DISSERTATION

A NEW DAWN FOR AURORA B REGULATION: SHINING LIGHT ON MULTIPLE DISCRETE POPULATIONS OF AURORA B KINASE AT CENTROMERES AND KINETOCHORES

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ABSTRACT

A NEW DAWN FOR AURORA B REGULATION: SHINING LIGHT ON MULTIPLE DISCRETE POPULATIONS OF AURORA B KINASE AT CENTROMERES AND KINETOCHORES

Cell division is a fundamental biological process that is essential for all eukaryotes to divide the replicated genome with high fidelity into individual daughter cells. Improper segregation of replicated DNA results in chromosome instability, a characteristic that is deleterious to most cells. Critical to the proper segregation of mitotic chromosomes is attachment to spindle microtubules, which are dynamic cytoskeleton filaments that drive the movement of chromosomes during mitosis. A complex network of proteins, collectively called the kinetochore, mediates microtubule attachments to chromosomes. Kinetochores are recruited to individual chromosomes through a specialized heterochromatin domain known as the centromere. Centromeric heterochromatin is comprised of both canonical, H3-containing nucleosomes as well as nucleosomes that contain the histone H3 variant CENP-A. Centromeres serve as a central point of organization in mitotic cells, recruiting both structural and regulatory kinetochore proteins to chromosomes.

This extensive protein/DNA network ensures the accurate segregation of chromosomes by regulation of proper kinetochore-microtubule attachments in mitosis. Kinetochore-microtubule interactions are regulated by Aurora B kinase, which phosphorylates outer

kinetochore substrates to promote release of erroneous attachments. Although Aurora B kinase substrates at the kinetochore are defined, little is known about how Aurora B is recruited to and evicted from kinetochores, in early and late mitosis, respectively, to regulate these essential interactions.

We set out to determine how Aurora B kinase is regulated during mitosis. We found that, contrary to the current model, Aurora B kinase and the Chromosomal Passenger Complex are recruited to distinct regions within the centromere and kinetochore. Furthermore, we found that accumulation of Aurora B kinase at centromeres is independent from Aurora B localization and activity at outer kinetochores. These results lead us to hypothesize a new model for Aurora B kinase regulation. In the direct recruitment model, a population of the kinase is recruited directly to kinetochores in early mitosis, then as mitosis progresses and kinetochore-microtubule attachments are stabilized, architectural changes within the kinetochore result in the eviction of outer-kinetochore localized Aurora B kinase and the stabilization of kinetochore-microtubule attachments.

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Chapter 1: Introduction

1.1 Mitosis

Mitotic cell division is a biological process essential for all eukaryotes to accurately divide the replicated genome with high fidelity into individual daughter cells. Improper segregation of replicated DNA results in chromosome instability, a characteristic that is deleterious to most cells [1, 2]. Successful cell division depends on a series of highly coordinated events in order to create identical daughter cells each with exact copies of the replicated genome.

Activation of CDK1 by the mitotic cyclin B starts a cascade of phosphorylation events that initiate the first phase of cell division—prophase [3]. In prophase the interphase microtubule cytoskeleton disassembles and reforms the mitotic spindle mainly from centrosomes which serve as the spindle poles in animal cells. Sister chromatin condenses to form mitotic chromosomes; and phosphorylation of nuclear envelope proteins and nuclear lamins results in breakdown of the nuclear envelope. Prometaphase follows and is marked by complete disassembly of the nuclear envelope. Then microtubules of the mitotic spindle begin forming attachments to mitotic chromosomes. Forces from microtubule plus end dynamics move chromosomes to the spindle equator in metaphase, where eventually stable bi-polar attachments between sister chromatids and spindle microtubules from opposite spindle poles form. Stable attachment results in silencing of the spindle assembly checkpoint and degradation of cohesin, which links sister chromatids before the onset of anaphase. In anaphase, microtubule depolymerization drives the now separated sister chromatids towards opposite spindle poles and the cell creates a new nuclear envelope around each set of

segregated chromosomes. During cytokinesis the cytoplasm of the cell is divided by pinching of the cell membrane at the cleavage furrow, creating two cells with identical sets of genomic material (Figure 1.1) [3, 4].

The Mitotic Spindle

One of the most important aspects of mitosis is formation of the mitotic spindle, which is a bi-polar array of highly dynamic microtubules (Figure 1.1). Microtubules are composed of alpha and beta tubulin heterodimers that bind head-to-tail forming polarized protofilaments that then associate in parallel to form a tubular structure typically composed of 13 protofilaments [3, 5-7]. Due to the nature in which protofilaments associate, a polarized track is formed with structural differences between the beta tubulin-exposed plus end and alpha tubulin-exposed minus end.

Both alpha and beta tubulin can bind guanosine triphosphate (GTP) however, only beta tubulin can hydrolyze GTP to guanosine diphosphate (GDP). The critical concentration of subunit addition to GTP-bound heterodimers in the microtubule lattice is more favorable than GDP bound heterodimers. Conversely, the dissociation of GDP heterodimers from the microtubule lattice is energetically more favorable than heterodimer addition. Therefore, periods of growth and shrinkage and the stochastic switching between the two states, termed dynamic instability, are defined by the GTP bound state of tubulin heterodimers within a microtubule lattice. GTP-bound tubulin heterodimers are added to GTP-capped protofilaments resulting in periods of rapid growth; however, when beta tubulin hydrolizes GTP in the microtubule lattice, resulting in GDP-bound heterodimers at the plus end of microtubules, protofilaments rapidly disassemble, resulting in microtubule catastrophe [5]. In mitosis, the minus ends of

microtubules are anchored to microtubule organizing centers at centrosomes that nucleate microtubule growth from the gamma tubulin ring complexes towards mitotic chromosomes [3, 5, 8]. Recent work has demonstrated that microtubules can also be nucleated from organizing centers within the microtubule lattice and from chromatin, contributing to microtubule density within the spindle [8]. Plus ends of microtubules bind mitotic chromosomes at kinetochores, that track both growing and shrinking ends [3, 5, 8]. The dynamic growth and shrinkage of microtubules helps drive mitotic chromosome movement during cell division.

While the dynamic instability of microtubules can generate force for chromosome movements, the structure and dynamics of the spindle are also regulated by microtubule associated motors and other microtubule associated proteins [8, 9]. There are several classes of microtubule associated proteins (MAPs) that contribute to the dynamics of microtubules within the mitotic spindle. For example, End Binding Proteins (EBs) bind the growing plus ends of microtubules and function to promote microtubule growth and minimize catastrophe, therefore regulating the length of microtubules within the spindle. EBs also form complexes with critical regulators of kinetochore-microtubule attachment proteins needed specifically at the plus ends of microtubules to facilitate kinetochore binding [10]. Another class of MAPs are polymerases that promote microtubule stability. Microtubule polymerases such as ch-TOG or CLASP1/2 promote polymerization either by directly binding to and stabilizing the microtubule lattice or increasing the local concentration of tubulin dimers to increase the rate of microtubule polymerization [10]. Conversely, depolymerizing MAPs such as the kinesin13 family member, mitotic centromere associated kinesin (MCAK), also exist within the spindle.

The functions of different MAPs are coordinated throughout mitosis to regulate microtubules thereby maintaining the structure and dynamics of the mitotic spindle [3, 10].

Also essential to maintenance of the spindle are microtubule associated motors. As the spindle is newly forming, centrosomes that nucleate microtubule polymerization are separated to opposite sides of the plane of division and anchored to the cell cortex. Both of these events are driven by microtubule motors. There is a large family of plus end directed motors, called kinesins, that function to balance the shape and dynamics of the mitotic spindle [10].The homotetrameric kinesin 5 (Eg5) crosslinks anti-parallel microtubules and walks towards plus ends, thereby sliding microtubules and generating poleward force that separates centrosomes [10, 11]. Once centrosomes are separated, Eg5 cross-links microtubules and functions to help balance forces so the spindle does not collapse [10]. Another important microtubule motor is dynein, the major minus end directed motor in cells, that helps position the spindle by anchoring centrosomes to the cell cortex [10, 12]. The coordinated regulation of microtubule associated motors and MAPs maintain the shape and dynamics of the mitotic spindle critical to successful cell division [3, 10].

Centromeres and the constitutive associated centromere network

The centromere is a specialized chromatin domain formed at the primary constriction site of mitotic chromosomes that is epigenetically inherited and contains the histone H3 variant CENP-A [13-15]. Centromeres mediate chromosome attachment to spindle microtubules by serving as a scaffold capable of recruiting important structural and regulatory proteins. Centromeric DNA is organized by nucleosomes that contain the

histone H3 variant CENP-A along with canonical nucleosomes containing histone H3 [16-21]. Nucleosomes contain 147 base pairs (bp) of DNA wrapped around a histone octamer that is typically composed of two histone H2A-H2B dimers and two histone H3-H4 dimers. However, at centromeres a fraction of nucleosomes contains CENP-A-H4 dimers in place of H3-H4 dimers [14, 20, 22]. CENP-A directs kinetochore assembly through interactions with the constitutive centromere associated network (CCAN) along with serving as a point of cohesion between mitotic chromosomes during cell division [19, 23, 24]. The CCAN serves as a dynamic framework to assemble the outer kinetochore complex when cells enter mitosis (Figure 1.2 adapted from Cleveland et al., 2003 [18]).

The simplest centromere, termed a point centromere, is found in budding yeast and contains one CENP-A/Cse4 nucleosome positioned on 125 bp of DNA per chromosome [18, 25-28]. In contrast, in humans and most other multicellular organisms, the centromere is complex and spans anywhere from 3-5 megabases of DNA, termed a regional centromere. Regional centromeres in humans contain repeating units of 171 base pairs that arrange to form higher order repeats [18, 27, 28]. DNA at regional centromeres is neither necessary nor sufficient to drive CENP-A deposition, instead the centromere is defined by epigenetic factors [18, 27]. While the sequence of DNA at centromeres is highly divergent across metazoans, similarities in DNA composition include the repetitive nature, higher order repeats and length of the monomer units [18, 20, 27]. Interestingly, regional centromeres encompass areas occupied with CENP-A containing nucleosomes interspersed with H3 containing nucleosomes [29] and it is not understood how compact mitotic chromosomes are organized in this region [27, 30].

While the structure of centromeric chromatin remains unresolved, molecular reconstitution of the CCAN has provided immense insight to how CENP-A nucleosomes interact with CCAN components and direct the formation of the outer kinetochore [24, 31, 32].

CENP-A containing nucleosomes bind directly to CENP-N and CENP-C of the CCAN network [31-34]. CENP-C is critical and organizes the CCAN while also directly recruiting the kinetochore-microtubule network [32]. CENP-T is another critical component of the CCAN that functions in parallel with CENP-C to recruit the KMN network, but does not directly interact with CENP-A [35]. The CCAN forms through multiple, interdependent and cooperative binding between CENP complexes and serves as a mechanical bridge between CENP-A on mitotic chromosomes and proteins directly involved in microtubule attachment. Collectively the CCAN components comprise the inner kinetochore region and the “kinetochore-microtubule network” (KMN) comprises the outer kinetochore (Figure 1.2 adapted from Cleveland et al., 2003) [18]. In budding yeast, point centromeres recruit a single CCAN complex and KMN network that bind one microtubule, however, in humans with regional centromeres, many copies of the CCAN are recruited to centromeres and multiple KMN networks are present that bind many microtubules at kinetochores forming microtubule bundles.

In addition to CCAN recruitment, another important aspect of centromeres is maintaining a point of contact between sister chromatid throughout the course of mitosis [36, 37]. From S phase, when DNA is replicated, until mitosis, it is essential to maintain points of contact between replicated chromatin, and this is achieved by the multi-subunit complex cohesin that forms ring-like structures around sister strands of DNA [36, 38-

40]. In early prophase cohesin complexes are cleaved along chromosome arms through a mechanism called the “prophase pathway”, but cohesin complexes at the centromere are retained, thus forming the iconic X-shaped chromosomes in mitosis [37]. Briefly, in the prophase pathway Aurora B kinase and Polo like kinase 1 phosphorylate subunits of the cohesin complex, recruiting the protein WapL that separates the ring complex, resulting in dissociation of cohesin from chromosome arms. However, and importantly, cohesin complexes at the centromere region of mitotic chromosomes are protected until anaphase onset [36, 37]. Protection of centromere cohesin complexes is a result of Shugosin (Sgo1) proteins that are recruited to centromeres and bind protein phosphatase 2A (PP2A) to inhibit WAPL interactions at the centromere [41, 42]. As mitosis progresses and the spindle assembly checkpoint is silenced, Separase, a cysteine protease, is activated by the anaphase promoting complex and cleaves cohesin complexes at centromeres [40, 43]. Centromeres are thus critical to maintaining sister chromatid cohesion as well as serving as a point of assembly for outer kinetochores.

Kinetochores

The kinetochore is comprised of over 100 proteins that mediate chromosome segregation during mitosis by providing a physical link between chromatin and microtubules. Sister kinetochores assemble on centromeric chromatin at the beginning of mitosis and bind the plus ends of microtubules. Following correct amphitelic attachment, microtubule depolymerization drives poleward movement of chromosomes when kinetochores track depolymerizing plus end tips.

Several protein complexes comprise the kinetochore-microtubule binding interface including the NDC80, KNL1, and MIS12 complexes (KMN) and each contributes to kinetochore-microtubule binding *in vivo* (Figure 1.3A,B adapted from Wimbish and DeLuca 2020) [44-48]. Depletion of the NDC80 complex severely impairs kinetochore-microtubule interactions, suggesting this is an essential component for the binding of microtubules to kinetochores and therefore a target for regulation of kinetochore-microtubule interactions [48, 49]. NDC80 contains four subunits, Spc24, Spc25, Hec1, and Nuf2, that arrange to form a dumbbell-shaped protein complex (Figure 1.3 C adapted from Wimbish and DeLuca 2020) [48, 50-55]. Spc24 and 25 anchor NDC80 to kinetochores by binding at the inner kinetochore [53-55] while Hec1 and Nuf2 form a globular structure extended to the outer kinetochore that directly binds microtubules [45, 48, 50]. The microtubule binding activity of NDC80 is attributed to the calponin homology domain (CH) and the highly basic “tail” domain of Hec1 that binds the acidic tails on tubulin [24, 48, 50]. KNL1 also contains a microtubule binding domain, however unlike NDC80, KNL1 binding to microtubules is not essential for cell division [45]. Additional factors are recruited to kinetochores as microtubules accumulate to help mature and stabilize attachments such as the SKA complex and Astrin/Skap [56-59]. While the kinetochore-microtubule network directly links chromosomes to microtubules, many more protein complexes are recruited to kinetochores to regulate and maintain these essential interactions [60].

KNL1 directly binds microtubules but also serves as a kinetochore scaffold that recruits important regulatory phosphatases and kinases along with components of the spindle assembly checkpoint [61]. The spindle assembly checkpoint is a diffusible

molecular signal that propagates from unattached kinetochores to inhibit the anaphase promoting complex and therefore initiation of anaphase until all kinetochores are properly attached to microtubules [62-70]. Upon correct kinetochore-microtubule attachment, SAC proteins are evicted from kinetochores and the spindle assembly checkpoint is silenced resulting in the onset of anaphase [71].

1.2 Aurora B kinase regulates kinetochore-microtubule attachment stability in mitosis[1]

Equal division of genetic material during mitosis requires that each sister chromatid of a mitotic chromosome stably attach to spindle microtubules emanating from each of the two opposite spindle poles. Successful chromosome segregation also requires the precise regulation of kinetochore-microtubule attachment stability. In early mitosis, the mitotic spindle begins to form and establish its proper geometry at the same time that microtubules begin to dynamically probe for chromosomes, and as a result, erroneous kinetochore-microtubule attachments are likely to form (Figure 1.4) [19, 46, 72-75]. In order to prevent premature stabilization of kinetochore-microtubule attachments and to limit the accumulation of erroneous attachments, microtubule

[1] The work presented in the following sections of the introduction to my thesis, was submitted as a research review on March 16, 2020. Figures and material presented has been amended from the published version for clarity.

AJB and JGD wrote the review, JGD made all figures.

Broad AJ, and DeLuca JG. The right place at the right time-Aurora B kinase localization at centromeres and kinetochores. Essays in Biochem. Submitted March 2020.

turnover at kinetochores is high during early mitosis; conversely, as mitosis progresses and chromosomes begin to bi-orient, kinetochore-microtubule turnover decreases and stably-bound microtubules accumulate at kinetochores [1, 48, 72, 75-77]. These stable attachments allow kinetochores to harness the forces generated from depolymerizing microtubule plus ends to power chromosome movements and to silence the spindle assembly checkpoint, which delays anaphase until all kinetochores are properly connected to microtubules [48, 71, 72, 75, 78, 79].

The Chromosomal Passenger Complex (CPC) is comprised of INCENP, Borealin, Survivin and Aurora B kinase, the enzymatic component of the complex which phosphorylates multiple substrates on mitotic chromosomes to ensure proper chromosome segregation (Figure 1.5 adapted from Krenn and Mussachio 2015) [80-83]. One of the numerous functions of Aurora B kinase is to regulate kinetochore-microtubule attachment stability [21, 80, 84-86]. For this purpose, Aurora B phosphorylates outer kinetochore-associated substrates, including Hec1 of the NDC80 complex, which directly links kinetochores to microtubules [45, 47, 48]. Phosphorylation of Hec1 decreases the affinity of NDC80 complexes for microtubules, and as a result, reduces kinetochore-microtubule attachment stability [87-90]. As mitosis progresses, Aurora B kinase-mediated phosphorylation of Hec1 and other outer kinetochore substrates decreases, resulting in increased stabilization of kinetochore-microtubule attachments, which in turn promotes chromosome congression and silencing of the spindle assembly checkpoint [71, 79, 87-90].

Aurora B kinase

Aurora B kinase is a member of the Aurora family of serine/threonine kinases essential for cell division [80, 91]. Humans and most other vertebrate eukaryotes contain three Aurora kinases A, B, and C [91]. While the kinase domain of the Auroras is conserved, differences in the N and C terminal domains result in specific subcellular targeting during mitosis for independent roles [91]. The C-terminal domain is responsible for binding to co-factors that induce structural changes in the kinase and promote activity [51, 91]. Aurora C is expressed during meiosis in germ cells, while Aurora A and B are highly expressed during somatic cell division [80]. Differences in co-factor interactions result in differential sub cellular localization of Aurora A and B during mitosis [91]. Aurora A predominately localizes to centrosomes where it is activated by a microtubule binding protein, TPX2, and functions in centrosome separation, chromosome congression and phosphorylation of pole localized kinetochore substrates such as Hec1 and CENP-E [92-96]. Recent studies highlight a role for Aurora A at the kinetochore in late mitosis to phosphorylate Hec1 for proper chromosome congression [93], suggesting regulation of the Aurora kinases is not only dependent on differences in sub-cellular localization. How the Aurora kinases coordinate multiple, overlapping functions in cell division is an ongoing area of interest.

Aurora B localizes prominently to mitotic chromosomes in early mitosis and is re-located to the spindle midzone during cytokinesis [81, 97-102]. Aurora B localization and activity are directed by its co-factor, INCENP, that binds and activates Aurora B allosterically. Binding induces a conformational change in the phosphorylated activation loop (pThr248) and opening of the catalytic cleft of Aurora B leading to intermediate kinase activity [51, 100]. Full kinase activity occurs when Aurora B phosphorylates the

TSS motif of INCENP, resulting in further conformational changes that fully activate Aurora B kinase [51, 103-105]. Aurora B kinase is essential for chromosome condensation, chromosome alignment, and finally formation of the cleavage furrow during cytokinesis for successful cell division [80, 91].

INCENP

INCENP (inner-centromere protein) serves as a scaffold for the CPC, linking Aurora B to Survivin and Borealin along with regulating the localization of the complex. The C-terminal IN box of INCENP binds Aurora B while the N-terminal centromere targeting domain forms a three-helix-bundle with Survivin and Borealin [51, 100, 106]. INCENP is directed to centromeres by binding Survivin and heterochromatin protein HP1 alpha [80, 107]. The N- and C-termini of INCENP have well defined roles in CPC regulation but the large unstructured central domain of INCENP is largely not understood [21, 108-110]. The central domain of INCENP is regulated by phosphorylation and implicated in targeting the CPC to microtubules. While CPC binding to microtubules is essential for cytokinesis, how microtubules influence CPC activity in early mitosis is largely controversial [99, 108, 111-118]. Along with targeting Aurora B kinase, the CEN domain of INCENP plays a role in cohesion protection at centromeres [109, 110]. INCENP is a pivotal component of the CPC.

Survivin

The third component of the CPC was first discovered as an inhibitor of apoptosis that accumulates in G2 before the onset of mitosis, therefore gaining the name, Survivin [119]. While Survivin has been studied extensively as an inhibitor of apoptosis in cancer cells, our focus will be limited to the role Survivin has during cell division and binding the

CPC. Survivin targets the CPC to centromeres by directly interacting with phosphorylated histone, H3 [120-125] discussed in further detail below. Survivin has a zinc-coordinated N-terminal baculovirus inhibitor of apoptosis repeat (BIR) domain and C-terminal helical extension that interacts with Borealin and INCENP to form a three-helix bundle [106, 121, 122, 124]. The BIR domain of Survivin is critical for binding to phosphorylated histone, H3, and directs accumulation of the CPC at centromeres [106, 121, 122, 124, 126]. Although Survivin is required for targeting the CPC to centromeres and binding INCENP, the role of Survivin in centromere targeting is not essential for Aurora B kinases role during cell division [120, 126-128].

Borealin

Borealin was the last of the identified CPC subunits [97], and is implicated in bridging Sgo1 binding to the CPC [42, 125, 129, 130]. Borealin can be divided into functional domains much like the other members of the CPC. The N-termius forms an alpha helix that simultaneously binds Survivin and INCENP forming the three-helix bundle [106, 124] and the C-terminus contains a dimerization domain for self-association [131, 132]. The central region of Borealin is implicated in binding Sgo1 [42, 125, 129, 130] however this interaction has never been directly observed in human cells. Recently, Borealin dimerization has been implicated in maintaining centromere CPC in the absence of Survivin [131], though the mechanisms of this phenomena are not well understood. Finally, Borealin binds microtubules [111] which may be important for maintaining error correction at kinetochores with merotelic attachments [73, 111]. In sum, Borealin is a conserved member of the CPC important for Aurora B localization during mitosis.

It is well documented that the CPC forms a 1:1:1:1 complex *in vitro* [97, 104, 106, 132]. Nevertheless, given the evidence that individual subunits can associate with centromeres and kinetochores independent of the other CPC subunits, and there are additional supplementary proteins besides the canonical members of the CPC implicated in traveling with and regulating the complex [117, 126-128, 131, 133], important questions remain regarding sub-complexes of the CPC that may function *in vivo*.

Sgo1 is a critical CPC bridging factor

Although Sgo1 is not a member of the CPC, it is a conserved adaptor from yeast to humans and bridges CPC localization to histone modifications at the centromere [42, 125, 129, 130]. Sgo1 has long been known to play an important role as the “protector” of cohesin at the centromere in mitosis but Sgo1 also binds the CPC and has a role in recruiting Aurora B kinase to centromeres [41, 134]. Sgo1 is directly recruited to centromeres via an interaction with cohesin where it can recruit protein phosphatase 2A (PP2A) to de-phosphorylate cohesin subunits preventing degradation by WapL [2, 135]. Reduction of the inner-centromere pool of Sgo1 results in early sister chromatid separation by premature cohesin degradation; however, discrepancies in the literature exist explaining the effect of pH2A-T120, a histone modification implicated in Sgo1 and CPC recruitment to centromeres [41, 136]. Early reports found that the phosphorylation of H2A-T120 by Bub1 kinase is necessary to maintain an inner-centromere pool of Sgo1 [41, 42]. In yeast Sgo1 directly binds pH2A-T120 and the CPC, therefore bridging nucleosome modification at the centromere to CPC recruitment [41, 42, 125, 130, 135-137].

The Hongtao Yu lab described two distinct pools of Sgo1 that are recruited to the centromere and kinetochore-proximal centromere independently [41]. The Yu lab tested two mutants of Sgo1: a phosphodeficient-T346A that interrupts Sgo1 binding cohesin and K492A, a mutatant that is disrupted for pH2A-T120 binding. Interestingly, when expressed in cells, Sgo1^{T346A} could not localize to centromeres, and this resulted in a Sgo1 pool that was retained at kinetochores and co-localized with Bub1, an outer kinetochore protein [41]. When expressed in cells, Sgo1^{K492A} retained some centromeric localization but spread along chromosome arms unlike wild-type (WT) Sgo1 [41]. Expression of Sgo1^{K492A} substantially suppressed cohesin defects observed in Sgo1 deficient cells, while Sgo1^{T346A} was unable to rescue the observed phenotype [41]. These data suggest that the inner-centromere Sgo1 pool is largely responsible for cohesin protection and the outer kinetochore Sgo1 pool has an independent function [41]. In addition, the Yu lab recently published data indicating Sgo1 is recruited to kinetochore-proximal outer centromeres in early mitosis then subsequently recruited to inner-centromeres in a stepwise manner [130]. This could explain why Sgo1^{K492A} did not fully restore the centromeric pool of Sgo1, resulting in the cohesin defects, and importantly, implies there exists a second pool of Sgo1 at the kinetochore-proximal outer centromere that is directly recruited by pH2A-T120. A relationship between Sgo1, a cohesin protector, and the CPC, the major kinetochore-microtubule attachment regulator, may imply cooperative molecular mechanisms that lead to proper chromosome segregation. Sgo1 binds the CPC in yeast and interacts with modified nucleosomes yet little is understood about how these interactions are important at the

kinetochore and in human cells. How Sgo1 regulates and recruits the CPC to centromeres and kinetochores is examined in the following chapters [126-128].

1.3 Current models for kinetochore-microtubule attachment regulation by Aurora B kinase

Although proper regulation of kinetochore-microtubule attachments requires that Aurora B phosphorylate substrates at outer kinetochores, the kinase itself, along with the rest of the CPC, resides prominently at inner centromeres during mitosis [97, 99-102]. To explain how the centromere-localized kinase is capable of regulating the function of outer kinetochore proteins, researchers have proposed the “spatial positioning” model (Figure 1.6), which posits that activated Aurora B kinase emanates from the inner centromere as a diffusible gradient to phosphorylate its substrates [44, 85, 138-142]. In this model, Aurora B is recruited to and activated at inner centromeres in early mitosis, prior to formation of kinetochore-microtubule attachments [44, 99, 107, 108, 111-118, 139-144]. Kinetochores in this case lack pulling forces from attached microtubules and are physically close to the inner centromere. As such, kinetochore substrates are situated within the reach of the kinase and are highly phosphorylated. As kinetochore-microtubule attachments are generated, microtubule-based pulling forces stretch kinetochores away from centromeres and outside the boundaries of the Aurora B gradient, which results in decreased phosphorylation of kinetochore substrates and subsequent stabilization of kinetochore-microtubule attachments [44, 85, 138-142, 145]. In support of this model, Liu et al. [141] demonstrated that an ectopically targeted FRET sensor capable of detecting Aurora B kinase activity was phosphorylated when positioned at centromeres, but not at kinetochores, when kinetochores were properly bi-oriented. Additionally, ectopically

targeting Aurora B to kinetochores using a Mis12-INCENP fusion protein destabilized kinetochore-microtubule attachments and delayed spindle assembly checkpoint silencing. This led the authors to conclude that stabilization of attachments in metaphase results from the spatial separation of outer kinetochore substrates from centromere-localized Aurora B kinase [141]. It is important to point out however, that other models describing Aurora B regulation of attachment stability (discussed below) predict that irreversibly targeting Aurora B to kinetochores in metaphase, when kinase activity at this region is known to be low, would lead to destabilization of attachments. Thus, the data presented by Liu et al., [141] do not necessarily rule out other mechanistic models for Aurora B regulation of kinetochore-microtubule attachment stability [141].

The spatial positioning model described above is rooted in the idea that Aurora B kinase emanates from the inner centromere as a steep, diffusible gradient capable of differentially phosphorylating substrates within a short distance (~50-100 nm) [44, 88, 89, 141, 145-147]. The presence of such a fine-tuned gradient is debated, and the mechanisms for how the proposed gradient is established and maintained remain unknown [21, 142, 145, 146, 148, 149]. Similar to the spatial positioning model, the “dog leash” model accounts for differential activity of Aurora B kinase towards its substrates based on their distance from the centromere but does not rely on a diffusible gradient of the kinase. In this proposed mechanism, Aurora B’s “zone” of activity is restricted by its interaction with the CPC component INCENP, which contains a long single α-Helix (SAH) coiled-coil domain that may be capable of extending up to 80 nm to reach kinetochore substrates in early, but not late mitosis [21, 108, 144]. Consistent with this model, expression of a mutant version of chicken INCENP containing a shortened SAH domain

in human cells resulted in decreased phosphorylation of outer kinetochore-, but not centromere-localized Aurora B substrates [108].

A growing number of studies in both budding yeast and human cells have demonstrated that Aurora B kinase localizes not only to centromeres, but also to kinetochores, suggesting an alternative regulatory mechanism for controlling kinetochore-microtubule attachment stability, in which Aurora B kinase is recruited directly to kinetochores in early mitosis to phosphorylate its substrates and in turn, is evicted from kinetochores as stable attachments form (Figure 1.7) [21, 87, 117, 126-128, 131, 133, 150, 151]. In the following chapters we discuss results that shed light on the localization and functional properties of the CPC at both centromeres and kinetochores and how these new findings may lead to refinement of the current model for Aurora B kinase regulation of kinetochore-microtubule attachments during mitosis.

Aurora B and the CPC are recruited to centromeres via phosphorylated histones

Aurora B kinase and its CPC cofactors are recruited to the centromere region of mitotic chromosomes just prior to nuclear envelope breakdown [80, 116, 152], and this recruitment depends on phosphorylation of histones H3 and H2A (Figure 1.8) [125]. A significant body of work has demonstrated that Haspin kinase phosphorylates histone H3 at Thr3 (pH3-T3), which recruits the CPC component Survivin [121, 122, 125, 153]. The BIR domain of Survivin directly interacts with pH3-T3 [101, 106, 121, 123, 126], while a separate helical domain of Survivin forms a three-helix bundle with Borealin and INCENP, which is connected to Aurora B through the C-terminal IN-Box of INCENP [100, 106]. It has also been demonstrated that Bub1 phosphorylates histone H2A at Thr120 which recruits the Shugoshin proteins Sgo1 and Sgo2 to centromeres, which in

turn recruit the CPC [42, 125, 129, 130, 136]. In metazoans, a number of studies have suggested that this linkage is mediated through Borealin [42, 125, 129, 130, 136]. Antibodies to both pH3-T3 and pH2A-T120 localize to centromeres, and loss of either phospho-mark reduces centromeric CPC localization, which has led to a model in which Aurora B kinase and the CPC are recruited to regions of centromeric chromatin where the two marks overlap [123, 125, 129, 154]. This concentrated pool of centromere-localized Aurora B kinase is proposed to phosphorylate both centromere and kinetochore substrates to ensure proper chromosome congression and segregation [44, 121, 125, 136, 139, 141, 155, 156].

1.4 Thesis rationale

Although Aurora B kinase substrates at the kinetochore are defined, little is known about how Aurora B is recruited to and evicted from kinetochores, in early and late mitosis, respectively, to regulate these essential interactions. The current model describing the recruitment of Aurora B Kinase to centromeres proposes that two histone modifications are required. Specifically, phosphorylation of histone H3 (pH3-T3) and histone H2A (pH2A-T120) is suggested to localize Aurora B to the inner centromere. A major premise of the current model is that histone modifications pH3-T3 and pH2A-T120 spatially overlap, and this region of overlap defines the inner centromere and directs Aurora B recruitment. Contrary to the proposed model, however, immunostaining experiments reveal that pH3-T3 localizes to the inner centromere, while pH2A-T120 distinctly localizes to the kinetochore-proximal outer centromere. Thus, major questions remain, including how histone modifications affect the binding of Aurora B to regulate kinetochore-microtubule attachments.

We aim to determine the molecular basis for Aurora B kinase recruitment to both centromeres and kinetochores. A greater understanding of Aurora B kinase activity at the kinetochore will lead to a greater understanding of the regulation of kinetochore-microtubule attachments and the underlying basis for chromosome segregation errors. Most cancer types display structural changes in their chromosomes such as aneuploidy, deletions, or translocations that are thought to lead to tumorigenisis. Nonetheless, the underlying mechanisms that contribute to these errors are not entirely clear [2, 157]. Aberrant kinetochore-microtubule attachments, which are regulated by Aurora B kinase, are a common feature in most cancer cells [2, 157, 158]. The following chapters provide insight to the molecular mechanisms by which Aurora B kinase regulates chromosome alignment.

Figures 1

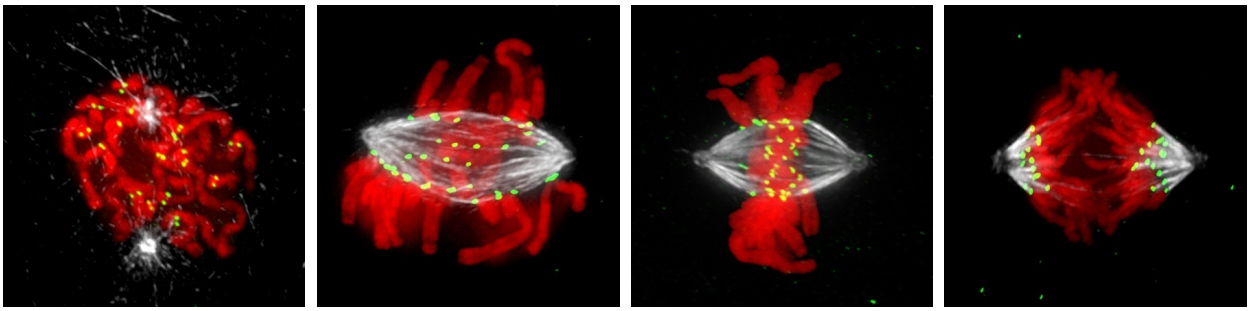


Figure 1.1. Phases of mitotic cell division. Mitotic chromosomes are shown in red, microtubules of the mitotic spindle are white, and kinetochores are green.

