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Regulation of Chromosome Segregation by the Chromosomal Passenger Complex

Boo Shan Tseng

REGULATION OF CHROMOSOME SEGREGATION BY THE CHROMOSOMAL PASSENGER COMPLEX

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Boo Shan Tseng

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Boo Shan Tseng, Ph.D.

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Life depends on faithful DNA segregation. The molecular underpinnings controlling this segregation are not fully understood. Here I describe the role of the chromosomal passenger complex (CPC) in the regulation of chromosome segregation, using *Xenopus* egg extracts.

The CPC contains the kinase Aurora B, which is well known for its phosphorylation of serine 10 on histone H3 (H3S10). While this phosphorylation is a hallmark of M-phase, its functional significance is enigmatic. In the first part of my thesis, I, with my collaborators, provide a molecular function for this phosphorylation in the chromosomal dissociation of HP1 (heterochromatin protein 1) in M-phase. The phosphorylation of H3S10 by Aurora B ejects HP1 from its stably methylated histone H3 lysine 9 (H3K9) chromatin binding site, clearly demonstrating the existence of a "methyl/phos switch." While the significance of this chromosomal HP1 removal is unclear, I propose that it functions in proper M-phase chromosome compaction.

In eukaryotes, chromosome segregation depends on spindle formation. Chromosome-induced spindle assembly requires the chromosomal recruitment and activation of Aurora B. How this chromosome-activated kinase spatially disseminates signals that lead to spindle formation remains unclear. In the second part of my thesis, I show that CPC must detect chromosomes and

microtubules to support spindle assembly. While the CPC is enriched on chromosomes in metaphase, I establish that a fraction of the CPC is targeted to the metaphase spindle. I demonstrate that this understudied metaphase CPC-microtubule interaction is required to target chromosomally activated Aurora B to emerging microtubules near chromosomes for spindle assembly. I propose that the dual detection of chromosomes and microtubules by the CPC ensures that spindle formation is spatially limited to the vicinity of chromosomes.

The spindle assembly checkpoint is vital for faithful chromosome segregation, as the checkpoint monitors the proper attachment of chromosomes to the spindle. In the third part of my thesis, based on preliminary data, I discuss the possibility that the dual detection of chromosome and microtubules may also function in the signaling of this checkpoint.

Together, my work demonstrates three different functions of the CPC that impinge on chromosome segregation and genomic integrity.

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LIST OF ABBREVIATIONS

aa	amino acid
AurB	Aurora B
BTB	Bric-a-brac/Tramtrack/Broad complex
CB	cytochalasin B
CC domain	putative coiled-coil domain
CPC	chromosomal passenger complex
CSF	cytostatic factor
DsrA	Dasra A
DS domain	Dasra/Survivin interaction domain
FL	full length
FRAP	fluorescence recovery after photobleaching
FRET	fluorescence (or Förster) resonance energy transfer
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
H3K14	lysine 14 of histone H3
H3K9	lysine 9 of histone H3
H3S10	serine 10 of histone H3
H3T3	threonine 3 of histone H3
HCG	human chorionic gonadotropin
HP1	heterochromatin protein 1
IAP	inhibitor of apoptosis protein
INC	INCENP
INCENP	inner centromeric protein

Ipl1	increase-in-ploidy-1
LPC	leupeptin, pepstatin, chymostatin
MCAK	mitotic centromere-associated kinesin
MKLP	mitotic kinesin-like protein
Nbl1	novel Borealin-like protein
Op18	oncoprotein 18
PMSG	pregnant mare's serum gonadotropin
SMC	structural maintenace of chromosome
Sur	Survivin
Tlk-1	Touseled-like kinase

CHAPTER 1. GENERAL INTRODUCTION

The origins of the spindle

In the mid-seventeenth century, using a compound microscope, Robert Hooke coined the term "cells" after seeing structures in a piece of cork that resembled a honey-comb (Hooke, 1665). For two hundred years, it was not clear how new cells were created, until finally Walther Flemming described the process of "mitosis" (Figure 1-1)(Flemming, 1882). While great advances have been made in the field of cell division in the last 130 years, we still do not fully understand the details of how those chromosome threads Flemming originally described are segregated into two new cells.

One major aspect of segregating DNA into two cells involves generating the forces necessary to move the genetic material. In eukaryotic cells, the structure that provides this force is the spindle. Flemming, along with other early cytologists, observed spindles as a network of fibrils, which he suspected was responsible for the transport of the chromosome threads via their filamentous nature (Paweletz, 2001). It was not until 1963 that these fibrils were identified as hollow tubes and thus named "microtubules" by Ledbetter and Porter, who imaged the structure using electron microscopy (Ledbetter and Porter, 1963).

Dynamic instability of microtubules

Similar to Flemming, Inoue and Sato in the 1960s postulated that chromosome movement was due to the polymerization dynamics of the microtubules (Inoue and Sato, 1967), but there was no clear evidence supporting the hypothesis until approximately fifteen years later when microtubules were

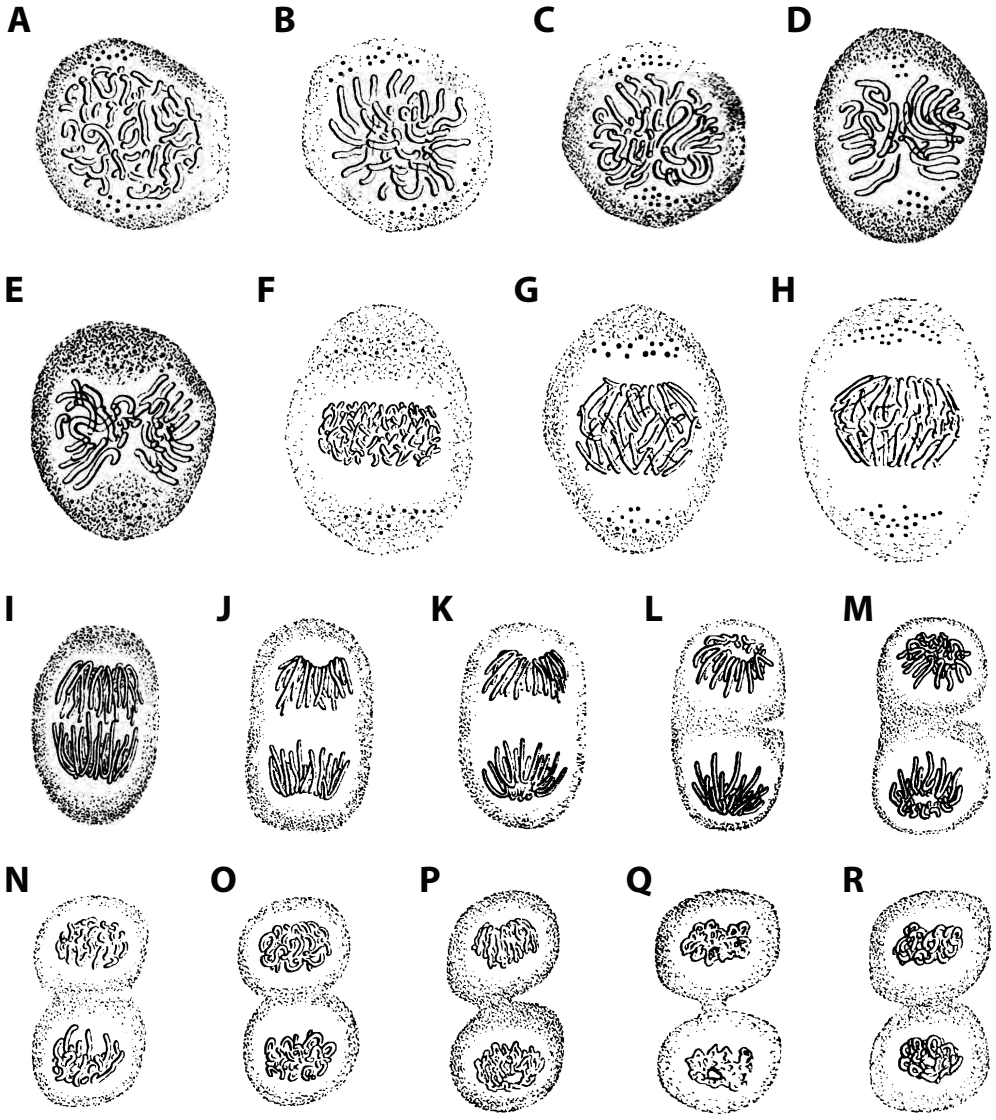


Figure 1-1. Flemming's observations.

(A-R) Sequential drawings of a dividing cell. Images from Flemming, 1882.

shown be dynamically unstable (Mitchison and Kirschner, 1984; Horio and Hotani, 1986). Microtubule dynamics are governed by four rates (Figure 1-2A, right): the rate of growth, the rate of shrinkage, the rate of catastrophe (switch from growth to shrinkage), and the rate of recovery (switch from shrinkage to growth)(Cassimeris et al., 1987). In addition to these four rates, microtubule polymerization is controlled by nucleation, which is the assembly of enough tubulin heterodimers (approximately 6-12) to seed a microtubule and is a rate-limiting step for microtubule assembly (Job et al., 2003). During mitosis, the rate of microtubule nucleation increases, as does the dynamic instability of the microtubules. The dynamic nature of these microtubules is postulated to produce the force needed to move chromosomes (Valiron et al., 2001). While the ability of depolymerizing microtubules to move chromosomes *in vitro* was shown in 1988 (Koshland et al., 1988), how exactly the forces produced by the microtubules are transmitted to chromosomes is still unclear.

The classic search-and-capture mechanism of spindle assembly

The classic image of a spindle contains chromosomes aligned in the middle of a bipolar, football-shaped, microtubule-containing structure. In addition to the chromosomes and spindle structure, Flemming saw that at each pole was an organizing structure, which was later named a centrosome by Boveri (Wilson, 1925). Centrosomes nucleate and spatially organize microtubules (Bettencourt-Dias and Glover, 2007). In spindle formation, the microtubules nucleated from the centrosomes are proposed to find chromosomes via a random search-and-capture mechanism, in which microtubules that are radially nucleated from centrosomes are highly dynamic until the microtubules contact

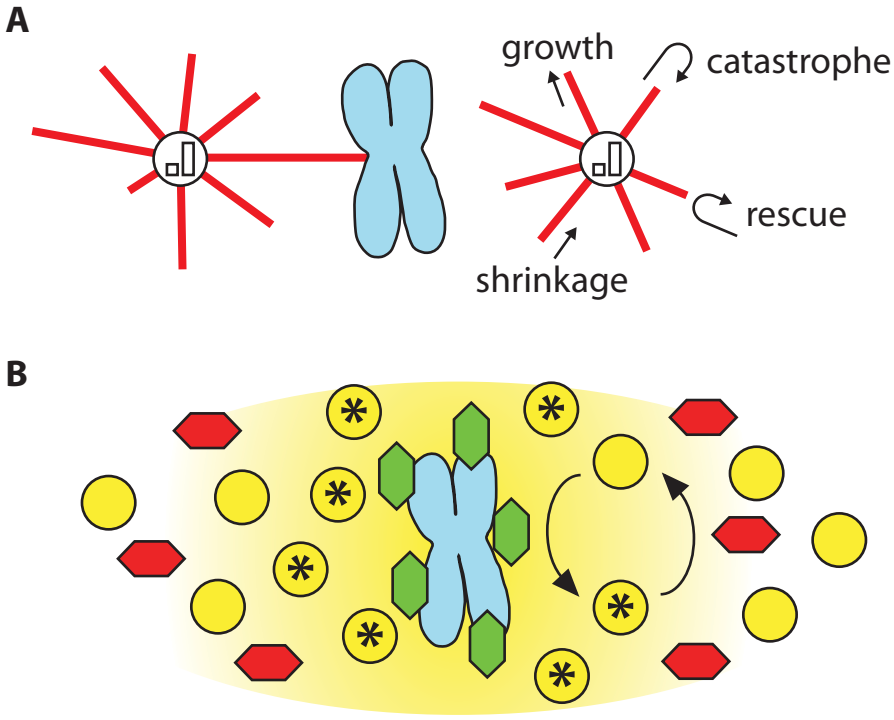


Figure 1-2. Two models of spindle assembly.
(A) The classic centrosome model with a search-and-capture based mechanism. Centrosomes (white circles with rectangles inside) nucleate microtubules that are dynamically unstable and search the cytoplasm for the chromosomes. Once a microtubule attaches to a chromosome, it is stabilized. On the right, the four rates that governed dynamic instability are shown. Arrow away from centrosome, growth; arrow towards centrosome, shrinkage; looped arrow pointing away from the centrosome, rescue; and looped arrow pointing to the centrosome, catastrophe. **(B)** The chromosome-induced self-assembly model with a reaction-diffusion gradient-based mechanism. Activating proteins (green hexagons) bind to chromosomes (blue) and activate effector proteins (yellow circles with asterisk), which then diffuse away from the chromosomes. In the cytoplasm, the effector proteins are inactivated (those without the asterisk) by inactivating proteins (red hexagons). Since the activating protein is locally concentrated at chromosomes, there is a higher concentration of active effector near chromosomes than farther away from it (gradient of yellow in the background).

and are capped by the kinetochore of a chromosome (Figure 1-2A)(Kirschner and Mitchison, 1986). While microtubule capture by a kinetochore has been directly visualized (Hayden et al., 1990; Rieder and Alexander, 1990), there are two conceptual problems with the model. First, spindle assembly does not require centrosomes (Heald et al., 1996; Khodjakov et al., 2000; Basto et al., 2006), suggesting that centrosome-independent mechanisms can regulate spindle formation. Second, mathematical modeling strongly suggests that a simple search-and-capture mechanism is too inefficient (Wollman et al., 2005). Instead, a biased search-and-capture mechanism was suggested, in which microtubules growing in the direction away from chromosomes are more likely to depolymerize than those growing towards chromosomes. Both of these observations suggest that a non-random search-and-capture-based mechanism should exist.

Chromosome-induced self-assembly of spindles

Indeed, a self-directed mechanism for spindle assembly does exist. Microtubules can be assembled locally near chromosomes and organized into an anti-parallel array to create the bipolar spindle independent of centrosomes (Karsenti and Vernos, 2001). Chromosomes stimulate this self-assembly by locally promoting microtubule polymerization through two signaling cascades: the Ran-GTP pathway and the chromosomal passenger complex (CPC) pathway (Kelly et al., 2007; Walczak and Heald, 2008). Both pathways are suggested to promote local microtubule assembly via a gradient of active effectors centered on chromosomes (Niethammer et al., 2004; Caudron et al., 2005; Bastiaens et al., 2006). This gradient is set up by a reaction-diffusion mechanism: effectors are

activated on chromosomes, diffuse away from chromosomes, and are subsequently inactivated in the cytoplasm (Figure 1-2B). Such effector gradients have been visualized using fluorescence (or Förster) resonance energy transfer (FRET)-based technologies for both the Ran-GTP and the CPC pathways (Kalab et al., 1999; Niethammer et al., 2004; Caudron et al., 2005; Bastiaens et al., 2006; Kalab et al., 2006; Fuller et al., 2008).

Ran-GTP pathway

The GTPase Ran cycles between a GDP and a GTP bound state, which is regulated by a guanine nucleotide exchange factor (GEF) and a GTPase activating protein (GAP) with its cofactor, RanBP1 (Gorlich and Kutay, 1999). During interphase, Ran directs the transport of proteins in and out of the nucleus (Figure 1-3A). Since the Ran GEF RCC1 is sequestered in the nucleus while RanGAP and RanBP1 are in the cytoplasm, Ran-GTP is high in the nucleoplasm and low in the cytoplasm. This arrangement creates a steep Ran-GTP/Ran-GDP gradient across the nuclear membrane, which Ran uses to control nuclear transport. Nuclear import factors bind to their cargo in the cytoplasm and transport them into the nucleus. Once in the nucleus, Ran-GTP binds to the nuclear import factor, induces the release of the cargo, and exits the nucleus with the import factor. In the cytoplasm, with the help of RanGAP and RanBP1, Ran-GTP is hydrolyzed to Ran-GDP, which releases the nuclear import factor to bind new cargo, completing the cycle. Ran also regulates the export of proteins from the nucleus through a reverse mechanism (Gorlich and Kutay, 1999).

A role for Ran in M-phase was uncovered approximately ten years ago. Multiple groups, using *Xenopus* egg extract, showed that misregulation of the

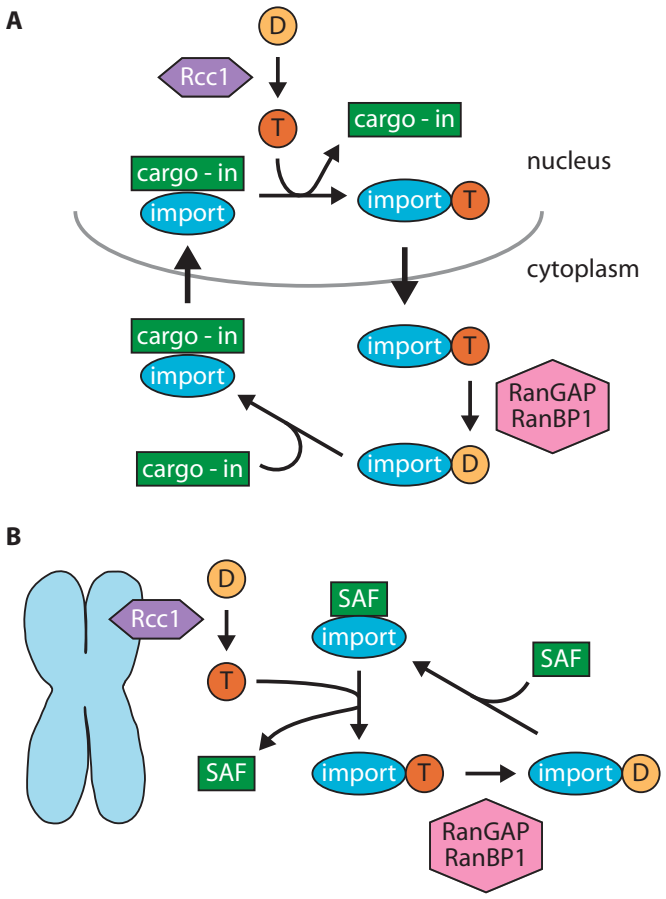


Figure 1-3. The Ran-GTP pathway.

(A) Ran-GTP controls nuclear import. **(B)** Ran-GTP promotes the release of the spindle assembly factors (SAF) near chromosomes to induce spindle assembly. Orange circles, Ran in the GTP state (T) and the GDP state (D); purple hexagon, Ran GEF Rcc1; pink hexagon, RanGAP and its cofactor RanBP1; blue oval, nuclear import factor; green rectangles, the cargo of the import factor (cargo-in or SAF); gray semicircle, nuclear envelope; light blue object, chromosome.

Ran nucleotide state leads to aberrant microtubule polymerization (Carazo-Salas et al., 1999; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999; Zhang et al., 1999). Spindle assembly is inhibited upon depletion of RCC1, addition of Ran mutants locked in the GDP-bound state, or addition of the RanGAP cofactor RanBP1. In contrast, addition of Ran mutants locked in the GTP-bound state lead to increased microtubule polymerization. Ran-GTP was later shown to promote microtubule assembly by releasing spindle assembly factors from inhibitory nuclear import proteins in M-phase (Figure 1-3B)(Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001). These spindle assembly factors, e.g. TPX2 and NuMA, promote spindle assembly in part via the nucleation and polymerization of microtubules (Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001; Trieselmann et al., 2003; Tsai et al., 2003; Ribbeck et al., 2007; Casanova et al., 2008). Since RCC1 binds to chromosomes in M-phase (Nemergut et al., 2001) while RanGAP and RanBP1 are localized in the cytoplasm, Ran-GTP levels are higher near chromosomes than elsewhere in the cytoplasm and a gradient of Ran-GTP is created. This gradient of Ran-GTP, which has been visualized (Kalab et al., 1999; Niethammer et al., 2004; Caudron et al., 2005; Bastiaens et al., 2006; Kalab et al., 2006), promotes localized microtubule polymerization by releasing spindle assembly factors near chromosomes. Though Ran-GTP is essential, this gradient is not strictly needed for spindle assembly around chromosomes, as the addition of two Ran mutants (one locked in the GTP-bound state and one locked in the GDP-bound state) to *Xenopus* egg extract flattened the Ran-GTP gradient but did not inhibit spindle assembly around chromosomes (Maresca et al., 2009).

Chromosomal passenger complex pathway

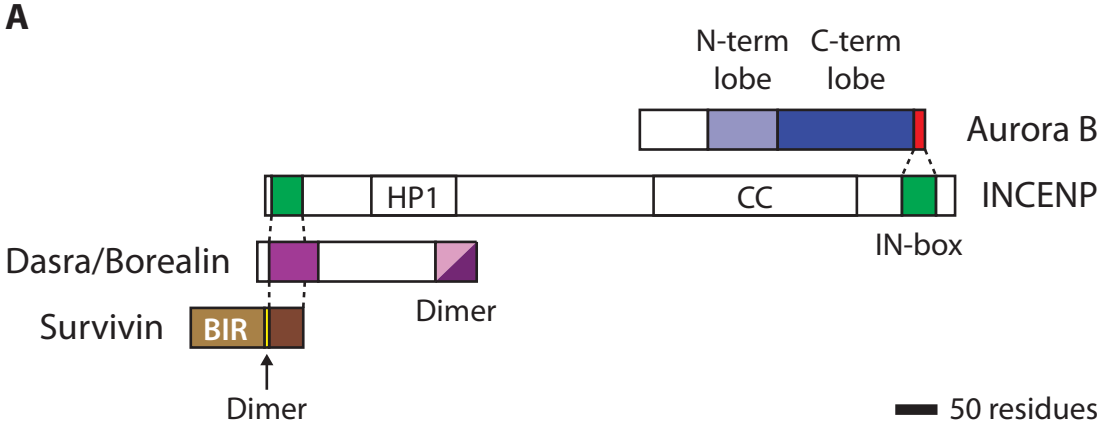
In addition to the Ran-GTP pathway, chromosomes also promote spindle assembly through the CPC, which is composed of the kinase Aurora B, INCENP, Dasra (also known as Borealin), and Survivin (Figure 1-4A) (Ruchaud et al., 2007). Like Ran-GTP, the CPC is required for chromosome-induced spindle assembly (Sampath et al., 2004) and a gradient of Aurora B-dependent phosphorylation has been suggested to spatially regulate cell division (Fuller et al., 2008). I will elaborate further on the CPC and its functions in M-phase in the following section.

The chromosomal passenger complex

The discovery of the CPC

Earnshaw and Bernat coined the term "chromosome passenger protein" to generally describe a protein that localizes to chromosomes and performs some non-chromosomal function late in mitosis (Earnshaw and Bernat, 1991). The members of the CPC are a subset of these proteins and form a complex together (Adams et al., 2001a). The founding member of the CPC was identified in 1987 based on immunohistochemistry (Cooke et al., 1987). In this paper, Earnshaw and colleagues created antibodies against the "mitotic chromosome scaffold" to identify new components. The monoclonal antibody they made was used to characterize INCENP (inner centromere protein). In this first study, INCENP was shown to have a cell-cycle-dependent localization. While INCENP stained the entire condensed metaphase chromosome, it was selectively enriched at the centromere in metaphase. At the metaphase-to-anaphase transition, INCENP

A



B

	Aurora B	INCENP	Dasra/Borealin	Survivin
mammals	Aurora B	INCENP	Borealin	Survivin
Xenopus	Aurora B	INCENP	Dasra A & B	Survivin/SIX
Drosophila	Ial	INCENP	Borealin	Deterin
C. elegans	AIR-2	ICP-1	CSC-1 ?	BIR-1
S. cerevisiae	Ipl1	Sli15	Nbl1	Bir1
S. pombe	Ark1	Pic1	Nbl1	Cut17

Figure 1-4. The chromosomal passenger complex.
(A) The members of the chromosomal passenger complex (CPC) are depicted. The structure has been solved for the areas in color but not for those in white. The colors correspond to those used in Figures 1-5 and 1-6 of the structures. For Aurora, the N- and C-terminal lobes of the kinase (N-term lobe and C-term lobe) are labeled; for INCENP, the IN-box, the putative HP1 binding domain, and the putative coiled-coil (CC) domain; and for Dasra and Survivin, the homodimerization domains (Dimer) and the BIR domain of Survivin. The dashed lines connect the domains that interact in the crystal structures. Not depicted is the "crown" interaction of the IN-box with the N-terminal lobe of Aurora B. Black bar, 50 residues. **(B)** The various homologues for the different CPC members are listed. Based on the minimal sequence similarity, whether CSC-1 is a Dasra/Borealin-like protein is unclear, as indicated by the question mark (?).

dramatically redistributed off the chromosomes to the central spindle and to the cortical region of the cleavage furrow (Cooke et al., 1987).

Following this initial discovery of INCENP, the Earnshaw laboratory showed that INCENP interacts with Aurora B, a serine/threonine kinase (Adams et al., 2000). The founding member of the Aurora kinase family was identified in a *Drosophila* screen for mitotic defects (Glover et al., 1995). Aurora was later recognized as the homologue of *S. cerevisiae* Ipl1 (increase-in-ploidy-1), which was identified in a screen for changes in chromosomal copy number (Chan and Botstein, 1993). While yeasts contain one Aurora-like gene (Ipl1 in *S. cerevisiae* and Ark1 in *S. pombe*), higher eukaryotes have up to three, which have been designated Aurora A, B, and C (Adams et al., 2001a). While Aurora A functions in centrosome maturation and bipolar spindle formation (Barr and Gergely, 2007), the function of Aurora C is less clear. A product of an Aurora B-gene duplication event (Brown et al., 2004), Aurora C appears to be testes specific (Tseng et al., 1998) and may be functionally similar to Aurora B (Slattery et al., 2009).

The third member of the CPC to be identified was Survivin. Initially suspected of being linked to Aurora B and INCENP due to their similar localization patterns and knockout phenotypes (Speliotes et al., 2000; Uren et al., 2000), Survivin was later shown to interact directly with Aurora B and INCENP *in vitro* (Wheatley et al., 2001a). Survivin, which contains only one BIR (baculovirus IAP repeat) domain, was originally identified as a member of the inhibitor of apoptosis protein (IAP) family (Ambrosini et al., 1997). While still controversial, Survivin is suggested to inhibit apoptosis through a mitochondria-

based mechanism (Altieri, 2006), in addition to its roles in mitosis as a member of the CPC.

Dasra (also known as Borealin and CSC-1) is the fourth, and the most recently identified, member of the CPC. The protein was originally identified in *C. elegans* as CSC-1 (Romano et al., 2003). Using two different screens for proteins that bind to metaphase chromosomes, two separate laboratories later identified Borealin and Dasra in humans and *Xenopus*, respectively (Gassmann et al., 2004; Sampath et al., 2004). While limited in homology, CSC-1 is proposed to be the Dasra/Borealin homologue in worms (Figure 1-4B). Similar to Survivin, Dasra was initially thought to be a member of the CPC based on its localization and then confirmed via interaction studies with Aurora B, INCENP, and Survivin (Romano et al., 2003; Gassmann et al., 2004; Sampath et al., 2004). While only one Dasra protein has been found in mammals (termed Dasra B or Borealin), two Dasra proteins exist in *Xenopus*, chicken, and zebrafish (termed Dasra A and B). Although a Dasra A-like protein may exist in mammals, it is suggested that Dasra A, in a role distinct from Borealin, may be important for the rapid embryonic cell cycles that exist in the organisms that have both proteins (Sampath et al., 2004). Unidentified for years in yeast, a Dasra/Borealin homologue, Nbl1p (novel borealin-like protein), was recently discovered in both budding and fission yeast (Bohnert et al., 2009; Nakajima et al., 2009).

The structure of the CPC

The crystal structure of the full complex has yet to be solved. The structures for the following parts of the complex, however, have been elucidated: Aurora B (aa 60-361) with a segment of INCENP (aa 790-847)(Sessa et al., 2005); a

subcomplex of Survivin, Borealin (aa 10-109), and INCENP (aa 1-68)(Jeyaprakash et al., 2007); Survivin and Borealin (aa 20-78)(Bourhis et al., 2007); Borealin (aa 207-280)(Bourhis et al., 2009); and full-length Survivin (Chantalat et al., 2000; Muchmore et al., 2000; Verdecia et al., 2000; Sun et al., 2005).

Aurora B (aa 60-361) was crystallized in the presence of a C-terminal INCENP peptide (aa 790-847; IN-box (Adams et al., 2000), Figure 1-4A) that is important for Aurora B activation (Kang et al., 2001; Bishop and Schumacher, 2002; Honda et al., 2003). Aurora B has a classic bi-lobe protein-kinase fold with an ATP binding pocket between the two lobes. The N-terminal lobe (aa 86-174; Figure 1-5, light blue) interacts with kinase regulators, while the C-terminal lobe (aa175-347; Figure 1-5, dark blue) interacts with substrates and directs phosphate transfer. The IN-box peptide (Figure 1-5, green) interacts with Aurora B at the N-terminal lobe, creating a "molecular crown" (Figure 1-5, left), and at an extension off the C-terminal lobe of Aurora B (Figure 1-5, right; red)(Sessa et al., 2005). While the IN-box does not directly interact with the kinase activation loop (Figure 1-5, yellow), INCENP is suggested to allosterically induce the extension of this loop, which is required for full activation. In addition, since the structure represents a partially active state with the catalytic cleft of the kinase closed, Musacchio and colleagues suggest that the phosphorylation of the IN-box TSS motif facilitates the opening of the catalytic cleft, which is required for the full activation of Aurora B. This TSS motif is phosphorylated by Aurora B. This phosphorylation, however, presumably occurs in trans by another Aurora B, since the TSS motif of INCENP cannot reach the catalytic pocket of the Aurora B it is bound to (Sessa et al., 2005), strongly suggesting that the activation of Aurora B is concentration dependent.

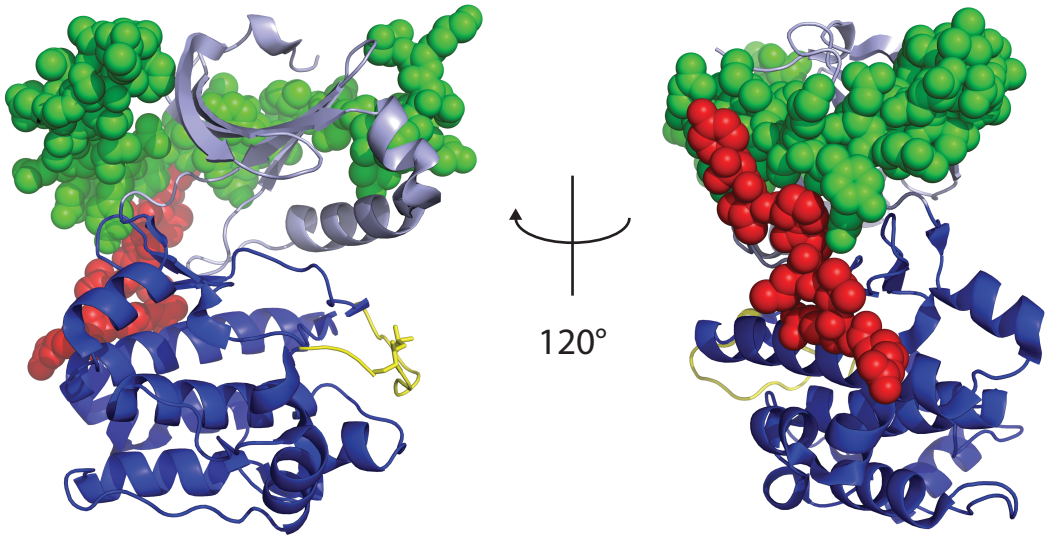


Figure 1-5. The structure of *X. laevis* Aurora B with the IN-box of INCENP. For Aurora, the N-terminal lobe of the kinase is in light blue; the C-terminal lobe, dark blue; the extension off the C-terminal lobe, red; the activation loop with the phosphorylation site in stick form, yellow. The IN-box peptide from INCENP is in green. The figure was made using PyMol with the published structure in the RCSB Protein Data Bank (ID: 2BFX)(Sessa et al., 2005).

While Aurora B interacts with the C-terminus of INCENP, the N-terminus of INCENP interacts with Dasra/Borealin and Survivin (Figure 1-4A)(Ruchaud et al., 2007). The N-terminal 58 residues of INCENP were crystallized with Borealin (aa 10-109) and Survivin (Figure 1-6A, B). Residues 8-46 of INCENP, 15-60 of Borealin, and 100-141 of Survivin form a three-helix bundle. While the helices of INCENP and Survivin stack in a parallel fashion, the Borealin helix is anti-parallel (Jeyaprakash et al., 2007). The crystal of structure of this helical bundle in the absence of INCENP was also solved. In the absence of INCENP, the Borealin (aa 20-60) helix still stacks anti-parallel to the Survivin C-terminal helix (aa 100-142)(Bourhis et al., 2007). This subcomplex of Borealin and Survivin, however, homodimerizes in the absence of INCENP, and the INCENP N-terminal helix appears to disrupt this oligomerization by interacting with Borealin and Survivin (Jeyaprakash et al., 2007).

The structure of the C-terminus of Borealin (aa 207-280) has also been solved (Figure 1-6C). Using NMR spectroscopy of the soluble protein, residues 226-277 of Borealin were well defined in the structure as a homodimer with C2 symmetry and anti-parallel monomers (Bourhis et al., 2009). The functional significance of this dimerization is currently unclear.

The structure of full-length Survivin was actually the first of the CPC components to be solved (Chantalat et al., 2000; Muchmore et al., 2000; Verdecia et al., 2000; Sun et al., 2005). While all three original papers revealed crystals of homodimerized Survivin, two potential dimer interfaces were identified among the studies (Chantalat et al., 2000; Muchmore et al., 2000; Verdecia et al., 2000). Subsequently, one of these dimer interfaces was confirmed by NMR studies of Survivin in solution (Sun et al., 2005). Survivin forms a bow-tie-shaped dimer

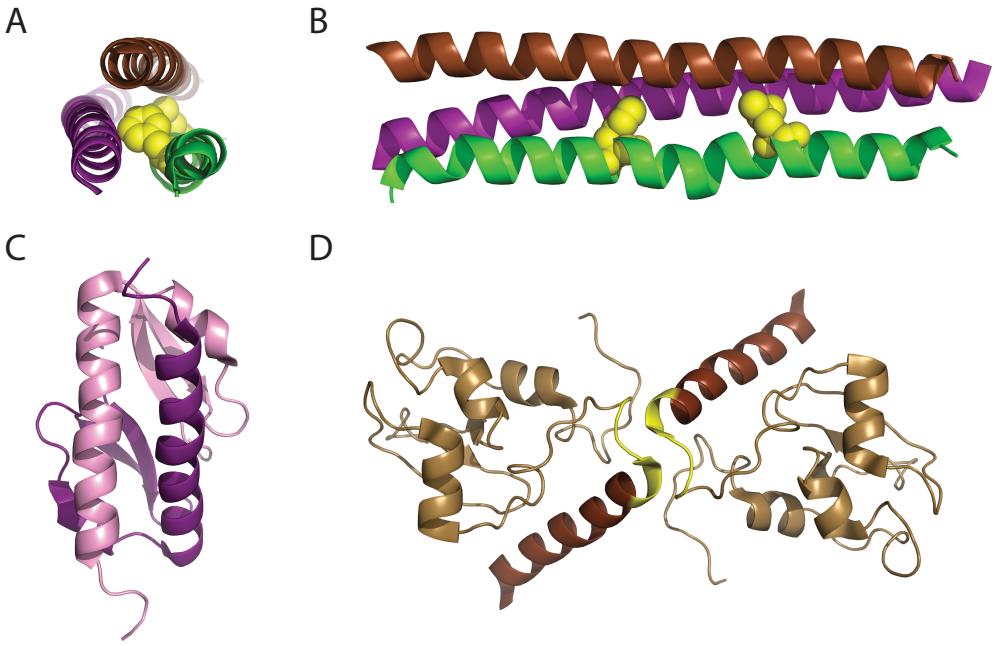


Figure 1-6. The structures of human Borealin, Survivin, and N-terminal INCENP.

(A and B) Two views of the three-helix bundle formed by INCENP (aa 1-57; green), Borealin (aa 15-60; purple) and Survivin (100-141; brown). F22 and L34 of INCENP are highlighted in yellow. **(C)** The homodimer formed by the C-terminus of Borealin (aa 226-277). Each monomer is in a different shade of purple. **(D)** The homodimer formed by Survivin. The BIR domain is in light brown; the C-terminal helix, dark brown; and the dimerization interface, yellow. The figure was made using PyMol with the published structures in the RCSB Protein Data Bank (for A and B, 2QFA; for C, 2KDD; and for D, 1XOX)(Sun et al., 2005; Jeyaprakash et al., 2007; Bourhis et al., 2009).

(Figure 1-6D), where a hydrophobic dimer interface is mainly created by the linker between the BIR domain and the C-terminal helix that interacts with Dasra and INCENP. This linker region (aa 94-99; Figure 1-6D, yellow) forms an intermolecular anti-parallel β-sheet (Chantalat et al., 2000; Verdecia et al., 2000). Interestingly, Survivin does not dimerize when in complex with Borealin and INCENP (Bourhis et al., 2007; Jeyaprakash et al., 2007). Part of Borealin appears to mimic the structure of the Survivin dimerizing linker region, which may explain how Borealin displaces half of the homodimer (Bourhis et al., 2007).

Transfer of the CPC from chromosomes to microtubules

As mentioned above, the CPC has very distinct localizations during M-phase (Figure 1-7). In prophase, the complex uniformly coats the chromosome arms. As the cell cycle progresses to prometaphase and metaphase, the CPC enriches at the centromere. At the metaphase-to-anaphase transition, the CPC moves from the chromosomes to the microtubules in the central spindle, where it stays in the midbody through telophase (Ruchaud et al., 2007). While the mechanisms that control the cell-cycle-dependent subcellular localizations of the CPC are not fully understood, some of the details that direct the transition from the metaphase chromosomes to the anaphase microtubules have been elucidated.

The knockdown of any one CPC member leads to a decrease in the protein levels of the other members and to general mislocalization (Adams et al., 2001b; Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003; Honda et al., 2003; Lens et al., 2003), as the localization of the CPC to the centromeres of (pro)metaphase chromosomes and to the microtubules of the anaphase central spindle depends on the interaction of INCENP with Dasra and Survivin (Klein et

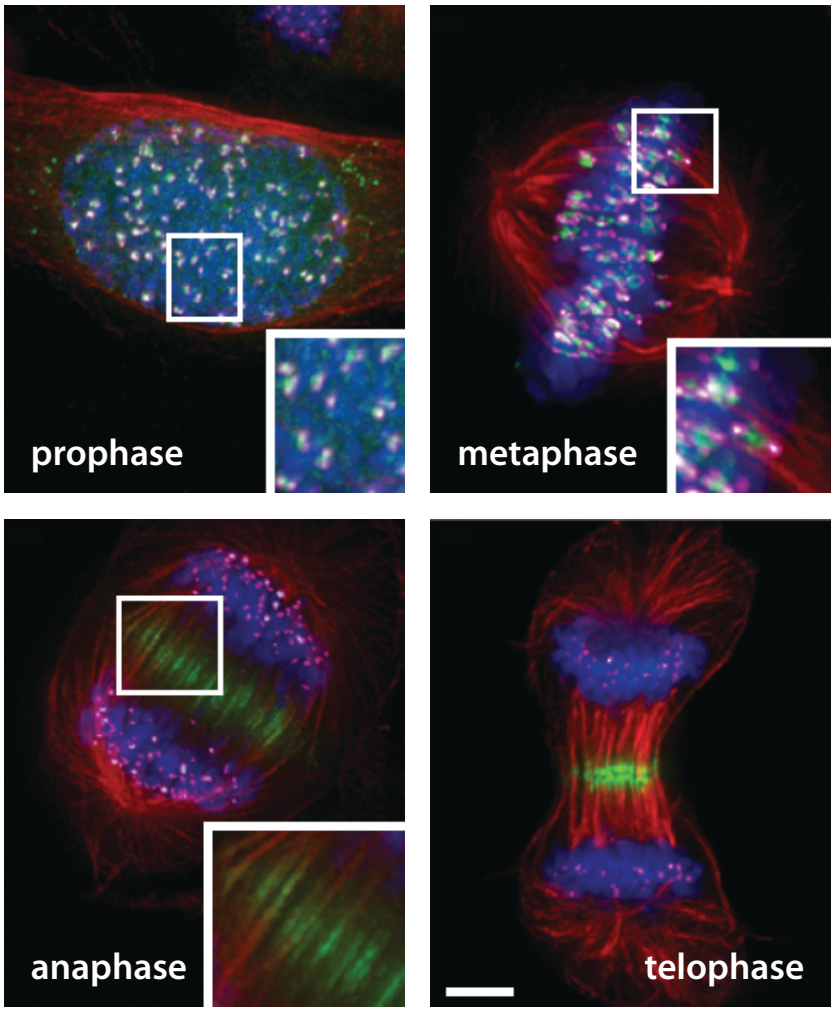


Figure 1-7. The localization of the CPC.
The localization of one CPC member, Aurora B is shown. The localization for the other members is the same. The images are of Aurora B immunofluorescence in HeLa cells during different phases of mitosis. Insets show magnifications of boxed area in larger image. In blue, the DNA; red, the microtubules; pink, centromeres; and green, Aurora B. Scale bar, 5 μm. The image is reproduced from Ruchard et al., 2007.

al., 2006; Jeyaprakash et al., 2007). Indeed, a double-point mutant of INCENP (F22R, L34R; see Figure 1-6A, B, in yellow) that abolishes the formation of the helical bundle with Dasra and Survivin inhibits localization to centromeres and the central spindle (Jeyaprakash et al., 2007). While one study suggests a role for Dasra in the centromeric localization of the CPC (Gassmann et al., 2004), others suggest that Survivin is the protein that actually targets the CPC to the different subcellular structures (Lens et al., 2006; Vader et al., 2006; Yue et al., 2008). Overexpression of a Borealin construct that lacks its C-terminus in HeLa cells interfered with the centromeric, but not central spindle, localization of the CPC, suggesting that the C-terminus of Dasra is important for the centromeric localization of the CPC (Gassmann et al., 2004). In cells depleted of Borealin, however, INCENP can target to centromeres and the midbody if it is fused to Survivin, suggesting that Dasra is not directly involved in the localization of the CPC (Vader et al., 2006). Furthermore, the deletion or mutation of the BIR domain of Survivin affects centromeric localization of the CPC (Lens et al., 2006; Yue et al., 2008). Together, these results suggest that Survivin plays a key role in directing the CPC to different subcellular structures. The role of Dasra in the targeting of the CPC, however, is less clear, and more work is needed to determine if Dasra plays a direct role or if it simply functions to stabilize the INCENP-Survivin interaction.

As mentioned above, the localization of the CPC to the anaphase central spindle microtubules depends on the interaction of INCENP, Dasra, and Survivin (Klein et al., 2006; Jeyaprakash et al., 2007). While Survivin has been implicated in the anaphase localization of the CPC (Vader et al., 2006; Yue et al., 2008), other factors also affect the localization of the CPC in anaphase, for

instance MKLP2 (mitotic kinesin-like protein-2). Depletion of MKLP2 inhibits the localization of the CPC to the central spindle in anaphase (Gruneberg et al., 2004). How MKLP2 controls the localization of the CPC, however, has not been determined.

In addition to the aforementioned factors, the dephosphorylation of the CPC at the metaphase-to-anaphase transition is important for its localization to the central spindle microtubules. In NRK cells, injection of a non-degradable cyclin B, which does not affect chromosome segregation, prevents Aurora B from dissociating from chromosomes and associating with microtubules in anaphase (Murata-Hori et al., 2002). Furthermore, in budding yeast, cells that lack Cdc14, the phosphatase that reverses mitotic Cdk1 phosphorylation events (Stegmeier and Amon, 2004), fail to localize Ip1 and Sli15, the Aurora B and INCENP homologues, to the anaphase spindle (Pereira and Schiebel, 2003). A mutant of Sli15 that cannot be phosphorylated by Cdk1 (S335A, S373A, S427A, S437A, S462A, and T474A) prematurely localizes to microtubules in metaphase (Pereira and Schiebel, 2003). Of the six mutated serine/threonine residues in the Sli15 phospho-mutant, none are conserved in other eukaryotes (Figure 1-8). One of these sites (S335) has a well-conserved serine-proline pair in a nearby region in higher eukaryotes. While this site could be phosphorylated by Cdk1, it does not fit the canonical Cdk1 phosphorylation consensus site, lacking the basic residue at the +2 position (Moreno and Nurse, 1990). Cdk1, however, does phosphorylate human INCENP at T57 and T388 (Goto et al., 2006). Since T388 of human INCENP is not near any of the mutated budding yeast sites, it is unclear if it plays a role in the anaphase localization of the CPC. T57, however, is important, as a phospho-mimetic mutant of INCENP (T57E) fails to localize to

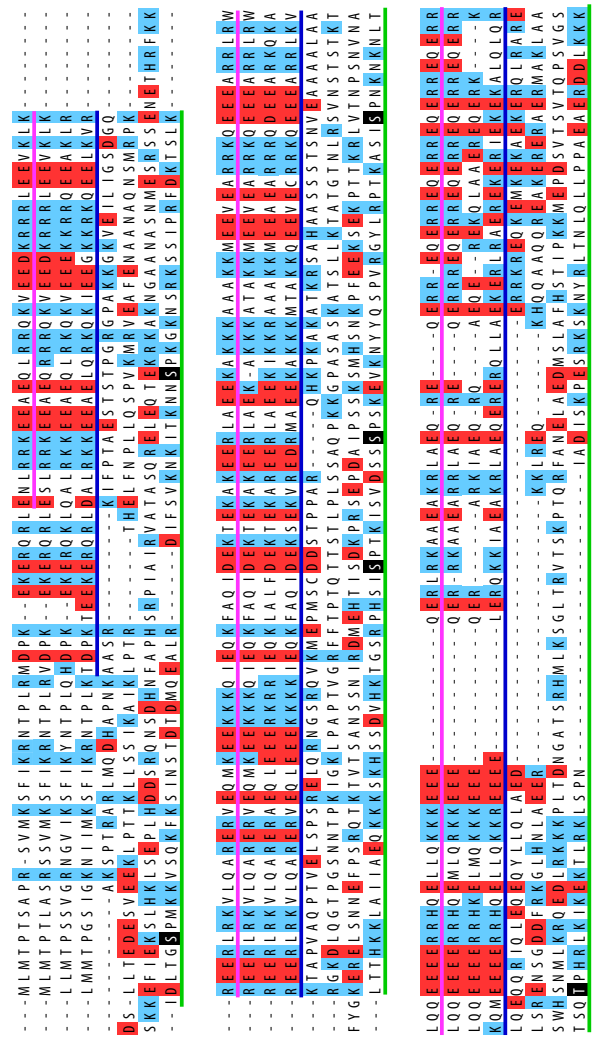


Figure 1-8. Alignment the putative coiled-coil domain of INCENP.

