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Control of Chromosome Segregation by the Aurora B Complex

A Thesis Presented to the Faculty of

The Rockefeller University

in Partial Fulfillment of the Requirements for

the degree of Doctor of Philosophy

by

Srinath Cidambi Sampath

June 2006

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ABSTRACT

Chromosome segregation during cell division requires spindle assembly around M-phase chromatin. In cells lacking centrosomes, such as those found in female meiosis, chromosomes themselves nucleate and stabilize microtubules in order to promote accurate spindle formation. Here we present a description of the composition and function of the vertebrate chromosomal passenger complex (CPC), known to include Incenp, Survivin, and the kinase Aurora B. We report the identification of Dasra A and Dasra B as two new components of the vertebrate CPC, and demonstrate that the CPC is required for chromatin-dependent spindle formation in *Xenopus* egg extracts. The failure of microtubule stabilization caused by depletion of the chromosomal passenger complex is rescued by codepletion of the microtubule-depolymerizing kinesin MCAK, whose activity is negatively regulated by Aurora B. We demonstrate that the Aurora B pathway is normally suppressed in the cytosol, but becomes activated by chromatin and centrosomes, leading to the phosphorylation of both histone H3 and the microtubule destabilizing protein Op18/Stathmin. Chromatin-mediated CPC activation and spindle assembly require Dasra protein-dependent chromatin binding by the CPC, but this function of Dasra proteins can be bypassed by adding anti-Incenp antibodies, which autonomously stimulate Aurora B pathway activity. Such inappropriate CPC activation leads to the formation of centrosomal spindles lacking chromosomes. These results demonstrate that Dasra proteins make the Aurora B pathway competent for chromatin-dependent activation, and provide a mechanism for the spatial regulation of spindle assembly.

That which seems like poison at first, but tastes like nectar in the end—that is the joy of sattva, born of a mind at peace with itself.

- *Bhagavad-Gita*, 18:37

ACKNOWLEDGEMENTS

It goes without saying that the work contained in this thesis would not have been possible without the support of my mentor, Hiro Funabiki. There is absolutely no doubt that Hiro's arrival at Rockefeller rescued my scientific career, and despite having worked in many labs before we met, my development as a scientist dates from that event. It was only by trying to emulate his many positive attributes that I developed whatever skills I now have. I already look back fondly on the time we spent working together, especially those early days in Founders' Hall, spent drinking espresso, talking incessantly, and doing wild experiments just for the fun of it. I will certainly remember it as one of the most challenging and personally fulfilling periods of my life.

I'm fortunate enough to have had two other outstanding mentors in my brief academic career. Sidney Orlov has acted as my informal advisor and advocate ever since my days in Ithaca. Her support has been invaluable, and her continual admonishment to dream big was a source of inspiration for me. My undergraduate lab mentor, Peter Bruns, was also instrumental in encouraging my love of science during a formative period of my training. He was a true role model, both as a scientist and as a gentleman.

I gratefully acknowledge my thesis committee (Drs. David Allis, Tarun Kapoor, and Prasad Jallepalli) for their thoughtful input throughout the course of our work. Their suggestions improved this thesis substantially, and led to me think more substantively about the strengths and weaknesses of the story taken as a whole. I also thank those whose groups kindly provided reagents (Rebecca Heald, Tatsuya Hirano, Tarun Kapoor, Kazuhisa Kinoshita, Ryoma 'Puck' Ohi), as well as Puck for an excellent long-term collaboration. Our work would not have been possible without all of their contributions.

Three of my labmates also need to be specially mentioned here. Oliver Leismann and I joined the lab (then a maze of unopened cardboard boxes) almost simultaneously in 2002 as the first postdoc and the first student, respectively. His amazing combination of sociability and humility were a lesson to me, and kept me grounded both when things went wrong and, more importantly, when they went right. Alex Kelly joined the lab after Oliver's departure, and did his best to make up for his absence. I think Alex and I instantly recognized something very familiar in each other; I'm sure he, Hari and I must

have been triplets in a past life (probably the hairiest triplets ever). Thanks go to Alex for being both a literal and figurative shoulder to lean on. And then there is Tapan Maniar, the man who officially put the "stud" in "rotation student", and the "bone" in "T-bone".

Which brings me to the final, and for me most significant, acknowledgements. These go of course to my brother Srihari and to my mother, both of whom have given me such incredible amounts of advice, encouragement, and support, that I am at a loss to know what to say. To paraphrase John Lennon, it's real love.

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CHAPTER 1

Introduction

All eukaryotic organisms face the fundamental challenge of accurately replicating and distributing their genetic material through successive cellular divisions. Failure in this regard can have catastrophic consequences not only for the individual cell, but also for the adult organism. For this reason, as well as due to the dramatic cytological rearrangements seen during cell division, the mechanisms controlling cellular proliferation have long held a special interest [reviewed in (Nasmyth, 2001)]. The study of chromosome segregation in particular has spanned most of the past 130 years, dating back to the initial observations on sister chromatid separation (Flemming, 1879) and the chromosomal basis of development (Boveri, 1888; 1907). Nonetheless it is only in the relatively recent past that meaningful progress has been made toward understanding in molecular detail how the cell and chromosome cycles are accurately and coordinately controlled.

The Logic of Cell Cycle Control

It is now understood that progression through the eukaryotic cell cycle is controlled through the sequential activity of cyclin-dependent kinases (CDKs), which, as implied by their name, are dependent on the presence of cyclin proteins for their function [reviewed in (Nurse, 2000)]. In animal cells, Cdk2 bound to S-phase specific cyclins (Cyclins E and A) acts to promote S-phase progression, whereas Cdk1 binding to M-phase cyclins (Cyclins A and B) allows entry into mitosis. The levels of cyclins themselves are

controlled through temporally restricted expression coupled to alternating rounds of ubiquitin-mediated proteolysis, ensuring that the cell cycle proceeds unidirectionally. Likewise, DNA replication is limited to one round per cycle through the control of pre-replication complex (pre-RC) assembly by CDK (Blow and Dutta, 2005). In higher eukaryotes, protein inhibitors of pre-RC formation also exist which are themselves degraded during anaphase of the preceding cell cycle (McGarry and Kirschner, 1998; Tada et al., 2001). The overall effect of such a system is to maintain an orderly progression of cellular events, culminating in cell division and a reinitiation of the clock. Moreover, since progression to each step of the cycle is largely dependent on the successful completion of the previous step, each transition provides a "checkpoint" to ensure that the genetic material is still intact [reviewed in (Hartwell and Weinert, 1989; Nyberg et al., 2002)].

Microtubule Dynamics and Dynamic Instability

Chromosome segregation occurs during M-phase, and requires the establishment of the spindle, a bipolar structure along which chromosomes segregate during anaphase. The spindle is comprised of microtubules, themselves polymers of αβ tubulin heterodimers [reviewed in (Desai and Mitchison, 1997)]. Tubulin dimers spontaneously assemble into protofilaments by longitudinal association, and each microtubule consists of a hollow ~25 nm tube formed by lateral association of approximately 13 tubulin protofilaments. The 'head-to-tail' orientation of α- and β-tubulin within each protofilament generates a natural polarity to microtubules, in which one end terminates in α-tubulin subunits, while the other terminates in β-tubulin subunits. By convention, the

former is referred to as the minus end (slow growing) and the latter as the plus end (fast growing).

Microtubules are dynamic structures, a property which is thought to aid in the capture of chromosomes during M-phase. The behavior exhibited by microtubules *in vivo* has been termed dynamic instability (Mitchison and Kirschner, 1984), which refers to the manner in which microtubules stochastically transition between phases of growth and shrinkage. According to this model, the dynamic properties of microtubules can be described by four parameters: growth rate, shrinkage rate, catastrophe frequency (the frequency of transitions from growth to shrinkage per unit time), and rescue frequency (the frequency of transitions from shrinkage to growth per unit time). GTP-bound tubulin dimers are added onto the growing microtubule end during the growth phase, which is however also associated with GTP hydrolysis by β-tubulin within the microtubule lattice (David-Pfeuty et al., 1977); it is the instability introduced into the lattice by GTP hydrolysis by β-tubulin that makes dynamicity possible. During shrinkage, free GDP-bound dimers or oligomers are released, at which point GTP can be exchanged for GDP, and the cycle can repeat (Figure 1-1).

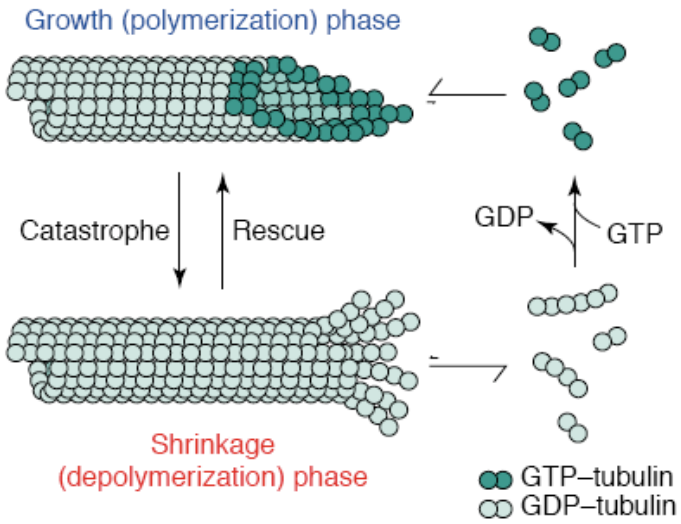

Figure 1-1. Mechanics of Microtubule Growth and Shrinkage
During microtubule growth, GTP-tubulin dimers are added to the growing end as GTP is hydrolyzed by β-tubulin within the microtubule lattice, while during shrinkage GDP-tubulin is released from shrinking ends. Reproduced from Kinoshita et al., 2002.

The values of the parameters of microtubule dynamics can be adjusted by numerous factors, including binding by Microtubule Associate Proteins (MAPs). For instance, it has been shown that XMAP215, a well-characterized MAP from *Xenopus*, can stimulate the growth rate of microtubules 8-fold (Gard and Kirschner, 1987), and also acts to inhibit catastrophes (Tournebize et al., 2000). The parameters of dynamic instability can also be modulated by the activity of members of the kinesin family of microtubule motor proteins, in particular the 'KinI' family, which contain an internal motor domain and seem to act as microtubule catastrophe promoting factors (Desai et al., 1999b). In addition, the oncoprotein 18 (Op18)/stathmin protein has been described to promote catastrophe (Belmont and Mitchison, 1996; Howell et al., 1999b), potentially by sequestration of tubulin dimers.

Pathways of Spindle Formation: Astral Spindle Assembly

Spindle formation is thought to occur by at least two pathways, one of which relies on the self-assembly of microtubules around chromatin during M-phase, and the other of which is organized by microtubule nucleation from centrosomes [reviewed in (Karsenti and Vernos, 2001)]. These pathways have been referred to as 'anastral' and 'astral', respectively, since they differ in their reliance on the astral microtubules nucleated by centrosomes (Varmark, 2004). In metazoa, the anastral chromatin-dependent pathway is thought to dominate during female meiosis (in which centrosomes are absent) and in higher plants. Conversely, the astral pathway plays a major role during somatic cell divisions in animals.

The astral pathway of spindle assembly is largely controlled by centrosomes in their capacity as microtubule organizing centers (MTOCs). Centrosomes are paired organelles each consisting of a pair of centrioles embedded in a proteinaceous matrix referred to as the pericentriolar material (PCM). During mitosis, one centrosome is typically found at each spindle pole, such that each daughter cell inherits one centrosome after cell division; this centrosome is then duplicated during S-phase of the next cell cycle, concomitant with DNA replication (Compton, 2000). As cells enter mitosis, the centrosomes separate and begin to nucleate highly dynamic astral microtubules. This increase in dynamicity is at least partly due to a dramatic increase in the catastrophe rate of microtubules (Inoue and Salmon, 1995). It has been suggested that the increase in dynamicity could provide a mechanism underlying a 'search-and-capture' mechanism for chromosome attachment to the mitotic spindle (Holy and Leibler, 1994; Kirschner and Mitchison, 1986). In this model, microtubules are proposed to randomly search space until they come in contact

with kinetochores, the proteinaceous structures formed on the centromeres of chromosomes during cell division, and which are required for spindle attachment; interaction between these 'kinetochore fiber' (K-fiber) microtubules and kinetochores would then stabilize K-fibers [see Figure 1-2; (Mitchison et al., 1986; Mitchison and Kirschner, 1985)]. Such a mechanism would require that microtubules be sufficiently dynamic to efficiently search for chromosomes throughout all of the volume of a mitotic cell.

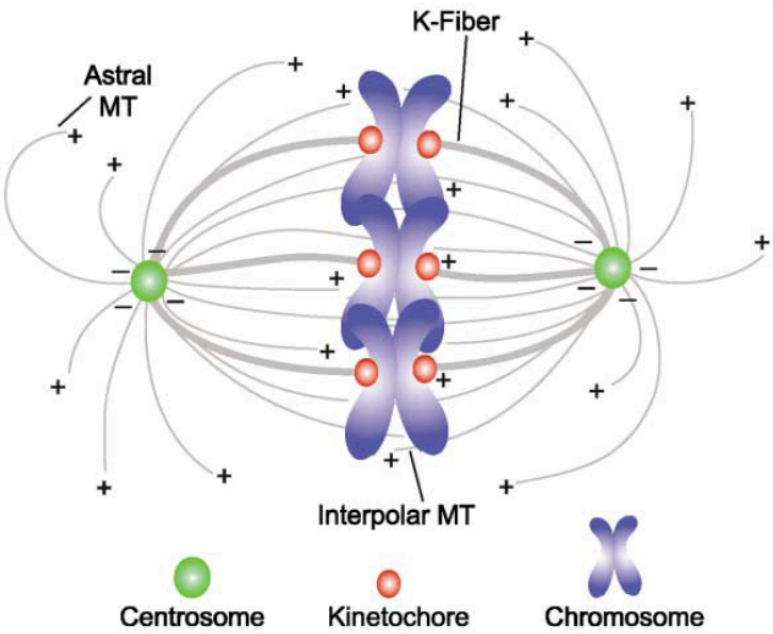

Figure 1-2. Structure of the Mitotic Spindle During Astral Spindle Assembly
Astral spindles are proposed to form by 'search-and-capture' mechanisms, in which dynamic plus-ends of astral microtubules nucleated from centrosomes stochastically search space, becoming stabilized on binding to kinetochores.

Chromosome capture by astral microtubules should usually lead to monopolar orientation of the chromosome, as it is extremely unlikely that a replicated chromosome will simultaneously attach each sister chromatid correctly. Monopolar orientation is

usually followed by poleward movement of the chromosome, which however eventually becomes properly orientated after capture of the previously unattached sister chromatid by microtubules emanating from the opposite spindle pole [reviewed in (Compton, 2000)]. Cells have developed elaborate mechanisms to ensure that anaphase is delayed until all chromosomes have become properly captured and oriented in this way (see below).

The microtubule nucleating capacity of centrosomes is crucial to their function in astral spindle formation. As mitosis initiates, centrosomes undergo a process termed maturation, in which factors important for microtubule nucleation are progressively accumulated into the PCM. These proteins include XMAP215 and the γ-tubulin ring complex (γTuRC), the latter being comprised of a tubulin variant which is involved in the initial stages of microtubule nucleation from centrosomes (Oakley and Oakley, 1989; Zheng et al., 1995). The process of centrosome maturation requires the function of the Aurora A kinase, which functions in multiple capacities to promote centrosome function. These include phosphorylation of transforming acidic coiled-coil (TACC) proteins, which has been demonstrated to recruit TACC proteins to centrosomes and promote their interaction with XMAP215. In turn, centrosomal XMAP215 promotes microtubule stability at nascent plus ends, allowing microtubule growth (Barros et al., 2005; Kinoshita et al., 2005). Consistent with these findings, loss of Aurora A leads to decreased γ-tubulin and XMAP215 accumulation, diminished centrosomal microtubule density and length, and abnormal centriole numbers (Berdnik and Knoblich, 2002; Giet et al., 2002; Gietz and Sugino, 1988; Hannak et al., 2001; Terada et al., 2003).

Pathways of Spindle Formation: Anastral Spindle Assembly

Recently at least three major pathways have been found to be essential for the chromatin-dependent pathway of spindle assembly. One of these involves Ran, a small GTPase which has been intensively studied due to its important role in nucleocytoplasmic shuttling during interphase [reviewed in (Harel and Forbes, 2004)]. It is now clear, however, that Ran also has a crucial function in cell division.

Ran controls nucleocytoplasmic shuttling by regulation of a group of nuclear transport proteins known as karyopherins, which includes molecules involved in nuclear import (importins) and others involved in nuclear export [exportins; (Weis, 2002)]. Importins themselves can be further subclassified into the α and β families, which regulate import by binding to the nuclear localization signal (NLS) of proteins destined for the nucleus. Binding of Ran-GTP to importins causes the release of their associated cargo, which therefore can become locally concentrated. During interphase, the levels of Importin-associated cargoes in the nucleus rises due to the action of RCC1, the guanine nucleotide exchange factor for Ran (Bischoff and Ponstingl, 1991; Kalab et al., 2002; Zheng, 2004). Since RCC1 itself binds histones (Nemergut et al., 2001; Ohtsubo et al., 1989), and is activated in the presence of chromatin (Nemergut et al., 2001), Ran-GTP levels become elevated within the nucleus. Moreover, the localization of RanGAP (the GTPase activating protein for Ran) to the cytoplasm ensures the directionality of cargo transport (Figure 1-3).

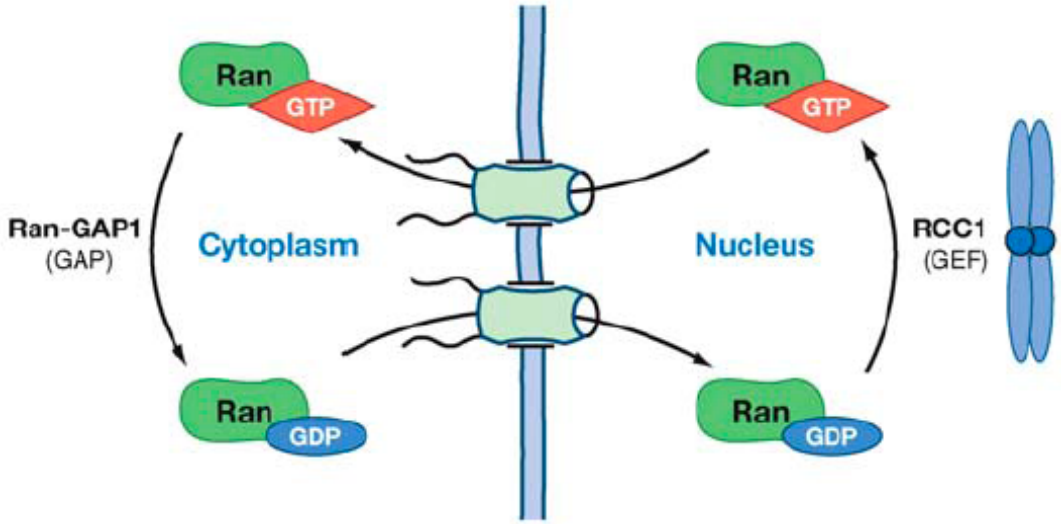

Figure 1-3. Production of a Nucleocytoplasmic Ran-GTP Gradient by RCC1 and RanGAP
Nuclear Ran-GTP levels are maintained at high levels by the action of chromatin-associated RCC1, which catalyzes GTP loading onto Ran, and cytoplasmic RanGAP, which promotes GTP hydrolysis by Ran. Reproduced from (Zheng, 2004).

As cells enter mitosis, the concentration of Ran-GTP in the nucleus leads to a locally high level of Ran-GTP in the vicinity of mitotic chromosomes; this enrichment has been directly visualized in both *Xenopus* egg extract (Kalab et al., 2002) and in mammalian cells (Li and Zheng, 2004a; Li and Zheng, 2004b). The high levels of chromatin-proximal Ran-GTP then lead to the release of 'spindle assembly factors' (SAFs), NLS-containing proteins bound to the Importins which function in assembly of the spindle during M-phase; such proteins include NuMA, TPX2, and XCTK2 [Figure 1-4; (Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001)]. These proteins play multiple roles in spindle assembly, including important functions in microtubule nucleation and spindle pole organization (Garrett et al., 2002; Gruss et al., 2001; Merdes et al., 2000; Merdes et al., 1996).

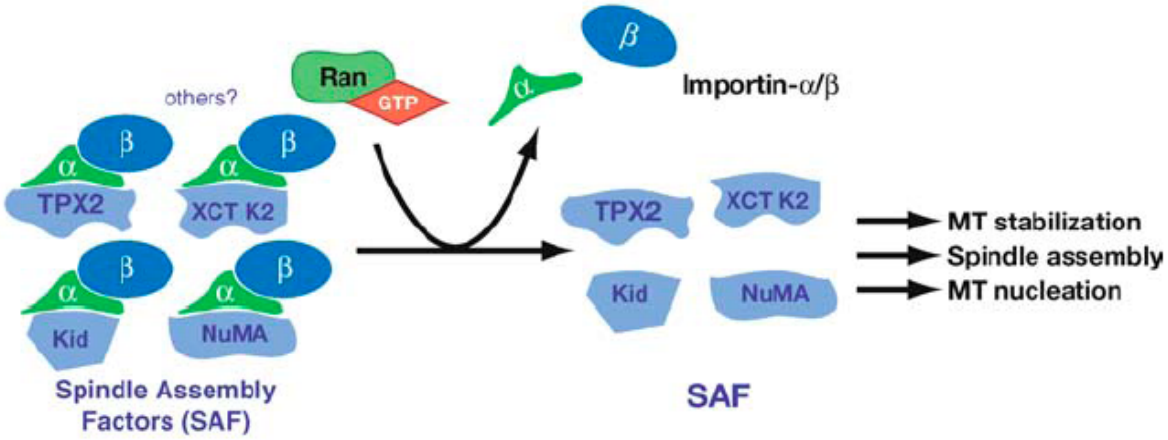

Figure 1-4. Ran-GTP Promotes Spindle Assembly Through the Release of Importin-bound Spindle Assembly Factors
Ran-GTP binding induces the release of Spindle Assembly Factors (SAFs) from their inhibitory binding to Importin α/β. Reproduced from Zheng, 2004.

Interestingly, when the upstream components of this pathway are bypassed in *Xenopus* egg extract by the direct introduction of non-hydrolysable Ran-GTP, bipolar spindles and asters are formed in the complete absence of centrosomes and chromosomes (Carazo-Salas et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999). Accordingly, it has been observed that Ran-GTP itself affects microtubule dynamics, inducing a ~3-fold increase in microtubule rescue frequency in *Xenopus* egg extract (Carazo-Salas et al., 2001; Wilde et al., 2001). Moreover, Ran-GTP increases the microtubule-nucleating activity of centrosomes (Carazo-Salas et al., 2001), and has been proposed to the source of the long-range communication described to occur between chromatin and centrosomes (Carazo-Salas and Karsenti, 2003). Ran has therefore emerged as a central player in multiple aspects of spindle formation, and is likely to play key roles both on chromosome arms, kinetochores (Joseph et al., 2002), and at centrosomes.

It has recently been proposed that the chromosomal Polo-like kinase Plx1 controls another pathway required for chromatin-dependent microtubule assembly (Budde et al.,

2001). In particular, it was found that depletion of Plx1from *Xenopus* egg extracts causes formation of aberrant spindle structures having reduced microtubule density around M-phase chromatin; interestingly, it was demonstrated that this effect of Plx1 depletion coincides with reduced phosphorylation of Op18/Stathmin, a small tubulin-binding protein previously described to be overexpressed in some types of tumors (Budde et al., 2001; Curmi et al., 2000).

Op18 exhibits an extremely complex phosphorylation pattern, and during M-phase becomes phosphorylated on three sites in a manner sensitive to chromatin and/or microtubule stabilization (Andersen et al., 1997; Kuntziger et al., 2001). Although the protein has no reported enzymatic activity, phosphorylation has been reported to antagonize an intrinsic microtubule destabilizing activity of Op18, such that introduction of non-phosphorylatable mutants perturbs microtubule stabilization and spindle formation (Andersen et al., 1997; Budde et al., 2001; Marklund et al., 1996). The actual mechanism by which Op18 destabilizes microtubules is controversial; both tubulin sequestration and direct catastrophe promotion have been proposed and supported by experimental evidence (Belmont and Mitchison, 1996; Howell et al., 1999a; Howell et al., 1999b; Larsson et al., 1997). Regardless of the downstream mechanism however, it is thought that chromatin-derived signals lead to Op18 phosphorylation and inhibition, allowing localized microtubule stabilization in the vicinity of chromosomes during M-phase (Figure 1-5). The question of whether Plx1 represents the only or even the main chromatin-dependent inhibitor of Op18-mediated microtubule destabilization currently remains unanswered.

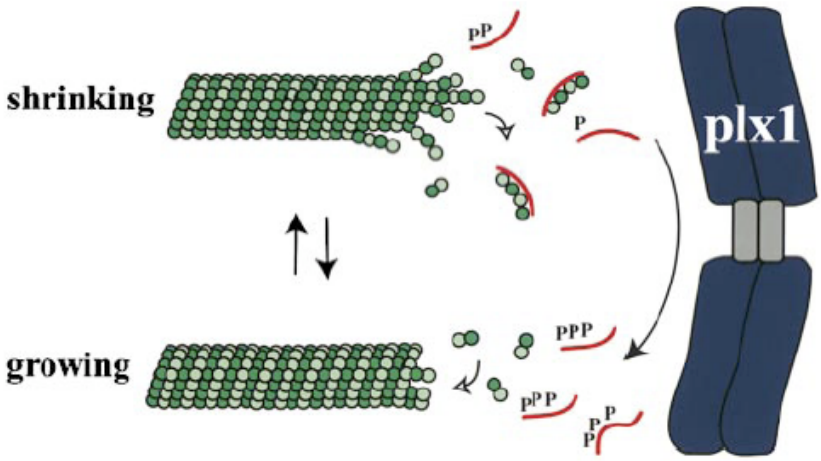

Figure 1-5. A Model For Chromatin-Dependent Inactivation of Op18/Stathmin
Chromatin-associated kinases, such as the *Xenopus* Polo-like kinase Plx1 are proposed to phosphorylate and inactivate the microtubule destabilizing activity of Op18/Stathmin (red line), allowing chromatin-dependent spindle assembly. Reproduced from Budde et al., 2001.

We recently described a third pathway required for chromatin-dependent spindle formation, which involves an evolutionarily conserved group of proteins referred to as the 'chromosomal passenger complex' [CPC; (Sampath et al., 2004)]. These proteins, which in higher eukaryotes include Aurora B, Incenp, Dasra A/B, and Survivin, form a stable complex, and share a dynamic localization pattern throughout M-phase (Bolton et al., 2002; Carmena and Earnshaw, 2003; Losada et al., 2002). Incenp (inner centromere protein) was originally isolated in a biochemical screen for antigens derived from purified 'chromosomal scaffold' preparations (Cooke et al., 1987), and was notable at the time for its striking relocalization from inner centromeres at metaphase to the spindle midzone at anaphase . In contrast, the budding yeast homolog of Aurora kinase family, Ipl1, was identified genetically as causing increased ploidy when mutated (Chan and Botstein, 1993). The appreciation of the functional interrelatedness of the passenger proteins came when it was found that all shared the same distinct localization patterns as Incenp, and

were interdependent for their correct localization (Adams et al., 2000; Kaitna et al., 2000). In addition to those previously mentioned, several other proteins have been suggested to behave as chromosomal passenger proteins. For instance, the recently discovered protein TD-60 has been reported to colocalize with the CPC, and is required for Aurora B and Survivin localization in mammalian cells (Mollinari et al., 2003). Likewise, the ICIS protein was isolated as a microtubule-binding protein which localizes to inner centromeres, physically interacts with the CPC, and activates the microtubule depolymerase MCAK, a member of the KinI family of kinesins (Ohi et al., 2003). Orc6, a component of the origin recognition complex (ORC), has also been proposed to serve as a chromosomal passenger protein, a speculation based largely on its distinctive CPC-type localization pattern during mitosis (Prasanth et al., 2002).

From numerous studies involving loss of function of individual CPC components, it has become clear that this complex has important roles in promoting proper chromosome orientation, spindle formation, spindle checkpoint signaling, and cytokinesis (Ditchfield et al., 2003; Hauf et al., 2003; Kaitna et al., 2000; Lampson et al., 2004; Mackay et al., 1998; Sampath et al., 2004; Tanaka et al., 2002). Nevertheless the question of how Aurora B can be regulated spatially and otherwise to impinge on such a diverse set of processes remains unanswered. Such regulation might be achieved through the function of other CPC components, as it has been reported that the C-terminal 'IN-box' of Incenp can allosterically activate Aurora B kinase activity (Honda et al., 2003; Sessa et al., 2005). Likewise, little is currently known regarding the Aurora B downstream effector proteins.