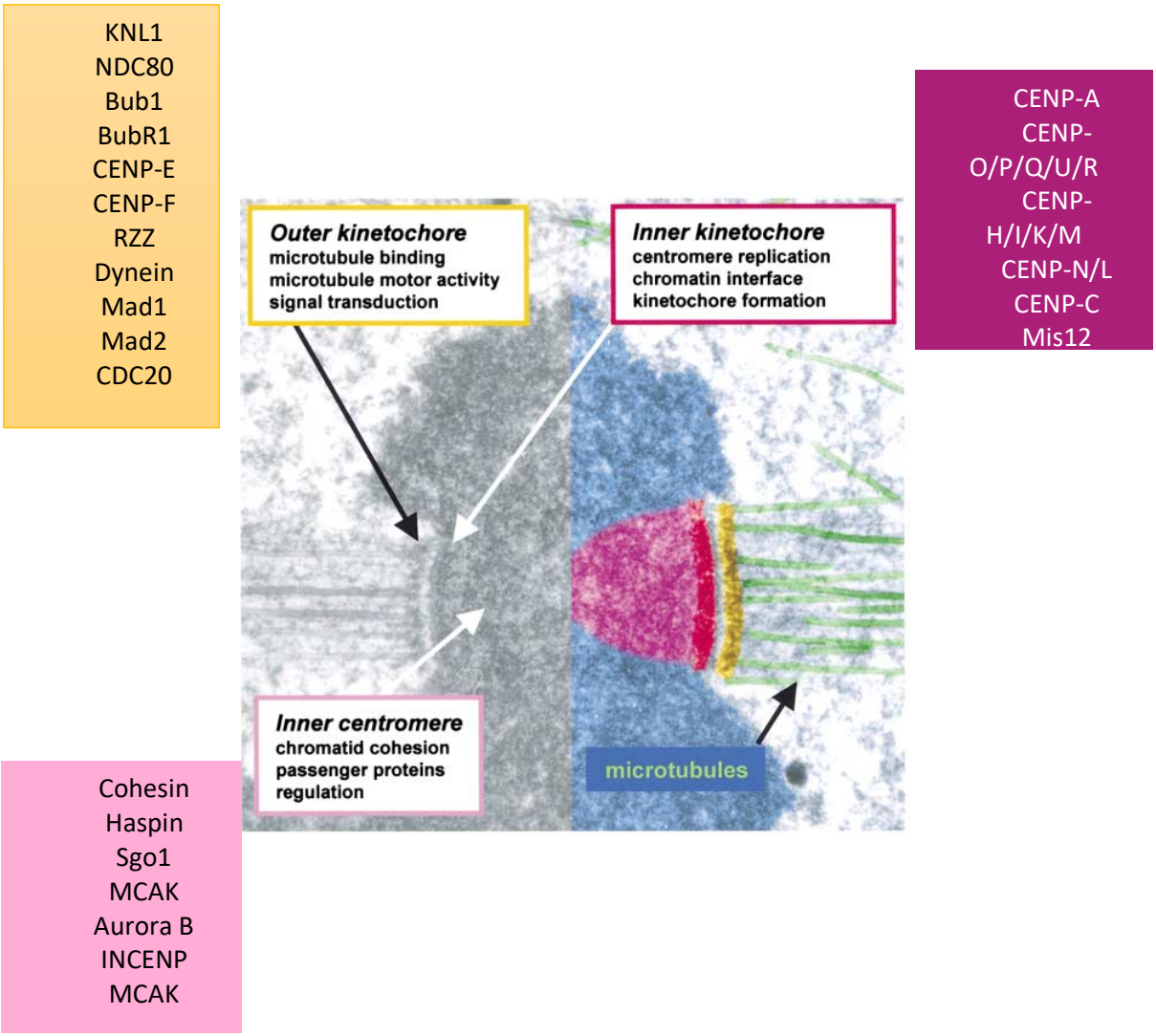


Figure 1.2. Organization of the centromere, inner kinetochore, and outer kinetochore. Electron micrograph with microtubules pseudo-colored green, the outer kinetochore yellow, the inner kinetochore dark pink, and the inner centromere light pink. Listed are representative proteins present at each position. Adapted from Cleveland et al., 2003 [18].

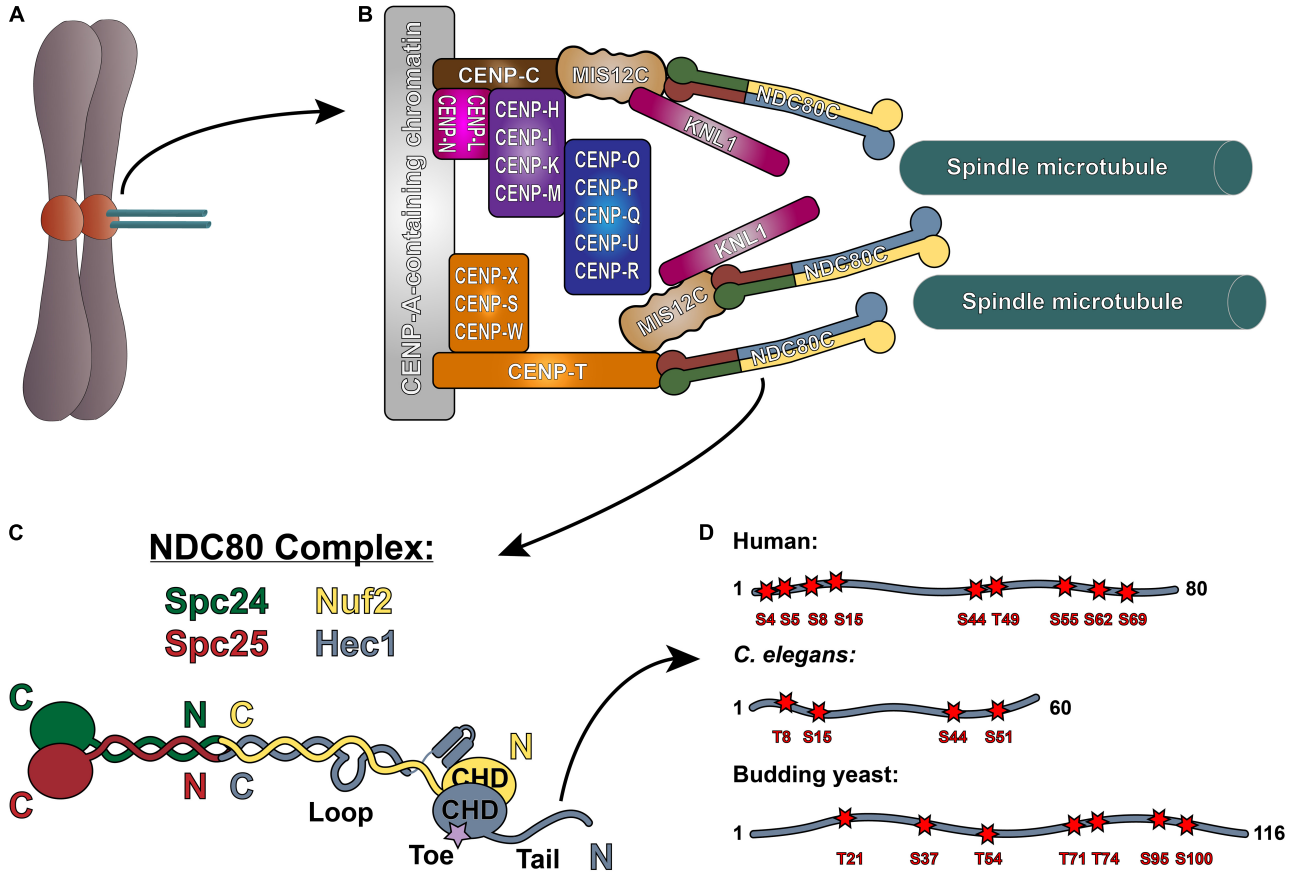


Figure 1.3. Organization of the kinetochore-microtubule interface. A) Cartoon image of mitotic chromosome. B) Organization of kinetochore-microtubule associated network. NDC80 is a critical outer kinetochore component that directly binds microtubules. C) Structural organization of the NDC80 complex. Calponin homology (CHD-purple star) and tail domains of NDC80 directly bind microtubules. D) Phosphorylation on the Hec1 tail by Aurora B kinase regulates kinetochore-microtubule attachments (red stars are phosphorylation sites). Adapted from Wimbish and DeLuca 2020 [52].

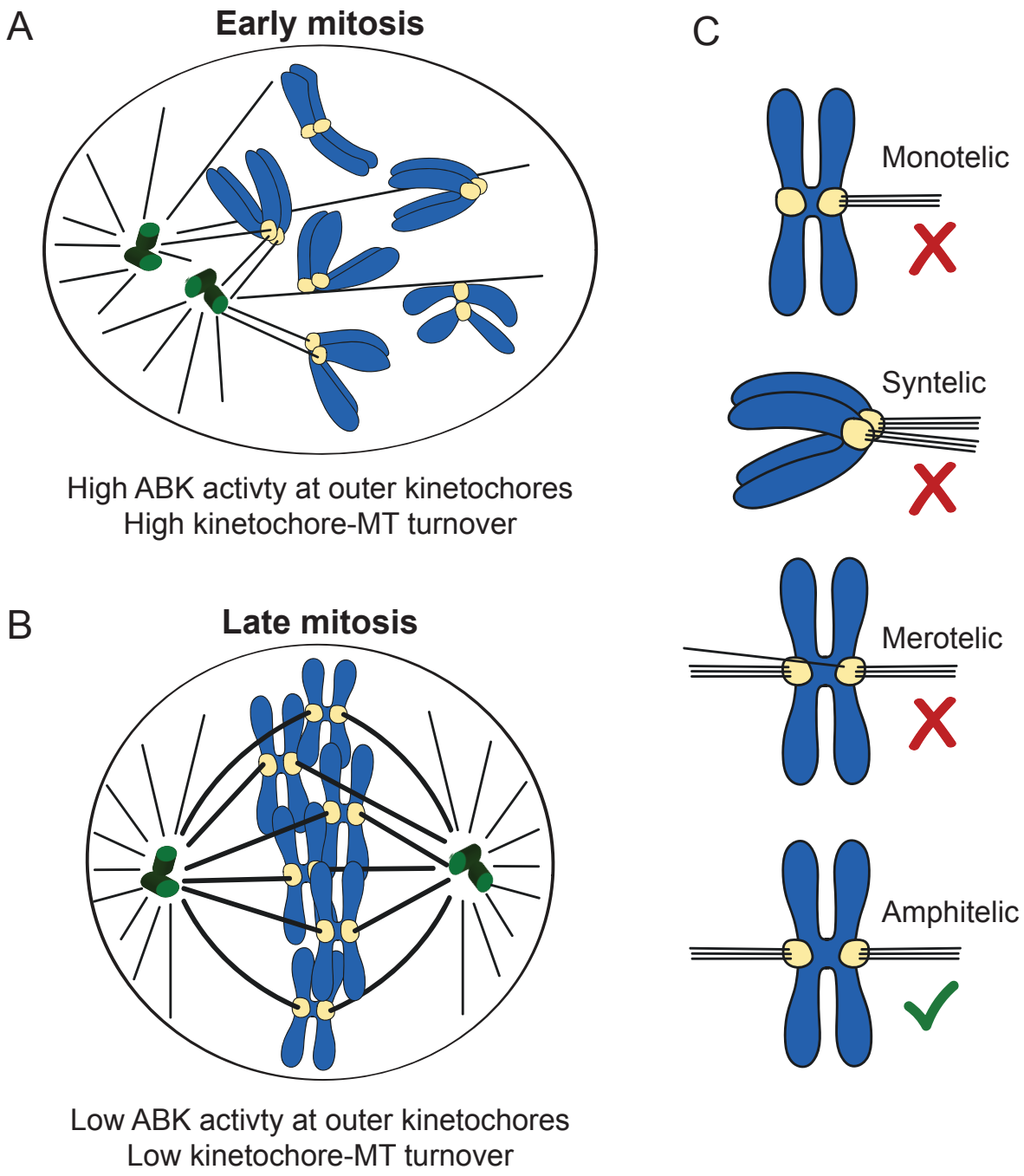


Figure 1.4. Kinetochore-microtubule attachments in mitosis. (A) In early mitosis, kinetochore-microtubule attachment errors are common, Aurora B kinase activity is high at outer kinetochores, and kinetochore-microtubule turnover is high to prevent premature stabilization of attachments. (B) In late mitosis, kinetochore-microtubule errors are infrequent, Aurora B kinase activity is low at outer kinetochores, and kinetochore-microtubule turnover is low to promote attachment stabilization. (C) Types of kinetochore-

microtubule attachments are shown. Erroneous attachments include monotelic, syntelic, and merotelic attachments. Monotelic attachments occur when one kinetochore is attached to microtubules emanating from one spindle pole, and its sister kinetochore is unattached. Syntelic attachments occur when both sister kinetochores are attached to microtubules emanating from the same spindle pole. Merotelic attachments occur when one sister kinetochore is attached to microtubules emanating from one pole, and its sister is attached to microtubules emanating from both spindle poles. Correct, amphitelic attachments, in which one sister is attached to microtubules emanating from one spindle pole and its sister is attached to microtubules emanating from the opposite spindle pole, are also shown.

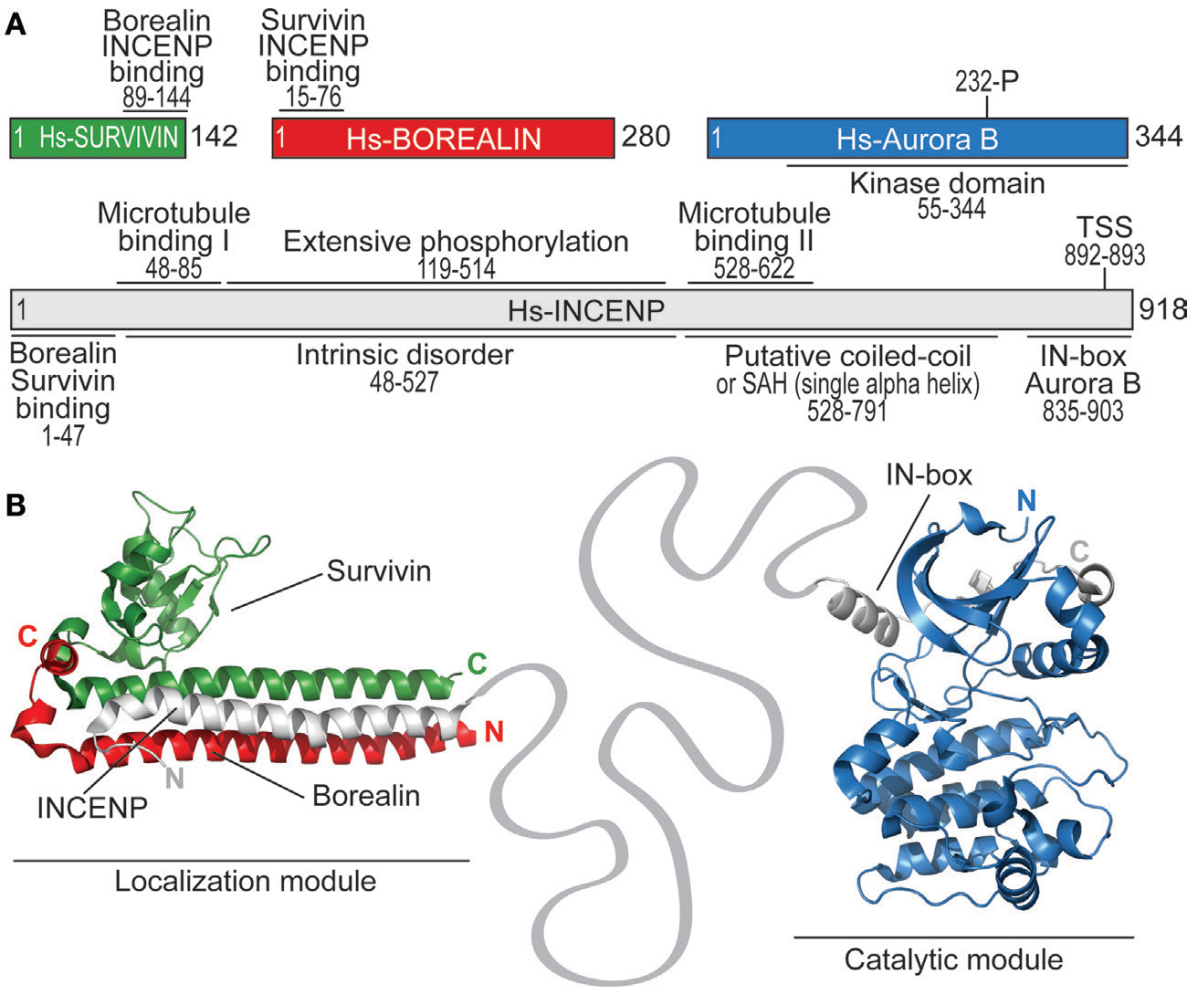


Figure 1.5. Organization of the Chromosomal Passenger Complex (CPC). A) Human CPC subunits with important domains highlighted. B) Structural organization of the CPC (PDB ID 2QFA and PDB ID 2BFX). Adapted from Krenn and Mussachio 2015 [21].

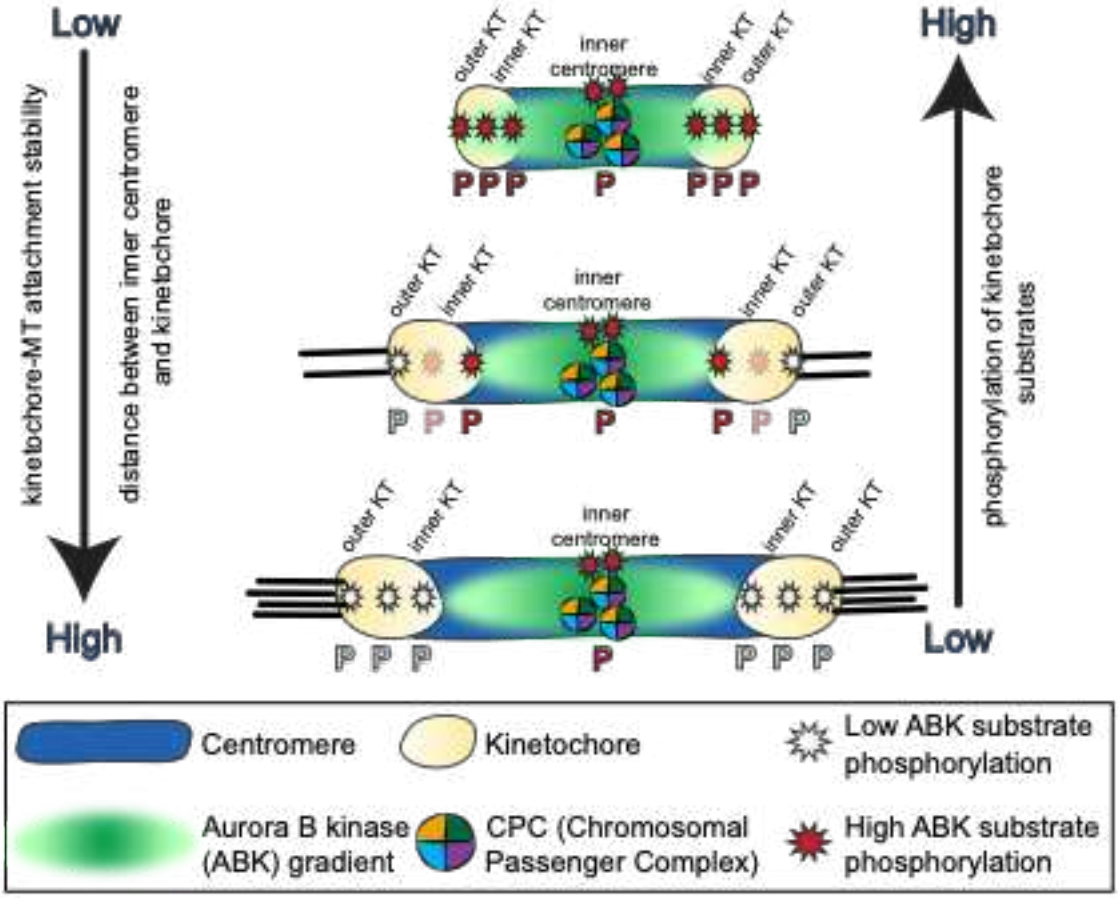


Figure 1.6. Spatial positioning model for Aurora B kinase-mediated regulation of kinetochore-microtubule attachment stability. In this proposed mechanism Aurora B kinase is recruited to and activated at the inner centromere, and active kinase emanates as a diffusible gradient outward towards kinetochores. In early mitosis, when kinetochores lack stable microtubule-attachments, kinetochores are physically close to the inner centromere, and the kinase gradient reaches and phosphorylates kinetochore substrates, which promotes kinetochore-microtubule turnover. As stable attachments form and kinetochores experience pulling forces from the dynamics of attached microtubule plus ends, kinetochores are stretched away from the inner centromere region and out of the reach of the active kinase. Kinetochore substrate phosphorylation decreases, and attachments to microtubules are further stabilized.

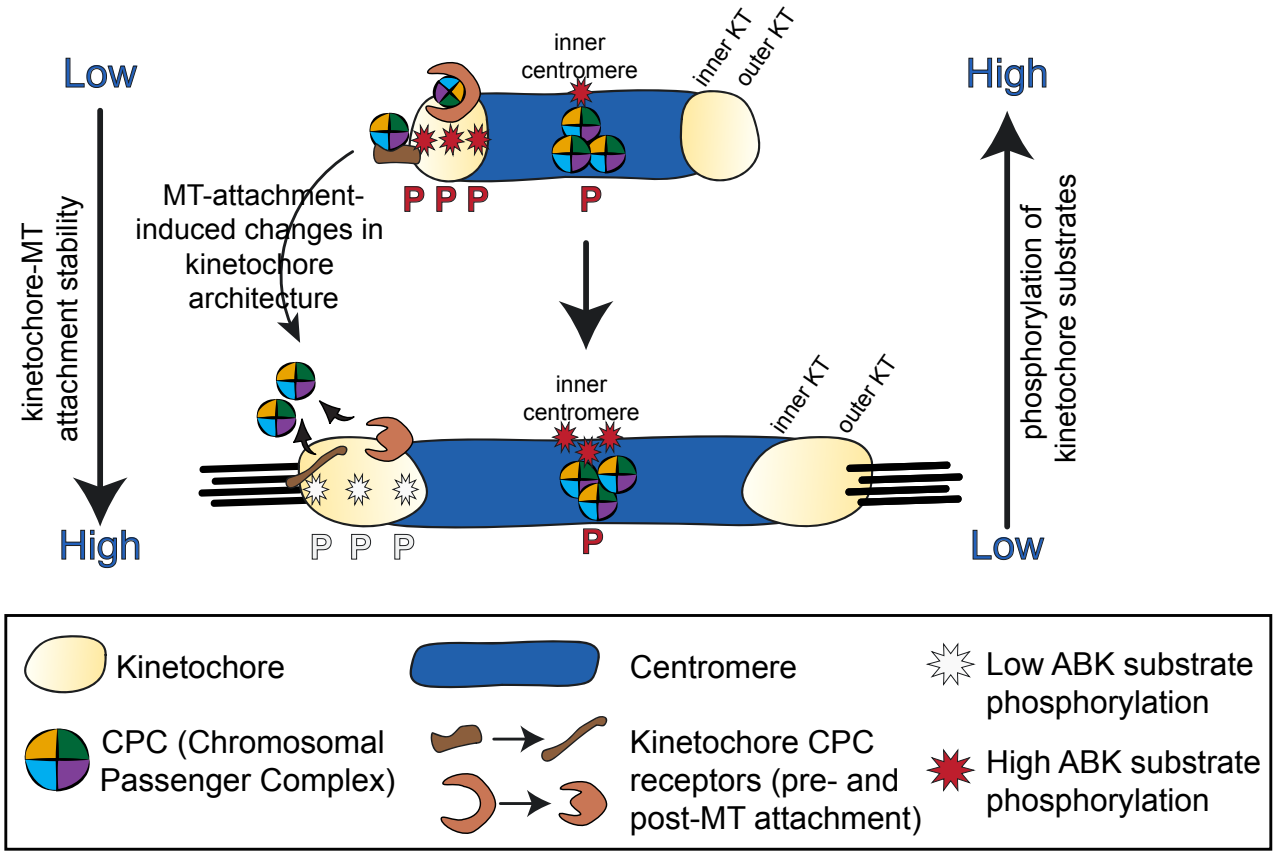


Figure 1.7. Direct recruitment model for Aurora B kinase-mediated regulation of kinetochore-microtubule attachment stability. In this proposed mechanism, Aurora B kinase is directly recruited to both centromeres and kinetochores. In early mitosis, the kinase and its CPC cofactors are recruited to the inner centromere (via the phospho-histone marks pH2A and pH3, see Figure 4) and to kinetochores through specific kinetochore receptors that likely reside in both the inner and outer kinetochore. As mitosis progresses and tension-generating kinetochore-microtubule attachments form, changes in kinetochore architecture (and in specific CPC receptor proteins) lead to loss of the kinetochore-associated Aurora B kinase/CPC binding sites and subsequent eviction of Aurora B and the CPC from kinetochores. Kinetochore substrate phosphorylation is reduced, and kinetochore-microtubule attachments are further stabilized. In this cartoon, while the CPC accumulates at inner centromeres in early mitosis, its activity increases in this region during later mitosis (see text for details). Activity on only one kinetochore of each pair is shown for clarity.

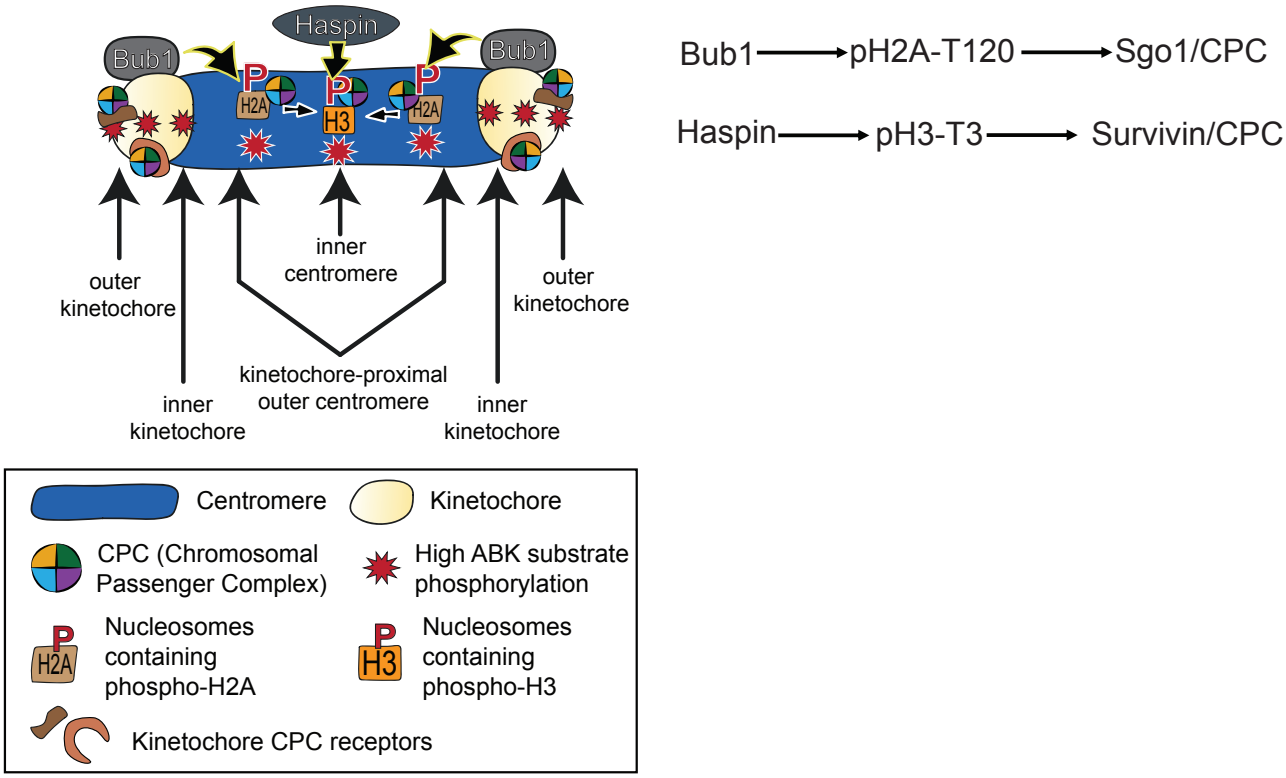


Figure 1.8. Recruitment pathways for centromere-localized Aurora B kinase. Phosphorylated histone H3 recruits the CPC to inner centromeres through direct binding of Survivin. Phosphorylated histone H2A recruits the CPC to kinetochore-proximal outer centromere regions through Sgo1-dependent recruit of the CPC, likely through direct interaction between Sgo1 and Borealin.

Chapter 2: Exploring the role of histone post-translation modifications in Chromosomal Passenger Complex recruitment at centromeres

2.1 Introduction to centromeres

The compaction of eukaryotic DNA is critically important during cell division. At the start of cell division, chromatin undergoes dynamic re-organization to form dense mitotic chromosomes that are mechanically stable enough to be moved by microtubules of the mitotic spindle [159]. The chromatin structure at the primary constriction site of mitotic chromosomes that is required for kinetochore formation and attachment to microtubules is called the centromere. Centromere specific proteins were first identified from patients that had scleroderma spectrum disease and produced anti-centromere antibodies for three antigens later named Centromere proteins- A, B and C (CENPs-A, B and C) [16, 17, 160]. Shortly after its discovery, CENP-A was determined to be an essential histone H3 variant that incorporates within nucleosomes at the centromere [161, 162].

CENP-A containing nucleosomes that reside at centromeres interact with the constitutive centromere associated network (CCAN), and at the onset of mitosis, kinetochore proteins associate with the CCAN to form a dynamic interface that physically links chromosomes to spindle microtubules [13, 159, 163, 164]. While CENP-A shares many of the characteristics of histone H3, variations in CENP-A structure provide a basis for recruitment of CCAN complexes (Figure 2.1 adapted from Pesenti et al., 2017) [32, 46]. In humans, CENP-A diverges from H3 in three main regions including the the CENP-A targeting domain (CATD) located in the histone fold, the loop1 region, and the C-terminal tail [31, 33, 34, 165-167]. CENP-A nucleosomes

simultaneously recruit CENP-C and CENP-N, through which CCAN complexes are recruited to serve as a dynamic framework that assembles the outer kinetochore complex [31, 32, 163, 168, 169]. Interaction of CENP-A nucleosomes with CENP-C is mediated by the C-terminal tail of CENP-A and the acidic patch of H2A-H2B [168]. In contrast, CENP-N binds the loop1 region on CENP-A and also makes extensive contacts with DNA [31, 34, 170]. CENP-A's CATD domain is not needed for kinetochore formation, but rather is necessary for the deposition of CENP-A histones within nucleosomes at the centromere region of chromosomes. The CATD is highly conserved across species and required to recruit the CENP-A specific histone chaperone, HJURP, which deposits new CENP-A molecules and therefore propagates centromere identity over many cell divisions [171].

X-ray crystallography studies have revealed that CENP-A and H3 mononucleosomes share many structural similarities, with only subtle differences between the two [172]. Compared to canonical nucleosomes, CENP-A nucleosomes contain a rigid core and wrap DNA less tightly [14, 165, 172]. Although *in vitro* both nucleosome types have similar structure, it is not known how CENP-A- containing chromatin is structurally arranged and how this contributes to the organization of nucleosomes within the centromere [27, 30, 159]. While the majority of chromatin at centromeres is comprised of H3 containing nucleosomes, there are interspersed regions of CENP-A containing nucleosomes (Figure 2.2 adapted from Cleveland et al., 2003) [18, 27]. About one in every 6-8 nucleosomes contains CENP-A; these nucleosomes are arranged in densely populated islets surrounded by H3 containing chromatin [27, 173, 174]. How these two histone variants are spatially organized relative

to each other is largely unknown; however, models propose that CENP-A-containing nucleosomes are positioned primarily in the kinetochore-proximal centromere region, while H3 containing nucleosomes primarily compose the inner centromere region (Figure 2.2 adapted from Cleveland et al., 2003) [18, 27, 30, 175].

It is without debate that the presence of CENP-A is necessary for proper kinetochore assembly and function, but a less appreciated characteristic of centromeric chromatin is the post-translational modifications present on histones that play a pivotal role in the regulation of mitotic processes. In particular, kinetochore-microtubule interactions are regulated by Aurora B kinase, the enzymatic component of the chromosome passenger complex (CPC) that is recruited to centromeres by two histone post translational modifications [21, 46, 136, 141]. Phosphorylation of histone H3 at threonine 3 (pH3-T3) and phosphorylation of histone H2A at threonine 120 (pH2A-T120) are proposed to recruit the CPC to centromeres [121, 122, 125, 129, 130, 136, 139, 142, 153, 176].

Haspin and Bub1 kinase phosphorylate nucleosomes at the centromere

Haspin kinase localizes to centromeres through an interaction with the cohesin complex [177] and phosphorylates the N-terminal tail of histone H3 on threonine 3 [121, 122, 153]. It is well documented that phosphorylation of H3-T3 directly recruits the CPC through its subunit Survivin, thereby recruiting Aurora B kinase to the centromere region [121, 122, 153]. Bub1 kinase is recruited to the kinetochore by KNL1 [68, 69] and phosphorylates histone H2A at threonine 120 to recruit Sgo1, a proposed CPC bridging factor [125, 130, 136]. A major tenet of the current model for CPC recruitment to centromeres states that histone modifications pH3-T3 and pH2A-T120 overlap and that

this overlap defines the inner centromere and optimal CPC recruitment [125, 178]. The region of overlap proposed for CPC recruitment could therefore be a region where H3 and CENP-A nucleosomes are modified, or a region where only H3 containing nucleosomes reside and are phosphorylated on both H3 and H2A. Furthermore, it is unknown how individual histone modifications affect the binding of CPC components and whether or not these modifications exist on the same nucleosome.

Contrary to the accepted model, it is clear from immunostaining experiments that pH3-T3 exists as a single focus marking the inner centromere, while pH2A-T120 displays a “paired dot” pattern, characteristic of kinetochore staining [125-128]. Moreover, Sgo1, the proposed CPC bridging factor that binds pH2A-T120 localizes to both the kinetochore-proximal outer centromere and the inner centromere (Figure 2.3) [41]. Given the localization of CENP-A nucleosomes to kinetochore-proximal outer centromeres, the staining pattern of pH2A-T120, and the localization of Sgo1, we set out to determine if differentially modified nucleosomes were individually capable of recruiting the CPC. To test if the CPC binds mononucleosomes containing CENP-A or H3 we purified a truncated version of the CPC (CPC^{mini}) and tested binding to reconstituted mononucleosomes *in vitro*. We also tested if Bub1 kinase preferentially phosphorylates CENP-A containing nucleosomes *in vitro* to determine if there is a basis for differentially modified nucleosomes within the centromere region. We hypothesize that the two histone modifications, pH3-T3 and pH2A-T120, are present on H3 and CENP-A containing nucleosomes respectively, that occupy distinct regions of the centromere, and can each individually recruit a population of the CPC

2.2 Results

Does Bub1 preferentially phosphorylate Thr120 in CENP-A containing mononucleosomes?

It is plausible that Bub1 kinase preferentially phosphorylates H2A-T120 in CENP-A containing nucleosomes compared to canonical H3 nucleosomes. This would create a basis for spatially distinct populations of modified nucleosomes that recruit individual populations of the CPC and Aurora B kinase to the centromere region. In order to determine if Bub1 differentially phosphorylates histone H2A-T120 in CENP-A or H3 containing nucleosomes, reconstituted mononucleosomes containing either CENP-A or H3 were tested for phosphorylation by Bub1 kinase. Reconstituted mononucleosomes were generated with either $(CENP\text{-}A/H4)_2$ $(H2A/H2B)_2$ or $(H3/H4)_2$ $(H2A/H2B)_2$ octamers and 207 bp DNA (601-Windom positioning sequence) then analyzed on 5% native polyacrylamide gels (Fig 2.4). Purified mononucleosomes were incubated with Bub1 kinase and phosphorylation was assessed by Western blotting with anti-pH2A-T120 antibodies. Contrary to our hypothesis that Bub1 would preferentially phosphorylate CENP-A containing nucleosomes, we found that Bub1 phosphorylates both H3 and CENP-A containing mononucleosomes *in vitro* (Figure 2.5).