INCENPs and INCENP homologues from eight organisms were aligned using ClustalW. Only the region corresponding to the coiled-coil (CC) domain is displayed. In red, acidic residues; in blue, basic residues; in black, residues that are mutated in Pereira et al., 2003; underlined in dark blue, the CC domain of *Xenopus* INCENP used in this thesis (aa 491-747); underlined in pink, the CC domain of human INCENP used in Vader et al., 2007 (aa 539-747); and underlined in green, the middle domain of Sli15 described in Kang et al., 2001 (aa 227-558).

the central spindle microtubules in anaphase (Hummer and Mayer, 2009). Dephosphorylation of this site, presumably by Cdc14, is important for the interaction with MKLP2 (Gruneberg et al., 2004; Hummer and Mayer, 2009). Whether INCENP is the only member of the CPC whose phosphorylation status affects the transfer to microtubules is unknown.

Another post-translational modification that is suggested to regulate the chromosome-to-microtubule transfer of the CPC is ubiquitinylation (Sumara et al., 2007; Maerki et al., 2009). Aurora B is ubiquitinylated by Cul3, which recognizes Aurora B via a BTB (Bric-a-brac/Tramtrack/Broad complex) adapter protein, KLHL21 (Maerki et al., 2009). While it is not clear if the ubiquitinylation of Aurora B itself is required for the chromosome-to-microtubule transfer of the CPC, knockdown of either Cul3 or KLHL21 reduces the chromosome removal of the CPC in anaphase and reduces the amount of CPC that localizes to the anaphase central spindle microtubules (Sumara et al., 2007; Maerki et al., 2009). While Cul3 and KLHL21 localizes to the central spindle microtubules (Sumara et al., 2007; Maerki et al., 2009), it is unclear if Aurora B targets them to the anaphase microtubules or vice versa. In addition to Aurora B, Survivin is also ubiquitinylated. While the de-ubiquitinylation of Survivin is not involved, whether the ubiquitinylation of Survivin plays a role in the chromosome-to-microtubule transfer of the CPC has not been studied (Vong et al., 2005).

Mobility of the CPC

In addition to changes in subcellular localization during the cell cycle, the mobility of the CPC at its different locations is variable and differs among the different members of the CPC, based on fluorescence recovery after

photobleaching (FRAP) data of Aurora B and Survivin. The mobility of Survivin changes drastically during the cell cycle (Beardmore et al., 2004; Delacour-Larose et al., 2004; Vong et al., 2005). While Survivin is very static on the chromosomes in G2 phase, it is highly mobile at the centromere in metaphase (Beardmore et al., 2004; Delacour-Larose et al., 2004; Vong et al., 2005). This mobility decreases if microtubules are disrupted or Aurora B kinase activity is inhibited (Beardmore et al., 2004; Delacour-Larose et al., 2004). Furthermore, the mobility of Survivin at centromeres is dependent on the deubiquitinylating enzyme hFam (Vong et al., 2005). In anaphase, Survivin on the central spindle microtubules, like in G2 phase, is not very mobile (Beardmore et al., 2004; Delacour-Larose et al., 2004).

The mobility of Aurora B is more controversial. While the static nature of Aurora B on chromatin in G2 phase and on central spindle microtubules in anaphase is not disputed, data from two studies as to whether Aurora B is mobile or not at centromeres in metaphase is conflicting (Murata-Hori and Wang, 2002; Delacour-Larose et al., 2004). Both studies show that Aurora B is less mobile than Survivin at centromeres. The FRAP data from Dimitrov and colleagues show that Aurora is not mobile (Delacour-Larose et al., 2004), while data from Murata-Hori and Wang show that the residence time of Aurora B at centromeres is approximately fifty seconds and, like that for Survivin, is sensitive to microtubule depolymerization and Aurora B kinase activity (Murata-Hori and Wang, 2002). This difference in Aurora B mobility may be due to the different techniques used to introduce fluorescently labeled Aurora B in each study: while Dimitrov and colleagues used a stably expressing cell line, Murata-Hori and Wang used a cell line that transiently expressed Aurora B (Delacour-Larose et al., 2004).

Activation of Aurora B

Aurora B requires multiple steps to become a fully activated kinase. First, Aurora B must interact with INCENP via its IN-box (Figure 1-5)(Kang et al., 2001; Bishop and Schumacher, 2002; Honda et al., 2003; Sessa et al., 2005). This interaction facilitates full activation by inducing the extension of the kinase activation loop (Sessa et al., 2005). Second, the kinase activation loop must become phosphorylated (Yasui et al., 2004). Third, the TSS motif on INCENP must be phosphorylated by Aurora B (Kang et al., 2001; Bishop and Schumacher, 2002; Honda et al., 2003; Sessa et al., 2005). This phosphorylation is suggested to occur in trans and induce the opening of the catalytic cleft of Aurora B (Sessa et al., 2005). All three steps are required to fully activate Aurora B.

The activation of Aurora B can be induced by chromosomes and microtubules that are stabilized by the drug taxol (Mackay et al., 1993; Kang et al., 2001; Kuntziger et al., 2001; Wheatley et al., 2001b; Kelly et al., 2007; Ruchaud et al., 2007; Rosasco-Nitcher et al., 2008; Tseng et al., in submission). While the CPC can bind to both of these structures, it is not clear how these structures activate Aurora B. A clustering-based mechanism has been suggested (Kelly et al., 2007). Since a trans-phosphorylation event by another Aurora B is required, full kinase activation should be concentration dependent (Sessa et al., 2005; Kelly et al., 2007). Since the CPC targets to chromosomes in (pro)metaphase and to microtubules in anaphase (Ruchaud et al., 2007), these structures may simply serve as platforms to increase the local concentration of CPC (Kelly et al., 2007). As further evidence for a concentration-driven activation mechanism, addition of an anti-INCENP antibody (with two antigen recognition sites and presumably

clusters two CPC) to *Xenopus* egg extract induces the activation of Aurora B, while addition of the Fab fragment of the same antibody (with only one antigen site) fails to activate Aurora B (Kelly et al., 2007). This result, in combination with the required trans-phosphorylation event, strongly suggests that chromosomes and microtubules activate Aurora B by increasing the local concentration of the CPC (Kelly et al., 2007).

As expected, since the activation loop of Aurora B and the TSS motif in the IN-box of INCENP both need to be phosphorylated to activate Aurora B (Kang et al., 2001; Bishop and Schumacher, 2002; Honda et al., 2003; Yasui et al., 2004; Sessa et al., 2005), *in vitro* dephosphorylation of the CPC by an exogenous phosphatase, such as lambda phosphatase, inactivates Aurora B (Bolton et al., 2002). While phosphatase activities balance the kinase activity of Aurora B in many of the functions the CPC regulates (Francisco et al., 1994; Hsu et al., 2000; Murnion et al., 2001; Pinsky et al., 2006; Emanuele et al., 2008; Toure et al., 2008; Wang et al., 2008; Vanoosthuyse and Hardwick, 2009), whether phosphatases directly inactivate Aurora B *in vivo* has yet to be clearly shown. Two candidate phosphatases that may inactivate Aurora B are PP1 and PP2A, as they interact with Aurora B *in vivo* and *in vitro* (Sugiyama et al., 2002), and incubation of the CPC with either PP1 or PP2A reduces Aurora B kinase activity *in vitro* (Sugiyama et al., 2002; Sun et al., 2008). Glc7, the *S. cerevisiae* homologue of PP1, however, does not seem to affect the *in vitro* kinase activity of Ip1, the *S. cerevisiae* Aurora B homologue (Pinsky et al., 2006). Further studies are required to determine if and which phosphatases inactivate Aurora B *in vivo*.

How the activation of Aurora B is regulated is not fully understood. Many proteins have been implicated in activating Aurora B. Interestingly, three kinases

have been shown to increase Aurora B kinase activity: Tlk-1 (Tousled-like kinase)(Han et al., 2005; Riefler et al., 2008), Mps1 (Jelluma et al., 2008), and Chk1 (Zachos et al., 2007). The mechanism that activates Aurora B by each kinase appears to be different. For Tlk-1-induced activation of Aurora B, Aurora B must first phosphorylate and activate Tlk-1 (Han et al., 2005). Once activated, Tlk-1 in a kinase-independent fashion can feedback and activate Aurora B (Han et al., 2005; Riefler et al., 2008). Mps1, on the other hand, activates Aurora B via the phosphorylation of Borealin (Jelluma et al., 2008). How the phosphorylation of Borealin activates Aurora B is unclear. Chk1 activates Aurora B only in response to a taxol-induced checkpoint via an unknown mechanism (see below in Signaling the spindle assembly checkpoint)(Zachos et al., 2007).

Other proteins that have been suggested to regulate Aurora B are EB1 (Sun et al., 2008), Cdc37 (Lange et al., 2002), TD60 (Rosasco-Nitcher et al., 2008), and BubR1 (Lampson and Kapoor, 2005). While EB1, Cdc37, and TD60 activate Aurora B, BubR1 inhibits the kinase (Lange et al., 2002; Lampson and Kapoor, 2005; Rosasco-Nitcher et al., 2008; Sun et al., 2008). EB1, a plus-end microtubule tracking protein, is suggested to inhibit PP1 inactivation of Aurora B (Sun et al., 2008), while Cdc37 with Hsp70, a chaperone, is suggested to stabilize Aurora B (Lange et al., 2002). How TD60 and BubR1 regulate Aurora B kinase activity is not clear. More work is necessary to elucidate the mechanisms by which these proteins regulate Aurora B activity.

Functions of the CPC in the cell cycle

The CPC has multiple functions throughout the cell cycle (Ruchaud et al., 2007). In early M-phase, the CPC has been shown to play a role in establishing

proper mitotic chromosome structure, promoting microtubule polymerization in spindle assembly, correcting aberrant kinetochore-microtubule attachments, and signaling the spindle assembly checkpoint. In late M-phase, the CPC functions in stabilizing the central spindle, defining the site of furrow ingression, and timing cytokinesis and nuclear envelope reformation. Below, I will describe the current knowledge of the CPC in these functions.

Chromosome structure. The mitotic chromosome is a highly compacted structure that is 500-fold more compressed than interphase chromatin (Georgatos et al., 2009). The CPC has been suggested to aid in the establishment of this compacted mitotic chromosome by inducing the mitotic release of interphase chromatin-bound proteins and recruiting other proteins that promote chromosome condensation.

Many proteins that bind to interphase chromatin are released from chromosomes in mitosis (Egli et al., 2008), which is suggested to aid in chromosome condensation. While the mechanisms that regulate the chromosomal dissociation for most of these proteins have yet to be determined, Aurora B has been implicated in the M-phase release for a few of these proteins (Losada et al., 2002; MacCallum et al., 2002; Fischle et al., 2005; Hirota et al., 2005; Terada, 2006; Hall et al., 2009; Loomis et al., 2009). The most mechanistically clear of these Aurora B-dependent chromosome releases is that of HP1 (heterochromatin protein 1), a protein that binds to chromatin via methylated lysine 9 of histone H3 (H3K9)(Bannister et al., 2001; Jacobs et al., 2001; Lachner et al., 2001; Nakayama et al., 2001; Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002). As I will describe in detail in Chapter 3, my work and that of others show

that in M-phase, the phosphorylation of serine 10 on histone H3 (H3S10) by Aurora B decreases the affinity of HP1 for its chromatin-binding site and ejects the protein from the chromosomes (Fischle et al., 2005; Hirota et al., 2005; Terada, 2006). The phosphorylation of H3S10, however, may not be the only mechanism that assists in the removal of HP1 from mitotic chromosomes (Mateescu et al., 2004; Terada, 2006; Loomis et al., 2009). The role of the CPC in the dissociation of other proteins from the chromosome during M-phase is less well characterized. While the CPC and Aurora B appears to be required for the chromosomal disassociation of these proteins (Losada et al., 2002; MacCallum et al., 2002; Terada, 2006; Hall et al., 2009; Loomis et al., 2009), it is unclear if, like in the removal of HP1, H3S10 phosphorylation is directly involved. The biological significance for the removal of any of these proteins has not been elucidated.

In addition to its functions in protein removal from mitotic chromosomes, the CPC is suggested to be involved in the recruitment of condensin (Giet and Glover, 2001; Morishita et al., 2001; Kaitna et al., 2002; Petersen and Hagan, 2003; Ono et al., 2004; Lipp et al., 2007; Takemoto et al., 2007), a protein complex that promotes chromosome condensation (Hirano, 2005). Condensin is a five-subunit complex composed of two SMC (structural maintenance of chromosome) proteins and three non-SMC proteins. While there is only one form of condensin in yeasts and nematodes, two forms of condensin (I and II) exist in most organisms (Hirano, 2005). While it is unclear how condensin compacts the chromosome, condensin I and II appear to have distinct functions in chromosome condensation (Ono et al., 2003; Hirota et al., 2004).

The exact role of the CPC in condensin recruitment and condensation is controversial. Inhibition of Aurora B kinase activity or loss of the CPC abrogates

the recruitment of condensin to chromosomes in some studies (Giet and Glover, 2001; Morishita et al., 2001; Kaitna et al., 2002; Petersen and Hagan, 2003; Lipp et al., 2007; Takemoto et al., 2007), while there is no effect on bulk condensin localization in others (Losada et al., 2002; MacCallum et al., 2002; Sumara et al., 2002; Ono et al., 2004). One study provides data showing that although condensin still loads on chromosome arms, the centromeric enrichment of condensin is lost in the absence of the CPC (Ono et al., 2004). Adding to the confusion, other data suggests that the CPC plays a role in late, but not early, chromosome compaction, which may or may not be condensin dependent (Lavoie et al., 2004; Maddox et al., 2006; Mora-Bermudez et al., 2007). While it is unclear why different studies using similar techniques in similar organisms show differences in condensin recruitment in the absence of the CPC, the controversy over the chromosome condensation effect may stem from interpretation. Albeit more "fuzzy" than in wild-type cells, chromosomes still compact in prometaphase of condensin-depleted cells (Kaitna et al., 2002; Ono et al., 2003; Hirota et al., 2004; Savvidou et al., 2005). Since there is no quantifiable measure of chromosome compaction in each of these studies, the severity of the phenotype is open to interpretation (Hirano, 2004). Therefore, the CPC may not affect gross chromosome compaction but may still be important for the proper condensation of chromosomes.

Microtubule assembly in spindle formation. While the CPC in mitotic somatic cells does not appear to play an important role in microtubule assembly during spindle formation (Adams et al., 2001a; Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003; Honda et al., 2003; Lens et al., 2003; Yue et al., 2008),

the CPC is essential for chromosome-induced microtubule polymerization in *Xenopus* egg extract (Sampath et al., 2004; Maresca et al., 2009). Activated by the chromosomes, Aurora B promotes microtubule polymerization via the phosphorylation of its substrates. One such substrate is MCAK (also known as XKCM1)(Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004; Zhang et al., 2007), a major microtubule depolymerizing enzyme (Walczak et al., 1996). Phosphorylation of MCAK by Aurora B inhibits its microtubule depolymerizing activity (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004), leading to microtubule polymerization. A second substrate of Aurora B is Op18 (also known as Stathmin)(Gadea and Ruderman, 2006; Kelly et al., 2007). Like MCAK, Op18 promotes the depolymerization of microtubules (Belmont and Mitchison, 1996) and its activity is suppressed by phosphorylation during mitosis (Marklund et al., 1996; Andersen et al., 1997; Di Paolo et al., 1997; Larsson et al., 1997; Tournebize et al., 1997; Budde et al., 2001). While Aurora B inhibits MCAK and Op18 to promote microtubule polymerization, other substrates of Aurora B may also play a role.

Although the CPC is not required for microtubule polymerization in somatic cells, some studies suggest that the CPC may still play a role in spindle formation. In addition to those originating at centrosomes, microtubules polymerize from kinetochores in somatic cells and contribute to spindle assembly (Witt et al., 1980; De Brabander et al., 1981; Maiato et al., 2004). While the CPC is not essential for the polymerization of these kinetochore-based microtubules (Tulu et al., 2006; Katayama et al., 2008; O'Connell et al., 2009), depletion of the CPC decreases the rate at which these microtubules form (Tulu et al., 2006; Katayama et al., 2008).

Correction of improperly attached kinetochore microtubules. The CPC is required for proper chromosome alignment in mitosis (Mackay et al., 1998; Adams et al., 2001b; Giet and Glover, 2001; Kaitna et al., 2002; Murata-Hori et al., 2002; Tanaka et al., 2002; Ditchfield et al., 2003; Hauf et al., 2003; Honda et al., 2003; Lens et al., 2003; Gassmann et al., 2004; Sampath et al., 2004). It is generally accepted that the CPC regulates congression via its ability to eliminate improperly attached kinetochore microtubules, allowing chromosomes to properly bi-orient and align (Biggins et al., 1999; Tanaka et al., 2002; Hauf et al., 2003; Lampson et al., 2004; Cimini et al., 2006). How the CPC regulates the detachment of these microtubules has not been fully determined. Three Aurora B substrates, however, have been shown to play a role in microtubule detachment: MCAK (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004), Ndc80 (also known as Hec1)(Cheeseman et al., 2006; DeLuca et al., 2006), and Dam1 (Cheeseman et al., 2002; Wang et al., 2007; Gestaut et al., 2008).

As mentioned above, MCAK is a microtubule destabilizing protein (Walczak et al., 1996). While Aurora B promotes spindle assembly by inhibiting MCAK (Sampath et al., 2004), the CPC also promotes the centromeric localization of MCAK via phosphorylation (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004; Zhang et al., 2007). This centromeric localization of MCAK is important for proper chromosome segregation (Maney et al., 1998; Kline-Smith et al., 2004; Wordeman et al., 2007), suggesting that Aurora B promotes microtubule turnover at kinetochores by targeting MCAK to the structure. How Aurora B-dependent inactivation of MCAK activity plays into this centromeric regulation is not known.

Ndc80 (also known as Hec1) is a kinetochore protein that complexes with three other kinetochore proteins, Nuf2, Spc24, and Spc25 (Janke et al., 2001; Wigge and Kilmartin, 2001). The Ndc80 complex is part of the KMN network (Cheeseman et al., 2006), which is essential for stable kinetochore-microtubule attachments (Kline-Smith et al., 2005). Ndc80 binds directly to microtubules *in vitro* via its N-terminal globular head domain (Cheeseman et al., 2006; DeLuca et al., 2006). Aurora B and Ipl1, the *S. cerevisiae* Aurora B homologue, can phosphorylate this head domain of Hec1 (from mammals) and Ndc80 (from budding yeast and *C. elegans*) *in vitro*, but do not appear to phosphorylate the other subunits of the Ndc80 complex (Cheeseman et al., 2002; Cheeseman et al., 2006; DeLuca et al., 2006). Phosphorylation of the Ndc80 complex by Ipl1 greatly reduces its affinity for microtubules *in vitro* (Cheeseman et al., 2006). *In vivo*, cells expressing a mutant of Hec1 (6A) that cannot be phosphorylated by Aurora B have defects in chromosome alignment and proceed into anaphase with chromosomes attached to both spindle poles (merotelic attachment) and hyper-stretched centromeres. Furthermore, in wild-type cells, addition of an anti-Hec1 antibody that inhibits Hec1 phosphorylation by Aurora B produces the same phenotype. In late metaphase of these antibody-injected cells, kinetochore-microtubules, but not non-kinetochore-microtubules, are stabilized, increasing their half-life by four fold (DeLuca et al., 2006). Together, these results suggest that phosphorylation of Ndc80 by Aurora B *in vivo* is important for promoting microtubule turnover and the error correction mechanism of the CPC.

Dam1 is a kinetochore protein that is part of the ten-member DASH (or Dam1) complex, which has been identified only in fungi thus far (Joglekar et al., 2010). Like the Ndc80 complex, the DASH complex is required for kinetochore-

microtubule attachment and is regulated by the CPC (Cheeseman et al., 2001). While Ipl1, the budding yeast Aurora homologue, phosphorylates multiple members of the DASH complex (Cheeseman et al., 2002), the phosphorylation of Dam1 is the best characterized (Kang et al., 2001; Cheeseman et al., 2002; Li et al., 2002). Ipl1 phosphorylates four sites on Dam1 (S20, S257, S265, and S292). In cells, mutation of these sites to alanine is lethal and leads to the mis-segregation of chromosomes, while mutation to aspartic acid greatly reduces growth and increases the number of lagging chromosomes during segregation (Cheeseman et al., 2002), suggesting that the CPC regulates kinetochore-microtubules in part through Dam1 and the DASH complex. The DASH complex binds to and stabilizes microtubules *in vitro* (Westermann et al., 2005) and the phosphorylation of Dam1 by Ipl1 decreases the ability of the DASH complex to stabilize these microtubules (Wang et al., 2007). Ipl1-mediated phosphorylation of Dam1 also affects its microtubule affinity, as a Dam1 S20A mutant inhibits the ability of Ipl1 to decrease its microtubule affinity *in vitro* (Gestaut et al., 2008). In addition to its effect on microtubule binding, Ipl1 regulates the interaction of Dam1 with its binding partners. An Ipl1 phospho-mimetic mutant of Dam1 has a decreased affinity for Ndc80 and Spc34, another member of the Dam1 complex (Shang et al., 2003). Together, these results suggest that *in vivo*, the CPC regulates kinetochore-microtubule interactions by regulating both the DASH and Ndc80 complexes.

Sensing aberrant kinetochore microtubule attachments. In order to correct improperly attached kinetochore-microtubules, the CPC must first sense that there are aberrant microtubules. How the CPC senses this state and how this

signal regulates Aurora B activity is unclear (Kelly and Funabiki, 2009). What is clear, however, is that tension plays a role. When a chromosome is properly bioriented with each sister kinetochore attached to opposite poles (amphitelic attachment), the pulling force generated by the opposing microtubules applies tension and stretches the centromere and the kinetochore. When only one sister kinetochore is attached to a pole (monotelic attachment) or when both sister kinetochores are attached to the same pole (syntelic attachment), no tension is generated across the kinetochore (Tanaka, 2005). This lack of tension appears to signal to the CPC that microtubules are improperly attached (Biggins and Murray, 2001; Tanaka et al., 2002).

One model of how this tension signal is read by the CPC is based on the physical separation of the sister kinetochores when under tension. Bi-orientation of a chromosome, and the tension it creates, physically separates the CPC from its kinetochore substrates, such as Ndc80 and Dam1, reducing the probability that Aurora B will phosphorylate them and induce microtubule turnover (Tanaka et al., 2002; Andrews et al., 2004). Consistent with this hypothesis, Dam1 is phosphorylated by Ipl1 in a tension-dependent manner (Keating et al., 2009). Furthermore, targeted FRET-based biosensors show that substrates of Aurora B in the centromere are phosphorylated in a tension-independent manner, while substrates in the kinetochore are phosphorylated in the absence, but not presence, of tension (Liu et al., 2009). This study also shows that targeting Aurora B closer to the kinetochore leads to the destabilization of kinetochore microtubules in a kinase-dependent manner (Liu et al., 2009).

Merotelic attachments, where one sister kinetochore is attached to both poles, present a unique problem. While monotelic and syntelic attachments have

no tension, and amphitelic attachments have full tension, merotelic attachments are somewhere in between (Tanaka, 2005). Cells are able to sense and correct merotely (Cimini et al., 2003; Cimini et al., 2004), and the CPC is suggested to aid in this correction (Kaitna et al., 2000; DeLuca et al., 2006; Knowlton et al., 2006). Aurora B enriches at and recruits MCAK to sites of merotelic attachment (Knowlton et al., 2006). Furthermore, addition of an anti-Hec1 antibody that inhibits Aurora B phosphorylation of Hec1 increases the number of merotelic attachments in a cell (DeLuca et al., 2006). Together, these results suggest that the CPC can sense small changes in tension and correct merotelic attachments via the Aurora B-mediated phosphorylation.

Signaling of the spindle assembly checkpoint. The spindle assembly checkpoint ensures that all chromosomes are bi-oriented before anaphase onset (Lew and Burke, 2003). While it is still controversial whether this checkpoint senses the attachment status of each kinetochore and/or the presence of tension across a pair of sister kinetochores (Pinsky and Biggins, 2005), the role of the CPC in the spindle assembly checkpoint is also not resolved. Two scenarios for the role of the CPC in the checkpoint exist. In the first model, since the CPC detaches kinetochore microtubules that are not under tension, the CPC may simply be responding to the lack of tension and creating unattached kinetochores that the checkpoint machinery then reads (Tanaka et al., 2002; Pinsky et al., 2006). Alternatively, the CPC may play a direct role in the spindle assembly checkpoint signaling (Biggins and Murray, 2001). Evidence suggests that while the CPC may create unattached kinetochores, the CPC also has a direct role (Ditchfield et al., 2003; Hauf et al., 2003; Vigneron et al., 2004; Vader et al., 2007).

The CPC recruits checkpoint proteins to the kinetochore. Which proteins are recruited by the CPC appears to be organism dependent. In *Xenopus*, in the absence of kinetochore-microtubule attachment, the CPC is important for the kinetochore localization of all the checkpoint proteins (Mps1, Bub1, Bub3, BubR1, CENP-E, Mad1/Mad2)(Vigneron et al., 2004). In contrast, the CPC in *S. pombe* is not required for the association of Bub1 or Mad3, the BubR1 homologue, but is required for maximal Mad2 localization (Petersen and Hagan, 2003). Likewise, in *S. cerevisiae*, the CPC is required for Mad2, but not Bub1, localization in response to kinetochores that are not under tension (Gillett et al., 2004). In human cells, in response to unattached or tension-less kinetochores, the CPC and Aurora B activity are required for the recruitment of only BubR1 (Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003). While Aurora B phosphorylates BubR1 (Ditchfield et al., 2003; Zachos et al., 2007), the function of this phosphorylation has yet to be elucidated. Data from *S. cerevisiae*, however, suggest that this phosphorylation plays a role in signaling the checkpoint in response to the lack of tension across sister kinetochores (King et al., 2007). While the CPC signals the checkpoint in part through BubR1, other CPC-dependent pathways in human cells may exist (Vader et al., 2007).

These CPC-dependent differences in the kinetochore localization of spindle checkpoint proteins most likely reflect differences in how the various organisms signal their spindle checkpoint. Supporting this hypothesis, the necessity of the CPC in the spindle checkpoint depends on the organism and whether the response is to unattached kinetochores (induced by the microtubule-destabilizing drug nocodazole) or to the lack of tension across kinetochores (induced by taxol). In mammalian cells and *S. cerevisiae*, the CPC is not required

for the nocodazole-induced checkpoint, suggesting that the CPC is unnecessary for the signaling associated with an unattached kinetochore (Biggins and Murray, 2001; Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003; Lens et al., 2003). The CPC, however, is required for the taxol-induced checkpoint in these organisms, indicating that the CPC is needed to sense the lack of tension across kinetochores (Biggins and Murray, 2001; Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003; Lens et al., 2003). In comparison, in *Xenopus* and *S. pombe*, the CPC is required for both checkpoints (Kallio et al., 2002; Petersen and Hagan, 2003).

Completion of cytokinesis. Upon anaphase onset, the CPC transfers from chromosomes to the central spindle microtubules, where Aurora B activity is important for the completion of cytokinesis (Ruchaud et al., 2007). At the central spindle, the CPC recruits MKLP1 (also known as Zen-4 in *C. elegans* and Pavarotti in *Drosophila*), a mitotic kinesin-like protein (Kaitna et al., 2000; Severson et al., 2000; Giet and Glover, 2001; Yang et al., 2004). MKLP1 is part of the centralspindlin complex with MgcRacGAP (also known as CYK-4)(Mishima et al., 2002). MgcRacGAP is the GAP for the GTPase RhoA (Minoshima et al., 2003), which is important in acto-myosin ring contraction during cytokinesis (Piekny et al., 2005). As a complex, MKLP1 and MgcRacGAP promote microtubule bundling and may play a role in the stabilization of the central spindle microtubules (Mishima et al., 2002). In addition, centralspindlin is required for proper cytokinesis (Glotzer, 2003).

At the central spindle, Aurora B phosphorylates both MKLP1 (Guse et al., 2005; Neef et al., 2006) and MgcRacGAP (Minoshima et al., 2003). While the

phosphorylation of MKLP1 by Aurora B is essential for maintaining centralspindlin at the central spindle (Neef et al., 2006), Aurora B-dependent phosphorylation of MgcRacGAP activates its GAP activity (Minoshima et al., 2003), which is important for the RhoA-dependent contraction of the cleavage furrow (Piekny et al., 2005). In addition to its enzymatic role on RhoA, centralspindlin at the central spindle is important for forming the contractile ring by recruiting RhoA (Yuce et al., 2005), anillin (Adams et al., 1998; Zhao and Fang, 2005), and myosin (Zhao and Fang, 2005).

The CPC plays an early role in marking the site that will become the cleavage plane. Besides the central spindle localization in anaphase, the CPC localizes to the cell cortex where the cleavage furrow will subsequently form (Cooke et al., 1987; Earnshaw and Cooke, 1991). This localization of the CPC to the cortical equator occurs before myosin targets to the cleavage furrow (Eckley et al., 1997) and depends on dynamic astral microtubules (Wheatley et al., 2001b; Murata-Hori and Wang, 2002; Shannon et al., 2005). While the function is unclear, this demarcation of the cleavage plane by the cortical CPC is not needed for furrow positioning (Shannon et al., 2005).

Timing of abscission. Cell cycle progression is regulated by many safeguard mechanisms that ensure the fidelity of genomic transmission. One such mechanism in budding yeast prevents DNA damage by inhibiting abscission until the chromosomes have cleared the bud neck (Mendoza et al., 2009). Cells that lack midzone microtubules or lack the ability to segregate their chromosomes prevent abscission by signaling the NoCut pathway (Norden et al., 2006; Mendoza et al., 2009). While the physiological structure that activates the

NoCut pathway is unknown, the CPC plays a role in its signaling by inhibiting abscission via the recruitment of two anillin-related proteins, Boi1 and Boi2, to the bud neck (Norden et al., 2006; Mendoza et al., 2009). Targeting of the CPC to the midzone microtubules in anaphase via the dephosphorylation of Sli15, the *S. cerevisiae* INCENP homologue, by Cdc14 is important for this signaling (Mendoza et al., 2009). The requirement for this microtubule localization in the activation of the NoCut pathway, however, can be bypassed. Artificial targeting of Ipl1, the *S. cerevisiae* Aurora, to chromosomes in cells that have no midzone defects inhibits abscission (Mendoza et al., 2009), suggesting that chromosome-induced activation of Ipl1 is sufficient to activate the NoCut pathway.

Abscission in human cells is also controlled by chromosome segregation, as cells with lagging chromosomes are delayed in abscission (Steigemann et al., 2009). While Aurora B plays a role in human cells, the mechanism may be different than in budding yeast. In cells connected by a chromosome bridge, inhibition of Aurora B activity at the midbody leads to cleavage furrow regression and polyploidy, instead of abscission. Therefore, instead of inhibiting abscission when chromosomes are in the cleavage plane, Aurora B appears to stabilize the cleavage furrow around the bridging chromosome to prevent polyploidy (Steigemann et al., 2009).

Nuclear envelope reformation. Upon anaphase onset, chromosomes segregate and the CPC localizes away from chromosomes to the central spindle microtubules (Ruchaud et al., 2007). As the chromosome masses pull away from each other, the chromosomes begin to decondense and the nuclear envelope starts to reform around the DNA. How these late M-phase processes are

regulated is not well understood, although the removal of the CPC from the chromosomes has been shown to be required (Ramadan et al., 2007). Cul3 mediates the ubiquitinylation of Aurora B, which is suggested to be important for the anaphase relocalization of the CPC from chromosomes to the central spindle (Sumara et al., 2007; Maerki et al., 2009). This process is mediated by the p97-Ufd1-Npl4 complex, which removes ubiquitinylated Aurora B, and presumably the rest of the CPC, from chromosomes post-anaphase in an ATP-dependent manner (Ramadan et al., 2007). Failure to remove the CPC from chromosomes by inhibiting the p97-Ufd1-Npl4 complex leads to defects in chromosome decondensation and nuclear envelope reformation upon exit from mitosis (Ramadan et al., 2007), suggesting that the removal of the Aurora B kinase activity from chromosomes may be important for the dephosphorylation of chromosome-bound Aurora B substrates that mediate these processes.

The *Xenopus* egg extract system

The *Xenopus laevis* egg extract system is perfect for studying the functions of the CPC in the cell cycle. Due to the size of the egg, large quantities of extract can be easily obtained. The egg extract is highly synchronous (Murray and Kirschner, 1989) and recapitulates many aspects of the cell cycle. DNA replicates, nuclei form, the nuclear envelope breaks down, chromosomes condense, the spindle forms, and chromosomes segregate in this cell-free system (Lohka and Maller, 1985; Blow and Laskey, 1986; Hutchison et al., 1987; Sawin and Mitchison, 1991; Shamu and Murray, 1992). Since the system is cell-free, it is easy to add and remove molecules from the extract, making manipulations for biochemical and cell biological analyses more feasible than in a cell-based

system. One particularly appealing aspect of the egg extract system is the ability to translate mRNA (Murray, 1991), which sidesteps the necessity for the purification of proteins recombinantly.

Open questions

While the CPC has been extensively studied, significant gaps in the knowledge of its function and regulation remain. The CPC is clearly important for proper chromosome segregation, which is critical for genomic integrity. How the CPC regulates and how the CPC itself is regulated to ensure proper segregation, however, is not fully understood. Here I describe my contribution to three aspects of chromosome segregation that involves the CPC: mitotic chromosome compaction, spindle assembly, and spindle checkpoint signaling. First, I will show the consequence of Aurora B-mediated phosphorylation for one substrate and suggest that this phosphorylation aids in forming the mitotic chromosome. Second, I will demonstrate that the kinase activity of Aurora B is spatially regulated in part by a mechanism that involves two of its inducers, and that this mechanism is important for spindle assembly. Third, I will describe preliminary data suggesting that a similar mechanism may play a role in spindle checkpoint signaling.

CHAPTER 2. MATERIALS AND METHODS

Reagents

Plasmids and primary antibodies used in this study are listed in Table 2-1 and 2-2, respectively.

Xenopus laevis extractology

Frog egg extracts

Meiotic metaphase II (CSF)-arrested *Xenopus laevis* egg extracts were prepared (Murray, 1991). Female frogs were injected with a total of 100 U PMSG (pregnant mare's serum gonadotropin). Two days (to up to two weeks) after the PMSG injection, the frogs were injected with 100 U HCG (human chorionic gonadotropin), which induced ovulation. Two hours after the HCG injection, the frogs were transferred to individual containers and incubated at 16°C for 14 hr. Eggs were collected, washed with MMR (5 mM HEPES pH 7.8, 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA), incubated with dejelly solution (2% cysteine w/v, 100 mM KCl, 1 mM $MgCl_2$, 45 mM NaOH) for a total of 10 min, and rinsed with XB (10 mM HEPES pH 8.0, 100 mM KCl, 1 mM $MgCl_2$, 0.2 M sucrose), then CSF-XB (XB, 5 mM EGTA), and finally CSF-XB containing 100 μg/ml each LPC (leupeptin, pepstatin, chymostatin) and cytochalasin B (CB). Eggs were crushed by centrifugation at 12K g for 15 min at 16°C in a swinging bucket rotor. The cytoplasmic layer was then extracted and supplemented with 10 μg/ml LPC, 10 μg/ml CB, and 1 mM $MgCl_2$. This CSF extract was stored on

Table 2-1. Plasmids used in this study.

Name	Description	Promoter
ID6326004	Image clone of xHP1β (Open Biosystems)	T7
ID6864895	Image clone of xHP1γ (Open Biosystems)	T7
ID6865720	Image clone of xRad21 (Open Biosystems)	SP6
ID6877643	Image clone of xHP1α (Open Biosystems)	T7
pAFS210	GFP (A. Straight)	SP6
pBT001	GST-xHP1α-GFP	tac
pBT005	xHP1α	SP6
pBT007	xHP1αW57A	SP6
pBT008	xHP1β	SP6
pBT025	GFP-INCENP (1-871)	SP6
pBT026	GFP-Aurora B	SP6
pBT043	INCENP (55-871)	SP6
pBT044	INCENP (58-871); ΔDS	SP6
pBT045	INCENP (138-871)	SP6
pBT046	INCENP (242-870)	SP6
pBT047	INCENP (Δ134-241); ΔHP1	SP6
pBT051	GFP-INCENP (491-871)	SP6
pBT052	GFP-INCENP (748-871)	SP6
pBT055	INCENP (479-871)	SP6
pBT059	INCENP (Δ491-747); ΔCC	SP6
pBT061	GFP-INCENP (Δ491-747)	SP6
pBT066	GFP-INCENP (491-747)	SP6
pBT072	GFP-INCENP (58-871)	SP6
pBT073	GFP-INCENP (Δ1-57, Δ491-747)	SP6
pBT080	INCENP (Δ578-871)	SP6
pBT081	INCENP (Δ619-747)	SP6
pBT083	INCENP (1-871) with linkers flanking 491-747; ΔCC∇CC	SP6
pBT085	INCENP (Δ491-576, Δ663-747)	SP6
pBT086	INCENP (Δ491-576)	SP6
pBT088	INCENP (Δ491-618)	SP6
pBT089	INCENP (Δ491-661)	SP6
pBT103	INCENP (Δ491-747, ∇PRC1)	SP6
pBT107	INCENP (Δ491-747, ∇tau4)	SP6
pBT108	GFP-INCENP (Δ491-747, ∇PRC1)	SP6
pBT109	GFP-INCENP (Δ491-747, ∇tau4)	SP6
pBT112	INCENP (Δ491-747, ∇GCN4)	SP6
pBT115	INCENP (Δ491-747, ∇tau1)	SP6
pBT119	GFP-INCENP (Δ491-747, ∇tau1)	SP6
pBT120	GFP-INCENP (Δ1-57, Δ491-747, ∇PRC1)	SP6
pBT121	GFP-INCENP (Δ1-57, Δ491-747, ∇tau4)	SP6
pBT124	GFP-INCENP (Δ1-57, Δ491-747, ∇tau1)	SP6
pcDNA3-tau-eGFP	tau (Rappoport et al., 2003)	
pCS2-Aurora B	xAurora B	SP6
pCS2-Dasra A	xDasra A	SP6
pCS2-Dasra B	xDasra B	SP6
pCS2-INCENP	xINCENP (1-871)	SP6
pCS2-Rcc1	Rcc1	SP6
pCS2-SIX	SIX	SP6
pCS2-Survivin	xSurvivin	SP6
pET9c-RanDM	RanDM	tac
pGEX6p	N-terminal GST tagging	tac
pGEX6p-AurB	GST-Aurora B (A. Kelly)	tac
pLT22-TAof15	PRC1 (273-621)(L. Tan)	
pSCS040	xHP1α-GFP (S. Sampath)	SP6

Table 2-2. Antibodies used in this study.

	Source	Dilution for immunoblot
α-tubulin	DM1 (Sigma)	1/10,000
Aurora B	Funabiki Lab (Sampath et al., 2004)	5 μg/ml
cyclin B	Ab10839 (Abcam)	2.5 μg/ml
Dasra A	Funabiki Lab (Sampath et al., 2004)	4 μg/ml
GFP	gift from T. Kapoor	
GFP	Roche	
H3	Ab1791 (Abcam)	0.5 μg/ml
H3K9me3	#07-442 (Upstate)	1/1000
H3S10p	#05-598 (Upstate)	0.5 μg/ml
H3T3p	#07-424 (Upstate)	1/200
HP1α*	Funabiki Lab (Fischle et al., 2005)	1/333
HP1α*	Funabiki Lab (antigen: MDASDTSTGPRPNRESC)	20 μg/ml
ICIS	gift from R. Ohi	
INCENP	Funabiki Lab (Sampath et al., 2004)	7 μg/ml
Ku80	Funabiki Lab (Postow et al., 2008)	1 μg/ml
MCAK	gift from R. Ohi	1 μg/ml
Op18	gift from R. Heald	0.2 μg/ml
phospho-Aurora	#2914 (Cell Signaling Tech)	1/200
Plx1	Funabiki Lab	1.5 μg/ml
SMC3	Funabiki Lab	10 μg/ml
Survivin*	Funabiki Lab (antigen: WEPDDDPWTEHSKRSANC)	15 μg/ml
TD60*	Funabiki Lab (antigen: CEPEHSKEKIKLEGSKAKG)	7.5 μg/ml
xKid	Funabiki Lab (Funabiki and Murray, 2000)	3 μg/ml

Asterisk, antibodies I generated and characterized.

ice or at 16°C. Maintaining the extract at 16°C extended the usable life of the extract to past 12 hr and made the extract more translation competent.

Immunodepletion

To make antibody-bound beads, Protein A Dynabeads (Invitrogen) were washed four times with TBS and incubated on a rotator with 1 μg antibody per 10 μl beads for 10-30 min at room temperature or for 1 hr to overnight at 4°C. To crosslink the antibodies to the beads, the antibody-bound beads were washed three times each with TBS and conjugation buffer (0.1 HEPES pH 8.0, 150 mM KCl), and then incubated with 6.8 mM BS^3 (Bis[sulfosuccinimidyl]suberate; Pierce) in conjugation buffer for 30-45 min at room temperature on a rotator. The cross-linking reaction was quenched with two 15 min incubations with 1 M Tris pH 7.5. The beads were then washed three times with TBS and stored at 4°C until use (up to 24 hr). The beads were washed three times with SDB (5mM HEPES pH 8.0, 100mM KCl, 1 mM $MgCl_2$, 150 mM sucrose) right before use. Antibody-cross-linked beads were incubated with an equal volume of extract for 60-90 min at 4°C or 16°C and then removed to yield the depleted extract.

Reconstitution

mRNAs encoding the CPC were made using the SP6 mMessage mMachine RNA transcription kit (Ambion) according to the manufacturer's instructions. The final concentration of the mRNA was 8-15 mg/ml. All INCENP-based mRNAs were added to extract at 0.3 μg/ml. Dasra A and

Survivin mRNAs were added at 0.1 μg/ml. For Aurora B, either 0.3 μg/ml mRNA or 500 nM recombinant protein was added.

Chromosome purification assay

Chromosomes were purified from extract as described previously (Funabiki and Murray, 2000; Sampath et al., 2004). Extract was supplemented with 5 μM biotin-dUTP, 2000 demembranated sperm per μl extract, and 100 μg/ml cycloheximide. 0.3 mM $CaCl_2$ was added to the extract to release the metaphase arrest. The extract was incubated for 80 min at 20°C (interphase incubation). Fresh CSF-arrested extract (half the original volume of the reaction), 50 μg/ml cyclin BΔ90 (Glotzer et al., 1991), and 33 μM nocodazole were then added to bring the extract back to a metaphase state. This reaction (approximately 180 μl) was incubated for another 80 min at 20°C (metaphase incubation). The extract was then flash-frozen and stored at -80°C.

To purify the chromosomes, frozen extracts (approximately 180 μl) were quickly thawed and diluted with 540 μl DB2 (10 mM HEPES pH 8.0, 50 mM β-glycerophosphate pH 7.3, 50 mM NaF, 2 mM EDTA, 20 mM EGTA, 0.5 mM spermin) containing 200 mM sucrose and 1 mM PMSF. The diluted extract was layered over a 0.5 ml cushion of DB2 containing 60% sucrose (w/v), 1 mM PMSF, and 0.05% Triton X-100, and then centrifuged in a swinging bucket rotor at 16.1K g for 30 min at 4°C. The chromosome pellet was resuspended in approximately 200 μl of the cushion and added to 15 μl M-280 streptavidin Dynabeads (Invitrogen) that were previously washed three times in DB2 containing 30% sucrose (w/v). This mixture was rotated for 1 hr at 4°C. The chromosome-bound

beads were then washed with DB2 containing 30% sucrose (w/v), 1 mM PMSF, and 0.05% Triton X-100 and resuspended in 30 μl Laemmli buffer for analysis by SDS-PAGE.

For chromosome purification assays containing radiolabeled protein, the radiolabeled proteins were added 50 min into the interphase incubation. For Figure 3-3B, 7 μl Rcc1 and 10 μl HP1s were added. For Figure 3-5A, 10 μl of each radiolabeled protein was used. For Figure 3-6B, 1 μl Rcc1 and 10 μl xRad21 were added. After electrophoresis, the gel was stained with Coomassie, dried, imaged with a PhosphorImager (Fuji).

For the chromosome purification followed by phosphatase treatment (Figure 3-4), instead of Laemmli buffer, purified chromosomes were resuspended in 30 μl RQ1 DNase buffer (40 mM Tris-HCl, 10 mM $MgSO_4$, 1 mM $CaCl_2$) and treated with 1 μl RQ1 RNase-Free DNase (Promega) at 37°C for 10 min. The buffer was then exchanged for 30 μl PP1 buffer (50 mM HEPES pH 7.0, 0.1 mM Na_2EDTA, 5 mM DTT, 0.025% Tween 20, 1 mM $MnCl_2$), and the chromosomes were heated at 65°C for 3 min. After the reaction had cooled to 30°C, 4 μl PP1 (New England Biolabs), for a concentration 0.29 U/μl, was added to the chromosomes, and the reaction was incubated at 30°C for 1 hr.