With regard to spindle formation, a major recent advance was the finding that Aurora B could phosphorylate MCAK, thus inhibiting its activity (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004). This finding was important given the previously described roles of MCAK in spindle formation, chromosome alignment and orientation, and regulation of microtubule dynamics (Kline-Smith et al., 2004; Ohi et al., 2004; Walczak et al., 2002; Walczak et al., 1996). We have proposed a model in which spindle formation around M-phase chromatin in *Xenopus* egg extract is regulated partly by Aurora B-dependent MCAK phosphorylation (Sampath et al., 2004).

In addition to microtubule nucleation and stabilization by the pathways discussed above, spindle formation also requires a balance of forces provided by the activity of both microtubule plus- and minus-end directed motor proteins [reviewed in (Compton, 2000; Heald, 2000)]. These proteins have been postulated to promote spindle formation at least in part by cross-linking antiparallel microtubules, thus separating the spindle poles, and by sliding parallel microtubules, leading to pole focusing (Compton, 2000; Nedelec et al., 2003; Walczak et al., 1998). As a result a bipolar structure is formed along which chromosomes can segregate equally.

The M-Phase Spindle Checkpoint

In addition to formation of a bipolar spindle, accurate chromosome segregation also requires correct chromosome orientation, in which each sister chromatid is connected to microtubules emanating from a single spindle pole, and vice versa. Eukaryotic cells have developed elaborate mechanisms to distinguish this 'amphitelic' orientation from malorientations, including 'syntelic' orientation, in which both chromatids attach to a

single pole, and 'merotelic' orientation, in which a single chromatid is attached to both spindle poles. It has been proposed that amphitelic orientations can be detected due to the physical tension that such a configuration induces across the paired centromeres (Nicklas, 1997; Tanaka, 2005). In the absence of tension and/or microtubule occupancy, a spindle checkpoint signal is propagated which causes cells to delay the metaphase-anaphase transition, allowing time for chromosome capture and reorientation (Figure 1-6).

The molecular mechanisms underlying spindle checkpoint activity are under intensive investigation, but several major themes have emerged. Kinetochores form the interface between chromosomes and the spindle, and therefore serve as the platform at which spindle checkpoint signaling occurs. Many of the proteins involved in the checkpoint localize to the kinetochore at least transiently, including Mad1, Mad2, Bub1, Bub3, BubR1, and Mps1, several of which were originally identified in screens for genes involved in the promoting cell cycle arrest in response to spindle poisons (Hoyt et al., 1991; Li and Murray, 1991). In *Xenopus*, recruitment of the Aurora B/Incenp complex to unattached kinetochores seems to be the most upstream component of a signaling cascade, eventually resulting in the recruitment of Mad1/Mad2 (Vigneron et al., 2004). These proteins, acting in concert with Cdc20 and the BubR1 kinase, are thought to mediate inhibition of the Anaphase Promoting Complex (APC), a multisubunit ubiquitin ligase responsible for ubiquitination and degradation of cyclin and securin [reviewed in (Musacchio and Hardwick, 2002)]. Thus, activation of the spindle checkpoint coordinately inhibits both the metaphase-anaphase cell cycle transition and sister chromatin separation (Figure 1-6).

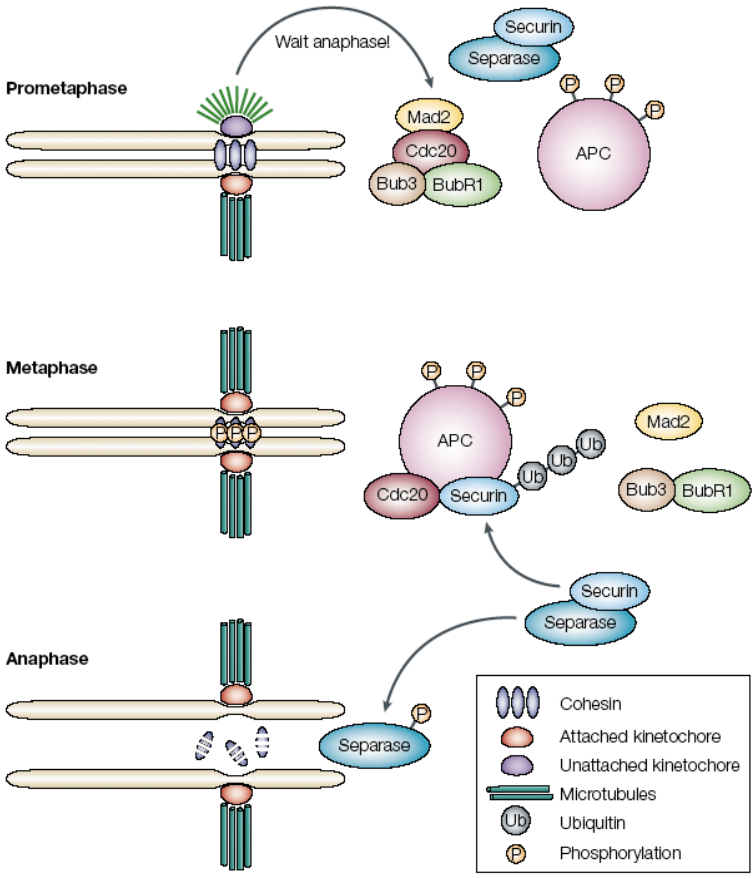

Figure 1-6. Inhibition of Sister Chromatid Separation by the Spindle Checkpoint Unattached kinetochores or the absence of tension activates the spindle checkpoint through the inhibition of the Anaphase Promoting Complex (APC), a ubiquitin ligase responsible for triggering anaphase through the ubiquitination of Securin and cyclins. Reproduced from Musacchio and Hardwick, 2002.

The process of chromosome reorientation likely involves Aurora B-dependent microtubule disassembly (Lampson et al., 2004; Tanaka et al., 2002), and the spindle assembly checkpoint likewise requires CPC function (Gadea and Ruderman, 2005; Vigneron et al., 2004). Given the recently established functional relationship between Aurora B and MCAK, and the similar accumulation of malorientations seen in Aurora B-

and MCAK-deficient cells, much attention is also now being focused on the ways in which the interplay between these two molecules might regulate spindle formation, chromosome orientation, and spindle checkpoint signaling.

Structure and Function of Aurora Kinases

Mammalian genomes encode three related Aurora-family kinases (Aurora A, B, and C), while only Aurora A and B homologs have been identified in frogs and nematodes, and only one Aurora homolog exists in budding and fission yeasts (Brown et al., 2004). All three kinases share a similar domain structure, with an N-terminal regulatory domain, a central kinase domain, and a short C-terminal domain. The N-terminal domain is relatively poorly conserved between the Aurora family members, and is thought to confer specificity in protein-protein interaction (Carmena and Earnshaw, 2003).

As discussed above, Aurora A is a crucial regulator of centrosome maturation, centrosome separation, and cytokinesis. In fact, the *aurora* mutant in *Drosophila* was initially described to cause formation of monopolar spindles, presumably as a result of failed centrosome separation (Glover et al., 1995). Since then, it has become clear that loss of Aurora A function leads to multiple defects in centrosome function, including impaired accumulation of pericentriolar material (Berdnik and Knoblich, 2002; Hannak et al., 2001) and defective astral microtubule formation (Giet et al., 2002).

Aurora A function is tightly regulated, at least in part by binding to the protein TPX2 (targeting protein for Xklp2). It has been demonstrated that during mitosis, chromosomal Ran-GTP promotes the localized release of TPX2 in the vicinity of chromosomes; this released TPX2 can then bind to Aurora A, regulating its localization to centrosomes and

microtubules (Kufer et al., 2002). The catalytic activity of Aurora A is also stimulated on TPX2 binding due to increased autophosphorylation and inhibition of protein phosphatase 1-dependent dephosphoryation (Eyers et al., 2003; Tsai et al., 2003). Interestingly, PP1 was also described to negatively regulate Ipl1 (Francisco et al., 1994), the single yeast Aurora protein, as well as vertebrate Aurora B (Murnion et al., 2001; Sugiyama et al., 2002).

As discussed previously, Aurora B has multiple functions in chromosome orientation, spindle checkpoint signaling, and cytokinesis [reviewed in (Carmena and Earnshaw, 2003)]. Like Aurora A, Aurora B activity and localization are governed by binding to an activating protein, Incenp. Recent structural data indicates that Aurora B becomes activated on binding to a C-terminal region of Incenp, which allosterically induces the active conformation of the Aurora B T loop; phosphorylation of Incenp by Aurora B then promotes full activation of the kinase (Sessa et al., 2005). Despite these insights, it remains unclear how this complex series of events is spatially and temporally regulated, and how it is affected by the presence of other CPC components. Likewise, the extent of functional overlap between Aurora A and Aurora B is currently unclear. For instance, while it is clear that Aurora is essential for histone H3 serine 10 phosphorylation (Giet and Glover, 2001; Murnion et al., 2001), recent data indicates that both kinases are required for serine 7 phosphorylation of Cenp-A, the centromeric H3 variant histone (Kunitoku et al., 2003). Recent data do indicate however that Aurora B in particular is involved in the removal of chromosome-bound heterochromatin protein 1 (HP1) isoforms during mitosis, although the functional relevance of this activity is completely unknown (Fischle et al., 2005; Hirota et al., 2005).

The issue of functional redundancy becomes more complicated in light of the recent discovery of mammalian Aurora C, a protein with extensive structural and sequence similarity to Aurora B. Aurora C can physically associate with CPC components, and its overexpression leads to mitotic defects reminiscent of those seen in CPC-deficient cells (Yan et al., 2005). Moreover, Aurora C is activated by binding to Incenp (Li et al., 2004), and expression of Aurora C can rescue the loss of Aurora B expression (Yan et al., 2005), suggesting the possibility for extensive functional overlap *in vivo*.

Xenopus Egg Extract As a Model For Studying Chromosome Segregation

Cytosolic extracts prepared from unfertilized *Xenopus laevis* eggs have served as a powerful model system in which to study the processes governing chromosome condensation, spindle assembly, spindle checkpoint signaling, and sister chromatid segregation. Since these unfertilized eggs are naturally arrested at metaphase II of meiosis (due to the so-called 'cytostatic factor', or CSF, activity), induction of anaphase results in a quasi-mitotic segregation event which recapitulates many of the aspects of somatic cell mitosis [reviewed in (Desai et al., 1999a; Murray, 1991)].

Addition of sperm nuclei to CSF-arrested extract results in the rapid reorganization of the sperm chromatin into a condensed M-phase configuration without intervening DNA replication. The metaphase-to-anaphase transition is under experimental control, and can be induced simply by adding calcium, which mimics the biochemical events occurring naturally after fertilization. Calcium addition induces cyclin degradation, loss of Cdk activity, and subsequent entry into interphase, which is accompanied by DNA replication. Murray and Kirschner demonstrated that resynthesis of cyclin is sufficient to switch

interphase extract back into an M-phase (high Cdk) state (Murray and Kirschner, 1989; Murray et al., 1989), and therefore addition of either fresh CSF extract or non-degradable cyclin B (cyclin $B^{\Delta 90}$) can be used to induce M-phase entry and chromosome recondensation in interphase extracts. Such 'cycled' extracts contain replicated, paired sister chromatids, and assemble spindles which, upon calcium addition, undergo anaphase A-type chromosome segregation (Funabiki and Murray, 2000). All of these events can be observed by fluorescence microscopy in either fixed or live specimens.

The use of this cell-free system also allows the use of powerful biochemical methodologies. For example, mutant proteins can be added to extracts and their dominant effects can be observed. More importantly, individual proteins and/or their associated protein complexes can be immunodepleted and replaced with mutant forms (Funabiki and Murray, 2000). In addition, interfering antibodies can be added to block endogenous protein function (Ohi et al., 2003). Taken together, these techniques make the *Xenopus* egg extract system an ideal one in which to investigate the complex spatial, temporal, and biochemical events regulating metazoan chromosome segregation.

CHAPTER 2

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

Results

Identification of A Novel Vertebrate Chromosome Binding Protein

The *Xenopus* egg extract system has previously been used to identify proteins which copurify with condensed, M-phase chromosomes (Hirano and Mitchison, 1994). This approach was limited by its ability to identify only those chromosomal proteins which were extremely abundant, and whose identity could be deduced by microsequencing. We set out to identify new vertebrate chromosome-binding proteins using a method not subject to these limitations. Specifically, we undertook an expression screen using *Xenopus* egg extracts, and utilizing a normalized cDNA library derived from *Xenopus laevis* eggs. Individual clones were arrayed into 384-well microtiter plates, and each 384 well plate was then reformatted into four 96 well plates, from which row, column, and plate pools of plasmids were derived (Figure 2-1A). These pools of plasmids were then translated *in vitro* and added to *Xenopus* egg extract containing sperm nuclei, which were cycled through interphase to metaphase in the presence of biotinylated dUTP. The condensed mitotic chromosomes were then partially purified by centrifugation through a sucrose cushion, followed by further purification with streptavidin-coated magnetic beads. Copurifed labeled proteins were examined by SDS-PAGE. Using this method, we identified a protein which bound to purified *Xenopus* sperm chromosomes; this protein was temporarily named $p34^{4B8}$ due to its apparent molecular weight and microtiter plate location (Figure 2-1B).

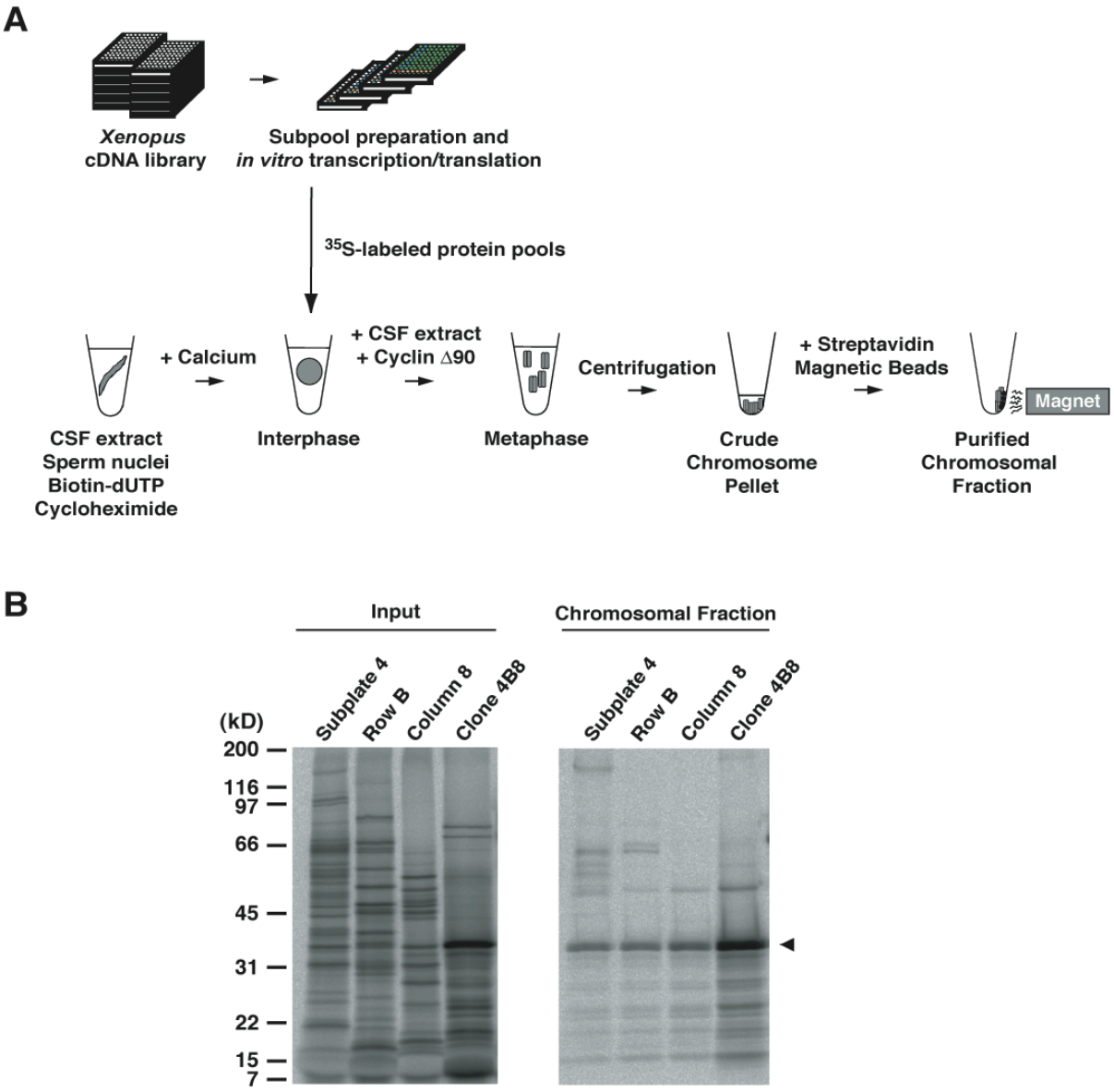

Figure 2-1. A Screen for Metaphase Chromosome-binding Proteins Identifies p34^{4B8}

(A) Schematic of the expression screening method used to screen an arrayed *Xenopus* egg cDNA library for mitotic chromosome-binding proteins. See Methods section for details. (B) Results of pooled expression screening. Each 364-well plate from the library was first reformatted into four 96-well plates; one "subplate" consists of 96 individual clones. Pools of cDNA clones were transcribed and translated in rabbit reticulocyte lysates (as described in Methods). Aliquots of input *in vitro* translations are shown at left, with the fraction co-purifying with mitotic chromosomes shown at right. Arrowhead indicates the position of clone p34^{4B8}.

To investigate the localization of the $p34^{4B8}$ protein, we generated a $p34^{4B8}$-GFP fusion protein, mRNA encoding which was transcribed *in vitro* and added to *Xenopus* egg extract containing sperm nuclei. When the localization of the $p34^{4B8}$-GFP protein was examined by live microscopy, we observed that the protein localized throughout metaphase chromosomes, with enrichment at the primary constriction (Figures 2-2A and 2-2B). Variable localization to telomeres was also noted (data not shown). When calcium was added to induced anaphase onset, we observed that the $p34^{4B8}$-GFP signal became redistributed from metaphase chromosomes to the spindle midzone (Figure 2-2C). This dynamic localization pattern was reminiscent of that of the 'chromosomal passenger' complex, which was known to include the proteins Aurora B, Incenp, and Survivin, and which has been described to bind to inner centromeres in metaphase and the spindle midzone in anaphase [reviewed in (Carmena and Earnshaw, 2003)].

Sequence analysis demonstrated that $p34^{4B8}$ did not encode a protein homologous to these or any other annotated protein, but rather was a member of a novel protein family comprising two proteins: $p34^{4B8}$ (hereafter referred to as Dasra A, see below) and a related protein which we designated Dasra B. Orthologues of *Xenopus laevis* Dasra A and Dasra B can be found in both *Xenopus tropicalis*, *Gallus gallus*, and *Danio rerio*, whereas only Dasra B-type sequences can currently be found among mammalian genomic DNA and EST databases (Figure 2-3). Likewise, only one protein containing any homology to Dasra proteins could be found in *Caenorhabditis elegans* sequence databases; this protein, CSC-1, contains little or no homology to Dasra A, and only limited homology to Dasra B, most of which is confined to a C-terminal patch (Figure 2-

3, arrowheads). The previously described imperfect direct repeat found in CSC-1 is absent in both Dasra A and Dasra B [Figure 2-3, underlined; (Romano et al., 2003)].

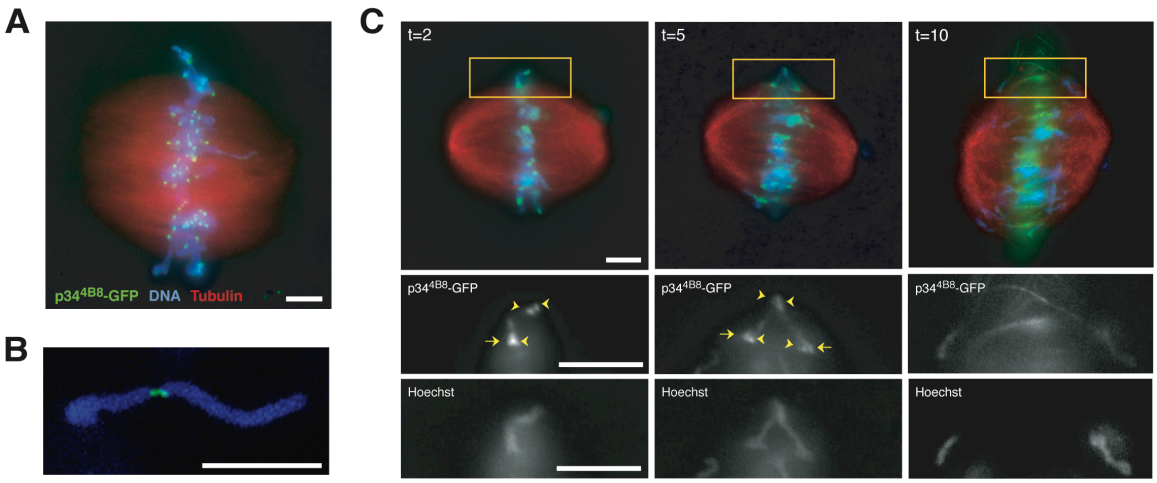

Figure 2-2. p34^{4B8}-GFP Localizes to the Inner Centromere at Metaphase and to the Spindle Midzone at Anaphase

(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.

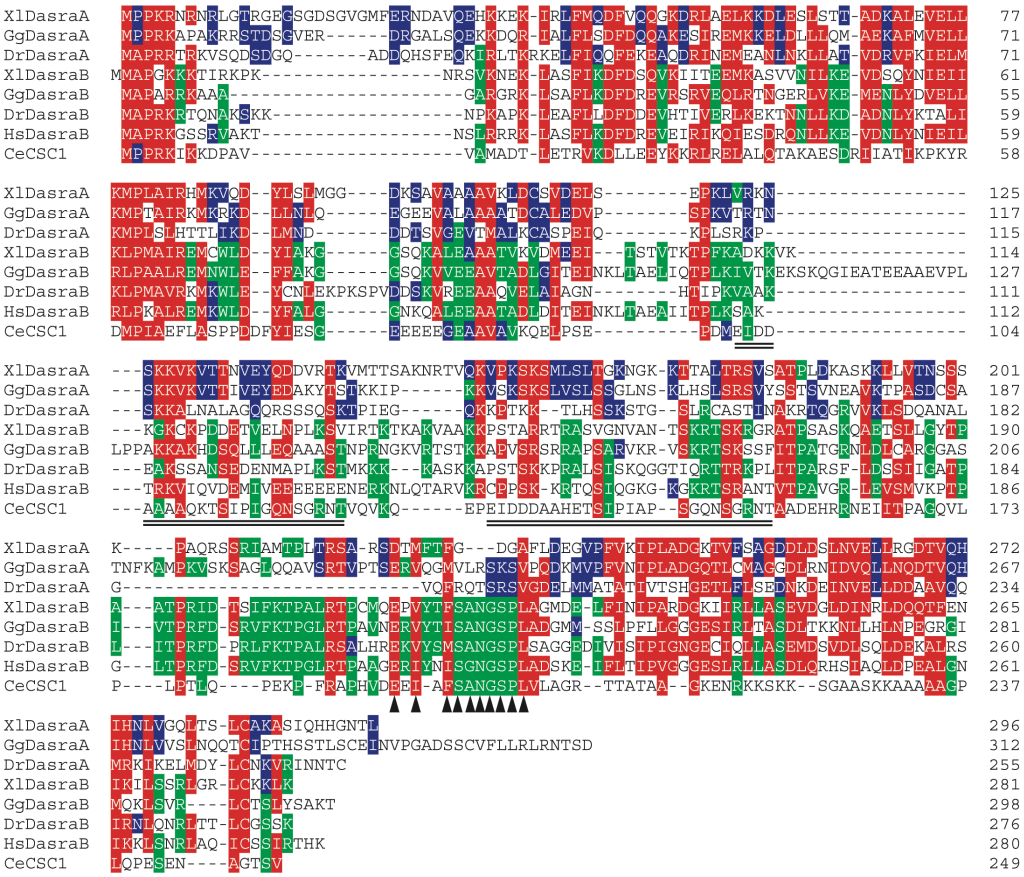

Figure 2-3. Sequence Alignment of Dasra Family and Dasra-like Proteins
Sequence alignment of *Xenopus laevis* Dasra A, *Gallus gallus* Dasra A, *Danio rerio* Dasra A, *Xenopus laevis* Dasra B, *Gallus gallus* Dasra B, *Homo sapiens* Dasra B/CDCA8, and *Caenorhabditis elegans* CSC-1. Identical or conserved 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.

To begin to address the expression patterns of *Xenopus* Dasra A and Dasra B, we performed Northern blot analysis on total RNA purified from either eggs (arrested at meiotic metaphase II) or the XTC *Xenopus* fibroblast tissue culture cell line. We observed that Dasra A and Survivin were specifically expressed in egg, with little or no detectable mRNA present in fibroblasts (Figure 2-4). This is in keeping with previous findings that Survivin levels are highest during embryogenesis and substantially lower in

somatic cells. In contrast, Dasra B, Aurora B, and Incenp all demonstrated approximately equivalent mRNA levels between eggs and fibroblasts (Figure 2-4), suggesting the Dasra B may be the predominant or only Dasra family member present in the CPC of *Xenopus* somatic cells. No evidence was found to suggest that either Dasra A, Dasra B, or Incenp is subject to alternative splicing, however both Aurora B and Survivin displayed minor bands which may correspond to alternatively spliced products.

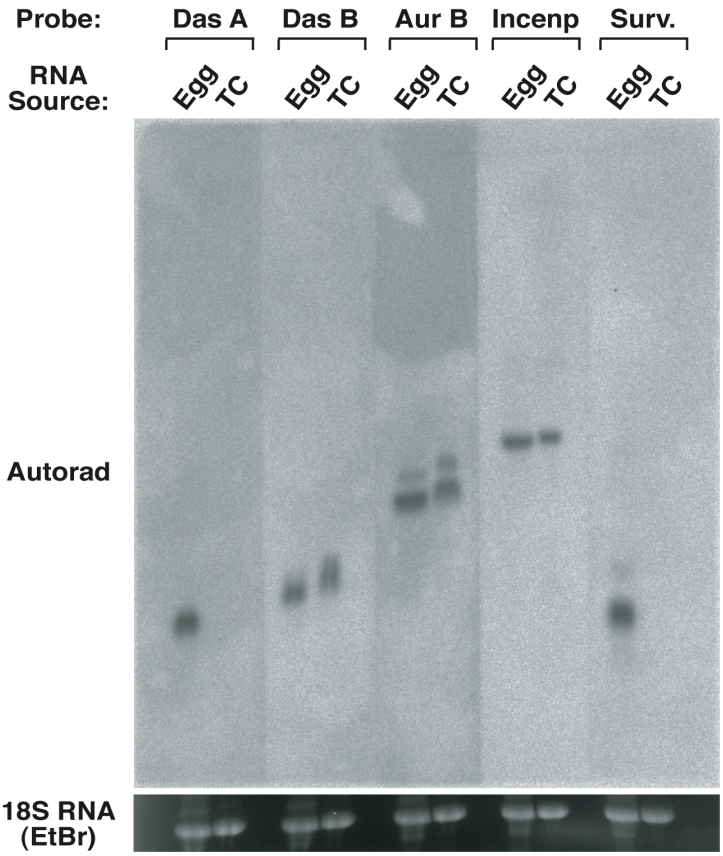

Figure 2-4. Dasra A, But Not Dasra B, is Preferentially Expressed in *Xenopus* Eggs
Total RNA was purified from unfertilized *Xenopus* eggs ('Egg') or from the XTC *Xenopus* fibroblast tissue culture cell line ('TC'). 20 micrograms of total RNA per sample were separated on a formaldehyde agarose gel, transferred to nitrocellulose, and hybridized with labeled probes produced by PCR of full-length xDasra A, xDasra B, xAurora B, xIncenp, or xSurvivin clones. Membranes were then washed and exposed to a PhosphorImager. Ethidium bromide staining of 18S rRNA was used to demonstrate equal loading.

To confirm the lack of expression of Dasra A in somatic cultured cells, we performed indirect immunofluorescence on XTC cells using Dasra A- or Incenp-specific antibodies (see below for description of antibodies). As expected, Incenp localized to centromeres at metaphase and the spindle midzone/midbody at anaphase/telophase; in contrast, no significant signal was seen with the Dasra A-specific antibodies (Figure 2-5). We conclude that Dasra A expression is likely limited to the period of embryonic development, consistent with our observations that Dasra A ESTs are less abundant in libraries derived from adult tissues (H.F., unpublished observations).