Although Bub1 phosphorylates both CENP-A and H3 containing nucleosomes *in vitro*, it is possible that *in vivo* kinetochore-localized Bub1 preferentially phosphorylates CENP-A containing nucleosomes that are located in the kinetochore-proximal outer centromere in cells. It is also possible the structure of CENP-A nucleosomes within centromeric chromatin contribute to Bub1 substrate specificity in a manner that is not mirrored in mononucleosomes. Therefore, whether Bub1 phosphorylates CENP-A and H3 containing nucleosomes in the context of centromeric chromatin remains unknown.

CPC^{mini} purification and in vitro binding to mononucleosomes

We next set out to determine if the CPC differentially binds CENP-A or H3 containing mononucleosomes *in vitro*. In order to determine if the CPC preferentially binds CENP-A or H3 mononucleosomes *in vitro,* we purified a truncated version of the CPC (termed CPC^{mini}) from *E. coli* cells. Briefly, the CPC^{mini} was expressed in *E. coli* using a dicistronic vector encoding for GST-ABK-INCENP^{mini} co-transformed with a plasmid containing Survivin and His-Borealin and the complex was purified using a GST purification scheme (Figure 2.6). To determine if the CPC^{mini} binds to either CENP-A- or H3-containing mononucleosomes, increasing concentrations of purified CPC^{mini} was incubated with either CENP-A- or H3-containing mononucleosomes, and binding was analyzed by electrophoretic mobility shift assays (EMSA). *In vitro*, the CPC^{mini} bound free 207 bp DNA, CENP-A- and H3-containing mononucleosomes (Figure 2.7). These results suggest that without phospho-modifications present on mononucleosomes, the CPC^{mini} has affinity for both CENP-A and H3 containing mononucleosomes along with free DNA *in vitro*.

In cells the CPC localizes to chromosome arms in early prophase to release cohesin complexes, then as the nuclear envelope breaks down, the CPC re-localizes from chromosome arms to the centromere region [36, 37]. Thus, it is probable that phospho-histone modifications at the centromere drive the centromere specific accumulation of the CPC in the later stages of prophase and the beginning of prometaphase. The presence of histone phospho-modifications at the centromere likely creates a higher affinity binding site for the CPC at centromeres compared to chromosome arms. So, the binding between mononucleosomes and CPC^{mini} observed in the EMSA likely captures the inherent affinity the complex has for chromatin. In the

future binding assays that contain either pH3-T3, pH2A-T120, or both modifications will be tested to determine if the CPC has higher affinity for phospho-modified mononucleosomes compared to un-modified mononucleosomes. It will also be interesting to understand if CPC binding phospho-modified nucleosomes composed of octamers with either CENP-A or H3 is different. Another potential explanation for our results is the use of the CPC^{mini} construct. A large region of INCENP that may impact CPC binding to nucleosomes is absent in the recombinant CPC^{mini} which may impact CPC binding nucleosomes (Figure 2.6). We found that at least *in vitro*, the CPC^{mini} can bind both CENP-A and H3 containing mononucleosomes independent of phoshpho-histone modifications.

Immunoprecipitation of the CPC with reconstituted mononucleosomes

To address the issues mentioned above, we reasoned that we could test the CPC interaction with modified mononucleosomes using immunoprecipitation assays with mitotically enriched cell lysate that contains full-length CPC complexes and proposed bridging factors (Figure 2.8). Sgo1 is a CPC bridging protein that binds pH2A-T120 and the CPC at centromeres. Consequently, we expect Sgo1 to enhance the binding affinity of the CPC to pH2A-T120 modified nucleosomes [125, 130, 136]. To this end, we immobilized FLAG-tagged mononucleosomes that were either phosphorylated or not by Bub1 kinase in an *in vitro* kinase assay on beads and incubated the nucleosome-bound beads with mitotically enriched human cell lysate, then assessed CPC binding by Western blotting with anti-Aurora B kinase antibodies. H3 containing mononucleosomes that were immobilized on anti-FLAG affinity beads

bound Aurora B in both the presence and absence of phosphorylation of H2A-T120 (Figure 2.9).

These data indicate that the CPC has, at least to some extent, binding affinity for both unmodified and modified mononucleosomes. In the future, *in vitro* binding assays that measure the relative binding affinity between full-length CPC, bridging factors, and various types of modified mononucleosomes will be needed to understand how the CPC is differentially recruited to centromeres. Furthermore, *in vitro* kinase assays result in a mixed population of phosphorylated and un-phosphorylated mononucleosomes. This mixed population of modified nucleosomes make it difficult to evaluate specific binding of the CPC. In the future it will be important to determine the level of phosphorylated mononucleosomes that result from *in vitro* kinase assays and to isolate only phospho-modified nucleosomes for use in downstream binding assays.

Purification of full-length CPC

In order to better understand how the CPC is recruited to centromeres and kinetochores it is important to purify a full-length complex for *in vitro* studies. To begin purification of full-length CPC, two approaches were attempted. In one method plasmids were designed with affinity tagged CPC subunits for expression in human ExpiHek293 cells. In the second strategy, a construct was designed for CPC purification from ExpiSF9 insect cells using the baculovirus system (Figure 2.10). The advantage of both techniques is the ability to grow a large number of eukaryotic suspension cells that produce recombinant human protein complexes containing post-translational modifications that may be required for correct folding. The hope is that these systems

allow for purification of full-length CPC and, in the future, CPC bridging factors such as Sgo1.

Using the ExpiHek293 cells, a plasmid encoding 3XFlag-6XHis-GFP-INCENP was transfected into cells in suspension (~4x10^6 cells/mL in ~30mL) (Figure 2.10 (A)). These cells were then harvested and lysed for protein purification using affinity chromatography. We suspected that if we transfected one component of the CPC, we may co-purify other components of the CPC from mitotically enriched cells. However, attempts to purify 3XFlag-6XHis-GFP-INCENP from ExpiHek293 cells were ineffective and further optimization will be needed. Particularly, it is not well established how efficiently the plasmid was transfected in ExpiHek293 cells. Therefore, we do not know how many cells were expressing our protein of interest. Moreover, the conditions for maintaining cell viability while simultaneously over-expressing exogenous protein were not optimized and after treatment with nocodazole overnight to mitotically enrich the cultures, cell viability was <50% which may not be optimal for protein purification.

We decided to try purification of full-length CPC from ExpiSf9 insect cells that produce a large copy number of recombinant proteins (Figure 2.10 (B)). The Bac-to-Bac Baculovirus expression system has been optimized by others in our lab and the Markus lab that established protocols to purify difficult protein complexes, making this system a logical candidate for CPC purification. Initially, we cloned each subunit of the CPC with multiple affinity tags into the Big1a plasmid (Weissmann et al., 2016; Jeanne Mick) that contains elements necessary for high-level expression in insect cells and generation of bacmid DNA (Weissmann et al., 2016). Once the Big1a CPC plasmid was cloned, bacmid DNA was generated by transforming Big1a CPC into DH10Bac *E. coli* cells that

contain helper plasmids to recombine the CPC components from the Big1a plasmid and bacmid DNA, thus creating recombinant bacmid DNA. Bacmid DNA was isolated from bacteria and transfected into ExpiSf9 cells to generate recombinant baculovirus. The initial recombinant baculovirus was harvested and amplified then used for infection of large scale ExpiSf9 cultures (300mL). After infection of the large-scale culture, cells were harvested (~3 days) when cell viability was <50%. The cell pellet was lysed, and clarified lysate was run over a nickel column. Unfortunately, the fractions collected did not show any indication of the CPC subunits and the cells did not tolerate CPC baculovirus well (they were mostly dead and not expressing as judged by GFP expression). So, I did not successfully purify the CPC from insect cells. While this was not the expected result, many steps in the purification can be optimized including the number of amplifications of recombinant baculovirus, the number of days cells are infected, and the number of cells infected for purification.

2.3 Discussion

It is possible that there is not a significant difference in Bub1 kinase phosphorylation of mononucleosomes *in vitro* because the substrate H2A is present and structurally similar in both types of mononucleosomes. *In vitro* CENP-A and H3 nucleosomes have very little structural differences [179] therefore, Bub1 substrate recognition may not be affected. *In vivo* kinetochore localized Bub1 may restrict the H2A modification primarily in CENP-A containing nucleosomes. It may also be that the structure of nucleosomes within the centromere contribute to preferential phosphorylation of nucleosomes. We found that, at least *in vitro*, Bub1 kinase phosphorylates both CENP-A and H3 containing mononucleosomes.

We wanted to investigate the interaction between the CPC and mononucleosomes *in vitro*. To this end we purified CPC^{mini} and assessed binding to mononucleosomes using EMSA and immunoprecipitation assays (Figure 2.7 and 2.9). We found that CPC^{mini} and CPC from mitotic cell extract interact with both unmodified and phospho-modified mononucleosomes. While these results were interesting, they were somewhat expected because the CPC has affinity for chromatin in early prophase [36, 37]. It is likely possible that mononucleosomes *in vitro* do not accurately mimic the environment of nucleosomes within the centromere that may create a higher binding affinity for the CPC than chromosome arms. In the future it would be informative to determine the binding affinity between the CPC and modified nucleosomes. However, as mentioned above, *in vitro* kinase assays result in a mixed population of phospho-nucleosomes and un-modified nucleosomes which in turn makes interpretation of binding results difficult. It will be important to determine the level of phosphorylated mononucleosomes so pure samples can be used for binding assays.

Purification of the full-length CPC complex from either human or insect cells will permit better studies of CPC binding proteins *in vitro*. Importantly, in purifications from human cells (ExpiHek293) the CPC may contain post-translational modifications required for specific interactions along with the potential to co-purify essential bridging factors such as Sgo1. Purification of full-length CPC from insect cells also has the prospective to produce the CPC if the protocol is optimized.

2.4 Future directions

While the *in vitro* results are interesting, we found that in cells neither histone modification was necessary for the kinetochore activity of Aurora B kinase and

furthermore that individually either histone modification alone was sufficient to recruit the CPC [126-128]. These data suggest that individually each histone modification is able to recruit a population of the CPC to centromeres however, how the two independent populations of CPC at the centromere cooperate for faithful cell division is largely not understood.

When Haspin kinase is inhibited in cells, a population of the CPC is detected in the kinetochore-proximal outer centromere where the pH2A-T120 modification resides [126-128]. Interestingly, this population is less frequently observed in unperturbed cells where the majority of the CPC resides at the inner centromere concurrent with the pH3-T3 modification [126-128]. Furthermore, when either histone modification alone is absent cells undergo faithful cell division. Interestingly however, when both pH2A-T120 and pH3-T3 are depleted cells undergo cell division with a high rate of chromosome segregation errors despite high Aurora B kinase activity at kinetochores [126, 128]. These results suggest both pH2A-T120 and pH3-T3 recruit the CPC to the centromere region but when both histone modifications are absent errors in chromosome segregation are high [126, 128]. The mechanism of how centromere localized CPC promotes faithful chromosome segregation is unknown and discussed in the following chapter. In the future *in vitro* assays that test how full-length CPC interacts with pH2A-T120 and pH3-T3 modified nucleosomes will be interesting to understand how both populations cooperate at the centromere region.

Although Bub1 does not preferentially phosphorylate H2A-T120 in CENP-A containing nucleosomes *in vitro*, in cells it is possible pH2A-T120 and pH3-T3 are present on different nucleosomes. To understand if the histone modifications are

present on the same or different nucleosomes, immunoprecipitation (IP) assays can be used. IP of Flag-CENP-A can be used to isolate centromeric chromatin, which can be digested to produce mononucleosomes. The resulting nucleosomes will be subject to a second round of IP using anti-H2A-T120 or anti-pH3-T3 antibodies and then evaluated for histone modifications by Western blot (Figure 2.11). If the hypothesis is correct, and histone modifications vary between CENP-A and H3 nucleosomes, we expect after the second IP with pH2A antibodies, CENP-A, but not pH3, will be detected by Western blot depicted in scenario 4 from Figure 2.11. Similarly, after the second IP with pH3 antibodies, pH2A-T120 will not be detected (Figure 2.11, scenario 2). However, we may find that both marks exist on the same nucleosomes (Figure 2.11 scenarios 1, 3, or 5), which will be equally informative. These experiments will provide unprecedented insight regarding histone modifications at centromeric heterochromatin. If the protocol for immunoprecipitation of centromeric chromatin is optimized, it might also be possible to send samples for mass spectrometry analysis to identify new post-translational modifications on nucleosomes within the centromere.

2.5 Methods

Nucleosome reconstitution in vitro

Nucleosomes were reconstituted *in vitro* as previously described [22, 180, 181]. Lyophilized histones were obtained from the Protein Expression and Purification facility (Dr. Mam) and resuspended in unfolding buffer (6M guanidium hydrochloride, 20mM Tris-HCl pH 7.5, 5mM DTT) for 45 min. The nanodrop was used to determine the concentration of each resuspended histone. Equimolar ratios of each histone were mixed and dialyzed in refolding buffer (2M NaCl, 10mM Tris-HCl pH 7.5, 1mM EDTA,

5mM BME) for 24 hours then concentrated using an Amicon-15 (30kD cut off). The refolded octamer was purified on a Superdex200 size exclusion column, fractions were collected and concentrated using an Amicon-15. Pure octamers were analyzed on an 18% SDS-polyacrylamide gel. To make recombinant nucleosomes, purified histone octamers were mixed with 207 bp (601 Windom) DNA at varying ratios and dialyzed at 4° overnight from high salt buffer (2M KCl, 10mM Tris-HCl pH 7.5, 1mM EDTA, 1mM DTT) to low salt buffer (0.25M KCl, 10mM Tris-HCl pH 7.5, 1mM EDTA, 1mM DTT) using a peristaltic pump (flow rate 1.5 mL/min). Nucleosomes were analyzed on a 5% native polyacrylamide gel. Reconstitution of Flag-tagged mononucleosomes were performed as above with the use of 3XFlag-H2A. In all nucleosome reconstitutions, human H2A, H3, and H2B histones were used and *Xenopous* histone H4.

Electrophoretic mobility shift assay (EMSA)

For electrophoretic mobility shift assays, reconstituted mononucleosomes were incubated with increasing concentrations of purified CPC^{mini} in binding buffer (20mM Tris-HCl pH 7.5, 1mM EDTA, 4mM $MgCl_2$, 150mM NaCl). Samples were then analyzed on a 5% native polyacrylamide gels. Native gels were made fresh and pre-run at 90 V for 10 min prior to sample addition. Bands were detected by staining the gel with syber gold (need to check this?). Shifts were determined by smeared high molecular weight bands and the disappearance of either free DNA or mononucleosomes.

In vitro kinase reaction

For *in vitro* kinase reactions Bub1 (SignalChem #B11-35G-20) or Haspin (SignalChem #G10-11G-10) kinases were incubated with 1X kinase buffer (27.5mM $MgCl_2$, 4mM MOPS, 9.17mM Beta-glycerol phosphate, 1.83mM EGTA, 0.37mM Sodium

orthovanadate) and mononucleosomes at 37° for 1 hour. Following the reaction samples were run on 15% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membrane (EMD Millipore), and then processed for Western blot. For detection of phosphorylation, blots were incubated with either anti-pH2A-T120 (Actif Motif) or anti-pH3-T3 (Dr. Hiroshi Kimura) overnight. Primary antibodies were detected using a horseradish peroxidase–conjugated anti–rabbit or anti-mouse secondary antibody at 1:10,000 (Jackson ImmunoResearch Laboratories, Inc.) and enhanced chemiluminescence (Thermo Fisher Scientific).

Immunoprecipitation of CPC using modified mononucleosomes

Reconstituted $(H3/H4)_2$/$(3XFlag\text{-}H2A/H2B)_2$ octamers were dialyzed with 207 bp DNA (described above) to make mononucleosomes. Mononucleosomes were either incubated with or without Bub1 kinase and bound to anti-Flag M2 affinity gel (Sigma-Aldrich). Briefly, the resin was washed with binding buffer (20mM Tris-HCl pH 7.5, 1mM EDTA, 4mM $MgCl_2$, 150mM NaCl) then incubated with mononucleosomes at 4° rocking for 1 hour. The resin was then washed (3X) and incubated with cell lysate at 4° for 2 hours. Cell lysate was prepared from HeLa cells arrested in nocodazole overnight. The resin was washed 3X with binding buffer. The remaining mononucleosomes and binding partners were eluted using 5X SDS-sample buffer. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting.

Figures 2

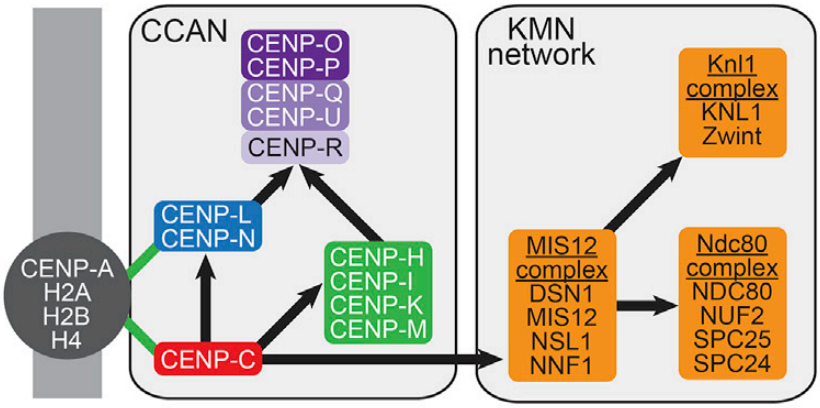


Figure 2.1. Interactions between CENP-A containing nucleosomes and the Constitutive Associated Centromere Network (CCAN). CENP-A nucleosomes can interact with CENP-C and CENP-N directly recruiting the CENP complexes and kinetochore-microtubule network. Adapted from Pesenti et al., 2018 [32].

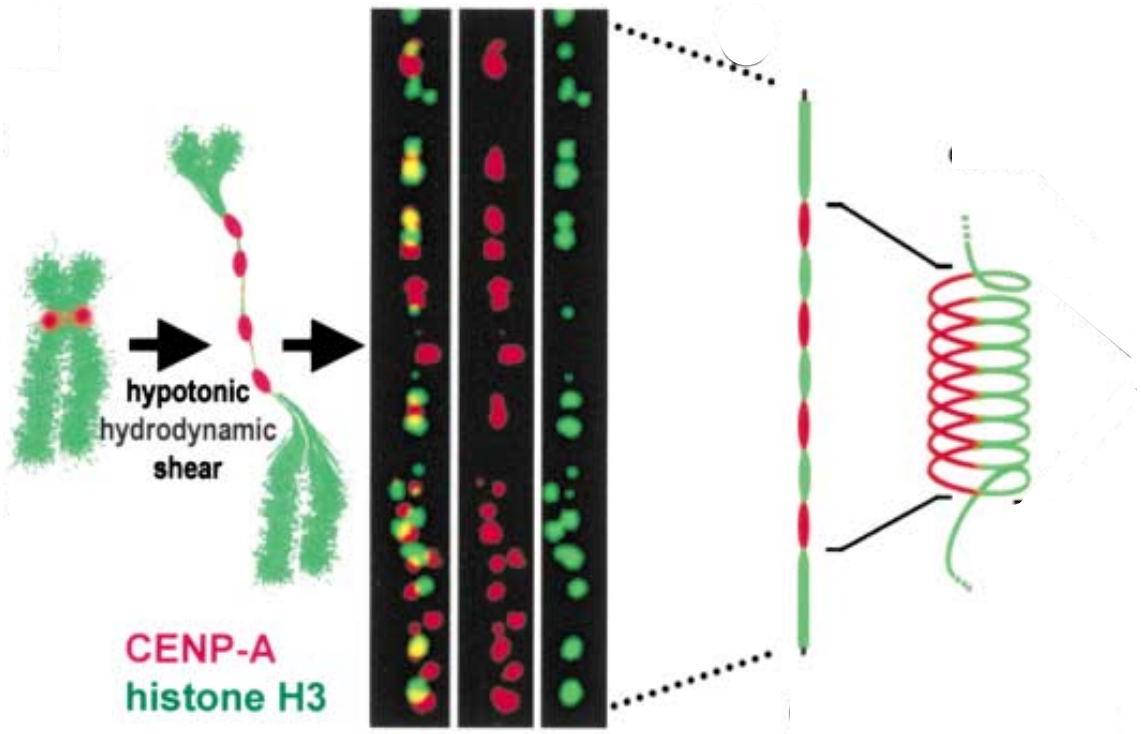


Figure 2.2. Model for the organization of centromeric nucleosomes. The structure of centromere chromatin is largely unknown; however, CENP-A nucleosomes are proposed to compose the kinetochore-proximal centromere region while H3 containing nucleosomes compose the inner centromere. Adapted from Cleveland et al., 2003 [18].

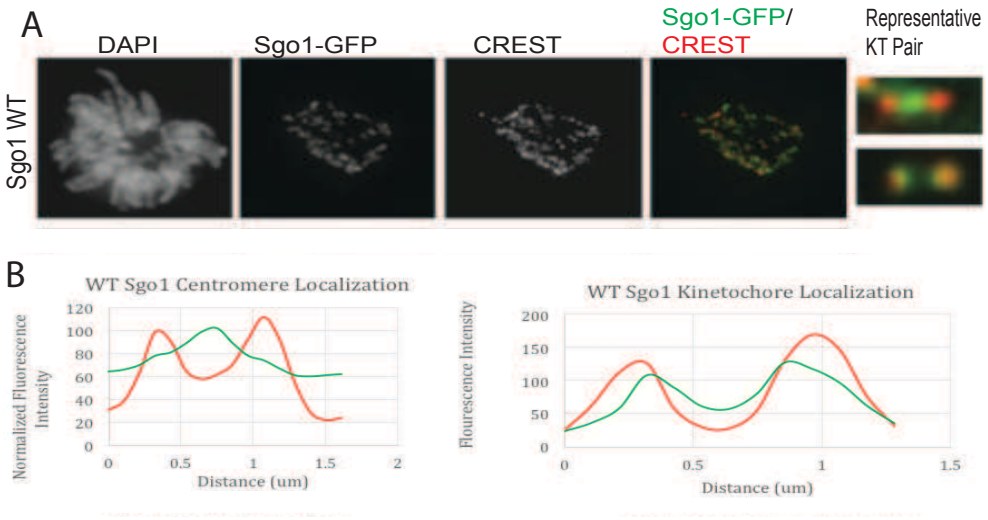


Figure 2.3. Sgo1-GFP localization in HeLa cells. A) Immunofluorescence image displaying $Sgo1^{WT}$-GFP localization relative to anti-centromere antibody (ACA). Insets show representative pairs used for line scans. B) Line scans of representative pairs for ACA (red) and Sgo1-GFP (green). Sgo1 the proposed CPC bridging factor localizes to both centromeres and kinetochores in cells.

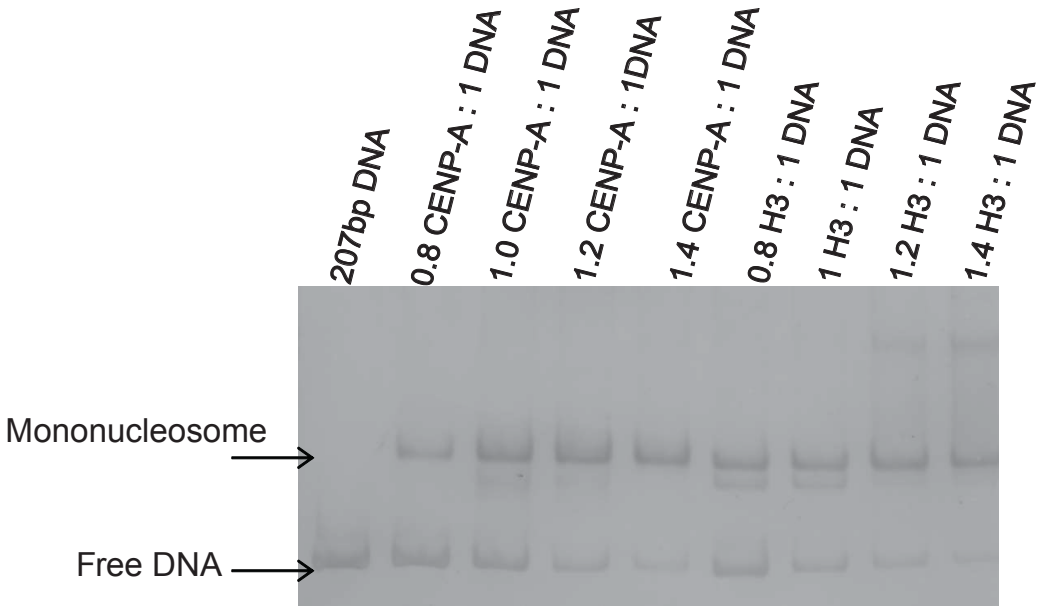


Figure 2.4. Reconstitution of CENP-A and H3 mononucleosomes. 5% Native polyacrylamide gel electrophoresis showing reconstituted mononucleosomes with 207 bp DNA.

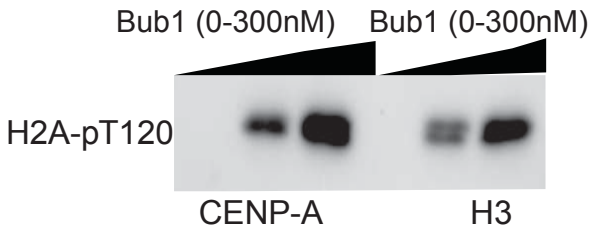


Figure 2.5 Western blot analysis of Bub1 phosphorylation of both CENP-A and H3 mononucleosomes *in vitro*.

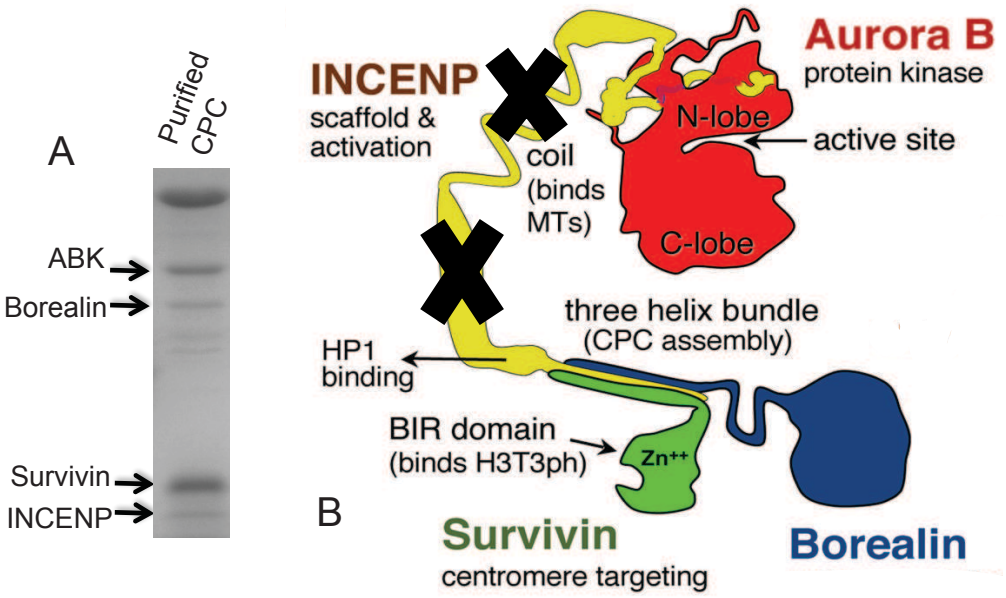


Figure 2.6. Model of the Chromosome Passenger Complex and CPC^{mini} purification. A) Purificiation from *E. coli* using a dicistronic encoding for GST-ABK-INCENP^{mini} co-transformed with a Survivin and His-Borealin plasmid. B) Adapted from Carmena et al., 2012 [80]. Diagram of the CPC with important domains of each component. Black X's represent regions of INCENP excluded in INCENP^{mini}.

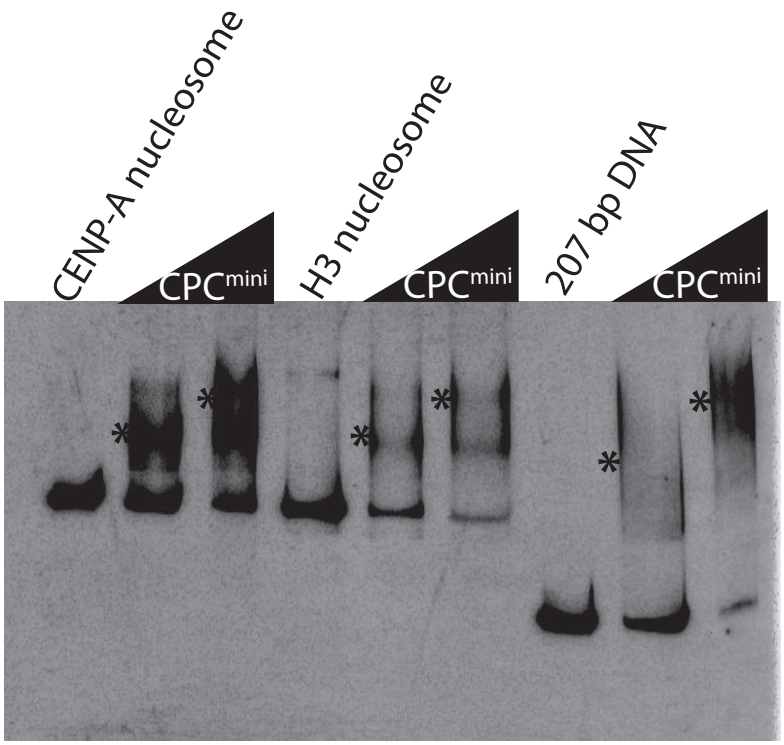


Figure 2.7. Electrophoretic mobility shift assay. Reconstituted mononucleosomes and free DNA incubated with increasing concentrations of CPC^{mini}. Asterisks indicate shifted bands in the presence of CPC^{mini}.

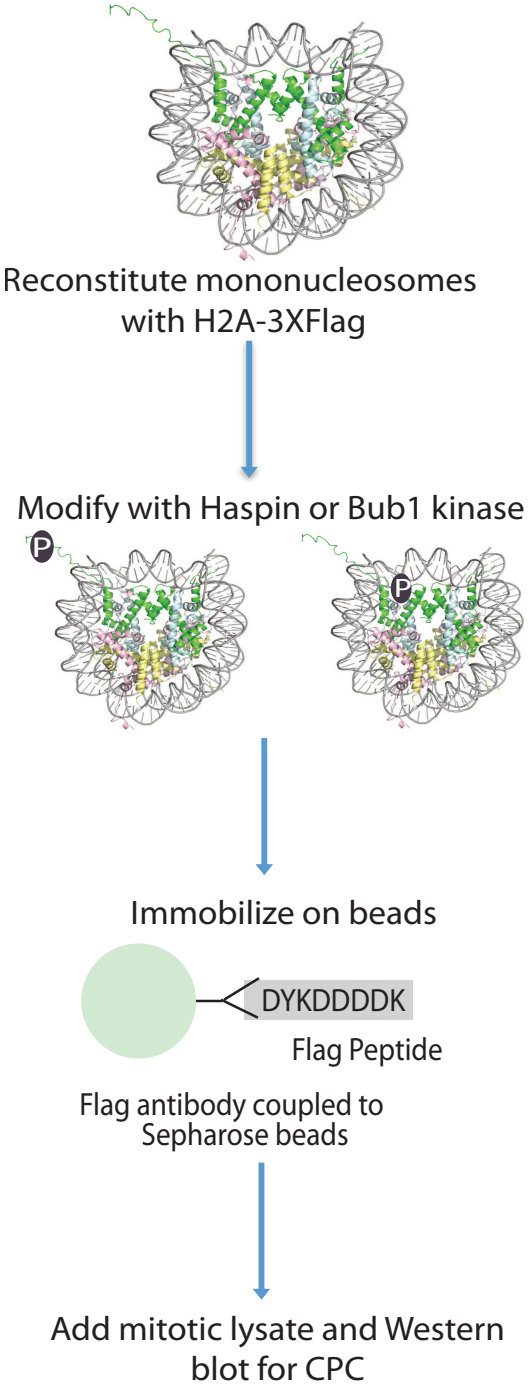


Figure 2.8 Workflow for immunoprecipitation. Co-immunoprecipitation of CPC components using reconstituted, phosphorylated mononucleosomes (PDB:1AOI Lugger et al., 1997) [22].

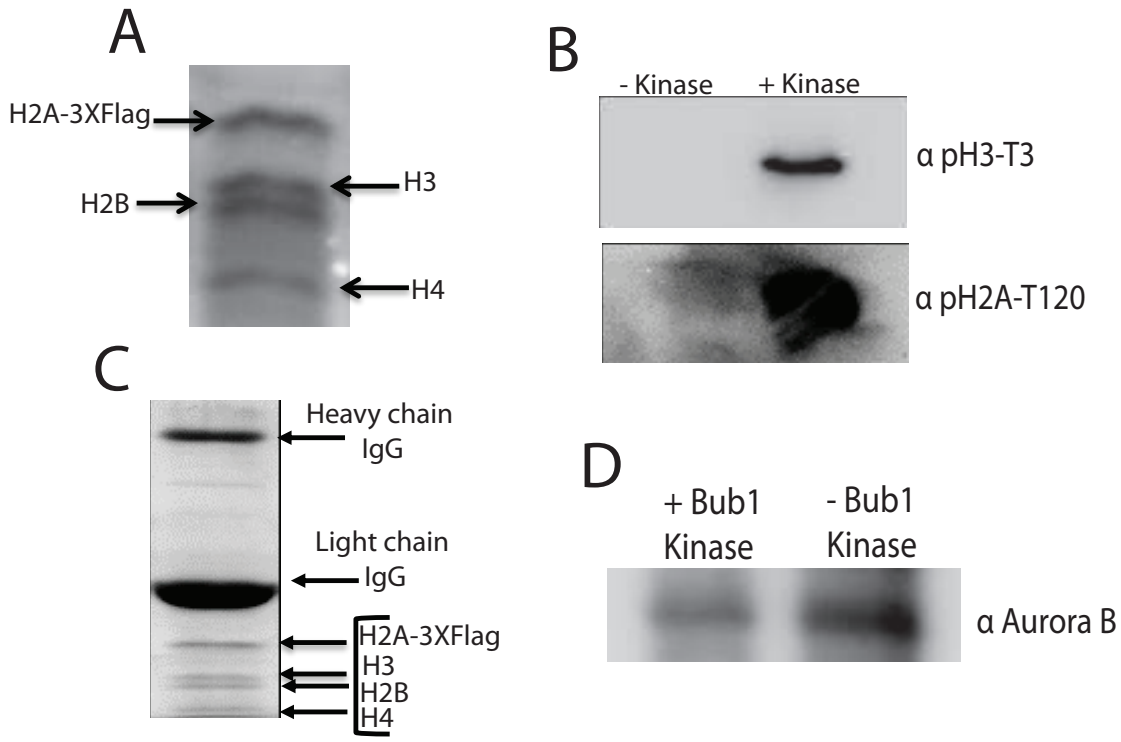


Figure 2.9. Immunoprecipitation of CPC using modified mononucleosomes as bait. A) Coomassie brilliant blue of H2A-3XFlag octamer reconstitution. B) Mononucleosomes containing $(H2A\text{-}3XFlag/H2B)_2$ $(H3/H4)_2$ histone octamers reconstituted with 207 bp DNA were incubated with Bub1 or Haspin kinase and evaluated for histone phosphorylation by Western blot with an anti-pH2A-T120 or anti-pH3-T3 antibody respectively. C) Coomassie brilliant blue of mononucleosomes coupled to anti-Flag affinity resin. D) Phosphorylated mononucleosomes bound to anti-Flag affinity resin were incubated with mitotic cell lysate, washed and run on an 12% SDS-PAGE for analysis by Western blot.