Spindle assembly assay

Extract, supplemented with 400 demembranated sperm per μl and doped with Rhodamine-tubulin, was cycled into interphase at 20°C for 90 min (or at 16°C for 120 hr), using 0.3 mM $CaCl_2$. Metaphase spindles were formed by adding three volumes of fresh CSF-arrested extract to the interphase extract and

incubating for 60 min at 20°C (or for 90 min at 16°C). To analyze the spindle phenotypes, 3 μl of Fix (MMR containing 50% glycerol and 11% formaldehyde)(Murray, 1991) was added to 1 μl extract and mounted on a slide. For live imaging, 2 μl extract was squashed without fix. The edges of the cover slip were sealed in Valap (equal volumes of lanolin, petroleum jelly and paraffin). Spindles were imaged using a Photometrics CoolSnap HQ-cooled CCD camera attached to a Carl Zeiss Axioplan 2 microscope and controlled by MetaMorph software (Universal Imaging). Images were acquired with a Plan Neofluar 40x objective. Spindle lengths were measured using MetaMorph.

For anti-INCENP antibody-induced spindle assembly in the absence of Dasra A and Survivin, a final concentration of 5 μg/ml anti-INCENP antibodies, unless otherwise noted, was added to extract before adding the calcium. For the peptide competition assay, CSF extract was incubated with 37.5 μg/mL xHP1α-GFP and 0.5 mg/mL H3 tail peptide for 30 min before demembranated sperm and calcium were added to the extract.

Checkpoint assay

33 μM nocodazole and 10,000 sperm per μl extract were added metaphase extract that had already been used to assemble spindles. The extract was incubated for 45 min at 20°C (or 70 min at 16°C) and 0.6 mM $CaCl_2$ was then added. Samples were taken every 30 min for 2 hr. To visualize the DNA morphology, a sample of the extract was squashed in Fix. To analyze the samples by immunoblot, samples were diluted in Laemmli buffer.

Aurora B activation assay

Extract, containing 25 μg/ml cyclin BΔ90 (Glotzer et al., 1991), was reconstituted with mRNA and incubated for 90 min at 20°C. The inducer (10 μM taxol, 1500/μl sperm nuclei with 33 μM nocodazole ("chromosomes"), or 100 μg/ml anti-INCENP antibodies) was then added and incubated for an additional 90 min at 20°C. For the activation with no added inducer, nothing or 33 μM nocodazole was added to the reconstituted extract and incubated as before. Samples were diluted in Laemmli buffer for analysis.

Immunofluorescence

Spindles were processed for immunofluorescence (Desai et al., 1999). 10-20 μl extract is diluted in Fix solution (BRB80 [80 mM K-pipes pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA], 30% glycerol (v/v), 2% formaldehyde) and incubated for 5 min at room temperature. The sample is then layered over a cushion of BRB containing 40% glycerol on top of a cover slip and spun 5K g for 15 min at 18°C. The cover slip is then collected and fixed in methanol for 3-5 min. After rehydrating the cover slip with two rounds of TBS containing 0.1% Triton X-100, the cover slip is blocked in AbDil (TBS, 0.1% Triton X-100 (v/v), 2% BSA (w/v), 0.1% NaN_3) for a minimum of 30 min. Primary antibody diluted in AbDil was incubated with the cover slip for 1 hr. For Figure 3-5, the anti-GFP antibody (gift from T. Kapoor) was used at 3 μg/ml. For Figure 4-10, the anti-GFP antibody (Roche) was used at 2 μg/ml. Cover slips were then washed three times with AbDil before incubation with the secondary antibody for 1 hr. Alexa-488-conjugated goat anti-mouse antibodies (Invitrogen) were used as a secondary at

1:1000. Cover slips were washed three times again in AbDil and then incubated for 5 min with 0.5 μg/ml Hoechst 33258 diluted in AbDil. Cover slips were rinsed again in AbDil and mounted on slides with 2 μl PBS containing 90% glycerol. The edges of the cover slip were sealed with nail polish.

In vitro microtubule pelleting assay

Frozen extract was thawed at 37°C, diluted 1:1 with XB, and precleared with a 6K g spin for 20 min at 4°C in a swinging bucket rotor. The supernatant was recovered and an input sample was taken. 30 μl extract was incubated with 10 μM taxol or 33 μM nocodazole for 30 min at 20°C. The extract was then diluted with 1.5 ml dilution buffer (30% glycerol, 0.25% IGEPAL, BRB80)(Desai et al., 1999), layered over a 3 ml cushion (40% glycerol, BRB80), and spun at 6K g for 20 min at 20°C in a swinging bucket rotor. The interface was washed twice with BRB80 and the pellet was washed once with BRB80 containing 10 μM taxol. The pellet was respun as before and all remaining wash solution was removed. The pellet was resuspended in Laemmli buffer for analysis.

Molecular biology

Immunoblots

After SDS-PAGE, the proteins in the gel were transferred onto nitrocellulose membrane overnight with 15 V. After the transfer, the membrane was rinsed with MilliQ water, stained with Ponceau S to determine the quality of the transfer, and dried. Prior to immunoblotting, the membrane was rehydrated in PBS for 5 min to remove the Ponceau S. The membrane was blocked in PBS

containing 4% milk for 1 hr. After washing the membrane with PBS for 5 min, primary antibodies were diluted (see Table 2-2) in PBS with 3% BSA and 0.05% NaN_3 were added and incubated for 1-2 hr. The blots were then washed three times for 5 min in PBS containing 0.05% Tween 20, and subsequently incubated with the secondary antibody for 1 hr. IRDye 680 or 800 goat anti-rabbit or anti-mouse IgG (Li-Cor) was used as the secondary antibody and diluted 1/20,000 in Li-Cor Blocking Solution (Li-Cor). Blots were detected and quantified using the Odyssey Infrared Imaging System (Li-Cor). Op18 hyperphosphorylation was measured by normalizing the fluorescence of the slowest-migrating band to the total fluorescence of all the Op18 bands.

Immunoprecipitations

Antibody beads were made in the same manner as those for immunodepletion. One volume of beads was incubated with one volume of extract for 30 min on ice. The beads were then washed (see washing conditions below) and resuspended in Laemmli buffer. In Figure A-2, extract was incubated with the mRNA for 90 min at 20°C before bead addition.

For Figure 3-8A, 1.5% radiolabeled xHP1α (v/v) was added to the extract. The beads were washed five times in EEWB (10 mM HEPES pH 8.0, 100 mM KCl, 1 mM $MgCl_2$, 50 mM sucrose, 50 mM NaCl, 0.1% Triton X-100). For Figure 3-8B, A-2 and A-3, the beads were washed twice in CSF-XB then three times in NP-40 buffer (10 mM sodium phosphate pH 7.2, 150 mM NaCl, 2 mM EDTA, 1% NP-40).

In vitro kinase assay

Antibody beads were made in the same manner as those for immunodepletion, except without cross-linking the antibody to the bead. One volume of antibody beads were incubated with one volume of thawed frozen extract for 1 hr on ice. Beads were collected, washed in three times in kinase buffer (20 mM HEPES pH 8.0, 5 mM $MgCl_2$, 10 mM DTT) with phosphatase inhibitors, and resuspended in a half volume of kinase buffer with phosphatase inhibitors and 1 mM EGTA. To 10 μl beads, 1.5 μM protein and 2 μl ATP mix (10% ^{32}P-γ-ATP, 10% cold ATP) were added and the reaction was incubated for 30 min at 30°C, mixing every 5 min. Laemmli buffer was added at the end of the incubation to stop the reaction. After electrophoresis, the gel was stained with Coomassie, dried, imaged with a PhosphorImager (Fuji).

Radiolabeled protein

Radiolabeled proteins were made using the TNT SP6 or T7 Coupled Reticulocyte Lysate System (Promega) with plasmids encoding the proteins (under an SP6 or T7 promoter), according to the manufacturer's instructions.

Protein purification

Aurora B. *Xenopus laevis* Aurora B was subcloned into the pGEX6p vector (GE Healthcare). GST-Aurora B was expressed in *E. coli* strain BL21 Rosetta competent cells (EMD Chemicals), and cultured in TBG-M9 medium (1% tryptone, 0.5% NaCl, 0.5% glucose, 1 mM $MgSO_4$, 0.1% NH_4Cl, 0.3% KH_2PO_4, 0.6% Na_2HPO_4). At OD_{600}=0.6, the cells were induced with 0.5 mM IPTG

(isopropyl-b-D-thiogalactopyranoside) and grown for 16 hr at 18°C. The cultures were spun down at 6.2K g for 20 min at 4°C. The pellet was rinsed in 30 ml cold PBS per liter of culture and repelleted at 5.5K g for 10 min at 4°C. The pellet was then frozen at -80°C. To purify the protein from one liter of culture, the pellet was thawed and resuspended on ice in 25 ml wash buffer (50 mM HEPES pH 7.6, 300 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol)(Sessa et al., 2005) containing 100 μg/ml LPC. To lyse the cells, 10 μg/ml lysozyme was added and incubated for 20 min on ice. 100 μM PMSF was then added, and the mixture was then sonicated at 4°C for 2.5 min at 30% amplitude with 10 s on/off cycles and mixed every 4 cycles. After sonication, the mixture was spun at 30.7K g for 15 min at 4°C. The supernatant was re-spun at 257.1K g for 20 min at 4°C. This cleared supernatant is passed through a 0.45 μm syringe filter. 1 ml Glutathione Sepharose 4B (GE Healthcare) that had been washed three times with wash buffer was added to the supernatant and incubated for 3 hrs at 4°C with rotation. The beads were then batch washed three times with 50 ml wash buffer. 400 U PreScission protease (GE Healthcare) was added to the beads, and the mixture was incubated overnight at 4°C. The beads were transferred to a column and 1 ml fractions were collected by adding fresh wash buffer to the column to push out the protein. The recombinant Aurora B was concentrated to 2 mg/ml using a Vivaspin column (Sartorius Stedim Biotech).

xHP1α-GFP. xHP1α-GFP was subcloned from pSCS040 into the pGEX6p vector (GE Healthcare) to create pBT001. GST-xHP1α-GFP was produced in *E. coli* BL21 cells containing the pBT001 plasmid, which were induced at OD_{600}=0.6

and grown overnight at 25°C with 0.1 mM IPTG in 2xYT (1.6% tryptone (w/v), 1% yeast extract (w/v), 85.6 mM NaCl, pH 7.0). The cultures were spun down at 6.2K g for 20 min at 4°C. The pellet was rinsed in 30 ml cold PBS per liter of culture and repelleted at 5.5K g for 10 min at 4°C. The pellet was then frozen at -80°C. To purify the protein from one liter of culture, the pellet was thawed and resuspended on ice in 25 ml lysis buffer (PBS, 10 mM EGTA, 10 mM EDTA) containing 100 μM PMSF, 100 μg/ml LPC, and 0.2 mg/ml lysozyme. The mixture was then sonicated at 4°C for 2.5 min at 30% amplitude with 10 s on/off cycles and mixed every 4 cycles. After sonication, 1 mM DTT was added and the mixture was spun at 17.2K g for 10 min at 4°C. The supernatant was re-spun at 164.5K g for 20 min at 4°C. This cleared supernatant is passed through a 0.45 μm syringe filter. Glutathione-sepharose beads (Amersham) that had been washed three times with PBS was added to the supernatant and incubated for 1.5 hrs at 4°C with rotation. The beads were then batch washed four times in PBS and loaded into a column. The beads were washed again with four bed volumes PBS. The PBS in the column was exchanged with one bed volume of elution buffer (50 mM Tris pH 8.0, 15 mM glutathione) and incubated for 5 min. More elution buffer was added to the column and 23-0.5 ml fractions were then collected. Peak fractions were pooled and dialyzed over night in cleavage buffer (50 mM Tris pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT). To produce xHP1α-GFP, the GST tag was removed from the protein via incubation with PreScission protease (Amersham) overnight at 4°C. The GST was separated from the xHP1α-GFP by adding glutathione-sepharose beads to the mixture, incubating overnight at 4°C with rotation, and collecting the supernatant. Finally, the supernatant was

dialyzed in SDB overnight at 4°C, yielding a 1.5 mg/ml solution of xHP1α-GFP in SDB.

RanDM. RanDM was produced in *E. coli* strain BL21 DE3 pLysS competent cells (Promega) containing pET9c-RanDM. The cells were cultured in 2xYT at 37°C and grown to OD_{600}=0.6 before 1 mM IPTG was added to induce expression. The cells were transferred to 24°C and grown for 24 hr. The cultures were spun down at 6.2K g for 20 min at 4°C. The pellet was rinsed in 30 ml cold PBS per liter of culture and repelleted at 5.5K g for 10 min at 4°C. The pellet was then frozen at -80°C. To purify the protein from one liter of culture, the pellet was thawed and resuspended on ice in 40 ml PBS. 100 μg/ml LPC and 1 mg/ml lysozyme was then added. The mixture was stirred for 15 min at 4°C. 100 μM PMSF was then added, and the cells were lysed by three cycles through a French press. The mixture was the spun at 30.1K g for 15 min at 4°C. The supernatant was poured into a beaker (to help approximate the volume). Solid ammonium sulfate is added to the supernatant to 1.4 M (35% maximum saturated) and stirred for 1.25 hr. The mixture was then spun at 15.1K g for 15 min at 4°C. The supernatant was recovered, and more solid ammonium sulfate was added to reach 2 M (55% maximum saturated). The mixture was stirred for another 1 hr and then spun at 15.1K g for 15 min at 4°C. The pellet was resuspended in PBS containing 0.5 mM GDP and 100 μg/ml LPC. The solution was dialyzed overnight in dialysis buffer (30 mM Tris pH 8.0, 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM GDP). The dialyzed material was diluted in one volume of dialysis buffer. Using the AKTA FPLC (GE Healthcare), the protein was then purified twice through HiTrap Q columns (GE Healthcare). In both purifications, buffer A (30

mM Tris pH 8.0, 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM GDP) and buffer B (buffer A with 1 M KCl) were the same, and the column was washed with 10 column volumes of each of buffer A, then B, and then A again before the sample as loaded. For the first purification, the elution profile was from 0-100% buffer B over 10 column volumes followed by 5 column volumes at 100% B. 1 ml fractions were collected at 1 ml/min. For the second purification, the elution profile started with 10% B for 10 column volumes, 25% B for 10 column volumes, a gradient of 25-100% B over 10 column volumes, and finally 100% B for 5 column volumes. The product of these purifications was further purified by size exclusion using a HiLoad Superdex 16/60 75 pg column (GE Healthcare). The column was washed with 1 column volume of water and 2 column volumes of buffer C (10 mM HEPES pH 7.0, 1 mM $MgCl_2$, 100 mM KCl, 0.5 mM GDP) before the sample was loaded. During the elution, the flow rate was 0.4 and 0.25 ml/min for the first and second column volumes, respectively. The purified RanDM was concentrated to greater than 5 mg/ml using a Vivaspin column (Sartorius Stedim Biotech). The protein was then loaded with GTP by adding 20 mM GTP and 3 mM EDTA. The mixture was incubated for 1 hr on ice. More $MgCl_2$ was then added to increase the concentration to 5.5 mM. The GTP-loaded RanDM was dialyzed overnight at 4°C in storage buffer (10 mM HEPES pH 8.0, 100 mM KCl, 1 mM $MgCl_2$, 150 mM sucrose, 0.2 mM GTP). The final GTP-loaded RanDM was 13 mg/ml.

CHAPTER 3. REGULATION OF HP1 BY THE CHROMOSOMAL PASSENGER COMPLEX

Introduction

Our genetic material undergoes a dramatic change in structure upon entry into mitosis. The extended interphase chromatin is compacted approximately 500-fold to create the mitotic chromosome (Georgatos et al., 2009). While the details involved in this process are still far from understood, the correlation between the phosphorylation of histones and chromosome compaction in mitosis was noted over 35 years ago (Gurley et al., 1974). Later work identified serine 10 on histone H3 (H3S10) as a major site of mitotic phosphorylation (Paulson and Taylor, 1982), which is still used today as a hallmark of mitosis. While the modification of this residue in mitotic cells was identified almost thirty years ago, the biological significance of H3S10 phosphorylation is still not yet fully understood.

In mitosis, H3S10 is phosphorylated by Aurora B (Hsu et al., 2000; Murnion et al., 2001; Petersen et al., 2001; Crosio et al., 2002; Sugiyama et al., 2002), a major mitotic kinase and a subunit of the chromosomal passenger complex (CPC), which also includes INCENP, Dasra (also known as Borealin), and Survivin (see Figure 1-4). The CPC is enriched on chromosomes from prophase to metaphase and is targeted to the midzone microtubules in anaphase (Ruchaud et al., 2007). In addition to a multitude of other functions, the CPC is suggested to play a role in chromosome condensation via the recruitment of condensin (Giet and Glover, 2001; Morishita et al., 2001; Kaitna et al., 2002; Petersen and Hagan, 2003; Ono et al., 2004; Lipp et al., 2007; Takemoto et al.,

2007), a protein complex that facilitates the compaction of chromosomes in mitosis (Hirano, 2005). While the phosphorylation of H3S10 by Aurora B is correlated with condensin recruitment, it is unclear if they are causally linked (Wei et al., 1998; Wei et al., 1999; de la Barre et al., 2001). In addition, because Aurora B phosphorylates many substrates during mitosis, it has been difficult to assign which phospho-substrates are responsible for each of the downstream functions.

Apart from the correlation with mitotic chromosome condensation, H3S10 phosphorylation has been hypothesized to play a molecular role in the binding of heterochromatin protein 1 (HP1). HP1 consists of three domains: an N-terminal chromo domain, which is highly conserved and binds to methylated lysine 9 of histone H3 (H3K9); a C-terminal chromo shadow domain, which is also well conserved and binds to a myriad of proteins, including itself; and a middle hinge domain that is not well conserved (see Figure 3-11)(Hiragami and Festenstein, 2005). While there is only one isoform in *Drosophila* and *S. pombe*, metazoans have three isoforms of HP1: α, β, and γ. HP1α and HP1β generally localize to heterochromatin, and HP1γ is found in euchromatin. In interphase, HP1 binds tightly to chromatin via its chromo domain interaction with methylated H3K9 and is important for transcriptional silencing and heterochromatin formation (Hiragami and Festenstein, 2005). During mitosis, HP1 dissociates from chromosomes (Wreggett et al., 1994; Kellum et al., 1995; Furuta et al., 1997; Minc et al., 1999; Murzina et al., 1999; Sugimoto et al., 2001; Hayakawa et al., 2003), while the methylation status of H3K9 is unchanged (Peters et al., 2002; Fischle et al., 2005; McManus et al., 2006). Prior to the work I

will describe in this chapter, the regulation of this mitotic removal was unclear. To explain this dichotomy, C. David Allis and colleagues had hypothesized that the phosphorylation of H3S10 next to a methylated H3K9 could regulate the binding of HP1 by decreasing the affinity of HP1 for the modified site (Figure 3-1)(Fischle et al., 2003). Others, however, suggest that other mechanisms regulate the release of HP1 from chromosomes (Fass et al., 2002; Mateescu et al., 2004; Terada, 2006; Wang et al., 2008). The functional significance of this HP1 release is still unknown.

In this chapter, I will first present data, which was published in 2005, indicating that Aurora B abrogates the binding of HP1 to metaphase chromosomes via the phosphorylation of H3S10 in *Xenopus* egg extract. In data not presented in this thesis from my collaborators in the laboratory of C. David Allis, similar findings were observed in mammalian cell lines and *in vitro* (Fischle et al., 2005). While I was unable to find a function for the M-phase dissociation of HP1 from metaphase chromosomes, I will present data showing that Aurora B phosphorylates HP1 and that the CPC interacts with HP1. My preliminary data implicate this CPC-HP1 interaction in the spindle assembly checkpoint, suggesting that HP1, apart from the enigmatic function associated with its mitotic removal from chromosomes, has unexplored roles in M-phase.

Results

xHP1α does not localize to metaphase chromosomes in Xenopus egg extract

HP1 localization changes dramatically during the cell cycle. In interphase, all three isoforms of HP1 localize to chromatin (Hiragami and Festenstein, 2005), but in mitosis, most of the HP1 is ejected from chromosomes, leaving a small

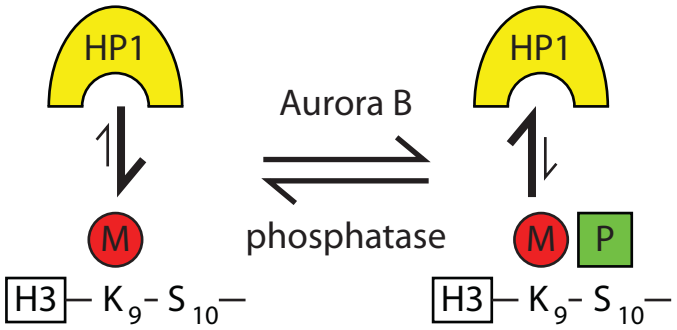


Figure 3-1. The methyl/phos switch.
Aurora B-mediated phosphorylation (green square marked with a "P") of serine 10 on histone H3 (S10) next to a methylated (red circle marked with an "M") lysine 9 on histone H3 (K9) regulates the binding of HP1 by decreasing the affinity of HP1 for the methylated site.

portion of HP1α at the centromere (Wreggett et al., 1994; Kellum et al., 1995; Furuta et al., 1997; Minc et al., 1999; Murzina et al., 1999; Sugimoto et al., 2001; Hayakawa et al., 2003). While the localization of HP1 has been examined in *Drosophila* and mammals, it has not been monitored in *Xenopus* egg extracts. To visualize xHP1α, I purified recombinant xHP1α C-terminally tagged with GFP. The localization of this protein from interphase to metaphase was observed by time-lapse microscopy (Figure 3-2). Similar to the *Drosophila* and mammalian systems (Wreggett et al., 1994; Kellum et al., 1995; Furuta et al., 1997; Minc et al., 1999; Murzina et al., 1999; Sugimoto et al., 2001; Hayakawa et al., 2003), xHP1α colocalized with the chromosomes in interphase, but was removed in metaphase. Unlike the other systems, however, xHP1α failed localize to the centromere. It is possible that no HP1 isoform localizes to centromeres in metaphase of *Xenopus* egg extract. Alternatively, one of the other isoforms of xHP1 could localize to centromeres. I, however, did not examine the localization of xHP1β or xHP1γ.

Depletion of the CPC inhibits the removal of xHP1α from metaphase chromosomes

HP1, via its chromo domain, binds to methylated H3K9 (Bannister et al., 2001; Jacobs et al., 2001; Lachner et al., 2001; Nakayama et al., 2001; Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002). While HP1 is released from chromosomes in M-phase, the methylation status of H3K9 is unchanged (see Figure 3-4)(Peters et al., 2002; Fischle et al., 2005; McManus et al., 2006). How then is the binding of HP1 regulated? It was proposed that the recruitment of a chromatin effector protein to a post-translationally modified residue can be regulated by the modification of neighboring residue on the histone tail,

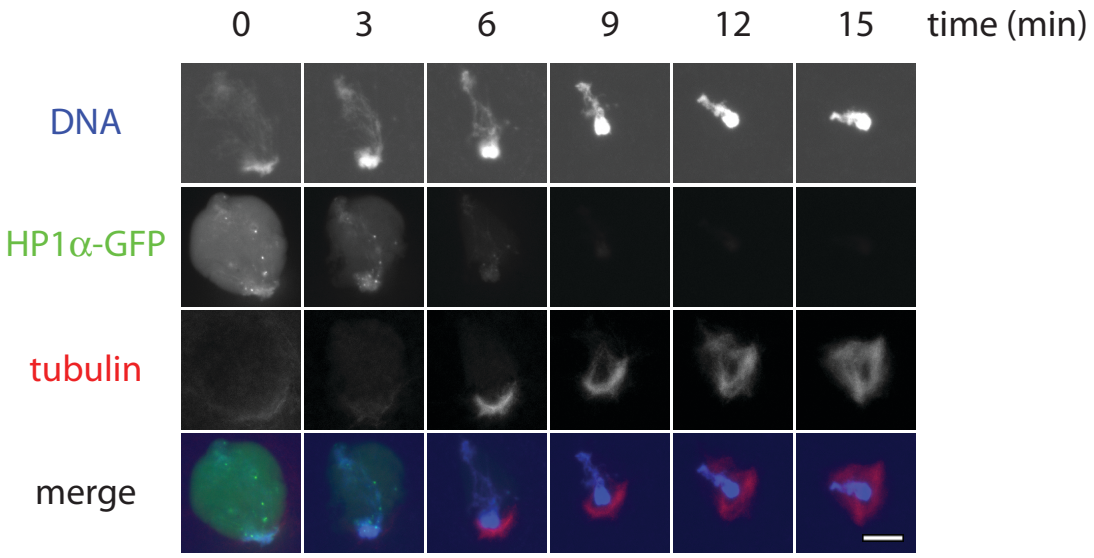


Figure 3-2. HP1α does not localize to metaphse chromosomes.
Control extract was supplemented with 740 nM HP1α-GFP (green) and cycled from interphase to metaphase. The sample was imaged every 3 min for 15 min. DNA (blue) and tubulin (red) were visualized with DAPI and rhodamine-labeled tubulin, respectively. Scale bar, 10 μm.

specifically that the binding of HP1 to methylated H3K9 is regulated by the phosphorylation of H3S10 (Figure 3-1)(Fischle et al., 2003).

Since Aurora B mitotically phosphorylates H3S10 (Hsu et al., 2000; Murnion et al., 2001; Petersen et al., 2001; Crosio et al., 2002; Sugiyama et al., 2002), I determined whether removal of Aurora B, which eliminates H3S10 phosphorylation, affected the loss of xHP1 from metaphase chromosomes (Figure 3-3). From extract lacking Aurora B, via the immunodepletion of the CPC with anti-INCENP antibodies (ΔCPC extract), I purified the metaphase chromosome fraction and then analyzed the associated chromosome-bound proteins by immunoblot. As expected (Cooke et al., 1987; Wreggett et al., 1994; Kellum et al., 1995; Furuta et al., 1997; Minc et al., 1999; Murzina et al., 1999; Sugimoto et al., 2001; Hayakawa et al., 2003), in control extract, INCENP copurified with the chromosomes, while xHP1α did not (Figure 3-3A). In extract lacking the CPC, however, xHP1α associated with metaphase chromosomes. The binding of histone H3, a control, was unaffected by the CPC depletion.

To examine if this CPC-induced binding to chromosomes was specific to xHP1α and to quantify the increase in binding, I examined if all three xHP1 isoforms stay bound to chromosomes in the absence of the CPC using radiolabeled protein (Figure 3-3B). While Rcc1, a chromatin-bound protein (Ohtsubo et al., 1989), was not affected by depletion of the CPC, all three isoforms of xHP1 bound to chromosomes in the absence, but not presence, of the CPC. At least seven-fold more xHP1 associated with metaphase chromosomes assembled in extracts lacking the CPC as compared to in control extracts. Since depletion of the CPC did not affect the methylation of H3K9 but greatly

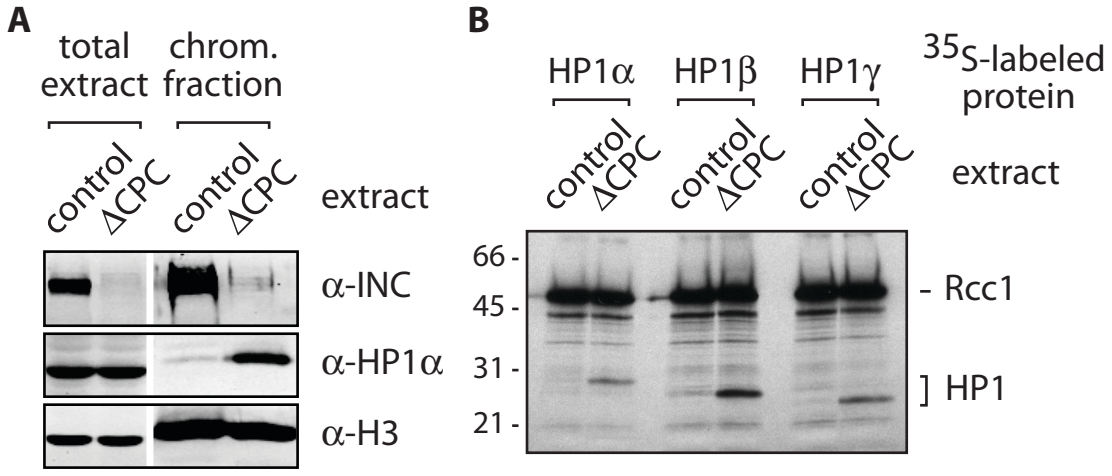


Figure 3-3. HP1 binds more strongly to chromosomes in the absence of Aurora B.

Control or ΔCPC extracts were cycled from interphase to metaphase with 1,333/μl sperm nuclei in the presence of 33 μM nocodazole at 20°C. The proteins bound to the chromosomes were purified 90 min after entry into metaphase. **(A)** The samples were analyzed by immunoblot with the indicated antibodies on the right. INC, INCENP. **(B)** The extract was supplemented with ^{35}S-labeled Rcc1 and xHP1α, β, or γ. The samples were analyzed by autoradiography. Molecular weight markers (in kD) are on the left.

decreased the phosphorylation of H3S10 (Figure 3-4), these results suggest that the CPC actuates the removal of HP1 from metaphase chromosomes via the phosphorylation of H3S10.

The enhanced binding of xHP1α to chromosomes in the absence of the CPC is via its chromo domain

If the removal of methylated H3K9-bound HP1 is due to the phosphorylation of H3S10 on metaphase chromosomes, then the chromo domain of HP1, which mediates the interaction with methylated H3K9 (Bannister et al., 2001; Jacobs et al., 2001; Lachner et al., 2001; Nakayama et al., 2001; Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002), should be critical to staying bound to chromosomes in the absence of the CPC. To test this hypothesis, I used two approaches. First, I mutated the chromo domain of HP1 to inhibit the interaction with methylated H3K9, and second, I competed the HP1-methyl H3K9 interaction with a methylated H3K9 peptide.

For the first approach, I mutated tryptophan 57, one of the three aromatic cage residues that create the binding pocket for methylated H3K9 in the chromo domain of HP1, to alanine (W57A; see Figure 3-11), which abrogates the *in vitro* binding of HP1 to trimethylated H3K9 peptides (Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002). xHP1αW57A bound to metaphase chromosomes 70-80% less efficiently than wild-type xHP1α in ΔCPC extract (Figure 3-5A).

In the second approach, I investigated whether binding of xHP1α to metaphase chromosomes in ΔCPC extracts could be inhibited by a peptide that competes with the methylated H3K9 on chromosomes for the chromo domain of

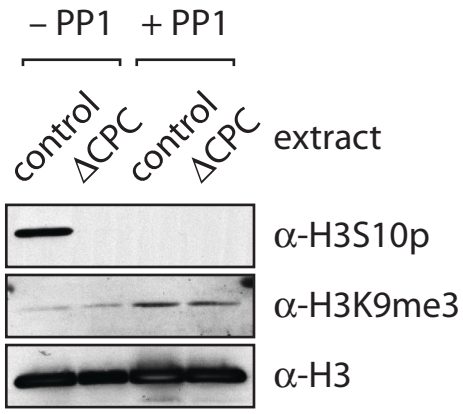


Figure 3-4. Depleting the CPC does not affect the methylation of histone H3 K9.

Control or ΔCPC extracts were cycled from interphase to metaphase with 1,333/μl sperm nuclei in the presence of 33 μM nocodazole at 20°C. The proteins bound to the chromosomes were purified 90 min after entry into metaphase, treated with (+) or without (–) 0.29 U/μL PP1, and analyzed by immunoblot with the indicated antibodies on the right. H3S10p, phosphorylated H3 serine 10; H3K9me3, trimethylated H3 lysine 9.

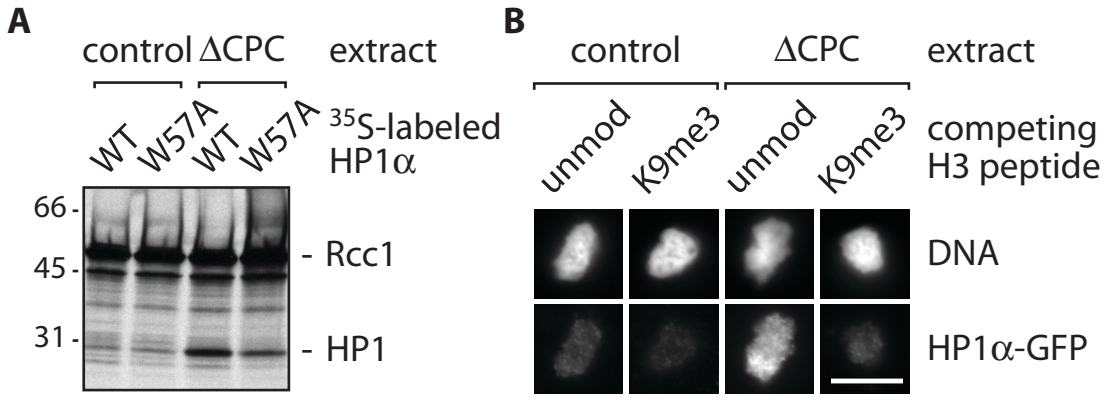


Figure 3-5. The enhanced binding of HP1α to chromosomes in the absence of Aurora B is via its chromo domain.

(A) Control or ΔCPC extracts were cycled from interphase to metaphase with 1,333/μl sperm nuclei in the presence of 33 μM nocodazole at 20°C. The extract was supplemented with ^{35}S-labeled Rcc1 and xHP1α ("WT") or xHP1αW57A. The proteins bound to the chromosomes were purified 90 min after entry into metaphase. The samples were analyzed by autoradiography. Molecular weight markers (in kD) are on the left. **(B)** Control or ΔCPC extract containing 740 nM HP1α-GFP and 227 μM H3 tail peptides (aa 1-20) were cycled from interphase to metaphase in the presence of 33 μM nocodazole at 20°C. Samples were processed for immunofluorescence 60 min after entry into metaphase using anti-GFP (bottom) antibodies. DNA (top) was visualized with Hoechst 33258. Scale bar, 10 μm. unmod, unmodified; K9me3, trimethylated lysine 9.

HP1. Neither an unmodified nor a trimethylated H3K9 peptide had any effect on the chromosomal binding of xHP1α-GFP in control extracts (Figure 3-5B, left). The addition of the methylated H3K9 peptide to ΔCPC extracts, however, significantly reduced the chromosomal binding of xHP1α-GFP, which was approximately 50% less than that with the unmodified control peptide ($P<0.0001$, $n=50$ in each of two independent experiments)(Figure 3-5B, right).

While these manipulations did not completely eliminate the binding of xHP1α to metaphase chromosomes in the absence of the CPC, they greatly reduced the association. Therefore, these results show that a significant portion of the chromosome-bound xHP1α in the absence of the CPC is dependent on the chromo domain. Together, these data imply that Aurora B regulates the release of HP1 proteins from chromosomes in M-phase via the phosphorylation of H3S10, which modulates the interaction between the chromo domain and the methylated H3K9-containing nucleosomes. These results were corroborated *in vitro* and in mammalian cells via a fruitful collaboration with the laboratory of C. David Allis (Fischle et al., 2005) and are consistent with the work of others (Hirota et al., 2005).

Enhanced binding of xHP1 to metaphase chromosomes in ΔCPC extracts is not correlated with an increase in cohesin binding

The CPC stimulates the removal of HP1 from metaphase chromosomes, but the biological significance of this release is unclear (Dormann et al., 2006). The *S. pombe* HP1 homologue Swi6 is essential for mitosis because it is required for proper cohesion at centromeres, but not along chromosome arms (Ekwall et

al., 1995; Bernard et al., 2001; Nonaka et al., 2002). At centromeres, Swi6 is suggested to recruit cohesin (Bernard et al., 2001; Nonaka et al., 2002), a multi-subunit complex that keeps sister chromatids attached until anaphase (Nasmyth and Haering, 2009). While cohesin on chromosome arms persists until anaphase onset in yeast, a majority of the chromosome arm cohesin in metazoans is released in prophase and only a small population of cohesin, which resides mainly at the centromere, remains until anaphase onset (Losada et al., 1998; Darwiche et al., 1999; Losada et al., 2000; Waizenegger et al., 2000; Warren et al., 2000). While this prophase removal of cohesin is not required for the proper segregation of chromosomes (Gimenez-Abian et al., 2004), it is partially dependent on the kinase activity of Aurora B (Losada et al., 2002; Gimenez-Abian et al., 2004). Therefore, one hypothesis for the function of the M-phase HP1 release is to aid in the prophase removal of cohesin.

To test if the retention of xHP1 on metaphase chromosomes is correlated with an increase in cohesin binding, I determined if the amount of cohesin that copurifies with metaphase chromosomes increases in extract lacking the CPC (Figure 3-6). While the depletion of the CPC led to an increase in the mobility of the chromosome-bound xSMC3, a subunit of the cohesin complex (Losada et al., 1998), the total amount of the protein that purified with metaphase chromosomes was unaffected (Figure 3-6A). To better quantify the response, I repeated the experiment with radiolabeled xRad21 (also known as Scc1 and Mcd1), another member of the cohesin complex (Losada et al., 1998). Like Rcc1, the amount of chromosome-bound xRad21 was not affected by the depletion of the CPC (Figure 3-6B). These results show that the CPC does not regulate the bulk binding of cohesin to metaphase chromosomes. Therefore, the HP1 release from metaphase

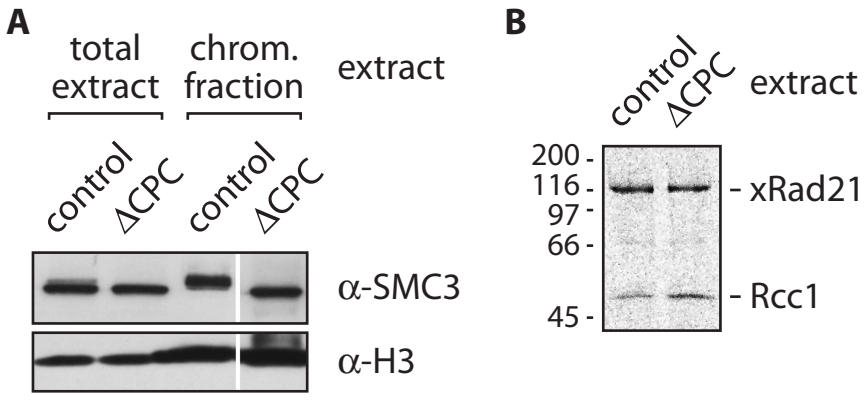


Figure 3-6. Depletion of the CPC does not affect the binding of cohesin to chromosomes.

Control or ΔCPC extracts were cycled from interphase to metaphase with 1,333/μl sperm nuclei in the presence of 33 μM nocodazole at 20°C. The proteins bound to the chromosomes were purified 90 min after entry into metaphase. **(A)** The samples were analyzed by immunoblot with the indicated antibodies on the right. **(B)** The extract was supplemented with ^{35}S-labeled Rcc1 and xRad21. The samples were analyzed by autoradiography. Molecular weight markers (in kD) are on the left.

chromosomes, which is regulated by the CPC, does not mediate the prophase removal of cohesin.

On a side note, the depletion of CPC changed the mobility of the chromosome-bound xSMC3 (Figure 3-6A). The only published post-translational modification of SMC3 is acetylation by Eco1, which is required for the establishment of cohesion (Ivanov et al., 2002; Ben-Shahar et al., 2008; Unal et al., 2008; Zhang et al., 2008). Since the CPC is not known to affect the establishment of cohesion, it seems unlikely that this shift in xSMC3 represents a change in acetylation. Since the CPC contains kinase activity, this increased mobility of chromosome-bound xSMC3 may represent direct phosphorylation by Aurora B. Alternatively, the CPC may indirectly regulate the phosphorylation or other post-translational modification of xSMC3. This change in mobility suggests that the CPC may regulate cohesin via an unidentified pathway.

Aurora B phosphorylates xHP1α in vitro

A small portion of xHP1α is retained on metaphase chromosomes in ΔCPC extracts independent of its chromo domain interaction with methylated H3K9 (Figure 3-5). How is this regulated? Since xHP1 isoforms are specifically phosphorylated in mitosis (Minc et al., 1999) and Aurora B is a major mitotic kinase (Ruchaud et al., 2007), one hypothesis is that, in addition to H3S10, Aurora B phosphorylates HP1 itself, which further ejects HP1 from metaphase chromosomes. To test this hypothesis, I determined if Aurora B could phosphorylate xHP1α *in vitro* (Figure 3-7). As a source of active Aurora B, I immunoprecipitated the CPC from *Xenopus* egg extracts using beads bound with

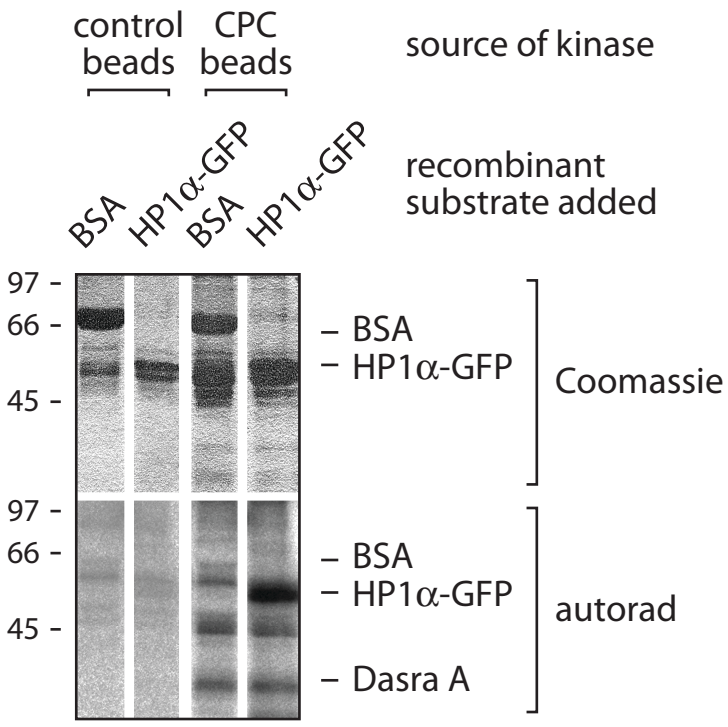


Figure 3-7. Aurora B phosphorylates HP1α.

1.5 μM BSA or HP1α-GFP were incubated with control or CPC-bound beads for 30 min at 30°C in the presence of ^{32}P-γ-ATP. Samples were analyzed by autoradiography (bottom). The Coomassie-stained gel (top) is shown as a loading control. Molecular weight markers (in kD) are on the left.

anti-INCENP antibody (CPC beads)(Kelly et al., 2007). As a control, I used beads with pre-immune rabbit IgG (control beads). These beads were incubated with the substrate (either xHP1α-GFP or BSA, as a control) in the presence of radiolabeled ATP. The entire reaction was analyzed by SDS-PAGE followed by autoradiography. While neither BSA nor xHP1α-GFP was phosphorylated when incubated with the control beads, xHP1α-GFP, but not BSA, was phosphorylated when incubated with the CPC beads, containing active Aurora B (Figure 3-7, bottom). As an internal control for active Aurora B on the CPC beads, Dasra A, which is also on the CPC beads, is shown, and it was also phosphorylated. This result shows that Aurora B can phosphorylate xHP1α *in vitro*.

Further characterization of this phosphorylation of HP1α by Aurora B was done by Holger Dormann in the laboratory of C. David Allis, with whom I collaborated on the M-phase release of HP1 from chromosomes via the Aurora B-mediated phosphorylation of H3S10 (Fischle et al., 2005). In the discussion of this chapter, I will elaborate on his results (Dormann, 2009).

xHP1α copurifies with the CPC

While a majority of HP1 is released from metaphase chromosomes, a small portion of HP1α remains on chromosomes at the centromere (Wreggett et al., 1994; Kellum et al., 1995; Furuta et al., 1997; Minc et al., 1999; Murzina et al., 1999; Sugimoto et al., 2001; Hayakawa et al., 2003). This localization is independent of the HP1 chromo domain (Hayakawa et al., 2003) and is suggested to be via the interaction of HP1α and INCENP, which enriches at centromeres (Ainsztein et al., 1998; Ruchaud et al., 2007). Since I did not observe

a centromeric localization for xHP1α-GFP in metaphase of *Xenopus* egg extract (Figure 3-2), I wondered whether this interaction of HP1α and INCENP exists in *Xenopus* egg extract. To examine the interaction, I immunoprecipitated endogenous CPC using anti-INCENP antibodies and observed the copurifying proteins. Both exogenously added radiolabeled xHP1α and endogenous xHP1α copurified with the CPC (Figure 3-8). These results suggest that the lack of centromeric xHP1α in metaphase is not due to a physical inability to interact with INCENP.

The putative HP1 interaction domain of INCENP is not required for spindle assembly

The CPC must interact with chromosomes (Kelly et al., 2007) and microtubules (see Chapter 4) to trigger spindle assembly in *Xenopus* egg extract. While these two essential interactions have been characterized, it is unknown if other interactions are also required for the function of the CPC in spindle assembly. Therefore, I tested if the interaction between xHP1α and INCENP is required for spindle formation.