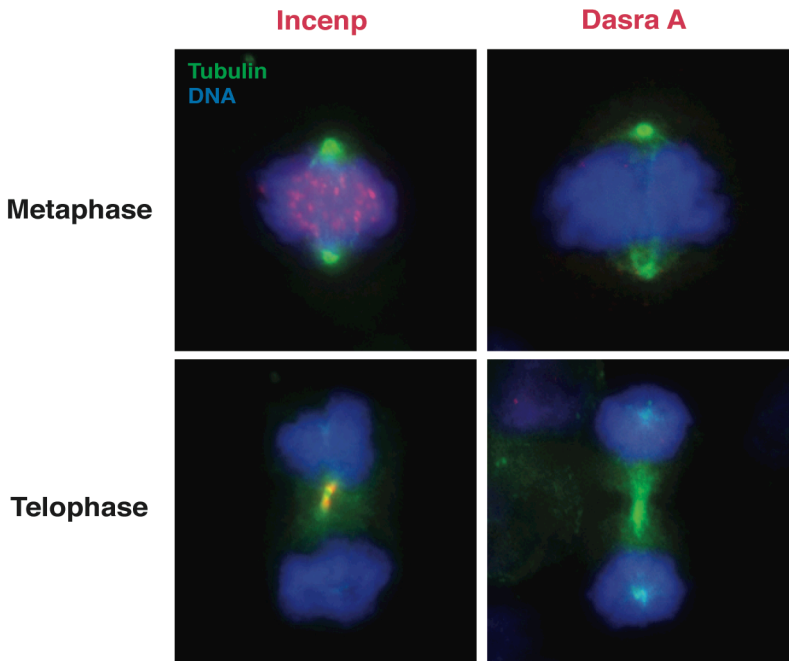

Figure 2-5. Dasra A is Not Expressed in Cultured *Xenopus* Somatic Cells
Asynchronous XTC cells were fixed with methanol and indirect immunofluorescence was performed with Dasra A- (red), Incenp- (red), and α-Tubulin (green)-specific antibodies. DNA (blue) was counterstained with Hoechst 33258.

Dasra Proteins are Novel Components of the Vertebrate Chromosomal Passenger Complex

The dynamic localization pattern of Dasra A was reminiscent of that of the 'chromosomal passenger' proteins Incenp, Aurora B, and Survivin [reviewed in (Carmena and Earnshaw, 2003)]. To investigate the relationship between the Dasra proteins and the vertebrate chromosomal passenger complex (CPC), antibodies were raised against peptides encoding the C-terminal 15 amino acids of *Xenopus* Dasra A and Incenp. These antibodies predominantly recognized one species each in CSF-arrested egg extract, which could be immunodepleted by >90% by treatment with anti-Dasra A or anti-Incenp beads, respectively (Figures 2-6A and 2-6B). Interestingly, immunodepletion of Incenp lead to >90% codepletion of Dasra A, Aurora B, and Survivin, whereas depletion of Dasra A lead to ~70% codepletion of Incenp (Figure 2-7). In agreement with these findings, immunoprecipitation of Dasra A could coprecipitate Incenp, Aurora B, and Survivin (Figure 2-6C), suggesting that Dasra A resides in the Incenp, Aurora B, and Survivin-containing CPC. This conclusion is supported by the finding that Dasra A cofractionates with the other CPC members by sucrose density centrifugation (H. Funabiki, data not shown). Moreover, Dasra A and Incenp colocalize by indirect immunofluorescence at both metaphase and anaphase (Figure 2-6D).

Together, these data demonstrate that Dasra A is a novel member of the *Xenopus* chromosomal passenger complex. We have named this protein family protein *Dasra* in reference to the twin deities of Hindu mythology, Dasra and Natsatya, who together act as harbingers of the goddess of the dawn (*Aurora* of Roman mythology).

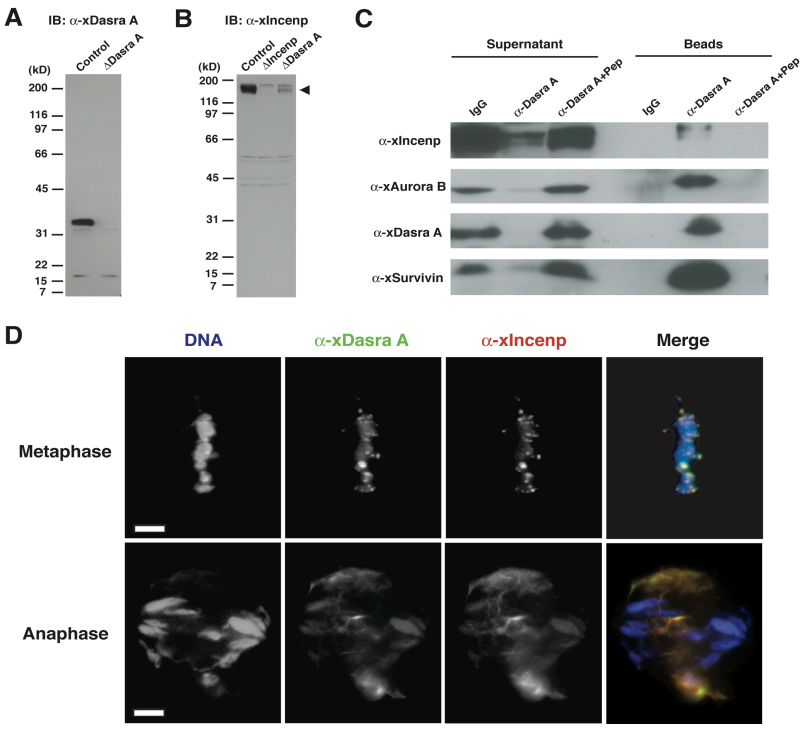

Figure 2-6. Dasra A and Dasra B are New Components of the Chromosomal Passenger Complex

p34^{4B8} is hereafter referred to as Dasra A (see text for details).

(A) Specificity of anti-Dasra A antibodies and immunodepletion of Dasra A from *Xenopus* egg extract. Metaphase low-speed extracts were depleted with either control IgG beads or anti-Dasra A beads. A Western blot of total protein from each is shown probed with anti-Dasra A antibodies (gift of H. Funabiki).

(B) Immunodepletion of Incenp from egg extract, and codepletion of Incenp with Dasra A. Metaphase low-speed extracts were depleted with either control IgG beads, anti-Incenp beads (gift of H. Funabiki), or anti-Dasra A beads. A Western blot of total protein from each is shown probed with anti-Incenp antibodies. Arrowhead indicates the position of Incenp; the minor crossreacting species are not depleted by either anti-Incenp or anti-Dasra A beads. Anti-Aurora B and anti-Survivin antibodies were gifts of T. Hirano.

(C) Dasra A physically interacts with the chromosomal passenger complex. High speed supernatants of metaphase *Xenopus* egg extracts were depleted with either control IgG beads, anti-Dasra A beads, or anti-Dasra A beads in the presence of Dasra 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) Dasra A and Incenp colocalize in metaphase and anaphase. Metaphase spindles were assembled on replicated sperm chromosomes in CSF extract. To induce anaphase, 0.5 mM calcium chloride was added to metaphase reactions. Assembled spindles were sedimented onto coverslips and processed sequentially for immunofluorescence using anti-Dasra A (green) and anti-Incenp (red) antibodies. DNA was stained with Hoechst 33258 (blue). Scale bars, 5 μm.

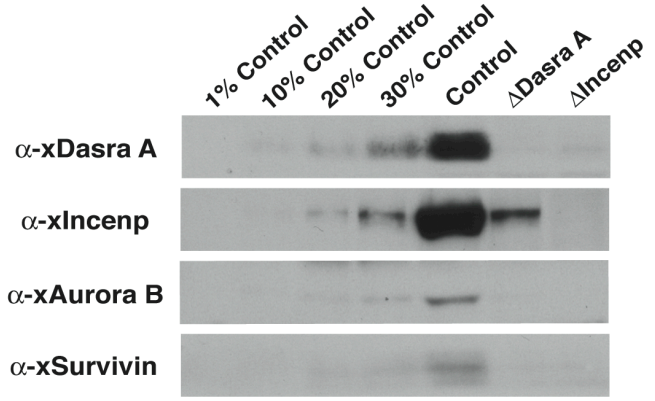

Figure 2-7. Dasra A and Incenp Reciprocally Immunodeplete From Egg Extract
(A) CSF extract was immunodepleted with either control IgG beads, anti-Incenp beads, or anti-Dasra A beads. Total protein from the supernatants of immunodepletion reactions was analyzed by Western blot using the indicated antibodies. Various fractions of the control depletion supernatant were loaded in order to quantify the percentage of immunodepletion achieved by either anti-xIncenp or anti-xDasra A bead treatment.

To determine whether Dasra B also behaves as a chromosomal passenger protein, the CPC was immunoprecipitated from extract containing ^{35}S-labeled Dasra B, using antibodies against either Incenp or Dasra A (Figure 2-8A). While Incenp immunoprecipitation coprecipitated significant amounts of Dasra B, only a relatively minor amount was precipitated with α-Dasra A beads; in contrast, Dasra A was efficiently precipitated with both antibodies. This demonstrates that exogenous Dasra B can be incorporated into the CPC, although the CPC may actually be comprised of distinct Dasra A- or Dasra B-containing populations. As expected for a chromosomal passenger protein, exogenous Dasra B-GFP fusion protein localizes to metaphase sperm chromosomes in egg extract, with enrichment observed at centromeres (Figure 2-8B).

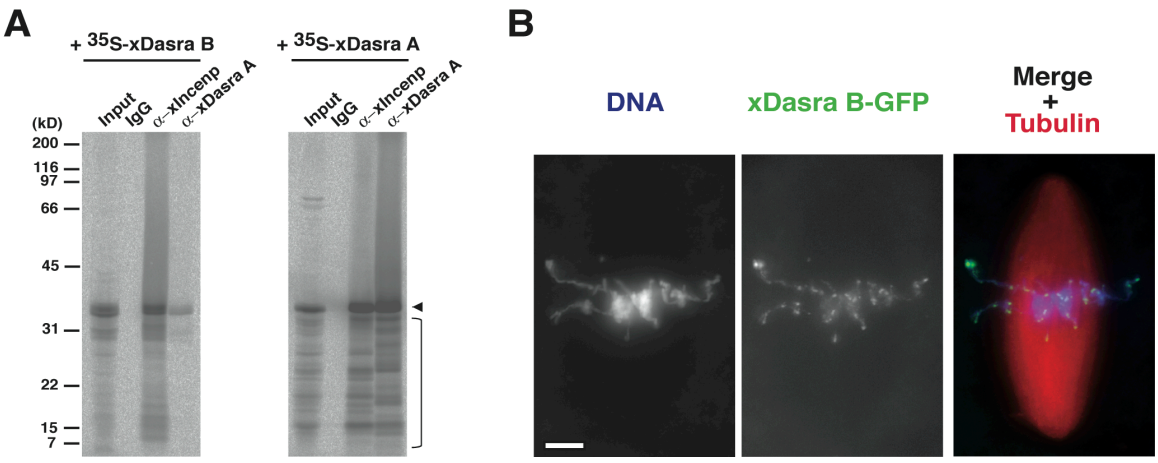

Figure 2-8. *Xenopus* Dasra B Behaves as a Chromosomal Passenger Protein
(A) Dasra B coprecipitates with Incenp and Dasra A in *Xenopus* egg extract. ^{35}S-labeled Dasra B (left) or Dasra A (right) protein was incubated for 30 min with CSF extract containing cycloheximide. The extract was then treated with either control IgG beads, anti-xIncenp beads, or anti-Dasra A beads, and the bead-bound fraction of each was analyzed by SDS-PAGE, followed by autoradiography. Arrowhead indicates the positions of Dasra B and Dasra A. Bracket indicates low molecular weight products from *in vitro* translation.
(B) Metaphase localization of Dasra B-GFP. mRNA encoding Dasra 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.

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

To examine the function of human Dasra B (hDasra B)/CDCA8, an antibody was raised 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 2-9A and 2-9B). Although this antibody did not work for immunoblotting (data not shown), it coimmunoprecipitated Aurora B and Survivin from mitotic cells, suggesting that hDasra B interacts with the chromosomal passenger complex (Figure 2-9C).

To examine whether hDasra B is required for proper function of the chromosomal passenger complex in human cells, 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, often with chromosomes that were no longer localized between the spindle poles (Figures 2-9B and 2-10A, quantified in Figure 2-10B, left); in such cells, anti-hDasra B, anti-Aurora B and anti-Survivin antibodies all failed to stain chromosomes by immunofluorescence (Figure 2-9B, and data not shown). hDasra B siRNA-treated cells also accumulated multiple interphase nuclei (Figure 2-10B, 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 2-9D), indicating that hDasra B is required to maintain normal levels of Survivin protein. Overall, these data correspond well with the findings of Gassmann and colleagues, who reported that hDasra B (referred to by them as Borealin) forms a complex with Aurora B, Incenp, and Survivin, and that siRNA-mediated depletion of hDasra B leads to chromosome misattachment and misalignment (Gassmann et al., 2004).

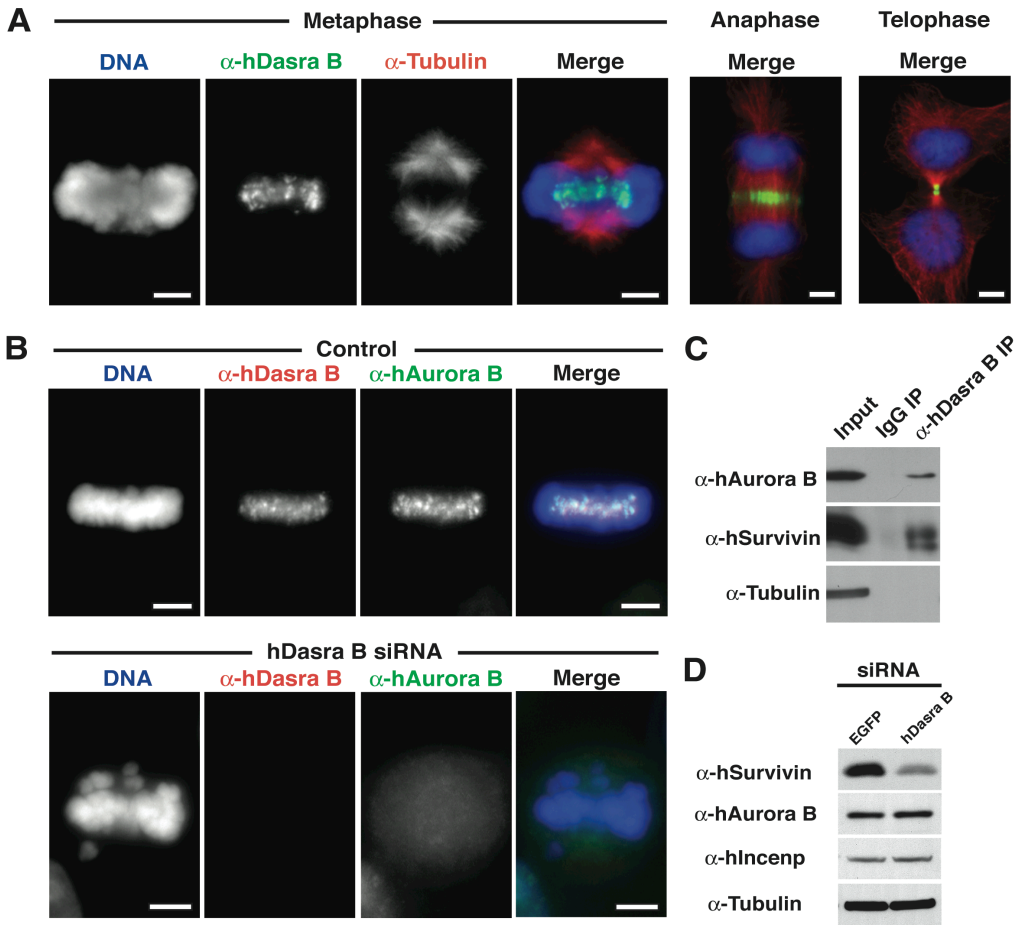

Figure 2-9. Human Dasra B (hDasra B) Is Associated with the Chromosomal Passenger Complex, and Is Required for Proper Metaphase Chromosome Alignment

(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 h, arrested with the proteasome inhibitor MG132 for 2 h, 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.

(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 h, cells were lysed and analyzed by Western blot using anti-hSurvivin, anti-hAurora B and anti-α-Tubulin antibodies.

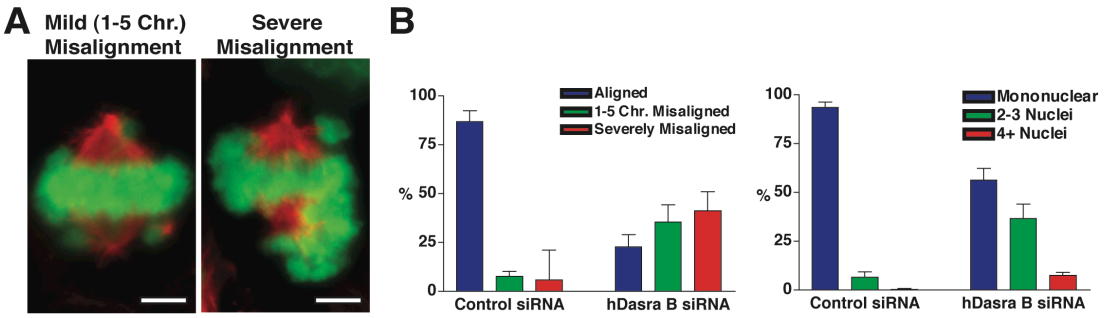

Figure 2-10. hDasra B Is Required for Proper Metaphase Chromosome Alignment
(A) Chromosome misalignment caused by loss of hDasra B. HeLa cells were treated with either a control or an hDasra B-specific siRNA oligo and analyzed by immunofluorescence with anti-α-Tubulin antibodies (red). DNA was stained with Hoechst 33258 (green). A cell was scored as "severely misaligned" if the chromosomal mass was either not organized into a metaphase plate, or was extended beyond the spindle or metaphase plate. Scale bars, 5 μm.
(B) 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 of chromosomes.

The Chromosomal Passenger Complex is Required for Bipolar Spindle Formation in *Xenopus* Egg Extracts

Note: The following work was conducted in collaboration with H. Funabiki.

To investigate the function of the Dasra A-containing CPC, we immunodepleted the complex from CSF-arrested egg extract using anti-Dasra A antibodies (ΔDasra A extract) and monitored spindle assembly around cycled sperm chromosomes. As expected, mock-depleted extract contained predominantly bipolar spindles by seventy minutes after entry into metaphase (Figure 2-11A, quantified in Figure 2-11B). To our surprise however, we found that 79% of the spindle structures formed in ΔDasra A extract were either monopolar spindles or asters (Figure 2-11A, quantified in Figure 2-11B). Given that Dasra A immunodepletion leaves ~30% of Incenp undepleted (Figure 2-7), we

speculated that this effect might itself represent only a partial CPC loss-of-function phenotype. To confirm and extend this finding, we therefore examined spindle formation in extract depleted using anti-Incenp antibodies (ΔIncenp extract). As would be expected if ΔDasra A depletion represents an intermediate CPC-depletion phenotype, ΔIncenp extract demonstrated an even more severe spindle formation defect (Figure 2-11A, quantified in Figure 2-11C), in which replicated sperm chromosomes were predominantly associated with either very few astral microtubules (42%) or no detectable microtubules at all (57%). This data suggests that the chromosomal passenger complex is required for efficient bipolar spindle formation.

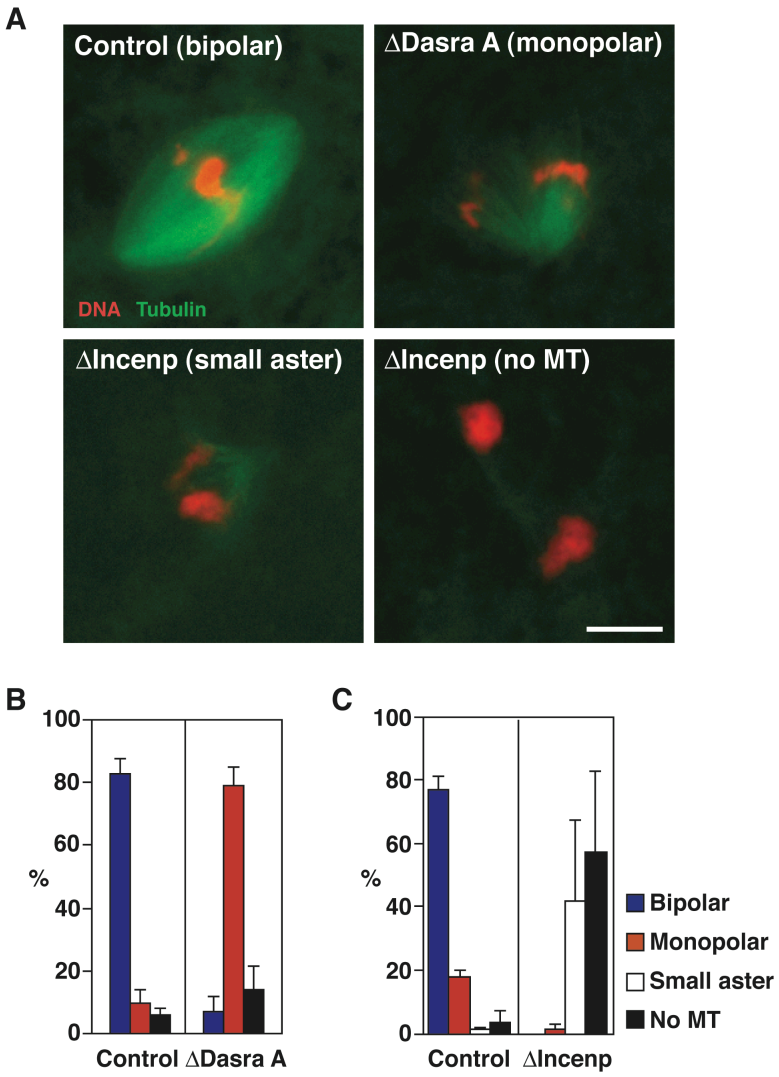

Figure 2-11. xDasra A and xIncenp Depletion Induce Defects in Spindle Formation in Egg Extracts

(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).

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

Note: The following experiments were conducted by H. Funabiki and R. Ohi.

Xenopus sperm nuclei contain both centrosomes and chromosomes, both of which can act as a source for microtubule assembly in egg extract. However, since microtubule polymerization from centrosomes precedes chromatin-induced spindle assembly, defects in these two pathways can be distinguished by monitoring spindle assembly over time. To determine whether the failure of spindle formation in ΔIncenp extract represents a defect in centrosomal and/or chromatin-induced spindle assembly, microtubule nucleation was monitored in control or ΔIncenp extract after addition of sperm nuclei to CSF extract. In control extract, rapid centrosomal microtubule nucleation was observed within 10 min after incubation at 15.5°C, which was progressively replaced by chromatin-oriented spindle assembly such that 96% of structures were either monopolar or bipolar spindles by 40 min (Figure 2-12, top row). By contrast, sperm nuclei in ΔIncenp extract displayed only centrosomal microtubule nucleation, after which spindle assembly failed to proceed, suggesting that chromatin-induced spindle formation was defective (Figure 2-12, bottom row).

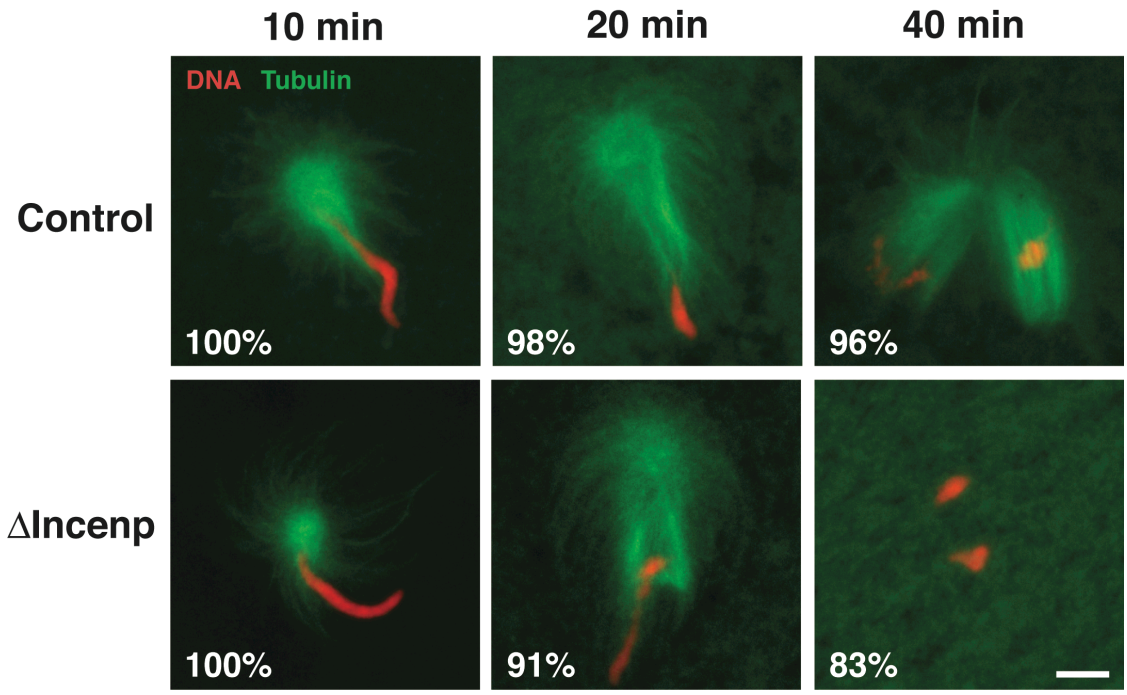

Figure 2-12. Microtubule Nucleation from Sperm Centrosomes and Chromosomes in ΔIncenp Extract
Microtubule nucleation was visualized from centrosomes associated with demembranated sperm nuclei in control or ΔIncenp extracts. Sperm nuclei were incubated in extracts at 15.5°C for the time indicated, and 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 timepoint. Scale bar, 10 μm.

To further test this possibility, spindle assembly was monitored around DNA-coated beads, which form chromatin in egg extract and can support bipolar spindle assembly (Budde et al 2001; Heald et al, 1996). In control extracts, bipolar spindles began to form around chromatin beads after ~20 min, whereas no microtubule assembly was observed in ΔIncenp extract (Figure 2-13A top and middle rows, quantified in Figure 2-14).