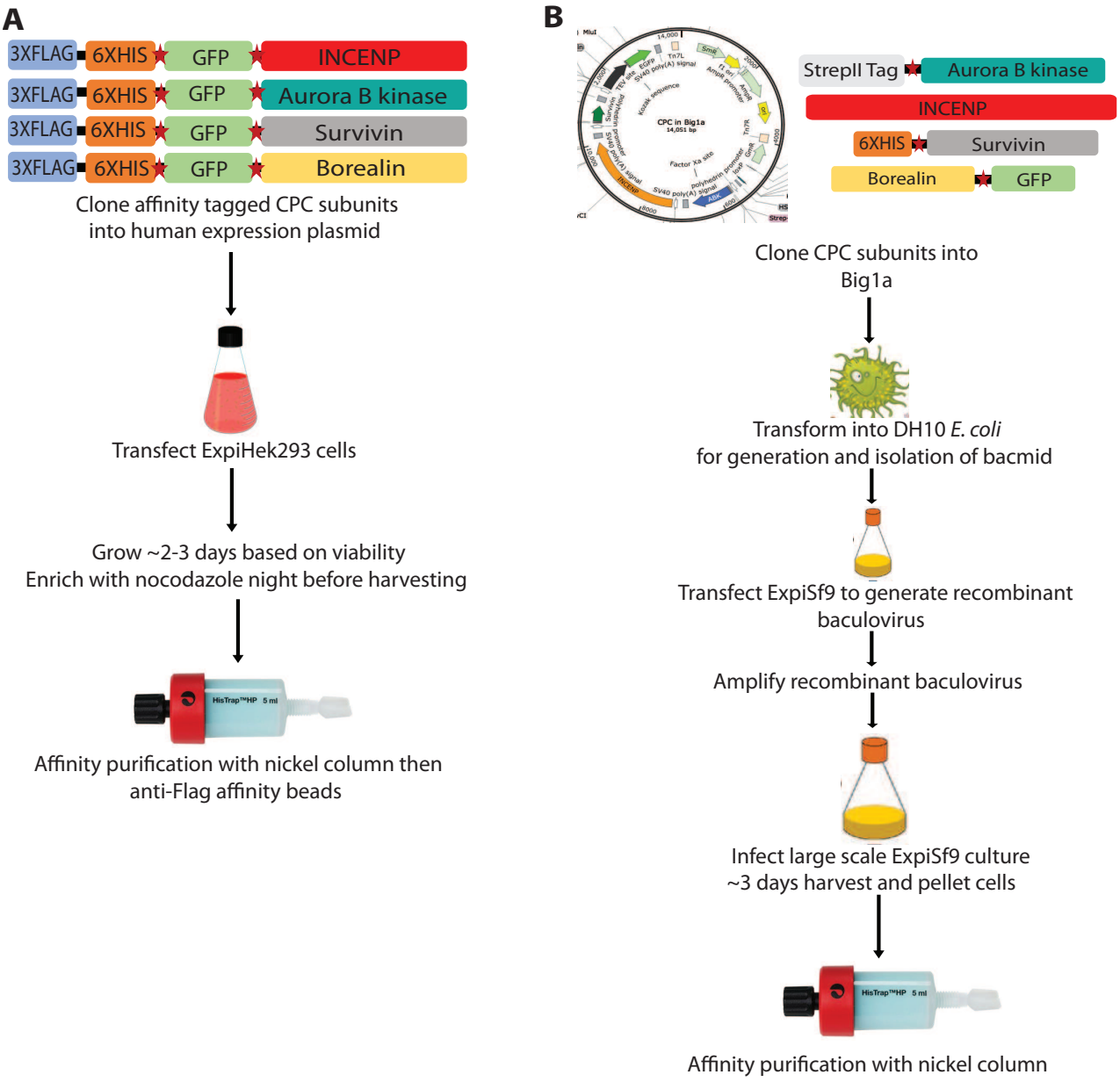


Figure 2.10. Workflow for purification of full-length CPC from A) ExpiHek293 and B) ExpiSf9 cells. Red asterisks indicate either TEV or HRV3C protease cut sites.

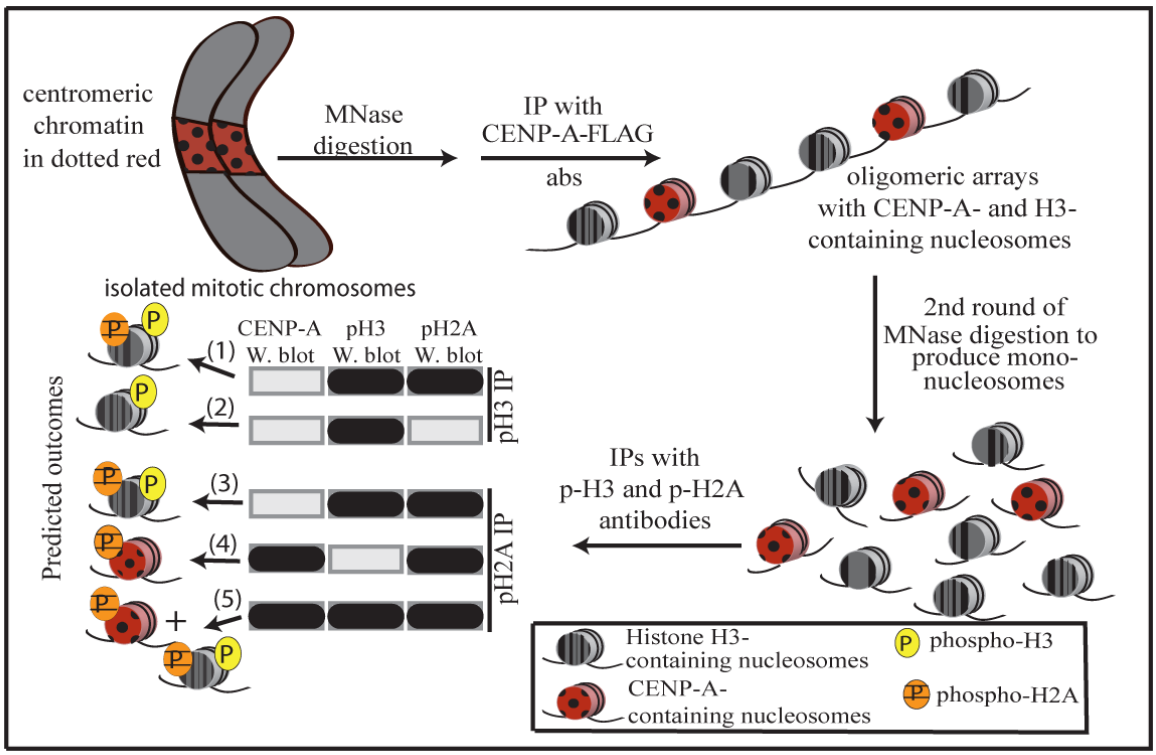


Figure 2.11. Immunoprecipitation of centromeric chromatin. Experimental outline that will be used in the future to determine if pH3-T3 and pH2A-T120 exist on the same or different nucleosomes in cells.

Chapter 3: Aurora B kinase is recruited to multiple discrete kinetochore and centromere regions in human cells[2]

3.1 Brief introduction

Aurora B kinase has a critical role in regulating attachments between kinetochores and spindle microtubules during mitosis. Early in mitosis, kinase activity at kinetochores is high to promote attachment turnover, and in later mitosis, activity decreases to ensure attachment stabilization. Aurora B localizes prominently to inner centromeres, and a population of the kinase is also detected at kinetochores. How Aurora B is recruited to and evicted from these regions to regulate kinetochore-microtubule attachments remains unclear. Here, we identified and investigated discrete populations of Aurora B at the centromere/kinetochore region. An inner centromere pool is recruited by Haspin phosphorylation of histone-H3, and a kinetochore-proximal outer-centromere pool is recruited by Bub1 phosphorylation of histone-H2A. Finally, a third pool resides ~20 nm

[2] The work presented in this chapter was published as a research article in 2020 under the same title. Figures presented in the original manuscript as supplementary figures, due to length constraints, are shown in this chapter as main figures.

AJB, KFD, and JGD conceived the project and JGD supervised the project. AJB and KFD carried out the experiments and analyzed the data. JGD helped with data analysis. AJB and JGD wrote the paper with input from KFD.

Broad AJ, DeLuca KF, DeLuca JG. Aurora B kinase is recruited to multiple discrete kinetochore and centromere regions in human cells. J Cell Biol. 2020;219(3). Epub 2020/02/07. doi: 10.1083/jcb.201905144. PubMed PMID: 32028528.

outside of the inner kinetochore protein CENP-C in early mitosis and does not require either the Bub1/pH2A/Sgo1 or Haspin/pH3 pathway for localization or activity. Our results suggest that distinct molecular pathways are responsible for Aurora B recruitment centromeres and kinetochores.

3.2 Introduction

During mitotic cell division, chromosomes must equally segregate into two daughter cells so that each new cell has an exact copy of the original genetic material. For this to occur, chromosomes connect to microtubules of the mitotic spindle at structures called kinetochores. In addition to forming kinetochore-microtubule attachments, successful chromosome segregation requires that cells precisely regulate the stability of these attachments [46]. In early mitosis, kinetochore-microtubule attachments are short-lived, and microtubule plus ends undergo repeated cycles of attachment and detachment [72, 73]. By maintaining a high level of microtubule turnover in early mitosis, kinetochores ensure that incorrect attachments do not accumulate [76, 77]. As mitosis progresses and chromosomes make their way to the spindle equator, attachments become long-lived, microtubules accumulate at kinetochores, and formation of these stable attachments leads to changes in kinetochore architecture that promote silencing of the spindle assembly checkpoint and anaphase onset [48, 71, 72, 75, 79, 182].

A critical regulator of kinetochore-microtubule attachment stability is Aurora B kinase, the enzymatic component of the Chromosomal Passenger Complex (CPC), also comprised of INCENP, Survivin, and Borealin [21, 80, 84, 85, 183]. In early mitosis, high Aurora B kinase activity towards kinetochore substrates inhibits the formation of stable

microtubule attachments, whereas in late mitosis, low activity promotes stabilization of attachments [44, 49, 88]. A key substrate of Aurora B is Hec1/Ndc80, a member of the four-subunit NDC80 complex and core component of the kinetochore-microtubule attachment interface [45, 48]. A gradual decrease in phosphorylation of the N-terminal Hec1 unstructured tail domain from early to late mitosis has been implicated in the cumulative stabilization of kinetochore-microtubule attachments [88-90].

Aurora B kinase activity towards Hec1 is temporally regulated to ensure that phosphorylation is high on unattached kinetochores and low on those kinetochores that have generated stable attachments to microtubules [49]. A prevailing model to explain this regulation posits that Aurora B is recruited to the inner centromere in early mitosis, and this population of the kinase is responsible for phosphorylating Hec1 and additional outer kinetochore substrates [145, 178]. Upon stable attachment to microtubules, as the outer kinetochore is pulled away from the centromere region by forces generated from microtubule plus-end dynamics, the model proposes that Aurora B kinase molecules concentrated at the inner centromere can no longer “reach” outer kinetochore substrates, resulting in their decreased phosphorylation. However, in addition to accumulating at the inner centromere, Aurora B kinase has also been observed at the kinetochore region of mitotic chromosomes, coincident with its kinetochore substrates [87, 184]. Thus, it is possible that Aurora B is responsible for phosphorylating kinetochore substrates independently of accumulation at inner centromeres and its distance from this region [90, 110, 117, 120, 133, 150, 151].

Recruitment of Aurora B and the CPC to the centromere is proposed to depend on two recruitment pathways initiated with distinct histone phosphorylation events. In

the first, Haspin kinase phosphorylates histone H3 at Thr3 (T3), which creates a binding site for the CPC component Survivin [121, 122, 125, 153]. In the second, Bub1 kinase phosphorylates histone H2A at Thr120 (T120) to recruit the mitotic protein Shugoshin1 (Sgo1), which in turn recruits the CPC, possibly through a linkage to Borealin in human cells [129, 130, 136]. Inhibition of either Haspin or Bub1 and consequent loss of phosphorylated histone H3-T3 (pH3-T3) or phosphorylated H2A-T120 (pH2A-T120), respectively, reduces centromeric accumulation of Aurora B and its CPC partners [121, 122, 125, 131, 153, 154, 185]. However, it is not clear how each of these histone marks impact the specific localization patterns and activity of different subpopulations of Aurora B kinase. Therefore, in this study, we set out to investigate the role of the Haspin/pH3 and Bub1/pH2A recruitment pathways in Aurora B kinase localization and activity at centromeres and kinetochores of mitotic chromosomes.

3.3 Results

Phosphorylated H2A-T120 and phosphorylated H3-T3 localize to distinct regions within mitotic centromeres

Previous studies have suggested that pH3-T3 and pH2A-T120 exhibit spatial overlap within the centromere of mitotic chromosomes, and this region of overlap is proposed to recruit Aurora B kinase and the rest of the CPC [125]. However, antibodies to pH3-T3 appear to recognize inner centromeres as a single focus, while antibodies to pH2A-T120 recognize two foci that flank the inner centromere region [121, 125, 136]. To further clarify this issue, we tested for co-localization of pH2A-T120 and pH3-T3 in HeLa and U2OS cells in early and late mitosis. We found under all conditions each mark localizes to a discrete region within mitotic chromosomes – pH3-T3 distinctly localizes to

the inner centromere as a single focus, whereas pH2A-T120 localizes in a “paired dot” pattern (Figure 3.1, A and B). Since this paired dot pattern suggested that phosphorylated histone H2A may localize to kinetochores, we wanted to more precisely determine where within the centromere or kinetochore region H2A is modified. For this we turned to two-color fluorescence localization microscopy [53] to map the position of pH2A-T120. We stained cells with antibodies to pH2A-T120, CENP-C (an inner kinetochore protein), and the N-terminus of Hec1 (located in the outer kinetochore) (Figure 3.1C). In metaphase cells, our measurements indicate that CENP-C is located 76 +/- 10 nm inside of the N -terminus of Hec1 (Figure 3.1, D and E; Table 1), consistent with previously published work [53, 186]. Our measurements further revealed that the pH2A-T120 signal is 170 +/- 32 nm inside of the N-terminus of Hec1 and 95 +/- 24 nm inside of CENP-C (Figure 3.1, D and E; Table 1). We also analyzed cells in late prophase/early prometaphase (prior to formation of kinetochore- microtubule attachments) and found that the N-terminus of Hec1 was closer to CENP-C in this population of cells (30 +/- 9 nm) than in metaphase cells (Figure 3.1, D and E; Table 1), which is consistent with previous findings demonstrating that chromosome bi-orientation results in an increase in the inner-to-outer kinetochore distance [140, 146]. However, we noted that pH2A-T120 remained ~94 nm on the inside of CENP-C in prometaphase cells, similar to its metaphase position (Figure 3.1, D and E; Table 1). Together, these results suggest that the centromeric chromatin modified by Bub1 kinase resides significantly inside of the inner kinetochore, but spatially distinct from the inner centromere. Based on these results, we agree that this chromosome region should be

referred to as the “kinetochore-proximal outer centromere,” as suggested by Hindriksen et al. [109].

Phosphorylated H3-T3 and H2A-T120 are each capable of recruiting Aurora B kinase and the CPC

Based on the distinct localization patterns of pH3-T3 and pH2A-T120, we tested if each phospho-histone mark was, on its own, sufficient to recruit the CPC. For these experiments, we expressed blue fluorescent protein (BFP)-tagged Haspin kinase or green fluorescent protein (GFP)-tagged Bub1 kinase fused to the Lac repressor (LacI) in U2OS cells containing an ectopic Lac operator (LacO) array stably integrated in the short arm of chromosome 1, distant from the centromere [187]. As expected, in cells expressing LacI-GFP-Bub1, the chromatin surrounding the LacO array was positive for pH2A-T120 and negative for pH3-T3 (Figure 3.2, A and C). In addition, binding of LacI-GFP-Bub1 to the LacO array promoted robust recruitment of Sgo1 (Figure 3.2, A and C), consistent with reports demonstrating a pH2A-Sgo1 interaction [41, 130, 136]. Also, in line with expectations, the chromatin surrounding the LacO array in cells expressing LacI-BFP-Haspin was positive for pH3-T3 and negative for pH2A-T120 (Figure 3.2, B and D). Targeting either kinase to the LacO array resulted in robust recruitment of Aurora B kinase and Survivin, indicating that each histone phospho-mark individually is capable of recruiting Aurora B kinase and the CPC in the absence of the other histone modification (Figure 3.2). In the case of pH2A, we found that targeting LacI-GFP-Sgo1 to the LacO array was sufficient to supersede the requirement for Bub1 and phosphorylation of H2A in CPC recruitment (Figure 3.3), in line with previous reports that Sgo1 serves as a bridging factor between pH2A-T120 and the CPC [41, 130, 136].

We also found that targeting LacI-GFP-Bub1 to the LacO array failed to promote robust recruitment of Aurora B kinase in cells depleted of Sgo1 (Figure 3.3), further supporting the idea that Sgo1 is important for Bub1/pH2A-T120-mediated recruitment of the CPC. Together, our results indicate that each histone phospho-modification is able to recruit the CPC, and raises the possibility that each mark has a unique function in localizing Aurora B kinase to a particular region of the centromere or kinetochore to carry out its roles in regulation of mitotic processes.

The pH2A-T120/Sgo1 pathway contributes to centromere accumulation of Aurora B but does not contribute to Aurora B kinase activity at the outer kinetochore

Previous studies have demonstrated that depletion of Haspin or Bub1, or inhibition of their respective kinase activities, results in delocalization of Aurora B kinase from the centromere region [123, 125, 142, 185, 188, 189]. Considering that pH2A is proximal to the kinetochore and the pH2A-T120/Sgo1 module is capable of recruiting Aurora B kinase independently of Haspin and pH3-T3, we hypothesized that the Bub1/pH2A/Sgo1 pathway may be responsible for recruitment of Aurora B specifically to kinetochores to phosphorylate kinetochore substrates. To test this, we depleted Sgo1 from cells and determined the levels of Aurora B at kinetochores using an antibody to active, phosphorylated Aurora B (pABK-T232) that prominently localizes to outer kinetochores in early mitosis. We found that while centromeric Aurora B levels decreased by ~30% upon Sgo1 depletion (Figure 3.4, A and B), which is consistent with previous reports [42, 190], pABK-T232 levels were unchanged after Sgo1 depletion (Figure 3.4, C and D). Furthermore, depletion of Sgo1 did not reduce kinetochore levels of pHec1-S44 (Figure 3.4, E and F), indicating that the activity of Aurora B at

kinetochores remains high in spite of Sgo1 depletion and perturbation of the pH2A/Sgo1 recruitment pathway. Consistent with these results, overexpression of wild-type Sgo1, or mutant versions of Sgo1 that either cannot bind cohesin and localize primarily to kinetochores (Sgo1-T346A), or cannot bind pH2A-T120 and localize primarily to chromosome arms (Sgo1-K492A) [41], did not drive accumulation of kinetochore-associated pABK-T232 or lead to an increase in phosphorylation of Hec1-S44 (Figure 3.4, I-N). These results suggest, contrary to our hypothesis, that while the Bub1/pH2A/Sgo1 pathway contributes to the localization of Aurora B kinase at the centromere, it does not significantly impact recruitment or activity of kinetochore-associated Aurora B kinase. These results also support the idea that loss of centromere-associated Aurora B does not strictly correlate to a reduction in Aurora B kinase activity at the kinetochore [98, 111, 117, 120, 133].

Bub1 depletion partially reduces Aurora B kinase activity at outer kinetochores

It has been previously established that Bub1 kinase is required for efficient accumulation of Aurora B at centromeres through the pH2A/Sgo1 recruitment pathway [121-123, 125, 142, 153, 154, 185, 188]. Consistent with these studies, we find that Bub1 depletion results in a decrease in centromere-associated Aurora B kinase by ~55% compared to levels measured in control cells (Figure 3.5). The remaining 45% of centromere Aurora B is presumably recruited through the Haspin/pH3-T3 pathway (see below and Figure 3.9). We also tested if depletion of Bub1 affected kinetochore-associated Aurora B kinase, and found that in cells treated with Bub1 siRNA, levels of pABK-T232 and pHec1-S44 at kinetochores were reduced to approximately 30% of levels measured in control cells (Figure 3.5; [133]). Since depletion of Bub1 reduced the

level of Aurora B kinase activity at kinetochores, we asked if this reduction was specifically due to loss of Bub1's kinase activity. To this end, we treated cells with a Bub1 inhibitor, BAY320 at 10 uM, which was sufficient to inhibit phosphorylation of H2A-T120, but had no effect on phosphorylation of H3-T3 (Figure 3.6) [154]. As shown in Figure 3.7A, and consistent with prior results, centromere accumulation of Aurora B was reduced after BAY320 treatment to ~68% of control levels [154]. To determine if activity of centromeric Aurora B was affected by this loss of accumulation, we generated and stained cells with a phospho-specific antibody to the TSS motif of human INCENP (Figure 3.8), a well-characterized Aurora B substrate [51, 105, 123]. Cells inhibited for Bub1 kinase activity exhibited a modest reduction in pINCENP-S893, S894 levels at centromeres (to ~77% of control levels), suggesting that the Aurora B recruited to centromeres via the Haspin/pH3-pathway retains activity (Figure 3.7B). We then tested if the activity of Aurora B at kinetochores was reduced upon kinase inhibition of Bub1. Kinetochore-associated pABK-T232 levels remained high in cells treated with BAY320 (Figure 3.7C), and consistent with this result, phosphorylation of Aurora B kinetochore substrates pHec1-S44 and pKnl1-S24 were not significantly decreased by treatment with BAY320 (Figure 3.7, D-E). We did note a small but significant decrease in phosphorylation of Dsn1-S109, an Aurora B kinase target site that is implicated in kinetochore assembly rather than error correction [35, 96, 191-193], to ~75% of control levels (Figure 3.7F). In contrast, treatment of cells with ZM447439, an Aurora B kinase inhibitor [123, 194], resulted in a near-complete loss of kinetochore-associated pABK-T232, pHec1-S44, pKnl1-S24, pINCENP-S893, S894, and pDsn1-S109 (Figure 3.7, D-F). Finally, we tested the efficiency of error correction in the presence of BAY320 using

a monastrol washout assay. In this assay, cells are arrested in monastrol, which results in the formation of monopolar spindles containing a large number of attachment errors in which both sister kinetochores are attached to the single spindle pole [195]. Upon washout of monastrol, bipolar spindles form, attachment errors are corrected in an Aurora B kinase-dependent manner, and chromosomes eventually align to the spindle equator and separate at anaphase [196]. To determine if loss of Bub1 kinase activity affects attachment error correction, we measured both the time from monastrol washout to anaphase onset and the percent of cells exhibiting chromosome segregation errors in cells treated with BAY320. As expected, upon inhibition of Aurora B kinase with the small molecule ZM447439, error correction was severely impaired and cells entered anaphase, on average, 49 min post monastrol-washout with a large number of uncorrected, erroneous attachments, resulting in severe chromosome segregation defects (Figure 3.7, G and H). Consistent with our finding that phosphorylation of kinetochore-associated Aurora B substrates are unaffected by Bub1 kinase inhibition, we did not observe statistically significant changes in the rate of chromosome segregation errors in the presence of BAY320 after monastrol washout or in the timing of anaphase onset compared to control cells (Figure 3.7, G and H), suggesting the presence of an in-tact error correction system. These results suggest that although Bub1 kinase activity is required for efficient centromere localization of Aurora B, it does not contribute to Aurora B activity at kinetochores. In contrast, depletion of the Bub1 protein from mitotic cells resulted in a decrease in kinetochore-associated Aurora B activity (Figure 3.5), which together with the BAY320 experiments, suggests that Bub1,

but not its kinase function, contributes to either the recruitment or activity of Aurora B at the kinetochore.

Inhibition of Haspin kinase re-localizes Aurora B from inner centromeres to kinetochore-proximal outer centromeres

Our results thus far suggest that although the Bub1/pH2A/Sgo1 pathway promotes efficient accumulation of Aurora B at the centromere, it is not required for localization or activity of Aurora B at kinetochores. This led us to ask if the Haspin/pH3 pathway, which is also required for efficient accumulation of Aurora B at the centromere [121, 122, 125, 153], is important for localization or activity of Aurora B at kinetochores. We therefore treated cells with the Haspin inhibitor, 5-iodotubercidin (5-ITu), and assessed recruitment of pABK-T232 to kinetochores and phosphorylation of multiple kinetochore Aurora B substrates. Unfortunately, addition of the inhibitor at concentrations that resulted in full inhibition of Haspin kinase activity towards pH3-T3 (1-10 uM) [122, 131, 185], resulted in decreased activity of Bub1 kinase, suggesting that the inhibitor exhibits some degree of non-specificity at the concentrations used (Figure 3.6; [131, 185]). Even so, we found that while treatment of cells with both 10 uM BAY320 and 10 uM 5-ITu led to a complete loss of accumulation and activity of Aurora B at the centromere (Figure 3.9), levels of kinetochore pABK-T232 were not decreased (Figure 3.9). Furthermore, phosphorylation levels of kinetochore-associated Aurora B kinase substrate Knl1-S24 were not significantly reduced and Hec1-S44 phosphorylation levels were only modestly decreased (Figure 3.9). Interestingly, we did measure a decrease in phosphorylation of Dsn1-S109 (an Aurora B kinase substrate implicated in kinetochore assembly) to ~45% of control levels upon treatment of cells

with both BAY320 and 5-ITu (Figure 3.9). We note that in an accompanying manuscript by Hadders et al., 2020 the authors report that phosphorylation levels of Dsn1-S109 are not reduced in Haspin knock-out cells or in Haspin knock-out cells treated with the Bub1 inhibitor BAY320 [128]. These findings suggest, consistent with our earlier results, that 5-ITu may induce off-target effects that differentially affect kinetochore functions at the concentrations required to inhibit Haspin activity in cells.

We noted that after treatment with 5-ITu and loss of centromere-localized Aurora B, a population of Aurora B, detected in cells expressing Aurora B-GFP, re-localized from the inner centromere to a “double dot” pattern, reminiscent of what we observed for pH2A-T120 staining (Figure 3.1). Linescans of kinetochore pairs from cells treated with 5-ITu suggest that this population of Aurora B resides inside of both inner and outer kinetochore markers (Figure 3.10, A and B). Indeed, we confirmed this by measuring the distances between Aurora B-GFP and both CENP-C and the N-terminus of Hec1. After 5-ITu treatment in early mitotic cells, Aurora B-GFP localizes approximately 65 ± 39 nm inside of CENP-C and 107 ± 48 nm inside of the N-terminus of Hec1 (Figure 3.11, C and D; Table 1). Therefore, after loss of the pH3-T3 binding site at the inner centromere, the kinase is still recruited to the kinetochore-proximal outer centromere, likely through the Bub1/pH2A/Sgo1 pathway (Figure 3.9).

Aurora B kinase and INCENP localize to both centromeres and kinetochores in mitotic cells

Two distinct pathways contribute to accumulation of Aurora B kinase at centromeres however, neither of these pathways impact Aurora B localization at kinetochores. These data suggest that the population of Aurora B at the kinetochore

may not only be functionally, but spatially distinct from both the inner centromere and kinetochore-proximal outer centromere populations of the kinase. To test this, we mapped the position of what we hypothesized to be “kinetochore-localized” Aurora B. Indeed, our measurements indicate that a population of Aurora B detected by the pABK-T232 antibody resides within the kinetochore proper and is on average ~22 nm outside of CENP-C, and ~ 8 nm inside of the N-terminus of Hec1 in early mitosis (Figure 3.11, A, C, and D; Table 1).

Finally, to confirm that the multiple populations of Aurora B detected at the centromere and kinetochore region are components of active CPC complexes, we stained cells with a phospho-specific antibody to INCENP (pINCENP-S893, S894), whose phosphorylation is required for activation of Aurora B and serves as an indicator of CPC activity [51, 105]. In late prometaphase and metaphase, pINCENP-S893, S894 localized prominently to the inner centromere, as previously reported ([197]; Figure 3.8). However, in early mitosis, we observed pINCENP-S893, S894 at multiple locations within the centromere and kinetochore. In the majority of kinetochore pairs, we observed robust localization of pINCENP-S893, S894 at the outer kinetochore (as determined by linescans), confirming that the outer kinetochore-localized Aurora B is indeed active (Figure 3.11B). In addition, although less frequently, we were able to detect populations of pINCENP-S893, S894 at the inner centromere and the kinetochore-proximal outer centromere in early mitotic cells (Figure 3.11B). Since pINCENP-S893, S894 localized to all three localizations, we were unable to accurately map its precise location within the centromere and/or kinetochore using the two-color fluorescence localization approach.

3.4 Discussion

Aurora B kinase localizes prominently to the inner centromere during mitosis. Several of its known substrates also localize to this region including its activator, INCENP, and the kinesin-13 microtubule motor protein MCAK, both of which have important roles in mitotic progression [51, 105, 155, 156]. Additionally, a population of Aurora B has been detected at the outer kinetochore in early mitosis, where it is proposed to have a role in phosphorylating substrates that regulate kinetochore-microtubule attachment stability [87, 90, 184]. Resolving how Aurora B is recruited to each of these regions is critical to understand how the kinase is regulated temporally during mitosis so that erroneous kinetochore-microtubule attachments are corrected and proper attachments are stabilized. Previous studies have shown that Aurora B and the CPC are recruited to centromeres through two phospho-histone modifications generated by Haspin-mediated phosphorylation of histone H3-T3 and Bub1-mediated phosphorylation of histone H2A-T120 [121, 122, 125, 130, 136, 153, 154, 198]. Both modifications are required for efficient Aurora B accumulation at centromeres, and it has been suggested that the region within the centromere where both phospho-marks overlap serves as the recruitment site for the CPC [125, 198]. However, we found that in both HeLa and U2OS cells in early and late mitosis, these two phospho-histone modifications are spatially distinct, with pH3-T3 localized to the inner centromere and pH2A-T120 localized to the kinetochore-proximal outer centromere. Additionally, both modifications are individually capable of recruiting Aurora B and CPC components, indicating that explicit overlap of the two marks within centromeric chromatin is not required for CPC recruitment. Based on these results, we hypothesized that the kinetochore-proximal outer centromere population of the CPC recruited by phospho-

H2A might be responsible for the localization and activity of Aurora B at kinetochores. We discovered, however, that this is not the case and the Bub1/pH2A/Sgo1 pathway is not required for kinetochore localization or activity of Aurora B. Rather, both histone marks, although spatially distinct, contribute to accumulation of Aurora B and the CPC at the centromere (Figure 3.10, A and B).

We found that in early mitosis, Aurora B -- detected by a pan-Aurora B antibody -- localizes predominantly to the inner centromere, and we rarely observed localization at the kinetochore-proximal outer centromere (Figure 3.10, A and B). However, upon inhibition of Haspin kinase with 5-ITu (which decreases, but does not completely eliminate Bub1 kinase activity), Aurora B localizes prominently to the kinetochore-proximal outer centromere region in early mitotic cells (Figure 3.10, A and B). A similar re-localization pattern was observed for the CPC component Borealin in cells treated with 5-ITu [131]. Based on these findings, we speculate that Aurora B and the CPC are recruited to the outer centromere region transiently. These results point to the possibility of a two-step loading process whereby a pool of the CPC is loaded directly to inner centromere binding sites provided by pH3, and a second population of the complex is initially recruited to the kinetochore-proximal outer centromere by pH2A and subsequently translocated to the inner centromere. This proposed two-step process is somewhat similar to what has been suggested for Sgo1, which has been observed to first load to kinetochores in early mitosis, after which it re-localizes to inner centromeres to protect cohesion and in turn, prevent premature sister chromatin separation [41, 110, 130]. Furthermore, a Sgo1 mutant unable to localize to kinetochores ($Sgo1^{K492A}$) cannot efficiently localize to centromeres [41, 130], suggesting that Sgo1 must first bind

kinetochores in order to eventually load onto centromeres [41, 110, 130]. This re-localization has been proposed to involve RNA Polymerase II transcription and "opening" of dense centromeric chromatin [130]. Finally, Sgo1 has been shown to de-localize from centromeres and re-localize to a region near CENP-C in Haspin knock-out cells [177], similar to what was observed for Borealin [131] and Aurora B (Figure 3.10, A and B) after Haspin inhibition. Based on these similarities, it is tempting to speculate that the CPC may be transported between different regions within the kinetochore and centromere, similar to what has been described for Sgo1. We note, however, that CPC "shuttling" is speculative, and that our data do not rule out other types of inter-dependencies between different CPC populations within the kinetochore and centromere.

Although the two distinct populations of the CPC recruited by histone phospho-modifications are required for efficient accumulation and activity of Aurora B at centromeres, our results suggest that neither modification is explicitly required for recruitment or activity of Aurora B at the outer kinetochore. Several studies corroborate this finding. (1) In chicken DT40 cells, a Survivin mutant lacking the centromere targeting domain is competent to rescue mitotic defects resulting from loss of inner centromere CPC after Survivin depletion [120]. (2) In human cells, ectopic targeting of the CPC to centromeres does not restore Aurora B activity at kinetochores in cells that are depleted of Knl1, which prevents recruitment of phosphorylated, active Aurora B to kinetochores [133]. (3) In budding yeast, a mutation in Sli5/INCENP that disrupts CPC centromere localization, but not microtubule binding, has a minimal effect on chromosome bi-orientation and Aurora B-mediated error correction [117] (4) Also in

budding yeast, two groups have recently demonstrated that the CPC binds directly to the kinetochore COMA complex (analogous to human CENP-O/P/Q/U), and is recruited to kinetochores in cells. Importantly, this kinetochore-associated population is sufficient for Ipl1/Aurora kinase activity at kinetochores in the absence of centromere-localized CPC [150, 151]. Together these results suggest that Aurora B activity at kinetochores can be uncoupled from centromere accumulation of the kinase. However, recent studies have also suggested that while centromere-localized Aurora B is not required *per se* for Aurora B phosphorylation of outer kinetochore substrates, it may be involved in properly regulating kinase activity in response to formation of stable kinetochore- microtubule attachments [90, 199]. How inner centromere-localized Aurora B impacts this regulation remains to be resolved.

In agreement with our results, an accompanying paper from the Lens lab also demonstrated that Bub1 and Haspin are individually capable of recruiting the CPC to distinct locations within the kinetochore, and furthermore, they find that neither recruitment pathway is required for kinase activity of Aurora B at kinetochores [128]. Interestingly, in their study, CRISPR/Cas9-mediated Haspin knockout combined with Bub1 kinase inhibition resulted in impaired error correction as detected by a monastrol washout assay. In light of high Aurora B substrate phosphorylation at kinetochores under these conditions, this finding could be explained by (1) a role for Haspin and/or Bub1 kinase activities in error correction separate from the kinetochore-associated Aurora B kinase-mediated mechanism [177]; (2) a role for centromere-localized Aurora B activity in error correction (*e.g.* mediated by phospho-MCAK) [155, 156]; or (3) a

requirement for centromere-localized Aurora B kinase (and the CPC) in cohesin protection and prevention of premature sister chromatid separation [110].