To study the function of the xHP1α-INCENP interaction, I sought to eliminate the interaction. Since I already had a system of depletion and reconstitution set up for INCENP, I chose to eliminate the interaction by mutating INCENP. The HP1α interaction domain in chicken INCENP has been previously mapped, using yeast two-hybrid assays (Ainsztein et al., 1998). I, therefore, removed the corresponding region of INCENP in *Xenopus* (INCENPΔHP1). The domain that I deleted is the presumed HP1 interaction domain (aa 134-241; see Figure 4-6, green box). It is important to note that I have

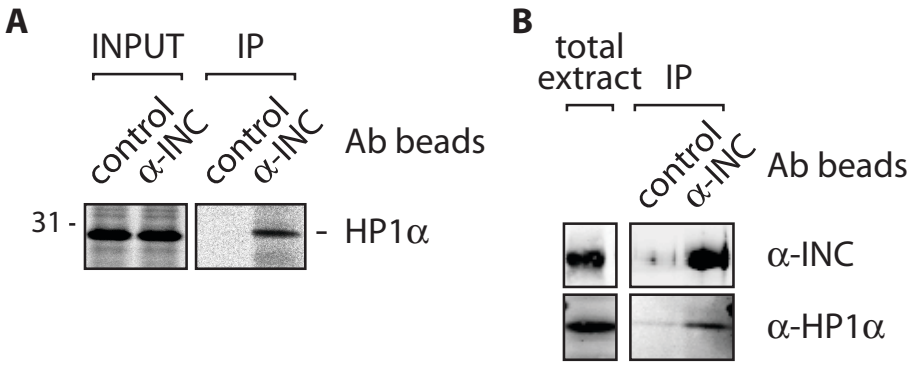


Figure 3-8. HP1α co-precipitates with the CPC.
Control or anti-INCENP antibody beads were incubated in extract for 30 min at 4°C and then purified. **(A)** The extract was supplemented with 35S-labeled HP1α. The samples were analyzed by autoradiography. Molecular weight markers (in kD) are on the left. **(B)** The samples were analyzed by immunoblot using the indicated antibodies on the right.

not yet tested if the deletion of this putative HP1 interaction domain on INCENP eliminates the interaction with xHP1α.

Nevertheless, I tested if INCENPΔHP1 had any effect on spindle assembly. I reconstituted ΔCPC extract with Aurora B, Dasra A, Survivin, and full-length or mutant INCENP (Figure 3-9). While extract that lacked INCENP did not support spindle assembly (#2), spindles did form in extract with INCENPΔHP1 (#4). Although I have not carefully measured the lengths of these spindles, they appeared to be similar to those in extract containing full-length INCENP (#3). Therefore, the presumed HP1 interaction domain of INCENP, and by inference the xHP1α-INCENP interaction, is not required for spindle assembly.

The putative HP1 interaction domain of INCENP is required for the spindle assembly checkpoint

At the centromere, the CPC is required for the recruitment of spindle checkpoint proteins, which inhibit anaphase onset until all chromosomes are properly bioriented on the spindle (Lew and Burke, 2003). While work in chicken cells suggests that the interaction between INCENP and HP1α is not required for the centromeric localization of INCENP (Ainsztein et al., 1998), it may be important for the function of the CPC at the centromere. To test this hypothesis, I examined if extract containing INCENPΔHP1 could properly signal the spindle assembly checkpoint. While the spindle assembly checkpoint is not normally signaled in response to microtubule depolymerization in *X. laevis* eggs (Hara et al., 1980; Gerhart et al., 1984), the checkpoint can be induced in the presence of

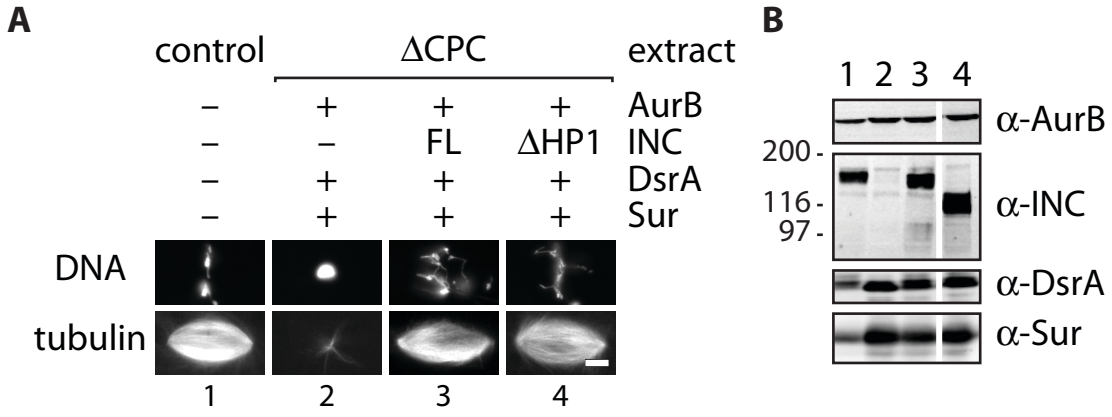


Figure 3-9. The putative HP1 interaction domain of INCENP is not required for spindle assembly.

(A) Control or ΔCPC extracts were reconstituted as indicated. Samples were cycled through interphase to metaphase with 100/μl sperm nuclei at 16°C. Samples were fixed and imaged 90 min after entry into metaphase. DNA (blue) and tubulin (red) were visualized with Hoechst 33258 and rhodamine-labeled tubulin, respectively. Scale bar, 10 μm. **(B)** Immunoblot of samples in (A) with the indicated antibodies on the right. Samples are numbered as in (A). Molecular weight markers (in kD) for INCENP are on the left. AurB, Aurora B; INC, INCENP; DsrA, Dasra A; Sur, Survivin.

high concentrations of DNA in *Xenopus* egg extract (Minshull et al., 1994). ΔCPC extracts that were reconstituted with Aurora B, Dasra A, Survivin, and full-length or mutant INCENP were incubated with a high concentration of sperm chromosomes, nocodazole, and calcium to initiate the spindle checkpoint (Figure 3-10). To test the functionality of the checkpoint, I used two assays. Using immunoblot, I monitored the degradation of cyclin B and the dephosphorylation of threonine 3 on histone H3 (H3T3), both of which occur upon mitotic exit (Murray and Kirschner, 1989; Polioudaki et al., 2004; Dai et al., 2005). Using direct fluorescence imaging, I monitored the nuclear morphology of the DNA stained with Hoechst 33258. As expected (Kallio et al., 2002), extract lacking a functional CPC degraded cyclin B, dephosphorylated H3T3, and had a swollen interphase nuclei (Figure 3-10A, B; #3), demonstrating that the checkpoint was not functional. While full-length INCENP did support an active spindle assembly checkpoint (#4), INCENPΔHP1 failed to hold the checkpoint (#5), suggesting that the interaction between the CPC and HP1 plays a role in the signaling of the spindle assembly checkpoint.

Discussion

A majority of HP1 proteins is removed from chromatin upon entry into mitosis (Wreggett et al., 1994; Kellum et al., 1995; Furuta et al., 1997; Minc et al., 1999; Murzina et al., 1999; Sugimoto et al., 2001; Hayakawa et al., 2003). While the function of this release is still unclear, my results indicate that the phosphorylation of H3S10 by Aurora B abrogates the binding of HP1 to methylated H3K9 in metaphase *Xenopus* egg extract. Similar results were seen *in*

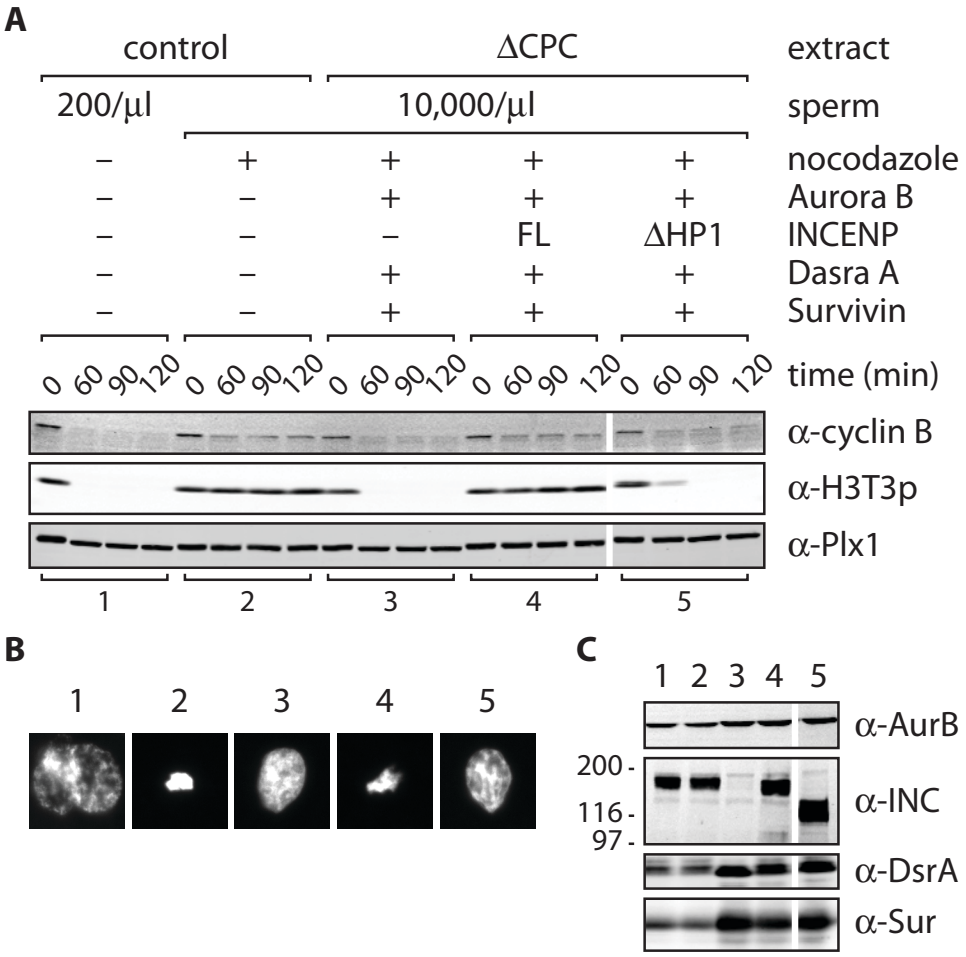


Figure 3-10. The putative HP1 interaction domain of INCENP is required for the spindle assembly checkpoint.

Control or ΔCPC extract was reconstituted as indicated and cycled from interphase to metaphase with 200/μl sperm nuclei at 16°C. At 90 min after entry into metaphase, 33 μM nocodazole and 10,000/μl sperm nuclei were added to samples 2-5. All samples were incubated for an additional 70 min. The extract was then released into interphase and samples were taken every 30 min for 2 hr. **(A)** Samples were analyzed by immunoblot using the indicated antibodies on the right. H3T3p, phosphorylated histone H3 threonine 3. The time course represents the time after release into interphase. **(B)** At 90 min, the samples were fixed and imaged using Hoechst 33258 to visualize the DNA. Scale bar, 10 μm. **(C)** Immunoblot of the 0 time point in (A) with the indicated antibodies on the right. Molecular weight markers (in kD) for INCENP are on the left. AurB, Aurora B; INC, INCENP; DsrA, Dasra A; Sur, Survivin. Samples are numbered as in (A).

vitro and in mammalian cells (Fischle et al., 2005; Hirota et al., 2005; Terada, 2006). I also show that the CPC interacts with and phosphorylates xHP1α. In addition, my results suggest that while the CPC-xHP1α interaction does not play a role in spindle assembly, it is needed for the activation of the spindle assembly checkpoint.

Regulated release of HP1 from metaphase chromosomes

While the regulation of the interaction between HP1 and methylated H3K9 accounts for the majority of the HP1 released in M-phase (Fischle et al., 2005; Hirota et al., 2005), it does not account for the full release of HP1 from metaphase chromosomes (Figure 3-5). While additional mechanisms may be regulating this removal, there is a trivial explanation as well. The possibilities that could have led to the incomplete M-phase release of HP1 are discussed below.

First, I will describe a possible trivial explanation. In the experiment, I manipulated the methylated H3K9-xHP1α interaction by mutating the chromo domain of HP1 (Figure 3-5A). The experiment, however, was done in extract that contains endogenous HP1 because reagents to remove all three isoforms of xHP1 do not exist. Since HP1 can dimerize via its chromo shadow domain (Platero et al., 1995; Ye et al., 1997; Brasher et al., 2000; Cowieson et al., 2000; Gaudin et al., 2001), the residual binding of xHP1αW57A to the chromosomes in ΔCPC extracts may simply be due to an interaction of the mutant with endogenous HP1 via their chromo shadow domains.

Alternatively, additional mechanisms may control HP1 removal from chromosomes in M-phase. For instance, since Aurora B phosphorylates xHP1α (Figure 3-7), the direct modification of HP1 proteins by Aurora B may play a role in their release. An extensive characterization of HP1α phosphorylation sites was done by Holger Dormann in the laboratory of C. David Allis. His doctoral thesis showed that Aurora B phosphorylates human HP1α on serine 92, a site that is not conserved in the *Xenopus* protein (Figure 3-11). Mutation of this human HP1α residue had no affect on its metaphase localization. He further mapped five other phosphorylation sites on human HP1α, which are not necessarily Aurora B phosphorylation sites. Mutation of all six sites had no affect on protein localization (Dormann, 2009). Only one of these sites is conserved in *Xenopus*, serine 118 (serine 110 in the human protein), which is in an Aurora consensus sequence (Cheeseman et al., 2002; Honda et al., 2003; Ferrari et al., 2005), suggesting it may be the site that Aurora B phosphorylates (Figure 3-7 and 3-11). Based on his thesis work, direct phosphorylation of xHP1α by Aurora B is unlikely to contribute to the M-phase release of HP1 proteins (Dormann, 2009).

In addition to the phosphorylation of histone H3, acetylation of histones has been suggested to play a role in the chromo domain-mediated mitotic removal of HP1 (Taddei et al., 2001; Mateescu et al., 2004; Li et al., 2006; Ma et al., 2008). How exactly acetylation plays a role, however, is confusing. General histone deacetylation is required for the mitotic enrichment of HP1 to the (peri)centromere (Taddei et al., 2001; Ma et al., 2008). However, since the localization of HP1 to the (peri)centromere is not dependent on the chromo domain (Hayakawa et al., 2003), this mechanism is unlikely to play a role in the

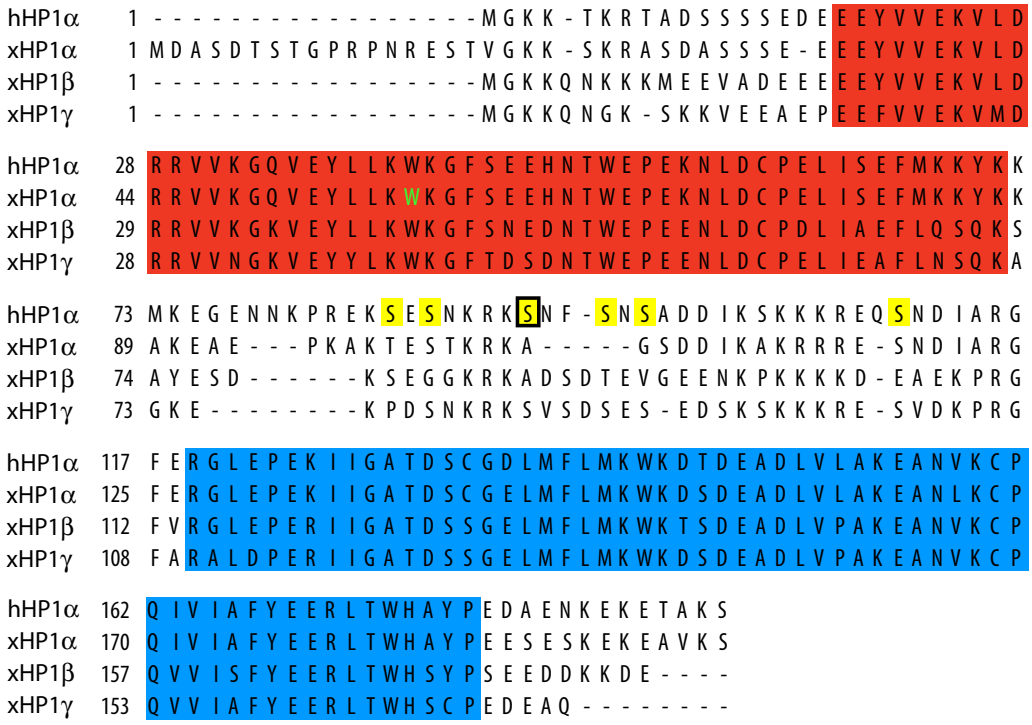


Figure 3-11. Alignment of human HP1α with *Xenopus* HP1s.

Human HP1α and the three *Xenopus* HP1s were aligned using ClustalW. In red, the HP1 chromo domain; in blue, the chromo shadow domain; region in between the red and blue, the variable hinge domain; W in green, the tryptophan (W57) that was mutated; and in yellow, the phosphorylation sites described in the thesis dissertation of Holger Dormann with a black box around the characterized Aurora B site (Dormann, 2009).

mitotic removal of HP1. In addition, since trichostatin A, a general inhibitor of histone deacetylases, was used for these studies, whether the effect of acetylation on HP1 localization is direct is unclear. To further add to the confusion, data from Muchardt and colleagues suggest that acetylation of lysine 14 on histone H3 (H3K14), in addition to phosphorylation of H3S10, is required for the removal of HP1 from chromosomes (Mateescu et al., 2004). Data from Lazar and colleagues, however, show that Aurora B preferentially phosphorylates hypoacetylated histone H3 tails. Furthermore, their data suggest that deacetylation of H3K14 is needed to properly phosphorylate H3S10 for the mitotic removal of HP1 (Li et al., 2006). Further studies are needed to clarify the role of histone acetylation in the M-phase release of HP1 proteins from chromosomes.

Functions of the M-phase HP1 release

Phosphorylation of H3S10 regulates the dissociation of HP1 from chromosomes in M-phase (Fischle et al., 2005; Hirota et al., 2005). But what is the biological significance of this HP1 removal from metaphase chromosomes? Below, I speculate on two possible functions for this release: removal of cohesin from chromosome arms and proper condensation/individualization of chromatids.

Cohesion. Since in *S. pombe*, the HP1 homologue, Swi6 is required for the recruitment of cohesin to centromeres in mitosis (Bernard et al., 2001; Nonaka et al., 2002), and in metazoa, Aurora B plays a role in the prophase removal of cohesin from chromosome arms (Losada et al., 2002; Gimenez-Abian et al., 2004),

I had hypothesized that the release of HP1 from metaphase chromosomes aided in this chromosome arm removal of cohesin. My results, however, show that Aurora B does not regulate the bulk amount of chromosome-bound cohesin (Figure 3-6), suggesting that the M-phase release of HP1 has no bearing on cohesin localization. Supporting this implication, HP1 and its centromeric localization are not required for cohesion in mammalian cells (Koch et al., 2008; Serrano et al., 2009).

Condensation and chromatid individualization. HP1 is proposed to compact chromatin by inducing heterochromatin formation via the cross-linking of nucleosomes in interphase (Hiragami and Festenstein, 2005). This HP1-mediated compaction of DNA, however, appears to be mechanistically distinct from mitotic chromatin compaction, since HP1 is largely removed from chromosomes in mitosis (Wreggett et al., 1994; Kellum et al., 1995; Furuta et al., 1997; Minc et al., 1999; Murzina et al., 1999; Sugimoto et al., 2001; Hayakawa et al., 2003). In comparison, condensin facilitates chromosome compaction in mitosis (Hirano, 2005). Therefore, one possible function for the M-phase HP1 dissociation from chromosomes may be related to condensin function. For instance, since HP1-induced heterochromatinization decreases DNA accessibility (Danzer and Wallrath, 2004), the removal of HP1 may facilitate the loading of condensin. Consistent with this hypothesis, the CPC and the phosphorylation of H3S10 have been implicated in the targeting of condensin to chromosomes (Giet and Glover, 2001; Morishita et al., 2001; Kaitna et al., 2002; Petersen and Hagan, 2003; Ono et al., 2004; Lipp et al., 2007; Takemoto et al., 2007). The aberrant maintenance of HP1 on metaphase chromosomes in the absence of the CPC and

H3S10 phosphorylation may inhibit proper chromosome condensation. Alternatively, since HP1 is suggested to cross-link nucleosomes (Hiragami and Festenstein, 2005) and linkages between sister chromatids must be resolved so that the chromatids can properly segregate in anaphase (Ghosh et al., 2006), the dissociation of HP1 in M-phase may simply remove cross-links that inhibit chromatid individualization. While studies have exogenously targeted HP1 to a specific locus to determine its effect on transcription (Li et al., 2003; Danzer and Wallrath, 2004; Verschure et al., 2005; Hines et al., 2009), the effect of general HP1 targeting to chromatin has not been examined. Experiments involving the bulk targeting of HP1 to metaphase chromosomes in the presence of the CPC need to be done to ascertain if any of the mitotic defects seen in cells lacking CPC are attributable to the retention of HP1 on chromosomes.

M-phase functions of HP1 in metazoa

While the role of HP1 in interphase is extensively studied (Hiragami and Festenstein, 2005), its functions in M-phase are less well understood. Though HP1 does not seem to be required for chromosome cohesion in mammalian cells (Koch et al., 2008; Serrano et al., 2009), it is needed in mitosis as mammalian cells depleted of HP1 have mitotic defects (Obuse et al., 2004; De Lucia et al., 2005; Serrano et al., 2009). Since cells devoid of HP1 do not properly segregate their chromosomes (Obuse et al., 2004; De Lucia et al., 2005; Serrano et al., 2009), HP1 may play a role in the proper biorientation of chromosomes. Consistent with this hypothesis, Mis12, a protein required for kinetochore assembly and for the stabilization of kinetochore-microtubules (Kline et al., 2006), is not properly localized to kinetochores in the absence of HP1 (Obuse et al., 2004). Interestingly,

the localization of Aurora B and the phosphorylation of H3S10 are also affected by HP1 depletion (De Lucia et al., 2005; Serrano et al., 2009), but not by HP1 mislocalization (Koch et al., 2008). While HP1 interacts with Mis12 (Obuse et al., 2004) and the CPC (Figure 3-8)(Ainsztein et al., 1998), how HP1 influences the localization of these two proteins and if their localizations are somehow linked are unknown. Since my results suggest that the CPC-xHP1α interaction is needed for the activation of the spindle assembly checkpoint (Figure 3-10) and Aurora B is suggested to correct improperly attached kinetochore-microtubules by phosphorylating Ndc80, a member of the Mis12-containing KMN network (Cheeseman et al., 2006; DeLuca et al., 2006), one appealing speculation is that HP1 plays a role in the error correction mechanism by linking the KMN network to the CPC. This speculative HP1 function, however, is either not dependent on its pericentromeric localization and/or not essential to the spindle checkpoint, as the proper localization of HP1 in both interphase and metaphase is not needed for viability in mice (Koch et al., 2008). Further studies are required to test this hypothesis and to elucidate if other M-phase functions for HP1 exist.

Functional significance of phosphorylating H3S10 in M-phase

While the phosphorylation of H3S10 regulates the dissociation of HP1 from chromosomes in M-phase (Fischle et al., 2005; Hirota et al., 2005), the functional significance of this phosphorylation is still not clear, since the purpose of the HP1 removal is unknown. Whether H3S10 phosphorylation plays a role in mitotic chromosome condensation is still controversial (Van Hooser et al., 1998; Wei et al., 1999; de la Barre et al., 2001; Georgatos et al., 2009), and other functions for H3S10 phosphorylation have not been studied. Ultimately, the

biological significance of phosphorylating H3S10 in M-phase remains an open question.

CHAPTER 4. TARGETING ACTIVATED AURORA B TO MICROTUBULES IS REQUIRED FOR SPINDLE ASSEMBLY

Introduction

In eukaryotes, chromosome segregation during mitosis and meiosis depends on a bipolar spindle. Although centrosomes can drive microtubule nucleation and assist in spindle pole formation, a functional spindle can form without centrosomes (Heald et al., 1996; Khodjakov et al., 2000; Basto et al., 2006). Instead, chromosomes, themselves, can promote spindle assembly by stimulating two signaling cascades: the Ran-GTP pathway and the chromosomal passenger complex (CPC) pathway (Kelly et al., 2007; Walczak and Heald, 2008). It has been proposed that these pathways promote local microtubule assembly by generating a spatial gradient of active effectors centered on chromosomes (Niethammer et al., 2004; Caudron et al., 2005; Bastiaens et al., 2006). The gradient in this model is created by a reaction-diffusion mechanism: an effector is chromosomally activated, diffuses away from chromosomes, and is cytoplasmically inactivated (see Figure 1-2). This simple reaction-diffusion mechanism, however, may not fully explain how spindle shape and size are controlled (Gaetz et al., 2006). Moreover, the regulation and integration of these signaling pathways in spindle assembly beyond the initial chromosomal stimulation remains unclear.

The CPC is composed of the kinase Aurora B, INCENP, Dasra (also known as Borealin), and Survivin (see Figure 1-4)(Ruchaud et al., 2007). The complex localizes to chromosomes and enriches at the inner centromeres from prophase to metaphase before re-localizing to the spindle midzone in anaphase.

In addition to this anaphase spindle localization, a relatively minor population of the CPC also localizes to the spindle microtubules in metaphase (Biggins et al., 1999; Kelly et al., 2007; Zhang et al., 2008; Tseng et al., in submission), when the CPC is most prominently localized to the chromosomes.

Chromosome-induced activation of Aurora B is required for spindle assembly in metaphase of *Xenopus* egg extracts (Sampath et al., 2004), in part by phosphorylating and suppressing the microtubule destabilizing factors MCAK (also known as XKCM1) (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004; Sampath et al., 2004; Gadea and Ruderman, 2005; Zhang et al., 2007) and Op18 (also known as Stathmin)(Tournebize et al., 1997; Budde et al., 2001; Gadea and Ruderman, 2006; Kelly et al., 2007). The CPC also plays a role in stabilizing microtubules near chromosomes during spindle assembly in *Drosophila* female oocytes (Colombie et al., 2008), *S. cerevisiae* (Kotwaliwale et al., 2007), and mammalian HeLa and LLC-PK1 cells (Tulu et al., 2006; Katayama et al., 2008). The importance of the spatial regulation of the CPC is highlighted in results suggesting that chromosomally localized CPC can support spindle assembly in the absence of the Ran-GTP gradient (Maresca et al., 2009).

In addition to chromosomes, taxol-stabilized microtubules can stimulate Aurora B activity in metaphase egg extracts (Kelly et al., 2007; Fuller et al., 2008; Rosasco-Nitcher et al., 2008). The function and necessity of this second activation pathway via microtubules in pre-anaphase stages of M-phase is enigmatic, as a relatively small population of the CPC localizes to microtubules during these cell cycle stages (Kelly et al., 2007; Zhang et al., 2008; Tseng et al., in submission). Since active Aurora B promotes microtubule assembly, a positive feedback loop may form if microtubules further stimulate Aurora B activity (Figure 4-1). This

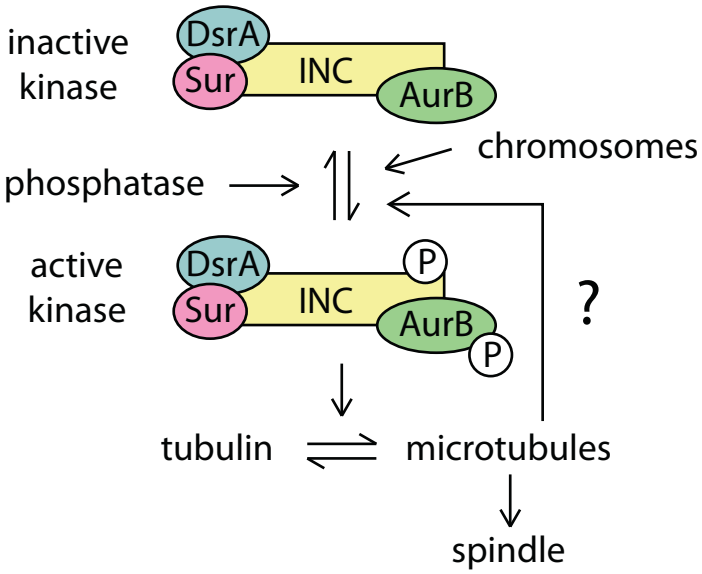


Figure 4-1. The potential positive feedback loop between Aurora B and microtubules.

Aurora B is activated by chromosomes via autophosphorylation and inactivated by cytoplasmic phosphatases. Active Aurora B promotes spindle assembly by supporting microtubule assembly. In turn, microtubules activate Aurora B. ?, potential positive feedback loop.

positive feedback, however, would need to be spatially restricted to the area around chromosomes. Otherwise, it may trigger chromosome-independent microtubule assembly, which could affect spindle organization and positioning.

In this chapter, I will discuss the importance of this seemingly minor CPC-microtubule interaction in spindle assembly. Using *Xenopus* egg extracts, I will show that chromosome-induced activation of Aurora B is insufficient and that this activated Aurora B must be targeted to microtubules via an interaction by INCENP to promote spindle assembly. Although microtubules have a capacity to activate Aurora B, I will present data suggesting that the CPC-microtubule interaction is modulated to prevent sporadically formed cytoplasmic microtubules from triggering the positive feedback between Aurora B and microtubules. Therefore, in the initial stages of spindle assembly, the functional INCENP-microtubule interaction must be limited to the vicinity of chromosomes, where the Ran-GTP pathway promotes microtubule nucleation. Altogether, I demonstrate that the CPC must interact with two structures, chromosomes and microtubules, to drive spindle assembly. I propose that detecting the coincident presence of chromosomes and emerging microtubules by the CPC within a confined space and time is a key mechanism in driving spindle assembly only around chromosomes.

Results

Microtubules activate Aurora B

In metaphase *Xenopus* egg extracts, taxol, which stabilizes microtubules, induces the phosphorylation of Aurora B substrates (Kuntziger et al., 2001; Kelly et al., 2007). To determine if the kinase activity of Aurora B itself is stimulated by

taxol-stabilized microtubules, I monitored the activation loop phosphorylation of Aurora B, since it is required for the full activation of the kinase (Yasui et al., 2004)(Figure 4-2A). As previously shown (Kelly et al., 2007), incubating metaphase egg extracts with taxol induced the hyperphosphorylation of Op18 (also known as Stathmin), as evident from the appearance of a slower migrating band in the immunoblot. Similarly, taxol stimulated the phosphorylation of Aurora B on its activation loop, which is normally suppressed in egg extract. The phosphorylations of both Aurora B and Op18 were eliminated by nocodazole, a drug that inhibits microtubule assembly, or by immunodepletion of the CPC using anti-INCENP antibodies. These results strongly suggest that taxol-stabilized microtubules activate Aurora B in metaphase *Xenopus* egg extracts. Since phosphorylation of Aurora B on its activation loop correlates well with the hyperphosphorylation of Op18, I primarily used Op18 hyperphosphorylation as an indicator for Aurora B activation in the rest of this chapter.

I further characterized the activation of Aurora B by taxol-stabilized microtubules using quantitative immunoblots (Figure 4-2B). I titrated the concentration of taxol and measured its affect on the hyperphosphorylation of Op18 during a three-hour time course. The percent of Op18 that was hyperphosphorylated was determined by normalizing the intensity of the slowest-migrating band to the total intensity of all the Op18 bands. Taxol at 10 μM induced approximately 20% of Op18 to become hyperphosphorylated at steady state, which was reached by approximately 90 min. Reducing the taxol concentration to 5 μM led to an overall reduction of Op18 hyperphosphorylation to approximately 15%. Concentrations of taxol less than 2 μM did not induce

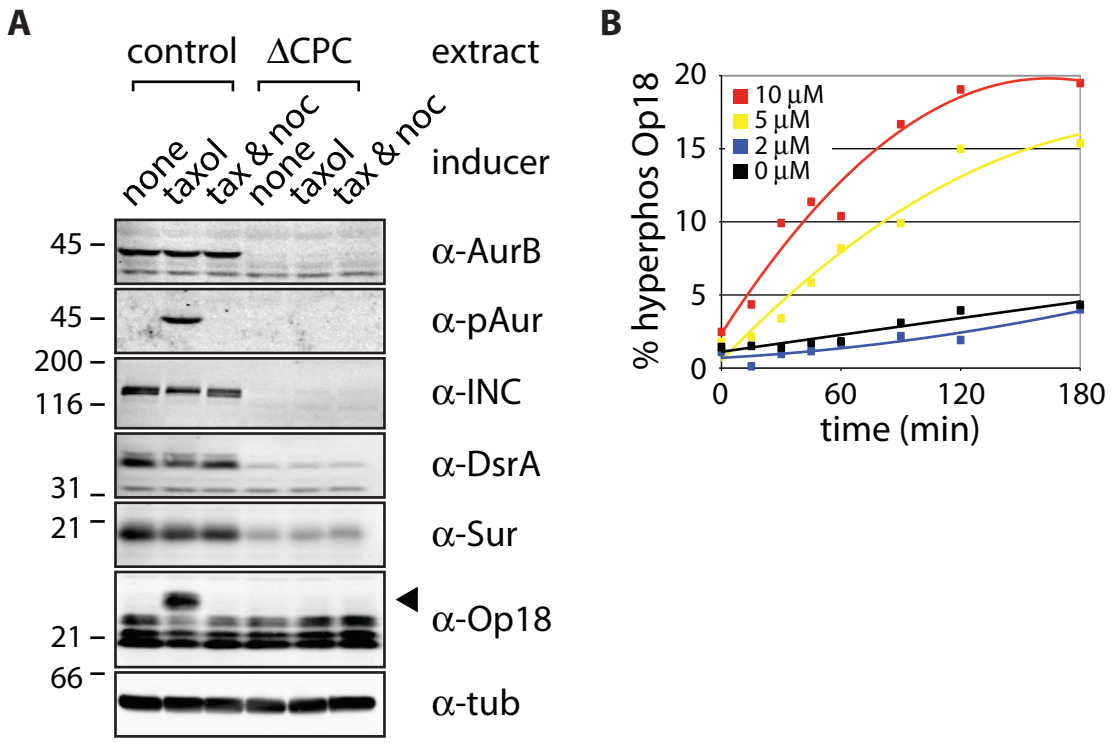


Figure 4-2. Taxol induces microtubule-dependent phosphorylation of Aurora B substrates in a CPC-dependent manner.

(A) Control or ΔCPC extract was incubated at 20°C for 90 min with nothing, 10 μM taxol, or 10 μM taxol with 33 μM nocodazole. Samples were analyzed by immunoblot with the indicated antibody on the right. Molecular weight markers (in kD) are on the left. AurB, Aurora B; INC, INCENP; DsrA, Dasra A; Sur, Survivin; pAur, Aurora B phosphorylated on its activation loop; arrowhead, the hyperphosphorylated form of Op18. **(B)** Control extract was incubated at 20°C for 180 min with the indicated concentration of taxol. Samples were taken at the indicated time points and analyzed by immunoblot with anti-Op18 antibodies. Hyperphosphorylation was measured by normalizing the intensity of the slowest-migrating band to the total intensity of all the Op18 bands. Red, 10 μM taxol; yellow, 5 μM taxol; blue, 2 μM taxol; black, 0 μM taxol.

Aurora B activation over the three-hour time course. Based on this analysis, I used 10 μM taxol and took a sample at 90 min after taxol addition for all assays involving taxol-induced activation of Aurora B.

Since taxol-stabilized microtubules and physiologically formed microtubules may differ in structure (Andreu et al., 1992), I tested if physiologically generated microtubules can also activate Aurora B. I used two methods to stabilize microtubules in egg extract: depletion of MCAK and addition of RanDM (G19V, Q69L), a Ran mutant that is locked in the GTP-bound state (Coutavas et al., 1993). MCAK is the major microtubule destabilizing protein in egg extract, and immunodepleting the protein leads to gross microtubule polymerization (Walczak et al., 1996). Congruent with the taxol result, depleting MCAK from extract stimulated the phosphorylations of Op18 and serine 10 of histone H3 (H3S10p), another Aurora B substrate (Hsu et al., 2000)(Figure 4-3). Similar to the induction with taxol, these phosphorylations were CPC-dependent, chromosome-independent, and nocodazole-sensitive. These results show that microtubules stabilized by the depletion of MCAK can induce Aurora B activation. Notably, in control extract containing chromosomes, more Op18 was hyperphosphorylated in the absence of nocodazole than in its presence. Therefore, microtubules contributed to the activation of Aurora B even when chromosomes are present, suggesting that microtubules can activate Aurora B in the physiological spindle context.

Ran, in its GTP-bound state, promotes microtubule assembly in egg extract by releasing spindle assembly factors from importin, an inhibitory nuclear import factor (Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001). Egg extracts incubated with Ran mutants that lack GTPase activity and are

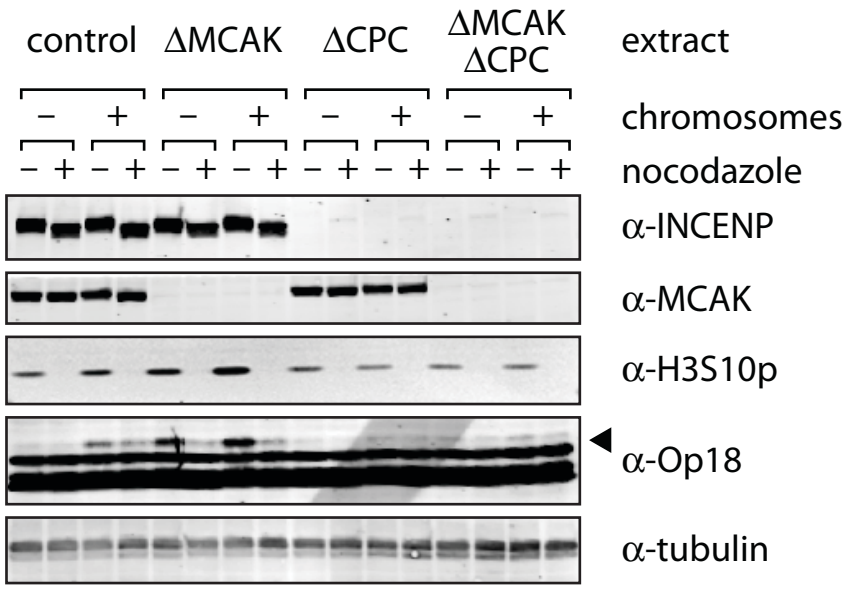


Figure 4-3. Depletion of MCAK induces microtubule-dependent phosphorylation of Aurora B substrates in a CPC-dependent manner. Control, ΔMCAK, ΔCPC, or ΔMCAKΔCPC extracts were cycled through interphase to metaphase with or without 100/μl sperm nuclei ("chromosomes") in the presence or absence of 33 μM nocodazole at 20°C. Samples were taken 45 min after entry into metaphase and were analyzed by immunoblot with the indicated antibody on the right. Arrowhead, the hyperphosphorylated form of Op18.

permanently GTP bound, like RanDM, form microtubule asters (Carazo-Salas et al., 1999; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999; Zhang et al., 1999). The microtubules formed by the addition of RanDM, however, failed to activate Aurora B (Figure 4-4). RanDM, however, was able to increase the amount of hyperphosphorylated Op18 in extract that was depleted of MCAK in nocodazole-sensitive manner. Therefore, while RanDM is unable to induce Aurora B activation when it is the sole method promoting microtubule assembly, RanDM is capable of polymerizing microtubules that activate Aurora B. Together, these three methods of microtubule stabilization show that microtubules induce Aurora B kinase activity. Since active Aurora B promotes microtubule stabilization (Sampath et al., 2004; Kelly et al., 2007), the ability of microtubules to activate Aurora B indicates the potential for a positive feedback loop (Figure 4-1).

Aurora B and INCENP constitute the minimum CPC subunits required for microtubule-induced kinase activation

All four members of the CPC are required for chromosome-induced activation of Aurora B (Kelly et al., 2007). If the primary role of the CPC for spindle assembly is to phosphorylate critical substrates, such as MCAK and Op18, at the vicinity of chromosomes, what is the functional significance of the microtubule-induced activation of Aurora B? Alternatively, does the microtubule-induced activation of Aurora B simply reflect the ability of the CPC to interact with microtubules? If so, is this interaction important for spindle assembly?

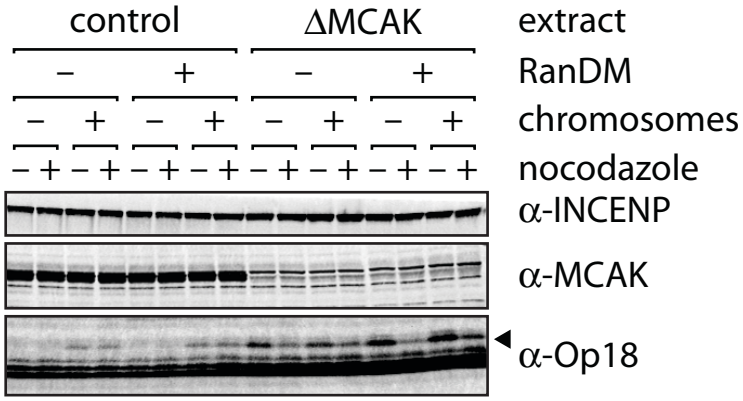


Figure 4-4. Addition of dominant active Ran induces microtubule-dependent phosphorylation of Op18.

Control or ΔMCAK extracts were cycled through interphase to metaphase with or without 100/μl sperm nuclei ("chromosomes") in the presence or absence of 33 μM nocodazole and/or 47.5 μM RanDM at 20°C. Samples were taken 45 min after entry into metaphase and were analyzed by immunoblot with the indicated antibody on the right. Arrowhead, the hyperphosphorylated form of Op18.

To address these questions, I sought to discover the minimal microtubule-sensing domain of the CPC, by first identifying the CPC subunits required for microtubule-induced activation of Aurora B. I tested extracts containing various combinations of the four CPC subunits for the ability of microtubules to activate Aurora B (Figure 4-5). I immunodepleted the endogenous CPC from egg extracts (ΔCPC) using anti-INCENP antibodies, and then reconstituted these extracts with *in vitro* transcribed mRNA encoding the various members of the CPC (Murray, 1991; Kelly et al., 2007). As previously published (Kelly et al., 2007), extracts lacking either Dasra A or Survivin failed to stimulate Op18 hyperphosphorylation in the presence chromosomes, while ΔCPC extracts reconstituted with all four members of the CPC were able to induce Aurora B activation by chromosomes. Aurora B and INCENP paired with Dasra B, the somatic homologue of Dasra A in *Xenopus* (Sampath et al., 2004), and SIX, a variant of Survivin that exists in *Xenopus* embryos (Song et al., 2003), also rescued chromosome-induced Aurora B activation (Figure 4-5, right). This result shows that Dasra B and SIX can complex with Aurora B and INCENP to produce a functional CPC in *Xenopus* egg extract. Together, these results are congruent with previously published data showing that Dasra A and Survivin are required for the chromosomal localization of the Aurora B (Kelly et al., 2007). In contrast, extracts lacking Dasra A/B and/or Survivin/SIX were able to induce Aurora B activation in the presence of taxol (Figure 4-5, left). Therefore, Aurora B and INCENP constitute the minimum CPC subunits required for microtubule-induced kinase activation and presumably contain the microtubule sensor

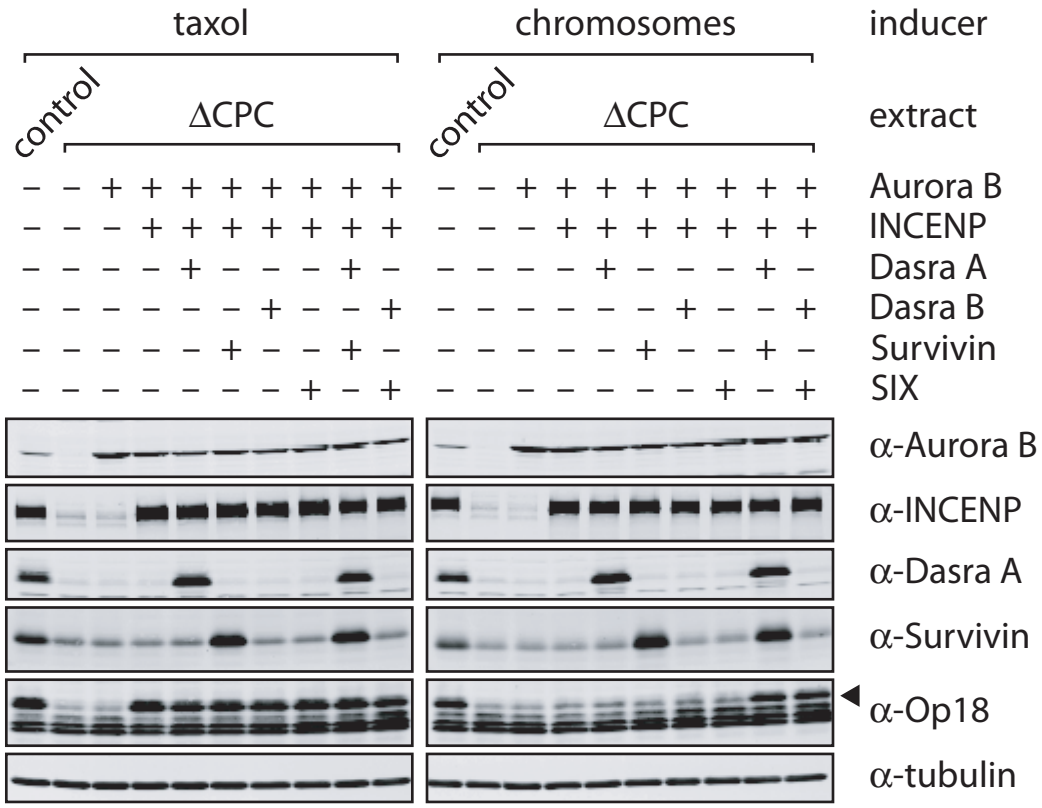


Figure 4-5. Dasra A and Survivin are not required for microtubule-induced phosphorylation of Op18.

Control or ΔCPC extracts were reconstituted as indicated and incubated at 20°C for 90 min with 10 μM taxol or with 2000/μl sperm nuclei and 33 μM nocodazole ("chromosomes"). Samples were analyzed by immunoblot with the antibodies indicated on the right. Arrowhead, the hyperphosphorylated form of Op18.

