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entitled

Analysis of Borealin-mediated Centromere Targeting of the Chromosomal Passenger Complex

by

Michael E. Bekier II

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Molecular and Cell Biology

________________________________________
Dr. William R. Taylor, Committee Chair

________________________________________
Dr. Song-Tao Liu, Committee Member

________________________________________
Dr. Malathi Krishnamurthy, Committee Member

________________________________________
Dr. John Bellizzi, Committee Member

________________________________________
Dr. Ivana de la Serna, Committee Member

________________________________________
Dr. Patricia R. Komuniecki, Dean
College of Graduate Studies

The University of Toledo

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An Abstract of

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The Chromosomal Passenger Complex (CPC) localizes to centromeres and monitors inter-kinetochore tension to activate the mitotic checkpoint and ensure faithful segregation of chromosomes during mitosis. The CPC subunit Borealin is implicated in centromere targeting of the complex, yet the details of targeting are not completely understood. We have used a combination of Borealin structure-function analysis, chemical genetics, FRAP, and an inducible FKBP dimerization system to investigate centromere targeting and function of the CPC. We provide evidence that Borealin dimerization plays two crucial roles in CPC targeting during mitosis. First, Borealin suppresses dynamic exchange at inner centromeres via dimerization of the CPC where slow exchange at centromeres is required for optimal CPC function. Second, the central region of Borealin targets the dimerized CPC to a region near kinetochores enabling feedback with Bub1 and activation of the mitotic checkpoint. The existence of a pool of CPC near kinetochores suggests that error correction is more complicated than predicted from the Aurora B phosphorylation gradient model. The critical role of Borealin dimerization in both inner centromere and kinetochore targeting of the CPC may simply
result from a doubling of CPC interactions with its centromere and kinetochore receptors.

Finally, we provide preliminary evidence that the CPC directly regulates Bub1 kinase activity at kinetochores, which is essential for localization of the CPC.
I would like to dedicate this dissertation to my father Michael Edward Bekier Sr. and my wife Sara Arno. Without your unwavering support and dedication to my pursuit of knowledge this dissertation would not be possible. I owe more than a dissertation to both of you and hope to make you proud with what this dissertation will bring. Finally, I would like to dedicate this dissertation to my son Michael Edward Bekier III: *Filius meus, vita mea.*
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List of Abbreviations

5Itu ..........................5-Iodotubercidin
AGC ...........................cAMP-dependent, cGMP-dependent, protein kinase C
APC/C .........................anaphase promoting complex/cyclosome
ATM ...........................Ataxia telangiectasia mutated
ATR ...........................Ataxia telangiectasia mutated-Rad3-related
BIR .........................baculoviral inhibitor of apoptosis
Bub ..........................budding-uninhibited by benzimidazole
BubR1 ........................budding uninhibited in response to benzimidazole-related 1
Cdc ...........................cell division cycle mutants
CDK1 ..........................Cyclin-dependent kinase-1
CDKs ..........................Cyclin-dependent kinases
Chk1 .........................Checkpoint kinase 1
Chk2 .........................Checkpoint kinase 2
CK2 ...........................Casein kinase-2
CO₂ ...........................Carbon dioxide
CPC ...........................Chromosomal Passenger Complex
DMSO .......................Dimethyl sulfoxide
ECT2 ..........................epithelial cell transforming gene
FRAP ..........................Fluorescence Recovery After Photobleaching
G1 ............................gap phase 1
G2 ............................gap phase 2
H3K9me3 ........................trimethylated lysine 9
HP-1 ..........................heterochromatin binding protein-1
IAP ..............................inhibitor of apoptosis
INCENPs .....................inner centromere proteins
Mad ..........................mitotic-arrest deficient
MCC..........................Mitotic Checkpoint Complex
MELT..........................Met-Glu-Leu-Thr
MgcRacGAP ..................male germ cell Rac-GTPase activating protein
MKLP1 ......................mitotic kinesin-like protein-1
MKLP2 ......................mitotic kinesin-like protein-2
Mps1 .......................Monopolar Spindle 1

PBS .........................phosphate buffered saline
PBSP .......................PBS plus protein
pH2A$^{T120}$ ..................phosphorylated histone 2A at threonine 120
pH3$^{T3}$ .......................phosphorylated histone H3 at threonine 3
PVDF ........................polyvinylidifluoride