Several questions regarding the regulation of Aurora B localization remain to be addressed, including: What are the binding sites for kinetochore-localized Aurora B? And how is kinase recruitment regulated such that it binds kinetochores with immature microtubule attachments but is evicted from those with mature, stable attachments? While these questions remain unresolved, our work suggests that there are at least two pathways for Aurora B recruitment to the kinetochore– one that requires Bub1 (but not its kinase activity) and one that does not. Previous work has demonstrated that localization and activity of kinetochore-associated Aurora B requires the large kinetochore scaffolding protein Knl1 [133], which directly recruits a complex of Bub1/Bub3 [62, 67-69, 200]. Thus it is likely that the dependence of kinetochore-localized Aurora B on Knl1 is at least in part due to its recruitment of Bub1. Interestingly, we found in this study that in early mitosis, Aurora B binds to kinetochores at a location somewhere between CENP-C and the N-terminus of Hec1 (Figure 3.11). Of note, Knl1 resides in this vicinity of the kinetochore and has been suggested to undergo significant changes in conformation as kinetochores experience pulling forces from attached, dynamic microtubules [53, 201]. It is tempting to speculate that Knl1 and its conformational changes upon kinetochore-microtubule stabilization may play a role in Aurora B kinase recruitment to and eviction from kinetochores, possibly through Bub1-dependent and Bub1-independent mechanisms. Further experiments are needed to explore this possibility.

3.5 Materials and Methods

Cell culture and generation of cell lines

HeLa Kyoto cells were cultured in DMEM supplemented with 10% FBS, 1% antibiotic/antimycotic solution, 4 mM L-glutamine and maintained at 37°C in 5% CO_2. U2OS osteosarcoma cells expressing an ectopic Lac operator array stably integrated into the short arm of chromosome 1 (a gift from S. Janicki; Wistar Institute, Philadelphia, PA; Janicki et al., [187]) were maintained in McCoy's 5A medium supplemented with 10% FBS and 1% antibiotic/antimycotic solution and maintained at 37°C in 5% CO_2. Flp-In T-REx HeLa host cells (a gift from S. Taylor, University of Manchester, England, UK) were cultured in DMEM supplemented with 10% FBS, 1% antibiotic/antimycotic solution, 2 mM L-glutamine and maintained at 37°C in 5% CO_2. Stable cell lines expressing inducible WT-Sgo1-GFP, K492A-Sgo1 GFP, and T346A-Sgo1-GFP were generated from the Flp-In T-REx HeLa host cell line. Cells were grown to 50% confluency and transfected with 2.4 ug pOG44 recombinase-containing plasmid and 0.3 ug pcDNA5.FRT.TO-WT-, K492A-, or T346A- Sgo1-GFP plasmids with Fugene HD (Promega). The pcDNA5.FRT.TO-Sgo1 plasmids were generated through PCR amplification of WT Sgo1 (gift from Dr. Hongtao Yu, UT Southwestern, Dallas, TX; Liu et al., 2013), and then cloned into a pcDNA5.FRT.TO vector through In-Fusion cloning. After 48 h, cells were switched to media containing 100 ug/ml hygromycin (EMD Millipore) and grown in selection media for 2 weeks. Hygromycin-resistant foci were expanded and tested for inducible Sgo1-GFP expression. Cell lines expressing Sgo1 fusion proteins were cultured in DMEM supplemented with 10% FBS, 1% antibiotic/antimycotic solution, 2 mM L-glutamine, 100 ug/mL hygromycin and

maintained at 37°C in 5% CO_2. Gene expression was induced with 1 ug/mL doxycycline (Sigma-Aldrich) for 12-24 h.

Cell treatments and transfections

For live-cell imaging, cells were seeded and imaged in 35 mm glass bottomed dishes (MatTek Corporation) and imaged in Leibovitz's L-15 medium (Invitrogen) supplemented with 10% FBS, 7 mM Hepes, and 4.5 g/liter glucose, pH 7.0. For fixed-cell analysis, cells were grown on sterile, acid-washed coverslips in six-well plates. U2OS cells were transfected with either LacI-GFP, LacI-BFP, LacI-GFP-Bub1, LacI-BFP-Haspin, or LacI-GFP-Sgo1 vectors using Lipofectamine 2000 (Thermo Fisher Scientific). Transfection solutions were added to cells for 24 h then 9 uM RO-3306 (Sigma-Aldrich, Fisher Scientific) was added for an additional 20 h. Cells were then released from RO-3306 by washing with drug-free media for 30 min before fixation. For siRNA experiments, HeLa Kyoto cells were transfected with 8 uL of 20 uM stock siRNA solution using Oligofectamine (Thermo Fisher Scientific) and fixed 24 h post-transfection. siRNA sequences are as follows: Sgo1: CAGTAGAACCTGCTCAGAACC (Qiagen); Bub1: CAGCTTGTGATAAAGAGTCAA (Qiagen); INCENP: CCGCATCATCTGTCACAGTTA (Qiagen). For Sgo1 overexpression experiments, Sgo1-GFP constructs were induced using 1 ug/mL doxycycline for 24 h. For Aurora B-GFP over-expression, HeLa Kyoto cells were transfected with a plasmid containing full length human Aurora B fused to GFP using Lipofectamine3000 (Thermo Fisher Scientific). For Haspin inhibition, cells were incubated with 10 uM 5-Iodotubercidin (5-ITu, Sellechkchem) 30 min prior to fixation. For inhibition of Bub1 kinase, cells were treated with 10 uM BAY320 (provided by Dr. Gerhard Siemeister, Bayer AG, Berlin) 30

min before fixation. For Aurora B kinase inhibition, cells were incubated with 20 uM ZM447439 (Tocris Biosciences) for 30 min prior to fixation. For monastrol washout experiments, HeLa Kyoto cells were synchronized in early mitosis using a double thymidine block and release. Briefly, cells were treated with 2.5 mM thymidine for 16 h, washed with PBS, replaced with fresh medium and incubated for 8 h. For the second block, cells were again treated with 2.5 mM thymidine for 16 h. Cells were then washed with PBS, replaced with fresh medium, and after 8 h, 150 uM monastrol (Tocris Biosciences) was added for 2 h. After 2 h, cells were washed with drug-free media and placed into either control media or media containing 10 uM ZM447439 or 10 uM BAY320 and imaged for 2 h.

Immunofluorescence

For U2OS LacO/LacI targeting and siRNA experiments, cells were rinsed in 37°C PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 4 mM $MgSO_4$, pH 7.0) and lysed at 37°C for 3 min in freshly prepared lysis buffer (PHEM buffer + 1% Triton X-100), followed by fixation for 20 min in 4% paraformaldehyde in PHEM buffer (37°C). After fixation, cells were washed 3 X 5 min in PHEM-T (PHEM buffer + 0.1% Triton X-100) and blocked in 10% boiled donkey serum (BDS) in PHEM for 1 h at room temperature. Primary antibodies were diluted in 5% BDS and added to coverslips overnight at 4°C. See Table 2 for primary antibody information. After primary antibody incubation, cells were rinsed 3 X 5 min in PHEM-T and then incubated for 45 min at RT with secondary antibodies conjugated to either Alexa Fluor 488, Alexa Fluor 568, or Alexa Fluor 647 (Jackson ImmunoResearch Laboratories, Inc.) at 1:500 diluted in 5% BDS. Cells were rinsed 3 X 5 min in PHEM-T, quickly rinsed in PHEM followed by

incubation in DAPI (2 ng/ml) diluted in PHEM for 30 s. Slides were rinsed 3 X 5 min in PHEM-T, quickly rinsed in PHEM and then mounted onto glass slides with antifade solution (90% glycerol, 0.5% N-propyl gallate). Coverslips were sealed with nail polish and stored at 4°C. For two-color distance measurements and kinase inhibition experiments, cells were rinsed in 37°C PHEM buffer and permeabilized with a wash (30 s) in PHEM-T (0.5%) followed by fixation for 20 min in 4% paraformaldehyde in PHEM buffer (37°C). After fixation, cells were treated as described above. The double-affinity purified antibody against dual phosphorylated INCENP pS893 and pS894 was generated at 21st Century Biochemicals. For generation of the dual phospho-INCENP pS893, pS894 antibody, rabbits were immunized with a peptide corresponding to amino acids 887-901 of human INCENP phosphorylated at S893 and S894.

Imaging and data analysis

All fixed cell images were acquired on an IX71 inverted microscope (Olympus) incorporated into a DeltaVision Personal DV imaging system (GE Healthcare) using SoftWorx software (GE Healthcare). Slides were imaged using a 60X 1.42 NA differential interference contrast Plan Apochromat oil immersion lens (Olympus) and a CoolSNAP HQ2 camera. Images for two-color distance and fluorescence intensity measurements were acquired as z-stacks at 0.2-um intervals. For two-color distance measurements, a 1.6 magnification lens was inserted in the light path, providing a final magnification of 67 nm/pixel at the camera sensor (CoolSNAP HQ2; Photometrics/Roper Technologies), and analysis was carried out on kinetochore pairs that resided in a single focal plane from non-deconvolved images (with the exception of Aurora B-GFP images, which were deconvolved prior to analysis). The centroids of

each kinetochore- or centromere-localized test antibody signal were determined in MATLAB (Math Works) using SpeckleTracker, a customized program provided by Drs. Xiaohou Wan and Ted Salmon [53]. Distances were then calculated using the identified centroids. Kinetochore (and in the case of pH2A-T120, kinetochore-proximal outer centromere) fluorescence intensities of pABK-T232, pHec1-S44, pKnl1-S24, and pH2A-T120 were quantified from images in MATLAB using the customized SpeckleTracker program. Centromere fluorescence intensities were measured using MetaMorph software (Molecular Devices) by centering a 9-pixel diameter circle between two sister kinetochores, placing two additional 9-pixel circles adjacent to the first circle, and logging the total integrated intensities. For each centromere measurement, the average background fluorescence intensity was calculated by averaging the two adjacent circles and subtracting this value from the integrated intensity logged from the centromere circle. Whole cell intensity values were calculated by first generating maximum intensity projections from non-deconvolved z-stacks. A large, 150-diameter pixel circle was placed around the cell, and total fluorescence intensity was logged. In addition, 6 15-pixel diameter circles were placed outside the large circle and fluorescence intensities were logged. The background intensity was determined by averaging the fluorescence intensity values from the 6 smaller circles, calculating the per-pixel intensity value, and multiplying by the area of the large circle. Finally, the background-corrected whole cell intensity was calculated by subtracting the background value from the total integrated intensity of the large circle. For quantification of LacO/LacI experiments, recruitment of CPC components to the LacO array was determined by measuring fluorescence intensity within the LacI-positive region using maximum intensity projected images with

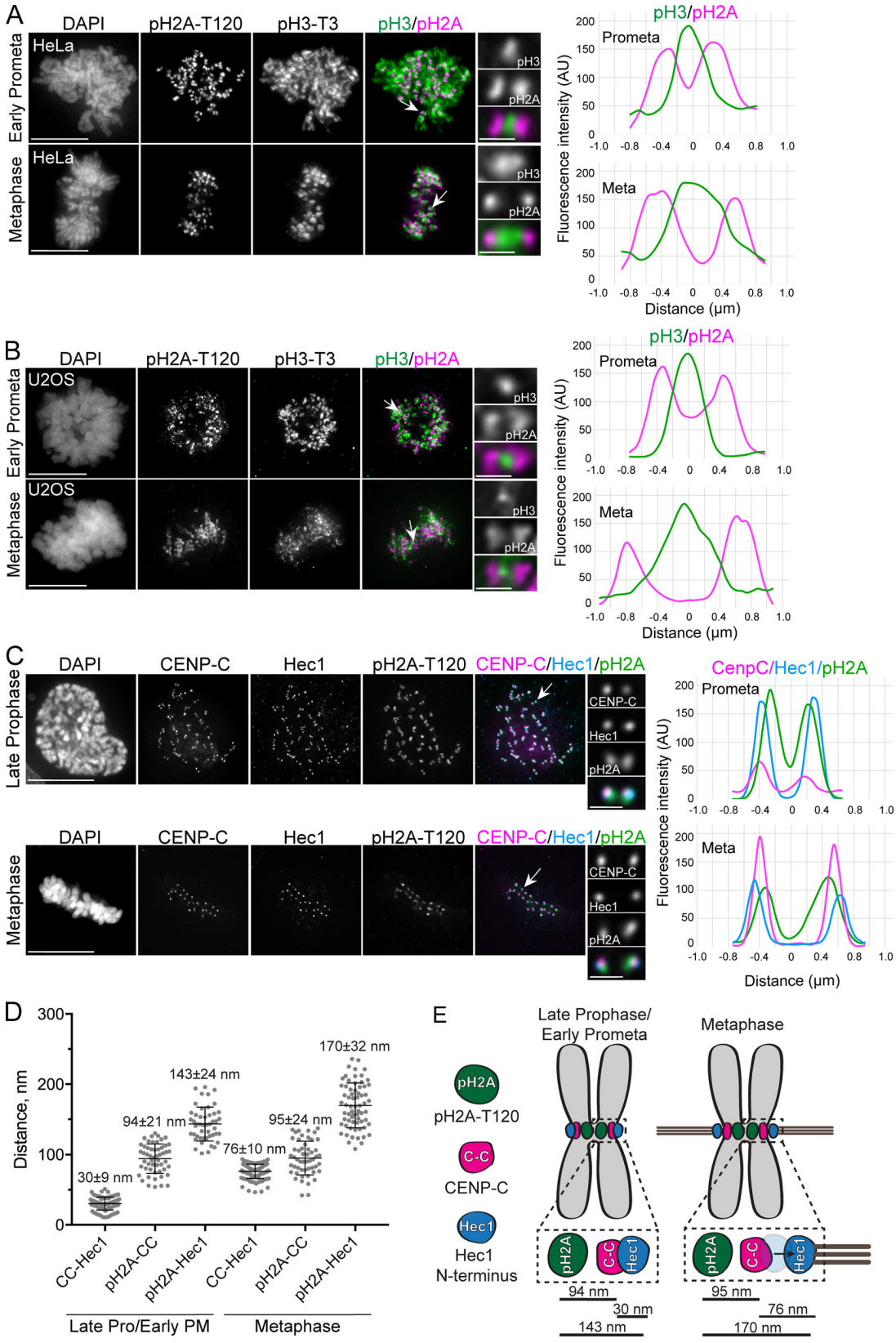
FIJI software. Background values were determined by measuring the average intensity of each test antibody at the LacI-GFP or LacI-BFP ectopic locus (measured in cells expressing LacI-GFP or LacI-BFP only, not fused to a test protein). In each experiment using a targeted LacI-fusion protein, cells were considered “positive” if the fluorescence intensity at the locus was higher than the average background values. For live cell experiments, images were collected on a Nikon Ti-E microscope equipped with a PLAN APO, DIC, 0.95 NA 40x TIRF objective, a Ti-S-E motorized stage, piezo Z-control (Physik Instruments), a spinning disc confocal scanner unit (CSUX1; Yokogawa) and an iXon DU888 cooled EM-CCD camera (Andor). Cells were imaged every 5 min for 2 h; 3 x 1 um z-sections were taken at each time point. All statistical analyses were performed in Prism software (GraphPad).

In vitro phosphatase assay

A complex of Aurora B kinase (amino acids 60-361) and INCENP (amino acids 1-68 and 834-900) was generated by expressing the constructs in BL21DE3 cells by induction with IPTG overnight at 18°. Cells were lysed by sonication and centrifuged at 45,000 rpm. The supernatant fraction was purified on glutathione resin (Pierce #16100), followed by cleavage of the GST moiety with HRV-3C protease. For the phosphatase assay, 1.7 uM Aurora B/INCENP complex was incubated with or without lambda protein phosphatase (2,400U) (NEB #P0753S) in 1X NEBuffer for protein metallophosphatases (PMP) and 1 mM $MnCl_2$, for 30 min at 30° C. Reactions were terminated with the addition of 5X SDS-PAGE sample buffer. Samples for pABK-T232 and pINCENP-S893, S894 were run on 12% and 15% SDS-polyacrylamide gels respectively, transferred to polyvinylidene difluoride membrane (GE 10600030) and processed for Western blotting.

For phospho-staining, blots were incubated with pABK-T232 and pINCENP-S893, S894 at 1:500 (1% BSA in TBS). Primary antibodies (see Table 2 for details) were detected using horseradish peroxidase-conjugated anti-rabbit secondary antibody at 1:10,000 (Jackson ImmunoResearch Laboratories, Inc.) and enhanced chemiluminescence. Reactions with and without lambda phosphatase were run on 15% SDS-polyacrylamide gels and stained with Coomassie brilliant blue for total protein.

Figures 3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143±24 nm
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170±32 nm
CC-Hec1
pH2A-CC
pH2A-Hec1
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76 nm
170 nm

Figure 3.1. pH3-T3 and pH2A-T120 occupy discrete locations within the centromere region in mitosis. (A) and (B) Immunofluorescence images of early prometaphase and metaphase HeLa (A) and U2OS (B) cells stained with antibodies to phosphorylated histone H3-T3 (pH3) and phosphorylated histone H2A-T120 (pH2A). Arrows point to the kinetochore pairs shown in the insets. Linescans through the centromere/kinetochore region are shown to the right of each panel. (C) Immunofluorescence images of late prophase and metaphase HeLa cells stained with antibodies to CENP-C, the N-terminus of Hec1 (antibody 9G3), and pH2A-T120. Arrows point to the kinetochore pairs shown in the insets. Linescans through the centromere/kinetochore region are shown to the right of each panel. (D) Plots of the mean distance between the indicated centromere or kinetochore proteins. Each point on the graph represents a distance measurement from a pair of sister chromatids. n values are listed in Table 1. Measurements on the left were obtained from late prophase or early prometaphase HeLa cells; measurements on the right were obtained from metaphase cells. Distance is in nm. (E) Summary of distance measurements from experiments in C and D. Scale bars are 10 um (whole cells) and 1 um (insets). Whole cell images shown are maximum intensity projections of z-stacks containing 64 planes and inset images shown are projections from 2-4 planes.

Table 1. Mean distance measurements for kinetochore and centromere components described in Figures 3.1 and 3.11. Shown for each are the average distances, standard deviations, and number of kinetochores/centromeres and cells measured for each condition.

	Distance, nm	SD	# Kinetochores	# Cells
Metaphase				
CENP-C to Hec1 N-term	76.2	10.2	113	19
pH2A to CENP-C	95.1	24.1	52	10
pH2A to Hec1 N-term	170.0	31.8	67	10
Late prophase/early prometaphase				
CENP-C to Hec1 N-term	30.4	9.3	86	18
pH2A to CENP-C	94.4	20.9	51	10
pH2A to Hec1 N-term	143.4	24.0	67	10
CENP-C to pABK	21.8	11.8	51	15
ABKGFP to CENP-C	64.7	39.4	96	27
ABKGFP to Hec1 N-term	107.4	48	81	22

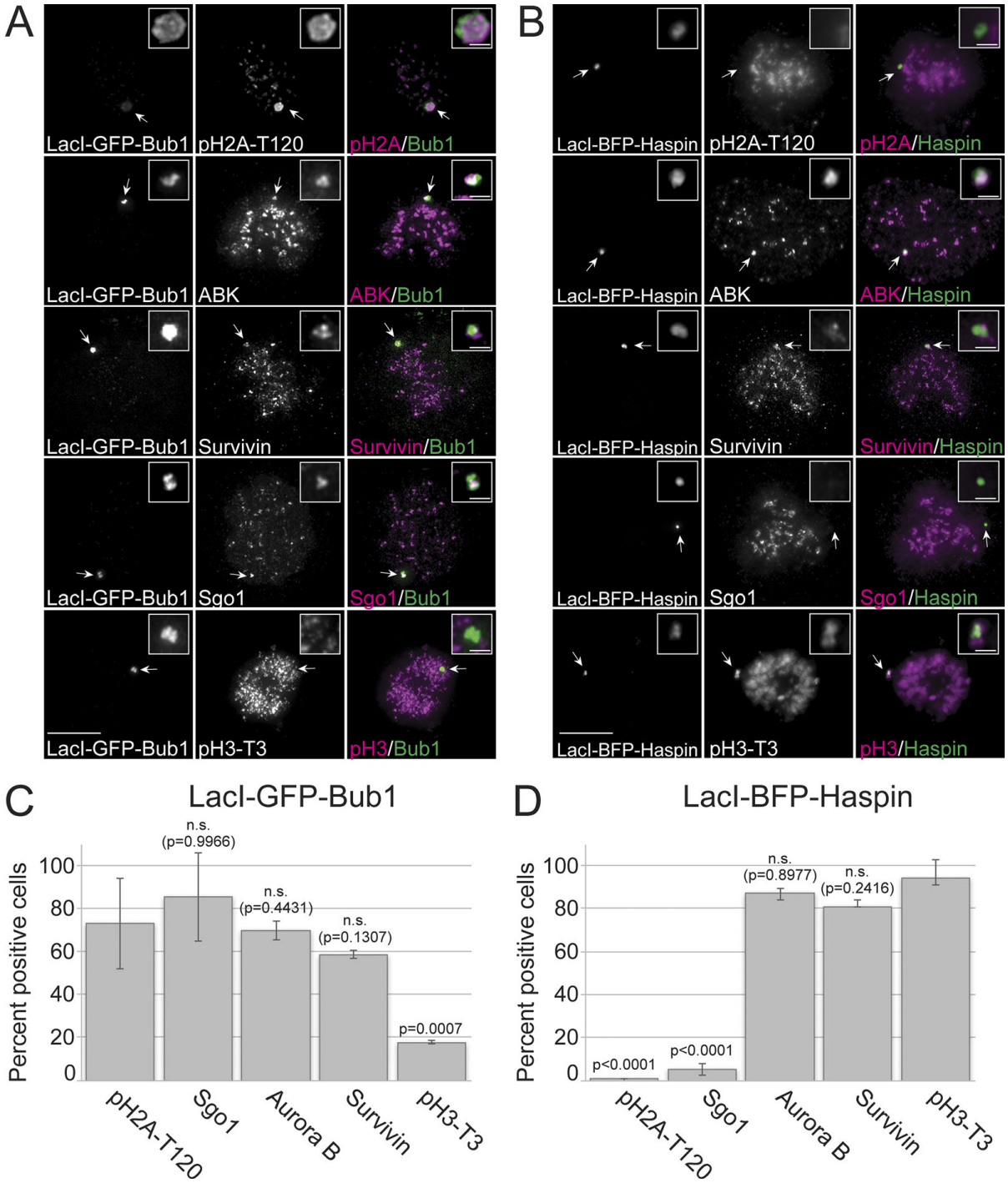


Figure 3.2. pH3-T3 and pH2A-T120 are each capable of recruiting Aurora B kinase and the CPC. (A) and (B) Immunofluorescence images of U2OS cells expressing LacI-GFP-Bub1 (A) or LacI-BFP-Haspin (B). Cells were immunostained for the indicated centromere/kinetochore proteins or phospho-specific epitopes. Arrows point to the LacO array with indicated LacI-fused protein shown in the insets. Cells were synchronized in G2 using RO-3306 and washed out of drug 30 min prior to fixation to enrich for early

mitotic cells. (C) and (D) Quantification of experiments in A and B. The average fluorescence intensity of each antibody was measured at the LacO array in cells expressing either LacI-BFP or LacI-GFP (not fused to a test protein), and average values were measured and used to calculate thresholds. Fluorescence intensities of each antibody were then measured in cells expressing LacI-BFP-Haspin or LacI-GFP-Bub1, and cells were scored as positive if intensity values were higher than the calculated thresholds. For each experiment, ≥20 cells were quantified from n ≥ 3 experiments. Error bars represent SD. Significance values were calculated from a one-way ordinary ANOVA test. Shown are significance values between experiments for each test antibody and either pH2A-T120 (C) or pH3-T3 (D). Scale bars are 10 um (whole cells) and 1 um (insets).

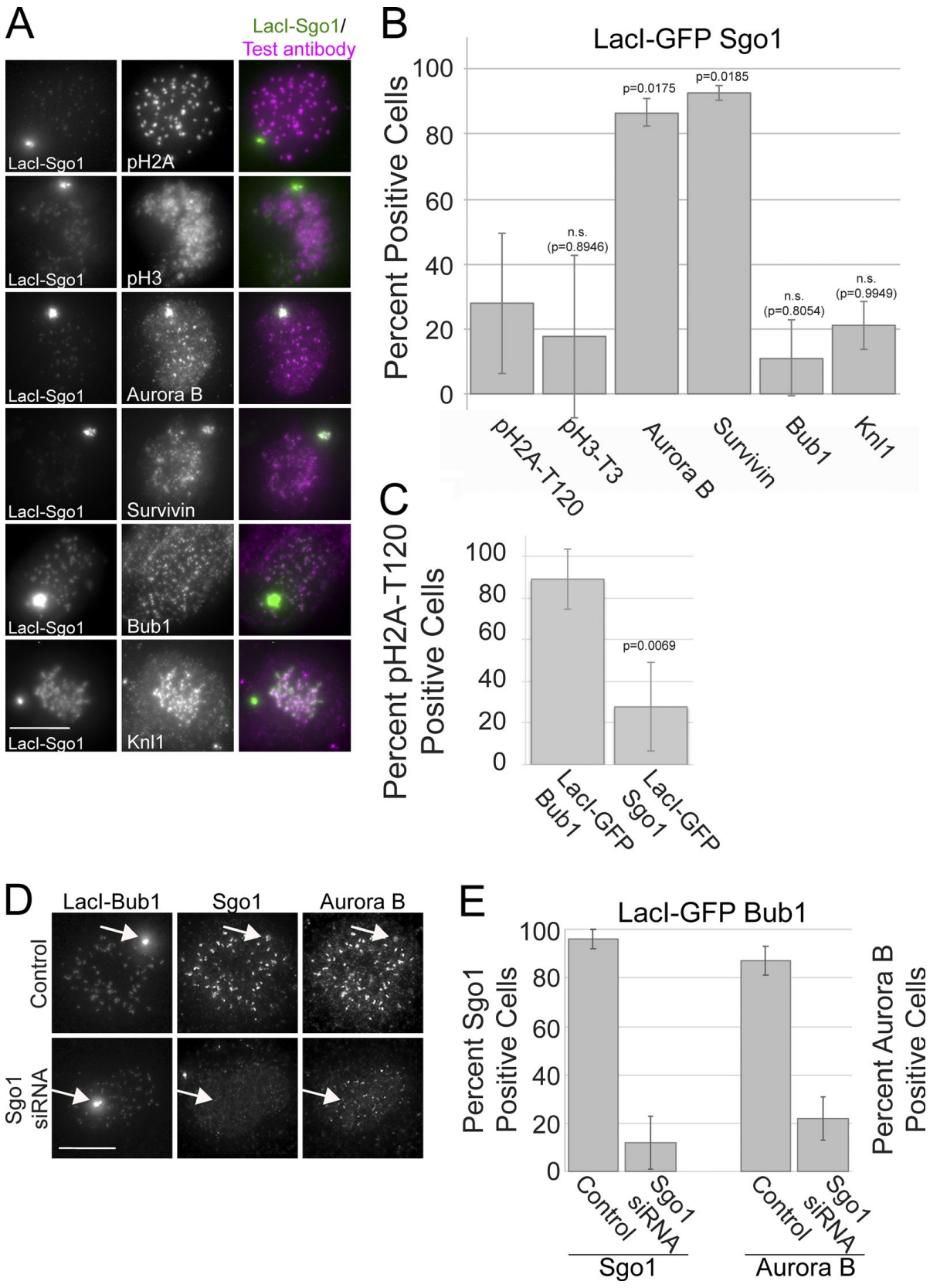

A
LacI-Sgo1/
Test antibody
LacI-Sgo1
pH2A
pH3
Aurora B
Survivin
Bub1
Knl1
B
LacI-GFP Sgo1
Percent Positive Cells
p=0.0175
p=0.0185
n.s.
(p=0.8946)
n.s.
(p=0.8054)
n.s.
(p=0.9949)
pH2A-T120
pH3-T3
Aurora B
Survivin
Bub1
Knl1
C
Percent pH2A-T120
Positive Cells
p=0.0069
LacI-GFP
Bub1
LacI-GFP
Sgo1
D
LacI-Bub1
Sgo1
Aurora B
Control
Sgo1
siRNA
E
LacI-GFP Bub1
Percent Sgo1
Positive Cells
Percent Aurora B
Positive Cells
Control
Sgo1
siRNA
Sgo1
Aurora B

Figure 3.3. Sgo1 recruits the CPC to an ectopic chromosome locus in human cells. (A) Immunofluorescence images of U2OS cells expressing LacI-GFP-Sgo1. Cells were immunostained for the indicated proteins or phospho-histone epitopes and analyzed for positive recruitment based on a threshold intensity determined from U2OS cells expressing LacI-GFP. (B) Quantification of the recruitment of indicated proteins or phospho-epitopes in LacI-GFP-Sgo1 expressing cells. (C) Quantification of pH2A-T120 recruitment in Bub1 or Sgo1-LacI expressing cells. For all conditions, cells were synchronized in G2 using RO-3306 and washed out of drug 30 min before fixation to enrich for mitotic cells. (D) Immunofluorescence images of U2OS cells expressing LacI-GFP-Bub1. Top row: control; bottom row: Sgo1 siRNA. (E) Quantification of percent Sgo1 positive cells (left) and percent Aurora B positive cells (right) in control and Sgo1 siRNA-treated cells. For each condition shown, ≥20 cells were quantified from at least 2 independent experiments. Error bars represent SD. For panel B, significance values were calculated using a one-way ordinary ANOVA test. Shown are significance values between experiments for each test antibody and pH2A-T120. For panel C, the significance value was calculated using an unpaired nonparametric Student's t test. Scale bars are 10 um.

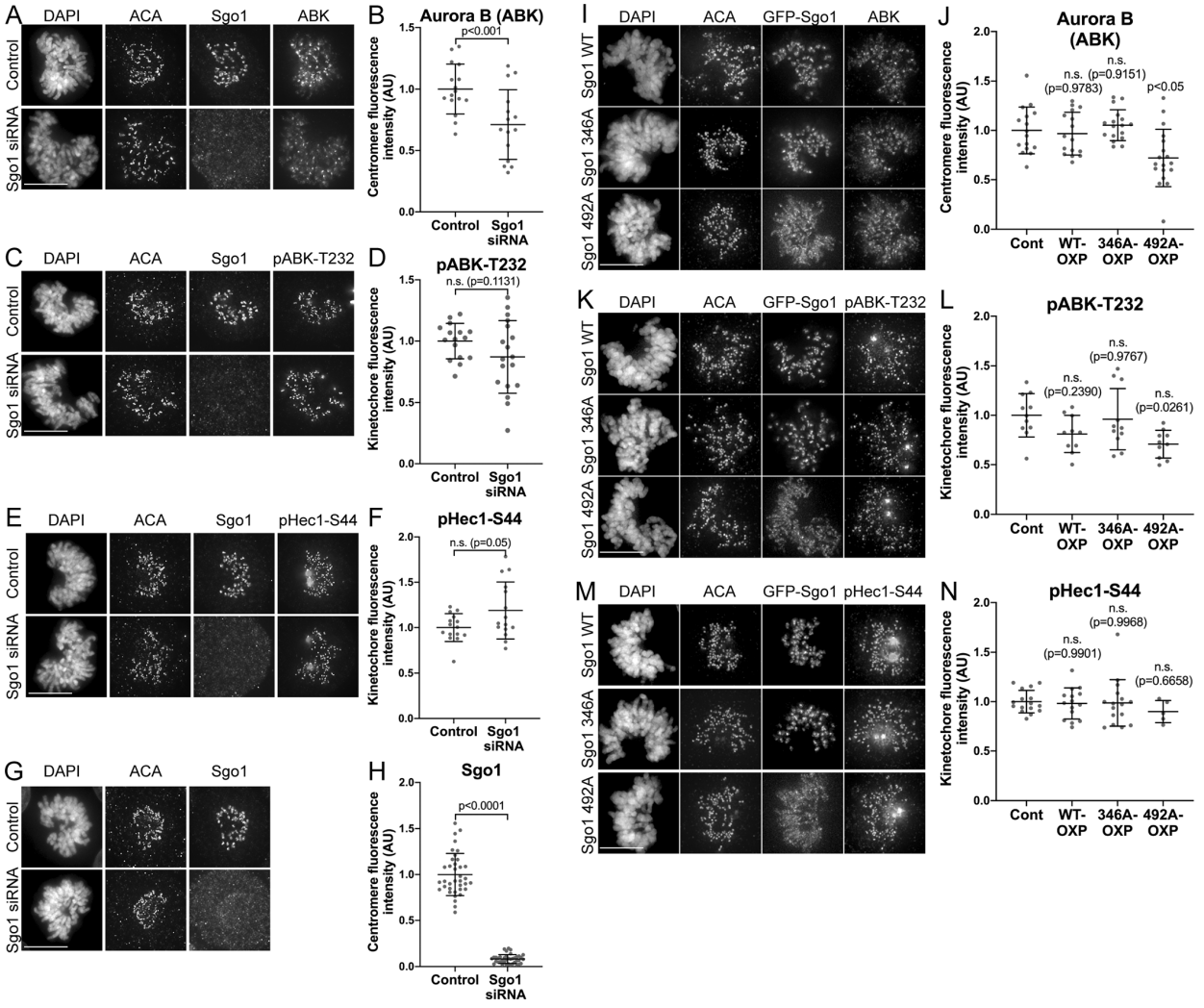


Figure 3.4 The pH2A-T120/Sgo1 pathway contributes to the accumulation of centromere-associated Aurora B but not localization or activity of kinetochore-associated Aurora B. (A), (C), (E), and (G) Immunofluorescence images of HeLa cells depleted of Sgo1 and stained for either Aurora B (ABK), active phosphorylated ABK (pABK-T232), phosphorylated Hec1-S44 (pHec1-S44), or Sgo1. (B), (D), (F), and (H) Quantification of centromere or kinetochore fluorescence intensities of Aurora B, pABK-T232, pHec1-S44, and Sgo1. For all conditions at least 120 kinetochores from 10 cells were measured from a total of 3 independent experiments. Fluorescence intensities for Sgo1 depletion were normalized to those calculated from control cells. (I) ,(K) , and (M) HeLa Flp-In TREx cells were induced to express either GFP-Sgo1-WT, GFP-Sgo1-K492A, or GFP-Sgo1-T346A fusion proteins [41] and immunostained for ABK, pABK-T232, or pHec1-S44. (J), (L), (N) Quantification of centromere (ABK) or kinetochore (pABK-T232, pHec1-S44) normalized fluorescence intensities. For ABK quantification, 120 centromeres were measured from 15 cells from 3 independent experiments. For pABK-T232 and pHec1-S44, at least 200 kinetochores were measured from at least 15 cells from 3 independent experiments. Fluorescence intensities for test conditions were normalized to those calculated from control cells. Error bars represent SD. For panels B, D, F and H, significance values were

calculated from unpaired nonparametric Student's t tests. For panels J, L, and N, significance values were calculated from a one-way ordinary ANOVA test, and shown are significance values between experiments using cells overexpressing WT or mutant Sgo1 constructs compared to control, untreated cells.