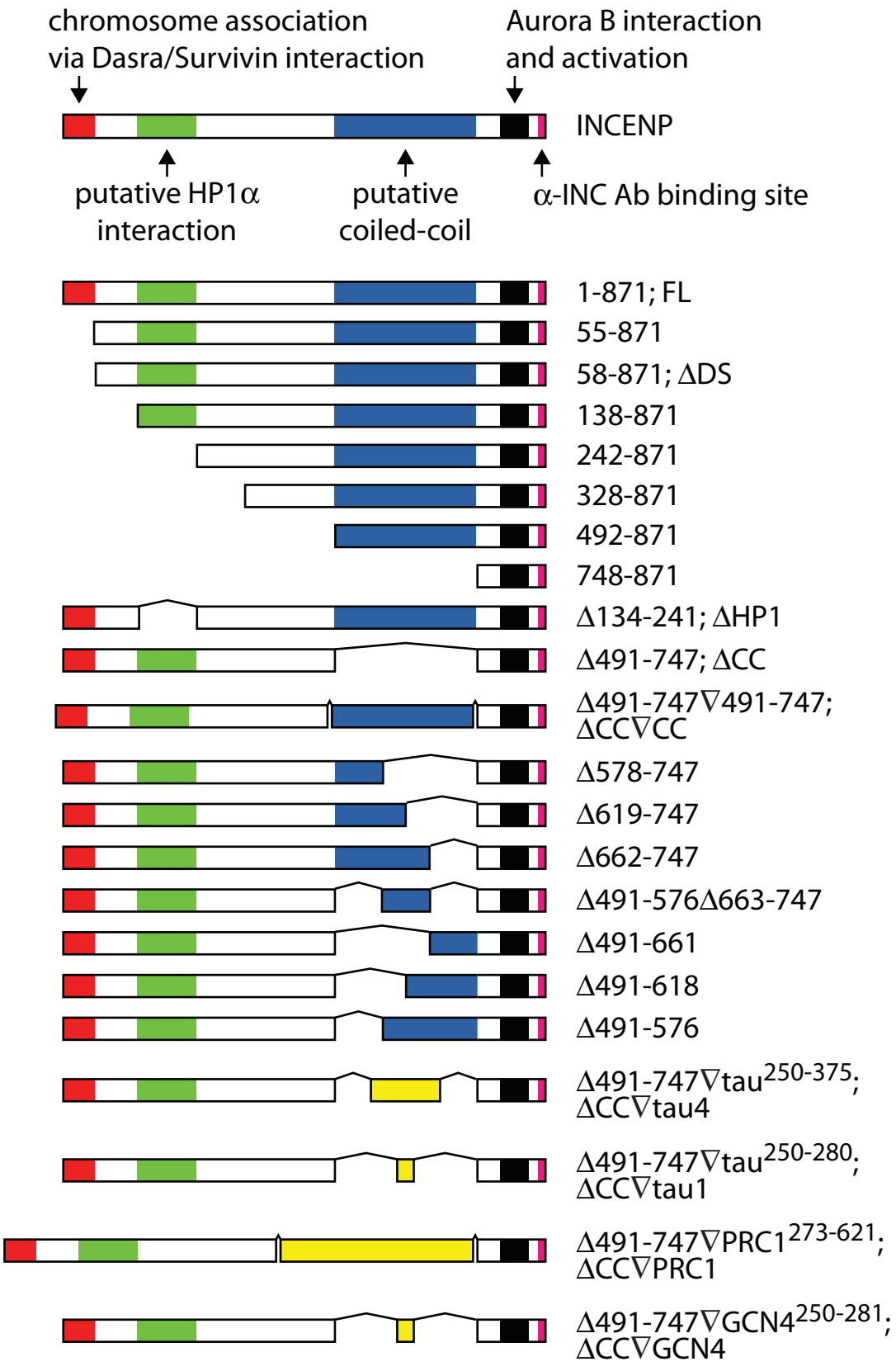
domain of the CPC, consistent with the conclusion obtained with the purified complex (Fuller et al., 2008; Rosasco-Nitcher et al., 2008).

The putative coiled-coil domain of INCENP is required for microtubule-induced Aurora B activation

Members of the CPC have been shown to bind to microtubules (Mackay et al., 1993; Kang et al., 2001; Wheatley et al., 2001; Sandall et al., 2006; Rosasco-Nitcher et al., 2008), and presumably, the interaction of the CPC with microtubules is required for microtubule-induced kinase activation. Since Dasra A and Survivin were dispensable for microtubule-induced Aurora B activation (Figure 4-5) and Aurora B is the activated kinase, I focused on determining whether INCENP contains a microtubule-interaction domain to allow for the manipulation of the CPC-microtubule interaction.

INCENP has four previously mapped domains. The N-terminal 57 amino acids of INCENP (Figure 4-6; red box, the DS domain) form a trimeric helix with Dasra and Survivin, and target the CPC to chromosomes, centromeres, and the spindle midzone (Mackay et al., 1993; Ainsztein et al., 1998; Klein et al., 2006; Jeyaprakash et al., 2007). INCENP has also been shown to complex with HP1α (green box, aa 138-241)(Ainsztein et al., 1998) and Aurora B (black box, the IN-box, aa 790-840)(Adams et al., 2000). While the function of the putative HP1α interaction domain is currently unknown, the IN-box of INCENP is required for the allosteric activation of Aurora B (Sessa et al., 2005). Furthermore, INCENP contains a putative coiled coil domain (blue box, CC domain, aa 491-747) that has been implicated in microtubule targeting in metazoans (Mackay et al., 1993;

Figure 4-6. Schematic of the constructs used in this study.
Red represents the Dasra A and Survivin interaction domain (DS domain, aa 1-57); green, the putative HP1α interaction domain (HP1 domain, aa 134-241); blue, the putative coiled-coil domain with a potential microtubule-targeting function (CC domain, aa 491-747); black, the Aurora B interaction and activation domain (IN box, aa 790-840); magenta, the antigen for the activating anti-INCENP antibody (aa 858-871); yellow, the exogenous domains that replaced the CC domain; caret, flexible linkers. The INCENP constructs contain or exclude (Δ) the indicated amino acids. For the chimeric constructs, ΔCC∇CC is an INCENP where the CC domain is replaced with the endogenous CC domain of INCENP, which serves as a chimera control for the linkers; ΔCC∇tau4, INCENP where the CC domain is replaced by the four binding cassettes of the microtubule binding domain of tau (250-375); ΔCC∇PRC1, INCENP where the CC domain is replaced by the microtubule binding domain of PRC1 (273-621); ΔCC∇GCN4, INCENP where the CC domain (Δ491-747) is replaced by the dimerization domain of GCN4 (250-281).


chromosome association
via Dasra/Survivin interaction
Aurora B interaction
and activation
INCENP
putative HP1α
interaction
putative
coiled-coil
α-INC Ab binding site
1-871; FL
55-871
58-871; ΔDS
138-871
242-871
328-871
492-871
748-871
Δ134-241; ΔHP1
Δ491-747; ΔCC
Δ491-747∇491-747;
ΔCC∇CC
Δ578-747
Δ619-747
Δ662-747
Δ491-576Δ663-747
Δ491-661
Δ491-618
Δ491-576
Δ491-747∇tau250-375;
ΔCC∇tau4
Δ491-747∇tau250-280;
ΔCC∇tau1
Δ491-747∇PRC1273-621;
ΔCC∇PRC1
Δ491-747∇GCN4250-281;
ΔCC∇GCN4

Vader et al., 2007). In budding yeast, the analogous domain of Sli15, the INCENP homologue, can bind to microtubules directly (Kang et al., 2001).

Using these four domains as a guide, I made serial N-terminal truncations of INCENP (Figure 4-6), and then tested their ability to activate Aurora B in the presence of taxol (Figure 4-7). All of the constructs should maintain their intrinsic ability to activate Aurora B, since they all still contain the IN-box. Indeed, an anti-INCENP antibody, which recognizes the C-terminus of INCENP (Figure 4-6; magenta box, aa 858-871) and activates Aurora B by clustering-mediated autophosphorylation (Kelly et al., 2007), was capable of inducing Op18 hyperphosphorylation in the presence of any of these constructs (Figure 4-7, right panels). Microtubules also induced Op18 hyperphosphorylation with all truncations of INCENP up to residue 491 (Figure 4-7A). Deletion of the first 747 residues, however, abolished the ability of microtubules to induce Op18 hyperphosphorylation (Figure 4-7B). These results suggest that residues 491-747 (Figure 4-6; the CC domain) are required for microtubule-induced kinase activation. Indeed, deletion of these residues (INCENPΔCC) failed to support Aurora B activation in the presence of taxol (Figure 4-7C). Furthermore, any tested truncation within the CC domain, while maintaining the intrinsic ability to activate Aurora B, could not activate the kinase in the presence of taxol (Figure 4-8). These results show that the CC domain of INCENP is required for microtubule-induced activation of Aurora B, and suggests that this domain is the microtubule-sensing domain of the CPC.

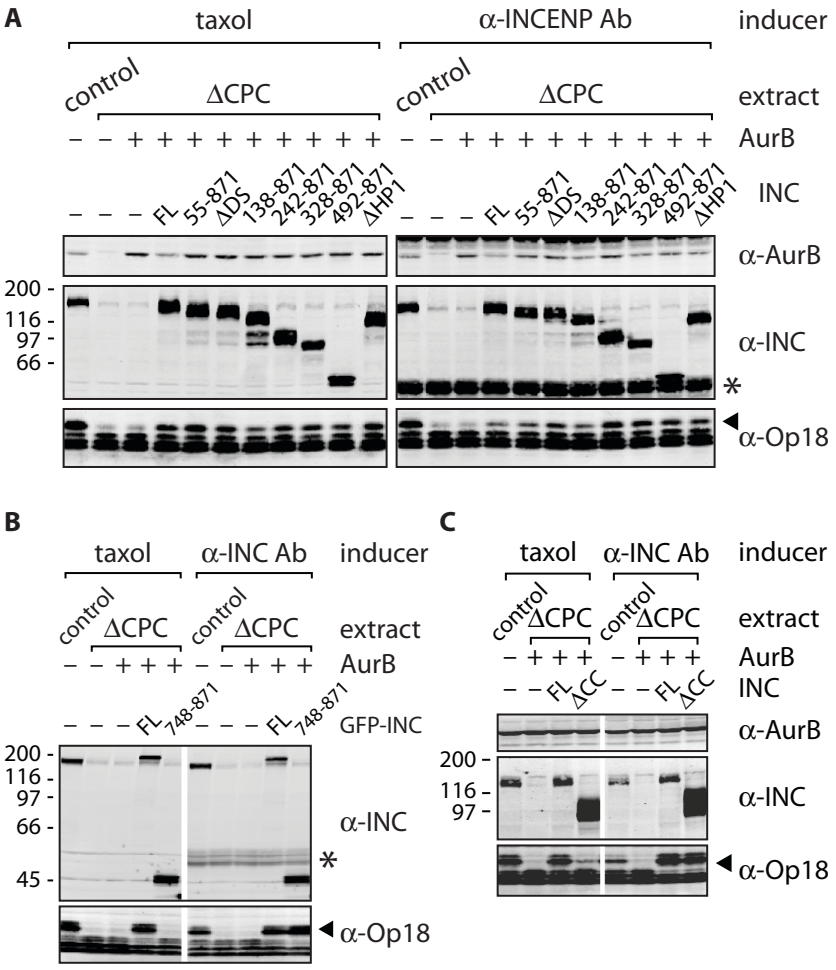


Figure 4-7. The CC domain of INCENP is required for taxol-induced kinase activation.

(A, B, C) Control or ΔCPC extracts were reconstituted as indicated and incubated at 20°C for 90 min with 10 μM taxol or 100 μg/ml anti-INCENP antibodies. Samples were analyzed by immunoblot with the indicated antibodies on the right. Molecular weight markers (in kD) for INCENP are on the left. AurB, Aurora B; INC, INCENP. Arrowhead, the hyperphosphorylated form of Op18; asterisk, anti-INCENP antibodies added to the extract.

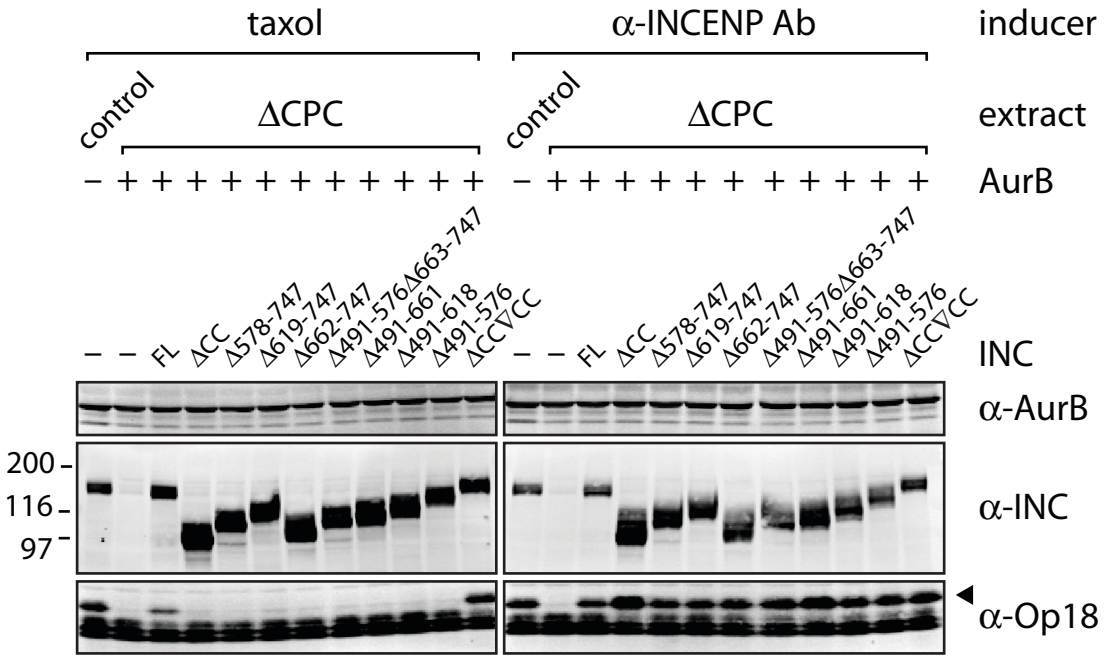


Figure 4-8. Partial deletion of the CC Domain is sufficient for disrupting microtubule-induced Aurora B activation.

Control or ΔCPC was incubated at 20°C for 90 min with 10 μM taxol or 100 μg/ml anti-INCENP antibody. Samples were analyzed by immunoblot with the indicated antibody on the right. Molecular weight markers (in kD) for INCENP are on the left. AurB, Aurora B; INC, INCENP. Arrowhead, the hyperphosphorylated form of Op18.

The putative coiled-coil domain of INCENP is not required for chromosome-induced activation of Aurora B

Although INCENPΔCC maintained its intrinsic ability to activate Aurora B, as assayed by the anti-INCENP antibody (Figure 4-7C, right), this 256-residue deletion of INCENP could lead to a severely misfolded protein that is unable to perform any of its normal physiological functions. To eliminate this possibility, the ability of chromosomes to activate Aurora B in the presence of INCENPΔCC was tested (Figure 4-9). In this assay, nocodazole, which depolymerizes microtubules, was added to eliminate the activation component induced by microtubules, and therefore, this assay should report on only the chromosome-induced activation. Consistent with previously published data (Kelly et al., 2007), extract containing Aurora B, Dasra A, Survivin, and full-length INCENP induced Op18 hyperphosphorylation in the presence of chromosomes, while extract containing an INCENP that lacks the chromosome-targeting Dasra/Survivin interaction domain (INCENPΔDS) failed to hyperphosphorylate Op18 when incubated with chromosomes. In contrast, INCENPΔCC induced Aurora B activity in the presence of chromosomes. Therefore, while the CC domain is required for microtubule-induced kinase activation, it is not required for the chromosome-induced pathway. These results suggest that INCENPΔCC does not severely misfold and maintains a subset of its physiological functions.

The putative coiled-coil domain of INCENP is required for spindle assembly

Since the CC domain is required for microtubule-induced activation of Aurora B, I sought to determine the role of this activation in spindle assembly by

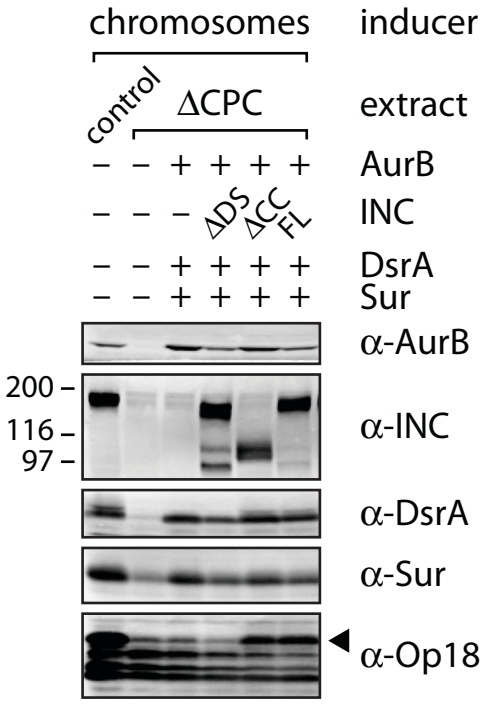
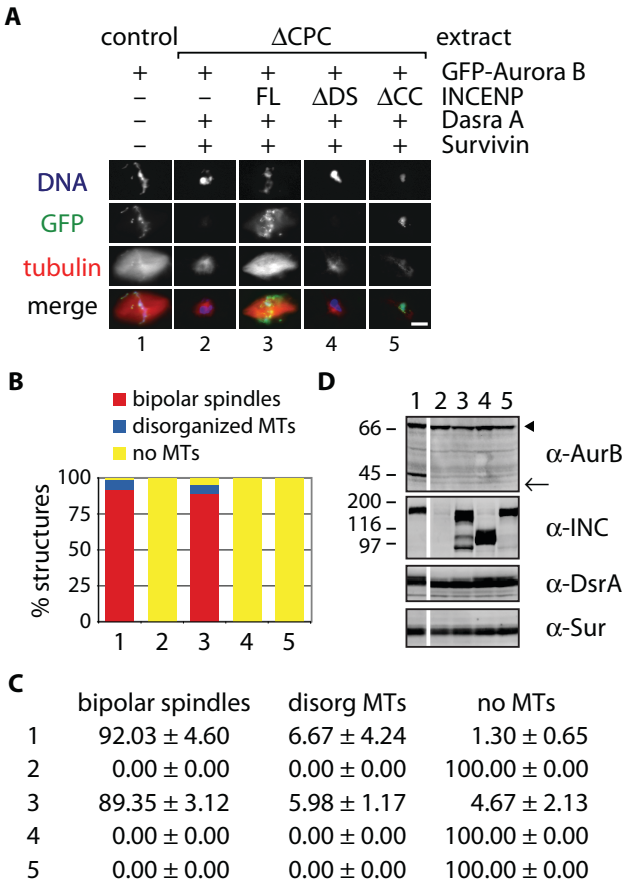


Figure 4-9. The CC domain of INCENP is not required for chromosome-induced kinase activation.

Control or ΔCPC extracts were reconstituted as indicated and cycled through interphase to metaphase with 1333/μl sperm nuclei ("chromosomes") in the presence of 33 μM nocodazole at 20°C. Samples were taken 90 min after entry into metaphase and were analyzed by immunoblot with the indicated antibodies on the right. Molecular weight markers (in kD) for INCENP are on the left. AurB, Aurora B; INC, INCENP; DsrA, Dasra A; Sur, Survivin. Arrowhead, the hyperphosphorylated form of Op18.

assessing the requirement for the CC domain of INCENP. I reconstituted ΔCPC extract with GFP-Aurora B, Dasra A, Survivin, and full-length or mutant INCENP (Figure 4-10). As expected (Kelly et al., 2007), while full-length INCENP (FL) targeted GFP-Aurora B to chromosomes and supported spindle assembly (#3), INCENP lacking the Dasra/Survivin interaction domain (ΔDS) failed to do so (#4). This result is consistent with a previous conclusion from our laboratory that the CPC-chromosome interaction, which is mediated by the DS domain of INCENP, is required for spindle assembly (Kelly et al., 2007). The CPC-chromosome interaction, however, is not sufficient: even though INCENPΔCC localized GFP-Aurora B to chromosomes, the microtubule assembly around chromosomes was severely impaired (#5). Furthermore, any tested smaller deletions of the CC domain, which all abrogated activation by microtubules (Figure 4-8), also failed to support spindle assembly (Figure 4-11). Therefore, in addition to the CPC-chromosome interaction and the resulting Aurora B activation, the CC domain of INCENP is required for the initial steps of spindle assembly.

While GFP-Aurora B appeared to localize to chromosomes equally in extract containing either full-length INCENP or INCENPΔCC, the chromosome morphology is different between these samples, which complicates the interpretation. To verify that INCENPΔCC properly targets the CPC to chromosomes, I biochemically purified chromosomes from extract containing INCENPΔCC (Figure 4-12). The chromosome-bound fraction from extract with full-length INCENP contained all four members of the CPC, while none of the four components bound to chromosomes in extract containing INCENPΔDS. In



C

	bipolar spindles	disorg MTs	no MTs
1	92.03 ± 4.60	6.67 ± 4.24	1.30 ± 0.65
2	0.00 ± 0.00	0.00 ± 0.00	100.00 ± 0.00
3	89.35 ± 3.12	5.98 ± 1.17	4.67 ± 2.13
4	0.00 ± 0.00	0.00 ± 0.00	100.00 ± 0.00
5	0.00 ± 0.00	0.00 ± 0.00	100.00 ± 0.00

Figure 4-10. The CC domain of INCENP is required for spindle assembly. **(A)** Control or ΔCPC extracts were reconstituted as indicated. Samples were cycled through interphase to metaphase with 100/μl sperm nuclei at 20°C. Samples were processed for immunofluorescence 60 min after entry into metaphase using anti-GFP (green) antibodies. DNA (blue) and tubulin (red) were visualized with Hoechst 33258 and rhodamine-labeled tubulin, respectively. Scale bar, 10 μm. **(B, C)** Quantitation and statistical analysis of chromosome-containing microtubule structures formed in the extracts described in and numbered as in (A). The average of 3 experiments with at least 250 structures counted per sample is represented. Red, spindles; blue, disorganized microtubules; yellow, weak/no microtubules. The numbers in (C) represent the percent of that structure ± S.E.M. **(D)** Immunoblot of experiments in (A) with the indicated antibodies on the right. Samples are numbered as in (A). Molecular weight markers (in kD) are on the left. Arrow, endogenous Aurora B; arrowhead, GFP-Aurora B. AurB, Aurora B; INC, INCENP; DsrA, Dasra A; Sur, Survivin.

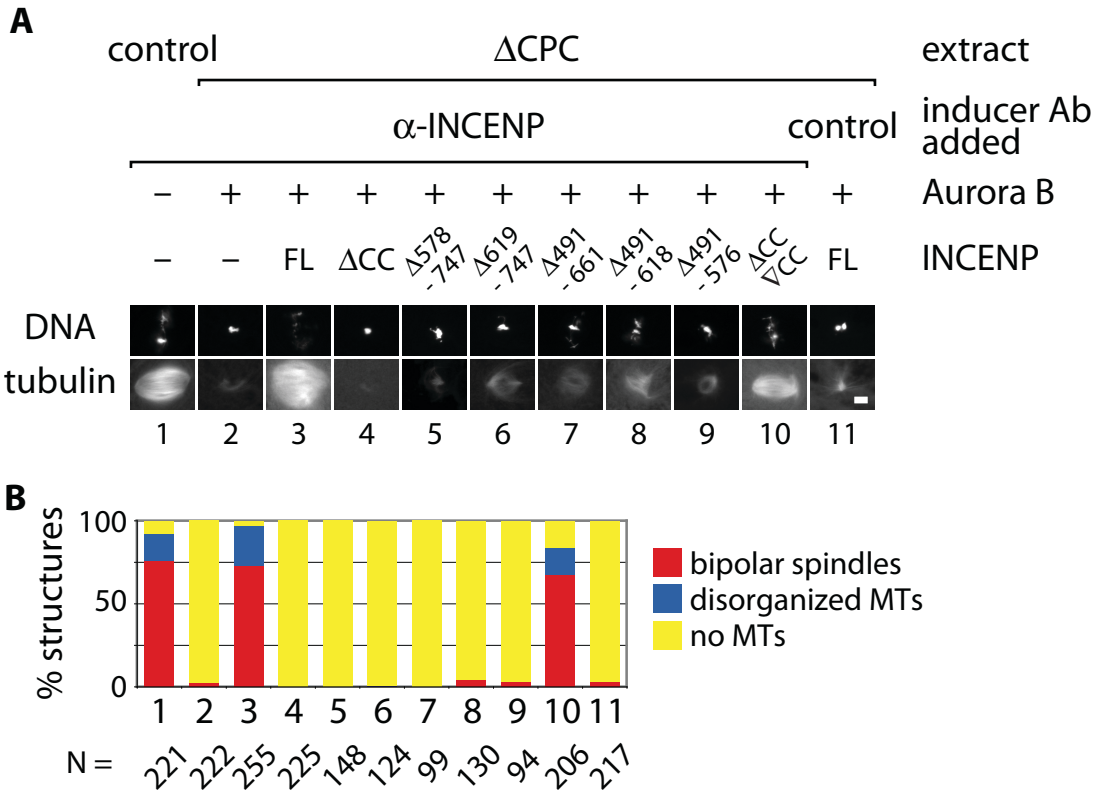


Figure 4-11. Partial deletion of the CC Domain inhibits spindle assembly.
(A) Control or ΔCPC extracts were reconstituted as indicated and cycled through interphase to metaphase with 100/μl sperm nuclei and 5 μg/ml anti-INCENP antibody at 20°C. Samples were fixed 60 min after entry into metaphase and imaged. DNA (blue) and tubulin (red) were visualized with Hoechst 33258 and rhodamine-labeled tubulin, respectively. Scale bar, 10 μm. **(B)** The chromosome-containing microtubule structures were counted. Samples are numbered as in (B). N, number of structures counted. Red, spindles; blue, disorganized microtubules; yellow, weak/no microtubules.

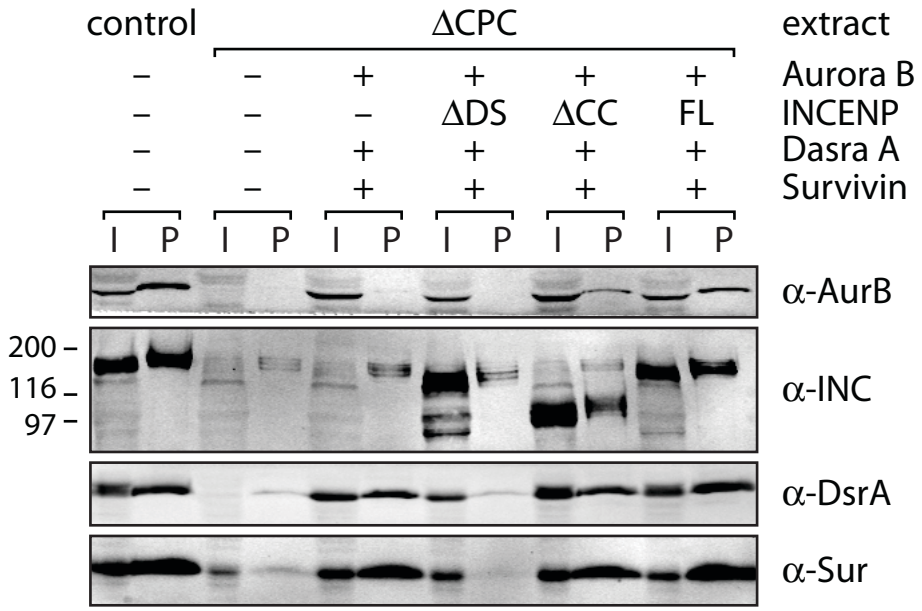


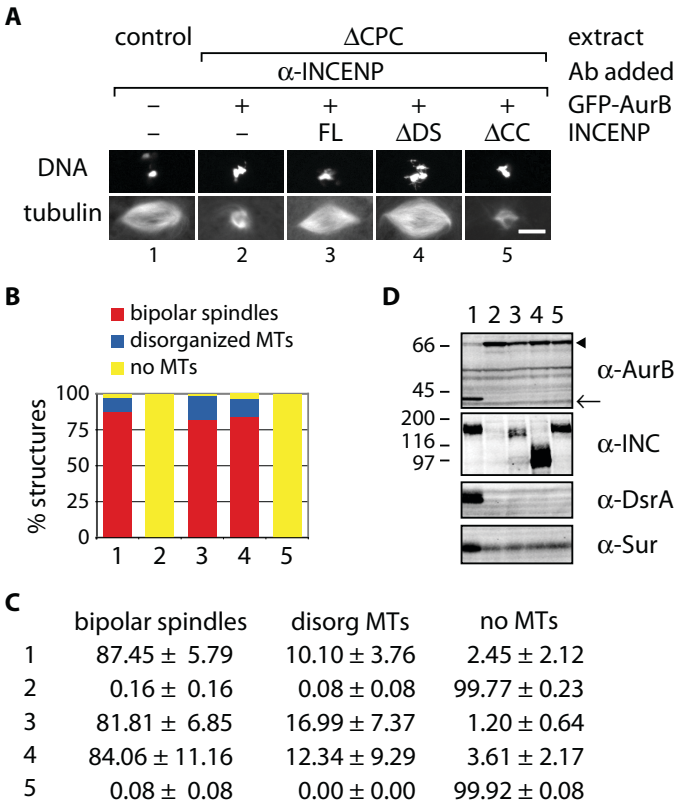
Figure 4-12. The CC domain of INCENP is not required for chromosomal binding.

Control or ΔCPC extracts were reconstituted as indicated and cycled through interphase to metaphase with 1333/μl sperm nuclei in the presence of 33 μM nocodazole at 20°C. Samples were taken 90 min after entry into metaphase. The proteins bound to chromosomes were purified and analyzed by immunoblot with the indicated antibodies on the right. Molecular weight markers (in kD) for INCENP are on the left. AurB, Aurora B; INC, INCENP; DsrA, Dasra A; Sur, Survivin; I, input of unfractionated extract; P, chromosomal bound proteins.

extract containing INCENPΔCC, all members of the CPC purified with chromosomes to a similar level as that of extract containing full-length INCENP. These results confirm that INCENPΔCC properly targets Aurora B to chromosomes.

Since the CC domain is responsible for microtubule-mediated activation of Aurora B, I asked if microtubules, in addition to chromosomes, must activate Aurora B in order to trigger spindle assembly. To address this question, I investigated if activating Aurora B by other means could bypass the necessity for the CC domain. Spindle assembly in ΔCPC extract reconstituted with Aurora B and full-length or mutant INCENP in the presence of the activating anti-INCENP antibody was monitored (Figure 4-13). As previously shown (Kelly et al., 2007), artificial activation of Aurora B, by the clustering anti-INCENP antibody, bypassed the requirement of Dasra A (#3) and the DS domain (#4) for spindle assembly. These results confirmed that the primary role of the CPC-chromosome interaction in spindle assembly is to activate Aurora B. As long as Aurora B becomes activated, however, the chromosomal localization of the CPC, per se, is not essential for microtubule assembly. In contrast, even in the presence of the anti-INCENP antibody, INCENPΔCC failed to support spindle assembly (#5). These results suggest that while activation of Aurora B is required for spindle assembly, the CC domain is critical for an additional process beyond kinase activation, possibly by targeting chromosomally activated Aurora B to microtubules.

Supporting the hypothesis that the CC domain functions in a process beyond kinase activation, further stimulation of Aurora B activity by two



C

	bipolar spindles	disorg MTs	no MTs
1	87.45 ± 5.79	10.10 ± 3.76	2.45 ± 2.12
2	0.16 ± 0.16	0.08 ± 0.08	99.77 ± 0.23
3	81.81 ± 6.85	16.99 ± 7.37	1.20 ± 0.64
4	84.06 ± 11.16	12.34 ± 9.29	3.61 ± 2.17
5	0.08 ± 0.08	0.00 ± 0.00	99.92 ± 0.08

Figure 4-13. Activating Aurora B with the anti-INCENP antibody does not bypass the requirement for the CC domain of INCENP in spindle assembly. **(A)** Control or ΔCPC extracts were reconstituted as indicated with 5 μg/ml anti-INCENP antibodies and cycled through interphase to metaphase with 100/μl sperm nuclei at 20°C. Samples were fixed and imaged at 60 min after entry into metaphase. DNA and tubulin were visualized with Hoechst 33258 and rhodamine-labeled tubulin, respectively. Scale bar, 10 μm. **(B, C)** Quantitation and statistical analysis of chromosome-containing microtubule structures formed in the extracts described in and numbered as in (A). The average of 3 experiments with at least 250 structures counted per sample is represented. In (B), red, spindles; blue, disorganized microtubules; yellow, weak/no microtubules. The numbers in (C) represent the percent of that structure ± S.E.M. **(D)** Immunoblot of samples in (A), numbered as such, with the indicated antibodies on the right. Molecular weight markers (in kD) are on the left. Arrow, endogenous Aurora B; arrowhead, GFP-Aurora B. AurB, Aurora B; INC, INCENP; DsrA, Dasra A; Sur, Survivin.

different methods still did not bypass the requirement for the CC domain in spindle assembly. First, the extract was incubated with higher levels of anti-INCENP antibodies. To bypass the requirement for the CPC-chromosome interaction in spindle formation, 5 μg/ml anti-INCENP antibody is sufficient. In the ΔCPC extracts supplemented with INCENPΔCC, 100 μg/ml anti-INCENP antibody still failed to support spindle assembly (Figure 4-14C, D), even though this extract achieved Op18 hyperphosphorylation to at least the same level as that of extract containing full length INCENP with 5 μg/ml anti-INCENP antibody (Figure 4-14A, B). Second, the CC domain of INCENP was replaced with the dimerizing GCN4 coiled-coil domain (250-281, INCENPΔCC∇GCN4, Figure 4-6)(O'Shea et al., 1989). Consistent with the activation of Aurora B after clustering the CPC (Kelly et al., 2007), INCENPΔCC∇GCN4 converted the majority of Op18 to the phosphorylated form even in the absence of an inducer, but failed to form spindles (Figure 4-15). Together, these results demonstrate that, unlike the case of the DS domain, global activation of Aurora B cannot bypass the requirement for the CC domain in spindle assembly, suggesting that the function of the CC domain is not simply to activate Aurora B in the initial steps of spindle assembly.

The putative coiled-coil domain of INCENP targets the CPC to microtubules

The CC domain of INCENP in metazoans has been implicated in microtubule targeting (Mackay et al., 1993; Vader et al., 2007). In addition, the analogous domain of *S. cerevisiae* INCENP, Sli15, can directly bind to microtubules (Kang et al., 2001). Furthermore, my data suggests that the CC

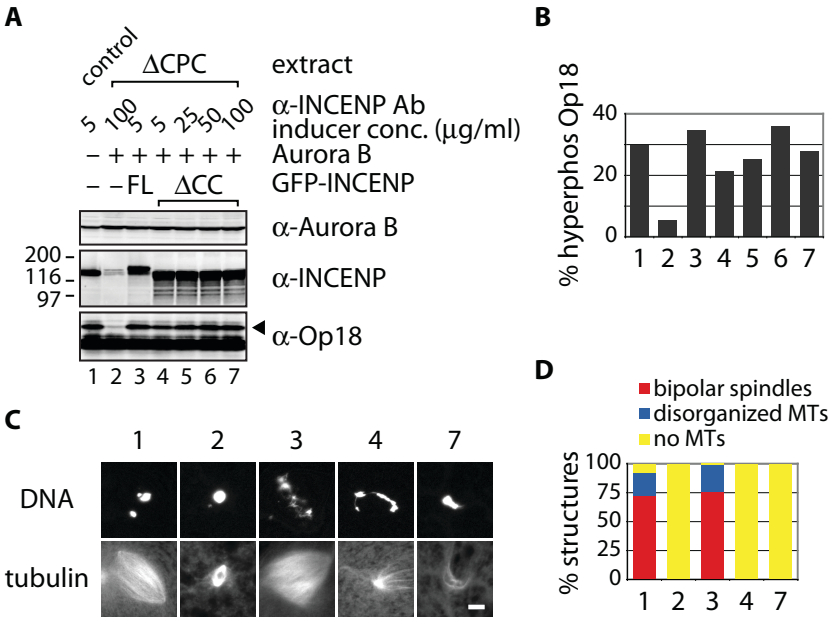
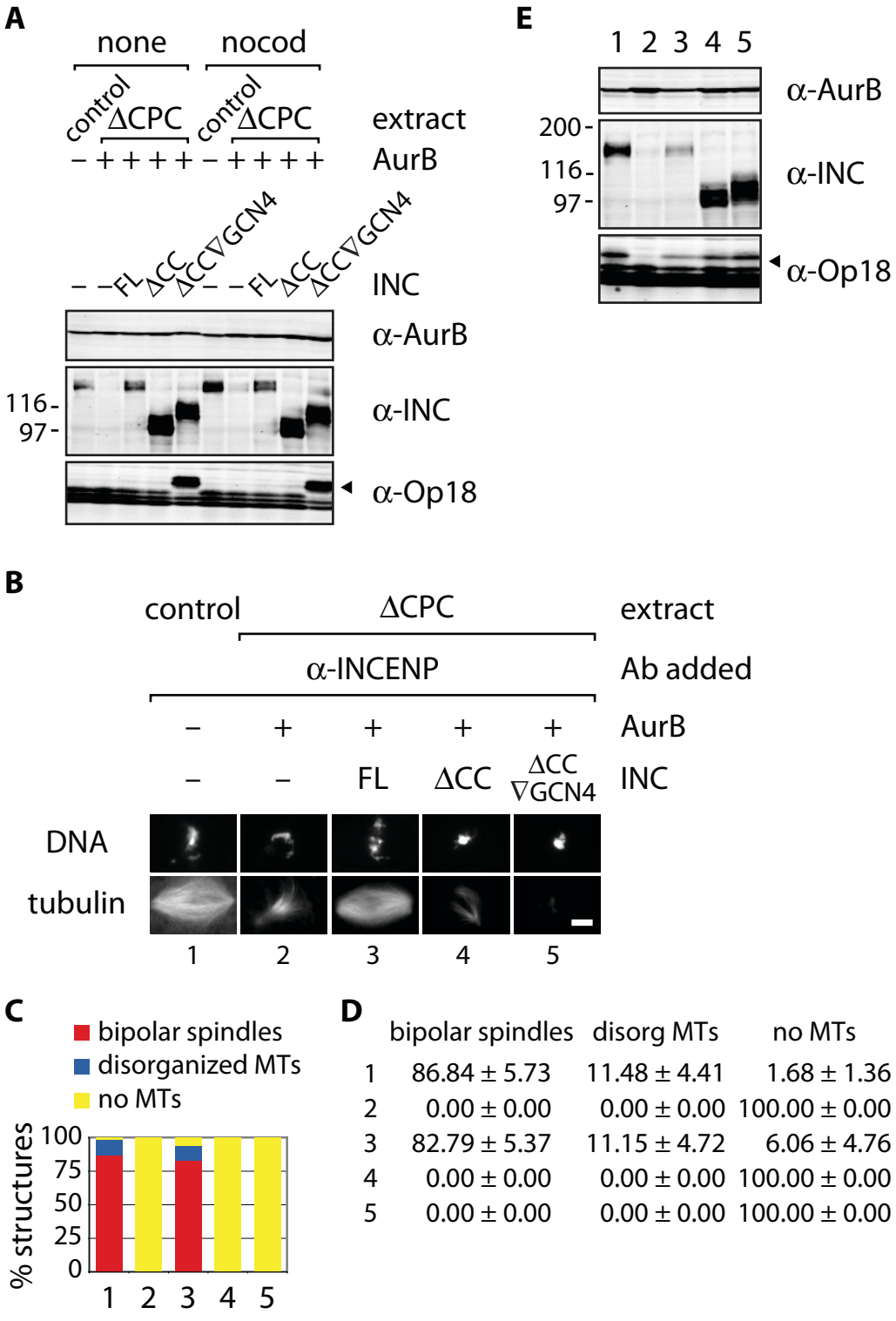


Figure 4-14. Increasing the amount of anti-INCENP antibody does not rescue spindle assembly.

Control or ΔCPC extracts were reconstituted as indicated and cycled through interphase to metaphase with 100/μl sperm nuclei and the indicated amount of anti-INCENP antibodies at 20°C. Samples were taken 60 min after entry into metaphase. **(A)** Samples were analyzed by immunoblot using the indicated antibodies on the right. Molecular weight markers (in kD) for INCENP are on the left. Arrowhead, the hyperphosphorylated form of Op18. **(B)** The percent of hyperphosphorylated Op18 was measured by normalizing the intensity of the slowest-migrating band to the total intensity of all the Op18 bands. **(C)** Samples were fixed and imaged. DNA and tubulin were visualized with Hoechst 33258 and rhodamine-labeled tubulin, respectively. Scale bar, 10 μm. **(D)** Quantitation of chromosome-containing microtubule structures formed in these samples. At least 200 structures were counted per sample. Red, spindles; blue, disorganized microtubules; yellow, weak/no microtubules. Samples numbered as in (A).

Figure 4-15. Dimerization of INCENP does not rescue spindle assembly.
(A) Control or ΔCPC extracts were reconstituted as indicated and incubated at 20°C for 90 min with or without 33 μM nocodazole. Samples were analyzed by immunoblot with the indicated antibodies on the right. **(B)** Control or ΔCPC extracts were reconstituted as indicated and cycled through interphase to metaphase with 100/μl sperm nuclei and 5 μg/ml anti-INCENP antibodies at 20°C. Samples were fixed and imaged 60 min after entry into metaphase. DNA and tubulin were visualized with Hoechst 33258 and rhodamine-labeled tubulin, respectively. Scale bar, 10 μm. **(C, D)** Quantitation and statistical analysis of chromosome-containing microtubule structures formed in the extracts described in (B). The data is the average of 3 experiments with at least 250 structures counted per sample. In (C), red, spindles; blue, disorganized microtubules; yellow, weak/no microtubules. The numbers in (D) represent the percent of that structure ± S.E.M. **(E)** Immunoblot of samples in (B), with the indicated antibodies on the right. Molecular weight markers (in kD) are on the left. Arrowhead, the hyperphosphorylated form of Op18; AurB, Aurora B; INC, INCENP. Samples numberered as in (B).


A
none
nocod
control
ΔCPC
control
ΔCPC
extract
– + + + + – + + + +
AurB
– – FL ΔCC ΔCC∇GCN4 – – FL ΔCC ΔCC∇GCN4
INC
α-AurB
116-
97-
α-INC
α-Op18
E
1 2 3 4 5
α-AurB
200-
116-
97-
α-INC
α-Op18
B
control
ΔCPC
extract
α-INCENP
Ab added
– + + + +
AurB
– – FL ΔCC ΔCC ∇GCN4
INC
DNA
tubulin
1 2 3 4 5
C
bipolar spindles
disorganized MTs
no MTs
% structures
100
75
50
25
0
1 2 3 4 5
D
bipolar spindles disorg MTs no MTs
1 86.84 ± 5.73 11.48 ± 4.41 1.68 ± 1.36
2 0.00 ± 0.00 0.00 ± 0.00 100.00 ± 0.00
3 82.79 ± 5.37 11.15 ± 4.72 6.06 ± 4.76
4 0.00 ± 0.00 0.00 ± 0.00 100.00 ± 0.00
5 0.00 ± 0.00 0.00 ± 0.00 100.00 ± 0.00

domain may function in microtubule sensing (Figure 4-7). To test this hypothesis, I first examined the localization of the different INCENP constructs when they are expressed on top of endogenous INCENP in wild-type extract (Figure 4-16A, B). In control extract, GFP-tagged full-length INCENP enriched on chromosomes but also localized to the spindle microtubules (#2), as previously observed with endogenous INCENP (Kelly et al., 2007). Consistent with the requirement of Dasra A for the chromosomal targeting of the CPC (Kelly et al., 2007), GFP-INCENPΔDS showed reduced chromosomal localization, while still localizing to the spindle (#3). In contrast, GFP-INCENPΔCC lost microtubule localization, but maintained robust chromosomal localization (#4), consistent with my other data (Figure 4-10 and 4-12). GFP-INCENPΔCC also had a reduced ability to bind taxol-stabilized microtubules relative to full length GFP-INCENP (Figure 4-17). Therefore, in addition to its role in microtubule-induced activation of Aurora B, the CC domain targets the CPC to microtubules, which is consistent with a model of localization-coupled kinase activation (Kelly et al., 2007).