Rb ..........................Retinoblastoma protein
Rev ..........................Reversine
Rho-GEF .....................Rho-guanine nucleotide exchange factor

SDS-PAGE ..................Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sgo1/2 ......................Shugoshin proteins 1/2
S-phase ........................synthesis phase

TD-60 ........................telophase disk protein
TSS .........................threonine-serine-serine
ZM ..........................ZM447439
List of Symbols

°............................Degrees

µg..............................microgram
µL..............................microliter
µM..............................micromolar
Φ..............................hydrophobic residue

C..............................Celsius
mM..............................millimolar
t..............................time
g..............................relative centrifugal force
Chapter 1

Introduction

1.1 Significance

From single-cell to multi-cellular organisms, life as we know it relies on the process of cell division, or creating two cells from a single cell. Cell division, also termed the cell cycle, is a unidirectional process that can be divided into two distinct modules: interphase and mitosis (Figure 1-1). Interphase begins with an initial gap phase (G1) followed by DNA replication (S-phase) and a second gap phase (G2). Exit from G2 signals entry into mitosis where duplicated chromosomes are condensed and segregated into two daughter cells (Figure 1-1). Successful cell division warrants that DNA replication and chromosome segregation are tightly regulated to ensure that both daughter cells receive an unaltered, equal complement of the duplicated genome. Roughly 8.7 million extant species share similar network topologies that regulate cell cycle progression and unregulated cell division can directly impact human health as various disorders and diseases such as Down syndrome and cancer have been linked to defects in genes that regulate cell division [1-3]. Therefore, understanding the molecular basis of cell division is essential for understanding the persistence of cellular life on the planet.
Figure 1-1. The Cell Cycle
Cells prepare to divide by synthesizing proteins necessary for DNA synthesis in G1. Initiation of DNA synthesis signals entry into S-phase where the genome of the cell is duplicated with high fidelity. Completion of DNA synthesis signals entry into G2 where cells grow and synthesize proteins essential for mitosis. Entry into mitosis is morphologically distinguished by chromosome condensation, nuclear envelope breakdown and chromosome congression at the center of the cell (metaphase plate). When duplicated chromosomes have bi-oriented, individual sister chromatids are segregated to opposing spindle poles while the cleavage furrow, which signifies the plane of cell division, begins to form. When chromosomes reach the spindle poles the nuclear envelope begins to reform and abscission of the plasma membrane eventually results in the production of two daughter cells with equal DNA content.
1.2 History of the cell cycle

The molecular synthesis of the cell cycle model that describes the ordered series of events that take place during cell division initially came from a collection of temperature-sensitive mutants, termed cell division cycle mutants (cdc), that arrested the development of the yeasts *Saccharomyces cerevisiae* and *Saccharomyces pombe* [4-12]. The interdependence of DNA synthesis, nuclear division, and cell separation was obtained by observing which events do not occur at a restrictive temperature. Organizing the collection of mutants allowed Hartwell and colleagues to conclude that the highly reproducible temporal sequence of events during cell division occur via a dependent pathway where an earlier event in the cell cycle must be completed before a later event occurred. In general, cdc mutants with an initial defect in a particular phase fail to complete any other events which normally occur later in the cell cycle. Thus, completion of each event observed in the cell cycle is a necessary prerequisite for the occurrence of the immediate succeeding event.

Subsequent work in yeast and other eukaryotic cells provided evidence for the existence of control mechanisms that enforce dependency in the cell cycle, termed checkpoints. Scientists noticed that eukaryotic cells require complete synthesis of DNA to enter mitosis [8, 9, 13] However, the dependency on DNA replication for entry into mitosis is relieved upon inactivation of key regulatory genes. For example, temperature sensitive cdc mutants that are deficient in DNA replication, such as cdc9 which encodes a DNA ligase, do not normally undergo mitosis at the restrictive temperature [14, 15].
However, *cdc*-*rad* double mutants continue through mitosis at the restrictive temperature. Similarly, gamma irradiation prevents yeast cell progression from G2 into mitosis and mutations in the *rad* gene allow cells to proceed to mitosis despite DNA damage induced by gamma irradiation during G2. Thus, when DNA replication is impaired in S-phase or when cells experience DNA damage in G2 the *rad* gene product prevents entry into the next phase of the cell cycle. Another example of a checkpoint stemmed from observations that cells with impaired chromosome alignment during mitosis do not initiate chromosome segregation to daughter cells, or anaphase, until all chromosomes align at the metaphase plate. Scientists speculated that a checkpoint existed to halt the initiation of anaphase until chromosomes became aligned to ensure accurate chromosome segregation [16-18]. The observations that cells exert control mechanisms that check the status of particular events during each phase of the cell cycle led to the term checkpoints.