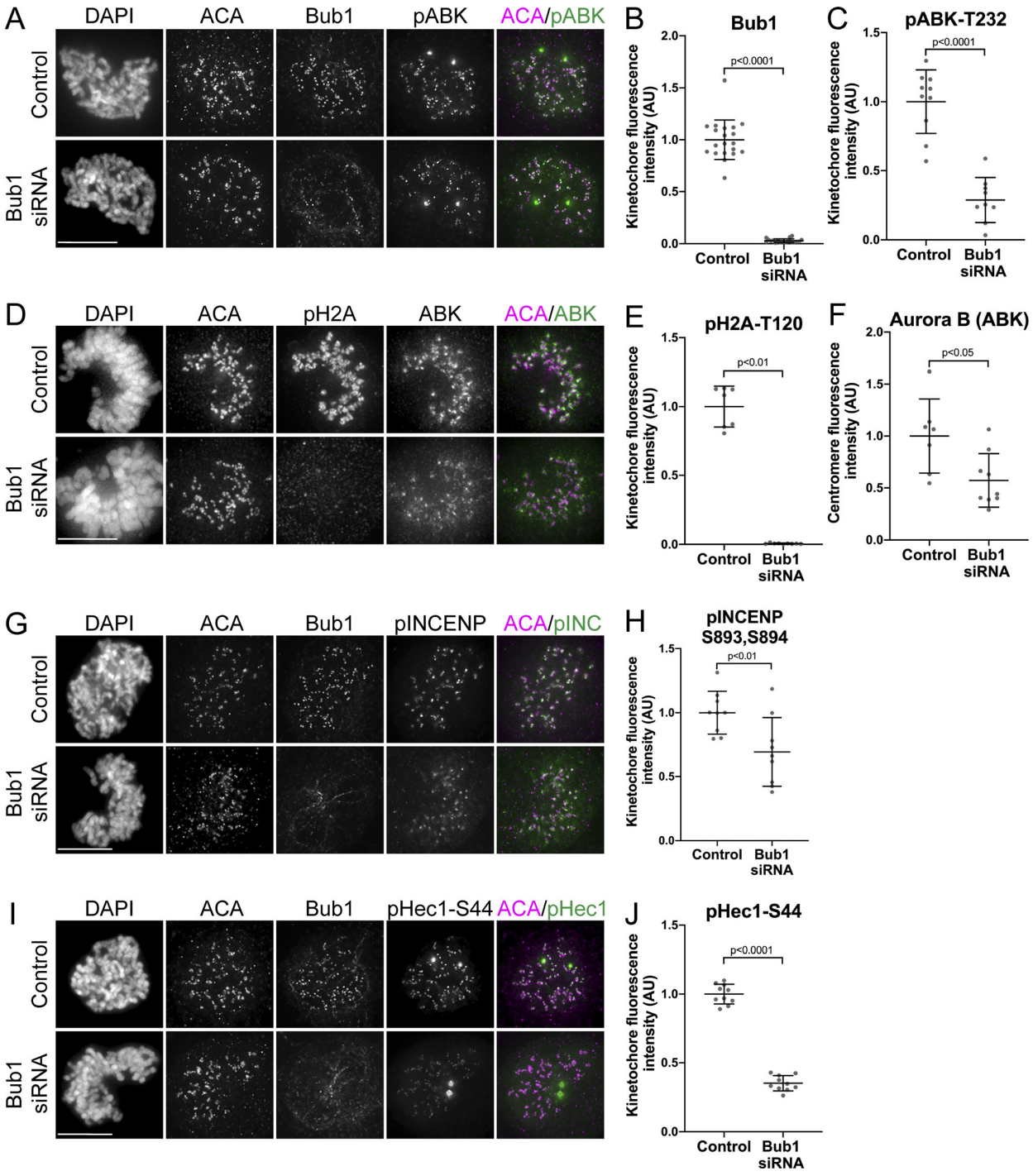


Figure 3.5. Depletion of Bub1 protein results in reduction of both centromere and kinetochore-associated Aurora B in human cells. (A), (D), (G) and (I) Immunofluorescence images of HeLa cells depleted of Bub1 and immunostained for the indicated antibodies. (B), (C), (E), (F), (H), and (J) Quantification of kinetochore or centromere fluorescence intensities of indicated antibodies. Error bars indicate SD. For all conditions at least 120

kinetochores from 10 cells were measured from a total of 3 independent experiments. Error bars represent SD. Significance values were calculated using unpaired nonparametric Student's t tests. Scale bars are 10 um.

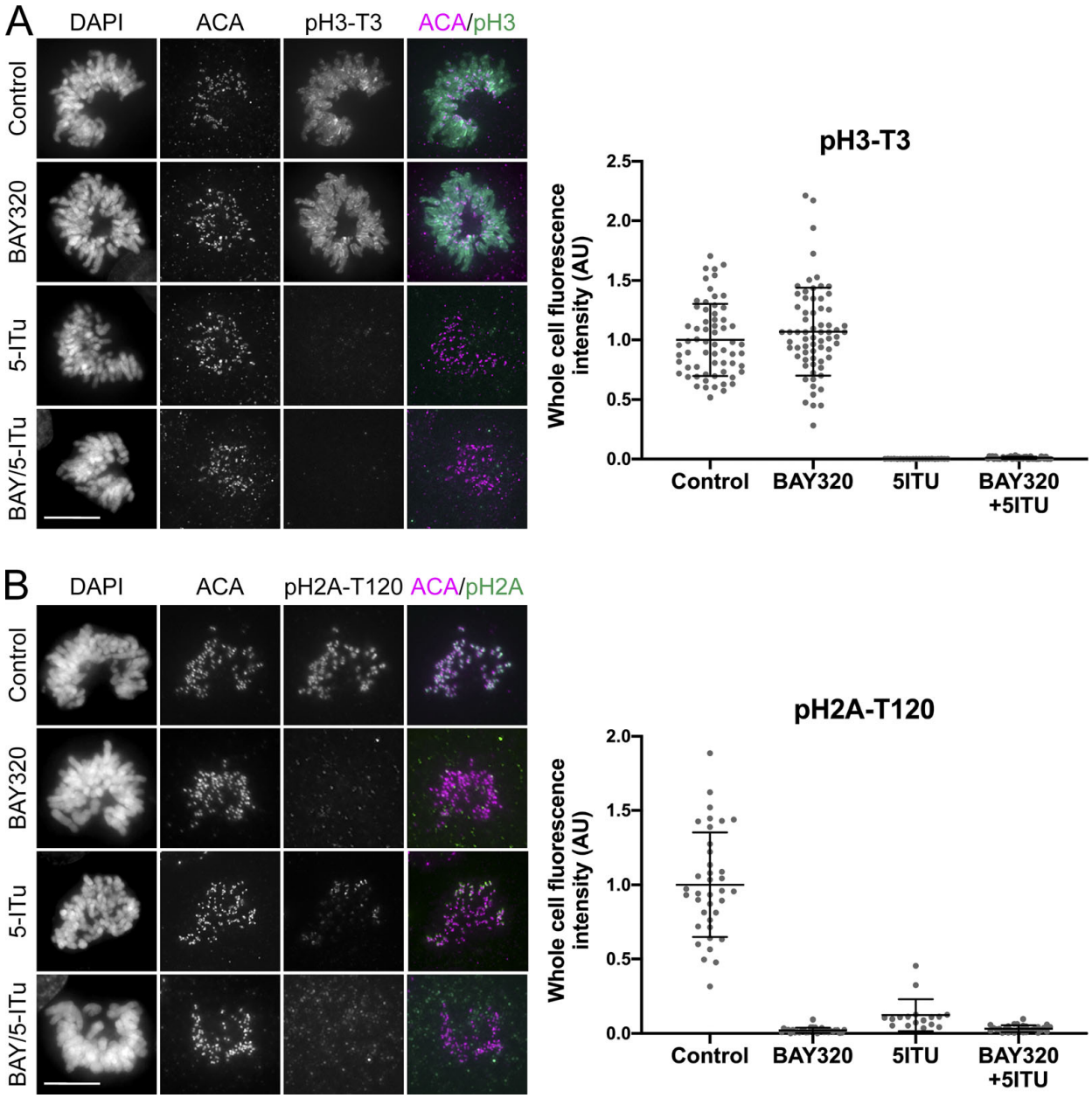


Figure 3.6. Specificity of Bub1 and Haspin kinase inhibitors. (A) Immunofluorescence images of control HeLa cells and cells treated with 10 uM BAY320, 10 uM 5-ITu, or both kinase inhibitors for 30 min and then immunostained for pH3-T3. Quantification of whole cell fluorescence intensity for each condition is shown to the right. Fluorescence intensities for test conditions were normalized to those calculated from control cells. For all conditions, at least 16 cells from 3 independent experiments were measured. Error bars indicate SD. (B) Immunofluorescence images of control HeLa cells and cells treated with 10 uM BAY320, 10 uM 5-ITu, or both kinase inhibitors for 30 min and then immunostained for pH2A-T120. Quantification of centromere fluorescence intensity for

each condition is shown to the right. Fluorescence intensities for test conditions were normalized to those calculated from control cells. For all conditions, at least 14 cells from 3 independent experiments were measured. Error bars indicate SD. Error bars represent SD. Significance values were calculated using a one-way ordinary ANOVA test. Shown are significance values between each experiment and control cells. Scale bars are 10 um.

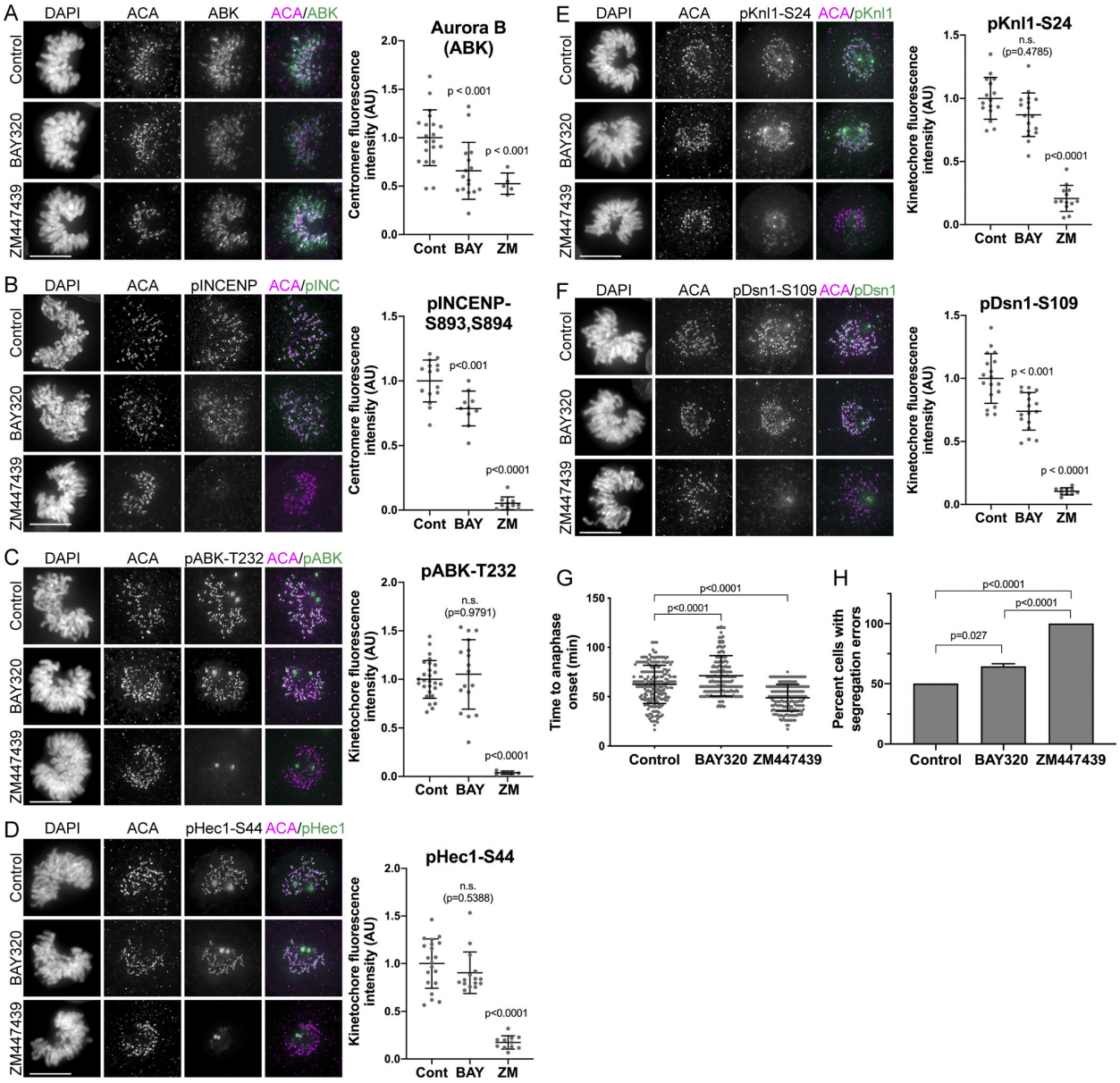


Figure 3.7 Bub1 kinase activity is not required for localization or activity of Aurora B at kinetochores. (A-F) Immunofluorescence images of control HeLa cells or HeLa cells treated with either 10 uM Bub1 kinase inhibitor BAY320 or 20 uM Aurora B kinase inhibitor ZM447439. Cells were fixed and stained with the indicated antibodies. To the right of each immunofluorescence panel is the quantification of centromere or kinetochore fluorescence intensity. Fluorescence intensities for test conditions were normalized to those calculated from control cells. n values for each experiment follow. ABK: Control (n=153 centromeres; 15 cells; 3 experiments); BAY320 (n=226 centromeres; 13 cells; 4 experiments); ZM447439 (n=222 centromeres; 11 cells; 3 experiments). pINCENP: Control (n=349 centromeres; 15 cells; 3 experiments); BAY320 (n=245 centromeres; 10 cells; 3 experiments); ZM447439 (n=179 centromeres; 10 cells; 3 experiments). pABK-T232: Control (n=621 kinetochores; 42 cells; 4 experiments); BAY320 (n=350

kinetochores; 24 cells; 4 experiments); ZM447439 (n=219 kinetochores; 6 cells; 3 experiments). pHec1-S44: Control (n=384 kinetochores; 16 cells; 3 experiments); BAY320 (n=272 kinetochores; 15 cells; 3 experiments); ZM447439 (n=224 kinetochores; 11 cells; 3 experiments). pKnl1-S24: Control (n=363 kinetochores; 16 cells; 3 experiments); BAY320 (n=348 kinetochores; 17 cells; 3 experiments); ZM447439 (n=281 kinetochores; 14 cells; 3 experiments). pDsn1-S109: Control (n=400 kinetochores; 18 cells; 3 experiments); BAY320 (n=368 kinetochores; 17 cells; 3 experiments); ZM447439 (n=209 kinetochores; 10 cells; 2 experiments). (G) Quantification of time (in min) to anaphase onset after washout of monastrol. HeLa cells were treated with 150 uM monastrol for 2 hr. Cells were washed out into control media, or media containing either 10 uM BAY320 or 20 uM ZM447439. The time from initiation of washout to anaphase onset was scored. For each condition, at least 200 cells were quantified from 3 experiments. (H) Quantification of chromosome segregation errors in cells treated with 150 uM monastrol for 2 hr, and washed out into control media, or media containing either 10 uM BAY320 or 20 uM ZM447439. Errors in chromosome segregation were quantified from anaphase cells. For panels A-F, significance values were calculated from an unpaired nonparametric students t tests. For panels G and H, significance values were calculated from one-way ordinary ANOVA tests. Scale bars are 10 um.

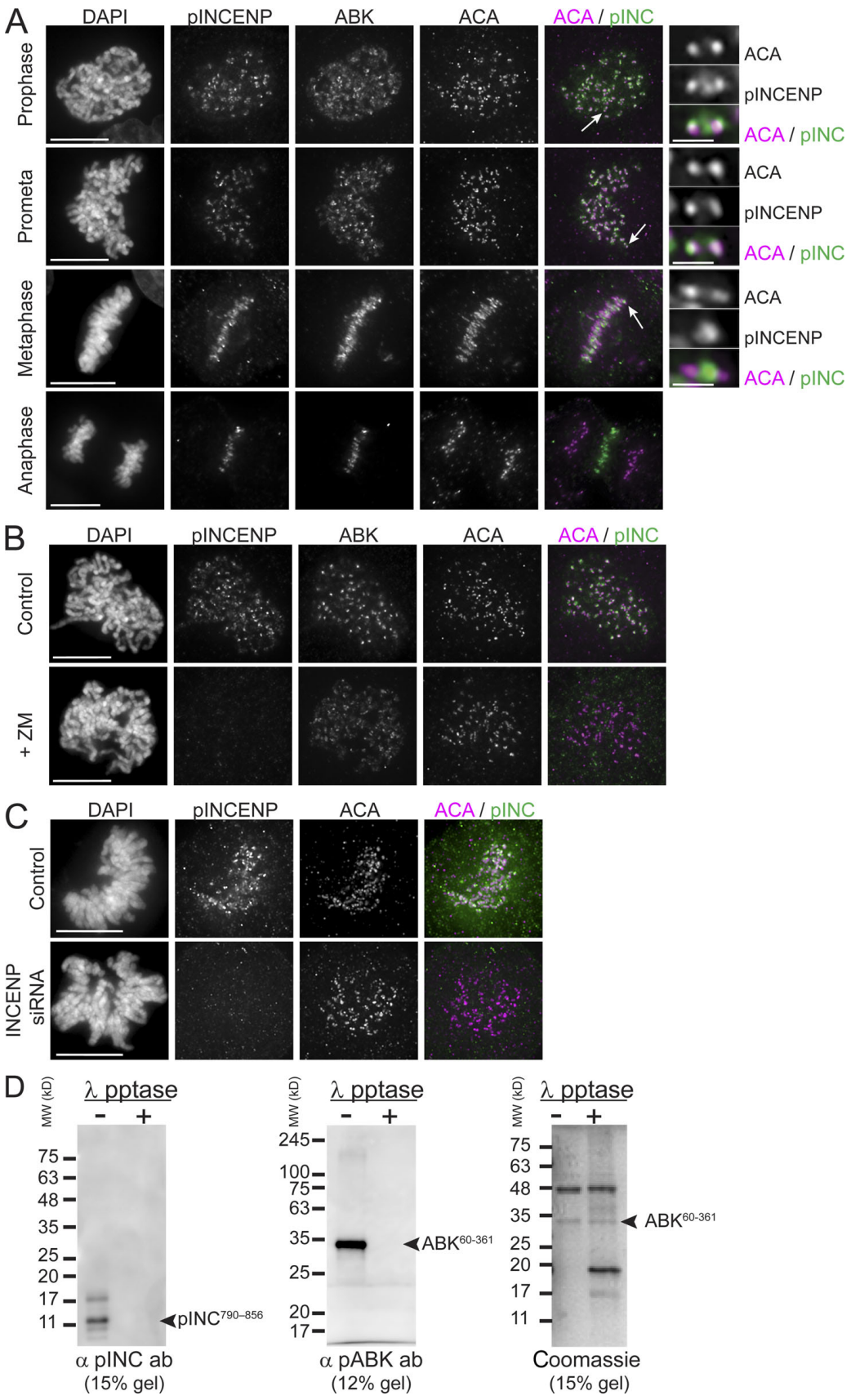

A
DAPI
pINCENP
ABK
ACA
ACA / pINC
Prophase
Prometa
Metaphase
Anaphase
ACA
pINCENP
ACA / pINC
B
DAPI
pINCENP
ABK
ACA
ACA / pINC
Control
+ ZM
C
DAPI
pINCENP
ACA
ACA / pINC
Control
INCENP siRNA
D
λ pptase
- +
MW (kD)
75
63
48
35
25
20
17
11
pINC790–856
α pINC ab
(15% gel)
245
100
75
63
35
25
20
17
ABK60-361
α pABK ab
(12% gel)
Coomassie
(15% gel)

Figure3.8. Characterization of pINCENP-S893,894 antibody. (A) Immunofluorescence images of HeLa cells in various stages of mitosis stained with pINCENP-S893, S894, Aurora B, and ACA. Arrows point to the kinetochore pairs shown in the insets on the right. (B) Immunofluorescence images of a control HeLa cell and a HeLa cell treated with 10 uM ZM447439. (C) Immunofluorescence images of a control cell and HeLa cell treated with INCENP siRNA. (D) A complex of Aurora B (amino acids 60-361) and INCENP (amino acids 1-68 and 834-900) was incubated with or without lambda protein phosphatase and reactions were terminated with the addition of SDS-PAGE sample buffer. Images show Western blots of reactions probed with pINCENP-S893, S894 (left) and pABK-T232 (middle). A Coomassie stained gel is shown to the right. Molecular weight markers are indicated. Scale bars are 10 um (whole cells) and 1 um (insets). Whole cell images shown are maximum intensity projections of z-stacks containing 64 planes and inset images shown are projections from 2-4 planes.

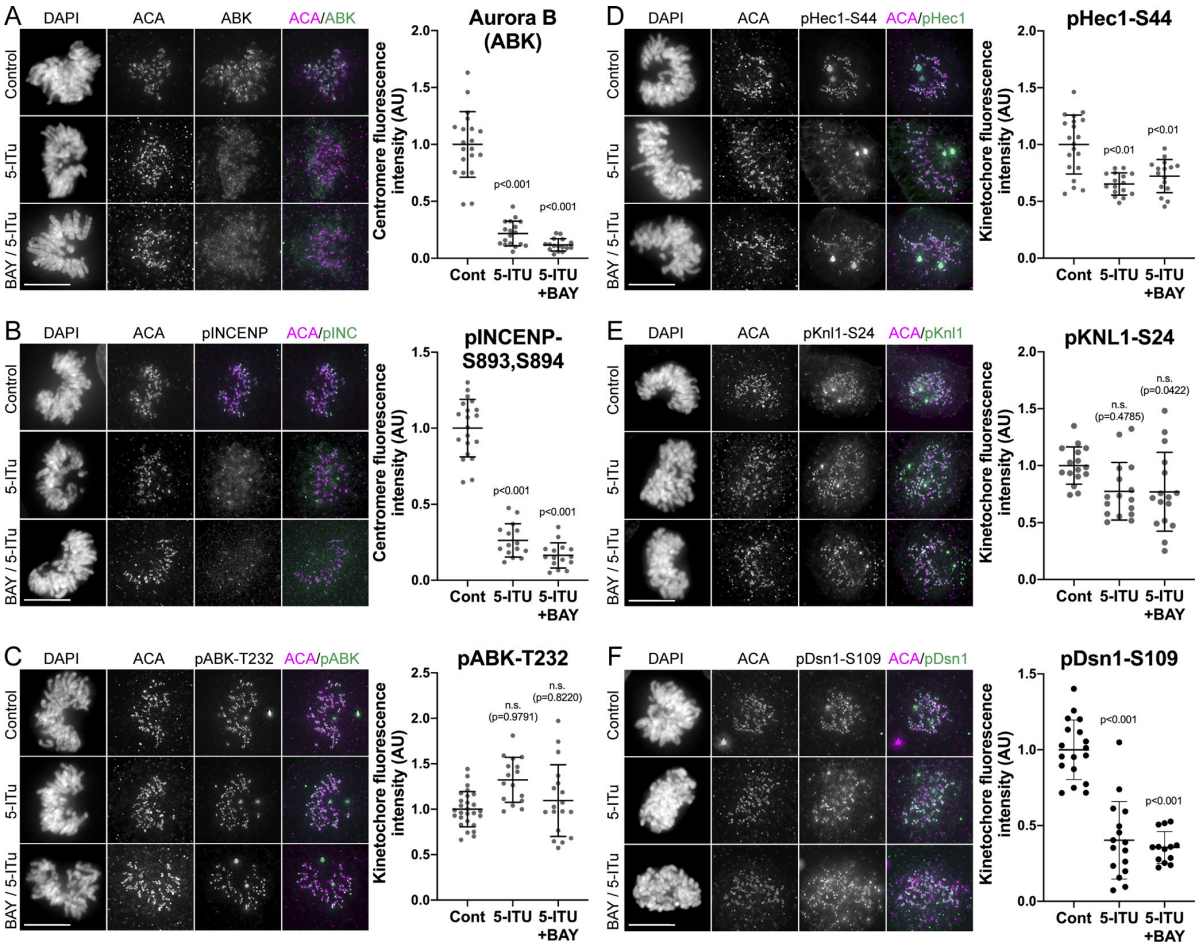


Figure 3.9. 5-ITu-treatment prevents accumulation and activity of Aurora B at centromeres, but not kinetochores. (A-F) Immunofluorescence images of control HeLa cells or HeLa cells treated with either 10 uM 5-ITu or 10 uM 5-ITu plus 10 uM BAY320 for 30 min. Cells were fixed and stained with the indicated antibodies. To the left of each immunofluorescence panel is the quantification of fluorescence intensities. Fluorescence intensities for test conditions were normalized to those calculated from control cells. n values for each experiment follow. ABK: Control (n=153 centromeres; 15 cells; 4 experiments); 5-ITu (n=161 centromeres; 14 cells; 3 experiments); 5-ITu + BAY320 (n=115 centromeres; 11 cells; 3 experiments). pINCENP: Control (n=349 centromeres; 15 cells; 3 experiments); 5-ITu (n=210 centromeres; 10 cells; 3 experiments); 5-ITu + BAY320 (n=195 centromeres; 10 cells; 3 experiments). pABK-T232: Control (n=621 kinetochores; 42 cells; 4 experiments); 5-ITu (n=359 kinetochores; 38 cells; 4 experiments); 5-ITu + BAY320 (n=279 kinetochores; 40 cells; 4 experiments). pHec1-S44: Control (n=384 kinetochores; 16 cells; 3 experiments); 5-ITu (n=374 kinetochores; 16 cells; 3 experiments); 5-ITu + BAY320 (n=336 kinetochores; 16 cells; 3 experiments). pKnl1-S24: Control (n=363 kinetochores; 16 cells; 3 experiments); 5-ITu (n=337 kinetochores; 16 cells; 3 experiments); 5-ITu + BAY320 (n=309 kinetochores; 18 cells; 3 experiments). pDsn1-S109: Control (n=400 kinetochores; 18 cells; 3 experiments); 5-ITu

(n=295 kinetochores; 16 cells; 3 experiments); 5-ITu + BAY320 (n=315 kinetochores; 16 cells; 3 experiments). Error bars represent SD. Significance values were calculated using a one-way ordinary ANOVA test. Shown are significance values between experiments for each test condition compared to control cells.

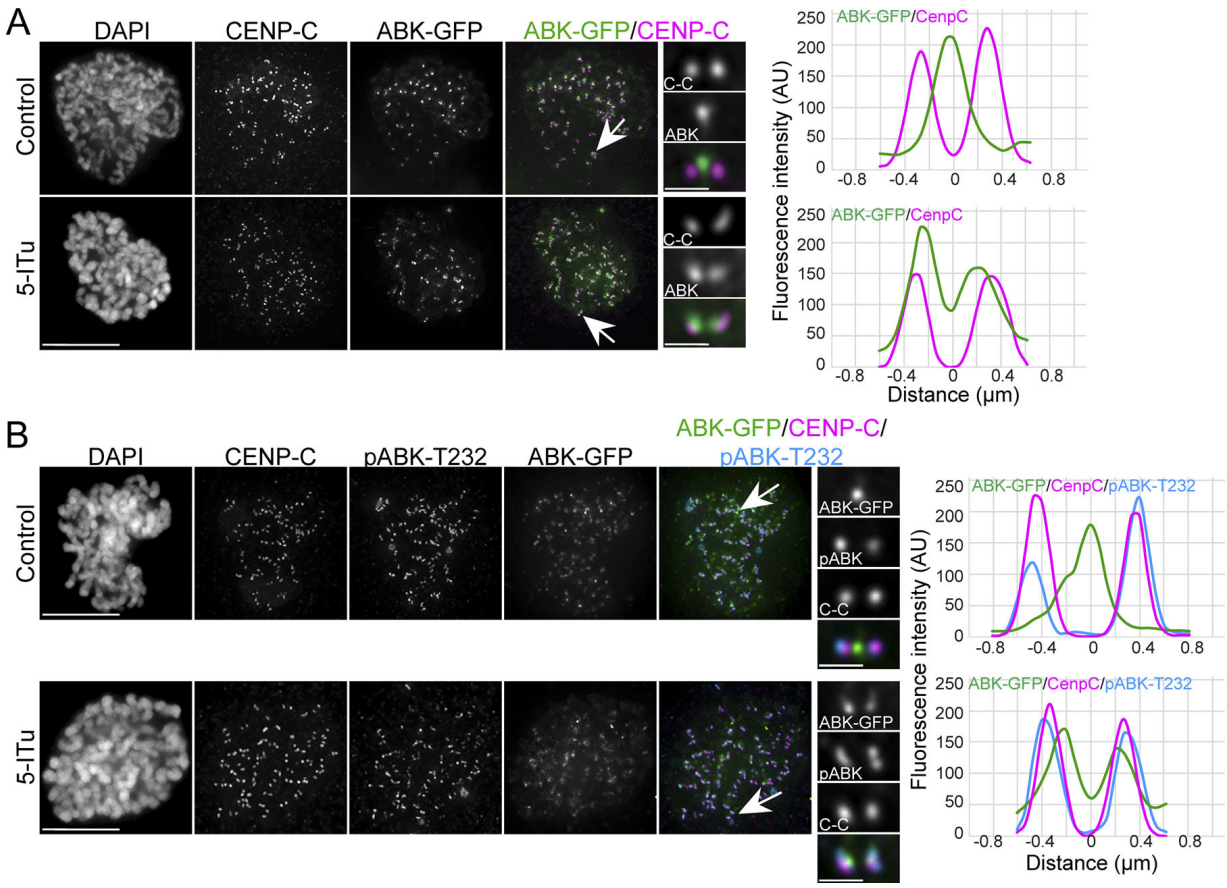


Figure 3.10. Inhibition of Haspin kinase results in re-localization of Aurora B from inner centromeres to kinetochore-proximal outer centromeres. (A) Immunofluorescence images of control and 5 uM 5-ITu-treated early prometaphase HeLa cells expressing Aurora B kinase-GFP (ABK-GFP) and stained with antibodies to CENP-C. Arrows point to the kinetochore pairs shown in the insets. Linescans through the centromere/kinetochore region are shown to the right of each panel. (B) Immunofluorescence images of control and 5 uM 5-ITu-treated early prometaphase HeLa cells expressing ABK-GFP and stained with antibodies to pABK-T232. Arrows point to the kinetochore pairs shown in the insets. Linescans through the centromere/kinetochore region are shown to the right of each panel. Scale bars are 10 um (whole cells) and 1 um (insets). Whole cell images shown are maximum intensity projections of z-stacks containing 64 planes and inset images shown are projections from 2-4 planes.

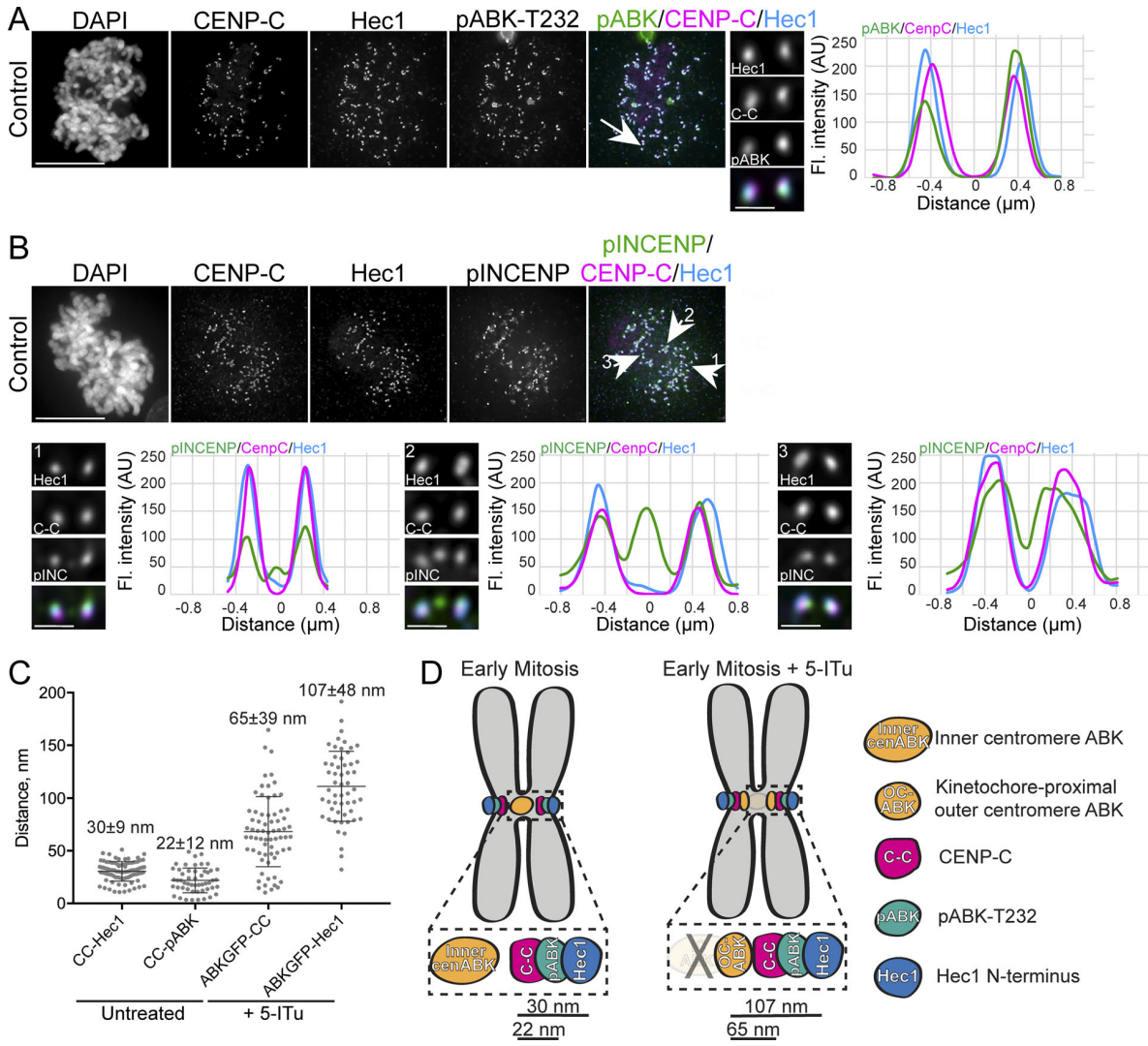


Figure 3.11. Aurora B kinase and INCENP localize to both centromeres and kinetochores in mitotic cells. (A) Immunofluorescence images of an early prometaphase control HeLa cell stained with antibodies to pABK-T232, CENP-C, and the N-terminus of Hec1 (antibody 9G3). Arrows point to the kinetochore pairs shown in the insets. A linescan through the centromere/kinetochore region is shown to the right of the panel. (B) Immunofluorescence images of an early prometaphase control HeLa cell stained with antibodies to pINCENP, CENP-C, and the N-terminus of Hec1 (9G3). Numbers indicate kinetochore pairs shown in the insets, and linescans through the centromere/kinetochore region are shown to the right of each panel. pINCENP is localized to either the kinetochore (pair 1), the centromere and kinetochore (pair 2), or the kinetochore-proximal outer centromere (pair 3). (C) Plots of the mean distance between the indicated kinetochore proteins. Each point on the graph represents a distance measurement for a pair of sister

chromatids and the mean value is indicated. n values are listed in Table 1. (D) Summary of distance measurements from experiments in the study. Scale bars are 10 um (whole cells) and 1 um (insets). Whole cell images shown are maximum intensity projections of z-stacks containing 64 planes and inset images shown are projections from 2-4 planes.