To show that the CC domain itself could target to microtubules, I expressed a GFP-tagged INCENP construct that contains only residues 491 to 747 (GFP-INCENP^{CC}). This construct localized strongly to the spindle microtubules relative to the GFP control (Figure 4-16B, C), suggesting that this domain of INCENP is sufficient to target the CPC to microtubules. Interestingly, the spindles formed in wild-type extract expressing GFP-INCENP^{CC} were longer (Figure 4-16D). This result suggests that the CC domain of INCENP can stabilize microtubules independent of its interaction with Aurora B and its affiliated kinase activity, since GFP-INCENP^{CC}, which lacks the IN-box domain, should not interact with the kinase (Kang et al., 2001; Bishop and Schumacher, 2002; Honda

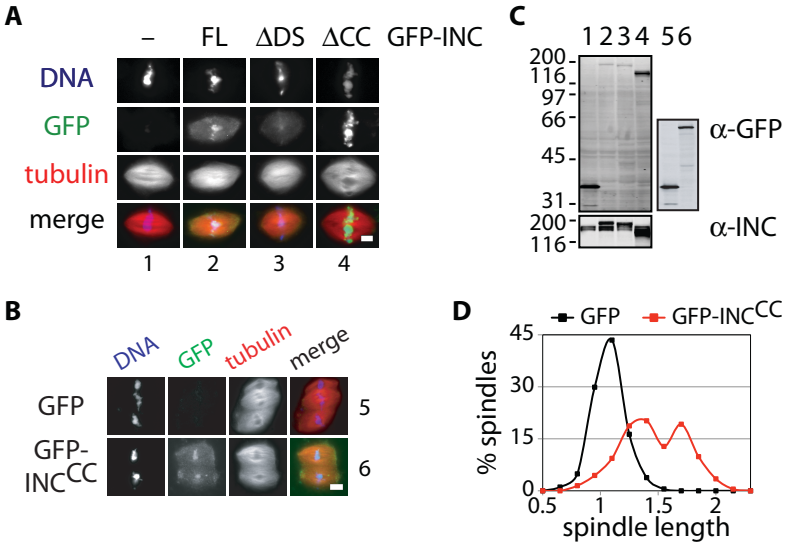


Figure 4-16. The CC domain of INCENP targets the CPC to the spindle microtubules.

(A, B) Control extracts were supplemented as indicated and cycled through interphase to metaphase with 100/μl sperm nuclei at 20°C. Samples were processed for immunofluorescence with anti-GFP (green) antibodies 60 min after entry into metaphase. DNA (blue) and tubulin (red) were visualized with Hoechst 33258 and rhodamine-labeled tubulin, respectively. Scale bar, 10 μm. INC^{CC}, the CC domain of INCENP. **(C)** Immunoblot of experiments in (A) and (B), numbered as such, with the indicated antibodies on the right. Molecular weight markers (in kD) are on the left. INC, INCENP. **(D)** A histogram of the measured spindle lengths in (B). Each spindle length was normalized to the average spindle length in the GFP population. The lengths were then binned and the number of spindles in each bin was normalized to the total number of spindles measured (greater than 200 per sample). Black, GFP sample; Red, GFP,-INC^{CC} sample.

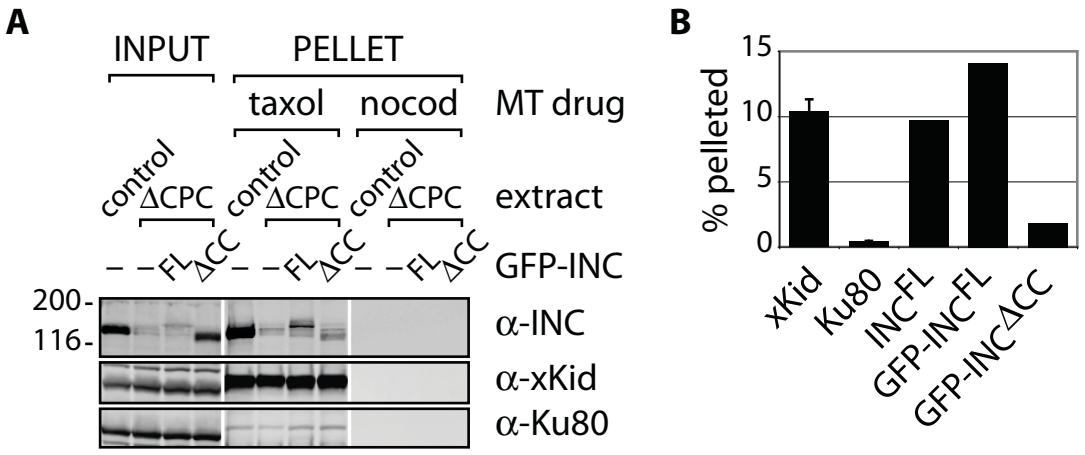


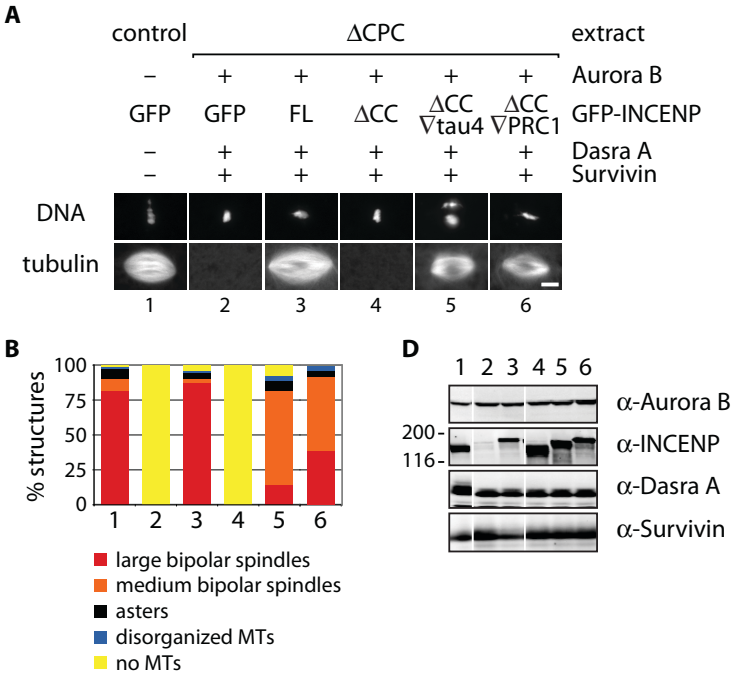
Figure 4-17. INCENPΔCC does not bind to microtubules *in vitro*.
Control or ΔCPC extracts were reconstituted as indicated and cycled through interphase to metaphase in the absence of sperm at 16°C. Samples were frozen at -80°C 90 min after entry into metaphase. The thawed samples were diluted, precleared, and incubated with 10 μM taxol or 33 μM nocodazole for 30 min. The samples were pelleted through a sucrose cushion. **(A)** The pellets were analyzed by immunoblot with the indicated antibody on the right. xKid and Ku80 are the positive and negative control for microtubule binding, respectively. **(B)** Quantification of the percent of protein that pelleted with microtubules in (A). The percent of pelleted protein was determined by normalizing the intensity of the band that pelleted with taxol-stabilized microtubules to the intensity of the band in the input. Error bars represent SEM.

et al., 2003; Sessa et al., 2005). Together, these results suggest that the CC domain of INCENP is necessary and sufficient for targeting the CPC to microtubules.

Exogenous microtubule-binding domains can replace the function of the putative coiled-coil domain of INCENP in spindle assembly

The results above suggest that the CPC-microtubule interaction, mediated by the CC domain of INCENP, is critical for spindle assembly. To test if the function of the CC domain in spindle assembly is to mediate the CPC-microtubule interaction, I created chimeric INCENPs in which a known exogenous microtubule-binding domain, flanked by flexible linkers, replaced the CC domain (Figure 4-6). If an unrelated microtubule-binding domain can replace the function of the CC domain in spindle assembly, then the central function of the CC domain is most likely to mediate the microtubule interaction. Two different microtubule-binding domains were used: tau (INCENPΔCC∇tau4) and PRC1 (INCENPΔCC∇PRC1). The microtubule-binding domain (250-375) of tau, which consists of four binding cassettes, has weak nucleation activity, but no bundling activity (Gustke et al., 1994). The microtubule-binding domain (273-621) of PRC1, which is required for stabilizing the anaphase spindle midzone, can bundle microtubules in interphase but not in metaphase (Mollinari et al., 2002).

Strikingly, both microtubule-binding INCENP chimeras (Figure 4-18, #5 and #6) supported spindle assembly in ΔCPC extract reconstituted with Aurora B, Dasra A, and Survivin. These spindles, however, were slightly shorter. Spindles in extracts containing INCENPΔCC∇tau4 and INCENPΔCC∇PRC1



C

	large bipolar spindles	med bipolar spindles	asters	disorg MTs	no MTs
1	81.87 ± 9.34	8.73 ± 1.76	6.62 ± 5.11	1.85 ± 1.55	0.93 ± 0.93
2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	100.00 ± 0.00
3	87.26 ± 5.18	2.89 ± 0.06	4.41 ± 0.62	1.78 ± 1.36	3.67 ± 3.25
4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	100.00 ± 0.00
5	14.26 ± 5.28	66.91 ± 16.75	7.79 ± 4.26	3.75 ± 3.75	7.30 ± 3.45
6	38.85 ± 6.05	52.93 ± 3.66	4.41 ± 2.66	3.18 ± 0.61	0.63 ± 0.34

Figure 4-18. Exogenous microtubule-binding domains can replace the function of the CC domain in spindle assembly.

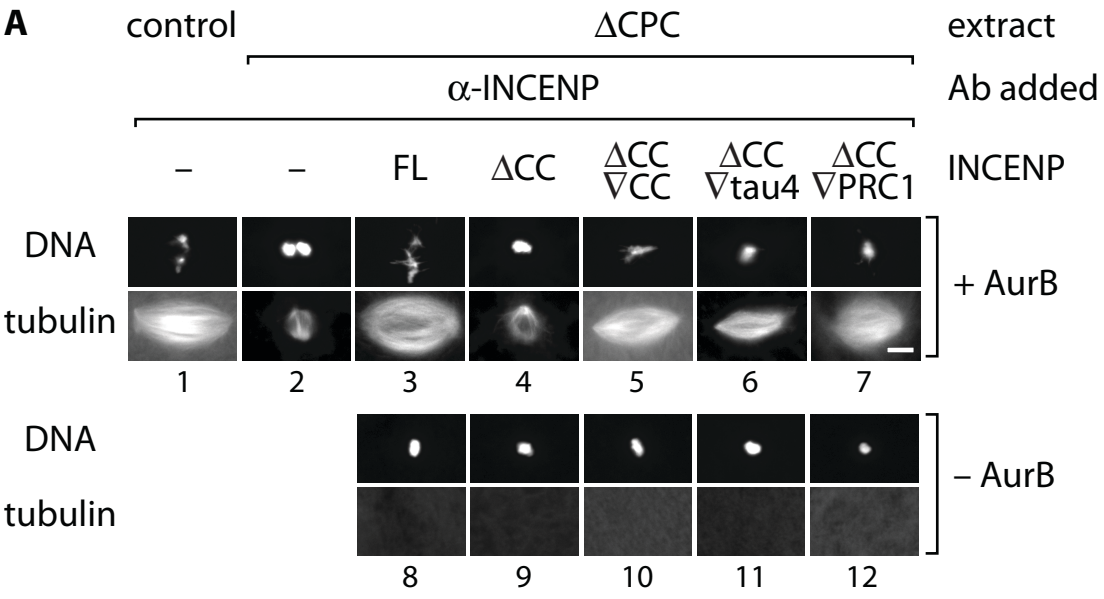
(A) Control or ΔCPC extracts reconstituted with various INCENP constructs and Aurora B, Dasra A, and Survivin were cycled through interphase to metaphase with rhodamine-labeled tubulin at 20°C. Samples were fixed 60 min after entry into metaphase. DNA was visualized with Hoechst 33258. Bar, 10 μm. **(B, C)** Quantitation and statistical analysis of chromosome-containing microtubule structures formed in the extracts described in and numbered as in (A). The average of 2 experiments with at least 250 structures counted per sample is represented. In (B), red, large bipolar spindles; orange, medium bipolar spindles; black, asters; blue, disorganized microtubules; yellow, weak/no microtubules. The numbers in (C) represent the percent of that structure ± S.E.M. **(D)** Immunoblot of the samples in (A) using the indicated antibodies on the right. Molecular weight markers (in kD) for INCENP are on the left.

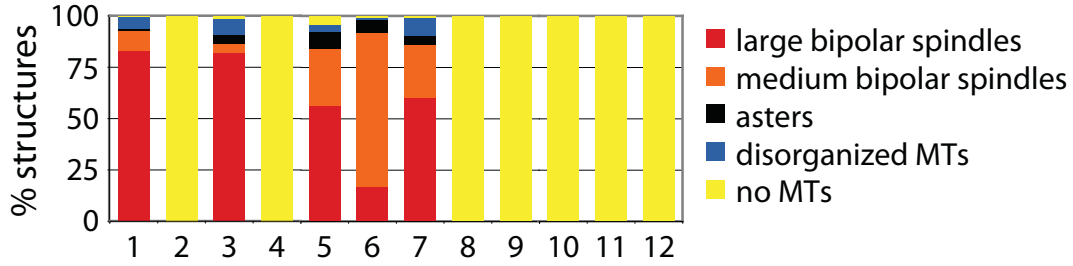
were, respectively, 61.5 ± 8.2% and 69.0 ± 5.9% (where the error represents 1 standard deviation) the length of spindles in extract containing full-length INCENP. To eliminate the possibility that this change in spindle size was due to differences in Aurora B activation, I monitored spindle assembly in ΔCPC extract reconstituted with the chimeric INCENPs, Aurora B, and 5 μg/ml anti-INCENP antibodies (Figure 4-19). As expected, while the CC domain was required for spindle assembly (#4), replacing this domain with a microtubule-binding domain (#6 and #7) rescued spindle assembly. These spindles, however, were still shorter than that of full-length INCENP. This discrepancy in spindle length is not due to insufficient Aurora B activation, since Aurora B was activated to at least the same level in these chimeric INCENP-containing extracts as that in full-length INCENP-containing extracts (Figure 4-19D). In addition to this difference in spindle length, large achromosomal microtubule structures formed in the presence of these chimeras (data not shown, but see Figure 4-21), which may explain the change in spindle length (see Discussion).

Although the microtubule-binding domains of tau and PRC1 may stabilize microtubules (Aizawa et al., 1989; Brandt and Lee, 1993; Mollinari et al., 2002), no microtubule structures were observed in the absence of Aurora B (Figure 4-19A, bottom), confirming that the microtubule assembly is still dependent on Aurora B. Furthermore, the microtubule-binding activity of the exogenous domain was well correlated with the ability to assemble spindles. While INCENPΔCC∇tau4 containing four microtubule-binding cassettes of tau was able to support spindle assembly, an INCENP chimera containing a single microtubule-binding cassette of tau (INCENPΔCC∇tau1), which has a decreased

Figure 4-19. Activating Aurora B with anti-INCENP antibodies does not rescue the spindle length in extract containing microtubule-binding INCENP chimeras.

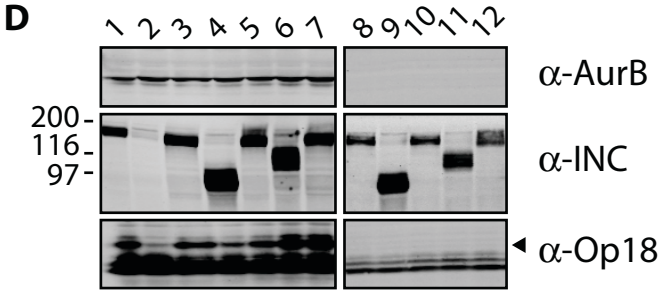
(A) Control or ΔCPC extracts were reconstituted with various INCENP constructs, 5 μg/ml anti-INCENP antibody and with or without Aurora B. The extracts were cycled through interphase to metaphase with rhodamine-labeled tubulin at 20°C. Samples were fixed 60 min after entry into metaphase. DNA was visualized with Hoechst 33258. Bar, 10 μm. **(B, C)** Quantitation and statistical analysis of chromosome-containing microtubule structures formed in the extracts described in (A). The data represents the average of 2 experiments with at least 250 structures counted per sample. In (B), red, large bipolar spindles; orange, medium bipolar spindles; black, asters; blue, disorganized microtubules; yellow, weak/no microtubules. The numbers in (C) represent the percent of that structure ± S.E.M. **(D)** Immunoblot of the samples in (A) using the indicated antibodies on the right. Molecular weight markers (in kD) for INCENP are on the left. Arrowhead, the hyperphosphorylated form of Op18. Samples are numbered as in (A).





C

	large bipolar spindles	med bipolar spindles	asters	disorg MTs	no MTs
1	83.14 ± 1.21	9.41 ± 0.56	1.30 ± 0.06	5.81 ± 1.05	0.34 ± 0.34
2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	100.00 ± 0.00
3	82.15 ± 8.02	4.25 ± 1.14	4.63 ± 3.19	7.64 ± 4.76	1.33 ± 1.06
4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	100.00 ± 0.00
5	56.28 ± 9.48	27.65 ± 8.67	8.59 ± 3.17	3.57 ± 1.54	3.90 ± 3.90
6	16.97 ± 11.83	74.65 ± 6.38	6.73 ± 5.13	1.06 ± 0.27	0.59 ± 0.59
7	60.49 ± 8.04	25.50 ± 13.31	4.49 ± 1.45	8.65 ± 7.60	0.87 ± 0.87
8-12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	100.00 ± 0.00



affinity for microtubules (see Figure 4-22)(Butner and Kirschner, 1991), did not support spindle assembly (Figure 4-20). Since two unrelated microtubule-binding domains that are of different size and structure were able to replace the function of the CC domain, it is unlikely that the primary sequence or the physical length of the CC domain is important for its function. Instead, these results show that the critical role of the CC domain of INCENP in spindle assembly is to mediate the interaction between microtubules and the CPC.

The microtubule-binding INCENP chimeras trigger Aurora B activation and microtubule polymerization in the absence of chromosomes

If the CPC-microtubule interaction leads to Aurora B activation, which in turn drives microtubule assembly, then a stochastically generated microtubule in the cytoplasm may trigger positive feedback between Aurora B and microtubules, causing further assembly of chromosome-independent microtubules. Since I observed achromosomal microtubule structures in the extracts expressing the microtubule-binding INCENP chimeras (INCENPΔCC▽tau4 and INCENPΔCC▽PRC1), I examined if these chimeras can promote such a feedback in the absence of chromosomes. Remarkably, Aurora B was activated with no inducer in the presence of these chimeras (Figure 4-21A). Unlike the case of the dimerization-induced activation by INCENPΔCC▽GCN4 (Figure 4-15A), this Aurora B activation was nocodazole-sensitive, suggesting that this inducer-free activation in the presence of these microtubule-binding INCENP chimeras resulted from the formation of microtubules. Indeed, large microtubule asters were visible in these extracts (Figure 4-21B). Since active

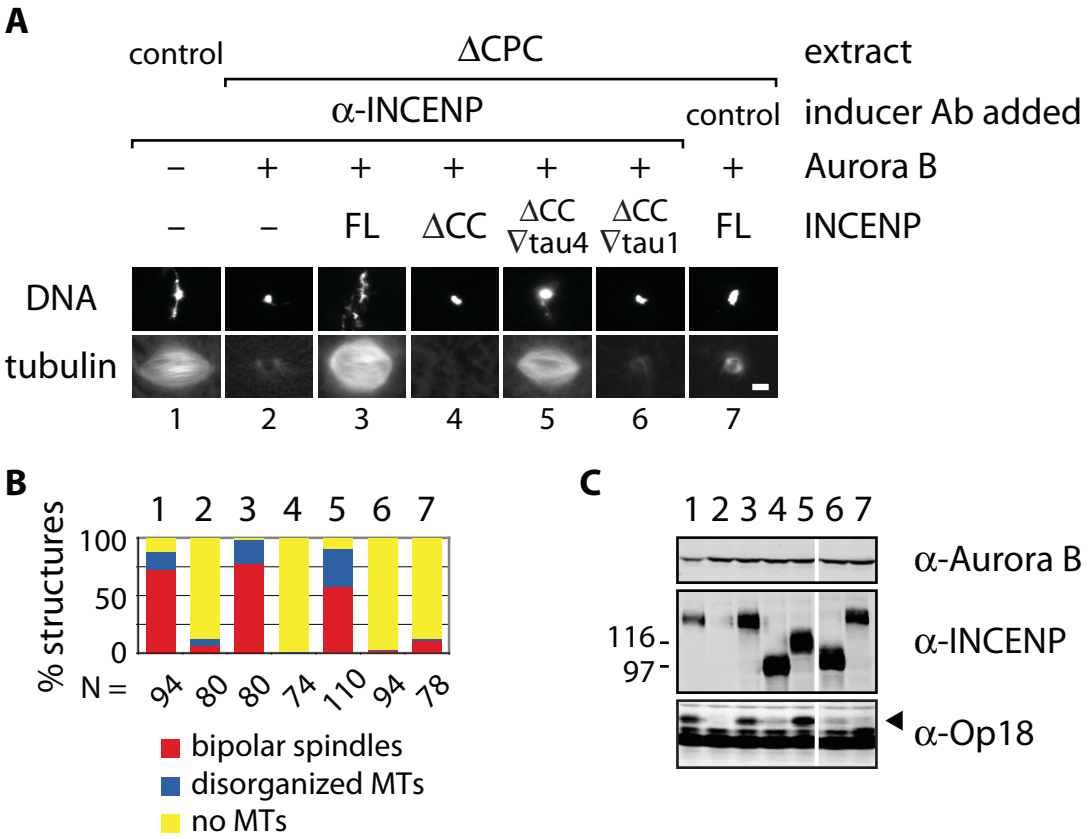


Figure 4-20. Decreasing the microtubule affinity of the microtubule-binding INCENP chimera inhibits its ability to rescue spindle assembly.

Control or ΔCPC extracts that were reconstituted as indicated with 5 μg/ml anti-INCENP antibody and were cycled through interphase to metaphase (red) at 20°C. **(A)** Samples were fixed and imaged 60 min after entry into metaphase. DNA and tubulin were visualized with Hoechst 33258 and rhodamine-labeled tubulin, respectively. Scale bar, 10 μm. **(B)** Quantitation of the chromosome-containing microtubule structures in (A). N, number of structures counted. Red, spindles; blue, disorganized microtubules; yellow, weak/no microtubules. **(C)** Immunoblot of the samples in (A) using the indicated antibodies on the right. Molecular weight markers (in kD) for INCENP are on the left. Arrowhead, the hyperphosphorylated form of Op18. Samples are numbered as in (A)

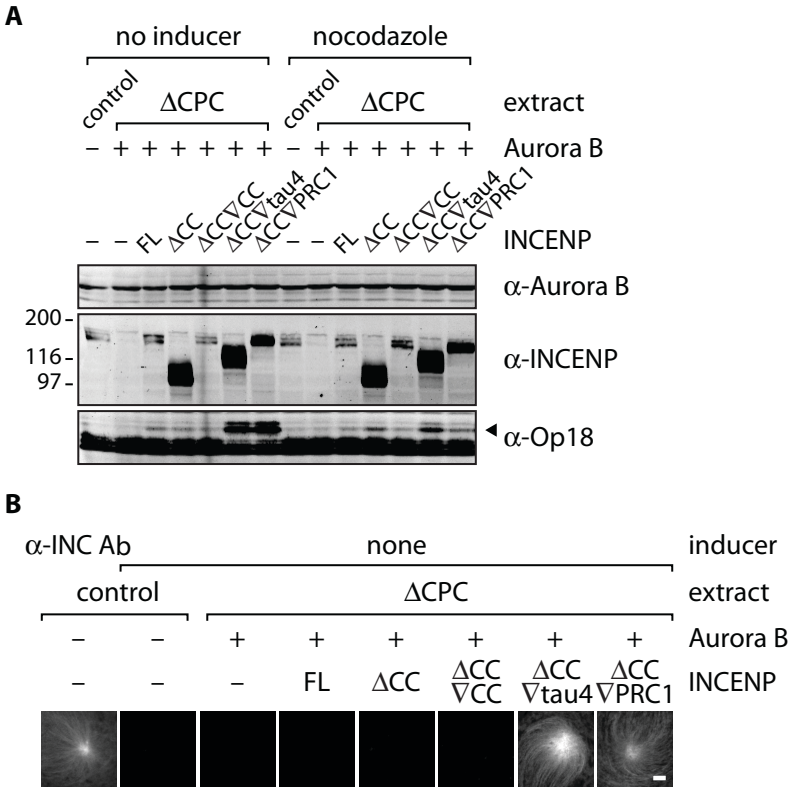


Figure 4-21. Microtubule-binding INCENP chimeras promote spontaneous activation of Aurora B and microtubule assembly.

(A) Control or ΔCPC extracts were reconstituted as indicated and incubated at 20°C for 90 min with or without 33 μM nocodazole. Samples were analyzed by immunoblot with the indicated antibodies on the right. Molecular weight markers (in kD) for INCENP are on the left. Arrowhead, the hyperphosphorylated form of Op18. **(B)** Microtubule formation was monitored with rhodomine-tubulin in extracts as prepared in (A) in the absence of nocodazole. For the anti-INCENP antibody-induced activation, the antibody was added to 100 μg/ml. Scale bar, 10 μm.

Aurora B stabilizes microtubules (Sampath et al., 2004; Kelly et al., 2007), these INCENP chimeras appeared to trigger a positive feedback loop, resulting in chromosome-independent microtubule assembly. These results suggest that while the interaction between Aurora B and microtubules can potentially promote spontaneous microtubule assembly via positive feedback, the endogenous CPC-microtubule interaction is normally ineffective and does not promote microtubule assembly in the absence of chromosomes.

INCENP and the microtubule-binding chimeras target to spindle microtubules differently

Replacing the endogenous CC domain of INCENP with an exogenous microtubule-binding domain from tau or PRC1 appears to trigger the positive feedback between Aurora B activation and microtubule polymerization independent of chromosomes. One explanation for this phenotype is a difference in microtubule-binding affinity between the chimeras and the wild-type INCENP. To test if these chimeras bind to microtubules with a higher affinity than wild-type INCENP, I attempted to purify these microtubule-binding domains recombinantly from bacteria to perform *in vitro* microtubule pelleting assays. I, however, was unable to produce appreciable amounts of any protein because the recombinant proteins were highly unstable and proteolytically degraded (data not shown). As an alternative, I determined if the microtubule-binding chimeras targeted to spindle microtubules more robustly than wild-type INCENP (Figure 4-22A, C). I expressed GFP-tagged constructs in wild-type extracts that contain a full complement of endogenous CPC. Congruent with the data shown in Figure 4-16, the GFP-tagged full-length INCENP localized to both

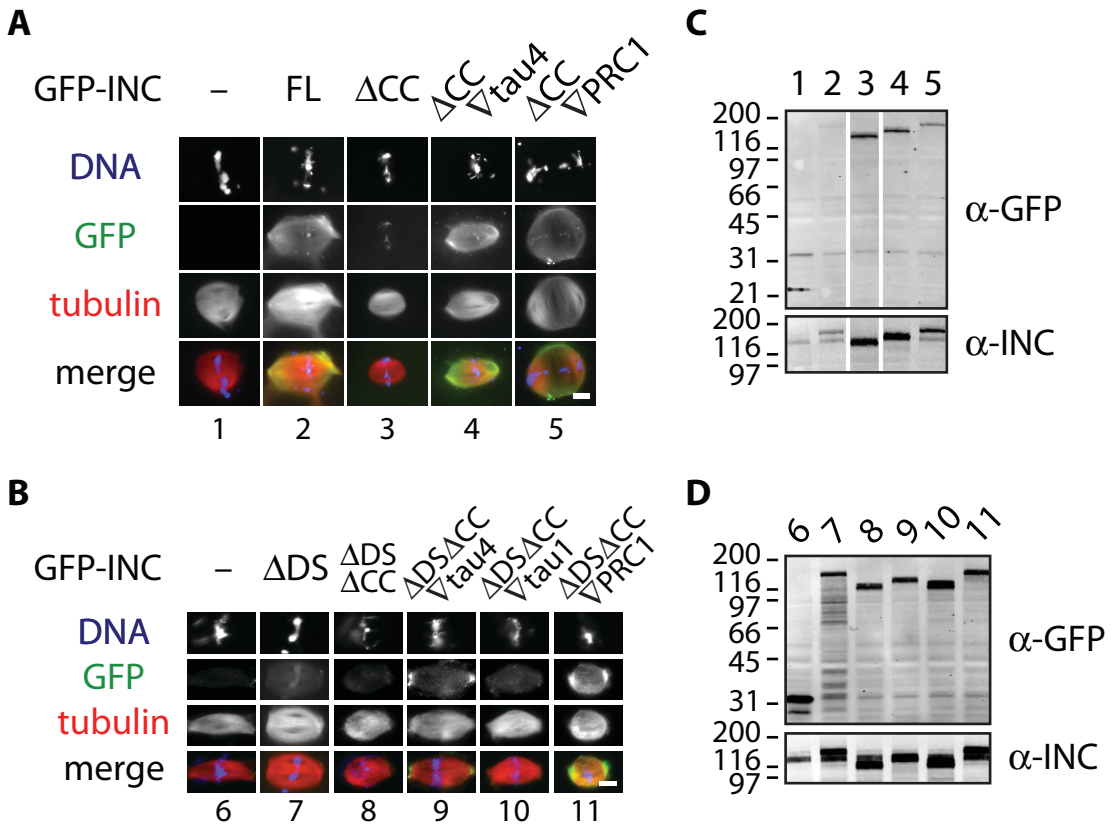


Figure 4-22. The microtubule-binding INCENP chimeras localize to spindle microtubules.

(A, B) Control extracts were supplemented as indicated and cycled through interphase to metaphase with 100/μl sperm nuclei at 20°C. Samples were processed for immunofluorescence with anti-GFP (green) antibodies 60 min after entry into metaphase. DNA (blue) and tubulin (red) were visualized with Hoechst 33258 and rhodamine-labeled tubulin, respectively. Scale bar, 10 μm. INC^{CC}, the CC domain of INCENP. **(C, D)** Immunoblot of samples in (A) and (B), numbered as such, with the indicated antibodies on the right. Molecular weight markers (in kD) are on the left. INC, INCENP.

chromosomes and spindle microtubules (#2), while INCENPΔCC localized only to the chromosomes (#3). As expected from the spindle rescue phenotype, the microtubule-binding INCENP chimeras localized to both chromosomes and microtubules (#4 and #5). Based on the immunofluorescence staining of GFP, however, these chimeras did not qualitatively target to microtubules appreciably more than wild-type INCENP. This result suggests that the microtubule affinity of INCENPΔCC∇tau4 and INCENPΔCC∇PRC1 is not grossly greater than that of wild-type INCENP. The effects of these constructs on the spindle length, however, complicate this interpretation. Expression of GFP-INCENP, GFP-INCENPΔCC∇tau4, and GFP-INCENPΔCC∇PRC1 over endogenous INCENP qualitatively appeared to slightly increase spindle length, while expression of GFP-INCENPΔDS and GFP-INCENPΔCC appeared to decrease spindle length (Figure 4-16A and 4-22A). Since the quantitative effect of these constructs on spindle length was not evaluated, whether these constructs all produced the same change in length is unknown. This change in spindle length, which obviously affects the amount of microtubules in a spindle, may have affected the targeting of the INCENP constructs. Therefore, whether the microtubule-binding INCENP chimeras bind to microtubules better than wild-type INCENP is not clear from this experiment.

In an attempt to remedy this problem, I hypothesized that this change in spindle length is a consequence of the chromosomal targeting. I, therefore, repeated the previous experiment with INCENP constructs that lack the DS domain and do not target to chromosomes (see Figure 4-16). While this manipulation did not fully relieve the spindle length issue, it did highlight

differences in microtubule binding among these constructs. GFP-INCENPΔDSΔCC∇tau4 and GFP-INCENPΔDSΔCC∇PRC1 enriched on the spindle poles (#9 and #11), while GFP-INCENPΔDS did not (#7). In addition, the localization of both chimeric constructs within the spindle was much more speckled than that of GFP-INCENPΔDS. For INCENPΔDSΔCC∇tau4, this speckled, spindle pole enriched localization was dependent on microtubule binding, as the localization of GFP-INCENPΔDSΔCC∇tau1 was less speckled and not enriched at the spindle poles (#10). Together, these results suggest that the microtubule-binding INCENP chimeras bind to microtubules in a manner different from wild-type INCENP containing the CC domain. It is tempting to speculate that this difference in localization reflects an increased binding to microtubule ends in the chimeras relative to wild-type INCENP, which may account for the chromosome-independent triggering of the positive feedback loop in the presence of these chimeras.

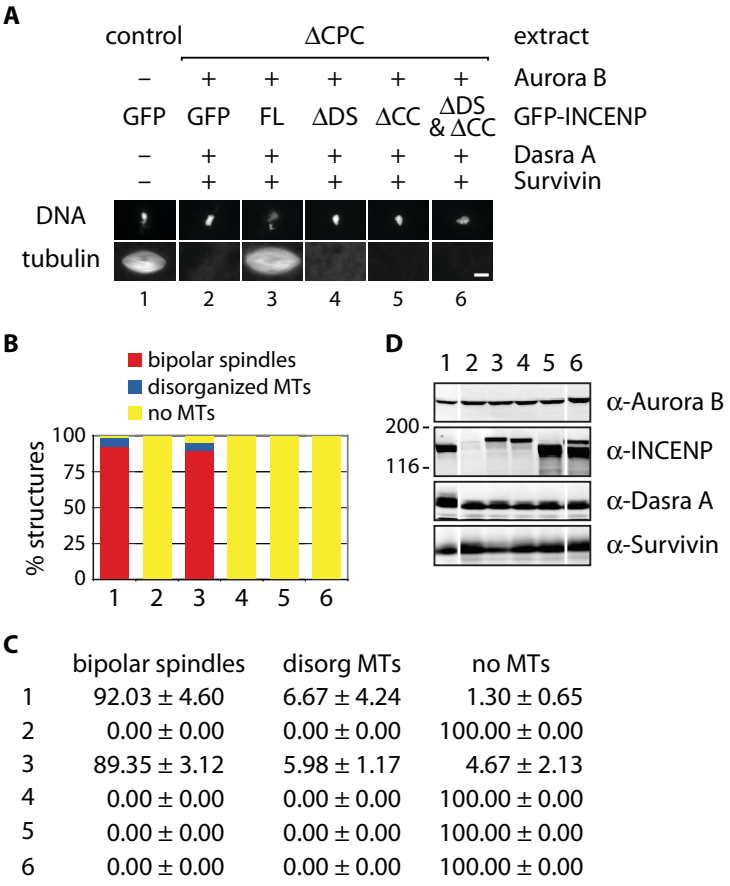
Active Aurora B must interact with microtubules to trigger spindle assembly

These results show that the chromosome-induced Aurora B activation and the CPC-microtubule interaction are both required for spindle assembly. Can these two functions of the CPC support spindle assembly even when they are spatially or temporally unlinked? Uncoupling of the two functions would be possible if Aurora B is first activated on chromosomes to promote an initial step of microtubule formation, and then the CPC-microtubule interaction subsequently promotes a second Aurora B-independent step in spindle assembly. Since INCENPΔCC can be activated by chromosomes but cannot

interact with microtubules and INCENPΔCEN can interact with microtubules but cannot be activated by chromosomes, the co-expression of these constructs should be able to support spindle assembly in such a sequential model. In contrast, if these functions must be physically linked together, co-expression of these proteins would still fail to support spindle assembly. Consistent with the latter hypothesis, reconstituting ΔCPC extract with both INCENPΔCEN and INCENPΔCC together with Aurora B, Dasra A and Survivin failed to rescue spindle assembly (Figure 4-23, #6). This result suggests that the CPC carrying chromosomally activated Aurora B must interact with microtubules to promote spindle assembly (Figure 4-24 and 4-25A) and emphasizes the importance of detecting chromosomes and microtubules by a single molecule of the CPC within a confined space and time in the initial steps of spindle assembly.

Discussion

This study demonstrates the importance of the previously overlooked interaction between the CPC and spindle microtubules in pre-anaphase stages of M-phase. While previous studies have shown the enrichment of the CPC on centromeres and chromosomes during these mitotic stages (Ruchaud et al., 2007), I have demonstrated that the CPC interacts with spindle microtubules during metaphase and that this interaction is required for spindle assembly. Furthermore, these results reveal that activated Aurora B must be linked to microtubules to trigger spindle assembly, which is suggested to aid in the spatial restriction of spindle assembly to the vicinity of chromosomes.



C

	bipolar spindles	disorg MTs	no MTs
1	92.03 ± 4.60	6.67 ± 4.24	1.30 ± 0.65
2	0.00 ± 0.00	0.00 ± 0.00	100.00 ± 0.00
3	89.35 ± 3.12	5.98 ± 1.17	4.67 ± 2.13
4	0.00 ± 0.00	0.00 ± 0.00	100.00 ± 0.00
5	0.00 ± 0.00	0.00 ± 0.00	100.00 ± 0.00
6	0.00 ± 0.00	0.00 ± 0.00	100.00 ± 0.00

Figure 4-23. The DS and CC domains of INCENP must be physically linked to promote spindle assembly.

Control or ΔCPC extracts that were reconstituted as indicated and were cycled through interphase to metaphase at 20°C. Samples were fixed 60 min after entry into metaphase. DNA and tubulin were visualized with Hoechst 33258 and rhodamine-labeled tubulin, respectively. Bar, 10 μm. **(B, C)** Quantitation and statistical analysis of chromosome-containing microtubule structures formed in the extracts described in (A). The data represents the average of 3 experiments with at least 250 structures counted per sample. In (B), red, spindles; blue, disorganized microtubules; yellow, weak/no microtubules. The numbers in (C) represent the percent of that structure ± S.E.M. **(D)** Immunoblot of the samples in (A) using the indicated antibodies on the right. Molecular weight markers (in kD) for INCENP are on the left. Samples are numbered as in (A).

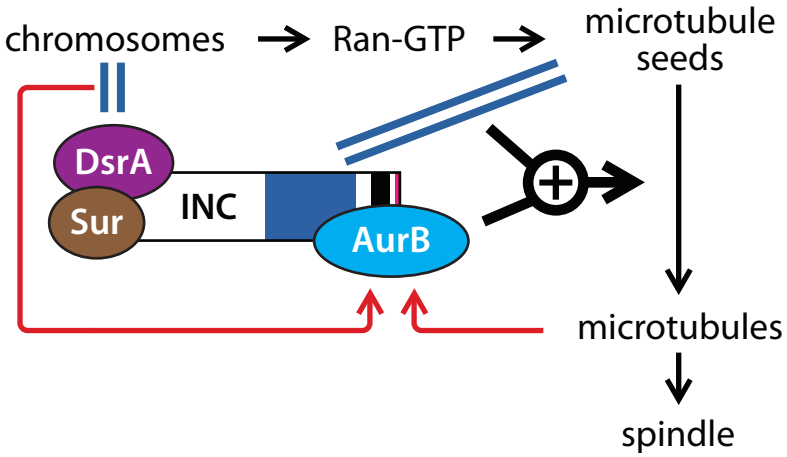


Figure 4-24. Chromosomally activated Aurora B must interact with microtubules to drive spindle assembly.

The model with emphasis on requiring both chromosomes (via activation of Aurora B) and microtubules. The CPC with chromosomally activated Aurora B must detect emerging microtubules, which are nucleated by the Ran-GTP pathway, near chromosomes. Dark blue double lines, protein-protein interactions; red single-headed arrow, Aurora B activation; plus sign, "AND" gate; AurB, Aurora B (deep blue); INC, INCENP (green in B); DsrA, Dasra A (purple); Sur, Survivin (brown).

Modulation of the CPC-microtubule interaction

The CPC can interact with both chromosomes and microtubules, but its relative affinities for these structures changes dramatically at the metaphase to anaphase transition, when the chromosome-bound CPC is relocalized to the spindle midzone (Ruchaud et al., 2007). Although the enrichment of the CPC on microtubules is significantly weaker than that on chromosomes before anaphase, these results show that this relatively weak interaction contributes to spindle assembly and may be important for restricting microtubule assembly near chromosomes. These results demonstrate that the CC domain can be functionally replaced with an exogenous microtubule-binding domain, implicating the interaction between the CC domain and microtubules as an essential step in spindle assembly.

The differences between extracts containing wild-type INCENP and the microtubule-binding INCENP chimeras, however, highlight the importance of the proper adjustment of the CPC-microtubule interaction. If INCENP does not bind microtubules, no spindle forms. If an exogenous microtubule-binding domain, like that from tau or PRC1, mediates the CPC-microtubule interaction in place of the endogenous domain, microtubules assemble in a chromosome-independent context by triggering a positive feedback loop between Aurora B and microtubules. These chromosome-independent microtubules may reduce the concentration of free tubulin available for spindle assembly, which may explain the shorter spindles that form in the presence of these INCENP chimeras (Figure 4-18 and 4-19). It is tempting to speculate from the localization differences of these INCENP constructs on spindle microtubules (Figure 4-22) that the chromosome-independent emergence of microtubules in the presence of the

chimeras is due to an increase in microtubule affinity. Further mechanistic understanding of the CPC-microtubule interaction is needed to reveal how the triggering of the potential positive feedback loop is suppressed in the absence of chromosomes.

The microtubule structure sensed by the CPC

These results suggest that through this weak CPC-microtubule interaction, microtubules in the physiological spindle activate Aurora B (Figure 4-3 and 4-4). Indeed, data from Lei Tan in the laboratory of Tarun Kapoor shows Aurora B-dependent phosphorylation of a substrate on the mitotic spindle microtubules of mammalian cells (Tseng et al., in submission). Not all microtubules, however, seem to be able to activate Aurora B. Microtubules formed from the addition of a Ran mutant that is constitutively in the GTP-bound state (RanDM) failed to activate Aurora B on its own, but this treatment could contribute to Aurora B activation in a MCAK-depleted background (Figure 4-4). Two possible explanations for this result are discussed below.

First, RanDM did not stimulate enough polymerized microtubules to activate Aurora B. The addition of taxol or the depletion of MCAK promotes gross polymerization of microtubules (Schiff et al., 1979; Walczak et al., 1996), while the addition of RanDM promotes a less robust formation of microtubules (data not shown). However, it seems unlikely that these microtubules do not activate Aurora B, since in extract depleted of MCAK, there is an increase in Op18 hyperphosphorylation upon addition of RanDM (Figure 4-4), suggesting that RanDM does stabilize enough microtubules to activate Aurora B. The level of RanDM-induced Aurora B activation appears to be lower than that of MCAK

depletion. In concert with the soluble cytoplasmic substrate read-out that I am using (hyperphosphorylation of Op18), this reduced level of Aurora B activation, and Op18 hyperphosphorylation, may fall within the noise of the assay and be viewed as not activated.

The second possibility, which is more speculative, is that a specific microtubule structure activates Aurora B. Since taxol-stabilized microtubules may differ in structure from physiologically formed microtubules (Andreu et al., 1992), one possibility is that the physiological microtubules formed in the presence of RanDM cannot activate Aurora B. This situation is unlikely, however, since physiologically formed microtubules in the absence of MCAK can activate Aurora B. One difference between the microtubules formed by MCAK depletion and that formed by RanDM addition is the presence of anti-parallel microtubules. GTP-bound Ran induces the formation of microtubule asters in *Xenopus* egg extracts (Ohba et al., 1999; Wilde and Zheng, 1999). While microtubule asters can interact to produce achromosomal bipolar spindle-like structures with anti-parallel microtubules (Wilde and Zheng, 1999), I generally did not see such structures in the RanDM-containing extract (data not shown) and assume, therefore, that in my experiments, RanDM did not induce anti-parallel microtubule formation. In the MCAK-depleted extract, however, large clusters of interacting microtubule asters are induced, which presumably contain anti-parallel microtubules. This difference in microtubule structure may account for the difference in Aurora B activation. Furthermore, since addition of RanDM to MCAK-depleted extracts appears to increase the amount of aster clustering (data not shown), this anti-parallel microtubule-based theory would explain why

RanDM activates Aurora B in the MCAK-depleted background but not on its own.

Activation of Aurora B by anti-parallel microtubules is an appealing hypothesis. The *Xenopus* spindle contains a large array of overlapping anti-parallel microtubules (Danuser et al., 2000; Tirnauer et al., 2004; Mitchison et al., 2005; Yang et al., 2007; Yang et al., 2008), which could account for the general spindle localization of the CPC (Figures 4-10 and 4-16). In addition, since microtubules overlap at the chromosome-containing metaphase plate, where Aurora B is already localized, the specific recognition of anti-parallel microtubules by the CPC may play a role in the initiation of spindle assembly (see below) or in the spindle assembly checkpoint (see the Discussion in Chapter 5).

CPC-microtubule interaction before and after anaphase onset

The mechanism that regulates the CPC-microtubule interaction seems to be different before and after anaphase onset. In anaphase, Dasra and Survivin are required for the proper localization of the CPC to the central spindle (Lens et al., 2006; Jeyaprakash et al., 2007; Yue et al., 2008). In contrast, the CC domain of INCENP, and not Dasra and Survivin, mediates the CPC-microtubule interaction in metaphase (Figure 4-16). Furthermore, in anaphase, Cdc14 dephosphorylates Sli15, the *S. cerevisiae* INCENP homologue, which relocalizes the protein to the central spindle (Pereira and Schiebel, 2003). Since Cdc14 is activated in anaphase (Shou et al., 1999), such a mechanism cannot play a role in the spindle localization of INCENP in metaphase. Consistent with this difference in the molecular requirements for spindle localization between the pre- and post-

anaphase stages of M-phase, the CPC is uniformly localized to the entire pre-anaphase spindle (Figure 4-16A), in comparison to just localizing to the central spindle during anaphase (Ruchaud et al., 2007).

Function of the CPC-microtubule interaction in spatially limiting spindle assembly to the vicinity of chromosomes

Previous models explaining localized spindle assembly focused on a chromosome-based gradient of active effectors (Figure 1-2)(Caudron et al., 2005; Bastiaens et al., 2006; Athale et al., 2008). This model in and of itself however, fails to accommodate our results showing that the CPC-microtubule interaction is required for triggering spindle assembly and that simple phosphorylation of substrates by activated Aurora B near chromosomes is insufficient for the CPC to execute its role in spindle assembly. Here I propose a modified reaction-diffusion mechanism, in which the CPC must detect two structures, chromosomes and microtubules (Figure 4-24). My data suggests that chromosomally activated Aurora B must be targeted to emerging microtubules via the CPC-microtubule interaction. Moreover, the data also suggest that the nature of the CPC-microtubule interaction must be adjusted so that a positive feedback between Aurora B and microtubules is inhibited in the absence of chromosomes. Taken together, I propose a two-step model for the role of the CPC in spindle assembly (Figure 4-25). First, in the initial steps of spindle assembly, the CPC with chromosomally activated Aurora B must detect emerging microtubules near chromosomes (Figure 4-25A). Second, subsequent to this initial set-up of the spindle, an active Aurora B is targeted to the spindle microtubules to promote microtubule assembly farther from the chromosomes (Figure 4-25B).