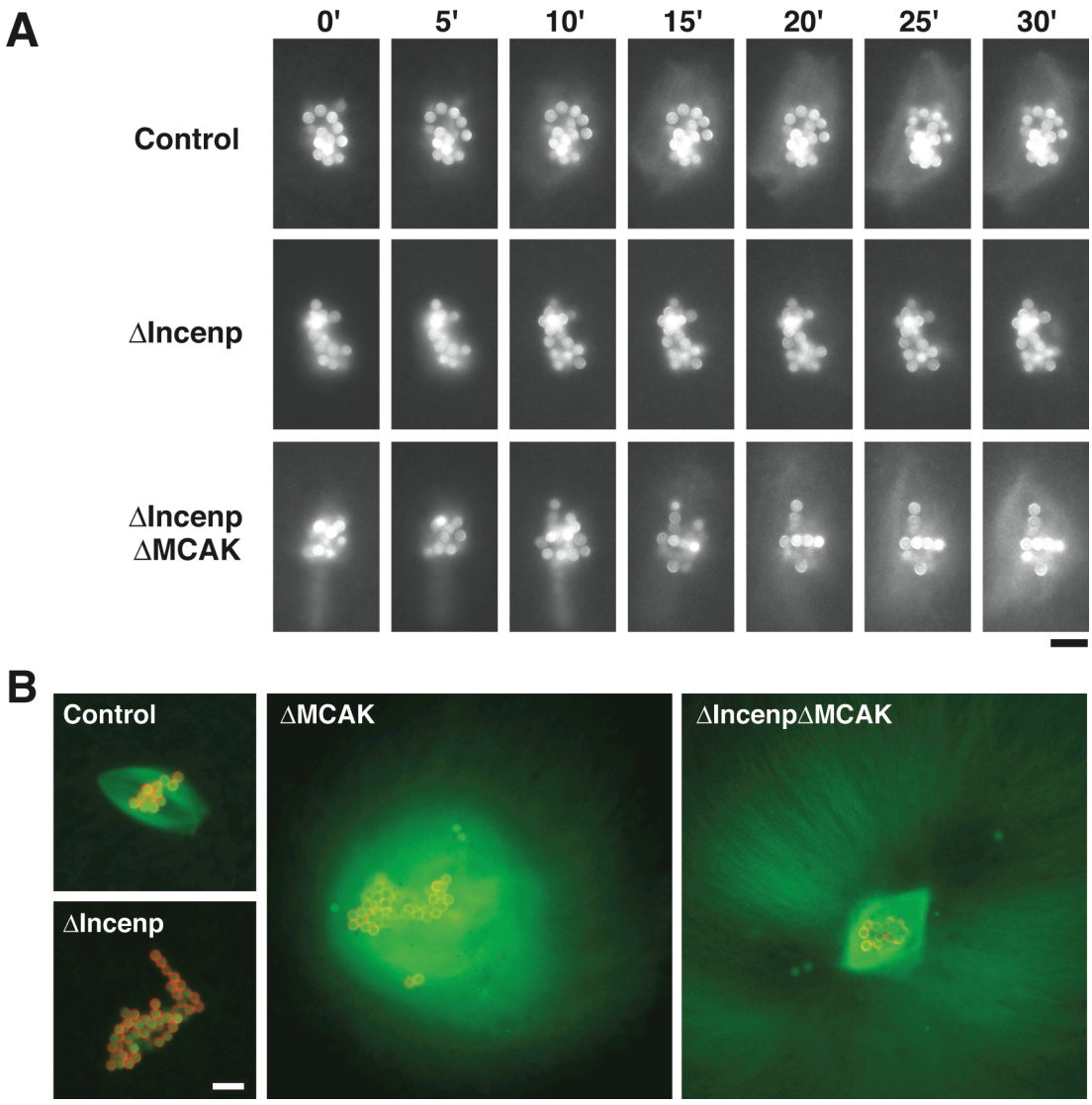

Figure 2-13. Microtubule Nucleation from Chromatin Beads in ΔIncenp Extract
(A) Spindle formation was monitored by time-lapse video microscopy immediately after adding control, ΔIncenp, or ΔIncenpΔMCAK extracts to DNA-coated beads at 20°C. Microtubules were visualized with rhodamine-tubulin, and chromatin 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.
(B) Microtubules assembled on chromatin beads in control extract, ΔIncenp extract, ΔMCAK extract, or ΔIncenpΔMCAK extract. 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.

Since Aurora B has been described to phosphorylate and inactivate the microtubule depolymerase MCAK, we hypothesized that the failure of chromatin-induced microtubule assembly in ΔIncenp extract might be due to MCAK hyperactivity. To test this possibility, spindle assembly was monitored in extract codepleted of MCAK and Incenp (ΔIncenpΔMCAK extract). Unlike the phenotype observed in ΔIncenp extract, microtubules were stabilized around chromatin beads in ΔIncenpΔMCAK extract (Figure 2-13A bottom row, Figure 2-13B, quantified in Figure 2-14), suggesting that the absence of chromatin-induced spindle assembly in ΔIncenp extract may be at least partially a consequence of unrestrained MCAK activity.

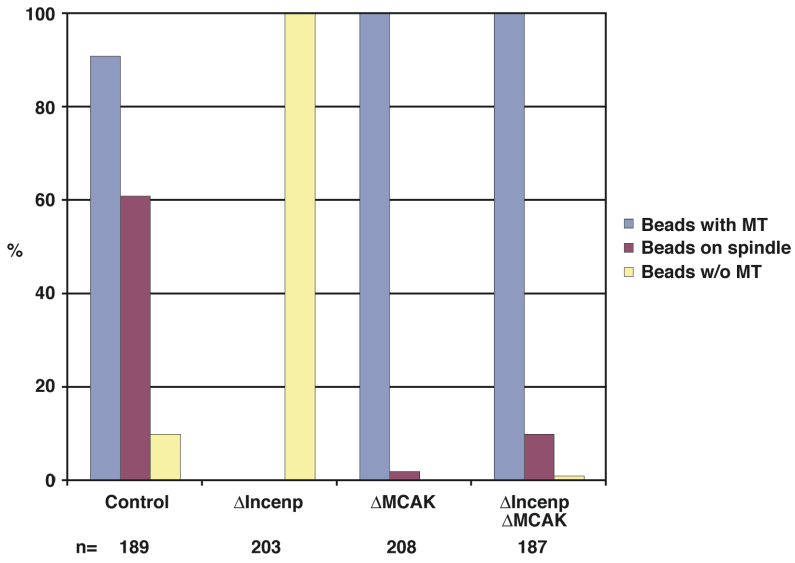

Figure 2-14. Quantitation of Microtubule Nucleation from Chromatin Beads in ΔIncenp Extract

Quantitation of microtubule structures assembled on chromatin beads as described in Figure 2-13. Structures associated with bead aggregates consisting of more than 6 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; "Beads w/o MT," chromatin beads are not associated with any visible microtubule fibers.

The Chromosomal Passenger Complex is Not Required for Ran-GTP-Dependent Microtubule Nucleation

Note: The following experiments were conducted in collaboration with H. Funabiki.

To determine whether the Ran-GTP pathway of microtubule nucleation is required for chromatin-induced spindle formation downstream of the CPC, we utilized a dominant-negative mutant of Ran, RanT24N, which blocks RCC1-mediated production of Ran-GTP (Carazo-Salas et al., 2001). While 100% of bead aggregates demonstrated microtubule nucleation in ΔIncenpΔMCAK extract, 98% of bead aggregates in ΔIncenpΔMCAK containing RanT24N lacked detectable microtubules (Figure 2-15). Microtubule polymerization around chromatin beads in ΔMCAK extract could likewise be suppressed by addition of RanT24N (Figure 2-15). These findings suggest that Ran-GTP can act to promote microtubule nucleation in the absence of the CPC.

To confirm that microtubule nucleation induced by Ran-GTP does not require the CPC, an activated, non-hydrolyzable Ran-GTP mutant was added to control or ΔIncenp extracts. We observed similar levels of microtubule aster formation and qualitatively similar aster morphology induced by Ran-GTP in control and ΔIncenp extract (Figure 2-16), again suggesting that microtubule nucleation by Ran-GTP does not require the CPC. In addition, this finding indicates that CPC-depletion in ΔIncenp extract does not either specifically or non-specifically codeplete other microtubule stabilizing factors working downstream of Ran-GTP.

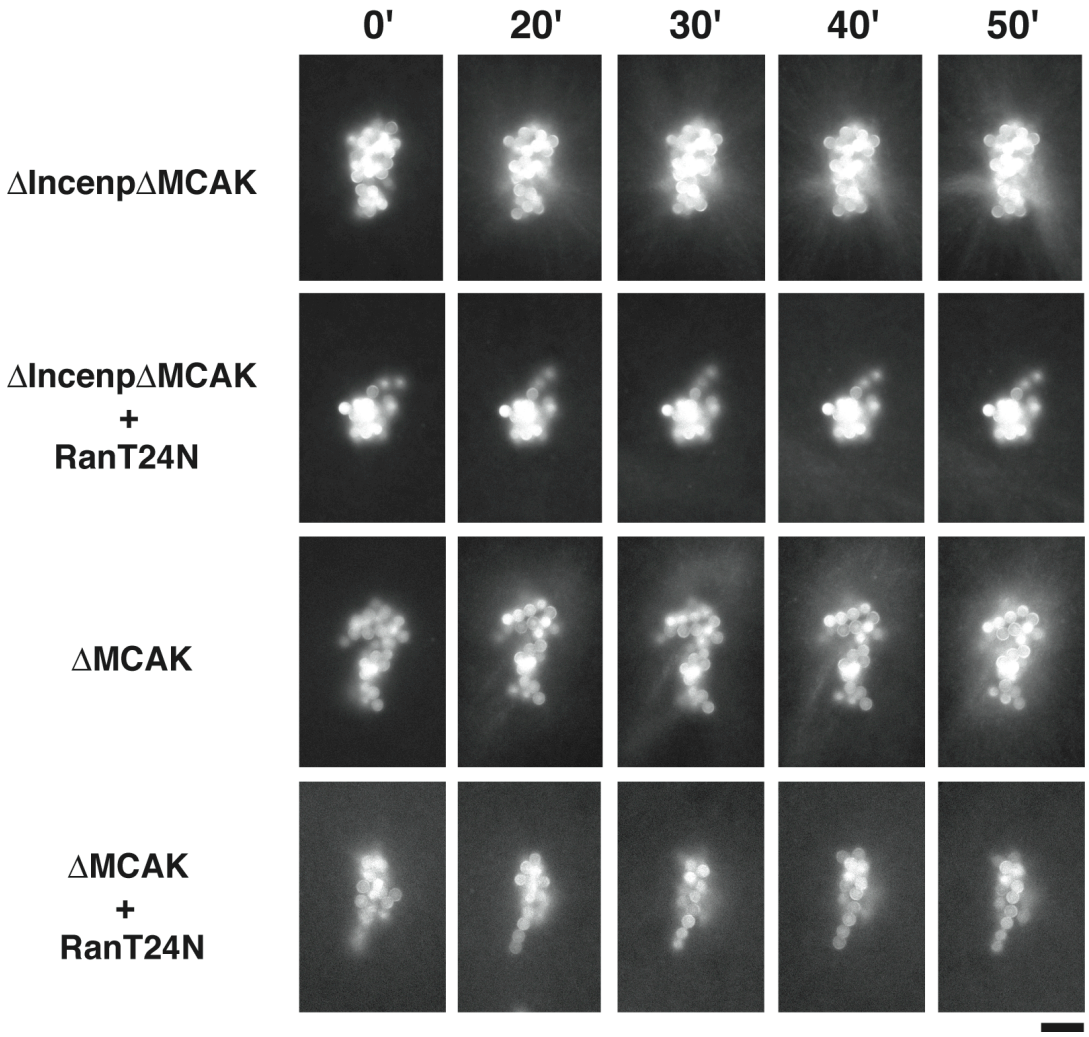

Figure 2-15. Ran-GTP Nucleates Microtubules in ΔIncenp Extract
Chromatin-induced microtubule nucleation in ΔIncenpΔMCAK extract depends on the Ran-GTP pathway. Spindle formation was monitored by time-lapse video microscopy immediately after placing ΔIncenpΔMCAK or ΔMCAK extracts, containing DNA-coated 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.

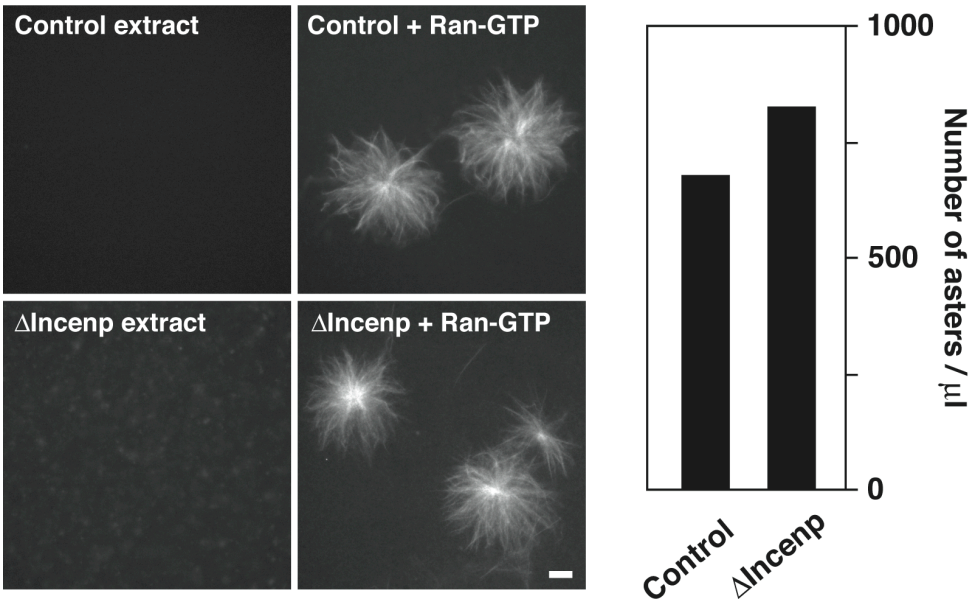

Figure 2-16. Ran-GTP Nucleates Microtubules in ΔIncenp Extract
Metaphase control or ΔIncenp extracts were incubated with or without 25 μM GTPase-defective Ran-G19V/Q69L (loaded with GTP; a gift of E. Coutavas and J. Gaetz) for 20 min at 15.5°C. Microtubules were visualized with rhodamine-labeled tubulin. 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.

Spindle Formation in Egg Extract Involves Dynamic CPC Binding to Chromatin

We sought to determine whether chromatin-induced spindle formation by the CPC involved stable loading of the complex onto mitotic chromosomes, or whether binding instead was dynamic. To address this question, we assembled cycled chromatin beads in either control or ΔIncenp extract, retrieved the beads with a magnet, and transferred the beads into fresh control or ΔIncenp extract in all combinations. Spindle formation was then monitored over time, and after 40 minutes of spindle assembly, aliquots of each reaction were fixed and processed for immunofluorescence using anti-Incenp antibodies (Figure 2-17).

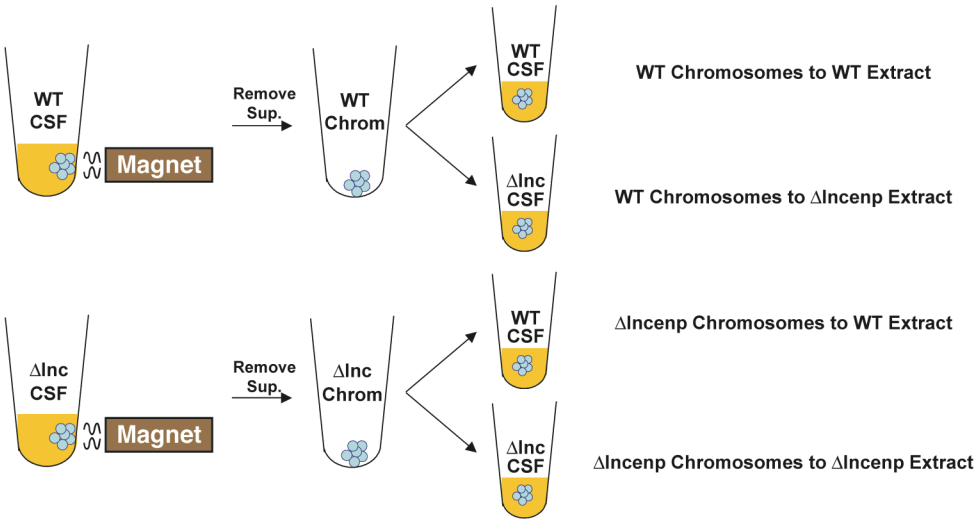

Figure 2-17. A Method for the Study of CPC Binding to Chromatin
(A) Schematic of the 'chromosome transfer' experiment. DNA beads were cycled through interphase to metaphase in control ('wild type') or ΔIncenp extract, washed in control or ΔIncenp extract, and transferred into both types of extract in all combinations. After 40 min, samples were fixed and processed for indirect immunofluorescence using anti-Incenp antibodies.

As expected, we observed that control bead aggregates ('chromosomes') transferred into control extract demonstrated robust spindle formation, whereas ΔIncenp chromosomes transferred into ΔIncenp extract failed to support spindle formation (Figure 2-18, quantified in Figure 2-19). Importantly, we found that when control chromosomes were transferred into ΔIncenp extract, microtubules became transiently stabilized around the beads before abruptly collapsing, eventually leading to a phenotype indistinguishable from the ΔIncenp chromosome/ΔIncenp extract phenotype. Unlike control chromosomes placed in control extract, control chromosomes placed in ΔIncenp extract failed to maintain Incenp on the beads as judged by immunofluorescence, and also did not display histone H3 serine 10 phosphorylation (data not shown). Interestingly, although ΔIncenp chromosomes placed into control extracts did eventually display bipolar spindle formation and Incenp loading, the kinetics of this process were delayed, such that at 30

min ~74% of structures in the ΔIncenp chromosome/control extract sample were asters, while ~85% of structures in the control chromosome/control extract sample were either bipolar or multipolar spindles (Figure 2-19). These findings suggest that the CPC is not stably associated with bulk chromatin during M-phase, but rather exchanges dynamically between the chromatin-bound and soluble states. Moreover, the finding that CPC-deficient chromosomes are delayed in the initiation of microtubule assembly suggests that spindle formation may require at least transient cycling of the CPC through mitotic chromatin.

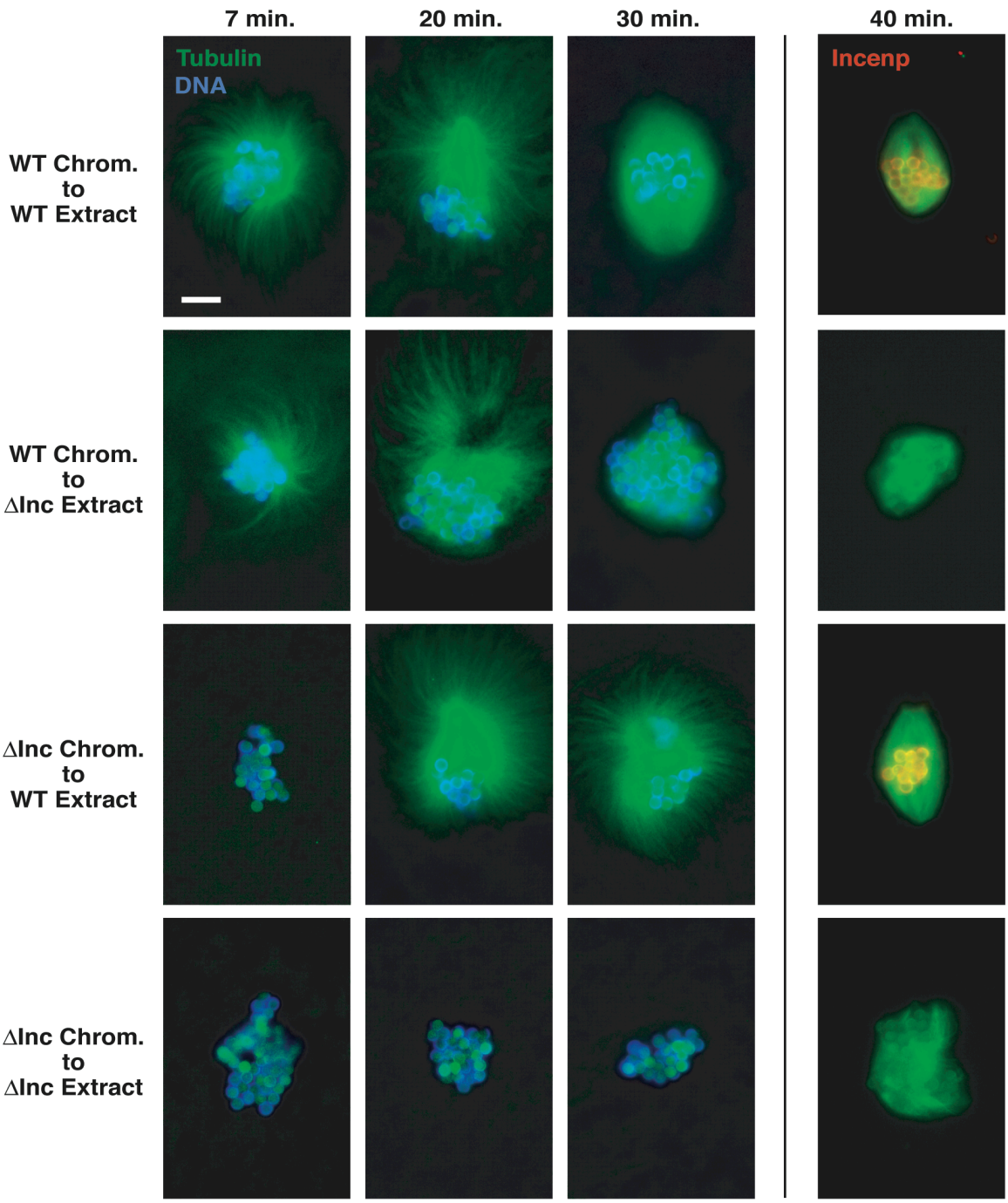

Figure 2-18. The CPC Binds Dynamically to Chromatin During Spindle Assembly
Fixed squashes (7 min, 20 min, 30 min) and anti-Incenp immunofluorescence (40 min) for the samples indicated in (A). Microtubules were visualized with rhodamine-tubulin (green), DNA beads with Hoechst 33258 (blue), and Incenp with anti-Incenp antibodies (red). Scale bar, 10 μM.

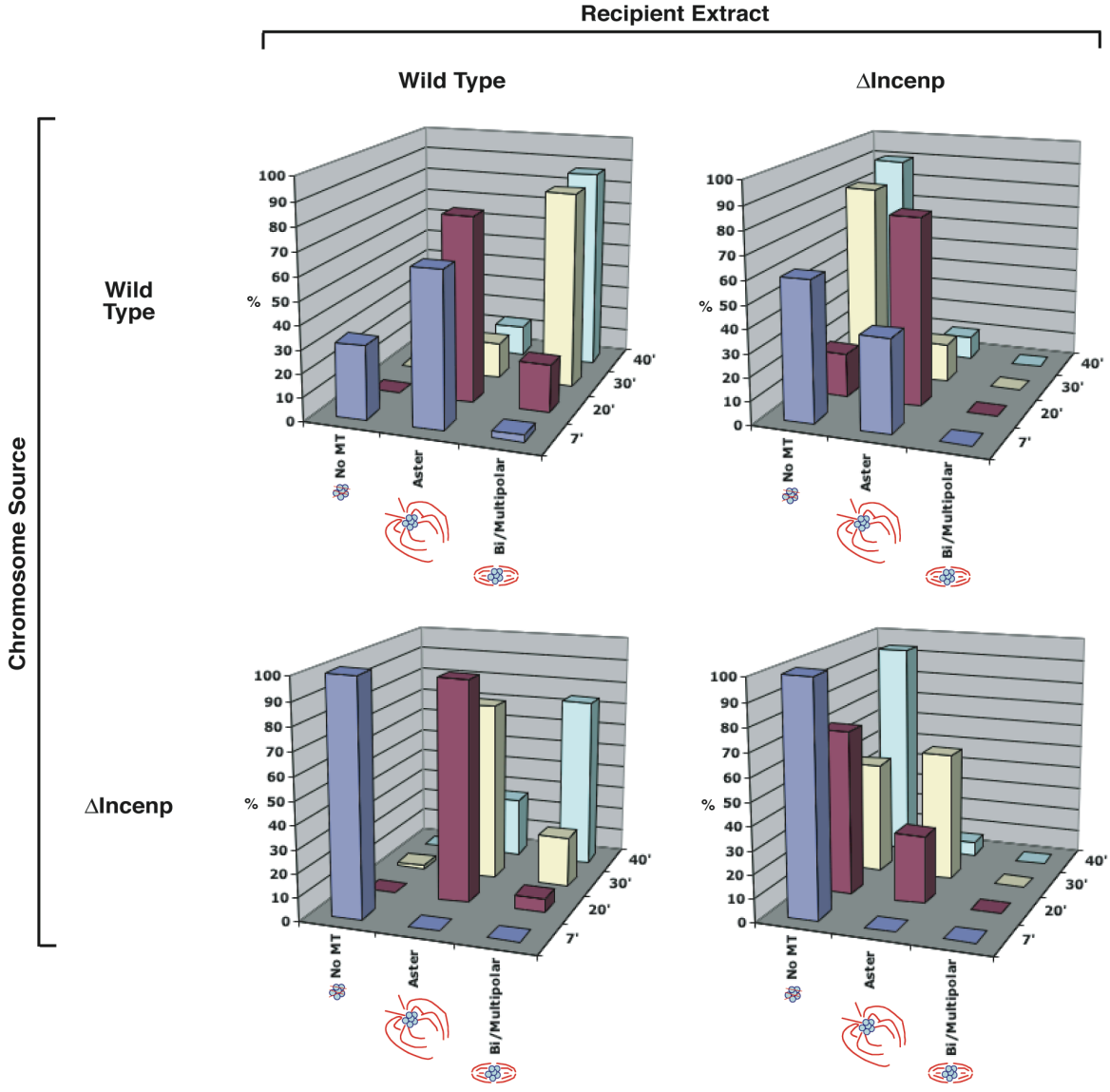

Figure 2-19. Spindle Formation Around Transferred Chromosomes
Quantitation of spindles from samples described above (Figure 2-18).

CHAPTER 3

Functional Analysis of the Vertebrate Chromosomal Passenger Complex

Results

Aurora B and Incenp Regulate Spindle Formation

We previously described the identification of Dasra A and Dasra B, two new components of the vertebrate chromosomal passenger complex (CPC), and reported that immunodepletion of the CPC from *Xenopus* egg extract leads to failure of bipolar spindle formation around M-phase chromatin (Sampath et al., 2004). Subsequently, our lab (work of K. Zelenova) found that a previously described homologue of Survivin, referred to as SIX [Survivin In *Xenopus*; (Song et al., 2003)], can bind to metaphase chromosomes (Figure 3-1). Due to our inability to rescue CPC-depleted (ΔIncenp) extract with epitope-tagged recombinant proteins, however, we were unable to perform further functional analysis of individual CPC components, including the Dasra proteins.

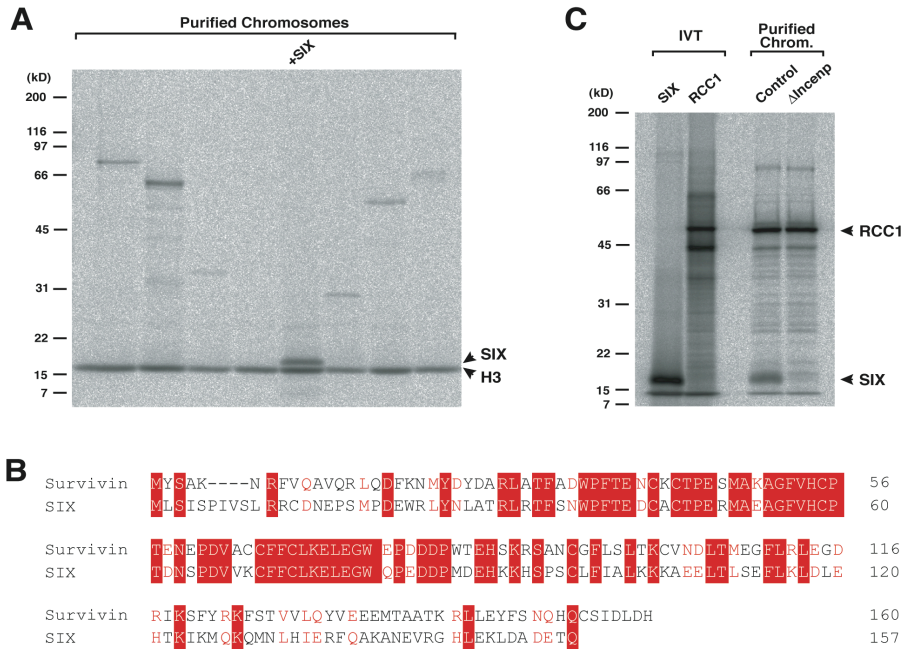

Figure 3-1. SIX Is a New Component of the Chromosomal Passenger Complex in *Xenopus*

(A) Identification of SIX as a chromosome binding protein. Individual clones identified in the course of a screen for new chromosome binding proteins were translated *in vitro* and tested for their ability to bind to mitotic chromosomes in *Xenopus* egg extract (Sampath et al., 2004). Recovery of *in vitro* translated histone H3 was used to judge chromosome recovery.

(B) Sequence alignment between *Xenopus laevis* Survivin and SIX. Red boxes indicate identity between amino acids, while red lettering indicates similarity.

(C) SIX binds to mitotic chromosomes in a CPC-dependent manner. Metaphase chromosomes were purified from control or ΔIncenp egg extract containing *in vitro* translated SIX or RCC1 (latter as a negative control). Copurifed proteins were analyzed by SDS-PAGE and autoradiography.

To bypass potential complications related to epitope tagging and purification of recombinant proteins, we adapted the previously described 'mRNA-dependent' extract system (Murray and Kirschner, 1989). In this method, egg extract is treated with a low dose of RNase A, leading to destruction of endogenous mRNAs while sparing ribosomes. After addition of RNase inhibitor and tRNA, introduction of exogenous mRNAs leads to resynthesis of the corresponding proteins. Using this approach, it was previously

demonstrated that cyclin is the only mRNA whose translation is required to permit cell cycle progression *in vitro* (Murray and Kirschner, 1989).

