligofectamine (Invitrogen). Target sequences were the following: hDasra B (5′-AAAGGUCAAGCCGUG CUAACA-3′) and EGFP (5′-AAGACGUAAACGGCCACAAGUUC-3′). After 24–36 hr, cells were treated with 20 μM MG132 (Peptides International) for 2 hr at 37°C, fixed, and analyzed by immunofluorescence. A mitotic cell was scored as "misaligned" if at least one chromosome was visibly separated from the metaphase plate or if the chromosome mass extended either throughout the interpolar region or beyond the spindle poles.

M Phase Arrest and Immunoprecipitation

HeLa cells were arrested with 2 mM thymidine for 18 hr, released into fresh medium for 3–4 hr, and blocked with 100 ng/ml nocodazole for 10–12 hr. Mitotic cells were shaken off, washed, transferred into fresh media for 1 hr, and lysed by incubation in NP-40 lysis buffer (0.5% NP-40, 20 mM Tris-HCl [pH 7.4], 500 mM NaCl, 0.5 mM EGTA, 10 mM β-glycerophosphate, 10 μg/ml LPC, 1 mM PMSF) on ice for 20 min. Precleared lysates were immunoprecipitated with 10 μg of control IgG or affinity-purified anti-hDasra B antibodies.

Western Blots

Immunoblots were blocked with PBS/4% nonfat dry milk. Primary and secondary antibodies were used at the following concentrations: 1 μg/ml anti-Dasra A, 1 μg/ml anti-Incenp, 1 μg/ml anti-Aurora B and anti-Survivin (gifts of T. Hirano), 1 μg/ml anti-hDasra B, 2 μg/ml anti-XMAP215 (a gift of M. Shirasu-Hiza), 1 μg/ml anti-AIM1 (BD Biosciences), 1 μg/ml anti-hSurvivin (R & D Systems), and 1:5000 anti-α-tubulin (DM1, Sigma). Antibodies were detected using either ECL or ECL-Plus (Amersham).

Sucrose Density Gradient Sedimentation

High-speed supernatant of CSF extract (95 μl) was loaded onto a 5 ml 5%–40% continuous sucrose gradient and spun for 15 hr at 36,000 rpm in a rotor (SW55Ti; Beckman) at 4°C. Fractions (192 μl) were collected from each gradient.

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Accession Numbers

The cDNA sequences for xDasra A and xDasra B have been deposited in GenBank with accession numbers AY644400 and AY644401, respectively.

Note Added in Proof

After acceptance of this manuscript, we learned that Gassmann et al. independently identified hDasra B, referred to as Borealin in their study (Gassmann, R., Carvalho, A., Henzing, A.J., Ruchaud, S., Hudson, D.F., Honda, R., Nigg, E.A., Gerloff, D.L., and Earnshaw, W.C. [2004]. Borealin: A novel chromosomal passenger required for stability of the bipolar mitotic spindle. J. Cell Biol., in press.).