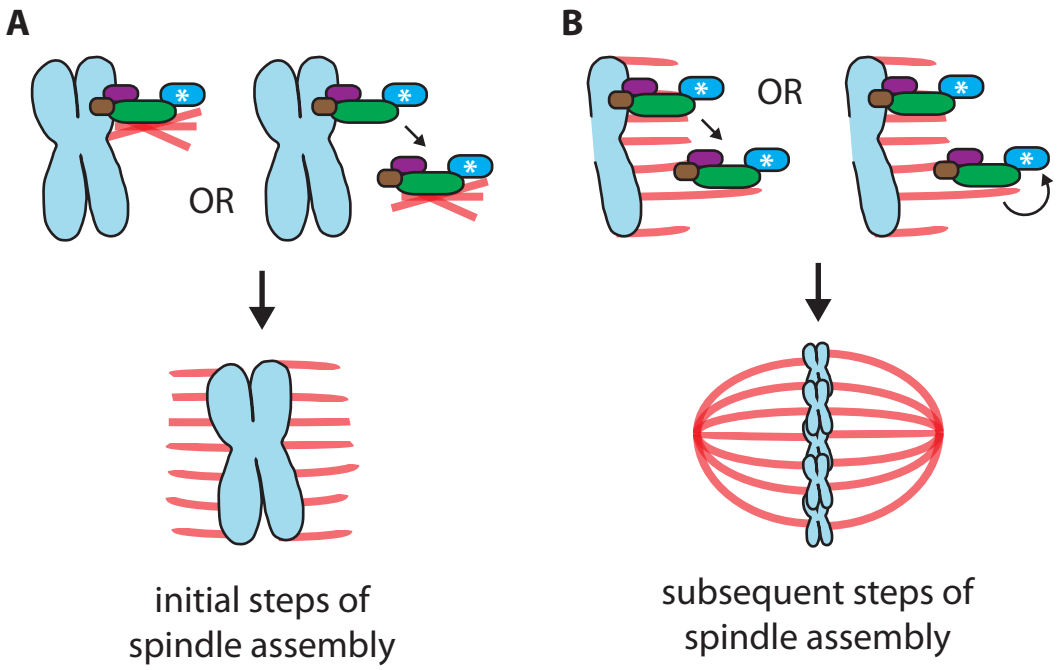


Figure 4-25. Spatial and temporal possibilities for the dual detection of chromosomes and microtubules by the CPC.

(A) In the initial steps of spindle assembly, chromosomally activated Aurora B must be targeted to the microtubules. The CPC may coincidently detect both structures (left). Alternatively, the CPC may be activated on chromosomes and then transferred to the emerging microtubules near the chromosomes (right). **(B)** Once microtubules have started to polymerize, the CPC may stabilize microtubules farther from the chromosomes by targeting active Aurora B to the microtubules. Either the CPC containing chromosomally activated Aurora B is transferred from chromosomes to the microtubules (left), or the CPC with inactive Aurora B targets to microtubules and the Aurora B is subsequently activated by the microtubules (right). Blue oval with asterisk, activated Aurora B; green oval, INCENP; purple oval, Dasra; and brown oval, Survivin.

Why is the CPC-microtubule interaction needed to initiate spindle assembly? I propose that detecting the coincident presence of chromosomes and emerging microtubules by the CPC is key in turning on spindle assembly around chromosomes in the initial steps of spindle assembly (Figure 4-25A). Although sparse sporadic microtubules could form in the cytoplasm, Aurora B is not activated in the absence of an inducer (Figures 4-2 and 4-21), suggesting that a functional CPC-microtubule interaction cannot be established in the cytoplasm. On chromosomes, the Ran-GTP pathway and the Aurora B pathway are independently activated (Sampath et al., 2004; Walczak and Heald, 2008). As Ran-GTP triggers microtubule nucleation, the avidity between the CPC and the locally nucleated microtubules increases enough to target activated Aurora B to the microtubule seeds, a process that is critical for bipolar spindle formation. Due to the proximity of the chromosomes and these emerging microtubules, I suggest that these two structures are coincidently detected by the CPC (Figure 4-25A, left). The physical bridging of chromosomes and microtubules by the CPC, however, is not required to support spindle formation, since artificial activation of Aurora B by anti-INCENP antibodies can bypass the necessity for the chromosome-CPC interaction (Figure 4-13)(Kelly et al., 2007). Therefore, it is possible that chromosomally activated Aurora B is transferred off chromosomes and onto microtubules (Figure 4-25A, right). This dissociation from chromosomes and re-association with microtubules should occur on microtubules adjacent to chromosomes to restrict microtubule assembly near chromosomes, as extract containing cytoplasmically activated Aurora B assemble spindles and microtubule asters independent of chromosomes (Kelly et al., 2007). I propose that with either method of targeting, the interaction of the CPC with

active Aurora B and microtubules must occur within a confined space and time for spindle assembly.

Functions of the CPC-microtubule interaction farther from chromosomes

Subsequent to this initial requirement for the interaction of chromosomally activated Aurora B and microtubules, the CPC-microtubule interaction may function in promoting microtubule polymerization farther from chromosomes. Since my data show that the CPC is localized to microtubules in an established bipolar spindle and suggest that this localization affects spindle length (Figure 4-16), I propose that subsequent to the aforementioned initial step, a small population of the CPC is targeted to microtubules to trigger the positive feedback loop, which aids in microtubule assembly farther from chromosomes and contributes to spindle elongation or maintenance (Figure 4-25B). Similar to the transfer of chromosomally activated Aurora B to microtubules in the initiation step of spindle assembly, this population of microtubule-bound CPC could have originated on chromosomes and contain chromosomally activated Aurora B (Figure 4-25B, left). Alternatively, the cytoplasmic pool of CPC may bind to the spindle and Aurora B may be activated by the spindle microtubules (Figure 4-25B, right).

Recently, the importance of microtubule nucleation within the spindle has been reported (Luders et al., 2006; Clausen and Ribbeck, 2007; Goshima et al., 2008; Zhu et al., 2008). Furthermore, most of the plus-ends of non-kinetochore microtubules are not attached to chromosomes, and they can be frequently found within a spindle (Tirnauer et al., 2004). To counteract the microtubule depolymerizing activities (e.g., MCAK) distal to chromosomes during spindle

elongation, it may be critical to target active Aurora B at those microtubules nucleated within a spindle to effectively phosphorylate critical microtubule-bound substrates. For instance, since MCAK binds to microtubule ends and processively promotes rapid microtubule depolymerization there (Hunter et al., 2003), activated Aurora B may have to be targeted to this specific population of MCAK to inhibit it. A more careful analysis of the possible roles of the CPC-microtubule interaction in spindle microtubule density is required.

Targeting active Aurora B to microtubules to promote spindle assembly

How does the CPC-microtubule interaction promote the initial steps of spindle assembly? Artificial stimulation of the kinase activity of Aurora B by the clustering antibodies bypassed the requirement for the CPC-chromosome interaction, but not for the CPC-microtubule interaction. Therefore, although microtubules have a capacity to activate Aurora B, I suggest that the role of the CPC-microtubule interaction for the initial steps of spindle assembly is not to activate Aurora B, but instead to target chromosomally activated Aurora B to emerging microtubules. While Aurora B can phosphorylate some of its substrates independent of its microtubule localization, targeting of active Aurora B to microtubules may be required for the effective phosphorylation of critical substrates that function on microtubules. Alternatively, the microtubule-targeting domain may harbor a kinase-independent function that affects microtubule dynamics. In either case, this function of the CC domain still must be physically linked to the activated Aurora B to promote microtubule assembly (Figure 4-24). As stated above, I propose that this requirement for the initial

coincidence detection of chromosomes and microtubules by the CPC is a key mechanism to turn on spindle assembly around, and only around, chromosomes.

CHAPTER 5. THE ROLE OF THE PUTATIVE COILED-COIL DOMAIN OF INCENP IN THE SPINDLE ASSEMBLY CHECKPOINT

Introduction

The proper segregation of chromosomes during M-phase is critical for genomic integrity. One key component in this process is the biorientation of chromosomes such that sister chromatids, via their kinetochores, attach to microtubules from opposite poles of the spindle. Monitoring this biorientation of kinetochores is the spindle assembly checkpoint. Through a highly conserved signal transduction pathway, this checkpoint functions to prevent anaphase onset until all the chromosomes are properly attached to the spindle (Lew and Burke, 2003). Whether this checkpoint ultimately senses the attachment status of the kinetochores or the tension created across attached kinetochores remains controversial (Pinsky and Biggins, 2005). Tension across kinetochores, however, does appear to be sensed by the chromosomal passenger complex (CPC), which destabilizes microtubules attached to kinetochores that lack tension (Tanaka et al., 2002; Lampson et al., 2004; Pinsky et al., 2006). How tension is converted into a signal that regulates the CPC is unclear (Kelly and Funabiki, 2009).

In metaphase, the CPC, composed of the kinase Aurora B, INCENP, Dasra (also known as Borealin), and Survivin (see Figure 1-4), is enriched at centromeres (Ruchaud et al., 2007). One of the centromeric functions of the CPC is to correct improperly attached microtubules. Aurora B is proposed to negatively regulate microtubule attachment by phosphorylating components of the outer kinetochore that interact with microtubules (Cheeseman et al., 2006; DeLuca et al., 2006). In addition, Aurora B regulates the centromeric localization

and activity of MCAK, a major microtubule-destabilizing enzyme (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004), but how this regulation relates to the error correction mechanism of the CPC is unclear. In addition to error correction, the centromeric CPC, via active Aurora B, recruits multiple spindle assembly checkpoint proteins to the kinetochore (Ditchfield et al., 2003; Hauf et al., 2003; Lens et al., 2003; Vigneron et al., 2004; Famulski and Chan, 2007). This recruitment of checkpoint proteins appears to be independent of the attachment status of the kinetochores, since BubR1 is recruited to kinetochores in mammalian cells treated with nocodazole, a microtubule-destabilizing drug, in an Aurora B activity-dependent manner (Ditchfield et al., 2003; Hauf et al., 2003). Both the correction of improperly attached kinetochore microtubules and the recruitment of checkpoint proteins are dependent on Aurora B kinase activity. While the CPC may have additional roles in the spindle assembly checkpoint (Vader et al., 2007), whether any function of the CPC in the checkpoint is independent of Aurora B kinase activity is unclear.

In this chapter, I will discuss the implications of a preliminary result showing that the CC domain of INCENP is required for the spindle assembly checkpoint in *Xenopus* egg extract. This result is consistent with published results from mammalian cells showing that the CC domain is required for the spindle assembly checkpoint but not the error correction mechanism of the CPC (Vader et al., 2007). Data from my work investigating the role of the CC domain in spindle assembly (see Chapter 4) suggest that the CC domain may function as a microtubule-binding domain in the spindle checkpoint. Furthermore, I propose that the function of CC domain is independent of the Aurora B kinase activity in

the spindle assembly checkpoint, similar to its suggested function in spindle assembly.

Results

The putative coiled-coil domain of INCENP is required for the spindle assembly checkpoint

In Chapter 4, I showed that the required function of the putative coiled-coil (CC) domain of INCENP in spindle assembly is to interact with microtubules. The CC domain of INCENP has also been shown to be critical for the taxol-induced spindle assembly checkpoint in mammalian cells (Vader et al., 2007). Since there are organismal differences in the role of CPC in the spindle assembly checkpoint (Biggins and Murray, 2001; Kallio et al., 2002; Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003; Lens et al., 2003; Petersen and Hagan, 2003)(see Differences between Xenopus laevis and other model organisms in Chapter 6), I tested if this domain is required for the spindle checkpoint in the *Xenopus* egg extract system, using a mutant of INCENP that lacks the CC domain (INCENPΔCC; Figure 4-6). I immunodepleted the endogenous CPC from egg extracts (ΔCPC) using anti-INCENP antibodies and reconstituted these extracts with Aurora B, Dasra A, Survivin, and full-length or mutant INCENP (Figure 5-1). The extract was then incubated with a high concentration of sperm chromosomes, nocodazole, and calcium to initiate the spindle checkpoint (Minshull et al., 1994). Two assays were used to determine if the spindle checkpoint was active. First, since cyclin B is degraded upon exit from mitosis (Murray and Kirschner, 1989), I monitored the degradation of cyclin

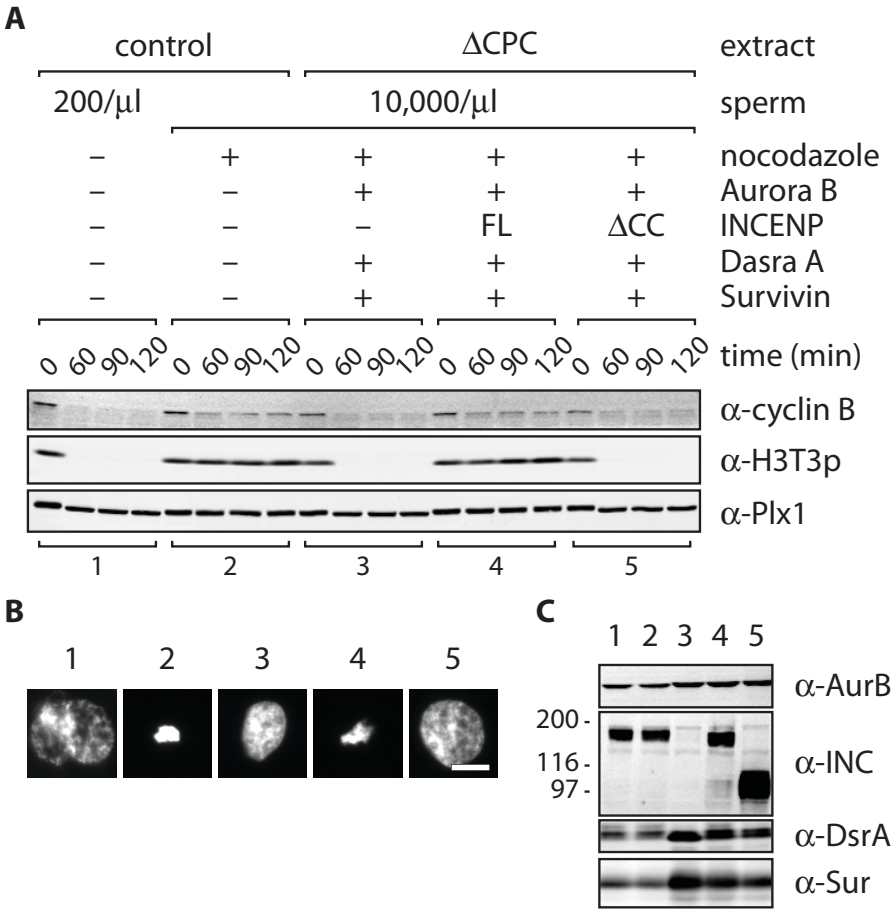


Figure 5-1. The CC domain of INCENP is required for the spindle assembly checkpoint.

Control or ΔCPC extract was reconstituted as indicated and cycled from interphase to metaphase with 200/μl sperm nuclei at 16°C. At 90 min after entry into metaphase, 33 μM nocodazole and 10,000/μl sperm nuclei were added to samples 2-5. All samples were incubated for an additional 70 min. The extract was then released into interphase and samples were taken every 30 min for 2 hr. **(A)** Samples were analyzed by immunoblot using the indicated antibodies on the right. H3T3p, anti-phospho histone H3 threonine 3. The time course represents the time after release into interphase. **(B)** At 90 min, the samples were fixed and imaged using Hoechst 33258 to visualize the DNA. Scale bar, 10 μm. **(C)** Immunoblot of the 0 time point in (A) with the indicated antibodies on the right. Molecular weight markers (in kD) for INCENP are on the left. AurB, Aurora B; INC, INCENP; DsrA, Dasra A; Sur, Survivin. Samples are numbered as in (A).

B by immunoblot. In addition, I monitored the phosphorylation of threonine 3 on histone H3 (H3T3), which is phosphorylated only in mitosis (Polioudaki et al., 2004; Dai et al., 2005). Second, I monitored the nuclear morphology. Consistent with previously published data (Kallio et al., 2002), extract lacking the CPC did not arrest in metaphase in the presence of nocodazole, as evidenced by the degradation of cyclin B, dephosphorylation of H3T3, and a swollen nucleus (Figure 5-1A, B; #3). While full-length INCENP supported an active spindle assembly checkpoint (#4), INCENPΔCC failed to hold the checkpoint (#5). These preliminary results show that, analogous to its role in mammalian cells (Vader et al., 2007), the CC domain of INCENP is required for a functional spindle assembly checkpoint in the presence of nocodazole in *Xenopus* egg extract.

Discussion

The CC domain as a microtubule-interaction domain in the spindle assembly checkpoint

This preliminary experiment shows that the CC domain of INCENP, in addition to its role in spindle assembly, is required for signaling the spindle assembly checkpoint. Whether the required function of the CC domain in spindle assembly is the same as that in the checkpoint, namely as a microtubule-interaction domain, is an intriguing question. To test this hypothesis, I will examine if the microtubule-binding chimeric INCENPs (INCENPΔCC∇tau4 and INCENPΔCC∇PRC1; Figure 4-6) can support the checkpoint. If the chimeras fail to rescue the checkpoint, then it suggests that the CC domain must have some other non-microtubule-interacting function. In this case, I will use the mutants that contain smaller deletions of the CC domain to determine if the checkpoint

activation function falls within a specific region of this domain. In addition, by replacing the CC domain with a rigid α-helical domain of the same amino acid length (see Is the CC domain of INCENP a coiled-coil in Chapter 6), I will test if the length of the domain plays a role in spindle checkpoint.

Alternatively, the microtubule-binding INCENP chimeras may support the activation of the checkpoint, suggesting that the CC domain of INCENP functions as a microtubule-interacting domain in the checkpoint as well as in spindle assembly. In the following two sections, I will further elaborate on the possible functions of the CC domain as microtubule-binding domain in the spindle checkpoint.

Activating Aurora B. Since the CC domain of INCENP is required for the microtubule-induced activation of Aurora B (Figure 4-7C), and Aurora B activity is required for the destabilization of improperly attached microtubules and for the proper localization of checkpoint proteins (Kelly and Funabiki, 2009), the function of CC domain may be simply to activate Aurora B for the spindle checkpoint. Since the CPC and presumably activated Aurora B are required for the kinetochore localization of checkpoint proteins in *Xenopus* (Vigneron et al., 2004), I will determine if these proteins are properly localized to kinetochores in the presence of INCENPΔCC.

The microtubule-induced activation of Aurora B via the CC domain of INCENP could provide a site-specific activation at the centromere. This proposed function suggests that localized activation of Aurora B on chromosomes should be sufficient to bypass the function of the CC domain in the

checkpoint. To test if artificial activation of Aurora B is sufficient to rescue the checkpoint, I will use the INCENPΔCC▽GCN4 construct (Figure 4-6). In this chimera, the endogenous CC domain of INCENP is replaced with the dimerizing GCN4 coiled coil (O'Shea et al., 1989). This construct robustly activates Aurora B in a microtubule-independent manner (Figure 4-15) and targets to chromosomes (data not shown). If the dimerizing INCENP chimera can support the checkpoint, then the function of the CC domain is simply to activate Aurora B, unlike its role in spindle assembly.

If the role of the CC domain in the checkpoint is only to activate Aurora B in the presence of microtubules (for instance, when tension is lacking), it is unclear what role this domain would play in a nocodazole-induced checkpoint, which lacks microtubules, as shown in Figure 5-1. One tantalizing speculation is that similar to the case in spindle assembly (Figure 4-24), the CPC must detect both chromosomes and microtubules to inactivate the checkpoint and allow for cell cycle progression. The CC domain of INCENP, by sensing if microtubules are attached to kinetochores, may signal the checkpoint via activation of Aurora B in response to a lack of attachment. This scenario, however, seems counterintuitive for two reasons. First, if the CC domain senses the presence of microtubules, then INCENPΔCC would not be able to sense microtubules, suggesting that the construct would constitutively activate the checkpoint. I saw, however, that INCENPΔCC failed to support checkpoint activation. Second, the presence, not the absence, of microtubules activates Aurora B (Figures 4-2, 4-3, and 4-4), suggesting that the lack of microtubules would inactivate Aurora B and the checkpoint, instead of activating them. Furthermore, if the CC domain senses

the microtubule attachment status of kinetochores in *Xenopus*, this function is most likely not conserved in mammals and budding yeast, as the CPC is not required for the spindle checkpoint signaled in response to such a situation in these organisms (Biggins and Murray, 2001; Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003; Lens et al., 2003).

A kinase-independent function. Since the CC domain sensing microtubules for the sole purpose of activating Aurora B in the spindle checkpoint seems counterintuitive, the simplest explanation is that the CC domain has a kinase-independent function in the checkpoint. While Aurora B kinase activity is required for the kinetochore localization of a growing number of checkpoint proteins (Ditchfield et al., 2003; Hauf et al., 2003; Lens et al., 2003; Vigneron et al., 2004; Famulski and Chan, 2007), it is unclear if any other function of the CPC is also needed. Published data showing that the CC domain of INCENP is required for a taxol-induced spindle assembly checkpoint suggest a kinase-independent function could exist (Vader et al., 2007). While the taxol-induced checkpoint was abrogated in mammalian cells containing an INCENP that lacked the CC domain (see Figure 1-8), improperly attached kinetochore microtubules were appropriately corrected and BubR1 was localized to kinetochores (Vader et al., 2007). These results suggest that Aurora B is active in these cells and able to phosphorylate at least a subset of its substrates. While the CC domain in the checkpoint may be important for targeting Aurora B to the a specific set of critical substrates, as suggested by Lens and colleagues (Vader et al., 2007), it is also possible that the CC domain has a function independent of Aurora B kinase activity. In light of my results in Chapter 4 and those of Lens

and colleagues (Vader et al., 2007), it seems unlikely that the CC domain of INCENP targets Aurora B to specific substrates for both microtubule stabilization and checkpoint signaling, since INCENP lacking the CC domain does not affect the phosphorylation of the tested substrates, which have roles in both microtubule stabilization and checkpoint signaling. The simplest explanation is that the CC domain functions in part via a kinase-independent mechanism. Ultimately, however, this hypothesis is difficult to test as presumably not all of Aurora B substrates have been identified. Further characterization of the CC domain in the spindle assembly checkpoint will provide insight as to whether such a kinase-independent function of the CPC exists.

CHAPTER 6. PERSPECTIVE

Here I have described three ways in which the CPC regulates different aspects of M-phase using the *Xenopus* egg extract system: the CPC aids in the M-phase disassociation of an interphase chromatin-bound protein (see Chapter 3), spatially restricts microtubule assembly to ensure spindle formation around chromosomes (see Chapter 4), and controls the activation of the spindle assembly checkpoint (see Chapter 5). In this chapter, I will place my work in a larger biological context by speculating on the significance of my work, elaborating on a few nuances of using *Xenopus* as a system, and describing future directions for this work. In addition, I will comment on the structure of the putative coiled-coil (CC) domain of INCENP.

Removing interphase chromatin-binding proteins from metaphase chromosomes

The plethora of proteins that are mitotically released from chromosomes is amazing. The DNA replication machinery, the RNA pol II and III transcriptional machinery, sequence-specific transcription factors, transcriptional insulators and chromatin-remodeling factors are among the classes of proteins that removed from chromosomes in M-phase (Egli et al., 2008). Interestingly, this mass displacement of proteins accompanies a drastic change in the nuclear and chromatin architecture. In interphase, the nucleus is highly organized with chromosomes occupying distinct territories and domains of transcriptional activation and repression (Lanctot et al., 2007). In mitosis, this structure is completely rearranged with the break down of the nuclear envelope,

condensation of the chromatin fiber, and metaphase alignment of chromosomes (Gerlich et al., 2003; Walter et al., 2003).

As discussed in Chapter 3, Aurora B induces the disassociation of HP1 from metaphase chromosomes by phosphorylating serine 10 of histone H3 (H3S10)(Figure 3-1)(Fischle et al., 2005; Hirota et al., 2005). In addition to HP1, the CPC and/or the phosphorylation of H3S10 by Aurora B have also been implicated in the mitotic removal of the following molecules: SUV39H1, the histone methyltransferase for lysine 9 of histone H3 (H3K9)(Terada, 2006); two ISWI-containing chromatin remodeling complexes (MacCallum et al., 2002); two SR protein mRNA splicing factors (Loomis et al., 2009); and XIST RNA, which functions in X chromosome inactivation (Hall et al., 2009). Both the biological significance and the mechanism by which the CPC regulates their release are unclear. Two speculative roles for the disassociation of proteins from metaphase chromosomes in facilitating mitotic chromatin re-structuring are discussed below.

Molecular memory of the interphase transcription state

As chromosomes condense and move from their interphase territories to the metaphase plate, transcriptionally active and repressed regions in interphase could encounter one another. In interphase, rearrangement of genetic loci within the nucleus accompanies changes in transcription (Lanctot et al., 2007). Such a change in transcription of chromosomal loci in mitosis, however, is unlikely, since the transcription of many genes is inhibited in mitosis (Prescott and Bender, 1962; Johnson and Holland, 1965). However, this mitotic chromosomal rearrangement may affect the memory of the transcriptional state before mitosis

of specific loci. To further elaborate, while many chromatin-bound proteins dissociate in mitosis, the covalent modifications of the chromatin persist through mitosis and may serve as a memory of the interphase transcriptional state (Stein et al., 1982; Peters et al., 2002). Similar to inducing transcriptional changes by gene kissing in interphase (Lanctot et al., 2007), the proteins that modulate these modifications, if retained on chromosomes during mitosis, may affect the modifications of a different locus as mitotic chromosomes rearrange and loci that were separated in interphase come in contact. Therefore, one speculative role of removing transcription factors and chromatin-remodeling proteins from mitotic chromosomes is to prevent changes in the memory of the interphase transcriptional state.

Mitotic chromosome structure

The second speculative role for this M-phase disassociation of proteins is to aid in the individualization and compaction of the mitotic chromosome. The mitotic chromosome is compacted 500-fold relative to the interphase chromatin (Georgatos et al., 2009). How this massive condensation is achieved is not fully understood. One protein complex that is known to play a role is condensin (Hirano, 2005), which is suggested to condense chromosomes by inducing positive supercoiling of the DNA (Kimura and Hirano, 1997; Kimura et al., 1999; Bazett-Jones et al., 2002; Stray and Lindsley, 2003; Stray et al., 2005). Removal of these interphase chromatin-bound proteins may facilitate the recruitment or function of condensin (Dormann et al., 2006). Along the same rationale, the release of these proteins may generally aid in the compaction of chromosomes by relieving DNA constraint. For instance, HP1 proteins in interphase are thought to

promote the highly compacted structure of heterochromatin by cross-linking nucleosomes (Hiragami and Festenstein, 2005), and the release of chromosomal HP1 in mitosis (Wreggett et al., 1994; Kellum et al., 1995; Furuta et al., 1997; Minc et al., 1999; Murzina et al., 1999; Sugimoto et al., 2001; Hayakawa et al., 2003) may loosen this interphase structure to allow chromatids to individualize and the mitotic chromosome structure to form (Dormann et al., 2006).

Coincidence detection as a general mechanism of spatially regulating the CPC

As demonstrated in Chapter 4, my results indicate that in *Xenopus* egg extract, chromosomally activated Aurora B needs to be targeted to emerging microtubules to support robust microtubule assembly in the early steps of spindle formation. From my results, I have suggested that the CPC must coincidently detect chromosomes and emerging microtubules in the physiological scenario as these two structures are physically close in the initial formation of a chromosome-induced spindle. Furthermore, I have proposed that the coincident detection of chromosomes and microtubules by the CPC via INCENP is important for spatially restricting robust microtubule assembly to the vicinity of chromosomes. Interestingly, the CPC regulates other functions where chromosomes and microtubules are involved and where the coincident detection of these two structures may moderate the response of the CPC. Two such scenarios are discussed below.

In the spindle assembly checkpoint

The coincident detection of chromosomes and microtubules by the CPC may play a role in the spindle assembly checkpoint. As shown in Chapter 5, my

results indicate that the putative coiled-coil (CC) domain of INCENP (see Figure 4-6) is required for the spindle assembly checkpoint in *Xenopus* egg extract. Similar results were seen in mammalian tissue culture cells, where deletion of the CC domain of INCENP prevents the cells from arresting in metaphase in response to taxol, suggesting that this domain is required for the CPC-dependent signaling of the spindle checkpoint (Vader et al., 2007). The mechanistic function of this domain in the spindle checkpoint was not demonstrated in this study. Based on my analysis of the CC domain as a microtubule-binding domain in spindle formation, I propose that the CC domain functions as a microtubule-binding domain in the checkpoint, which can be tested using the microtubule-binding INCENP chimeras (see the Discussion of Chapter 5).

If the microtubule-binding function of the CC domain is required for the CPC, it would suggest that the coincident detection of chromosomes and microtubules by the CPC may monitor the tension status of sister kinetochores. Indeed, Sli15 and Bir1, the *S. cerevisiae* INCENP and Survivin homologues, link centromeric DNA and microtubules *in vitro* (Sandall et al., 2006). Furthermore, this linkage requires the microtubule-binding domain of Sli15 (Sandall et al., 2006). In metazoans, however, how the CC domain of an inner centromere-bound CPC would encounter a microtubule is not clear. The CPC enriches in the inner centromere (Ruchaud et al., 2007), while kinetochore-microtubules terminate in the outer kinetochore (McEwen et al., 1998). In HeLa tissue culture cells, the outer kinetochore to outer kinetochore (interkinetochore) distance in a cell that is not under tension is approximately 750 nm (Wan et al., 2009), suggesting that there is a distance of approximately 375 nm between the kinetochore and the center of inner centromere. Approximately 60 nm of this

distance is covered by different kinetochore proteins, and CENP-A is approximately 80 nm from the outer kinetochore (as defined by the Ndc80/Hec1 localization)(Wan et al., 2009). Since targeting Aurora B away from the inner centromere to the centromere is sufficient to induce kinetochore-microtubule turnover and a checkpoint (Liu et al., 2009), the CPC must reside farther from the outer kinetochore than CENP-A. As the CPC is predicted to be no longer than approximately 50 nm (Kelly and Funabiki, 2009), it does not seem possible for one molecule of the CPC to interact with the inner centromere and a microtubule in the outer kinetochore at the same time.

One hypothesis to resolve this discrepancy is that Aurora B diffuses away after activation in the inner centromere, and if this activated Aurora B encounters a kinetochore microtubule via the CC domain of INCENP before the kinase activity is inactivated, the checkpoint is signaled. Supporting this hypothesis, the turnover of the CPC at the centromere is important for chromosome alignment (Vong et al., 2005; Sumara et al., 2007), suggesting that the CPC must be actively cycled off chromosomes to promote microtubule turnover and checkpoint signaling. Results from the Lens laboratory, however, are inconsistent with this hypothesis. While the CC domain in human cells is required for checkpoint signaling, it is not required for correcting kinetochore-microtubule attachments (Vader et al., 2007). These results suggest that the CC domain of INCENP may not be important in sensing tension, since presumably the CPC senses tension via the same mechanism for both functions. Further studies are required to determine if the CC domain of INCENP is important for the ability of the CPC to sense microtubules and tension in both the correction of kinetochore microtubules and the spindle assembly checkpoint.

In abscission

As mentioned in Chapter 1, the CPC functions in abscission. In budding yeast, the CPC inhibits abscission via the NoCut pathway until chromosomes have cleared the bud neck (Norden et al., 2006; Mendoza et al., 2009), and in human cells, the CPC functions later, stabilizing the cleavage furrow until chromosome bridges have been resolved (Steigemann et al., 2009). In both cases, the CPC is localized to the microtubules in the midzone, where some of the chromosome mass is aberrantly localized (Mendoza et al., 2009; Steigemann et al., 2009). Therefore, like on chromosome arms in spindle formation and at the centromere in spindle checkpoint signaling, the CPC is poised to sense both structures in cytokinesis, suggesting that the coincident detection of chromosomes and microtubules by the CPC regulates its function in abscission.

Active Aurora B is required for the function of the CPC in abscission (Norden et al., 2006; Mendoza et al., 2009; Steigemann et al., 2009). How Aurora B is activated by the presence of chromosomes and microtubules in cytokinesis is not clear. In budding yeast, artificial targeting of Aurora B, but not a kinase dead version, is sufficient to activate the NoCut pathway (Mendoza et al., 2009) and the NoCut pathway is activated when the midzone microtubules are disrupted (Norden et al., 2006), suggesting that the chromosome-induced activation of Aurora B in cytokinesis is sufficient and microtubule sensing may be dispensable, at least in budding yeast. Microtubules, however, may be important in human cells. In unperturbed cytokinesis, the microtubule bundles are disassembled and Aurora B is inactivated in the midbody remnant (Steigemann et al., 2009). In cells with chromosome bridges, on the other hand, Aurora B is

activated in the midbody. While the anaphase microtubule bundles are still disassembled in these cells, an anti-tubulin antibody stains the entire length of the bridge between cells (Steigemann et al., 2009), suggesting that microtubules line the structure. Since physical obstructions do not stabilize the cleavage furrow like the chromosome- and microtubule-containing bridges (Steigemann et al., 2009), chromosomes and microtubules likely play a role in abscission via Aurora B signaling.

Differences between *Xenopus* laevis and other model organisms

While *Xenopus* laevis is an ideal organism for studying the cell cycle (Murray, 1991), differences in the requirement for the CPC in various functions of the cell cycle exist between *Xenopus* laevis and other organisms. Below I will elaborate on two of these functions: microtubule assembly in spindle formation and checkpoint signaling in response to nocodazole.

Requirement of the CPC in microtubule assembly in spindle formation

While the CPC, and Aurora B kinase activity, is required for microtubule assembly in spindle formation of metaphase *Xenopus* egg extract (Sampath et al., 2004; Kelly et al., 2007), depletion or inactivation of Aurora B does not grossly affect microtubule polymerization in somatic tissue culture cells (Adams et al., 2001; Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003; Honda et al., 2003; Lens et al., 2003; Yue et al., 2008). This apparent lack of requirement for Aurora B may be due to differences in spindle assembly of the two systems (Compton, 2000). One major difference between the meiotic *Xenopus* egg extract system and mitotic mammalian somatic tissue culture system is the contribution

of kinetochores to spindle assembly (O'Connell et al., 2009). In a CPC-dependent manner, microtubules can polymerize and a spindle can form around DNA that lacks kinetochores in *Xenopus* egg extract (Karsenti et al., 1984; Sawin and Mitchison, 1991; Heald et al., 1996; Sampath et al., 2004). In comparison, microtubules polymerize from kinetochores, and not chromosome arms, of somatic tissue culture cells *in vivo* and *in vitro* (McGill and Brinkley, 1975; Snyder and McIntosh, 1975; Telzer et al., 1975; Gould and Borisy, 1978; Pepper and Brinkley, 1979; Bergen et al., 1980; Witt et al., 1980; De Brabander et al., 1981; Czaban and Forer, 1985; Maiato et al., 2004). While the CPC contributes to the assembly of microtubules near kinetochores (Tulu et al., 2006; Katayama et al., 2008), the CPC and Aurora B activity are not required (Tulu et al., 2006; Katayama et al., 2008; O'Connell et al., 2009). Together, these data suggest that in somatic tissue culture cells, the CPC and Aurora B activity are not required for microtubule assembly in spindle formation because they are not strictly needed for the kinetochore-based assembly of microtubules.

Requirement of the CPC in the nocodazole-induced spindle assembly checkpoint

There are also organismal differences in the exact role of the CPC in the spindle assembly checkpoint. In mammalian cells and *S. cerevisiae*, the CPC is required for the taxol-induced, but not the nocodazole-induced, checkpoint (Biggins and Murray, 2001; Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003; Lens et al., 2003), while in *Xenopus* and *S. pombe*, the CPC is required for both checkpoints (Kallio et al., 2002; Petersen and Hagan, 2003). These data suggest that in mammalian cells and budding yeast, the role of the CPC is to trigger the spindle assembly checkpoint in response to the lack of tension across

sister kinetochores, but not to the lack of microtubule attachment at kinetochores (Pinsky and Biggins, 2005). Consistent with this hypothesis, there are differences between *Xenopus* and other systems in the requirement of the CPC to recruit checkpoint proteins to the kinetochore (see Signaling the spindle assembly checkpoint in Chapter 1)(Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003; Petersen and Hagan, 2003; Gillett et al., 2004; Vigneron et al., 2004). In mammalian cells, however, the CPC is needed to recruit BubR1 in response to unattached kinetochores and is required to maintain the checkpoint if kinetochores remain unattached for long periods of time (Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003), suggesting that the CPC, while not required for initiating the checkpoint, does play a role in signaling the nocodazole-induced spindle checkpoint in these organisms.

Implications on the function of the CC domain

Since there are organismal differences in the requirement for the CPC, it would be interesting to examine the role of the CC domain of INCENP in somatic tissue culture cells. Data from the Lens laboratory shows that the CC domain is not required for the turnover of kinetochore-microtubules, but is required for signaling the taxol-induced spindle checkpoint (Vader et al., 2007). A caveat of these experiments, however, is that they were performed in cells transiently transfected with a plasmid encoding the mutant INCENP and the expression levels of the mutant INCENP, relative to the physiological level of endogenous INCENP, were never shown. Therefore, the conclusions of these experiments are tainted with the possibility that the mutant INCENP was overexpressed, occluding the observation of some of its functions. Therefore,

these experiments should be repeated in a stably expressing mutant INCENP clonal cell line, for which the expression level of the mutant INCENP has been determined. In collaboration with Lei Tan in the laboratory of Tarun Kapoor, I have started to create such cell lines.

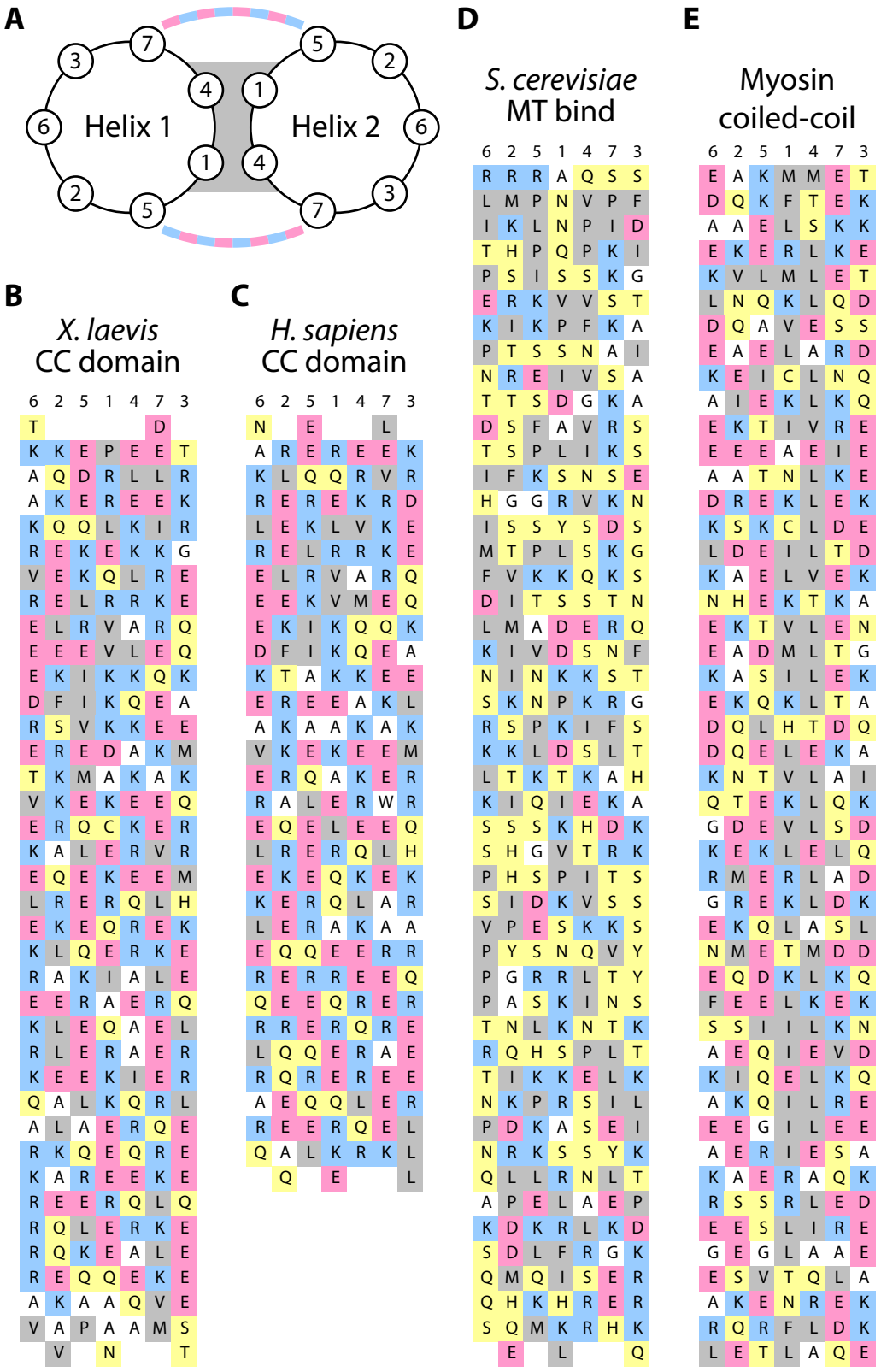
Is the CC domain of INCENP a coiled-coil?

α-Helices are not very stable in solution and, in proteins, often pack together via their hydrophobic side chains (Branden and Tooze, 1999). One such packing arrangement consists of two α-helices wrapping around one other to produce a coiled-coil. An α-helix in a coiled-coil contains seven residues every two turns of the helix and these seven residues often repeat. While the residues of this heptad repeat are typically labeled a-g, I will describe the positions by number (1-7) to prevent confusion with the single-letter amino acid code. The positions around the helix often correspond to residues of a specific chemical nature. Generally, residues at positions 1 and 4 are hydrophobic, packing against the other helix to create the coiled-coil core, while residues at positions 2, 3, and 6 are hydrophilic, extending into the solvent. Forming a salt bridge to stabilize the coiled-coil are the residues at positions 5 and 7, which are often charged (Figure 6-1A)(Branden and Tooze, 1999).

The Earnshaw laboratory originally commented on the CC domain of INCENP (Mackay et al., 1993). This region of chicken INCENP is highly α-helical and predicted to be in a coiled-coil (Mackay et al., 1993). Similar analyses (Lupas et al., 1991) of INCENPs from *X. laevis* and *H. sapiens* reveal a region of similar size and location with a propensity for adopting a coiled-coil structure for both

Figure 6-1. The CC domain of INCENP is not composed of canonical heptad repeats.

(A) The top view of the canonical coiled-coil heptad repeat structure. The two helices that wrap around each other are represented as large circles with the seven positions (#1-7) of the amino acids marked for two full turns of helices. The primary sequence of the residues is sequential. Gray area, hydrophobic core; red and blue line, salt bridge. **(B-E)** The CC domain of INCENP from *X. laevis* (B; aa 491-747) and *H. sapiens* (C; aa 539-747, Vader et al., 2007) are shown, as is the homologous microtubule-binding domain of *S. cerevisiae* Sli15 (D; aa 227-558, Kang et al., 2001) and part of the coiled-coil of myosin (aa 851-1187). The residues are arranged to represent their spatial distribution if the sequence were arranged in a coiled-coil structure. Each helical turn is one row. Each column represents one position on the helix with the residues in vertical order. The helical position of the first residue for the frog and human CC domains was determined by the Marcoil program (Delorenzi and Speed, 2002). Highlighted in gray are hydrophobic residues (FILMPV); white, amphiphilic residues (AGW); yellow, hydrophilic residues that are neutral or slightly charged (CHNQSTY); blue, basic residues (KR); and red, acidic residues (DE).


A
Helix 1
Helix 2
B
X. laevis
CC domain
C
H. sapiens
CC domain
D
S. cerevisiae
MT bind
E
Myosin
coiled-coil
6 2 5 1 4 7 3

proteins (data not shown). These regions approximately correspond to the CC domains used in this study and that from the Lens laboratory (Vader et al., 2007). In contrast, Sli15, the *S. cerevisiae* INCENP homologue, contains a very small region near its C-terminus that may be coiled-coil in nature (data not shown). This region, however, does not correspond to the microtubule-binding domain of INCENP (Kang et al., 2001) and does not align with the CC domains of *X. laevis* and *H. sapiens* (see Figure 1-8).

While predicted to contain a heptad repeat, the CC domain of INCENP from neither *X. laevis* (aa 491-747) nor *H. sapiens* (aa 539-747) appears to contain a canonical heptad repeat based on analysis of the primary sequence. To better analyze the secondary structure of the CC domain, I created a schematic to better visualize the charge distribution on the helix based on the assumption that the domain is an α-helix with 3.5 residues per turn (Figure 6-1B-D). I used the Marcoil program (Delorenzi and Speed, 2002) to determine the frame of the heptad repeat for both the frog and human CC domains. Since the Sli15 microtubule-binding domain is not predicted to be a coiled-coil, I place the first residue of the delineated domain in position 1 of the helix for this analysis. I then arranged the residues of each domain to represent their positions around the helix. To further describe the figure, I will use the frog CC domain as an example. The first 15 residues for the CC domain of *X. laevis* (aa 491-505) are TDPKTEEKERQRLDA. T491 is predicted to be at position 6 of the helix (Figure 6-1A)(Delorenzi and Speed, 2002). Therefore, in the schematic (Figure 6-1B), T491 is in first column, which represents position 6, and in the first row, which represents the first heptad repeat of the helix. Similarly, K498 and A505 are in the

second and third row of the first column (position 6); D492 and E499 are in the first and second row of the sixth column (position 7); and so on.