Over time, biochemical characterization of the *cdc* yeast mutant genes and homologous genes discovered in metazoans led to the realization that eukaryotic cell cycle checkpoints rely on the complex regulation of Cyclin-dependent kinases (CDKs) [19]. CDKs require binding to cognate co-activators known as Cyclins and the abundance of specific Cyclins is modulated throughout the cycle to regulate a particular CDK, or set of CDKs. Additionally, the activity of CDKs is also regulated post-translationally, providing an additional level of checkpoint regulation. For example, the G1/S checkpoint regulates entry into S-phase from G1 in the presence of DNA damage or when environmental conditions are unfavorable by inhibiting initiation of DNA replication [20-22]. Growth factor signaling and environmental cues initiate cell division and ultimately
lead to expression of Cyclin D, which activates CDK4 and CDK6. CDK4/6 activation leads to inactivation of Retinoblastoma protein (Rb) family proteins Rb, p107, and p130 which releases E2F, a transcription factor required for Cyclin E and Cyclin A expression. Cyclin E and Cyclin A, along with the phosphatase Cdc25, activate CDK2. CDK2 activation initiates DNA replication and progression through S-phase. DNA damage prior to S-phase initiation activates the serine/threonine kinases Ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR). Activated ATM/ATR activates Checkpoint kinase 1 and 2 (Chk1/2), as well as the transcription factor p53. Activated Chk1/2 inactivates Cdc25 leading to CDK2 inactivation, while p53 up-regulation increases expression of p21, which inhibits CDK2 and CDK4/6. Thus, DNA damage prevents CDK-mediated initiation of DNA replication and progression from G1 to S-phase by modulating Cyclin levels and post-translational modifications of CDKs. Another example of checkpoint regulation in higher eukaryotes is the G2/M checkpoint, which prevents passage from G2 into mitosis in response to incomplete DNA synthesis or DNA damage [23, 24]. Entry into mitosis requires Cyclin B binding to CDK1 and activation of CDK1 by Cdc25. DNA damage in G2 activates ATM/ATR which leads to Chk1/Chk2 activation. Chk1/2 activation leads to Cdc25 inhibition resulting in CDK1 inactivation. Additionally, DNA damage in G2 activates p53, which down-regulates genes that are essential for entry into and progression through mitosis. Thus, DNA damage during G2 prevents CDK1-mediated mitotic entry by modulating the abundance of Cyclin B and phosphorylation of CDK1. Cell cycle research has only recently focused on the mitotic checkpoint as a major control point in cell cycle regulation and disease prevention [25].
1.3 History of the Mitotic Checkpoint