We treated CSF-arrested egg extract with RNase and RNase inhibitor, and then immunodepleted with either control or anti-Incenp antibodies (Figure 3-2A). Pools of *in vitro* transcribed CPC mRNAs were added with sperm nuclei to these extracts, which were released to interphase and subsequently cycled back to metaphase by the addition of fresh RNase-treated, immunodepleted CSF extract. Labeling of the reconstituted extracts with ^{35}S-methionine demonstrated efficient translation of all exogenous mRNAs (Figure 3-2B); note that the 'Aurora B^{p35}' species represents a product of internal translation initiation, which also seems to exist endogenously (T. Maniar and S.C.S, unpublished observations; (Honda et al., 2003). Immunoblots with antibodies recognizing Incenp, Aurora B, Dasra A, and Survivin indicated that all of these proteins were reconstituted to approximately endogenous levels (Figure 3-3B).

When spindle assembly was monitored by the addition of rhodamine-tubulin to the reconstituted extracts, we found that CPC-depletion led to the expected absence of chromatin-associated microtubules, whereas addition of a pool containing all CPC mRNAs efficiently rescued the assembly of bipolar spindles (Figure 3-3A, top row; quantified in Figure 3-3C). By contrast, addition of an mRNA pool containing only Dasra A/B and Survivin/SIX did not support spindle formation, demonstrating that Aurora B and Incenp are required for chromatin-induced microtubule assembly in this system. Moreover, since spindle formation was observed following the addition of pools lacking both Dasra proteins, we conclude that Dasra A/B are dispensable for bipolar spindle formation in egg extract under the conditions for immunodepletion used here (see

below). We were not able to definitively determine the requirement for Survivin/SIX in spindle formation, due to the fact that ~25% of Survivin was consistently undepleted in ΔIncenp extracts (Figure 3-3B, and data not shown). Consistent with these results, when pools lacking individual CPC components were tested in this assay, only Aurora B and Incenp were found to be individually required for bipolar spindle formation (Figure 3-4). However, as will be described below, the dispensability of Dasra proteins for spindle formation likely reflects leaching of αIncenp antibody into the immunodepleted extracts.

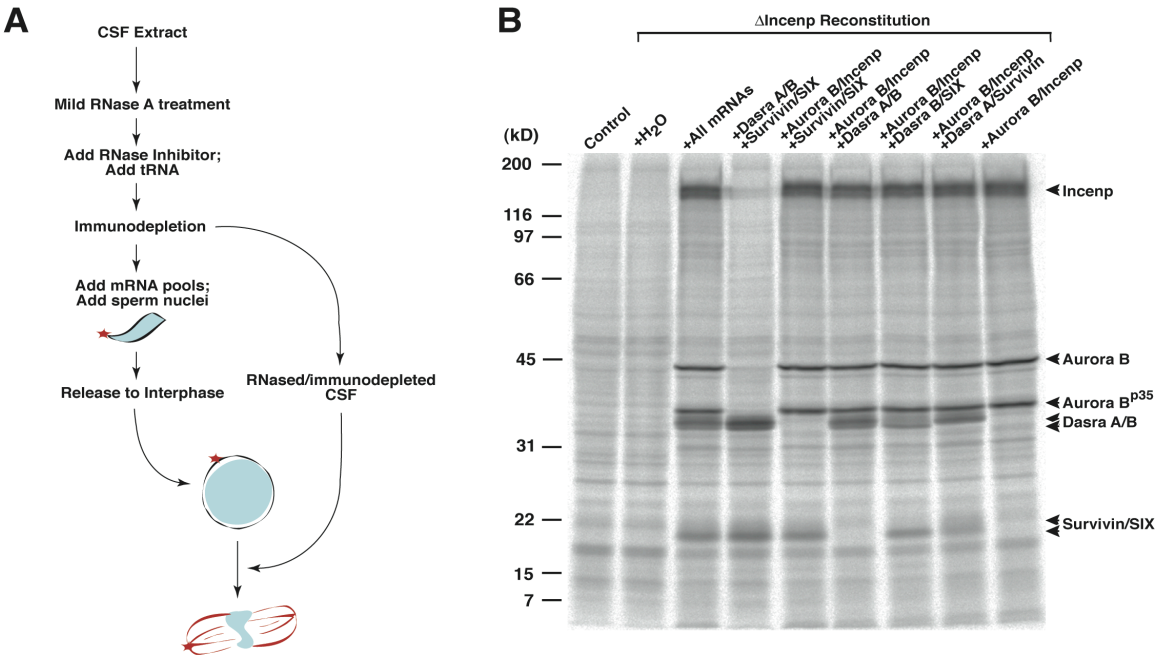

Figure 3-2. A Method for the Reconstitution of ΔIncenp Extract
(A) Schematic for the reconstitution of CPC-immunodepleted (ΔIncenp) egg extract with mRNA pools encoding CPC components. Chromosomes (blue) and centrosomes (red) are diagrammed.
(B) Top panel: Metabolic labeling of reconstituted egg extract. RNase-treated control or ΔIncenp extracts were reconstituted with mRNA pools and supplemented with ^{35}S-methionine, cycled through interphase to metaphase, and analyzed by SDS-PAGE and autoradiography.

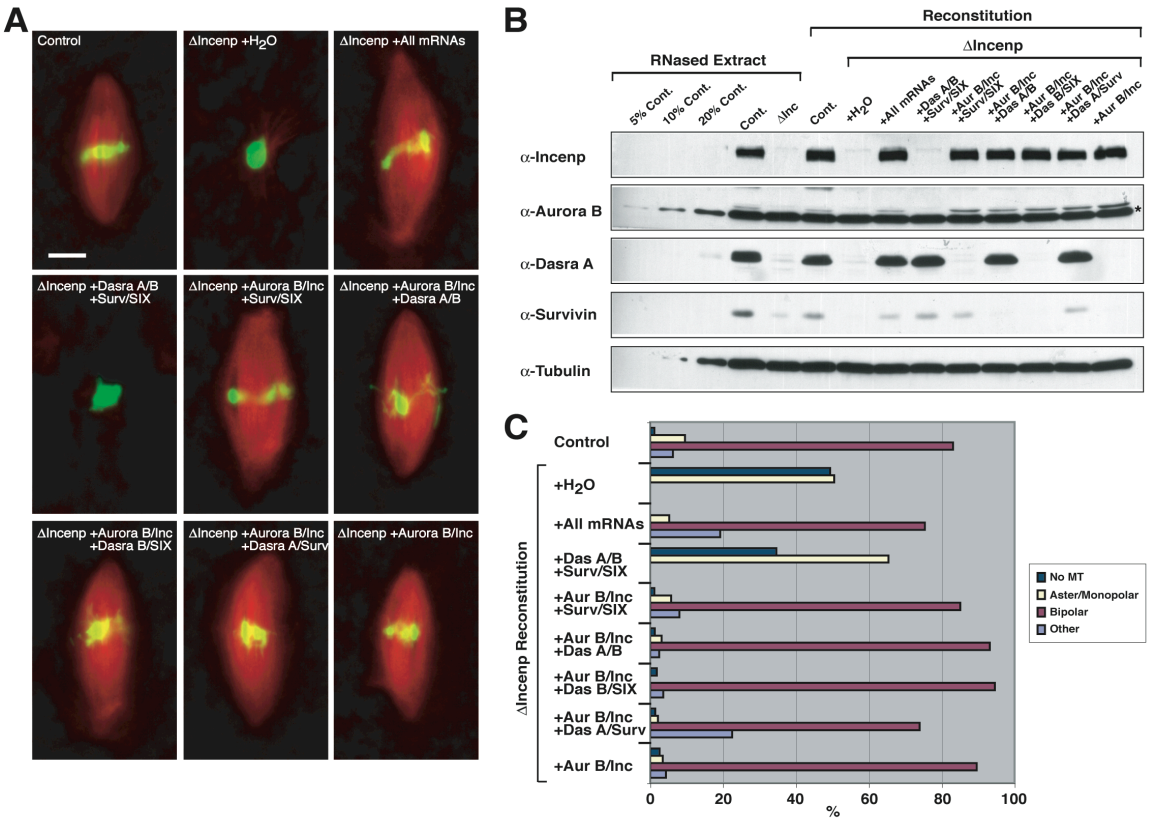

Figure 3-3. Aurora B and Incenp are Required for Chromatin-Dependent Spindle Formation in *Xenopus* Egg Extract

(A) Spindle assembly in reconstituted egg extracts. Control or ΔIncenp extracts reconstituted as described in (A) were cycled through interphase to metaphase, and rhodamine-tubulin (red) was added to visualize microtubules; extracts were fixed 45 min after entry into metaphase. Hoechst 33258 (green) was used to visualize DNA. Scale bar, 10 μm.

(B) Western blot of total proteins from the cycled extracts described above (top panel) using the indicated antibodies.

(C) Quantitation of structures formed in the control, ΔIncenp, and reconstituted ΔIncenp extracts described in (A) and (C).

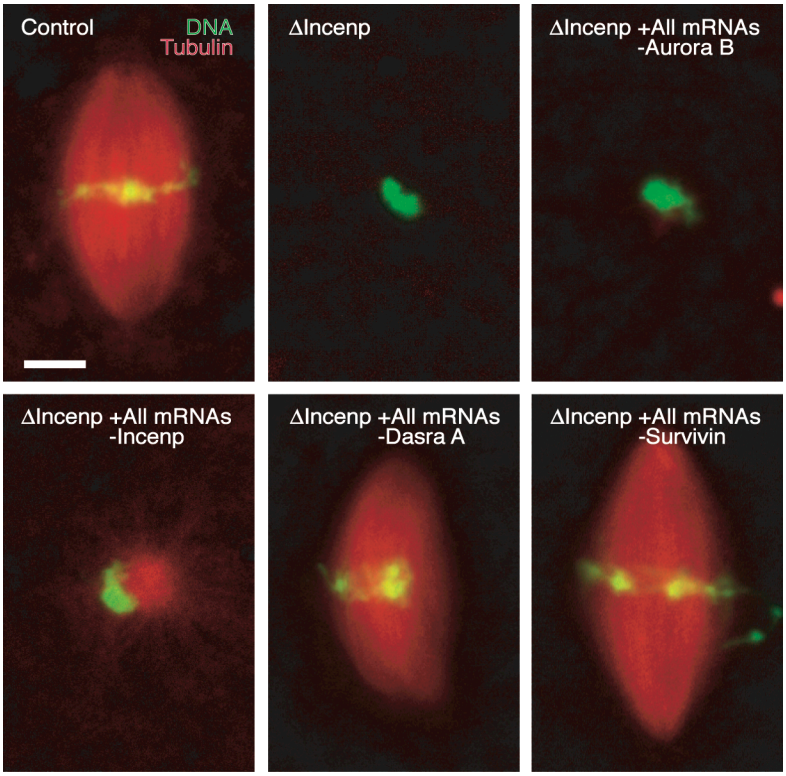

Figure 3-4. Aurora B and Incenp are Individually Required for Chromatin-Induced Spindle Formation
Control, ΔIncenp, or reconstituted ΔIncenp extracts containing sperm chromosomes were cycled through interphase to metaphase. Samples were fixed and spindle structures were examined 45 min after entry into metaphase; a representative image of the predominant (>70%) phenotypic class for each sample is shown. All reconstitutions included mRNAs encoding Aurora B, Incenp, Dasra A, Dasra B, Survivin, and SIX, unless otherwise noted. Rhodamine-tubulin (red) and Hoechst 33258 (green) were added to visualize microtubules and DNA, respectively. Scale bar, 10 μm.

A potential homologue of Dasra B is required for viability in *C. elegans* (Romano et al., 2003), and Survivin homologues are required for cell viability in both *C. elegans* and fission yeast (Fraser et al., 1999; Morishita et al., 2001; Rajagopalan and Balasubramanian, 1999), therefore we were surprised to find that depletion or substantial reduction in the levels of these proteins did not noticeably perturb spindle formation. In order to confirm this result, we generated a deletion mutant of Incenp (Incenp$^{\Delta 1\text{-}327}$) which retains binding to Aurora B, but which cannot bind to Dasra proteins or

Survivin/SIX [Figure 3-5; (Bolton et al., 2002)]. Pools of mRNAs containing Aurora B and full-length or truncated Incenp were added to RNase-treated, immunodepleted egg extracts as described above. We observed that Incenp$^{\Delta 1\text{-}327}$ still supported spindle formation at levels equivalent to full-length Incenp (Figure 3-6, quantified in Figure 3-7). Although these data seem to suggest that Aurora B and Incenp represent a minimal CPC subcomplex required for spindle formation, we subsequently found that this result in fact reflected the presence of αIncenp antibodies in the immunodepleted extract, which led to CPC activation. When immunodepletion was carried out under conditions which precluded antibody leaching, Dasra proteins were found to be essential for robust spindle assembly (A. Kelly, T. Maniar, and S.C.S., data not shown). Of note, we cannot formally exclude the possibility that the residual undepleted Survivin also has a function in spindle assembly.

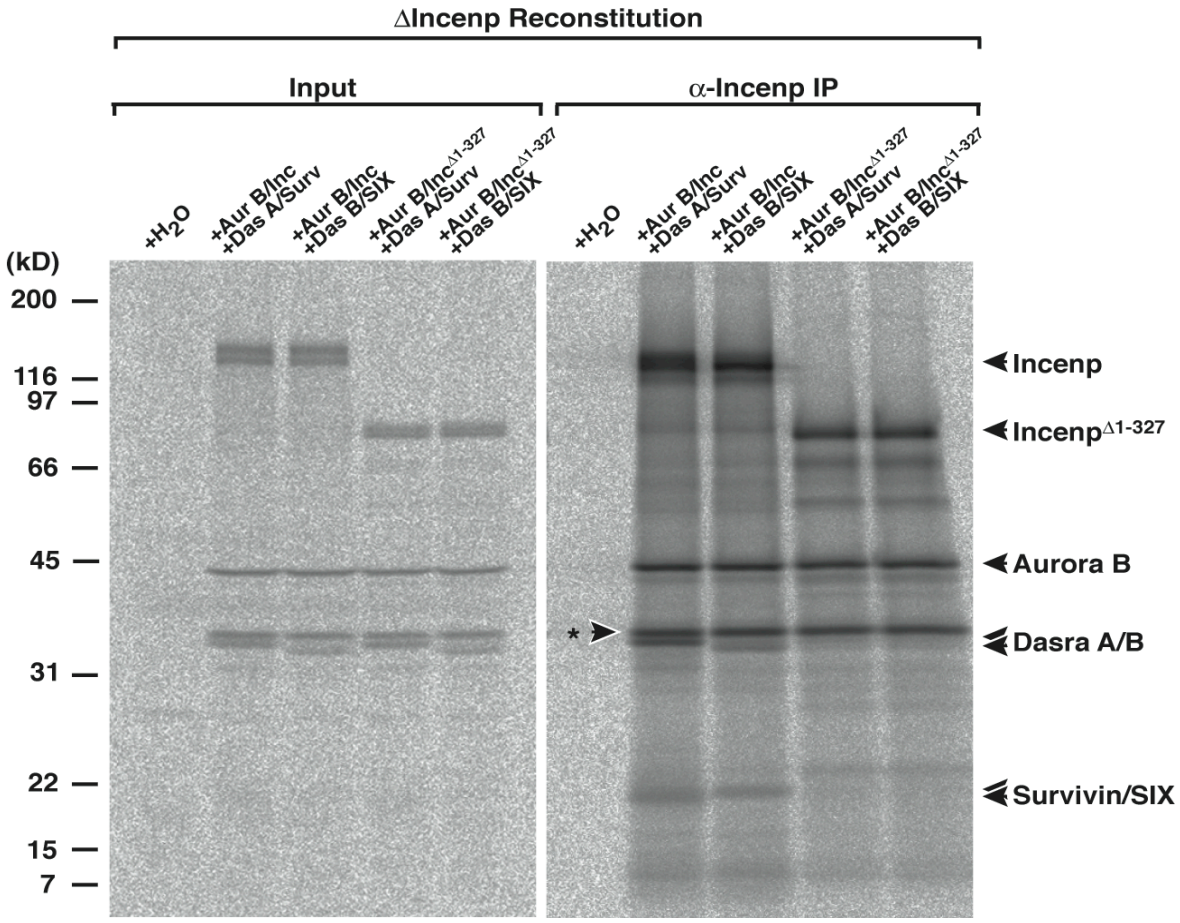

Figure 3-5. The Incenp$^{\Delta 1\text{-}327}$ Mutation Abolishes Incenp Interaction with Dasra A, Dasra B, Survivin, and SIX

Metabolically labeled control, ΔIncenp, or reconstituted ΔIncenp extracts containing ^{35}S-methionine were cycled through interphase to metaphase, and the CPC was immunoprecipitated with anti-Incenp beads. Copurifying proteins were examined by SDS-PAGE and autoradiography. Asterisk indicates the Aurora B^{p35} species, a product of internal translation initiation. Aliquots of the cycled and labeled pre-immunoprecipitation samples are shown at left.

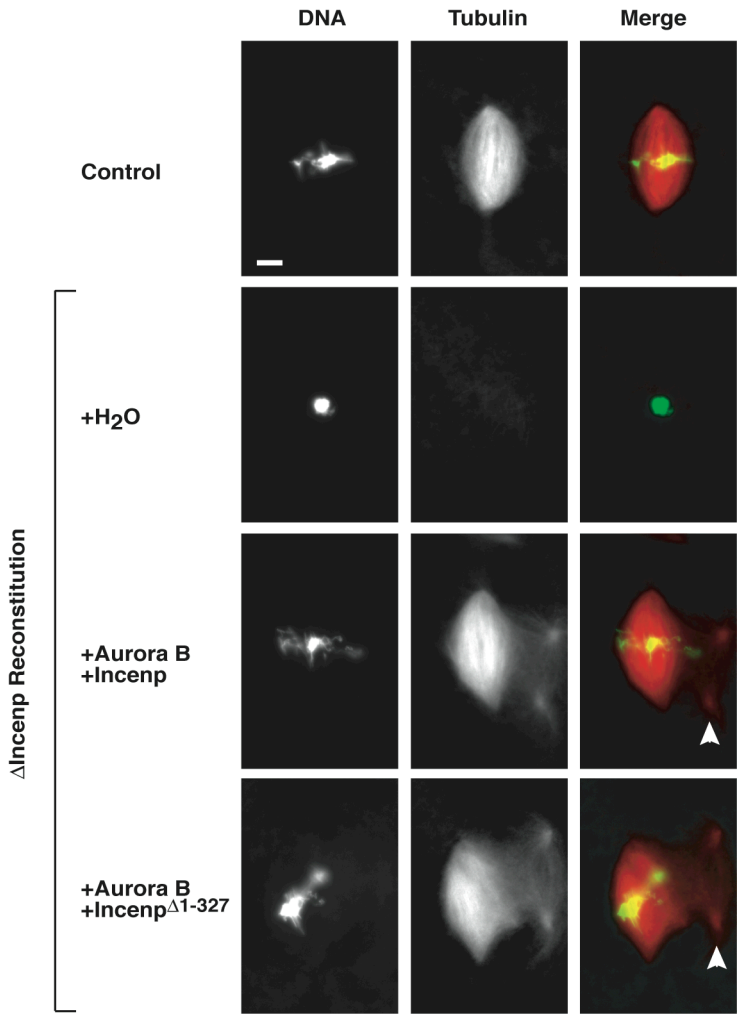

Figure 3-6. The N-terminal Domain of Incenp is Dispensable For Spindle Formation In the Presence of αIncenp Antibodies

Control, ΔIncenp, or reconstituted ΔIncenp extracts were cycled, and samples were fixed 45 min after entry into metaphase. Arrowheads indicate achromosomal spindles formed in reconstituted ΔIncenp extract. Rhodamine-tubulin (red) and Hoechst 33258 (green) were added to visualize microtubules and DNA, respectively. Scale bar, 10 μm.

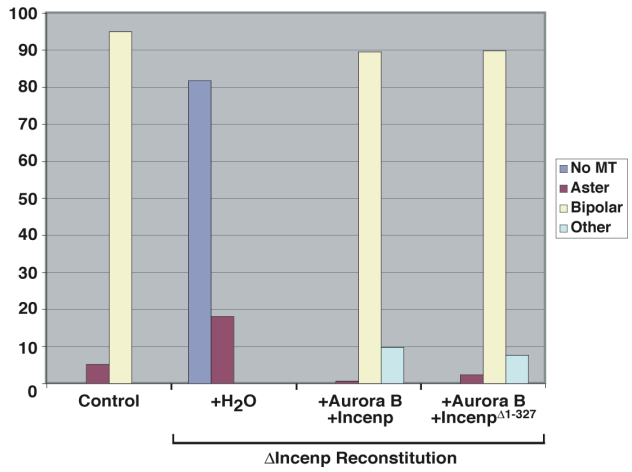

Figure 3-7. The N-terminal Domain of Incenp is Dispensable For Spindle Formation In the Presence of αIncenp Antibodies
Quantitation of DNA-containing structures formed in the extracts described in Figure 3-6; >250 structures were scored for each sample.

Achromosomal Spindle Formation is Induced by Aurora B and Incenp

On closer inspection of the structures formed in reconstituted ΔIncenp extract, we noticed the presence of numerous apparently bipolar structures lacking sperm chromosomes (Figure 3-6, arrowheads). These achromosomal bipolar spindles were observed in ΔIncenp extract reconstituted with Aurora B and either full-length Incenp or Incenp$^{\Delta 1\text{-}327}$, and could be seen both in the vicinity of chromosomal spindles and completely separated from them (Figures 3-6, 3-8). Although such achromosomal spindles comprised up to ~50% of all bipolar structures in reconstituted extracts, they could not be detected in either control depleted or unreconstituted ΔIncenp extracts (Figure 3-7). Interestingly, preliminary data suggests that the formation of achromosomal bipolar spindles by Aurora B and Incenp may be partially suppressed by the addition of mRNAs encoding Dasra A/Dasra B/Survivin/SIX, while this suppression was not seen in extracts expressing Aurora B and Incenp$^{\Delta 1\text{-}327}$ (data not shown).

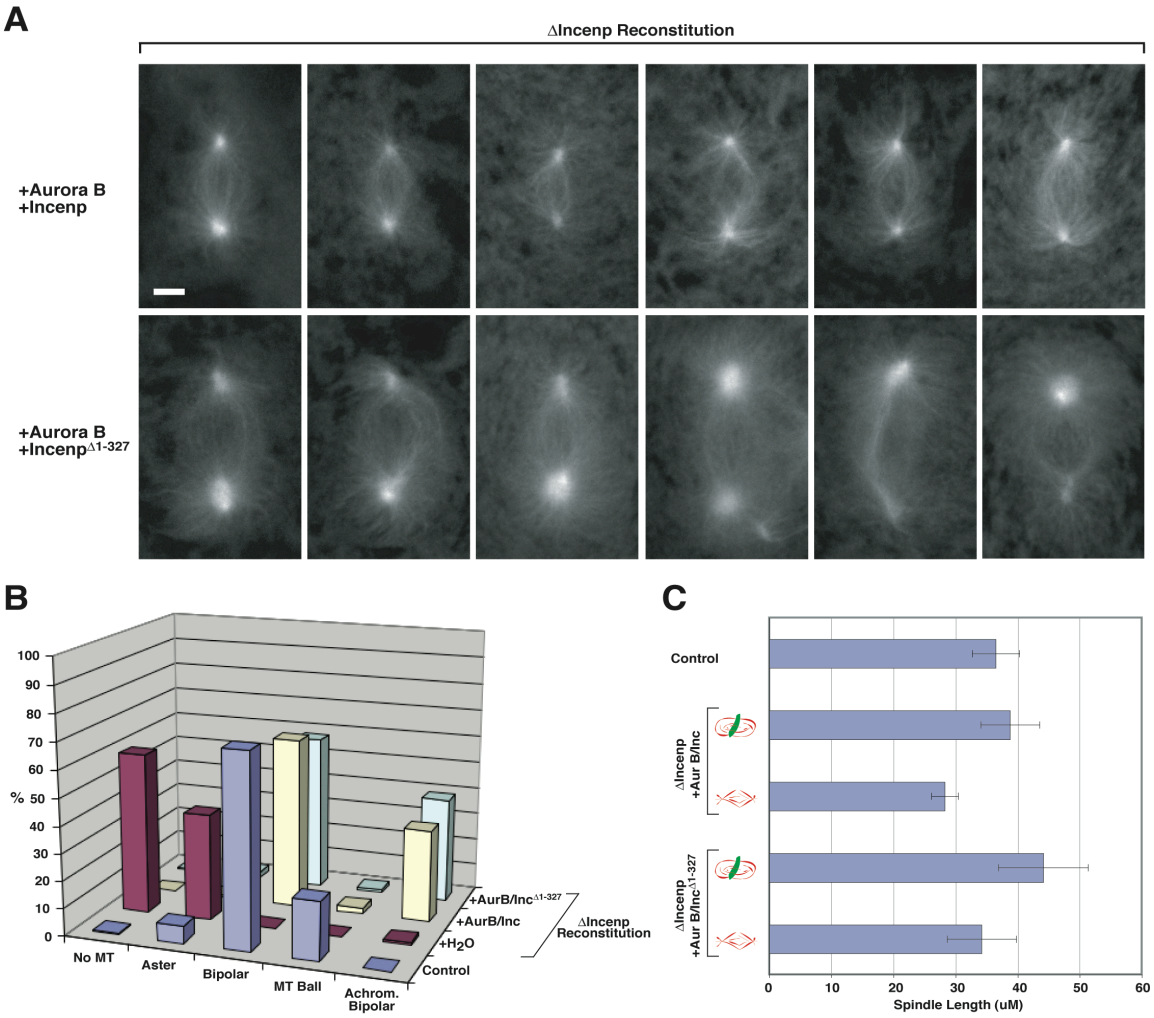

Figure 3-8. Addition of Aurora B and Incenp to ΔIncenp Extract Induces Achromosomal Spindle Formation

(A) Representative images of achromosomal spindles formed in cycled ΔIncenp extract reconstituted with Aurora B and either full length Incenp (top row) or the N-terminal truncation mutant Incenp$^{\Delta 1\text{-}327}$ (bottom row), which does not interact with Dasra A/Dasra B or Survivin/SIX. Rhodamine-tubulin (monochrome) and Hoechst 33258 were added to visualize microtubules and DNA, respectively. None of the depicted figures contained Hoechst-stainable material (data not shown). Scale bar, 10 μm.

(B) Quantitation of structures formed in control, ΔIncenp, and reconstituted ΔIncenp extract, including achromosomal bipolar spindles.

(C) Length of chromosome-containing and achromosomal spindles formed in control, ΔIncenp, or reconstituted ΔIncenp extracts. Pole-to-pole distance was measured for cycled spindles fixed 45 min after entry into metaphase. Mean plus standard deviation for >50 spindles of each class is included.

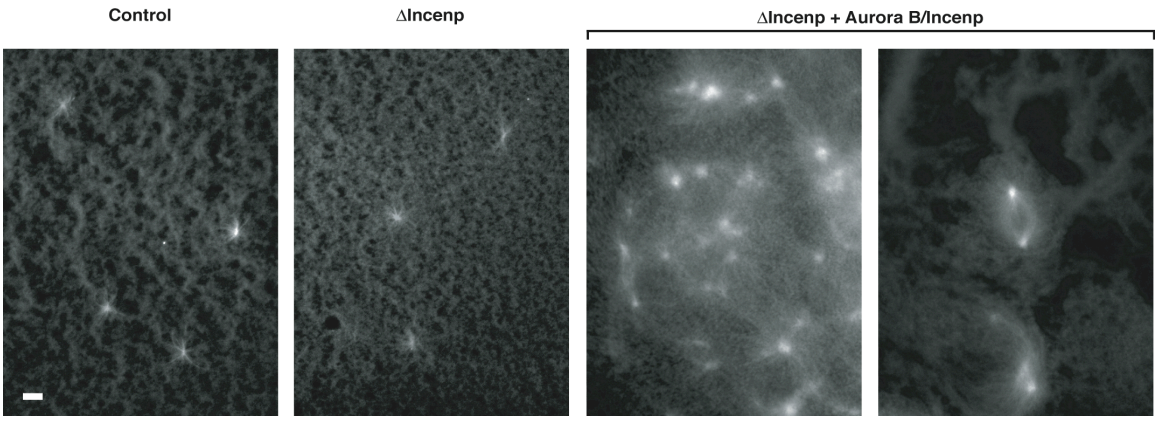

Figure 3-9. Addition of Aurora B and Incenp to ΔIncenp Extract Induces Achromosomal Spindle Formation
Spindle formation by purified centrosomes in reconstituted ΔIncenp extract. Purified centrosomes (a gift of K. Kinoshita) were added to control, ΔIncenp, or reconstituted ΔIncenp extract, cycled through interphase to metaphase, and fixed 45 min after entry into metaphase. Rhodamine-tubulin was added to visualize microtubules (monochrome). Scale bar, 10 μm.