Table 2. Details for all antibodies used in the study. For each antibody used for standard immunofluorescence experiments, two-color distance measurements, and Western blots, the table indicates the concentration used, antibody source, and fixation conditions.

Antibody	Dilution	Source	Conditions
Immunofluorescence			
Mouse anti-pH3-T3	1:10,000	Gift, Dr. Hiroshi Kimura (Kelly et al., 2010)	1% TX 3 min, 4% PFA 20 min
Mouse anti-Sgo1	1:500	Abcam 58023	1% TX 3 min, 4% PFA 20 min
Rabbit anti-ABK	1:500	Abcam 2254	0.5% TX 30 s, 4% PFA 20 min; 1% TX 3 min, 4% PFA 20 min
Mouse anti-Bub1	1:200	Abcam 54893	0.5% TX 30 s, 4% PFA 20 min; 1% TX 3 min, 4% PFA 20 min
Rabbit anti-Bub1	1:200	Bethyl Laboratories A300-373A	1% TX 3 min, 4% PFA 20 min
Rabbit anti-pH2A-T120	1:1,000	Active Motif 39391	0.5% TX 30 s, 4% PFA 20 min; 1% TX 3 min, 4% PFA 20 min
Rabbit anti-pABK-T232	1:500	(DeLuca et al., 2017)	0.5% TX 30 s, 4% PFA 20 min; 1% TX 3 min, 4% PFA 20 min
Rabbit anti-pDsn1-S109	1:500	Gift, Dr. Iain Cheeseman (Welburn et al., 2010)	0.5% TX 30 s, 4% PFA 20 min
Rabbit anti-pHec1-S44	1:1,000	(DeLuca et al. 2011)	0.5% TX 30 s, 4% PFA 20 min; 1% TX 3 min, 4% PFA 20 min
Rabbit anti-pINCENP-S893,S894	1:400	This study	0.5% TX 30 s, 4% PFA 20 min
Rabbit anti-pKNL1-S24	1:400	Gift, Dr. Iain Cheeseman (Welburn et al., 2010)	0.5% TX 30 s, 4% PFA 20 min
Rabbit anti-Survivin	1:500	Santa Cruz Biotechnology 10811	1% TX 3 min, 4% PFA 20 min
Two-color distance measurements for centromere/kinetochore mapping			
Mouse anti-Hec1 (9G3)	1:2,000	GeneTex GTX70268	0.5% TX 30 s, 4% PFA 20 min
Guinea pig anti-CENP-C	1:500	MBL PD030	0.5% TX 30 s, 4% PFA 20 min
Rabbit anti-pH2A-T120	1:1,000	Active Motif 39391	0.5% TX 30 s, 4% PFA 20 min
Rabbit anti-pABK-T232	1:500	(DeLuca et al., 2017)	0.5% TX 30 s, 4% PFA 20 min
Western blotting			
Rabbit anti-pABK-T232	1:500	(DeLuca et al., 2017)	
Rabbit anti-pINCENP-S893,S894	1:500	This study	

Chapter 4: Identification of the kinetochore receptor for Aurora B kinase and the Chromosomal Passenger Complex

4.1 Introduction

There is a growing amount of evidence suggesting Aurora B kinase activity and localization at kinetochores are uncoupled from Aurora B localization to centromeres [117, 120, 126-128, 133, 150, 151]. While two phospho-histone modifications, pH2A-T120 and pH3-T3, are required for robust centromere accumulation of the CPC and Aurora B, neither modification is required for Aurora B kinase activity at the outer kinetochore [126-128]. This leaves a major unanswered question- how is Aurora B recruited to outer kinetochores to phosphorylate kinetochore substrates in early mitosis and evicted as stable end-on kinetochore microtubule attachments are formed? We set out to determine the kinetochore receptor for Aurora B kinase.

Previously, Gina Caldas from our lab discovered that the N-terminus of KNL1, a large kinetochore scaffold protein, is required for Aurora B kinase activity at outer kinetochores [133]. Interestingly, in cells depleted of KNL1, artificially tethering the CPC to centromeres with a CENP-B-INCENP fusion did not rescue the activity of Aurora B at outer kinetochores [133]. In recent studies, we found that in addition to KNL1 depletion, Bub1 depletion, but not Bub1 kinase inhibition, resulted in a decrease in Aurora B activity at kinetochores [127]. These results suggest Bub1 and KNL1 contribute to Aurora B activity at kinetochores [127, 133, 188, 189]. We hypothesize that an interaction between Bub1 and KNL1 leads to a conformation that promotes Aurora B kinase activity and localization at the outer kinetochore. We therefore set out to

determine if KNL1 is a kinetochore receptor of Aurora B and furthermore, to determine if KNL1 and Bub1 cooperate to promote Aurora B activity at the outer kinetochore.

KNL1 (Kinetochore-null protein 1)

KNL1 is the largest protein in the kinetochore microtubule network and plays a pivotal role in cell division (Figure 4.1 adapted from Caldas et al., 2014) [61]. KNL1 is a kinetochore scaffold responsible for outer kinetochore assembly, chromosome congression, and spindle assembly checkpoint signaling. KNL1 has many conserved motifs that recruit both kinases that promote kinetochore-microtubule turnover and phosphatases that promote kinetochore microtubule attachment stability. Therefore, KNL1 integrates many signaling pathways at the kinetochore and likely contributes directly to the localization and activity of Aurora B at kinetochores [62, 65, 67, 68, 133].

KNL1 is recruited to kinetochores through an interaction between its C-terminus and the inner kinetochore Mis12 complex [63, 202]. Of particular importance, KNL1 is phosphorylated on a series of MELT (Methionine-Glutamate-Leucine-Threonine) repeats by the mitotic kinase Mps1. Phosphorylation of KNL1 on MELT repeats in the central region results in the recruitment of spindle assembly checkpoint protein complexes Bub3-Bub1 and Bub3-BubR1 [62, 65, 67, 68]. The spindle assembly checkpoint generates a molecular signal generated at KNL1 from unattached kinetochores that leads to inhibition of the anaphase promoting complex until kinetochores are properly attached to microtubules [60, 62, 64, 68, 70, 203-205]. Attachment of kinetochores to microtubules leads to the eviction of spindle assembly checkpoint proteins from KNL1 and silencing of the spindle assembly checkpoint, initiating the onset of anaphase [71, 79, 206-210]. In addition to the MELT motifs of

KNL1, KI1 and KI2 (Lysine-Isoleucine) motifs in the N terminus of KNL1 recruit Bub1 and BubR1 independent of Mps1 phosphorylation. However, the recruitment of Bub1 and BubR1 to the KI domains of KNL1 is not sufficient to maintain spindle assembly checkpoint signaling in the absence of phosphorylated MELTS [60, 63, 70]. The N-terminus of KNL1 harbors conserved RVSF/SILK motifs that are required to recruit protein phosphatase one (PP1) to kinetochores [211]. Aurora B phosphorylation of the RVSF/SILF motifs antagonizes PP1 recruitment [44, 211]. The N-terminus of KNL1 also contains a microtubule binding domain, however it is not essential to form stable end-on attachments in cells [45]. As mentioned above, KNL1 directly recruits the Bub complexes that recruit spindle checkpoint proteins and generate the mitotic checkpoint complex at unattached kinetochores [61, 212]. Both Bub1 and BubR1 have roles in chromosome congression independent of checkpoint signaling. We focused on investigating Bub1 and KNL1, because, as mentioned above, depleting either protein results in loss of Aurora B at kinetochores [127, 133].

Bub1 (budding uninhibited by benzimidazoles 1)

KNL1 is required for Bub1 localization to kinetochores, which contributes to Aurora B localization to centromeres as described in previous chapters [126-128, 136, 188, 189]. Interestingly, Bub1 kinase activity is required for centromere accumulation of the CPC; however, it is not required for Aurora B localization or activity at the kinetochore [126, 128, 213]. It is unknown how Bub1 contributes to Aurora B recruitment or activity at outer kinetochores. In cells depleted of Bub1 there is a slight loss of Aurora B at kinetochores [127] and furthermore, KNL1 depleted cells result in a significant decrease in Aurora B at kinetochores [133]. Therefore, it could be Bub1

localized to KNL1 or KNL1 alone that directly contribute to Aurora B localization and activity at kinetochores.

Bub1 was first discovered in budding yeast when cells were treated with a microtubule depolymerizing drug but failed to arrest during cell division, and consequently was given the name- budding uninhibited in benzimidazoles one [214]. Since its discovery, studies have uncovered diverse roles for Bub1 in both spindle assembly checkpoint signaling and chromosome congression [212, 215]. Much like other kinetochore proteins, Bub1 contains functional domains required to recruit various mitotic regulators to kinetochores (Figure 4.2 adapted from Ashgar and Elowe 2016) [212]. The N-terminus of Bub1 contains a tetratricopeptide repeat (TPR) domain, a Gle2- binding sequence (GLEBS domain), and a loop region. The TPR, GLEBS and loop domains are needed for binding to KNL1 and therefore, Bub1 localization to kinetochores [60, 62-64, 68, 69, 216]. Bub1 localized to KNL1 contributes to BubR1 kinetochore localization through a BubR1 localization domain and the loop domain [200, 216]. Bub1 is involved in recruiting CENP-F and CENP-E to kinetochores, although the details of these interactions are less well-understood [217]. The central region of Bub1 contains domains that recruit spindle assembly checkpoint factors to form the mitotic checkpoint complex at unattached kinetochores. The ABBA domain binds CDC20 and the conserved motif 1 (CD1) binds Mad1/Mad2 [200, 218-220] all of which are essential components of mitotic checkpoint signaling. The C terminus of Bub1 is a highly conserved serine/threonine kinase domain [188, 189, 221-224]. Bub1 autophosphorylation leads to optimal kinase activity and promotes its own localization and retention at kinetochores [225]. In addition to autophosphorylation, Bub1

phosphorylates histone H2A at threonine 120 to recruit Shugoshin proteins (Sgo1 and Sgo2) [41, 136, 188]. Sgo1 in turn binds protein phosphatase 2A to protect cohesin and serves as a bridging factor for the Chromosomal Passenger Complex (CPC) at the kinetochore-proximal outer centromere [41, 126-128]. Recently, we found that Bub1 but not Bub1 kinase activity, is required for Aurora B kinase at the outer kinetochore [127].

KNL1-Bub1 as kinetochore receptors for Aurora B kinase?

Though the overall organization of KNL1 at kinetochores is unknown, it resides in the outer kinetochore region [53], and undergoes a significant conformational change upon kinetochore-microtubule attachment [201]. Therefore, we hypothesize that Bub1 interacts with KNL1 at kinetochores and promotes a confirmation that contributes to Aurora B activity and localization at the outer kinetochore. Upon kinetochore microtubule attachment, KNL1 undergoes a conformational change resulting in the eviction of Bub1 and Aurora B kinase from outer kinetochores, in turn recruiting protein phosphatases to stabilize end-on kinetochore-microtubule attachments.

We set out to determine how depletion of KNL1 mechanistically leads to a decrease in Aurora B activity at the kinetochore. To this end we built on previous work that Gina Caldas initiated in lab and generated various truncation mutants of KNL1 to test which region of KNL1 interacts with Aurora B by co-immunoprecipitation and mass spectrometry analysis. In addition, we designed new mutants of KNL1 in order to build stable cell lines for future immunofluorescence and immunoprecipitation experiments. Finally, we depleted kinetochore proteins CENP-E and F that are recruited to KNL1 and Bub1 and measured Aurora B activity at kinetochores. In a parallel approach, we designed a construct containing Aurora B linked to the promiscuous biotin ligase, BirA,

[226] in order to try and capture transient interactions that might be missed in traditional immunoprecipitation assays.

4.2 Results

Co-Immunoprecipitation of Aurora B with KNL1 fragments suggests an interaction at outer kinetochores

While many of the multiple binding partners of KNL1 have been identified, it remains unknown how the N-terminal region of KNL1 contributes to Aurora B activity at kinetochores [133]. A construct containing the middle 300-800 amino acids of KNL1 was sufficient to rescue Aurora B activity at kinetochores in KNL1 depleted cells to wild-type levels. Similarly, a construct containing the N terminal 300 amino acids of KNL1 was sufficient to partially rescue Aurora B activity at the kinetochore in KNL1 depleted cells, and the C-terminal 1500 amino acids of KNL1 did not rescue Aurora B activity at the kinetochore in cells (Figure 4.3 adapted from Caldas et al., 2014) [61, 133]. Based on these data, we wanted to test if the N- terminal region of KNL1 interacts with Aurora B kinase and the CPC in early mitosis with the use of co-immunoprecipitation and mass spectrometry analysis. We expected that the N-terminal fragments that rescued Aurora B activity at kinetochores in cells would co-precipitate Aurora B and the CPC while the C-terminal region would not. To this end, Hek293T cells that stably express FLAG-KNL1 constructs were grown to ~70% confluency in T500 (X4) square dishes to obtain a large cell volume. Cells were synchronized using a double thymidine block and after the first arrest, expression of the FLAG-KNL1 constructs was induced with the addition of doxycycline. Upon entry into mitosis cells were treated with low doses of nocodazole to maintain an early population of mitotically enriched cells (Figure 4.4). We found that

immunoprecipitation of FLAG-KNL1 fragments 1500C, 300-800N and 300N all co-precipitated Aurora B kinase to some extent (Figure 4.4). In addition to Aurora B, other known proteins that interact with these regions of KNL1 were identified (Figure 4.4).

Interestingly, these results suggest Aurora B may interact with KNL1 or potentially, proteins that are recruited to KNL1 at the outer kinetochore [61, 133]. These results suggest Aurora B is recruited to the outer kinetochore in a KNL1 dependent manner. In the future, optimization of the immunoprecipitation assay will be needed to understand which regions of KNL1 are required for Aurora B at the kinetochore and how other KNL1 interacting proteins contribute to Aurora B recruitment at kinetochores.

KNL1-mini mutants to assess Aurora B recruitment and activity at kinetochores

To better understand the regions of KNL1 that are required for Aurora B at the outer kinetochore we designed a set of FLAG-HIS-GFP-tagged KNL1 truncation mutants and cloned them into a lentiviral vector to generate stable cells (Figure 4.5). As discussed above, KNL1 undergoes a change in conformation when kinetochores attach to microtubules [201] and the N-terminus of KNL1 is required for Aurora B localization to outer kinetochores [133]. Therefore, to map which regions of KNL1 are important for Aurora B at kinetochores, we designed a “mini” version of KNL1 that stitches together many of the functional motifs of KNL1 required for downstream recruitment of protein complexes while at the same time significantly shortening the length of KNL1 from 2,317 amino acids to 758 amino acids (Figure 4.5). We then made mutations within the functional regions of KNL1-mini to understand how each motif contributes to Aurora B localization. We generated KNL1-mini-11A, which is a construct that contains alanine instead of threonine in all of the repeat MELT motifs. This prevents the phosphorylation

of KNL1 and therefore recruitment of Bub3-Bub1, Bub3-BubR1 complexes. We also made two different truncations to the N-terminus of KNL1-mini that encompass both the microtubule binding domains and the motifs important for PP1 recruitment (KNL1-mini ΔN^{22} and KNL1-miniΔN^{86}). We expect that if KNL1-mini can recruit Aurora B to the outer kinetochore but cannot undergo a conformational change due to the shortened length, then Aurora B might be retained at kinetochores in late mitosis when it is typically evicted. However, it might be that KNL1-mini can recruit Aurora B and function similar to endogenous KNL1 despite the shortened length. If this interaction depends on Bub1 then we expect KNL1-mini11A will not be able to support Aurora B at kinetochores. It may also be that the N-terminus of KNL1 alone is important for Aurora B and therefore, in the N-terminal deletions of KNL1 we expect to see a loss in Aurora B at kinetochores.

These KNL1 mutants will give important insight to how KNL1 and Bub1 contribute to Aurora B activity and localization at outer kinetochores in early mitosis. We are currently making stable cells using the lentiviral constructs. Once the stable cell lines are generated, endogenous KNL1 will be knocked down using siRNA and expression of the KNL1-mini mutants will be induced by the addition of doxycycline. Then cells can either be evaluated by immunofluorescence or immunoprecipitation to determine how each mutant differentially contributes to Aurora B localization or activity at outer kinetochores.

Neither CENP-E nor CENP-F are not required for Aurora B activity at kinetochores

Kinetochore localization of CENP-E and F depend on KNL1 and Bub1 at kinetochores [217]. CENP-F is part of the fiborous corona formed in early mitosis to aid in capturing microtubules [227] and is also proposed to bind dynein at kinetochores

[228]. CENP-E is a plus end directed microtubule motor involved in a number of events at kinetochores including congression of polar chromosomes and converting lateral kinetochore-microtubule attachments to end-on attachments [229]. We wanted to test if Aurora B activity was perturbed in Bub1 and KNL1 depleted cells due to loss of CENP-E or CENP-F at kinetochores. To this end, we depleted CENP-E or -F from HeLa cells and measured Aurora B localization at outer kinetochores with an antibody that recognizes active phosphorylated Aurora B (α-pABK-T232). In both cases, Aurora B levels at the kinetochore are similar to wild-type in cells depleted of CENP-E of -F (Figure 4.6). These results suggest that the loss of Aurora B from kinetochores in KNL1 or Bub1 depleted cells is not due to loss in CENP-E or F.

Proximity-dependent ligation assay to capture transient interactions between Aurora B and its kinetochore receptor

In order to determine the interaction between Aurora B and its kinetochore receptor that may be weak or transient, we designed a construct containing Aurora B fused to the promiscuous biotin ligase, BirA [200, 226]. In this assay, BirA will biotinylate proteins that are in close proximity when biotin is added to media [226]. We reasoned that because we recently uncovered ways to localize Aurora B and the CPC to particular regions within the centromere and kinetochore by inhibiting Bub1 or Haspin kinase then we could use these conditions to determine which receptors are differentially biotinylated in drug treated cells (Figure 4.7). We expect that in cells where both Bub1 and Haspin kinase are inhibited then the BirA-Aurora B fusion protein will localize predominately to outer kinetochores [126-128] and biotinylate the kinetochore receptor(s) of Aurora B. Then biotinylated proteins will be purified using streptavidin-

conjugated beads, and because the biotin-streptavidin interaction is very strong, purification can be done under stringent conditions without loss of proteins that have weak binding interactions. As an initial test, a set of cells will be either uninhibited, Haspin inhibited, Bub1 inhibited, or Bub1 and Haspin inhibited while expressing BirA-Aurora B, then when cells enter mitosis they will be subject to drug treatments to establish Aurora B localized at distinct locations, finally biotin will be added to the media (Figure 4.7B). The cells will be lysed, and lysate will be incubated with streptavidin-conjugated beads. The samples will be run on SDS-PAGE and transferred to membranes for evaluation by Western blot. Biotinylated proteins will be detected by streptavidin-conjugated-HRP antibodies and we expect that in the differentially drug treated cells we will see different bands representing the various receptors for Aurora B at these locations (Figure 4.6C). Once the protocol is optimized, purified biotinylated proteins can be sent to mass spectrometry for identification. The use of this system will help capture the kinetochore receptor for Aurora B especially if as we suspect, the interaction is weak or transient. However, the timing of the various drug treatments used to perturb Aurora B localization and the addition of biotin to the media will need further optimization.

4.3 Discussion

These results suggest KNL1 is, at least to some extent, required for Aurora B activity and localization to outer kinetochores. Fragments of FLAG-KNL1 co-immunoprecipitated Aurora B from mitotically enriched cells further supporting the idea that KNL1 is a kinetochore receptor for Aurora B and the CPC [133]. However, in these assays Aurora B is present in low amounts and inconsistently. We suspect that either

the conditions of cell treatments and expression of the FLAG-KNL1 constructs or the co-immunoprecipitation protocols need optimization. It could also suggest that the interaction with Aurora B and KNL1 is transient or weak and will not be detected with standard immunoprecipitation assays. For this reason, we designed a construct containing Aurora B fused to a promiscuous biotin ligase, BirA. The BirA-Aurora B construct can be expressed in cells and will biotinylate proteins nearby, thus immunoprecipitation of proteins within distance of Aurora B will be possible even if interactions are transient [200, 226]. KNL1 is required for Bub1 localization to kinetochores, and interestingly depletion of Bub1 results in loss of Aurora B at kinetochores [127]. Furthermore, we found that loss of Aurora B at kinetochores in Bub1 depleted cells is not due to the subsequent loss of CENP-E or F (Figure 4.6). Interestingly, FLAG-KNL1 fragments that contained Aurora B also co-immunoprecipitated Bub1. In the future it will be important to understand how Bub1 and KNL1 individually contribute to Aurora B activity at kinetochores. To this end, we designed KNL1-mini constructs to test how various motifs within KNL1 contribute to Aurora B at the outer kinetochore. These constructs were designed in lentivirus backbones and recombinant lentiviral virus was made to infect Kyoto-TetR cells for both immunoprecipitation and immunofluorescence assays that test Aurora B activity at outer kinetochores. In the future, we hope to determine the kinetochore receptors for Aurora B and the CPC using the various tools described above.

4.4 Methods

Co-immunoprecipitation of FLAG-KNL1 from Hek293T cells

T500 dishes were treated with 1M HCl shaking overnight, then washed with water multiple times and sterilized before seeding cells. For each mutant four T500 dishes were grown per prep. When cells were ~70% confluent thymidine was added, then washed out 16 hours later with the addition of doxycycline to induce FLAG-KNL1 expression. Thymidine was added again after 9 hours and washed out after 16 hours. 1 hour before cells go into mitosis (9 hrs after the washout) nocodazole was added for a total of 2 hours (5uM). Cells were collected in lysis buffer (75mM HEPES, 150mM KCl, 1.5mM EGTA, 1.5mM $MgCl_2$ 0.1% NP40, 1mM PMSF, 1X phosphatase inhibitor, 1X proteast inhibitor). Cells were lysed by passing through a 25-gauge needle many, many times and lysate was clarified by spinning at 13,000RPM for 10 minutes. The concentration of clarified lysate was approximated using the nanodrop absorbance at 280nm so a normalized amount of lysate for each mutant was bound to beads. FLAG M2 affinity beads were prepared by washing 3 times in lysis buffer. Normalized cell lysates were added to FLAG-beads for 1 hour at 4°C rocking. The beads bound by lysate were washed once, to preserve transient interactions and protein bound to FLAG beads was eluted using Glyc-HCl pH 3. Elutions were collected and concentrated using a spin column. Once the volume was ~40uL, samples were mixed with 1X SDS sample buffer and run on a 12% SDS-PAGE. Before the sample passed the stacking portion of the gel, the bands were cut out and sent to CU Boulder for mass spectrometry analysis.

Generation of stable cells expressing KNL1-mini constructs

KNL1-mini constructs were cloned into the pLCN (plasmid-lentiviral, CMV promoter, neomycin/G418 resistant) backbone. Recombinant lentivirus was generated by transfecting Hek293T cells with KNL1-pLCN and lentiviral donor plasmids MD2.G and

psPAX2. Briefly, a 10cm poly-lysine treated dish was seeded with 300,000 Hek293T cells/mL. Cells were transfected using lipofectamine3000 (2ug pLCN-KNL1, 5.5ug psPAX2, 1.5ug MD2.G) and transferred to DMEM for generation of virus particles. After 48 hours, the lentivirus was harvested by collecting the media from transfected Hek293T cells. The lentivirus-containing media was centrifuged at 2,000 RPM for 8 minutes for clarification and polybrene was added (10ug/mL). The lentivirus can then be stored at -80° C. KNL1-mini11A was used to infect Kyoto-TetR (Tet Repressor) cells. TetR expressing cells repress expression of genes that contain a Tet-operator (TetO) until doxycycline is added. TetO was included in all pLCN-KNL1 constructs. Kyoto-TetR cells were seeded in a T24 well dish at either 10,000 or 15,000 cells/mL and infected with 100ul or 500ul of lentivirus containing media for two consecutive days. After 24 hours cells were moved to 10cm dishes and incubated with 0.2mg/ml G418 for selection of positive colonies. Positive colonies were picked and transferred to 24-well dishes then grown up and frozen for future characterizing. Recombinant lentivirus for all KNL1-mini clones were made at stored at -80° C.

Figures 4

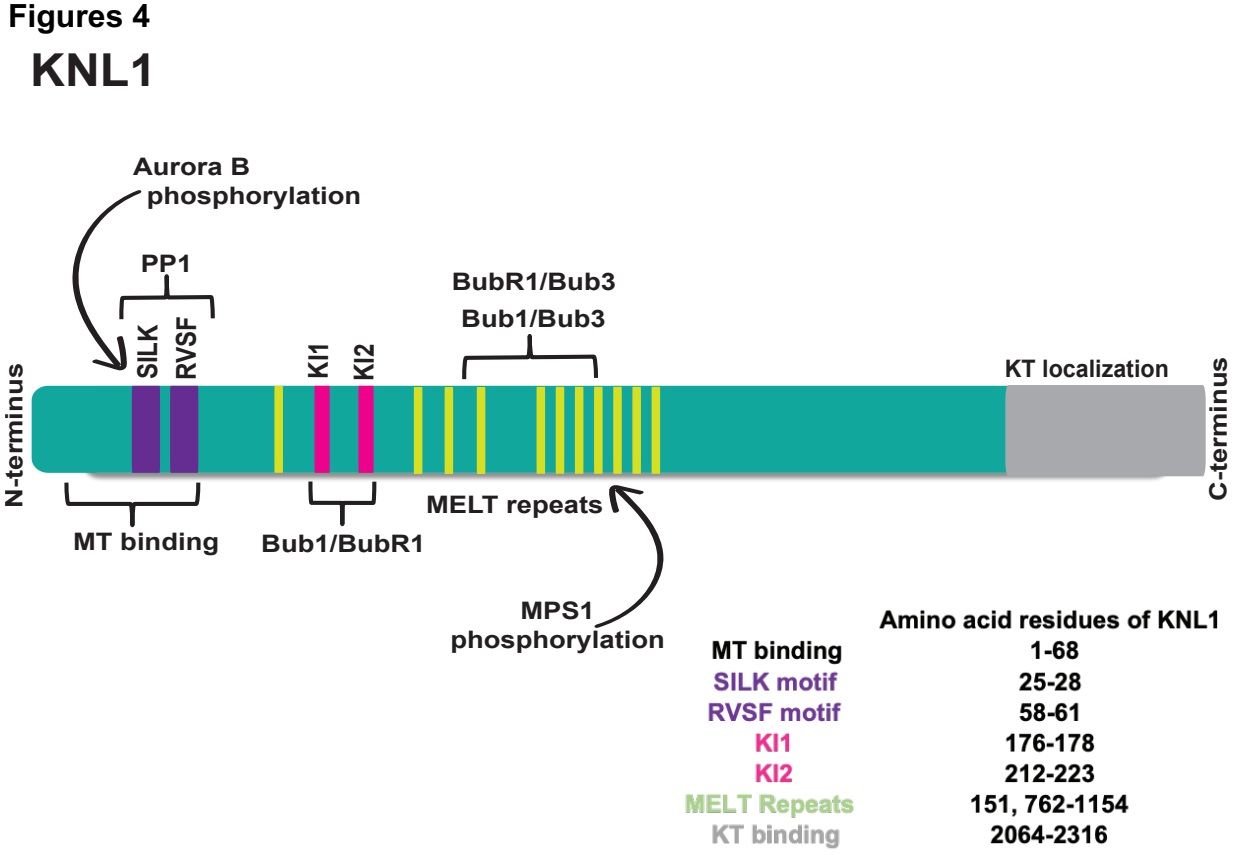


Figure 4.1. Map of KNL1. Displays important motifs of KNL1 and interacting partners. Listed are amino acid residues for important motifs. Adapted from Caldas et al., 2014 [61].

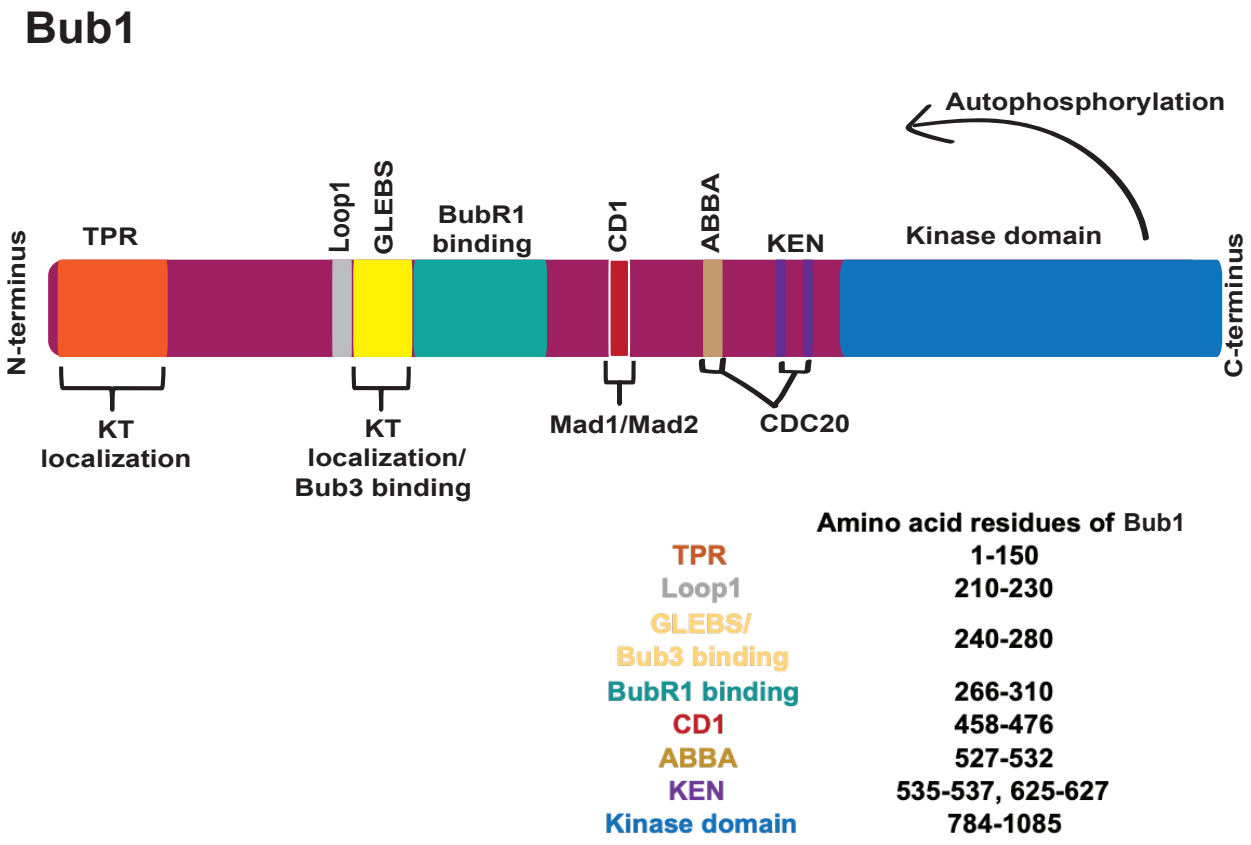
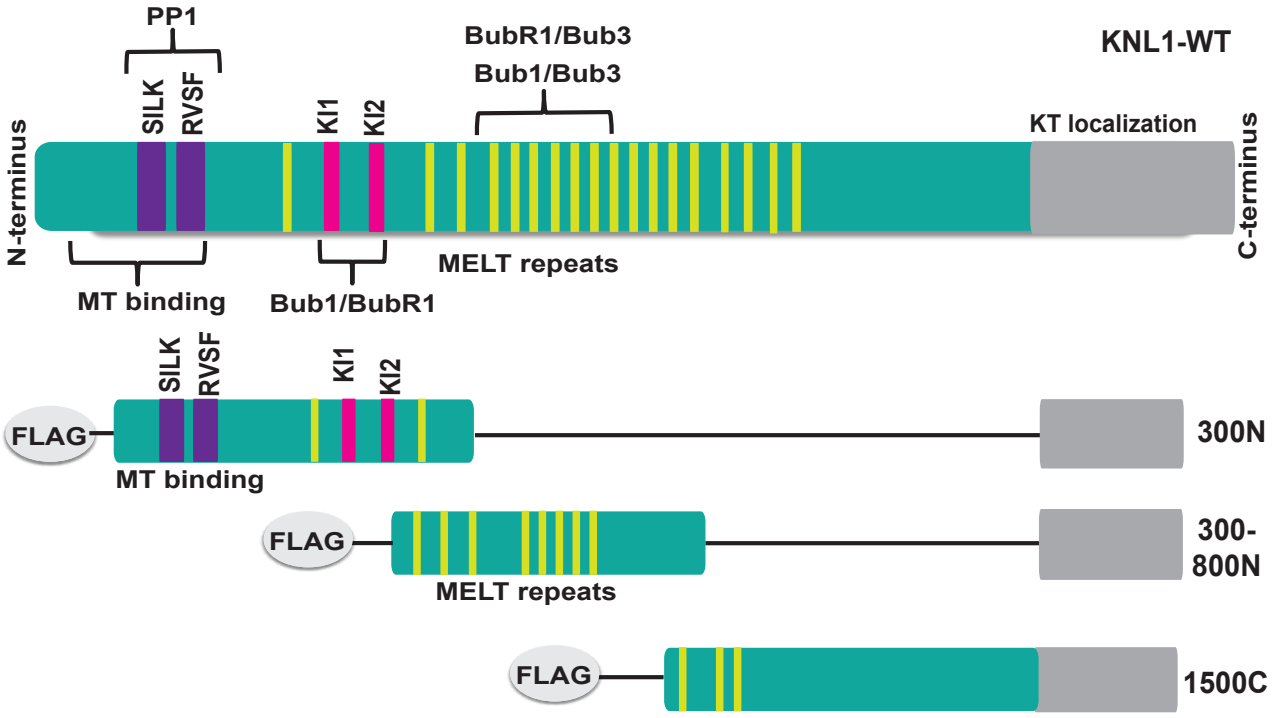


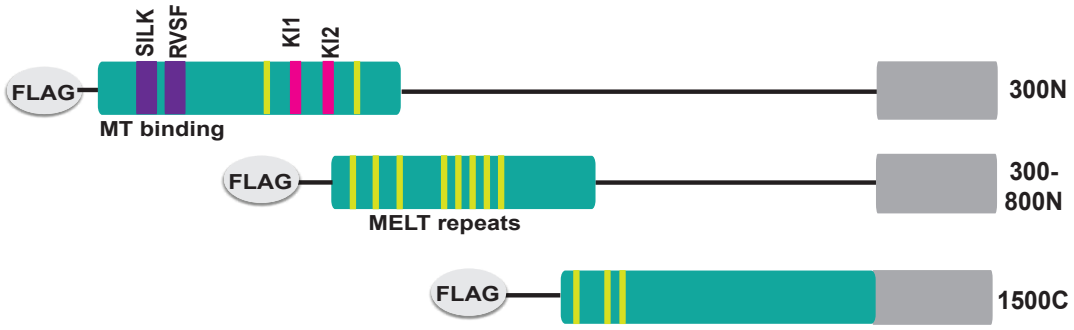
Figure 4.2. Map of Bub1 kinase. Listed are important amino acid residues for given motifs. Adapted from Ashgar and Elowe 2016 [212].