Early research in yeast describing cell cycle progression had established that the gene product \( cdc2 \), known as Cyclin-dependent kinase-1 (CDK1) in higher organisms, was essential for mitotic entry \([26-28]\). Sustained activity of \( cdc2 \) was accomplished via Cyclin B binding and Cyclin B was known to be degraded upon chromosome segregation by an E3 ubiquitin ligase known as the anaphase promoting complex/cyclosome (APC/C) \([29-31]\). Observations of mitotic metazoan cells indicated that initiation of chromosome segregation correlated with alignment of chromosomes at the metaphase plate and scientists observed that inhibiting spindle assembly with small molecules that disrupt the dynamic instability of microtubules ultimately halted cell cycle progression at mitosis. These observations led to speculation of a checkpoint which served to couple cell cycle progression to chromosomal alignment and spindle assembly. Two seminal papers describing yeast mutants that fail to respond to microtubule disrupting agents and complete mitosis, termed mitotic-arrest deficient (\( mad \)) and budding-uninhibited by benzimidizole (\( bub \)), led to the idea that mitosis is indeed under checkpoint control \([32, 33]\). Additional research in yeast and metazoan cells established that the fidelity of centromeres and kinetochores, the multi-protein complexes that connect chromosomes to the spindle, was essential for proper chromosome segregation \([34-36]\). An elegant study that realized the importance of kinetochores in regulating the mitotic checkpoint showed that laser ablation of the last unattached kinetochore rapidly induced mitotic exit indicating that unattached kinetochores are the primary signal that activates the mitotic
checkpoint[37]. Not surprisingly, subsequent biochemical and cytological evaluation of Mad and Bub gene products indicated that many Mad and Bub proteins associate with kinetochores [38-42]. In particular, the homologous mad2 gene product in *Xenopus laevis*, XMAD2, was described as a dynamic kinetochore associated protein that bound to unattached kinetochores, where XMAD2 localization to kinetochores was reduced upon chromosome alignment, suggesting that Mad2 was at the apex of mitotic checkpoint activation [43]. Subsequently, genetic and biochemical interactions between Mad2 and the APC/C in *Saccharomyces pombe* and *Xenopus laevis* were described, which led to the discovery that Mad2 binds and inhibits the APC/C which prevents degradation of Cyclin B [44, 45]. Further characterization of homologous mad and bub genes in higher eukaryotes eventually led to the general conclusion that unattached kinetochores catalyze the formation of an inhibitory complex known as the Mitotic Checkpoint Complex (MCC), composed of Mad2, Bub-related 1 (BubR1), Bub3 and the APC/C co-activator Cdc20 (Figure 1-2). The MCC effectively inhibits the APC/C, which leads to Cyclin B stabilization and mitotic checkpoint activation via sustained CDK1 activity (Figure1-2). Formation of the MCC ceases only when sister kinetochores bind to microtubules from opposing spindle poles, which allows Cdc20 to activate the APC/C. Consequently, activation of the APC/C leads to Cyclin B degradation, which inactivates CDK1 and induces mitotic exit.
Figure 1-2. The Mitotic Checkpoint

A.) Prior to anaphase initiation, unattached kinetochores generate an inhibitory complex composed of Mad2, BubR1, Bub3, and Cdc20, known as the MCC. The MCC effectively sequesters Cdc20, which is a co-activator of the E3 ubiquitin ligase APC/C. B.) When chromosomes become bi-oriented and kinetochores are stably attached to spindle microtubules the MCC disassembles and Cdc20 is free to activate the APC/C. Active APC/C targets essential mitotic substrates such as Cyclin B and securin for proteasomal degradation ultimately leading to anaphase initiation and mitotic exit.
1.4 Mitotic Checkpoint Activation and Inter-kinetochore Tension.

Because the mitotic checkpoint appeared to ensure proper attachment of kinetochores to spindle microtubules, scientists wondered how improper kinetochore-microtubule attachments were sensed and regulated. For instance, initiating mitotic exit when merotelic (a single sister-kinetochore is bound to the both spindle poles) or syntelic (both sister-kinetochores are bound to microtubules from the same spindle pole) attachments persist often leads to chromosome missegregation and the generation of daughter cells with unequal DNA content, known as aneuploidy [46]. Scientists initially hypothesized that the physical tension that arises from bi-orientation of chromosomes, or sister-kinetochore attachment to opposite spindle poles, regulated the mitotic checkpoint given that bi-orientation is consistent with anaphase initiation and successful cell division [37, 47]. Indeed, application of tension on improperly attached chromosomes leads to anaphase initiation earlier compared to the absence of tension[48]. Additionally, Gorbsky and colleagues observed that increased tension between sister kinetochores and anaphase initiation correlated with dephosphorylation of kinetochore proteins indicating that kinetochore chemistry is sensitive to tension and may link mitotic forces to checkpoint activation [49]. Further characterization of Mad and Bub proteins indicated that their kinetochore localization was regulated differentially in response to either kinetochore-microtubule attachment or sister-kinetochore tension [43, 50-53]. Together, these observations led to speculation that either microtubule occupancy or physical tension induced by microtubule capture negatively-regulated mitotic checkpoint activation. Yet again, observations of cell cycle dynamics in yeast mutants led to a molecular link
between tension and mitotic checkpoint activation. The observation that yeast *cdc6*
mutants, which are allowed to progress into mitosis without replicating their genome,
activate the mitotic checkpoint despite attaching all kinetochores to microtubules was
evidence that lack of tension between sister-kinetochores was the primary activator of the
mitotic checkpoint [54]. Furthermore, the discovery that mutating the gene *Ipl1*, which
had been described as a regulator of microtubule turnover at kinetochores, overcame the
tension-less arrest in *cdc6* mutants suggested that tension was sensed by the *Ipl1* gene
product [55, 56]. All together, these observations led to a more refined model of mitotic
checkpoint regulation where unattached kinetochores initially activate the mitotic
checkpoint and the checkpoint signal is inactivated when microtubules bind kinetochores
(Figure 1-3). However, kinetochore-microtubule interactions that do not result in the
physical separation of sister kinetochores are destabilized resulting in MCC formation
and mitotic checkpoint activation (Figure 1-3). Only when kinetochores capture
microtubules from opposing spindle poles is inter-kinetochore tension high enough to
separate sister kinetochores and silence MCC production (Figure 1-3). Thus, activation of
the mitotic checkpoint is sensitive to kinetochore-microtubule attachment, but also inter-
kinetochore tension. Not surprisingly, the tension-regulated error-correction mechanism
is regulated by a complex of proteins bound to inner centromeres between sister
kinetochores, known as the Chromosomal Passenger Complex (CPC).
Figure 1-3. Inter-kinetochore tension and Error-correction