Qualitatively, the achromosomal spindles observed in reconstituted extracts were unlike normal spindles assembled around sperm chromatin, since they possessed prominent astral microtubules, sparse overlapping interpolar microtubules, and reduced overall length and tubulin density (Figure 3-8A, 3-8C). These characteristics led us to speculate that they were generated via interaction of centrosomal rather than chromatin-derived microtubules. To test whether achromosomal spindles could be produced in the complete absence of chromatin, we added purified centrosomes to ΔIncenp extract reconstituted with Aurora B and Incenp. While little or no interaction was observed between centrosomes in control or ΔIncenp extract, abundant centrosome association was seen in the reconstituted ΔIncenp extract, leading to the generation of both bipolar structures and much larger, mesh-like networks of centrosomes (Figure 3-9). As expected, achromosomal bipolars also formed in the presence of sperm nuclei in these experiments. In contrast, achromosomal spindle formation was never observed in

reconstituted extract alone (data not shown). Taken together, these findings indicate that the formation of achromosomal spindles in this system either directly or indirectly involves the activity of Aurora B and centrosomes.

Op18/Stathmin Phosphorylation is Increased in Reconstituted ΔIncenp Extract

To begin to address the molecular events underlying the achromosomal spindle formation observed in reconstituted ΔIncenp extract, we considered the possibility that depletion and reconstitution might in some way lead to hyperactivation of one of the known pathways of spindle formation; these include Aurora B-dependent MCAK phosphorylation (Ohi et al., 2004; Sampath et al., 2004) and phosphorylation of Op18/Stathmin (Andersen et al., 1997; Budde et al., 2001). Since activation of these two pathways should lead to increased phosphorylation of MCAK and Op18, respectively, we immunoprecipitated MCAK and Op18 from metabolically labeled control or ΔIncenp extract, with or without reconstitution of Aurora B and Incenp, and quantified the degree of labeling of each protein following SDS-PAGE and exposure to a PhosphorImager. We observed that control, reconstituted control, and ΔIncenp extracts showed no differences in the level of MCAK and Op18 labeling (Figures 3-10A, 3-10B). In contrast, reconstitution of ΔIncenp extract with Aurora B and Incenp lead to a relatively modest increase in MCAK phosphorylation (~40%; Figure 3-10A), but a substantial increase in Op18 phosphorylation (~150%; Figure 3-10B).

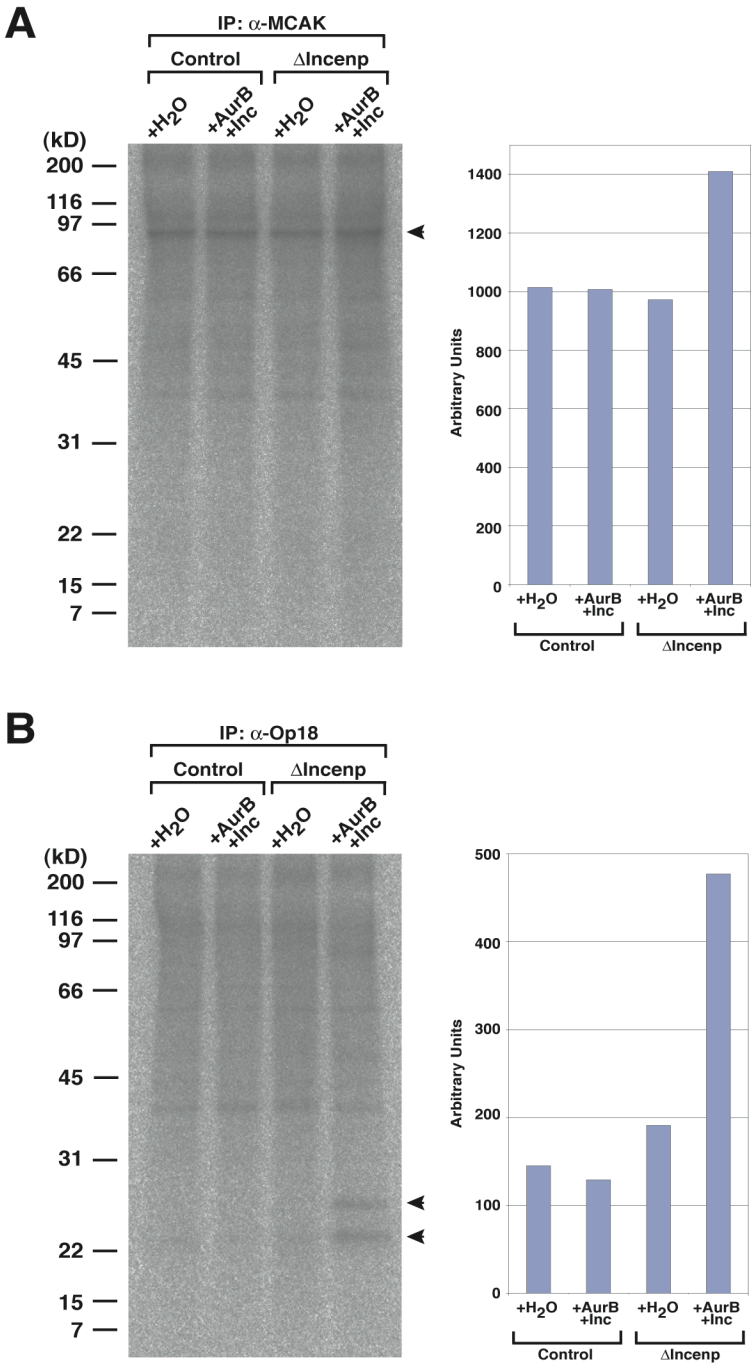

Figure 3-10. Reconstitution of ΔIncenp Extract Leads To Hyperphosphorylation of Op18/Stathmin

Control and ΔIncenp extract were cycled through interphase to metaphase with or without reconstitution of Aurora B and Incenp. Metaphase samples were labeled with γ-^{32}P-ATP and immunoprecipitated with either α-MCAK (A) or α-Op18 (B) beads. The bead-bound fraction was washed extensively in the presence of phosphatase inhibitors, separated by SDS-PAGE, and exposed to a PhosphorImager for quantitation of the indicated bands (right).

The dramatic increase in Op18 phosphorylation observed after reconstitution of ΔIncenp extract was surprising, since no DNA or any other source for microtubule stabilization was added to the reactions, and Op18 has been previously reported to become hyperphosphorylated in response to microtubule stabilization (Kuntziger et al., 2001). These findings therefore prompted us to further investigate the regulation of Op18 by Aurora B.

Op18 Hyperphosphorylation is CPC-Dependent, and is Activated in Reconstituted ΔIncenp Extract

Op18 has been reported to become phosphorylated on serine 16 in response to microtubule stabilization mediated by sperm nuclei, centrosomes, or isolated chromatin (Kuntziger et al., 2001), however it has not been reported to be phosphorylated by Aurora B. To establish whether Op18 hyperphosphorylation is dependent on the CPC, we added increasing doses of sperm nuclei, centrosomes, or DNA beads to control or ΔIncenp extract, and monitored Op18 phosphorylation by Western blot. We found that the slowest migrating, most highly phosphorylated form of Op18, corresponding to the serine 16-phosphorylated form (Kuntziger et al., 2001), was induced by all three stimuli in a dose-dependent manner, but was completely absent in ΔIncenp extract (Figure 3-11). This result demonstrates that the CPC is required either directly or indirectly for Op18 phosphorylation. Importantly, as suggested above by the induction of centrosomes interaction in reconstituted ΔIncenp extracts, this finding again indicates that the Aurora B pathway is activated by centrosome- or microtubule-derived signals.

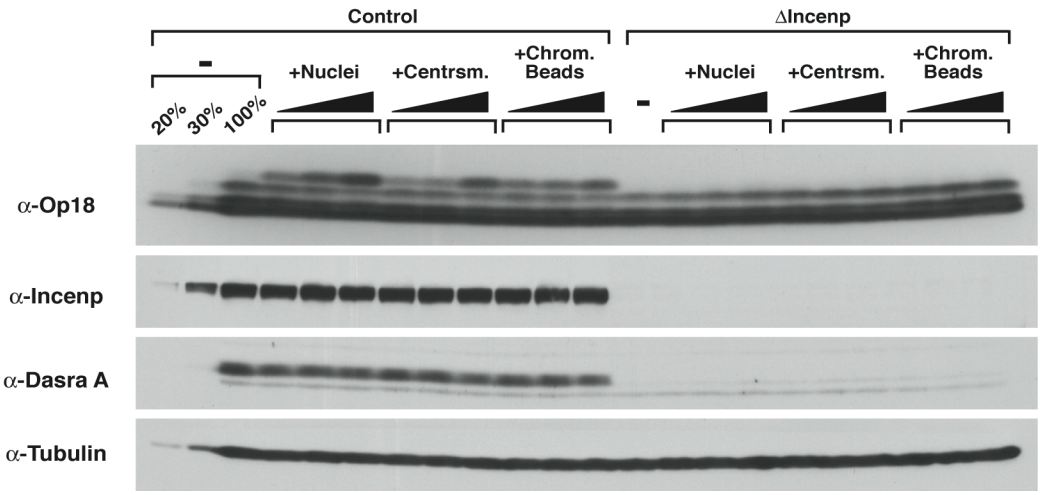

Figure 3-11. Op18 Hyperphosphorylation is CPC-Dependent, and is Activated in Reconstituted ΔIncenp Extract

Op18 hyperphosphorylation is CPC-dependent. Control and ΔIncenp extracts containing increasing amounts of sperm nuclei (500, 2000, or 10000/μl), centrosomes (250, 1000, or 10000/μl), or DNA beads (1, 2, or 5 μl) were added to CSF arrested control or ΔIncenp extract, and were cycled through interphase to metaphase. Samples were taken 45 min after entry into metaphase and analyzed by Western blot using the indicated antibodies, including α-Op18 (a gift of R. Heald).

The finding that Op18 becomes constitutively hyperphosphorylated following reconstitution of ΔIncenp extract with Aurora B/Incenp, but not by Aurora B/Incenp expression in control extract, suggested that CPC depletion might lead to codepletion of an unknown inhibitory factor. One potential source of inhibition might be Dasra proteins or Survivin/Six, particularly given that ~30% of Survivin usually remains undepleted, and therefore might serve to inhibit exogenous protein. To investigate this possibility, we examined Op18 phosphorylation by Western blot in control or ΔIncenp extracts reconstituted with Aurora B and Incenp$^{\Delta 1\text{-}327}$, which cannot bind to Dasra proteins or Survivin/SIX (Figure 3-5). We observed that Op18 still became hyperphosphorylated in reconstituted extract in the presence of Incenp$^{\Delta 1\text{-}327}$ (Figure 3-12), suggesting that, if it existed, the unknown inhibitory factor was not Dasra A, Dasra B, Survivin, or SIX.

Moreover, the fact that Aurora B and Incenp expression in control extract does not lead to Op18 hyperphosphorylation indicates that this effect is not due to CPC overexpression.

We eventually found that, as with spindle formation, both the constitutive activation of Op18 phosphorylation and achromosomal spindle formation in reconstituted ΔIncenp extract were dependent on the presence of leached αIncenp antibody in the immunodepleted extract (A. Kelly and S.C.S., data not shown). Direct addition of αIncenp antibodies to control extracts induces both CPC-dependent Op18 phosphorylation, as well as achromosomal spindle formation by centrosomes (A. Kelly, data not shown).

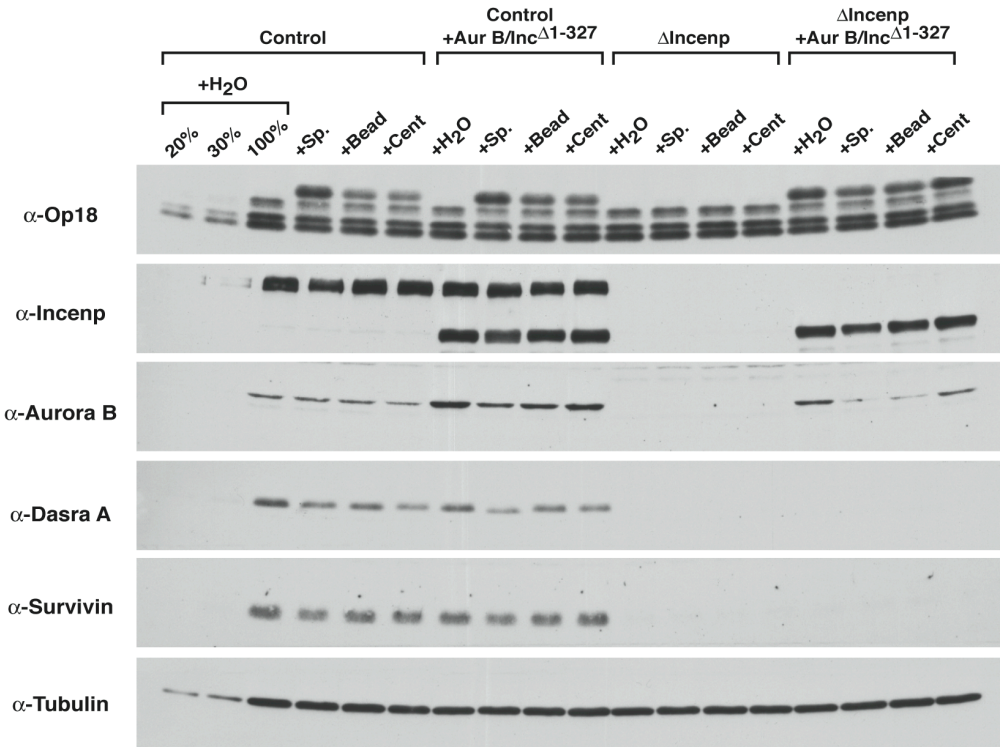

Figure 3-12. Op18 Hyperphosphorylation is CPC-Dependent, and is Activated in Reconstituted ΔIncenp Extract
Reconstitution of ΔIncenp extract leads to Op18 hyperphosphorylation. Control or ΔIncenp extracts containing water, sperm nuclei (10000/μl), centrosomes (3000/μl), or DNA beads (5 μl) with or without reconstitution were cycled through interphase to metaphase. Samples were taken 60 min after entry into metaphase and analyzed by Western blot using the indicated antibodies.

Op18-mediated microtuble destabilization has been shown to be regulated by phosphorylation (Gavet et al., 1998; Horwitz et al., 1997; Larsson et al., 1997; Marklund et al., 1996), and serine 16 phosphorylation in particular has been shown to inhibit its microtubule-destabilizing activity (Melander Gradin et al., 1997). The data presented here therefore suggest that at least part of the mechanism by which reconstituted Aurora B induces the formation of achromosomal spindles by centrosomes is via constitutive inhibition of Op18-mediated microtubule destabilization.

Dasra Proteins Regulate Spindle Checkpoint Activity and CPC Chromosome Binding

The above data define distinct roles for Aurora B and Incenp in promoting spindle formation, including functions both at chromosomes and on centrosomes. To further investigate the spatial regulation of CPC function, we examined the requirements for mitotic spindle checkpoint activity, which is thought to require localization of the complex to the centromere (Vigneron et al., 2004). Aurora B-dependent mitotic checkpoint activity can be activated in egg extract by the addition of high concentrations of sperm nuclei in the presence of nocodazole (Gadea and Ruderman, 2005; Vigneron et al., 2004). Accordingly, we tested the ability of reconstituted ΔIncenp extracts to support checkpoint activity after cycling and adding 9000 sperm nuclei per microliter of extract. Checkpoint activity can be monitored by analyzing chromosome morphology and cyclin degradation after the addition of calcium to induce anaphase; in the presence of an activated checkpoint, chromosomes should remain condensed and cyclin levels should

remain stable. As expected, we found that chromosomes from control depleted extract remained condensed after calcium addition, whereas chromosomes in ΔIncenp extract had an interphase morphology (Figure 3-13A). This was reflected in the maintenance and loss, respectively, of cyclin B1 levels in control and ΔIncenp extract (Figure 3-13B). Importantly, addition of all CPC mRNAs to ΔIncenp extract rescued the spindle checkpoint defect, and both mitotic chromosomes condensation and cyclin B1 levels were maintained. As observed for spindle assembly, mRNA pools lacking Aurora B and Incenp were unable to rescue spindle checkpoint function. Surprisingly however, we found that the absence of Dasra proteins also led to the complete abrogation of spindle checkpoint activity (Figure 3-13A, 3-13B). Addition of either Dasra A or Dasra B to the mRNA pools was sufficient to restore checkpoint activity. In contrast, Survivin/SIX deficiency did not affect checkpoint activity, though we cannot rule out the possibility that the residual ~25% of undepleted Survivin protein is sufficient to confer checkpoint function.

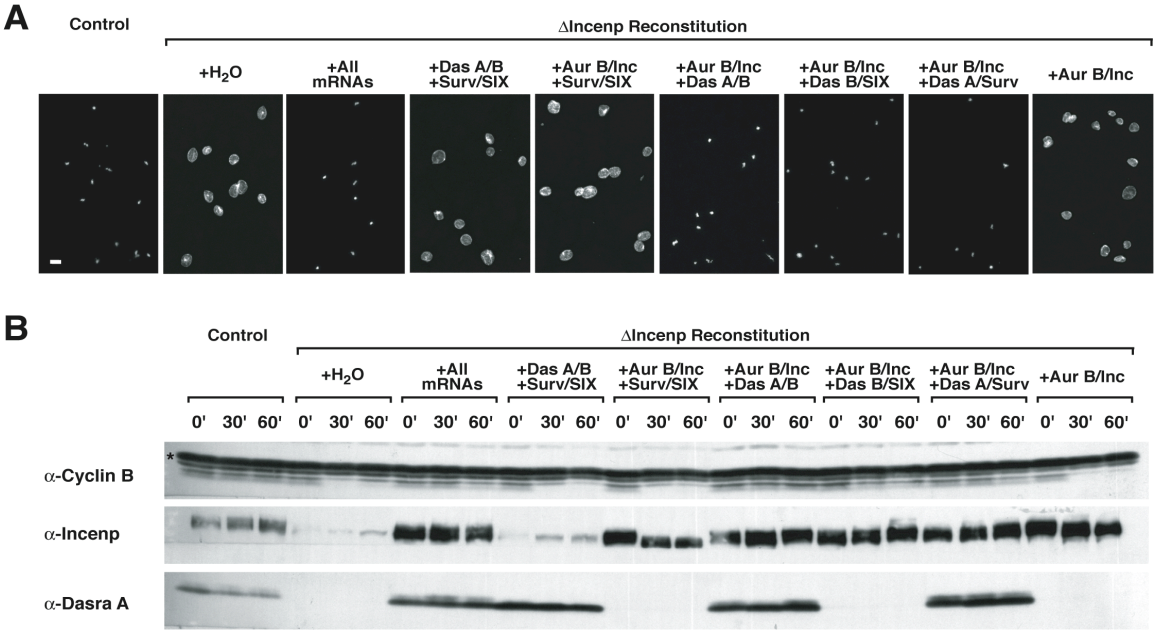

Figure 3-13. Dasra Proteins Are Required for Spindle Checkpoint Activity in *Xenopus* Egg Extract

(A) Chromosome morphology assay for spindle checkpoint activity. CSF arrested control, ΔIncenp, or reconstituted ΔIncenp extracts were cycled through interphase to metaphase, and 10000 sperm nuclei/μl egg extract were added. Extracts were then incubated for 30 min in the presence of nocodazole, calcium was added to induce exit from metaphase, and chromosome morphology was examined in fixed samples 60 min after calcium addition. Note that the compact metaphase morphology is due to the presence of nocodazole, whereas interphase nuclei are decondensed and swollen. Hoechst 33258 was added to visualize DNA (monochrome). Scale bar, 10 μm.
(B) Western blot analysis of control, ΔIncenp, or reconstituted ΔIncenp extracts following checkpoint activation and release from CSF arrest. Aliquots of the cycled, nocodazole-treated samples described in (A) were taken at 0 min, 30 min, or 60 min after the addition of calcium to release CSF arrest. Western blots of total proteins were performed using the indicated antibodies. Asterisk (upper band) indicates a cross-reactive species.

We previously demonstrated that loss of hDasra B function in mammalian cells inhibits proper localization of the passenger complex during mitosis (Sampath et al., 2004). We therefore investigated whether the lack of spindle checkpoint function in Dasra-deficient extract might be due to lack of CPC binding to mitotic chromosomes. Metaphase chromosomes were purified from reconstituted ΔIncenp extract which had

been metabolically labeled with ^{35}S-methionine, and the extent of copurification of the labeled CPC members was examined by SDS-PAGE (Figure 3-14A). We observed that ~9-fold less Incenp and Aurora B, and 25-fold less Survivin were recovered when Dasra proteins were absent than in their presence. Chromosomes purified in the absence of both Dasra proteins and Survivin/SIX showed no additive effect on suppression of Aurora B and Incenp chromosomal loading. To confirm and extend this result, we repeated the experiment using chromatin beads rather than sperm chromosomes as the source of DNA. Essentially the same effect was seen on purified chromatin beads, which contained ~20-fold less Incenp, 15-fold less Aurora B, and 20-fold less Survivin in the absence of Dasra proteins than in their presence (Figure 3-14B).

It is important to note that a fraction of Aurora B and Incenp were still recovered in the absence of Dasra A/B and Survivin/SIX using both sperm chromosomes and chromatin beads, which may indicate the existence of a parallel pathway for the loading of this subcomplex onto metaphase chromosomes. One possibility in this regard is chromosomal recruitment through the previously described interaction between Incenp and Heterochromatin Protein 1 (HP1), which involves the hinge region of HP1 (Ainsztein et al., 1998). Alternatively, this effect may also reflect the presence of αIncenp-dependent constitutive Aurora B activation.

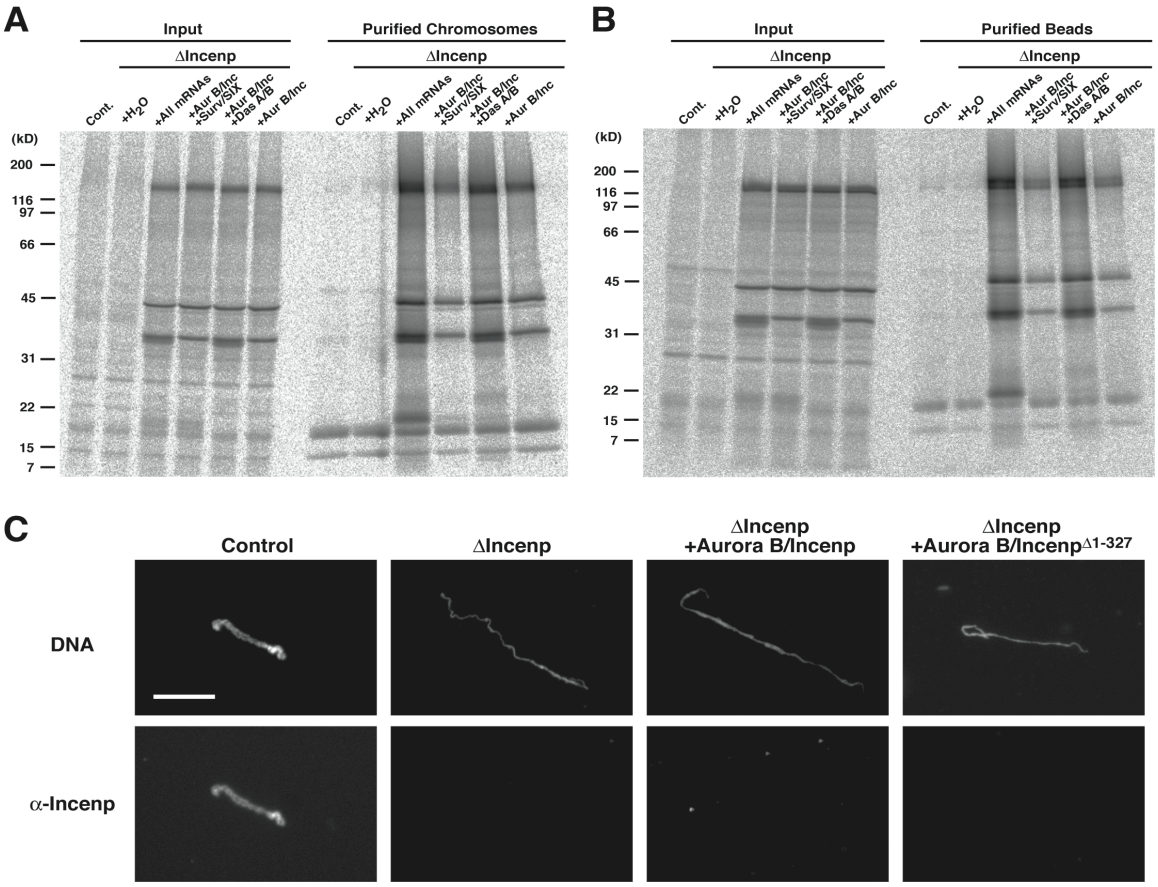

Figure 3-14. Dasra Proteins Are Required for Efficient CPC Chromosome Binding in *Xenopus* Egg Extract

(A) Dasra proteins are required for efficient CPC binding to chromosomes. Metabolically labeled control, ΔIncenp, or reconstituted ΔIncenp extracts containing sperm nuclei, ^{35}S-methionine, and biotin-dUTP were cycled through interphase to metaphase, and chromosomes were purified using streptavidin-coated magnetic beads. Copurification of labeled proteins was analyzed by SDS-PAGE and quantified using a PhosphorImager. Note that copurifed labeled histones are evident due to incomplete degradation of their abundant mRNAs during RNase treatment, and indicate equal chromosome recovery, as does Coomassie staining (data not shown).

(B) Dasra proteins are required for efficient CPC binding to chromatin beads. Metabolically labeled control, ΔIncenp, or reconstituted ΔIncenp extracts containing DNA beads and ^{35}S-methionine were cycled through interphase to metaphase, and the beads were recovered using a magnetic particle separator. Copurification of labeled proteins was analyzed as described above (C).

(C) Incenp localization on individual chromosomes. Control, ΔIncenp, or reconstituted ΔIncenp extracts containing sperm nuclei were cycled through interphase to metaphase, diluted with chromosome dilution buffer, and processed for indirect immunofluorescence using anti-Incenp antibodies (bottom), and Hoechst 33258 to visualize DNA (top). Scale bar, 10μm.

Because chromatin beads do not contain the repetitive centromeric sequences required for kinetochore assembly, they are thought to mimic chromosome arms in composition. Indeed, we found that *in vitro* translated histone H3, but not the centromeric H3 variant Cenp-A, copurified with chromatin beads incubated in egg extract (Figure 3-15). The failure of the CPC to load onto chromatin beads in Dasra-deficient extract therefore suggests that Dasra proteins are required for CPC binding to chromosome arms during mitosis. Consistent with this model, immunofluorescence on individual chromosomes revealed lack of Incenp staining on both centromeres and chromosome arms in the absence of Dasra proteins (Figure 3-14C).

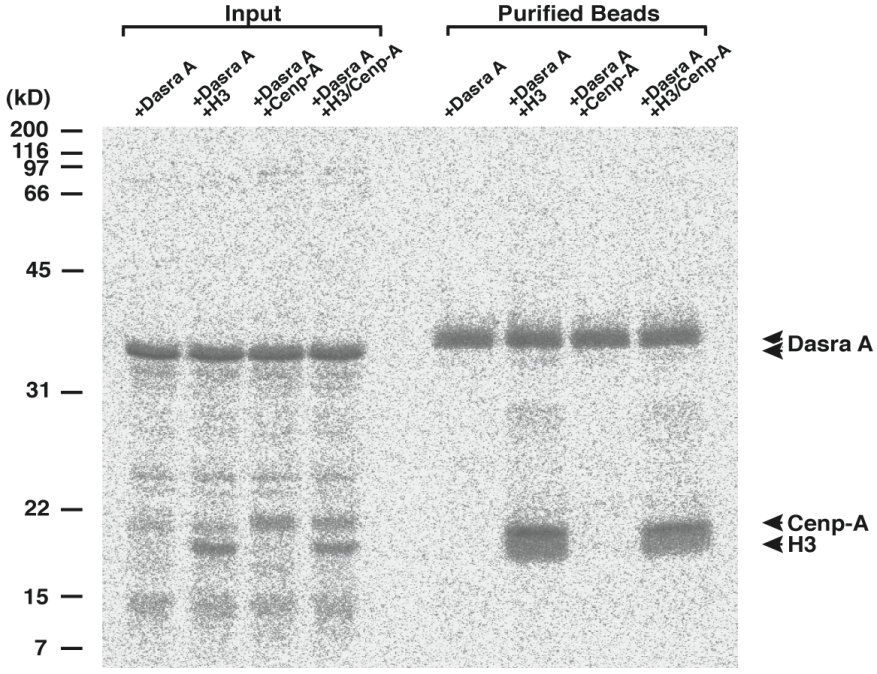

Figure 3-15. Chromatin Beads Bind Histone H3, But Not the Centromeric H3 Variant Cenp-A
Egg extract containing DNA beads and the indicated *in vitro* translated proteins were cycled through interphase to metaphase, the beads were recovered using a magnetic particle separator, and copurifying proteins were examined by SDS-PAGE and autoradiography. Aliquots of the cycled and labeled samples before bead purification are shown at left. Note that both H3 and Dasra A shift to higher molecular weight after becoming chromatin-associated.