This analysis shows that while predicted to be a coiled-coil with a heptad repeat, the CC domain of INCENP from neither *X. laevis* nor *H. sapiens* follows the canonical heptad repeat of a coiled-coil: I did not observe two columns of hydrophobic "core" residues flanked on each side by a column of charged residues for either the frog or human CC domain (Figure 6-1B, C). Such an arrangement was observed for a known coiled-coil in the protein myosin (Figure 6-1D). Instead, the helix of the CC domains is littered with basic and acidic patches. In comparison, similar analysis of the microtubule-binding domain of Sli15 (aa 227-558)(Kang et al., 2001), which is not predicted to be helical or in a coiled-coil, shows little similarity to either CC domain. This analysis shows that although the microtubule-binding domain of Sli15 aligns with the CC domain of metazoans (Figure 1-8), the chemical composition of the region is very different (Figure 6-1D, Table 6-1). Together, these data suggest that the CC domain of INCENP is not a coiled-coil. Since the coiled-coil prediction program also identifies α-helical bundles in proteins (Lupas et al., 1991), the CC domain, instead of a coiled-coil, may form an α-helical bundle.

Alternatively, the CC domain may form a 38.4 nm-long extended α-helix with 3.6 residues per turn. While α-helices are often stabilized by tertiary interactions, proteins containing extended α-helices do exist (Wang et al., 1991; Knight et al., 2005; Sivaramakrishnan et al., 2008; Spink et al., 2008). Seventy to eighty percent of the residues in these extended helices are glutamic acids (E), arginines (R) and lysines (K). These residues are interspersed with hydrophobic

	X. laevis	*H. sapiens*	*S. cerevisiae*
Alanine	9.34	8.61	3.92
Cysteine	0.39	0.00	0.00
Aspartic acid	1.56	0.96	4.22
Glutamic acid	26.07	28.23	3.92
Phenylalanine	0.39	0.48	2.41
Glycine	0.39	0.00	2.71
Histidine	0.39	0.48	3.01
Isoleucine	1.95	0.96	6.93
Lysine	16.73	13.40	15.06
Leucine	8.17	9.09	6.02
Methionine	1.56	0.96	1.51
Asparagine	0.39	0.48	5.42
Proline	0.78	0.00	6.93
Glutamine	10.89	12.92	3.92
Arginine	15.18	20.10	6.93
Serine	0.78	0.00	15.66
Threonine	1.56	0.48	6.33
Valine	3.50	2.39	3.61
Tryptophan	0.00	0.48	0.00
Tyrosine	0.00	0.00	1.51
Hydrophobic	16.34	13.88	27.41
Amphiphilic	9.73	9.09	6.63
Hydrophilic	14.40	14.35	35.84
Basic	31.91	33.49	21.99
Acidic	27.63	29.19	8.13

Table 6-1. Amino acid composition of the CC domain of INCENP.
The amino acid composition for the CC domain of INCENP from *X. laevis* (aa 491-747) and *H. sapiens* (aa 539-747, Vader et al., 2007) was determined, as was that for the microtubule-binding domain of *S. cerevisiae* Sli15 (aa 227-558, Kang et al., 2001). The numbers represent the percent occurrence for each residue (top) or for each class of residues (bottom). The hydrophobic residues are FILMPV; amphiphilic residues, AGW; hydrophilic residues that are neutral or slightly charged, CHNQSTY; basic residues, KR; and acidic residues, DE.

residues (alanine, leucine, isoleucine, valine, and methionine) as well as glutamines and aspartic acids. The helix is stabilized by charged side chain interactions, namely E interacts with an R or K at the -4 and +3 positions (Sivaramakrishnan et al., 2008). To determine if the human and *Xenopus* CC domains form extended α-helices, I counted the occurrences where an R or K is in the -4 or +3 position relative to an E (ER/K OR)(Table 6-2). For comparison, I included the myosin VI medial tail domain, which forms an extended α-helix (Spink et al., 2008). I did not perform this analysis on the microtubule-binding domain of Sli15 as the entire 331-residue domain contains only 13 glutamic acids. Since E, R and K compose a lower percentage of the residues in the CC domains (approximately 60%) relative to the medial tail domain (approximately 71%), I divided the number of "ER/K OR" occurrences by two times the number of glutamic acid residues. This manipulation also offsets biases due to the varying domain sizes. While lower than that for the medial tail domain, the incidence of "ER/K OR" for both CC domains is substantial, suggesting that the charged side chains do interact and the domain forms an extended α-helix.

Since extended α-helices often contain chains the charged residue interactions (Sivaramakrishnan et al., 2008), I counted the incidence of glutamic acids with an arginine or lysine at both the -4 and +3 positions (ER/K AND). I multiplied this number by two and then divided by the "ER/K OR" occurrences to determine the proportion of the "ER/K OR" occurrences that actually have arginines and/or lysines at both the -4 and +3 positions. A high "ER/K AND" percentage would suggest a more stable extended α-helix. While the frog CC domain contains approximately the same "ER/K AND" incidence as the medial

	X. laevis CC domain	*H. sapiens* CC domain	Myosin VI medial tail
total residues	256	208	72
number of E's	67 (26.2%)	59 (28.4%)	23 (31.9%)
number of R's and K's	82 (32.0%)	70 (33.7%)	28 (38.9%)
ER/K OR	72 (53.7%)	58 (49.1%)	29 (63.0%)
ER/K AND	19 (52.8%)	10 (34.5%)	9 (62.1%)

Table 6-2. Extended α-helix analysis of the INCENP CC domain.
The incidence of an arginine (R) or lysine (K) at the -4 and/or +3 position relative to a glutamic acid (E) was determined for the CC domain of *X. laevis* and *H. sapiens*, as well as for the medial tail domain of myosin VI (aa 908-980). ER/K OR, where a R/K is at the -4 or +3 position relative to an E; ER/K AND, where R/K are at both the -4 and +3 positions relative to an E. The absolute number represents the number of occurrences. For the number of E's or R's and K's, the percentage is the number of occurrences divided by the total number of residues in the domain (% composition of those amino acids). For the "ER/K OR" row, the percentage is the number of occurrences divided by twice the number of glutamic acids. For the "ER/K AND" row, the percentage is twice the number of occurrences divided by total number of occurrences in the "ER/K OR" row.

tail domain, the human CC domain contains only about half as much. Together, these analyses suggest that the CC domains of frogs and humans may form extended α-helices. Based on the amino acid composition, however, the CC domain would form a less stable extended α-helix than that of the published examples.

Conclusions

In this dissertation, I have described three functions of the CPC in the cell cycle. While one of these functions is conserved in mammalian systems (Fischle et al., 2005; Hirota et al., 2005), more work is needed to determine whether and to what extent the other two functions are conserved. Published data suggests that to some extent the function of the CC domain of INCENP that I observed in *Xenopus* may be conserved in other organisms (Sandall et al., 2006; Vader et al., 2007; Tseng et al., in submission). Importantly, my work shows that the simple reaction-diffusion gradient does not fully explain how the CPC is spatially and temporally regulated. Further studies are needed to examine this spatial and temporal aspect of CPC regulation in other organisms to determine if a similar mechanism is used, since the regulation of the CPC is important for understanding its functions in chromosome segregation and ultimately genomic integrity.

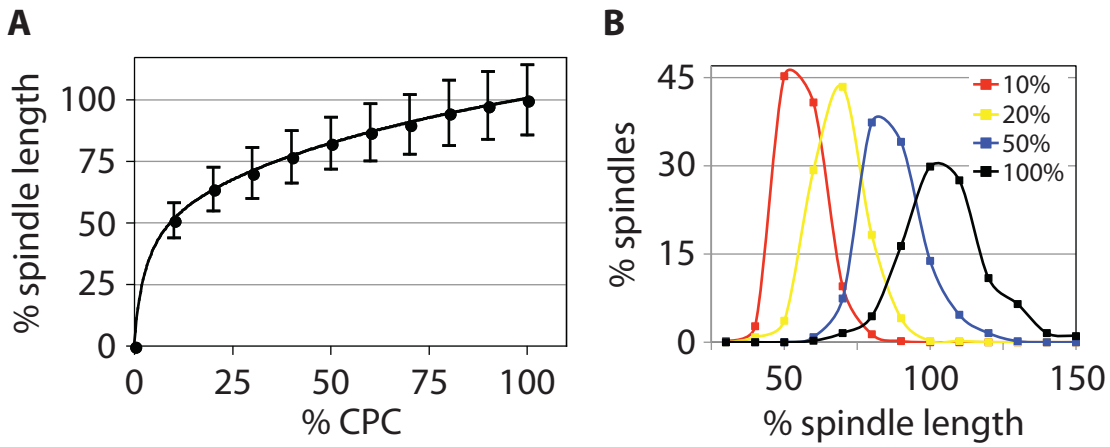


Figure A-1. The CPC scales spindle length.

(A) Control and ΔCPC extract were mixed to produce extract with varying amounts of CPC. The extract was then cycled through interphase to metaphase at 20°C. Samples were fixed and imaged 60 min after entry into metaphase from which spindle lengths were measured. Each spindle length was normalized to the average spindle length in the 100% CPC population. Error bars represent 1 S.D. **(B)** A histogram of the measured lengths in (A). The spindle lengths were binned and the number of spindles in each bin was normalized to the total number of spindles measured (greater than 400 per sample). Red, 10% CPC; yellow, 20% CPC; blue, 50% CPC; black, 100% CPC.

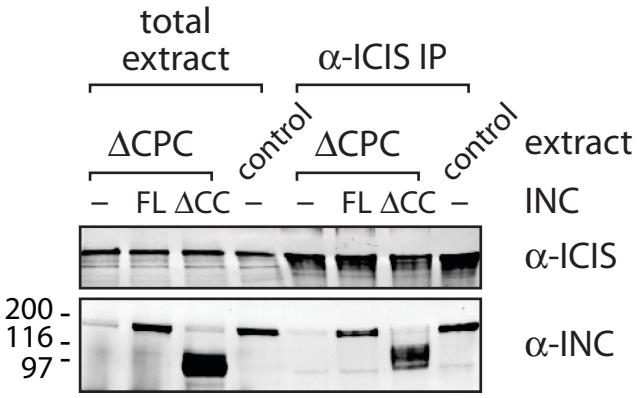


Figure A-2. The CC domain of INCENP is not required for ICIS interaction. Control or ΔCPC extract was reconstituted with INCENP as indicated for 90 min at 20°C and incubated with anti-ICIS antibody beads for 30 min at 4°C. The beads were then purified and analyzed by immunoblot using the indicated antibodies on the right. Molecular weight markers (in kD) for INCENP are on the left. INC, INCENP.

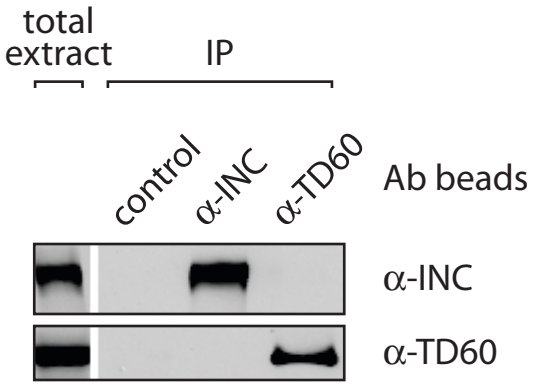


Figure A-3. TD60 and the CPC do not co-precipitate.
Control, anti-INCENP, or anti-TD60 antibody beads were incubated with extract for 60 min at 4°C, then purified, and analyzed by immunoblot with the indicated antibodies on the right. INC, INCENP.

Table A-1. Proteins that co-precipitate with full length INCENP, but not INCENPΔCC.

Accession No.	Annotation	Protein Description	Mass	Diff	S_ FL	S_ CC	M_ FL	M_ CC
gi|62860244	ribosomal protein	hypothetical protein LOC549409	14864	941	941	0	33	0
gi|5326785	RACK1; ribosomal protein?	activated protein kinase C receptor; RACK1	35544	813	813	0	35	0
gi|148224536	ribosomal protein	MGC82844 protein	13101	791	791	0	27	0
gi|155369251	ribosomal protein	ribosomal protein S1a protein	27156	756	756	0	37	0
gi|147904232	ribosomal protein	ribosomal protein L30	13101	755	791	36	27	3
gi|147904649	ribosomal protein	acidic ribosomal protein P0	34247	679	735	56	37	2
gi|27735393	ribosomal protein	Similar to ribosomal protein S3	27170	679	679	0	35	0
gi|46015116	H3	Chain B, Crystallographic Studies Of Nucleosome Core Particles Containing Histone ~sin~ Mutants	11202	574	719	145	50	34
gi|3745759	H3	Chain B, Complex Between Nucleosome Core Particle (H3,H4,H2a,H2b) And 146 Bp Long Dna Fragment	9962	544	787	243	55	39
gi|148232254	ribosomal protein	ribosomal protein S9	22621	524	573	49	46	14
gi|122936398	H4	Unknown (protein for MGC:160678)	11531	472	713	241	50	36
gi|148233630	ribosomal protein	MGC82808 protein	14956	470	503	33	38	26
gi|148223451	ribosomal protein	ribosomal protein S19	16040	469	469	0	35	0
gi|132743	ribosomal protein	60S ribosomal protein L4-A (L1A)	45192	456	456	0	44	0
gi|148238092	ribosomal protein	ribosomal protein S3a	30150	425	482	57	39	9
gi|58331994	ribosomal protein	ribosomal protein S16	16434	414	414	0	24	0
gi|148224857	ribosomal protein	ribosomal protein L10	24981	385	385	0	12	0
gi|132942	ribosomal protein	60S ribosomal protein L35a (L32)	12710	317	317	0	18	0
gi|148228673	ribosomal protein	ribosomal protein L3	46354	314	314	0	28	0
gi|147900456	ribosomal protein	ribosomal protein L13	24241	287	287	0	22	0

gi | 148227832	ribosomal protein	hypothetical protein LOC414688	16211	285	285	0	22	0
gi | 147905658	ribosomal protein	hypothetical protein LOC735172	28768	269	269	0	21	0
gi | 147905368	ribosomal protein	ribosomal protein L9	22056	266	266	0	20	0
gi | 41152459	ribosomal protein	ribosomal protein S12	14799	262	262	0	13	0
gi | 5032051	ribosomal protein	ribosomal protein S14	16434	256	256	0	11	0
gi | 147899358	ribosomal protein	hypothetical protein LOC431998	29858	253	253	0	25	0
gi | 4759160	snRNP	small nuclear ribonucleoprotein polypeptide D3	14021	251	310	59	9	3
gi | 62860010	ribosomal protein	ribosomal protein S13	17240	244	279	35	20	3
gi | 125858794	ribosomal protein	Unknown (protein for MGC:160337)	29994	241	241	0	21	0
gi | 147899497	ribosomal protein	MGC83421 protein	24321	237	237	0	26	0
gi | 147903835	ribosomal protein	ribosomal protein L12	18011	227	283	56	12	5
gi | 148223950	protein 2 methyltransferase	MGC84169 protein	27217	226	226	0	8	0
gi | 27924365	fibrillarin	LOC398434 protein	36286	220	496	276	35	22
gi | 148235098	ribosomal protein	hypothetical protein LOC100037184	46364	220	220	0	15	0
gi | 52346118	ribosomal protein	ribosomal protein L26	17179	219	219	0	18	0
gi | 147905470	ribosomal protein	similar to ribosomal protein S20	13461	217	217	0	11	0
gi | 148233658	laminin receptor?	67kD laminin receptor precursor	34208	202	202	0	8	0
gi | 148228908	ribosomal protein	ribosomal protein S11	18580	193	224	31	19	8
gi | 147903795	ribosomal protein	MGC85348 protein	17663	192	192	0	8	0
gi | 147898797	ribosomal protein	hypothetical protein LOC100049136	11177	190	190	0	10	0
gi | 148235809	ribosomal protein	MGC85232 protein	16116	178	178	0	18	0
gi | 147904981	ribosomal protein	MGC85310 protein	20336	173	173	0	26	0
gi | 128414	nucleophosmin	Nucleophosmin (NPM) (Nucleolar phosphoprotein B23) (Numatrin) (Nucleolar protein NO38)	33626	151	151	0	12	0
gi | 2454534	HP1 gamma	heterochromatin protein 1 gamma	19706	149	233	84	9	13
gi | 148226660	ribosomal protein	ribosomal protein S6	28862	144	144	0	15	0
gi | 147900221	ribosomal protein	ribosomal Protein, Small subunit (28.1	28830	144	144	0	24	0

		kD) (rps-6)						
gi|153791553	ribosomal protein	hypothetical protein LOC100101273	15598	143	143	0	17	0
gi|148236420	ribosomal protein	ribosomal protein L19	23580	137	137	0	10	0
gi|147901081	ribosomal protein	hypothetical protein LOC734509	14960	128	128	0	14	0
gi|148226228	ribosomal protein	hypothetical protein LOC379427	15601	127	127	0	18	0
gi|148230110	ribosomal protein	MGC86356 protein	13370	125	125	0	20	0
gi|148234180	ribosomal protein	ribosomal protein L13a	26475	124	124	0	23	0
gi|71051864	ribosomal protein	Rpl34 protein	13482	120	120	0	10	0
gi|148226202	ribosomal protein	ribosomal protein S21	9323	116	116	0	10	0
gi|147904776	ribosomal protein	ribosomal protein L10a	24931	113	113	0	22	0
gi|62859151	ribosomal protein	hypothetical protein LOC548934	15817	112	112	0	8	0
gi|148224359	ribosomal protein	ribosomal protein L27a	16697	108	108	0	9	0
gi|148237484	ribosomal protein	MGC82841 protein	15571	108	108	0	7	0
		laminin receptor 1 (ribosomal protein						
gi|147902645	ribosomal protein	SA, 67 kDA)	34145	106	202	96	8	2
gi|148237328	ribosomal protein	hypothetical protein LOC779230	11648	102	102	0	7	0
gi|148237794	ribosomal protein	hypothetical protein LOC379490	22916	100	100	0	14	0
		ubiquitin and ribosomal protein L40						
gi|4507761	ribosomal protein	precursor	15004	100	100	0	14	0
		ubiquitin and ribosomal protein S27a						
gi|4506713	ribosomal protein	precursor	18296	94	94	0	12	0
gi|148223553	unknown	MGC83386 protein	29532	93	148	55	7	6
	protein-L-							
gi|148230102	isoaspartate	hypothetical protein LOC495685	24896	90	145	55	17	5
gi|148227306	ribosomal protein	similar to ribosomal protein L5	34318	88	88	0	11	0
gi|52346074	ribosomal protein	ribosomal protein S25	13878	85	85	0	13	0
		PREDICTED: similar to 40S ribosomal						
gi|149692855	ribosomal protein	protein S29 isoform 1	6886	84	84	0	7	0
gi|9988777	Aurora B	protein kinase AIRK2	41979	78	109	31	6	6
gi|929913	ribosomal protein	ribosomal protein S8	19450	78	78	0	18	0
gi|148232341	ribosomal protein	ribosomal protein S2e	30540	75	75	0	11	0
gi|148228991	ribosomal protein	MGC80163 protein	11682	73	73	0	5	0

gi|122936412	ribosomal protein	Rps2e protein	38650	73	73	0	8	0
gi|62858613	ribosomal protein	hypothetical protein LOC549704	7890	72	72	0	5	0
gi|27769162	ribosomal protein	RPL18A protein	20938	64	64	0	26	0
gi|147906637	H1	hypothetical protein LOC100037078	22908	60	123	63	11	9
gi|148226616	H1	linker histone H1A variant	23654	60	123	63	55	52
gi|65068	ribosomal protein	ribosomal protein L14	21784	56	56	0	11	0
gi|148236843	ribosomal protein	ribosomal protein L15	24218	55	55	0	9	0
gi|6677779	ribosomal protein	ribosomal protein L28	15781	54	54	0	7	0
gi|148235024	ribosomal protein	40S ribosomal small subunit protein S10	18871	54	54	0	13	0
gi|147901606	phosphatase	receptor protein tyrosine phosphatase LAR	201400	53	53	0	5	0
gi|148237408	ribosomal protein	hypothetical protein LOC100037080	32931	53	53	0	5	0
gi|33186863	ribosomal protein	ribosomal protein L13	24348	46	46	0	11	0
gi|147901835	actinin?	actinin, alpha 4	104559	44	44	0	13	0
gi|148229543	K HMT Suvar 4-20	hypothetical protein LOC495826	90460	43	43	0	6	0
gi|148228277	calcium-reg; hypertension	COMM domain containing 5	25504	42	42	0	2	0
gi|133863	ribosomal protein	40S ribosomal protein S24 (S19)	15274	40	40	0	2	0
gi|147900069	ribosomal protein	ribosomal protein L8	28128	38	38	0	9	0
gi|148223762	splicing	hypothetical protein LOC734926	28772	36	36	0	2	0
gi|147902824	anchors Gcoupled proteins	gravin-like	269251	35	35	0	10	0
gi|148229332	GST	hypothetical protein LOC495981	16383	35	35	0	2	0
gi|147899418	unknown	DC12 homolog	38768	35	35	0	5	0
gi|171460964	ribosomal protein	similar to RNA-binding region (RNP1, RRM) containing 7	96205	33	33	0	13	0
gi|52346106	ribosomal protein	MGC89873 protein	12432	33	33	0	7	0
gi|147899858	insulinoma protein	hypothetical protein LOC548611	17021	33	33	0	8	0
gi|147902230	aminotransferase	hypothetical protein LOC398751	57133	33	33	0	6	0

gi|58802759	nuclear filament	TPR	226373	32	32	0	8	0
gi|47940216	intracellular junctions	LOC432146 protein	79275	32	32	0	6	0
gi|147900327	unknow	hypothetical protein LOC432102	50550	32	32	0	4	0
gi|27371057	ribosomal protein	Snrpn-prov protein	24286	31	53	22	7	5
gi|148227702	ribosomal protein	ribosomal protein S10	18899	31	31	0	13	0
gi|147902597	SMC5	structural maintenance of chromosomes protein 5	125943	29	29	0	76	0
gi|20977022	mitotic phosphoprotein 22	mitotic phosphoprotein 22	13896	29	29	0	14	0
gi|148237292	glu-fru oxreductase	hypothetical protein LOC379195	42861	29	29	0	4	0
gi|147903282	ubiquitin liages	E3 ubiquitin ligase Smurf2	87183	28	76	48	65	70
gi|148745071	Wdrepeat contain protein 43	LOC100101293 protein	72992	28	28	0	2	0
gi|147899432	ATPase, Ca++ transporting, plasma membrane 3	ATPase, Ca++ transporting, plasma membrane 3	133645	27	27	0	82	0
gi|147905652	p63	p63 DNA binding protein/tumor protein p73-like	41535	27	27	0	10	0
gi|120577606	Nusap1	Unknown (protein for IMAGE:6316729)	60118	27	27	0	6	0
gi|148226545	dehydrogenase	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit	50907	27	27	0	13	0
gi|117667435	myosin-2a	myosin-2a	30531	26	26	0	44	0
gi|49899736	tRNA synthetase	LOC445827 protein	58191	26	26	0	9	0
gi|148236031	thyroid protein	Tsh3	111314	26	26	0	2	0

gi|147900678	sulfotransferase	hypothetical protein LOC495289	35371	26	26	0	3	0
gi|60729664	NMDRG1	N-myc downstream-regulated gene 1 protein - African clawed frog	38541	25	25	0	18	0
gi|147898729	choline transporter-like protein 4	MGC83045 protein	80864	25	25	0	2	0
gi|148234823	cell fate decision gastrulation	xoct-91 protein	49460	25	25	0	6	0
gi|148235018	p53 binding domain; mdm4	similar to transformed mouse 3T3 cell double minute 4	54454	25	25	0	11	0
gi|148232403	keratin	hypothetical protein LOC432054	45441	25	25	0	7	0
gi|148224371	keratin	adult keratin XAK-A	45658	25	25	0	8	0
gi|147899980	keratin	hypothetical protein LOC100037084	47509	25	25	0	6	0
gi|148224399	LKB1 interactpro; S/T kinase 11 interactpro	hypothetical protein LOC432019	129430	25	25	0	46	0
gi|148224674	ribosomal protein	hypothetical protein LOC734664	14442	25	25	0	1	0
gi|148226698	pax7	myogenic specification paired-box transcription factor Pax7	55037	24	24	0	2	0
gi|148227856	PARP?	hypothetical protein LOC431935	103873	24	24	0	2	0
gi|148233215	Rho_GAP activating protein 9	MGC83212 protein	57482	24	24	0	4	0
gi|148230861	otoraplin (cytokine)	hypothetical protein LOC779155	15352	24	24	0	2	0
gi|148222816	tyr kinase SGK196	hypothetical protein LOC496338	40801	24	24	0	1	0
gi|34098593	wnt signalling pathway	Dapper 1-B (xDpr)	91871	24	24	0	35	0
gi|47124730	X-prolyl aminopeptidase 2	LOC431877 protein	79782	24	24	0	31	0
gi|148233560	RAB6 (Ras	MGC83971 protein	23635	23	23	0	4	0

	family)							
gi|148234427	cartiage; ECM	matrilin 2	68716	23	23	0	32	0
	MKI67 FHA domain-interacting nucleolar							
gi|32450283	phosphoprotein	LOC398645 protein	33129	23	23	0	24	0
gi|147903072	golgi related	hypothetical protein LOC496077	32375	23	23	0	35	0
gi|148234307	citrus tasting?	inv2	113037	23	23	0	29	0
	trx factor cell fate							
gi|148228639	decision	bHLH protein Hesr-1/Hey1	32172	23	23	0	161	0
	DEAH (Asp-Glu-Ala-His) box							
gi|148225756	polypeptide 8	MGC80994 protein	90564	23	23	0	25	0
gi|27882190	ribosomal protein	RPL17 protein	21896	23	23	0	8	0
		B4 protein (Linker histone B4)						
gi|114795	H1	(Histone H1-like protein)	29292	23	23	0	9	0
gi|148231189	arp2/3	hypothetical protein LOC432109	34432	23	23	0	7	0
	ubiquitin specific							
gi|171846441	peptidase 20	Unknown (protein for MGC:179984)	95800	23	23	0	9	0
		ATP-binding domain 1 family						
gi|148231847	ABC ATPase	member C	33549	23	23	0	1	0
	asparagine-linked							
gi|118835751	glycosylation 3	Alg3 protein	37502	23	23	0	3	0
gi|126631954	unknown	LOC100049117 protein	9755	23	23	0	7	0
gi|148232706	phosphatase	phosphoserine phosphatase	26702	23	23	0	4	0
	ctd phosphatase (ribosomal							
gi|76779483	protein)	MGC131093 protein	53258	23	23	0	16	0
	Histone deacetylase							
gi|148224576	complex	hypothetical protein LOC380449	20141	23	23	0	1	0

gi|148231953	sodium channel associated protein 2	hypothetical protein LOC431936	119353	22	22	0	3	0
gi|147903755	Tudor domain-containing protein 5	hypothetical protein LOC100036842	109560	22	22	0	4	0
gi|148228068	ribosomal protein	ribosomal protein S6 kinase, polypeptide 4	85937	22	22	0	10	0
gi|28278733	nuclear matrix protein; Tho trx factor	LOC398505 protein	76125	22	22	0	16	0
gi|52346094	ribosomal protein	MGC89823 protein	8258	22	22	0	2	0
gi|148232114	peroxisome biogenesis disorder protein 1	MGC79116 protein	135090	22	22	0	29	0
gi|49256367	ABC ATPase; G protein (activating peptide)	LOC443714 protein	53815	22	22	0	3	0
gi|147900271	ABC ATPase; G protein	hypothetical protein LOC399059	45063	22	22	0	2	0
gi|4808456	requiem (apoptosis protein?)	Requiem protein	23066	22	22	0	7	0
gi|4808454	requiem (apoptosis protein?)	Requiem protein	44776	22	22	0	6	0
gi|90819152	Scc2; cohesin	Scc2-1A	320113	22	22	0	18	0
gi|90819156	Scc2; cohesin	Scc2-2A	321905	22	22	0	21	0
gi|147905017	trx factor	Tcfap2a protein	48120	22	22	0	8	0
gi|51950233	PDZ and LIM domain 4	LOC446969 protein	39997	22	22	0	35	0

gi|148224746	rho-associated kinase	Rho-associated kinase alpha	160268	22	22	0	11	0
gi|148224862	potassium channel modulatory factor 1	MGC80548 protein	42601	22	22	0	2	0
gi|147907004	Frizzled-6 (wnt pathway)	hypothetical protein LOC495007	80240	22	22	0	2	0
gi|148229521	p90 autoantigen?; Sbc domain (ATPase in DNA damage repair)	hypothetical protein LOC494846	102631	21	21	0	6	0
gi|148237614	plastin 3 T isoform; actin-bundling proteins	hypothetical protein LOC399002	71486	21	21	0	3	0
gi|147899424	tyr kinase 1; IgG cell adhesion domains	hypothetical protein LOC379546	77155	21	21	0	96	0
gi|148232826	smad4 ub ligase	ectodermin	122250	21	21	0	11	0
gi|49257700	cdc42 GEF	LOC443708 protein	77786	21	21	0	8	0
gi|148230655	ectodermin	hypothetical protein LOC734959	60893	21	21	0	2	0
gi|21362964	StARD; cholesterol trafficking protein	Steroidogenic acute regulatory protein (StAR) (StARD1)	32331	21	21	0	6	0
gi|148232401	StARD; cholesterol trafficking protein	hypothetical protein LOC735015	31920	21	21	0	5	0
gi|147904583	electron transfer flavoprotein	electron transfer flavoprotein, alpha polypeptide	34256	21	21	0	2	0
gi|148225412	complement immunology	fourth component of complement	191354	21	21	0	11	0
gi|24363093	glucosyltransferas	UDP-glucose ceramide	45306	21	21	0	2	0

	e	glucosyltransferase						
gi|147903479	GINS; nuclear pore	MGC82660 protein	21368	21	21	0	3	0
gi|147898636	capE; condensin	(XCAP-E)	136656	21	21	0	9	0
gi|50417414	kif21	Kif21a protein	64914	21	21	0	4	0
gi|11119968	cadherin	cadherin-6	88859	21	21	0	1	0
gi|147906595	proteasome	MGC84123 protein	30389	21	21	0	7	0
gi|122108	H1	Histone H1.0-B (Histone H1(0)-2) (Histone H5A) (XlH5A) (H1D) (H1-SA)	21049	21	21	0	55	0
gi|148230569	T54 protein-like; g patch (RNA binding)	hypothetical protein LOC414461	54890	21	21	0	20	0
gi|147900468	vacuolar protein sorting 24	hypothetical protein LOC431872	24923	21	21	0	5	0
gi|148225140	calcium channel	putative transient receptor potential channel	169278	21	21	0	3	0
gi|12642955	aminotransferase	ornithine aminotransferase	48561	21	21	0	8	0
gi|147901772	aminotransferase	ornithine aminotransferase	48453	21	21	0	8	0
gi|148223183	3-beta-hydroxysteroid dehydrogenase	hypothetical protein LOC734818	42846	21	21	0	5	0
gi|27781329	wwc1?	Wwc1 protein	85696	21	21	0	11	0
gi|49119515	trx factor	LOC443681 protein	73372	21	21	0	3	0
gi|148224850	unknown	hypothetical protein LOC432030	131397	21	21	0	1	0
gi|148236801	polyhomeotic-like 1 early development regulation	hypothetical protein LOC779336	89705	21	21	0	2	0
gi|153792418	trx factor; G elongation factor, mitochondrial 1	hypothetical protein LOC100101315	84211	21	21	0	3	0

gi|148224024	cullin 3	MGC80402 protein	89469	20	20	0	5	0
gi|4096766	neurotrophin receptor B xTrkB-alpha	neurotrophin receptor B xTrkB-alpha	92275	20	20	0	3	0
gi|147902053	Mcm2	hypothetical protein LOC431809	128168	20	20	0	7	0
gi|148222361	ubiquinol-cytochrome c reductase	ubiquinol-cytochrome c reductase core protein II	48003	20	20	0	8	0
gi|147899071	complement immunology	similar to I factor (complement)	69333	20	20	0	6	0
gi|147907100	Peripherin-2; tetraspanin; cell signalling	MGC84108 protein	42185	20	20	0	3	0
gi|147902431	egg envelope	egg envelope component ZPAX	102512	20	20	0	3	0
gi|147903623	glucocorticoid modulatory element binding	hypothetical protein LOC495295	58592	20	20	0	2	0
gi|147904198	glutamine synthetase	glutamine synthetase	44641	20	20	0	6	0
gi|147907174	SEC23 interacting protein	MGC84165 protein	112469	20	20	0	9	0
gi|62825972	acyl-Coenzyme A dehydrogenase	LOC733224 protein	27128	20	20	0	28	0
gi|47507239	zinc binding alcohol dehydrogenase	LOC432094 protein	43091	20	20	0	7	0
gi|148224305	SH3 domain containing ring finger 2	hypothetical protein LOC432194	82239	20	20	0	1	0
gi|134025851	thyroid hormone receptor interactor 11	LOC100049140 protein	60897	20	20	0	5	0

gi|148231968	IFN regulatory factor	interferon regulatory factor 2 binding protein 2	57949	20	20	0	3	0
gi|147906749	transmembrane protein 161A	MGC81925 protein	56898	20	20	0	2	0
gi|148230804	RAD54	hypothetical protein LOC432195	101977	20	20	0	7	0
gi|147903865	G protein-coupled receptor 124	hypothetical protein LOC399052	147020	20	20	0	5	0
gi|148234927	golgi complex 6	hypothetical protein LOC398871	73076	20	20	0	1	0
gi|168693569	PNK	hypothetical protein LOC100137702	61174	20	20	0	9	0
gi|148224676	Btg5; inhibit cell prolif	hypothetical protein LOC734504	27813	20	20	0	3	0
gi|148237189	G protein	hypothetical protein LOC734430	41685	20	20	0	4	0
gi|58701955	unknown	LOC733166 protein	38255	20	20	0	25	0
gi|148232744	Wee1	wee1 homolog	62482	20	20	0	5	0
gi|1351421	Wee1	Wee1-like protein kinase	62504	20	20	0	6	0
gi|76779743	Xgalectin-iva; carbohydrate binding proteins	LOC733366 protein	50966	20	20	0	5	0
gi|153792667	small G protein signaling modulator 3	hypothetical protein LOC100101321	86727	19	55	36	89	66
gi|148227802	ankyrin repeat containing	hypothetical protein LOC734696	36392	19	37	18	79	44
gi|148231460	deoxythymidylate kinase	hypothetical protein LOC414549	24090	19	19	0	8	0
gi|27924367	clatherin coating	Ap1s1 protein	21428	19	19	0	3	0
gi|147899434	calcium ATPase	Calcium ATPase at 60A	110772	19	19	0	6	0
gi|147907326	calcium ATPase	ATP2A2 protein	117030	19	19	0	3	0
gi|13991829	neurabin; PP1 binding	neurabin	88188	19	19	0	10	0
gi|148231350	LReO_3 ; LTR	71 kDa protein	71590	19	19	0	2	0

	retrotransposon of the Gmr1 group?							
	solute carrier family 4 sodium bicarbonate cotransporter							
gi|148225418	member 7	hypothetical protein LOC431976	127137	19	19	0	6	0
	phosphatase	phosphatase regulatory subunit						
gi|168693595	regulation	B56alpha	55582	19	19	0	16	0
	mid-gastulation	heparin-binding growth factor						
gi|9457239	regulatory	midkine	16198	19	19	0	21	0
gi|148233996	metalloproteinase	metalloprotease/disintegrin	86066	19	19	0	4	0
gi|108743262	trx factor	LIM-domain-binding protein 2a	43347	19	19	0	1	0
gi|148228969	ribosomal protein	hypothetical protein LOC494769	104515	19	19	0	9	0
	ankyrin repeat							
gi|148227546	containing	hypothetical protein LOC734461	26221	19	19	0	3	0
gi|148233954	keratin	hypothetical protein LOC100036944	60712	19	19	0	8	0
gi|114145561	keratin	keratin complex 2, basic, gene 8	54531	19	19	0	8	0
gi|148224670	trx factor	hypothetical protein LOC100036877	51167	19	19	0	137	0
		GATA-binding factor 6-A						
gi|2494686	trx factor	(Transcription factor xGATA-6A)	42324	19	19	0	6	0
	methyl transferase							
gi|148233197	ASMTL	MGC80821 protein	70816	19	19	0	45	0
gi|148236329	dactylin; ub path	hypothetical protein LOC100037117	48305	19	19	0	2	0
		CCAAT box transcription factor p122						
gi|10834850	trx factor	subunit	98902	19	19	0	3	0
gi|148238211	cadherin	hypothetical protein LOC495024	78763	18	18	0	3	0
	carnitine/acylcar							
gi|147905676	nitine translocase	hypothetical protein LOC495458	33061	18	18	0	3	0
gi|148236601	translin; DNA	translin	26392	18	18	0	5	0

	damage response, RNA and trl control							
	UDP-glucose pyrophosphoryla							
gi|148222635	se 2	MGC85503 protein	9681	18	18	0	14	0
gi|148231147	APAF1	MGC80868 protein	143994	18	18	0	31	0
gi|148234743	cathepsin	hypothetical protein LOC494810	53526	18	18	0	3	0
	RNA binding							
gi|148227152	motif protein 18	MGC81467 protein	21762	18	18	0	24	0
gi|148229927	annexin	annexin A1	37841	18	18	0	2	0
	brevican; extracellular	brevican soluble core protein						
gi|148230821	matrix	precursor	128263	18	18	0	5	0
		serine/threonine kinase 10 (polo-like						
gi|147903353	Plkk1	kinase kinase 1)	112203	18	18	0	19	0
	calcium and integrin binding 1							
gi|147899167	(calmyrin)	hypothetical protein LOC496348	21855	18	18	0	3	0
		nuclear Y/CCAAT-box binding factor						
gi|3170223	immunology	A subunit NF-YA	33006	18	18	0	3	0
	proteinase	serine (or cysteine) proteinase						
gi|147905402	inhibitor	inhibitor, clade E, member 1	45318	18	18	0	9	0
	receptory tyr kinase in							
gi|1545821	macrophase diff	Xron	154449	18	18	0	2	0
gi|47939821	unknown	LOC432183 protein	61312	18	18	0	2	0
gi|148233656	unknown	hypothetical protein LOC379925	42629	18	18	0	13	0
gi|157423002	Rap GEF3	Unknown (protein for MGC:181775)	33906	18	18	0	2	0
	IFN							
	developmental	interferon-related developmental						
gi|148227722	regulator	regulator 2	48759	18	18	0	1	0

gi|148226567	IFN regulatory factor	MGC83957 protein	73394	17	17	0	2	0
gi|150383504	monophosphate kinase	UMP-CMP kinase (Cytidylate kinase) (Deoxycytidylate kinase) (Cytidine monophosphate kinase) (Uridine monophosphate/cytidine monophosphate kinase) (UMP/CMP kinase) (UMP/CMPK) (Uridine monophosphate kinase)	22320	17	17	0	3	0
gi|147907413	twinkle; helicase for mtDNA repl	hypothetical protein LOC414487	78133	17	17	0	9	0
gi|148236605	phosphate cytidylyltransfera se 1 (CTD)	hypothetical protein LOC734348	42026	17	17	0	8	0
gi|148228999	glycosyltransferas e 1 domain containing 1	MGC81837 protein	33143	17	17	0	5	0
gi|76780152	coiled-coil domain containing 113	LOC733369 protein	44747	17	17	0	3	0
gi|33417067	oxysterol binding protein	LOC398688 protein	50841	17	17	0	55	0
gi|33520299	adenylate cyclase	adenylate cyclase type VII	76335	17	17	0	1	0
gi|47124764	PHD finger protein 20	LOC431868 protein	71864	17	17	0	33	0
gi|147901319	tudor domain containing 3	hypothetical protein LOC431810	72981	17	17	0	10	0
gi|148224150	Pentatricopeptide repeat domain 1; RNA associated?		78937	17	17	0	4	0
gi|148226348	Rad23 (NER)	RAD23 homolog B	44005	17	17	0	43	0
gi|148225642	Rad23 (NER)	hypothetical protein LOC734481	45116	17	17	0	59	0
gi|147905864	neighbor of BRCA1 gene 1	hypothetical protein LOC379658	108801	17	17	0	1	0

gi|147901107	bystin; embryo implantation	hypothetical protein LOC432128	49500	17	17	0	3	0
gi|109157561	H2A	Chain H, 2.9 Angstrom X-Ray Structure Of Hybrid Macroh2a Nucleosomes	13616	17	17	0	8	0
gi|80479435	zinc finger protein 262	Unknown (protein for MGC:132098)	102688	17	17	0	5	0
gi|65184	unknown	unnamed protein product	21581	17	17	0	8	0
gi|148229989	nephronophthisis 1; cell-cell contacts?	hypothetical protein LOC432293	78754	17	17	0	12	0
gi|68534338	Sarcalumenin; calcium signalling	LOC733292 protein	54371	17	17	0	4	0
gi|147905348	IP5P	MGC83747 protein	47884	17	17	0	158	0
gi|52346104	ribosomal protein	ribosomal protein L36A	12738	17	17	0	12	0
gi|120538303	DEAH (Asp-Glu-Ala-His) box polypeptide 30; ribosomal?	LOC100036956 protein	75415	17	17	0	9	0
gi|147901005	metalloproteinase	disintegrin metalloproteinase	96350	17	17	0	9	0
gi|125858566	angiotensin I converting enzyme	LOC100037182 protein	98364	17	17	0	3	0
gi|160420137	insuloma associated	insuloma-associated 1	48577	17	17	0	14	0
gi|163914567	NMDA receptor	N-methyl-D-aspartate receptor subunit NR2A	165857	16	16	0	10	0
gi|148234024	zinc transporter	solute carrier family 30 (zinc transporter), member 9	62504	16	16	0	5	0
gi|27503878	zfr; embryogenesis	Zfr protein	79539	16	16	0	7	0
gi|148227186	tudor domain containing 7	hypothetical protein LOC414521	122364	16	16	0	23	0
gi|76779660	midnolin; has UB like	MGC52897 protein	49682	16	16	0	51	0
gi|49117075	nuclear cap binding protein subunit 2	Unknown (protein for MGC:80352)	17725	16	16	0	2	0
gi|3283070	katanin	p80 katanin	38776	16	16	0	24	0

gi|83405227	Xnf7 (1 pub on spindle integrity!)	Unknown (protein for IMAGE:4202738)	70483	16	16	0	5	0
gi|148224429	adenylate cyclase	adenylate cyclase 9	152886	16	16	0	7	0
gi|288990	H3	histone H3	15445	16	16	0	4	0
gi|3745758	H3	Chain A, Complex Between Nucleosome Core Particle (H3,H4,H2a,H2b) And 146 Bp Long Dna Fragment	13267	16	16	0	2	0
gi|148231957	ubiquitin aldehyde binding 2	hypothetical protein LOC495334	28501	16	16	0	4	0
gi|148234919	fibroblast growth factor 7	hypothetical protein LOC734697	22508	16	16	0	5	0
gi|147905430	slowmo2; intra-mito sorting	hypothetical protein LOC494772	21520	16	16	0	1	0
gi|49257602	heterogeneous nuclear ribonucleoprotein A/B	Unknown (protein for MGC:83385)	36143	16	16	0	12	0
gi|65253	beta-catenin	X-plakoglobin	17723	16	16	0	13	0
gi|147902896	splicing	splicing factor U2AF large chain	51656	16	16	0	9	0
gi|148224417	calsyntenin-3; cadherins	MGC84020 protein	103736	16	16	0	7	0
gi|62740093	chromodomain helicase DNA binding protein 1	LOC733207 protein	162333	16	16	0	9	0
gi|147900053	splicing	splicing factor 3a, subunit 2, 66kDa	43760	16	16	0	2	0
gi|1483621	secretogranin III; secretory sorting	secretogranin III	51872	16	16	0	2	0
gi|171460984	Glucosidase II beta subunit-like protein	hypothetical protein LOC495829	55331	16	16	0	6	0
gi|148233574	intracellular digestion of proteins; dymeclin	MGC80949 protein	76642	16	16	0	7	0
gi|120577672	unidentified no domains	LOC100037015 protein	53555	15	15	0	10	0
gi|147903457	glutamate receptor	unitary non-NMDA glutamate	53828	15	15	0	3	0

		receptor subunit U1						
gi|1333645	mad2	Mad2 gene product	53290	15	15	0	172	0
gi|148232986	aminolevulinate synthase	MGC81838 protein	69765	14	31	17	145	101
gi|148228523	calpastatin; membrane fusion?	calpastatin	97110	13	28	15	28	5
gi|148224534	Lambda-crystallin	hypothetical protein LOC100036971	36173	7	24	17	223	258
gi|147900165	keratin	hypothetical protein LOC495267	63295	4	20	16	10	3
gi|2852363	cadherin	NF-protocadherin	114043	4	19	15	4	9
gi|148222999	keratin	larval keratin	59904	3	19	16	9	4
gi|148230046	TRIO and F-actin binding protein; lipid binding and SMC domains	MGC83528 protein	63165	1	22	21	157	87
gi|148232984	galactose mutarotase (aldose 1-epimerase)	hypothetical protein LOC100036941	38111	1	17	16	57	27
gi|148230434	tyr kinase 2	MGC83617 protein	137181	-2	16	18	86	61

ΔCPC extract was reconstituted with INCENP (FL) or INCENPΔCC (CC) for 90 min at 20°C. The INCENPs, and their associated proteins, were then purified using anti-INCENP antibodies. The associated proteins that ran below 50 kD by SDS-PAGE were analyzed by mass spectrometry (MS). The hand annotation is based on a BLAST search of the sequence provided by the MS/MS. The list is sorted by the decreasing difference in abundance of a peptide that was used to identify the protein between INCENP and INCENPΔCC. Diff, the difference between the protein scores; S, protein score; M, protein match.

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