A.) Kinetochore-microtubule interactions that do not result in high tension between sister kinetochores (inter-kinetochore tension), such as merotelic or syntelic attachments, are erroneous and selectively destabilized, which enables MCC production and activation of the mitotic checkpoint B.) When sister kinetochores attach to microtubules from opposing spindle poles, or bi-orientation, inter-kinetochore tension is high and kinetochore-microtubule interactions are stabilized ultimately silencing MCC production which promotes mitotic exit.
1.5 History of the CPC

The first sub-unit of the CPC was identified by raising antibodies against the bulk proteins of the human mitotic chromosome scaffold. Consequently, scientists described an antibody that recognized two proteins that concentrate at the pericentromere and were provisionally termed inner centromere proteins (INCENPs) [57]. The INCENPs displayed dynamic localization during mitosis; localizing to inner centromeres before chromosome segregation and to the spindle midzone and midbody during cytokinesis and telophase, respectively. This led to the “chromosomal passenger” hypothesis which stated that various mitotic events, such as chromosome segregation and cytokinesis, were regulated by proteins that localize to chromosomes during early mitosis and transfer to the spindle midzone during late mitosis. Functional analysis revealed that INCENP played a role in chromosome congression and segregation [58]. Around the same time, Survivin, a protein that was previously characterized as a regulator of apoptosis, was described as a regulator of mitosis with a similar localization pattern to INCENP [59]. Eventually, scientists discovered the interactions between INCENP, Survivin, and Aurora B, the yeast Ipl1 homologue, which provided a direct link between chromosomal passenger proteins and mitotic checkpoint control [60-62]. Shortly thereafter, a proteomic screen for novel human proteins associated with histone-depleted mitotic chromosomes led to the discovery of Borealin, which interacts with and displays similar localization and functional phenotypes as INCENP, Survivin, and Aurora B [63]. Collectively, INCENP, Survivin, Aurora B, and Borealin are understood as the core-members of the CPC, which is highly conserved among eukaryotes ranging from yeast to human cells and
serves to regulate kinetochore-microtubule interactions and activation of the mitotic checkpoint (Figure 1-4).
Figure 1-4. Structure of the Chromosomal Passenger Complex (CPC)

The CPC is composed of INCENP (yellow), Aurora B (orange), Survivin (green), and Borealin (blue). Complex formation is facilitated by a three helix bundle between the N-terminus of INCENP, the C-terminus of Survivin, and the N-terminus of Borealin. The central region of INCENP contains an HP-1 binding site and a coiled-coil domain which can interact with microtubules. The C-terminus of INCENP contains a highly conserved IN-box motif that mediates the interaction with Aurora B. The C-terminus of INCENP also contains a conserved TSS motif which is phosphorylated by Aurora B and consequently stimulates Aurora B activity. The N-terminus of Survivin contains a conserved Zn$^{2+}$-binding baculoviral inhibitor of apoptosis (BIR) domain. The central region of Borealin contains multiple CDK1-mediated phosphorylation sites and the C-terminus of Borealin contains a conserved dimerization domain.
1.6 Structure of the CPC

A combination of structural and biochemical studies have indicated that the CPC is composed of a kinase module and localization module linked by the central region of INCENP. The C-terminus of Survivin and the N-terminus of Borealin bind the N-terminus of INCENP in a three-helix bundle to facilitate complex formation and mediate targeting of the CPC to various intra-cellular localizations[64, 65]. The kinase module is composed of Aurora B, which binds the highly conserved IN-box of INCENP at the C-terminus.