CHAPTER 4

Discussion

Our studies on the chromosomal passenger complex (CPC) began with our identification of Dasra A and Dasra B as two novel components of the CPC in vertebrates. In the process of exploring the functions of these two proteins, we made the unexpected finding that the CPC is strictly required for chromatin-dependent spindle assembly in *Xenopus* egg extracts. Nonetheless these initial studies left the actual functions of the Dasra proteins in doubt. Our subsequent studies on CPC function using depletion and add-back have allowed us to assign functions to the Dasra proteins, and serendipitously led us to uncover a new and unanticipated role of the CPC in the regulation of centrosome-derived astral microtubules. This finding in turn led us to define a new role for Aurora B in the phosphorylation of microtubule destabilizing protein Op18.

Dasra A and Dasra B Are Novel, Evolutionarily Conserved Components of the CPC

Our analysis indicates that, along with Aurora B, Incenp, and Survivin, Dasra A and Dasra B are components of the *Xenopus* chromosomal passenger complex. In addition to sharing the dynamic localization pattern observed for the known CPC components (Figures 2-2A, 2-2C, 2-5, 2-6D), Dasra A codepletes with the CPC (Figures 2-6B, 2-7), Dasra A and B coprecipitate with Incenp (Figures 2-6C, 2-8A), and Dasra A cosediments with Incenp, Aurora B, and Survivin by sucrose density centrifugation (Sampath et al., 2004). We detect these proteins (and possibly SIX), but no other major bands in precipitates of metabolically labeled CPC, and these are the major proteins absent on

Incenp-depleted chromosomes (Figure 4-1), therefore we believe that these five proteins comprise the major components of the CPC in *Xenopus*. We do not exclude the possibility, however, that other proteins may codeplete with the complex, although they may not bind at equal or near-equal molarity with the other components of the CPC (see below).

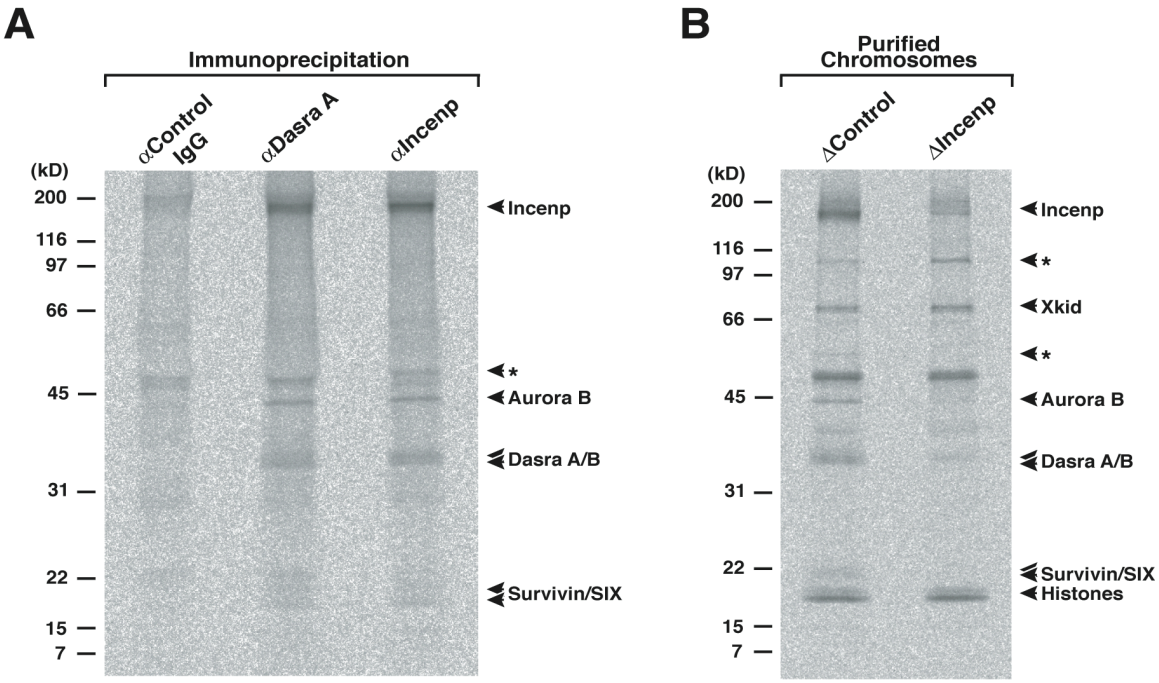

Figure 4-1. Incenp, Aurora B, Dasra A/Dasra B, and Survivin/SIX are the Primary Components of the CPC in *Xenopus* Egg Extract
(A) Metabolic labeling of CPC components. Egg extract was labeled with ^{35}S-methionine, immunoprecipitation was carried out with control IgG, anti-Dasra A, or anti-Incenp antibodies, and the precipitated proteins were resolved by SDS-PAGE and autoradiography. Asterisk indicates an unknown protein in the anti-Incenp IP.
(B) Protein composition of control and CPC-deficient chromosomes. Control and ΔIncenp egg extract were metabolically labeled with ^{35}S-methionine, chromosomes were purified, and the recovered chromosome-binding proteins were resolved by SDS-PAGE and autoradiography. Asterisk indicates unknown proteins which change in abundance between control and ΔIncenp chromosomes.

Xenopus Dasra A and Dasra B share only 26% identity at the amino acid level, similar to the level of identity between *Xenopus* Dasra A and human Dasra B (23%). In contrast, *Xenopus* Dasra A is 41% identical to chicken Dasra A, and *Xenopus* Dasra B is 42% identical to human Dasra B. It would thus seem to be the case that Dasra A and Dasra B are indeed evolutionarily (and thus functionally) distinct genes, rather than simply representing products of gene duplication within *Xenopus*. The precise functional differences between Dasra A and Dasra B remain mysterious; for instance, while we have established that Dasra proteins are required for spindle checkpoint activity in egg extract (Figure 3-13), both Dasra A and Dasra B are capable of fulfilling this function when added exogenously, a remarkable finding given the sequence dissimilarity between the two proteins. In the future, restoration of spindle checkpoint activity in ΔIncenp extract may provide an assay which can be used for domain-swap experiments, which may delineate the functionally conserved regions of both proteins. Structural data could also be particularly illuminating in this regard.

Dasra B, but not Dasra A, shows very limited sequence similarity to CSC-1, a component of the CPC in nematodes [Figure 2-3; (Romano et al., 2003)]. Much of the identity between xDasra B and CSC-1 is confined to a small C-terminal patch of unknown significance. In the future is will be important to investigate the function of this domain, potentially using spindle checkpoint activity as an assay as described above.

Chromatin-Dependent Spindle Formation by Aurora B and Incenp

As discussed in Chapter 1, several pathways are thought to be required for the induction of spindle formation around chromatin during M-phase; this includes, but is not

limited to, the Ran-GTP pathway (Carazo-Salas et al., 2001), and the Polo/Op18 pathway (Budde et al., 2001). Our findings demonstrate that depletion of the CPC leads to a profound block in spindle formation around chromatin; in ΔIncenp extract, sperm-associated centrosomes initially nucleate microtubules, but these microtubules fail to be incorporated and converted into chromatin-stabilized spindles (Figure 2-12). It has been previously demonstrated that centrosomes undergo long-range communication with chromatin, such that purified centrosomes added to egg extract containing chromatin beads preferentially grow towards the beads (Carazo-Salas and Karsenti, 2003). It therefore seems likely that Aurora B not only promotes microtubule stability around chromatin, but is also part of the mechanism underlying this communication between chromatin and centrosomes.

We show that the microtubule assembly defect observed in ΔIncenp extract can be at least partially reversed by codepletion of the microtubule depolymerase MCAK. Depletion of MCAK itself causes the formation of giant microtubule 'halos', and although some bipolar spindles can be formed on CPC/MCAK codepletion, most structures are qualitatively similar to those seen in ΔMCAK extract. This would seem to argue that MCAK depletion simply causes non-specific microtubule stabilization, and therefore that although Aurora B and MCAK have antagonistic activities, they do not necessarily act within a common pathway. Such a position is unlikely to be valid, however, since codepletion of MCAK with XMAP215, a well characterized microtubule stabilizing protein (Kinoshita et al., 2001; Tournebize et al., 2000), does not lead to microtubule 'halos'.

Alternatively, work from several groups now suggests that a functional relationship does indeed exist between Aurora B and MCAK. In particular, it has now been demonstrated that Aurora B can phosphorylate and inactivate the microtubule destabilizing activity of MCAK (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004). Spatially, such regulation seems plausible, since both Aurora B and MCAK reside at the inner centromere, and Aurora B-mediated phosphorylation appears crucial for localization of MCAK to the centromeres of metaphase chromosomes in *Xenopus* egg extract (Ohi et al., 2004). Interestingly, when ΔMCAK extract was reconstituted with a non-phosphorylatable mutant, bipolar spindle assembly was inhibited, and predominantly monopolar structures were formed (Ohi et al., 2004). This phenotype resembles the ΔDasra A depletion phenotype, suggesting that the partial loss of CPC function in ΔDasra A may phenocopy loss of MCAK regulation.

Aurora B, Incenp, and Dasra Proteins Regulate Chromatin-Dependent Spindle Assembly

We proposed on the basis of the findings presented in Chapter 1 that the CPC acts locally to promote microtubule stabilization around chromosomes (Sampath et al., 2004), however determination of the precise functions of each CPC component towards that function eventually required the use of the 'mRNA-dependent' extract system. Although Aurora B and Incenp have been previously described to form a subcomplex (Gassmann et al., 2004), our finding that these two proteins could together support spindle formation was surprising, since we provide evidence that Aurora B and Incenp bind only relatively weakly to chromosomes in the absence of Dasra proteins (Figure 3-14).

This discrepancy was eventually reconciled by the detection of αIncenp antibodies in the reconstituted ΔIncenp extracts; these antibodies induce dose-dependent CPC activation, suggesting that in the absence of Dasra proteins, antibody-induced CPC activation leads to spindle formation. This conclusion was subsequently supported by the finding that extracts depleted of the CPC using beads bearing covalently coupled antibodies are dependent on Dasra proteins for chromatin-dependent spindle formation. Overall, our findings suggest a model in which Dasra proteins promote CPC binding to and activation by chromatin, leading to spindle formation, while artificial activation of the CPC using αIncenp antibodies bypasses the necessity for Dasra proteins in spindle assembly. This model is supported by the recent finding that The molecular nature of this activating effect remain unclear, but the bivalent structure of immunoglobulin molecules suggests that clustering of CPC holocomplexes by antibody may promote their *trans*-phosphorylation and activation. Future experiments using a constitutively activated mutant of Aurora B (Eyers et al., 2005) may shed further light on the roles of the Dasra proteins in CPC localization and activation.

These data, as well as the finding that CPC-dependent Op18 phosphorylation is increased in the presence of sperm nuclei and chromatin beads (Figure 3-11), suggest that Aurora B may become activated by mitotic chromatin. Indeed, it has very recently been reported that hDasra B can bind directly to dsDNA *in vitro* (Klein et al., 2006), suggesting that Dasra-dependent DNA binding promotes CPC activation and spindle assembly. We have found that Aurora B binding to mitotic chromosomes is dynamic; when chromosomes purified from control extract are transferred to ΔIncenp extract, microtubules polymerize only briefly before collapsing (Figures 2-18, 2-19), likely due

to rapid exchange between the cytosolic and chromatin-bound CPC populations. We therefore propose that the activated form of Aurora B generated on chromatin creates a zone of localized MCAK inhibition, the extent of which would be limited by diffusion and the abundant phosphatase activity present in the cytosol.

In many ways this model resembles the emerging theory of Ran-GTP-dependent spindle formation, in which chromatin-bound RCC1 induces the formation of a high local concentration of Ran-GTP, which is required for spindle formation (Carazo-Salas et al., 1999; Kalab et al., 1999; Kalab et al., 2002). Recent studies indicate that long-range gradients of Ran-GTP/Importin-β are important for biased microtubule growth and spindle assembly in egg extracts (Caudron et al., 2005); such gradients can be modulated by the addition of RanGAP, RanBP1, or RCC1, which therefore perturb spindle formation (Caudron et al., 2005; Kalab et al., 1999). We speculate that Aurora B activation on chromatin may likewise break the symmetry of mitotic cytoplasm by generating a field of microtubule stabilization around chromatin, with the phosphatases present in the cytoplasm playing an antagonistic but essential role analogous to those of RanBP1 and RanGAP.

Multiple Pathways Contribute to Chromatin-Dependent Spindle Assembly

If the above model is correct, in what way would Aurora B function differ from that of Ran? Although many analogies may be drawn between the Ran and Aurora B pathways, many significant differences also exist. For instance, while Ran only requires binding to GTP for activation, Aurora B is dependent on continued binding to and phosphorylation of Incenp for its full activation (Sessa et al., 2005). This is reflected in the fact that, while

addition of Ran-GTP to extracts causes spontaneous aster formation and spindle assembly in the absence of chromatin (Carazo-Salas et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999), expression of Aurora B alone in egg extract has no significant effect on microtubule assembly (Figure 3-3, and data not shown). Likewise, preliminary data suggests that addition of a constitutively activated Aurora B mutant does not lead to spontaneous microtubule polymerization (T. Maniar and S.C.S., data not shown).

These findings underscore a more fundamental dissimilarity between the Ran and Aurora B pathways: while Ran-GTP is capable of initiating *de novo* microtubule nucleation, Aurora B seems largely to stabilize pre-nucleated structures. Taken together, it therefore appears likely that these two pathways must work together in order to organize chromatin-dependent spindle assembly, for instance through Ran-dependent microtubule nucleation followed by Ran- and Aurora B-dependent microtubule stabilization. Clearly, further investigation will be required to elucidate the downstream effectors of both pathways.

An additional layer of complexity is added by our finding that Aurora B also regulates Op18/Stathmin phosphorylation, a finding also recently described by others (Gadea and Ruderman, 2006); this function had previously ascribed to the chromatin-associated Polo-like kinase Plx1. We find that the CPC is indispensable for hyperphosphorylation of Op18 in response to a variety of stimuli, including sperm nuclei, centrosomes, chromatin beads, and taxol (Figure 3-11, and data not shown). Hyperphosphorylation of Op18 requires its phosphorylation on serine 16, which exists within a perfect Aurora B consensus site (KRAS; consensus [K/R][K/R]x[S/T]), suggesting that this site is a direct target of Aurora B kinase activity. Indeed, recent data demonstrate that the Aurora B-

containing CPC can phosphorylate serine 16 of Op18 *in vitro*. This is significant, given that non-phosphorylatable mutants of Op18 inhibit spindle formation, while 'pseudophosphorylation' mutants lead to enhanced microtubule stability (Andersen et al., 1997; Budde et al., 2001; Marklund et al., 1996). Interestingly, preliminary data demonstrate that, contrary to published findings, sperm nuclei, centrosomes, and chromatin are all capable of stimulating CPC-dependent Op18 phosphorylation in the presence of nocodazole, suggesting that in these cases microtubules are not necessary for the activation of Op18 phosphorylation (T. Maniar, data not shown). It is clear that investigating the exact role of Aurora B in the regulation of Op18-mediated microtubule destabilization will be an important avenue for future work.

Achromosomal Spindle Formation

One of the most striking findings of our study was the observation of achromosomal spindle formation in ΔIncenp extract reconstituted with Incenp and Aurora B. Such a phenotype has been reported in only a handful of situations to date: during spermatogenesis in *Drosophila* mutants exhibiting severe chromosome missegregation (Bucciarelli et al., 2003), in PtK cell homokaryons containing supernumerary centrosomes (Faruki et al., 2002), and after addition of a constitutively activated Ran mutant to *Xenopus* egg extract (Carazo-Salas et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999). Interestingly, the achromosomal spindles formed in these otherwise disparate scenarios share similar structural features. In particular, the spindles described here resemble the bipolar structures induced by activated Ran, including the presence of few interpolar microtubules, low overall tubulin density, and more prominent astral

microtubules (Carazo-Salas et al., 1999; Wilde and Zheng, 1999). Moreover, as Faruki and colleagues observed in PtK homokaryons, we also noted the presence of achromosomal spindles containing only a single prominent microtubule bundle (Faruki et al., 2002); data not shown). The instability of achromosomal spindles formed in PtK homokaryons is also a characteristic shared with achromosomal spindles induced by Ran-GTP (J. Gaetz, personal communication). These findings may indicate that the lack of chromosome-derived signals places defined constraints on certain aspects of spindle structure, such as the extent and stability of antiparallel microtubule overlap, and the number of astral microtubule bundles polymerized from each centrosome.

What is the molecular basis of achromosomal spindle formation in reconstituted ΔIncenp extract? We eventually found that the presence of activating αIncenp antibodies can account for the Aurora B- and Incenp- dependent formation of these structures in reconstituted ΔIncenp extracts; direct addition of αIncenp antibodies to control extracts also promotes centrosomal interaction, although the structures generated under these circumstances do not generally resemble the bipolar spindles illustrated in this work (A. Kelly, data not shown). Nonetheless, it seems clear that the formation of achromosomal bipolar spindles is a consequence of hyperactivation of the CPC. The precise mechanisms involved downstream of Aurora B remain unclear, but may include regulation of both Op18 and MCAK. Future work will also need to address the potential contributions of both Aurora A and the Ran pathway to this effect.

The finding that centrosome addition causes dose-dependent Op18 phosphorylation by Aurora B (Figure 3-11) indicates that Aurora B can ‘communicate’ with centrosomes, though in some currently ill-defined way. This conclusion is consistent with our earlier

findings that while centrosomes do nucleate microtubules in ΔIncenp, these microtubules fail to become associated with chromatin, a striking deviation from the 'long-range communication' which occurs between centrosomes and chromatin in undepleted extract [Figure 2-12; (Carazo-Salas and Karsenti, 2003)]. As stated above, nocodazole treatment does not block centrosome-induced, CPC-mediated Op18 phosphorylation; since taxol treatment does lead to CPC-dependent Op18 phosphorylation (T. Maniar, data not shown), it appears that both centrosomes themselves and the microtubules generated from them have the potential to activate Aurora B-dependent microtubule stabilization via Op18 phosphorylation. We believe that these observations can begin to explain the ability of the activated form of Aurora B generated after depletion and add-back to promote centrosomal interaction. Such an activity would not be apparent from the analysis of ΔIncenp extract, since in the absence of Aurora B no stable microtubules are formed in the vicinity of chromosomes. It is interesting to note in this context that reconstitution of ΔMCAK extract with a form that cannot be phosphorylated by Aurora B leads to formation of monopolar spindles around sperm chromosomes (Ohi et al., 2004).

What does the observation of achromosomal bipolar spindle formation indicate about the endogenous function of Aurora B? As described above, Karsenti and colleagues have advocated the view that chromatin initially generates spatial gradients of information which break the uniformity of mitotic cytoplasm; the developing spindle then progressively converts from a radially symmetric configuration centered on chromatin, to a bilaterally symmetric orientation after the recruitment of centrosomes and microtubule motors (Carazo-Salas and Karsenti, 2003; Caudron et al., 2005). These long-range gradients are speculated to produce a zone of microtubule stability around chromatin,

which would account for the ability of centrosomal asters to sense the presence of and migrate towards chromatin from a distance (Carazo-Salas and Karsenti, 2003).

We believe that by activating Aurora B after depletion and reconstitution, we have effectively produced a field which mimics and signals the homogeneous presence of chromatin. The effect of such a field would be to create a 'uniformly localized' zone of microtubule stability, to which centrosomes are predicted to be recruited through the long-range communication mechanism previously described (Carazo-Salas and Karsenti, 2003). This prediction is consistent with our results, which demonstrate that reconstituted ΔIncenp extract induces the widespread interaction of centrosomes. Theoretical studies suggest that, once recruited, microtubule motors would be capable of organizing centrosomes into bipolar arrays (Nedelec, 2002), consistent with our observation of achromosomal bipolar spindles in reconstituted extract. Remarkably, we show that achromosomal spindles can be formed in the absence of chromatin-dependent Ran activation, although the total independence of such achromosomal spindles from Ran-GTP has not yet been established. We propose that endogenously, Aurora B acts as part of the symmetry-breaking mechanism predicted to produce gradients of microtubule stability, and which allows centrosome recruitment and retention within the nascent spindle. Put differently, our data suggests that by spatially regulating microtubule stabilization near chromosomes and promoting centrosome interaction with these microtubules, Aurora B acts as a master regulator of spindle assembly, integrating its astral and anastral aspects. To date the only molecule thought to be able to fulfill such a function is Ran itself.

Although no specific role has been reported for Aurora B in mitotic spindle bipolarization in cultured mammalian cells (Ditchfield et al., 2003; Hauf et al., 2003), loss of Aurora B does perturb spindle formation more generally (Andrews et al., 2004). In particular, injection of anti-Aurora B antibodies into *Xenopus* somatic cells causes astral microtubule elongation and reduction of spindle microtubule density (Kallio et al., 2002), and introduction of a dominant-negative Aurora B mutant causes formation of spindle microtubule 'bundles', all similar to effects seen in achromosomal spindles from ΔIncenp reconstituted extract. Moreover, Aurora B has been reported to localize to spindle poles, in addition to centromeres, during metaphase (Murata-Hori et al., 2002). We therefore suggest that Aurora B has an unappreciated role in the regulation of centrosomal microtubule dynamics and the establishment of spindle bipolarity; future work will examine what the molecular nature of this regulation might be, including spatial control of MCAK function and modulation of proteins such as Eg5, a plus end-directed kinesin known to be required for spindle bipolarity (Kapoor et al., 2000; Mayer et al., 1999). It will also be crucial in the future to understand the similarities and differences between the functions of Aurora A and Aurora B at centrosomes.

Control of centrosome stability is crucial for the maintenance of genome stability. Aneuploidy (unequal chromosome segregation) is often observed in the presence of abnormal numbers of centrosomes, and a number of genetic diseases are associated with improper regulation of centrosome function [reviewed in (Badano et al., 2005; Nigg, 2002)]. Therefore it is clear that the initiation of centrosome formation must be tightly controlled. The association of centrosomes with chromatin must likewise be maintained in order to prevent the generation of multipolar spindles, a particular challenge in zygotic

cells, which contain an extraordinarily large amount of cytoplasm with relation to the size of the mitotic spindle. Our findings shed light on how these processes might be controlled.

Dasra Proteins and Spindle Checkpoint Activity

Even in the presence of αIncenp antibodies, we found that the Dasra proteins are required for spindle checkpoint function (Figure 3-13A, 3-13B). Recently it was reported that Aurora B is the most 'upstream' component of the spindle checkpoint pathway in *Xenopus* egg extract (Vigneron et al., 2004). The authors of that study also reported that addition of Aurora B, Incenp, and Survivin to CPC-depleted extract did not rescue checkpoint activity or proper localization of the complex, leading them to speculate the existence of an unknown component required for complex formation, centromeric localization and checkpoint activity (Vigneron et al., 2004). Given the data presented here, it seems clear that Dasra proteins represent the missing CPC component required for these processes.

Why are Dasra proteins required for checkpoint function? The answer to this question likely lies at least partially in the observation that Aurora B and Incenp require Dasra proteins for their efficient loading onto chromosomes (Figures 3-14A). Incenp does not become recruited to the inner centromere in the absence of Dasra A/B, as judged by immunofluorescence (Figure 3-14C), and to the extent that chromatin beads can serve as proxies for chromosome arms, Dasra proteins are also required for arm binding (Figure 3-14B). These findings may be related, since passenger proteins typically relocalize from chromosome arms in prophase to inner centromeres at metaphase [reviewed in (Adams et

al., 2001)]. The mechanism by which Dasra proteins promote chromosome binding is unknown, but may include recruitment to proteins having a similar dynamic and/or spatially restricted localization pattern (e.g. cohesin, centromeric or post-translationally modified histone proteins). In this regard, it is interesting to note that inhibition of Dasra B expression in mammalian cells leads to selective loss of Cenp-A phosphorylation, while H3Ser10 phosphorylation is unaffected (S.C.S., unpublished observations). However, the observation that hDasra B is capable of binding to dsDNA *in vitro* (Klein et al., 2006) suggests that Dasra dependent CPC-localization may be via direct chromosome binding to centromeric and/or non-centromeric DNA, a model not inconsistent with the evolutionary divergence of Dasra-family protein sequences.

Conclusions

Our functional analysis of the CPC has demonstrated the importance of spatial regulation of Aurora B function. Chromosomes, centrosomes, and microtubules likely all serve as sites of Aurora B activation towards substrates relevant for spindle formation (e.g. Op18 and MCAK; Figure 4-2A), a process which does not intrinsically require stable association of the CPC with metaphase chromosomes. Like Ran-GTP, activated Aurora B may then promote the formation a local zone of microtubule stability through regulation of both Op18, MCAK and other factors (Figure 4-2B), and would act on Ran-GTP-dependent microtubule seeds to promote microtubule polymerization within the nascent spindle. This microtubule stabilizing function could also facilitate centrosome interaction with the spindle, an activity that may be reflected in the dramatic ability of activated Aurora B to promote the formation of bipolar and multipolar structures by

centrosomes in the total absence of chromatin. Taken together, our findings shed new light on how Aurora B functions at multiple locations to promote accurate spindle formation and chromosome segregation.

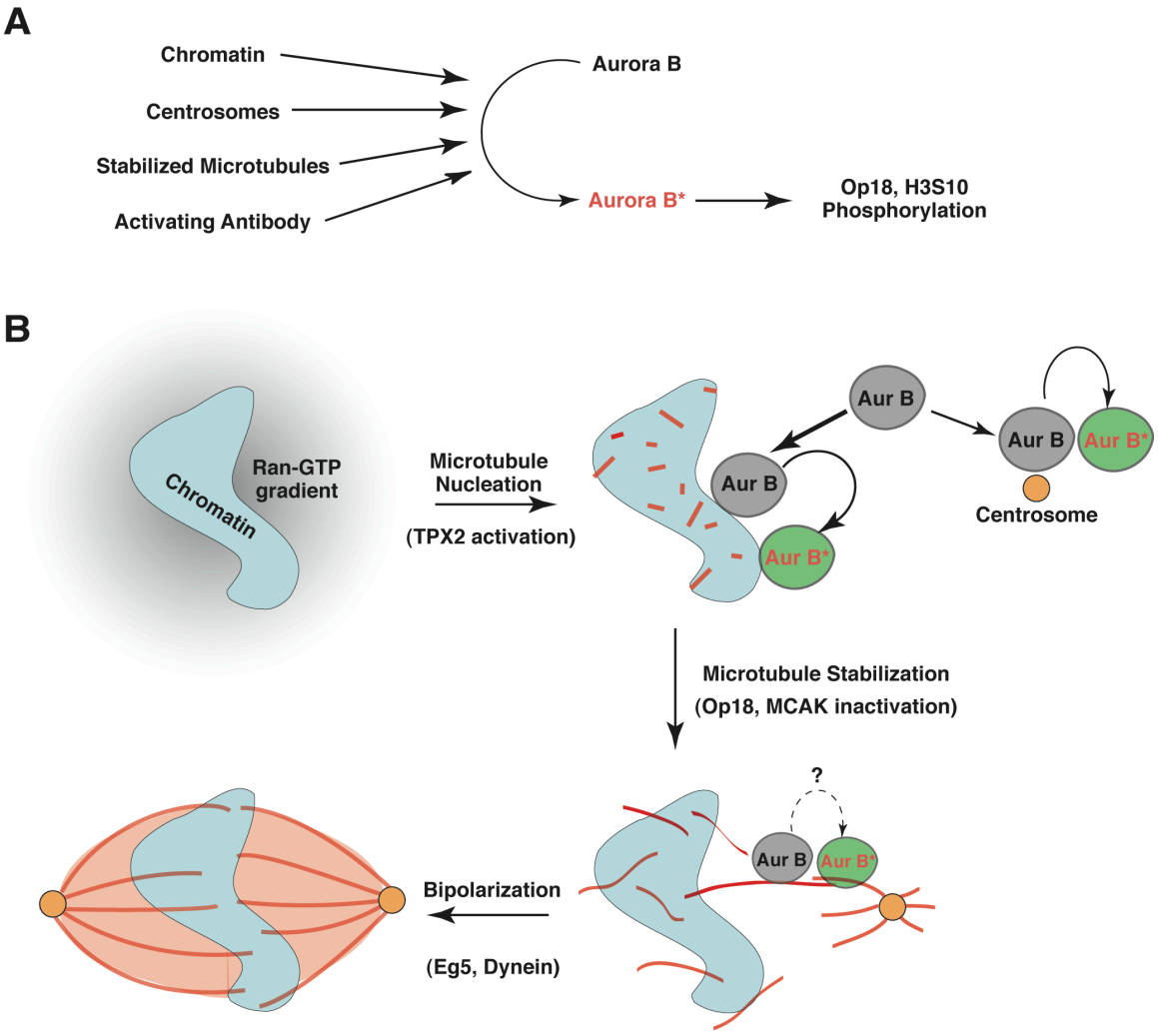

Figure 4-2. A Model for Chromatin-Induced Spindle Assembly in the Metazoa
(A) Chromatin, centrosomes, and microtubules can promote CPC activation; in the case of chromatin, this activation requires Dasra-dependent chromosome binding. CPC activation can be artificially induced by addition of αIncenp antibodies, which may act via localized CPC clustering. Activated CPC can then phosphorylate targets such as Op18, histone H3 serine 10, and MCAK.
(B) Following the generation of microtubule seeds by chromatin-proximal Ran-GTP, the activated CPC would promote localized microtubule stabilization via phosphorylation of microtubule destabilizing proteins such as Op18 and MCAK. The microtubules generated by this pool of activated CPC could likewise serve to anchor centrosomes to their associated spindle. Bipolarization would then be induced through the actions of microtubule motors and spindle associated proteins, including Eg5 and Dynein.