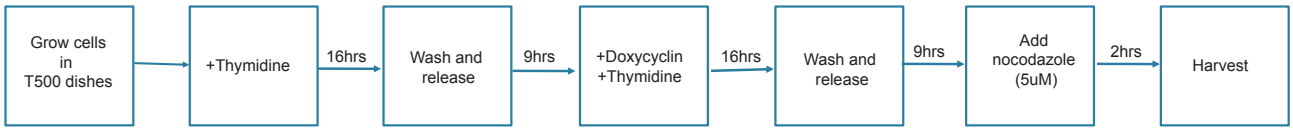
Knl1 Fragment	Amino acid residues
300N	1-300
300-800N	300-818
1500C	819-2316

Figure 4.3. FLAG-tagged KNL1 fragments used for co-immunoprecipitation. Adapted from Caldas et al., 2013 and Gina Caldas Dissertation 2014 [133].

A.


SILK
RVSF
KI1
KI2
FLAG
MT binding
300N
FLAG
MELT repeats
300-
800N
FLAG
1500C

B.


Grow cells in T500 dishes
+Thymidine
16hrs
Wash and release
9hrs
+Doxycyclin +Thymidine
16hrs
Wash and release
9hrs
Add nocodazole (5uM)
2hrs
Harvest

C.

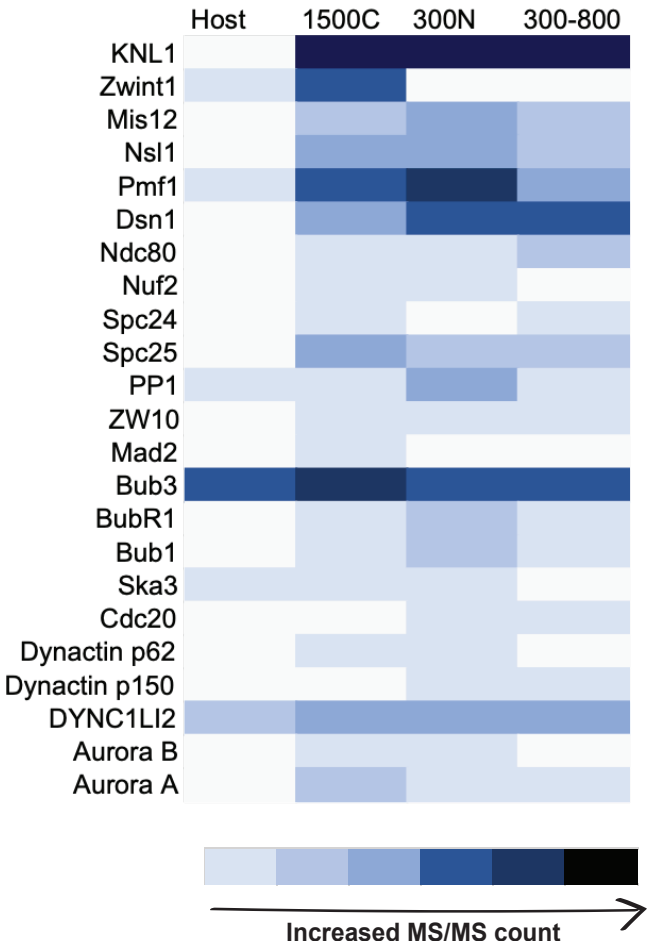

Host
1500C
300N
300-800
KNL1
Zwint1
Mis12
Nsl1
Pmf1
Dsn1
Ndc80
Nuf2
Spc24
Spc25
PP1
ZW10
Mad2
Bub3
BubR1
Bub1
Ska3
Cdc20
Dynactin p62
Dynactin p150
DYNC1LI2
Aurora B
Aurora A
Increased MS/MS count

Figure 4.4. Co-immunoprecipitation of FLAG-KNL1 fragments with kinetochore proteins. A) FLAG-tagged KNL1 constructs that are stably expressed in Hek293T cells. B) Workflow of cell treatment to generate samples for mass spectrometry analysis. C) Heat map of proteins that co-immunoprecipitated with FLAG-KNL1 fragments identified by mass spectrometry.

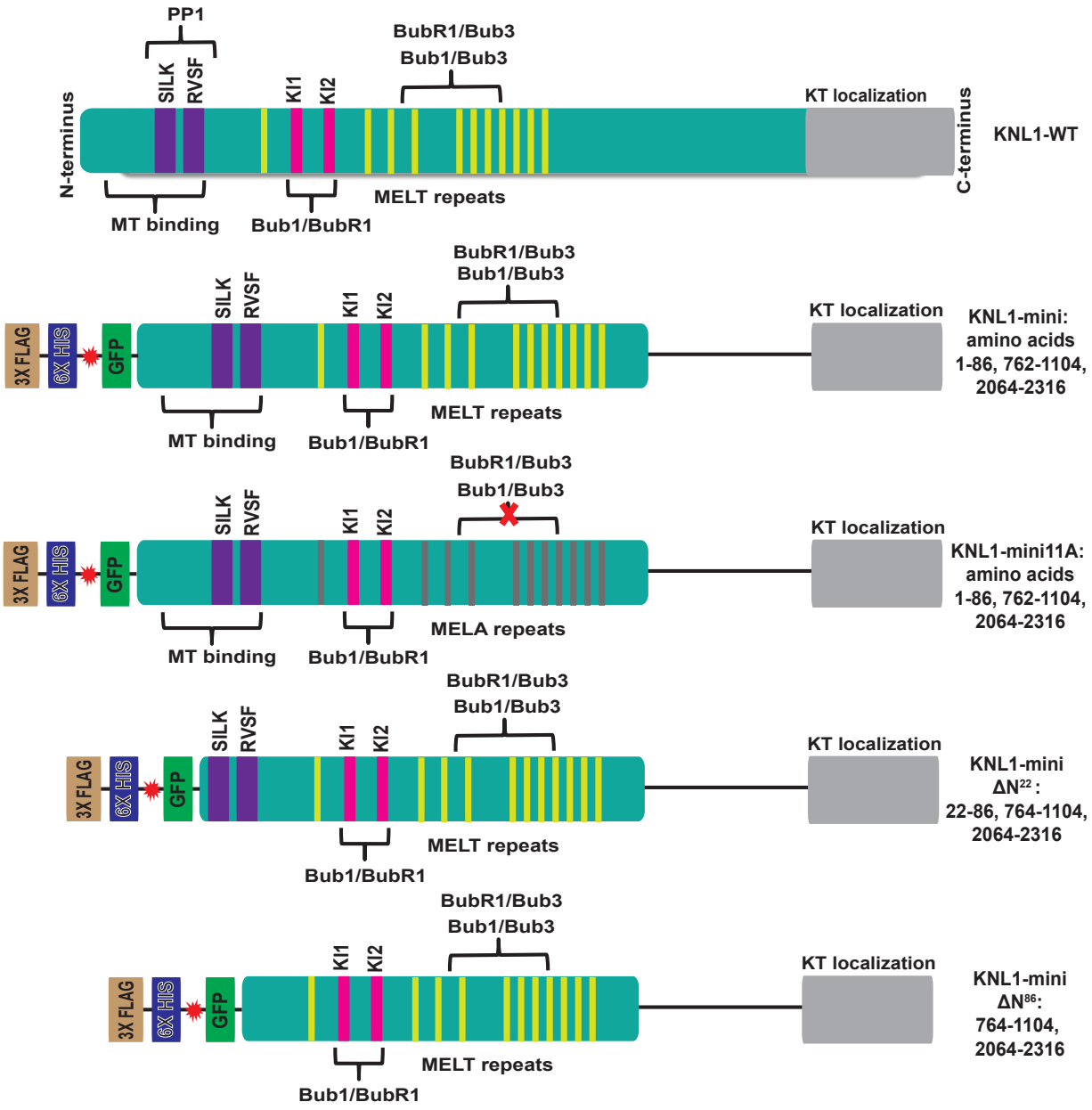


Figure 4.5. Maps of KNL1-mini mutants. Amino acid residues listed under mutant names were cloned into pLCN for the generation of recombinant lentivirus and stable cells. Red stars indicate HRV3C protease cut site.

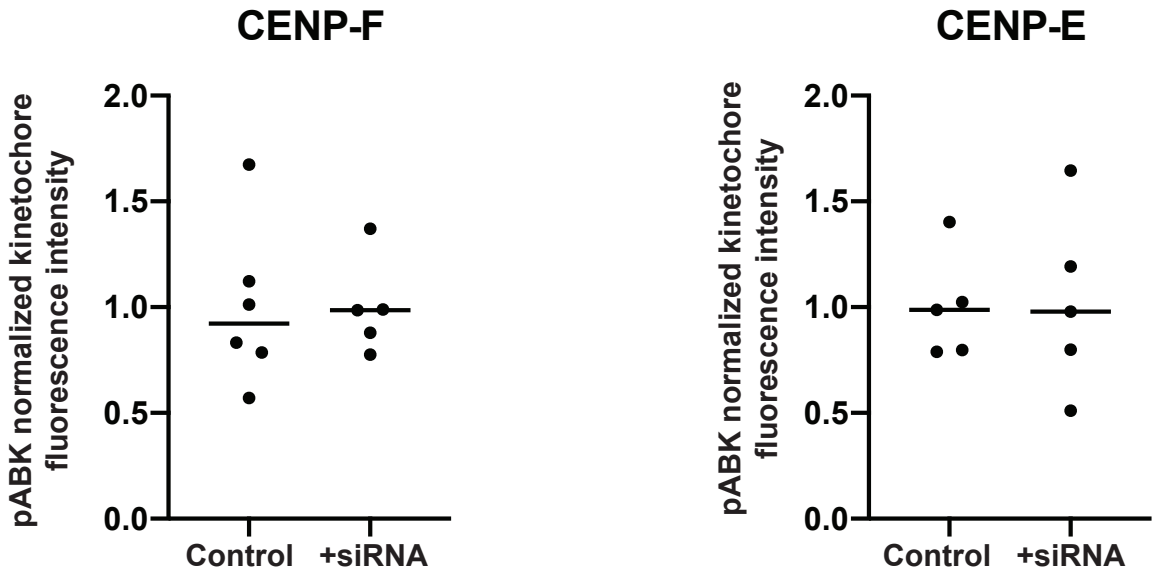


Figure 4.6. CENP-E and F are not required for localization or activity of Aurora B at kinetochores. Quantifications of Aurora B kinetochore fluorescence intensity in control HeLa cells or HeLa cells treated with either CENP-E or F siRNA.

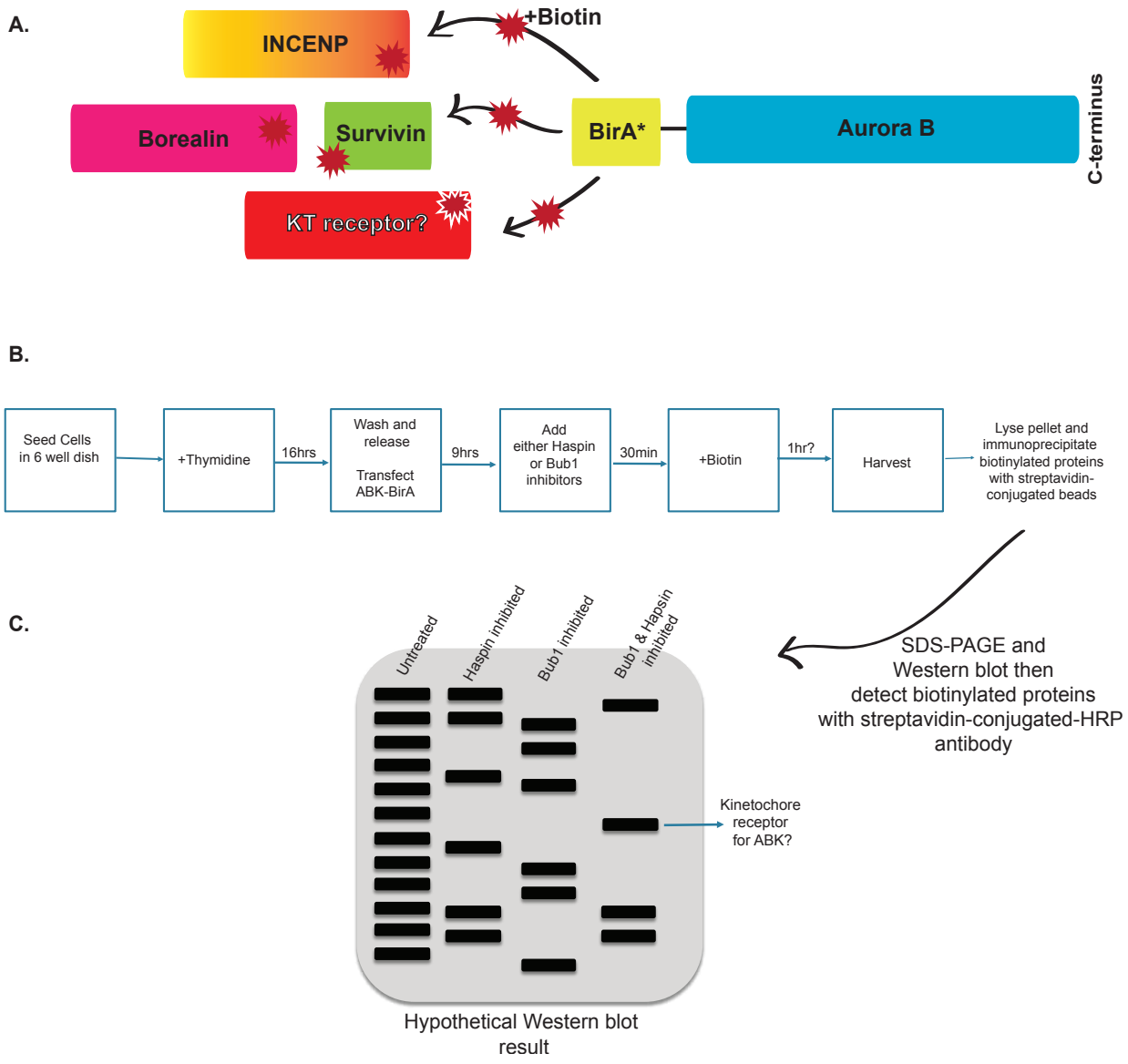


Figure 4.7. Proximity-based biotin ligation assay to capture transient interactions between Aurora B and its outer kinetochore receptors. A) Aurora B is tagged with the promiscuous biotin ligase, BirA. When expressed in cells, in the presence of biotin, BirA will biotinylate nearby proteins. B) Workflow for BioID assay. The amount of time drug-treated cells are incubated with biotin needs to be optimized. C) Hypothetical result from ligation assay. We expect that in drug treated cells, when Aurora B localizes to either the centromere or kinetochore, different proteins will be biotinylated and banding from Western blots will represent that. We expect that when both Bub1 and Haspin are inhibited and Aurora B localizes prominently to kinetochores, the kinetochore receptor of Aurora B will be biotinylated and identified.

Chapter 5: The right place at the right time- Aurora B kinase localization at centromeres and kinetochores[3]

5.1 Discussion

pH2A-T120 and pH3-T3 recruit the CPC to distinct locations within the centromere

In a series of recently published studies, three groups carried out experiments to directly test the model for CPC localization; that is, to determine if CPC recruitment to inner centromeres requires the overlap of pH3-T3 and pH2A-T120 in human cells [126-128]. Using antibodies to both phospho-marks, the authors of all three studies reported that pH2A-T120 and pH3-T3 did not show significant overlap in cells; instead, immunostaining revealed that pH3-T3 localized distinctly as a single focus at the inner centromere, while pH2A-T120 localized as a pair of dots flanking the inner centromere [126-128]. This is consistent with previous data suggesting distinct localization patterns of the two histone marks [21, 109, 121, 125, 131, 136]. Line-scans and two-color localization experiments further revealed that pH2A is localized inside of the inner kinetochore protein CENP-C, on the order of ~100 nm, in both prometaphase and metaphase cells, which places this mark within the “kinetochore-proximal outer

[3] The work presented in this chapter was submitted as a research review on March 16, 2020 under the same title. Figures and material presented has been amended from the published version for clarity.

AJB and JGD wrote the review, JGD made all figures.

Broad AJ, and DeLuca JG. The right place at the right time- Aurora B kinase localization at centromeres and kinetochores. Essays in Biochem. Submitted March 2020.

centromere" region, distinct from the pH3-T3-marked chromatin at the inner centromere (Figure 3.1) [126-128]. Importantly, all three studies reported that each histone mark individually was sufficient to recruit Aurora B kinase and the CPC. Each group analyzed U2OS cells containing an ectopic Lac operator (LacO) array stably integrated in the short arm of chromosome one that were expressing fusions of either LacI-Haspin or LacI-Bub1 [187]. In cells expressing LacI-Bub1, the chromatin surrounding the LacO array was positive for pH2A-T120 but not pH3-T3, and the single phosphorylation mark (pH2A-T120) was sufficient for recruitment of Aurora B kinase in a manner dependent on Sgo1 (Figures 3.2 and 3.3) [126-128]. Similarly, when Haspin was directed to the ectopic locus through expression of a LacI fusion protein, the local chromatin was positive for pH3-T3 but not pH2A-T120, and this single modification was also sufficient to recruit Aurora B kinase and its CPC partners (Figure 3.2) [126-128]. Moreover, each histone mark was sufficient to recruit a population of the CPC to spatially distinct regions within the centromere region of mitotic chromosomes in human cells (Figure 5.1). While depletion of Haspin or inhibition of its kinase activity resulted in loss of both the pH3-T3 mark and accumulation of Aurora B at inner centromeres, a population of Aurora B remained localized to the kinetochore-proximal outer centromere, coincident with pH2A-T120 (Figure 3.10 and 3.11) [126-128, 131]. Conversely, inhibition of Bub1 kinase activity resulted in loss of the pH2A-T120 mark, but Aurora B kinase and components of the CPC remained localized at the inner centromere coincident with pH3-T3 (Figure 3.7) [126-128, 154]. Inhibition of both Bub1 and Haspin kinase activities; however, resulted in no detectable Aurora B and CPC components at centromeres (Figure 3.6) [111, 126-128, 154].

In the studies described above, Aurora B kinase localized prominently to inner centromeres as a single focus, but was not clearly discernable at pH2A “marked” kinetochore-proximal outer centromere regions in control, unperturbed cells [126-128]. This population of the CPC became readily detectable however, when phosphorylation of H3-T3 was prevented through Haspin knockout or Haspin inhibition [126-128, 131], suggesting crosstalk between the two centromere-localized populations of the CPC. These results point to the possibility of a multifaceted loading process whereby a pool of Aurora B kinase is loaded directly to the inner centromere binding sites provided by pH3, and a second population of the complex is initially recruited to the kinetochore-proximal outer centromere by pH2A/Sgo1 and subsequently relocated to the inner centromere region. This mechanism is similar to what has been suggested for Sgo1, which first loads to kinetochores in early mitosis, which is required for its subsequent relocalization to the inner centromere, where it functions to protect cohesion and prevent premature sister chromatin separation [41, 109, 130, 142]. Furthermore, authors from the Liang et al. [126] study suggest that relocalization of Aurora B kinase from the pH2A-T120 binding sites to the inner centromere pH3-T3 binding sites in metaphase may be required to silence the spindle assembly checkpoint in response to kinetochore-microtubule attachment. The authors reported that experimentally-induced retention of Aurora B and the CPC at the kinetochore-proximal outer centromere in metaphase cells resulted in a small increase (by ~20%) in Aurora B kinase-mediated phosphorylation of the kinetochore scaffolding protein KNL1 which led to sustained checkpoint signaling [126]. In sum, three recent studies report the identification of discrete populations of Aurora B kinase within the centromere region that are recruited

by distinct histone modifications. These studies suggest that the different populations of the CPC functionally interact and cooperatively contribute to the robust accumulation of Aurora B kinase at the inner centromere of mitotic chromosomes.

A role for centromere-localized Aurora B kinase in chromosome segregation

In light of the finding that each of the two histone marks recruits a distinct population of Aurora B and the CPC to centromeres, the authors of the studies described above [126-128] tested if either population is required for Aurora B kinase activity at kinetochores or for proper chromosome segregation. The three studies reported that inhibition of either pathway alone did not result in chromosome segregation errors or reduced phosphorylation of kinetochore Aurora B kinase substrates [126-128]. However, in cells inhibited for both Bub1 and Haspin kinase activities, chromosome segregation was compromised, although it was noted that the defects were less severe than those observed in cells inhibited for Aurora B kinase itself [126, 128]. Thus, the inner centromere and kinetochore-proximal outer centromere populations of Aurora B likely have redundant roles in ensuring proper chromosome segregation. Strikingly, in cells inhibited for both Bub1 and Haspin kinase activities, which resulted in a complete loss of centromere-localized CPC, Aurora B kinase localization and substrate phosphorylation at kinetochores remained high (Figure 5.1) [126-128]. As such, the chromosome segregation defects resulting from loss of centromere-localized Aurora B could not be attributed to loss of phosphorylation of kinetochore substrates [126-128]. Importantly, these results provide evidence that centromere accumulation of the Aurora B kinase is not strictly coupled to Aurora B activity at kinetochores. This idea is consistent with a number of previous studies, the

first of which demonstrated that centromere-localized Aurora B is not required for the regulation of kinetochore-microtubule attachments in chicken DT40 cells [120]. Here, cells depleted of endogenous Survivin and expressing a mutant version of Survivin defective for centromere localization completed mitosis normally with no detectable chromosome segregation defects [120]. In addition, kinetochore-microtubule attachment defects observed in HeLa cells depleted of the large kinetochore-associated scaffolding protein KNL1, which resulted in loss of kinetochore-associated Aurora B kinase activity, could not be rescued by ectopic targeting of the CPC to centromeres [133]. Finally, several studies have suggested that centromere accumulation of the CPC is uncoupled from kinetochore-associated Aurora kinase activity in budding yeast. Campbell and Desai [117] reported that budding yeast cells expressing INCENP/Sli15 mutants that fail to localize to centromeres exhibited normal chromosome bi-orientation and Aurora/Ipl1 kinase-mediated error correction. Recent studies have further demonstrated that the CPC is recruited directly to kinetochores in budding yeast, and this population is sufficient for Aurora kinase/Ipl1 activity at kinetochores and error-free chromosome segregation in the absence of centromere-localized CPC [150, 151].

An obvious question that emerges from the recent studies in human cells [126, 128] is what causes the observed chromosome segregation errors in the absence of centromere-localized Aurora B if the kinase is still able to phosphorylate kinetochore-associated substrates normally? A plausible explanation is that centromere-localized Aurora B phosphorylates centromere-localized substrates to promote proper chromosome segregation. Previous studies have demonstrated that Aurora B kinase regulates the activity and localization of MCAK, a centromere-localized kinesin-13 motor

that promotes microtubule depolymerization, an activity that is implicated in correcting erroneous kinetochore-microtubule attachments [1, 73, 155, 156, 230-232].

Interestingly, the centromere localization of MCAK is perturbed in cells that are depleted of Haspin or inhibited for Bub1 kinase activity [121, 122, 128, 135, 233], consistent with the notion that alterations in MCAK activity and/or localization might contribute to the chromosome segregation errors observed in cells lacking centromere-localized Aurora B kinase. Further investigation into the role of MCAK and other centromere-localized Aurora B kinase substrates is needed to resolve this question. It is interesting to note that while antibodies to CPC components localize prominently to the inner centromere in early mitosis, antibodies to phosphorylated, active Aurora B (pT232) and phosphorylated, active INCENP (pS893/pS894) show minimal inner centromere localization in early mitosis, but these levels significantly increase as mitosis progresses [87, 127]. These results suggest that centromere substrates of Aurora B kinase that contribute to proper chromosome segregation may be phosphorylated and perhaps activated in late mitosis rather than in early mitosis.

Finally, it is important to point out that recent studies demonstrating that centromere-localized CPC is not explicitly required for phosphorylation of kinetochore substrates do not rule out the possibility that the centromere- and kinetochore-localized pools of the CPC may exhibit cross-talk and impact each others' localization or activity. In fact, studies have demonstrated that while delocalization of CPC from centromeres did not result in decreased activity of Aurora B at kinetochores in human cells and in M-phase *Xenopus* egg extracts, the regulation of kinase activity in response to kinetochore-microtubule attachment was compromised [90, 153, 199]. Why this is the

case is not clear, but in the future it will be important to resolve how the centromere pool of the CPC contributes to proper regulation of Aurora B kinase substrate phosphorylation at kinetochores in response to microtubule attachment.

5.2 Aurora B localization and activity at kinetochores

Aurora B kinase localizes to kinetochores

As previously mentioned, in addition to its centromere localization, Aurora B has also been detected at the kinetochore (Figure 3.11). Antibodies directed to active, phosphorylated Thr232, which resides in the T-loop of the kinase domain of Aurora B and is required for full activity of the kinase, recognize kinetochores in early mitosis, centromeres in late prometaphase and metaphase, and the spindle midzone in anaphase [87, 148, 184, 234, 235]. A similar localization pattern was observed for phosphorylated Aurora B Ser331, a site whose phosphorylation is required for optimal Aurora B activity [236]. Furthermore, antibodies to phosphorylated, active INCENP (pS893/pS894) recognize both kinetochores and centromeres in early mitosis, and this localization shifts primarily to centromeres in late prometaphase and metaphase and the spindle midzone in anaphase [127]. Together, these studies raise the possibility that a population of Aurora B kinase, and likely the entire CPC, is recruited directly to kinetochores in early mitosis where it phosphorylates its kinetochore substrates to promote kinetochore-microtubule turnover. In such a model, as mitosis progresses and kinetochores accumulate bound microtubules, Aurora B is evicted from kinetochores, resulting in decreased kinetochore substrate phosphorylation, stabilization of attachments, and silencing of the spindle assembly checkpoint (Figure 1.7) [71, 79, 127, 140, 201].

How is Aurora B kinase recruited to the kinetochore to phosphorylate kinetochore substrates?

A major task that remains to be tackled is identifying the kinetochore binding sites for the CPC. In budding yeast, two research groups have made considerable headway on this front by demonstrating that inner kinetochore COMA (Ctf19/Okp1/Mcm21/Ame1) complex recruits the CPC through a direct interaction between INCENP/Sli15 and Ctf19 [150, 151]. Importantly, this kinetochore-associated population is sufficient to support Ipl1/Aurora kinase activity and normal chromosome segregation in the absence of centromere-localized CPC [150, 151]. In metazoan cells, however; the kinetochore binding sites for Aurora B and the CPC remain unknown. In a recent study discussed above [127], authors approximated the location of Aurora B kinase in early mitotic cells to ~22 nm outside of the inner kinetochore protein CENP-C, which places it ~8 nm inside of the N-terminus of the outer kinetochore protein Hec1. Many kinetochore proteins localize in this region of the kinetochore, making it difficult to predict specific binding sites. A previous study reported that Aurora B kinase localization to kinetochores is dependent on the kinetochore protein KNL1 [133], and Broad et al. [127] demonstrated that Aurora B localization is at least partially dependent on Bub1. Moreover, a recent study found that KNL1 undergoes significant conformational changes upon kinetochore-microtubule attachment [201]. Together, these findings make it tempting to speculate that KNL1 may directly or indirectly provide binding sites for CPC components in early mitosis. As mitosis progresses and kinetochore-microtubule attachments accumulate, KNL1 may undergo conformational changes that occlude these sites, leading to eviction of Aurora B kinase and its CPC cofactors (Figure 1.7). This speculative model remains to be tested.

Importantly, the available data suggest that Aurora B kinase may be recruited to multiple locations within the kinetochore to facilitate different functions. Based on the recent studies described above, we predict that Aurora B is recruited directly to outer kinetochores to phosphorylate outer kinetochore substrates involved in kinetochore-microtubule attachment regulation [21, 87, 127, 133, 184]. However, as mentioned above, in budding yeast, the CPC is recruited to the inner kinetochore COMA complex, whose homolog in humans is the CENP-O/P/Q/U complex, a component of the Constitutive Centromere Associated Network (CCAN) [150, 151]. Indeed, Aurora B kinase has substrates at the inner kinetochore that are important for mitotic progression. For example, the Mis12 complex component Dsn1 is phosphorylated by Aurora B in early mitosis to promote kinetochore assembly by facilitating an interaction between the inner-kinetochore protein CENP-C and the Mis12 complex [35, 96, 191-193, 202, 237-240]. A recent study from Bonner et al. [238] demonstrated that delocalization of the CPC from centromeres in M-phase *Xenopus* egg extracts resulted in decreased Dsn1 phosphorylation and consequently, failure to assemble the outer kinetochore. The authors of this study found that the SAH domain of INCENP was required, in a microtubule-independent manner, for Aurora B kinase-mediated phosphorylation of Dsn1 and kinetochore assembly. Ectopic targeting of INCENP lacking its central SAH domain to the inner kinetochore protein Nsl1 and subsequent recruitment of the CPC to this region rescued both Dsn1 phosphorylation and kinetochore assembly [238]. The authors concluded that the INCENP SAH domain is critical for localizing the CPC to the inner kinetochore, in close proximity to the Mis12 complex so that Aurora B is able to efficiently phosphorylate Dsn1 to promote outer kinetochore assembly [238]. These

results also brings to light the idea that multiple kinetochore functions rely on kinetochore-associated Aurora B kinase activity, and the CPC may be recruited to different locations within the kinetochore to support these activities.

5.3 Closing comments

A growing number of studies has demonstrated that centromere-localized Aurora B kinase is not explicitly required for Aurora B kinase activity at kinetochores. Based on these studies and data from numerous model systems, the current model for Aurora B kinase-mediated regulation of kinetochore-microtubule attachment stability (i.e. the "spatial positioning model") should be re-evaluated. Many studies have mapped Aurora B kinase to kinetochores, and ectopically targeting the kinase to kinetochores in multiple cell types rescues loss of the centromere-localized population. In budding yeast, at least one kinetochore binding site for the CPC has been identified, which suggests that Aurora/Ipl1 kinase localizes to kinetochores to specifically phosphorylate kinetochore substrates. This straightforward mechanism for CPC function at budding yeast kinetochores, in which the kinase is recruited to the region of mitotic chromosomes where its substrates are located, is likely utilized in metazoan cells as well. The next major challenge is to identify the binding site, or more likely, binding sites, for the CPC at metazoan kinetochores.

Figure 5.0

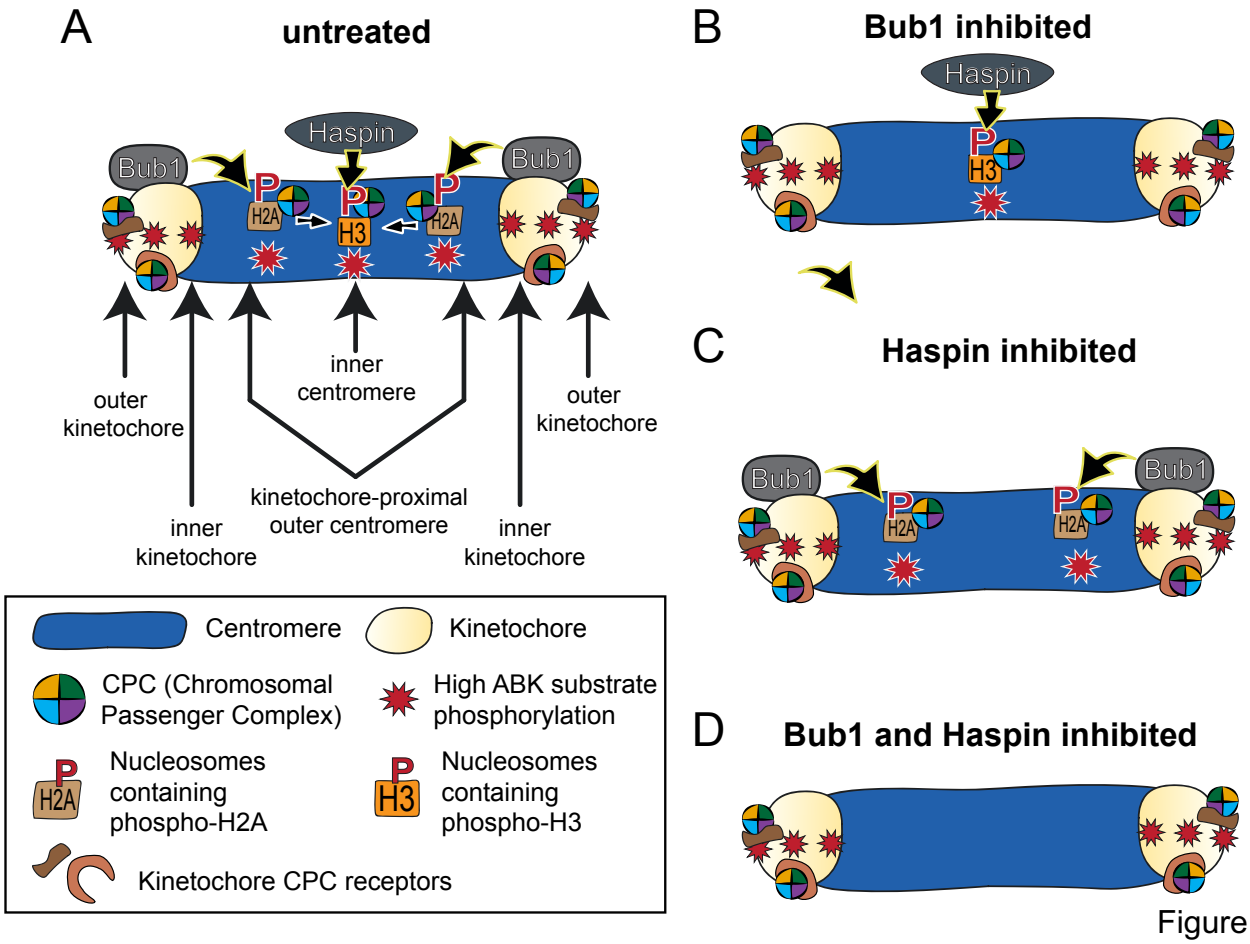


Figure 5.1. Recruitment pathways for centromere-localized Aurora B kinase. (A) Phosphorylated histone H3 recruits the CPC to inner centromeres through direct binding of Survivin. Phosphorylated histone H2A recruits the CPC to kinetochore-proximal outer centromere regions through Sgo1-dependent recruit of the CPC, likely through direct interaction between Sgo1 and Borealin. Once recruited to the outer centromere, this population of the kinase is possibly translocated to the inner centromere (indicated by inward directed arrows). (B) In Bub1 inhibited cells, phosphorylated histone H3 recruits the CPC to inner centromeres, and this activity is sufficient to support error-free chromosome segregation. (C) In Haspin inhibited cells, phosphorylated histone H2A recruits the CPC to kinetochore-proximal outer centromere regions. In this case, the kinase remains localized to these regions and is not relocated to inner centromeres. Similar to the scenario in (B), kinase recruitment here is sufficient to support error-free chromosome segregation. (D) In Bub1- and Haspin-inhibited cells, Aurora B fails to localize to centromeres, and chromosome segregation is impaired. In all cases (A-D), kinetochore-associated Aurora B kinase activity (in early mitosis) remains high, regardless of centromeric accumulation of the kinase. This model is based on data from references [126-128].

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