Aurora B is a member of the Aurora family of kinases that are related to the cAMP-dependent, cGMP-dependent, protein kinase C (AGC) family of serine/threonine kinases and is the enzymatic subunit of the CPC [66, 67]. The family of Aurora kinases is comprised of Aurora A, Aurora, B, and Aurora C. The Aurora family members share a consensus motif ([R/K]x[S/T]Φ) and substrate specificity is achieved by specific distribution patterns within the cell. For instance, Aurora A localizes to the mitotic spindle, centrosomes, and midbody while Aurora B localizes to chromosome arms, inner centromeres, the central spindle, and midbody. Localization of Aurora C is similar to Aurora B; however, Aurora C is largely expressed in germ cells and the morula during embryonic development. Interestingly, the differential localization of Aurora A and Aurora B during mitosis is achieved by a single amino acid difference that dictates binding to different receptors. For example, mutation of glycine 198 to asparagine in Aurora A changes its localization to that of Aurora B and rescues Aurora B depletion [68,
Basal activation of Aurora B is initially mediated by binding the conserved C-terminal IN box of INCENP. Aurora B then phosphorylates INCENP at conserved threonine-serine-serine (TSS) motif and threonine 232 in the activation loop of Aurora B resulting in full activation of Aurora B [70, 71]. Microtubules can also activate Aurora B and microtubule-mediated activation of Aurora B is stimulated by telophase disk protein 60 (TD-60) [72, 73].

INCENP is a large scaffolding protein that facilitates complex formation of the CPC. The N-terminus of INCENP binds to Borealin and Survivin via an N-terminal alpha helix, while the C-terminus bind Aurora B [74-76]. The N- and C-terminus of INCENP are separated by a large, unstructured region which contains a heterochromatin binding protein-1 (HP-1) binding motif and multiple CDK1 phosphorylation sites [58, 77, 78]. CDK1 phosphorylation prior to mitotic exit inhibits the interaction between INCENP and microtubules to prevent association with the spindle midzone until anaphase [79].

Survivin is a well-studied, multi-functional protein that contains an N-terminal Zn$^{2+}$-coordinated baculoviral inhibitor of apoptosis (BIR) domain and a C-terminal alpha helix that interacts with INCENP and Borealin [74-76]. Survivin was originally described as an inhibitor of apoptosis protein (IAP) that was proposed to negatively regulate cell death in mitosis; however, Survivin-null mutants in eukaryotes do not display significant defects in cell death responses [59, 80, 81]. However, Survivin has a nuclear export signal and scientists have proposed that cytoplasmic Survivin may inhibit cell death during interphase while CPC-bound Survivin in the nucleus regulates mitosis [82, 83]. Survivin forms a dimer in vitro; however the dimerization interface is disrupted when Survivin binds Borealin, thus inhibiting homodimer formation when in complex with the
CPC [75, 84, 85]. Survivin is phosphorylated by multiple kinases including Aurora B, CDK1, Plk1, and Casein kinase-2 (CK2) and CK2 phosphorylation might negatively regulate the interaction between Survivin and Borealin [86-88].

Borealin interacts with Survivin and INCENP via two N-terminal alpha helices [74-76]. Interestingly, the yeast homologue of Borealin, Nbl1, is roughly half the size of the vertebrate Borealin proteins; however all Borealin homologues contain the N-terminal helix that mediates triple-helix bundle formation with INCENP and Survivin [64]. The central region of Borealin contains multiple CDK1 phosphorylation sites which have been linked to centromere localization of the CPC [89, 90]. The central region of Borealin also interacts with the ESCRT-III subunit of the Shrb/CHMPC4 which regulates function of the CPC during anaphase [91, 92]. The C-terminus contains a dimerization domain whose structure has been solved and the dimerization domain has been linked to stability of the protein [93, 94]. The dimerization domain of Borealin is phosphorylated by the mitotic kinase Mps1 and mutation of this residue disrupts dimerization in vitro and impairs Aurora B activity and chromosomes alignment in vivo [93, 95]. Borealin is also sumoylated in a RanBP2-dependent manner during early mitosis and the isopeptidase SENP3 catalyzes removal of SUMO2/3 from Borealin [96]. However, the function of sumoylation is unclear given that mutation of all lysines on Borealin does not result in obvious mitotic defects.