Future Directions

The findings described here represent an initial description of the composition and function of the metazoan chromosomal passenger complex. Numerous avenues however exist for the further elucidation of CPC function in *Xenopus*. Notably, although we have now established the importance of Dasra proteins in chromosome binding, spindle formation, and spindle checkpoint activity, the molecular basis for these functions remains unclear. If, as suggested by recent data, Dasra proteins in fact mediate direct DNA binding (Klein et al., 2006), this may rationalize the radical divergence of Dasra-family protein sequences, since these would presumably reflect the observed divergence of centromeric DNA sequences. On the other hand, we have found that Dasra proteins are essential for efficient CPC binding to chromatin beads lacking centromeric DNA sequences (Figure 3-14), suggesting that the intrinsic binding of Dasra proteins to DNA may not be sequence specific. Greater understanding of this aspect of Dasra protein function will undoubtedly require structural and biophysical approaches in combination with the loss-of-function techniques described here. Such studies will also require consideration of the function of Survivin, as multiple lines of evidence suggest that Survivin and its homologs are functionally associated with Dasra proteins (Klein et al., 2006; Romano et al., 2003; Sampath et al., 2004; Vader et al., 2006).

The functional and evolutionary connection between Dasra A and Dasra B represents another area for future research. Despite intensive efforts, we were never successful in producing an antibody capable of recognizing endogenous *Xenopus* Dasra B in protein lysates derived from either eggs or somatic fibroblasts. This technical limitation precluded an analysis of Dasra B function in egg extracts, since it was not possible to

perform immunodepletion experiments, or indeed to confirm the existence of Dasra B in egg extracts. This issue remains one of compelling interest nonetheless, since although Dasra proteins have now been established as crucial regulators of CPC function, it remains unclear why some organisms possess two Dasra family members (e.g. *Xenopus*, chicken), while others require only one (as seen in mammals). This question gain even greater interest given that, despite the striking dissimilarity in sequence between *Xenopus* Dasra A and Dasra B, both are capable of supporting spindle checkpoint function in egg extracts. This observation suggests either that the functional constraints on the Dasra proteins do not translate into structural constraints, or that despite their dramatically different sequences, Dasra A and B assume similar tertiary and/or quaternary structures. While the latter possibility might be invoked to explain the observed differences in sequence between Dasra B homologs in organisms having differing centromeric sequences, the value of such a system within an individual species seems less obvious. True understanding of the functions of Dasra A and Dasra B will eventually require investigation in a physiological relevant context, namely embryogenesis, during which both embryonic and somatic functions can be observed and perturbed.

In addition to these questions related specifically to the Dasra proteins, many issues of (greater or lesser) interest exist in relation to the CPC as a whole. For example, the model that Aurora B functions endogenously to regulate centrosome integration into the nascent spindle deserves more careful examination. As with the functional analysis of the Dasra proteins, this would likely involve studying CPC function during early development, since it is only then that this issue is of physiological relevance. The role of the CPC in modulating microtubule dynamics (i.e. both upstream and downstream of

microtubule polymerization) is also of interest, although it has been reported that Aurora B inhibition has no effect on microtubule nucleation or dynamics during either interphase or mitosis (Rosa et al., 2006). Of perhaps greater importance are the potential functional connections between Aurora B and Aurora A, and between the CPC and Ran pathways for spindle formation. A precedent exists for the former, since it has been shown that Aurora B-dependent Cenp-A phosphorylation during metaphase requires prior Aurora A-dependent Cenp-A phosphorylation during prophase (Kunitoku et al., 2003).

By contrast, there currently is little data supporting a functional connection between the Ran-dependent and CPC-dependent pathways for spindle formation; indeed, we found that spindle formation in ΔIncenpΔMCAK extract requires Ran function (Figure 2-15), suggesting that these pathways operate in parallel. Given however the similarities between achromosomal spindle formation induced by activated CPC and activated Ran, it would be worthwhile to use this model to readdress the possibility of cross-regulation between these two critical pathways of spindle assembly.

CHAPTER 5

Methods

Chapter 2 Methods:

Frog Egg Extracts

Unless specifically noted, freshly prepared meiotic metaphase II-arrested (CSF) *Xenopus laevis* egg extracts were used (Murray, 1991). To obtain high speed supernatant (HSS), low-speed CSF extracts (supplemented with 10 μg/ml cytochalasin D) were centrifuged at 50000 rpm for 1 h at 4°C in a Beckman TLS55 rotor, lipids were removed from the top, and the remainder was recentrifuged at 50000 rpm for 1 h. The supernatants were then pooled and recentrifuged at 50000 rpm for 1 h. These supernatants were frozen in aliquots on liquid nitrogen, and thawed as needed.

Chromosome Binding Screen

A method described previously (Funabiki and Murray, 2000) was followed with modifications. *Xenopus* egg mRNA was isolated from low-speed egg extracts, and a full-length, normalized cDNA library (Carninci et al., 2000) was built in a modified pCS2 expression vector (Turner and Weintraub, 1994). Seven thousand bacterial colonies containing independent cDNA clones were robotically formatted into 384-well plates. The 5'- region of each clone was determined after amplification of the DNA using TempliPhi (Amersham). Each 384-well plate was robotically reformatted at the Rockefeller University Gene Array Resource Center into four 96-well subplates, and plasmid pools were prepared from columns, rows, and subplates, creating 24 pools per 384 well plate (12 column pools, 8 row pools, and 4 subplate pools). These plasmid

pools were added to a coupled transcription-translation system in rabbit reticulocyte lysate (Promega). For each chromosome binding reaction, sperm nuclei (final concentration 1400/µl), 100 µg/ml cycloheximide, and 5 µM biotin-21-dUTP (Clontech) were added to 120 µl of *Xenopus* CSF extract. Thirty minutes after adding 0.3 mM calcium chloride to allow entry into interphase, 12 µl of a labeled protein pool were added. After an additional 50 min at 22ºC, 60 µl of fresh CSF extract, 24 µg/ml cyclin $B^{\Delta 90}$ (Glotzer et al., 1991), and 10 µg/ml nocodazole were added. The extract was then incubated for 80 min at 22ºC, after which the samples were frozen on liquid nitrogen and stored at -80ºC.

To purify chromosomes, the thawed extracts were mixed with 540 µl of Dilution Buffer 2 [DB2; 10 mM K-HEPES (pH 7.7), 50 mM ß-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) in a centrifuge tube (Beckman #347357), and spun for 30 min at 14000 rpm in a refrigerated Beckman Coulter 22R microcentrifuge using an S241.5 swinging bucket rotor. The sucrose interface was washed three times with 400 µl DB2, and the crude chromosome preparation was resuspended in ~100 µl of residual 60SDB2 and incubated with 15 µl Dynabeads M-280 streptavidin (Dynal) pre-equilibrated with 30SDB2 (DB2 with 30% w/v sucrose). Chromosomes were captured by rotation with beads for 1 h at 4ºC, recovered using a magnetic particle separator (Boehringer) and washed five times with 100 µl 30SDB2. Samples were boiled in standard SDS-PAGE sample buffer, and the copurifying labeled proteins were resolved by 7.5-15% gradient SDS-PAGE, followed by exposure to a PhosphorImager screen (Fujifilm BAS-2500).

The individual clones responsible for bands observed in pooled screening were determined by identifying column, row, and subplate (96-well) pools demonstrating chromosome-binding proteins of similar molecular weight. Putative chromosome binding proteins were rescreened individually as described above, except that 4 µl of individual translated clones were added to the reaction.

Identification of p34^{4B8}/Dasra A and Dasra B

The sequence of the p34^{4B8}/Dasra A cDNA was deduced by 5' and 3' sequencing of the clone present in the arrayed library, followed by TBLASTN searches and alignment of multiple ESTs present in public databases (Unigene Cluster Xl.5274). This deduced cDNA predicts a translated protein of 34 kD, similar to the molecular weight observed after *in vitro* translation of the p34^{4B8} clone present in the arrayed library. TBLASTN searches using the predicted xDasra A ORF sequence identified a homologous human sequence (hDasraB; GenBank Accession Number BC008079, also known as CDCA8), which when used in TBLASTN searches of *Xenopus* EST sequences identified a second, more closely related *Xenopus* protein (xDasra B; partial sequence from GenBank Accession Number CA982386). Full length sequences of the cDNA clones encoding xDasra A and xDasra B were determined at the Rockefeller University DNA Sequencing Resource Center. Dasra A and Dasra B homologs of chicken, *Gallus gallus*, and zebrafish, *Danio rerio*, were obtained after TBLASTN searches using xDasra A and xDasra B protein sequences. The following EST sequences were used to assemble predicted open reading frames: *Gallus gallus* Dasra A (BU225566, BU328206, BU204597 and BU225870), *Gallus gallus* Dasra B (BU135762 and BX258302), *Danio*

rerio Dasra A (BM777478 and BM036885), and *Danio rerio* Dasra B (BU492956 and BQ616242).

Live Imaging of GFP Fusion Proteins

For C-terminal GFP-tagging, primers 4B8EcoRIAscI-1 (5' CCCGAATTCATGCCGCCCAAGAGGAACAG 3') and 4B8EcoRIAscI-2 (5' AAGGCGCGCCGAGGGTATTCCCGTGGTGCTG 3') were used to amplify the complete $p34^{4B8}$/Dasra A ORF using the arrayed library clone, and the product was cloned into pTGFC70 (H.F., unpublished; this plasmid contains the SP6 promoter driving expression of the GFP protein downstream of an in-frame *Asc*I site) to create pSCS012. xDasra B was amplified with primers 4LPEcoRI-Fwd (5' CCCGAATTCATGGCACCCGGGAAAAAGAAG 3') and 4LPAscI-Rev (5' AAGGCGCGCCCTTTAGTTTCTTGCAGAGCC 3') from a clone present in the arrayed library, and cloned into pTGFC70 to create pSCS021.

For live imaging, pSCS012 and pSCS021 were linearized with *Xba*I and *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 capped mRNA was added to 30 µl of *Xenopus* CSF extract containing sperm nuclei (final concentration 400/µl); 0.3 mM calcium chloride was added to release the extract into interphase, and the extract was incubated at 21ºC for 80 min. A 10 µl aliquot of fresh CSF extract was then added to 20 µl of this interphase extract to induce M-phase. After 30 min at 21ºC, DAPI was added to a final concentration of 0.05 µg/ml, rhodamine tubulin to ~100 nM, and the extract was incubated in the dark for a further 30

min. To induce anaphase, 0.5 mM calcium chloride was added, and 3.5 µl of this extract was immediately squashed under an 18x18 mm coverslip and sealed with Valap (Desai et al., 1999a). 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). Images were processed with MetaMorph and Adobe Photoshop.

Generation of Peptide Antibodies

Methods previously described (Field et al., 1998) were followed. Peptides corresponding to the C-termini of xDasra A (CAKASIQHHGNTL), xIncenp (CSNRHHLAVGYGLKY), and hDasra B (SNRLAQICSSIRTHK) were synthesized at the Rockefeller University Protein Resource Center. Peptides were conjugated to hemocyanin (Sigma B8556 or H9035) and polyclonal antibodies were raised in rabbits (Cocalico Biologicals, Reamstown, PA). Antibodies were affinity purified after coupling of the antigenic peptide to SulfoLink Coupling Gel (Pierce) according to the manufacturers's directions. Bound antibodies were eluted with 100 mM Glycine, pH 2.3, and peak fractions were pooled and dialyzed serially against PBS/50% glycerol, PBS, and PBS/50% glycerol. Affinity purified antibodies were stored at -30ºC.

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) for at least 1 h at 4ºC. Conjugated beads

were washed twice in cold TBS and three times in cold Sperm Dilution Buffer [5 mM K-HEPES (pH 7.7), 1 mM $MgCl_2$, 100 mM KCl, 150 mM sucrose]. Excess buffer was removed and 50 µl CSF extract containing 100 µg/ml cycloheximide and ~100 nM rhodamine-tubulin was added, followed by gentle tapping to mix. Extracts were depleted in 50 µl aliquots in 0.6 ml microcentrifuge tubes for 2 h on ice, with a gentle mixing after 1 h. Beads were removed using a magnetic particle separator, and residual beads were removed after pooling the supernatants from several tubes.

All incubations for spindle assembly using immunodepleted egg extracts were carried out at exactly 15.5ºC in a water bath, and manipulations were carried out in a temperature-controlled room at 20ºC. For spindle assembly on replicated chromosomes, 40 µl of immunodepleted egg extract containing sperm nuclei (final concentration 400/µl) and 0.3 mM calcium chloride were incubated for 120 min at 15.5ºC 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 15.5ºC 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 18x18 mm coverslip was placed on top.

For spindle assembly on demembranated sperm nuclei, sperm nuclei (final concentration 1500/µl) were added to 20 µl of fresh immunodepleted extracts. For spindle assembly on chromatin beads, 2 µl of DNA-coated beads, prepared following a method previously described (Heald et al., 1998) and washed once with 20 µl of depleted extract, were added to 66 µl of depleted extract containing cycloheximide and rhodamine-tubulin, and were incubated for 2 h at 15.5°C after adding 0.3 mM calcium.

To induce M phase entry, 24 μg/ml cyclin $B^{\Delta 90}$ 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 with a magnetic particle separator 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.

Immunoprecipitation from Egg Extract High-speed Supernatant (HSS)

20 μl of Protein A-sepharose (Sigma) bound to 5 μg of anti-xDasra A antibodies were washed with XBE2 (10 mM HEPES-KOH, pH 7.6, 100 mM KCl, 2 mM $MgCl_2$, 50 mM sucrose, 5 mM EGTA and 10 μg/ml LPC), and were added to 50 μl of HSS with or without 10 μl of 4 mg/ml xDasra A peptide (reconstituted in XBE2). The beads were incubated with HSS at 4°C for 80 min, washed 4 times with XBE2 + 0.3 M KCl, and washed twice with XBE2 without KCl. Beads were eluted with SDS-PAGE sample buffer, applied to a gradient SDS-polyacrylamide gel, and analyzed by Western blotting.

Immunofluorescence Microscopy

Spindles assembled in *Xenopus* egg extract were processed for immunofluorescence as described (Desai et al., 1999a; 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 h, and were visualized with FITC- or X-Rhodamine-conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch). DNA was counterstained with 0.25 μg/ml Hoechst 33258 in AbDil, and mounted in anti-fade mounting medium [90% glycerol, 0.5% p-phenylenediamine, 20 mM Tris-HCl (pH 8.8)].

For simultaneous immunofluorescence using anti-xDasra A and anti-xIncenp antibodies, slides were stained with 1 μg/ml anti-xDasra A antibodies as described above, washed three times with AbDil, incubated for 30 min with FITC-conjugated goat anti-rabbit Fab antibodies (Jackson ImmunoResearch), washed three times with Abdil, blocked for 1 h with 70 μg/ml unconjugated goat anti-rabbit Fab (Jackson ImmunoResearch), washed four times with AbDil, incubated with either 1 μg/ml anti-xIncenp antibodies or Abdil (the latter as a negative control for cross-reaction) for 1 h, washed three times with AbDil, and incubated with X-Rhodamine-conjugated goat anti-rabbit antibodies. Slides were then washed, counterstained, and mounted as described above. Negative control slides showed no rhodamine fluorescence when images were acquired with exposure times equal to those used for antibody-stained slides.

For immunofluorescence on cultured mammalian cells, HeLa cells grown on coverslips were fixed for 1.5 min with -20ºC methanol, rinsed twice in TBS, permeabilized in TBS+0.5% Triton X-100 for 10 min at RT, rinsed three times for 2 min each in Triton X-100, and blocked in AbDil for at least 30 min. 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. After washing 3 times in AbDil, rabbit antibodies were detected with X-Rhodamine-conjugated goat anti-rabbit antibodies, and mouse antibodies with FITC-conjugated goat anti-mouse (Jackson ImmunoResearch). Slides were stained with Hoechst and mounted as described above. 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 37ºC/5% CO_2. For siRNA treatment and immunofluorescence, $5x10^4$ cells were plated into each well of a 6 well plate containing 3-4 acid washed, poly-D-lysine coated coverslips, and were grown for 8-12 h in DMEM/10% FBS in the absence of antibiotics. siRNAs were used at 200 nM, and were transfected using Oligofectamine (Invitrogen) according to the manufacturers instructions. siRNA target sequences were as follows: hDasra B (5' AAAGGUCAAGCCGUGCUAACA 3'), EGFP (5' AAGACGUAAACGGCCACAAGUUC 3'). At 24-36 h post-transfection, cells were treated with 20 μM MG132 (Peptides International) for 2 h at 37ºC, fixed, and analyzed by immunofluorescence microscopy. Chromosomes were scored for misalignment by examining only those cells in mitosis. A 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

For mitotic arrest, $2x10^6$ HeLa cells were plated onto 15 cm plates, incubated for 12-16 h, treated with medium containing 2 mM thymidine for 18 h, released into fresh medium for 3-4 h, and blocked in medium containing 100 ng/ml nocodazole for 10-12 h. Mitotic cells were shaken off, washed twice with PBS, and transferred into fresh media for 1 h, after which they were 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 ß-glycerophosphate, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml chymostatin, 1 mM PMSF] on ice for 20 min.

Lysates were cleared by centrifugation at 4°C at 13200 rpm for 10 min, and the supernatant was diluted to 100 mM with NP-40 Lysis Buffer without salt. Diluted lysates were precleared with Protein A-sepharose (Roche) for 2 h at 4°C with rotation, the beads were removed, and 10 µg of control IgG or affinity purified anti-hDasra B antibodies were added. After rotation for 1 h at 4°C, 30 µl of fresh Protein A-sepharose was added, and the mixture was rocked for an additional 1 h. The beads were then washed five times with NP-40 Lysis Buffer, and samples were eluted with standard SDS-PAGE sample buffer.

Western Blots

Immunoblots were blocked with PBS/4% nonfat dry milk for 1 h at RT or overnight at 4ºC. Primary and secondary antibodies were diluted in PBS/4% nonfat milk 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), 1:5000 anti-α-Tubulin (DM1, Sigma). Antibodies were detected using either ECL or ECL-Plus (Amersham).

Sucrose Density Gradient Sedimentation

95 µl of CSF extract High Speed Supernatant was loaded onto a 5 ml 5-40% continuous sucrose gradient [in 10 mM HEPES-KOH (pH 7.7), 50 mM ß-glycerophosphate, 50 mM NaF] and spun for 15 h at 36,000 rpm in a rotor (SW55Ti; Beckman) at 4°C. In parallel 95 µl of calibration standards (BSA, 4.3S; catalase, 11.3S; and thyroglobulin, 19.4S) at 2

mg/ml each were run on a separate gradient. 192 µl fractions were collected from each gradient.

GenBank Accession Numbers

The mRNA sequences for *Xenopus* Dasra A and Dasra B have been deposited in GenBank, with accession numbers AY644400 (Dasra A) and AY644401 (Dasra B).

Chapter 3 Methods:

Frog Egg Extracts

Unless specifically noted, freshly prepared meiotic metaphase II-arrested (CSF) *Xenopus laevis* egg extracts were used (Murray, 1991).

Immunodepletion From RNase-treated *Xenopus* Egg Extracts

The preparation of RNase-treated extracts capable of supporting immunodepletion and cycling typically required the use of naïve frogs that had not been previously hormonally induced to lay eggs. To prepare RNase-treated extract, boiled RNase A was added to CSF-arrested extract at a final concentration of 0.11 µg/ml (10 µg/ml stock), and the extract was incubated for 15 min at 12ºC. RNase inhibitor (Super RNasin, Promega) was then added at 1:150 dilution, and the extract was further incubated 5 min at 12ºC. Extracts were then placed on ice, yeast tRNA was added to 0.05 µg/µl (5 µg/µl stock), and ~100 nM rhodamine-tubulin was added. RNase treatment was conducted in 300-400 µl aliquots, and the extracts were pooled after the addition of tRNA.

For each 50 µl immunodepletion reaction from RNase-treated CSF, 100 µl of antibody-conjugated Protein A-Dynabeads (Dynal) were used. Beads were conjugated overnight at 4ºC in 0.1M phosphate buffer (pH 8.0) at a ratio of 50 µl beads:5 µg affinity-purified α-Incenp, α-MCAK, α-Op18, or control rabbit IgG. Conjugated beads were washed once in phosphate buffer and five times in cold Sperm Dilution Buffer [5 mM K-HEPES (pH 7.7), 1 mM $MgCl_2$, 100 mM KCl, 150 mM sucrose]. Excess buffer was removed and RNase-treated CSF extract was added, followed by gentle tapping to mix. Extracts were depleted in 50 µl aliquots in 0.6 ml microcentrifuge tubes for 1.5 h on ice, with a gentle mixing after 1 h. Beads were removed using a magnetic particle separator (Dynal), and residual beads were removed after pooling the supernatants from multiple tubes.

Reconstitution of Immunodepleted Egg Extract

For the reconstitution of RNase-treated egg extract with >2 proteins, mRNA pools were prepared. Full length clones encoding *Xenopus laevis* Aurora B, Incenp, Dasra A, Dasra B, Survivin, and SIX were individually *in vitro* transcribed using the mMessage mMachine kit (Ambion). Following phenol/chloroform extraction and precipitation, capped mRNAs were pooled together, precipitated in the presence of 20 µg RNase-free glycogen, and resuspended in RNase-free water such that 2 µl of the resuspended mRNA contained sufficient mRNA for the reconstitution of 60 µl of ΔIncenp extract (final volume after cycling). The amount of mRNA needed for reconstitution of each protein was empirically determined in advance by Western blotting. Although these values varied slightly from extract to extract, in general the following amounts of mRNA

($OD_{260/280}$ ~1.7) were found to be sufficient to reconstitute ~60 µl (final volume) of ΔIncenp extract to physiological levels, when added at the initial release into interphase and as judged 60 min after entry into metaphase at 20°C: Incenp (10 µg), Aurora B (10 µg), Dasra A (2 µg), Dasra B (2 µg), Survivin (0.22 µg), SIX (0.22 µg).

For the reconstitution of 1-2 proteins, mRNA pools were not prepared, and mRNA encoding the proteins of interest were instead directly added to CSF-arrested RNase-treated extracts in the amounts listed above.

To monitor mRNA translation, 5 µl of CSF extract containing mRNA was taken immediately after addition of calcium to induce release into interphase, and was added to 0.25 µl of ^{35}S-methionine (Amersham #AG1594) diluted 1:1 with water. The extract was then cycled as described below, except that 10 µl of fresh extract was added to induce entry to M-phase. Samples were taken 60 min after M-phase entry, and translation efficiency was evaluated following SDS-PAGE and autoradiography.

Spindle Assembly in RNase-treated, Immunodepleted Egg Extracts

All incubations for spindle assembly using immunodepleted egg extracts were carried out at 20ºC in a water bath. For a typical spindle assembly on replicated chromosomes, 25 µl of RNase-treated control or immunodepleted egg extract containing sperm nuclei (final concentration 500-10000/µl) or purified centrosomes (a gift of K. Kinoshita), 2 µl mRNA, and 0.3 mM calcium chloride were incubated for 80 min at 20ºC to prepare interphase extracts, after first removing 5 µl of the reaction for ^{35}S-methionine labeling as described above. Metaphase spindles were assembled by the addition of 40 µl of fresh RNase-treated control or immunodepleted extract, followed by incubation at 20ºC for 60

min. To score phenotypes, 1 µl of extract was placed on a slide, 3 µl of Fix (Murray, 1991) was added, and an 18x18 mm coverslip was placed on top.

For spindle assembly and Western blot analysis using chromatin beads, 5 µl of DNA-coated beads per sample, prepared as previously described (Heald et al., 1998), were washed once with 20 µl of RNase-treated control or immunodepleted extract, added to 20 µl of RNase-treated control or immunodepleted extract containing rhodamine-tubulin, and incubated for 80 min at 20°C after addition of 0.3 mM calcium chloride. M phase entry was induced as described above.

Spindle Checkpoint Analysis

To assay spindle checkpoint activity in reconstituted extracts, cycled, reconstituted extracts were supplemented with sperm nuclei to a final concentration of 10000/µl extract, nocodazole was added (10 µg/ml final concentration), and the extracts were incubated for 45 min at 20°C. Calcium chloride was then added to 0.4 mM, extracts were placed at 20°C, and aliquots were taken on a time course for Western blot. Fixed squashes were simultaneously prepared for analysis of chromosome morphology.

Immunofluorescence Microscopy

Spindles assembled in *Xenopus* egg extract were processed for immunofluorescence as described (Desai et al., 1999a; Funabiki and Murray, 2000). Affinity purified anti-xIncenp antibodies were used at 1 μg/ml in AbDil (TBS/0.1%Triton X-100+2% BSA) for 1 h, and were visualized with Alexa 488-conjugated goat anti-rabbit antibodies

(Molecular Probes). DNA was counterstained with 0.25 μg/ml Hoechst 33258 in AbDil, and mounted in Mounting Medium [90% glycerol, 1X PBS].

Immunofluorescence on individual diluted chromosomes was performed essentially as described (Funabiki and Murray, 2000).

Microscopy

Hoechst 33258-stained chromosomes, rhodamine-tubulin labeled microtubules, and fluorescent antibodies were imaged using a Carl Zeiss Axioplan 2 microscope equipped with a Photometrics CoolSnap HQ cooled CCD camera, and controlled by MetaMorph software (Universal Imaging). Images were processed with MetaMorph and Adobe Photoshop.

Chromosome and Chromatin Bead Purification

Replicated M-phase sperm chromosomes were purified as described previously (Sampath et al., 2004). For purification of chromatin beads, chromatin beads assembled as described above were washed five times in cold Egg Extract Wash Buffer [10 mM K-HEPES (pH 8.0), 100 mM KCl, 1mM $MgCl_2$, 50 mM sucrose, 50 mM NaCl, protease inhibitors (leupeptin, pepstatin, chymostatin)], and resuspended in standard SDS-PAGE sample buffer.

Immunoprecipitation of Metabolically Labeled Proteins

For immunoprecipitation of labeled MCAK and Op18, RNase-treated control or ΔIncenp extracts with or without reconstitution were cycled as described above, and γ-^{32}P-ATP

(Amersham #AA0018) was added at 1:50 final dilution to 35 µl extract per sample. Extracts were then incubated at 20°C for 30 min, transferred to tubes containing 25 µl α-MCAK or α-Op18 (prepared as described above), and incubated for 90 min on ice. Beads were then retrieved with a magnet, washed four times with Egg Extract Wash Buffer containing phosphatase inhibitors (described above), and resuspended in standard sample buffer. The copurifying proteins were then separated by SDS-PAGE, and the dried gel was exposed to a PhosphorImager (Fujifilm BAS-2500) for quantitation.

Western Blots

Immunoblots were blocked with PBS/4% nonfat dry milk for 1 h at RT. Primary and secondary antibodies were diluted in PBS/4% nonfat milk at the following concentrations: 4 µg/ml α-Dasra A, 8 µg/ml α-Incenp, 1 µg/ml α-Aurora B and α-Survivin (gifts of T. Hirano), 0.2 µg/ml α-Op18, 1:5000 α-α-Tubulin (DM1, Sigma). Antibodies were detected using either ECL (α-Dasra A, α-Incenp, α-Op18, α-Tubulin; Amersham) or Visualizer (α-Aurora B, α-Survivin; Upstate).

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