1.7 Localization and Function of the CPC

During interphase, the CPC localizes to pericentromeric heterochromatin via INCENP binding to HP-1[57, 97-99]. An INCENP mutant that cannot bind HP-1 does
not localize to heterochromatin; however, expression of this mutant does not lead to mitotic defects. Localization of the CPC to pericentromeres correlates with H3 serine 10 phosphorylation, which reportedly disrupts HP-1 binding to trimethylated lysine 9 (H3K9me3). Disrupting HP-1 binding has been proposed to function as a switch to change from HP-1-mediated recruitment during interphase to mitotic modes of recruitment [99]. Additionally, pH3^{S10} phosphorylation requires POGZ, which removes HP-1 and the CPC from chromosome arms, thus promoting CPC enrichment at centromeres during mitosis [100].

Localization of the CPC is dynamic during mitosis and is an indication of the multiple roles the CPC plays in regulating mitotic progression and cell division. During early mitosis, the CPC is diffusely localized to chromosome arms where Aurora B, along with CDK1, contributes to sister chromatid resolution by phosphorylating the cohesion-stabilizing protein Sororin [101, 102]. Phosphorylated Sororin dissociates from the cohesion sub-unit Pds5, which ultimately results in Wapl-mediated release of cohesion from chromosome arms. As mitosis progresses the CPC concentrates at inner centromeres where the CPC regulates kinetochore-microtubule attachments and activation of the mitotic checkpoint until chromosomes become bi-oriented [64]. An attractive model that explains the role of the CPC in regulating the mitotic checkpoint, known as the spatial gradient model, suggests that Aurora B phosphorylates microtubule-binding kinetochore proteins under low inter-kinetochore tension which leads to dissociation of kinetochore-microtubule interactions (Figure 1-5) [73, 103-105]. As chromosomes become bi-oriented and sister kinetochores are under high tension, Aurora B is spatially separated from the kinetochore and can no longer destabilize kinetochore-
microtubule interactions. Indeed, sister chromosomes that have not bi-oriented display relatively unstable kinetochore microtubule interactions, compared to bi-oriented chromosomes, which requires Aurora B kinase activity [56, 104]. Additionally, the core microtubule-binding kinetochore proteins of the KMN network, which consists of the Knl1 complex, the Mis12 complex, and the Ndc80/Hec1 complex, have been identified as Aurora B substrates and Aurora B-mediated phosphorylation reduces their affinity for microtubules in vitro [105-107]. Moreover, an Aurora B FRET-sensor detects a gradient of Aurora B activity that is highest near inner centromeres (Figure 1-5)[103, 108]. Thus, according to this spatial gradient model of CPC-mediated checkpoint activation, inner centromere localization of the CPC is paramount for regulating error correction and mitotic checkpoint activation [103]. Localization of the CPC to inner centromeres during mitosis is common to yeast, zebra fish, frog, chicken, mouse and human cells suggesting that centromere accumulation is essential for the function of the CPC. However, scientists recently discovered that yeast cells expressing an INCENP mutant (Sli15ΔN) that does not bind Survivin, which effectively bypasses the centromere localization module of the CPC and fails to localize Aurora B to centromeres, are viable and can support accurate chromosome segregation [109]. This discovery is a direct challenge to the spatial-gradient model of CPC-mediated mitotic checkpoint activation; however, whether or not this applies to higher organisms remains to be determined. Expression of an INCENP mutant that cannot bind Survivin in chicken and human cells does not rescue Aurora B activity or a mitotic arrest induced by the microtubule-stabilizing drug taxol and expression of an INCENP mutant that cannot bind Survivin in human cells shows the same phenotype [80, 110, 111]. Together, this suggests that inner centromere localization
of the CPC is essential for mammalian cells and the viability of yeast cells expressing Sli15ΔN might indeed be specific for yeast.

The CPC translocates to the spindle midzone after inactivation of the mitotic checkpoint to stabilize the plane of cleavage furrow formation and to regulate constriction of the cleavage furrow [112]. The CPC translocates to the midzone after dephosphorylation of the central region of INCENP, which enables INCENP binding to midzone microtubules [79]. Localization of the CPC to the spindle midzone also depends on mitotic kinesin-like protein-1 (MKLP2), a kinesin-6 family member that also binds microtubules [113-115]. At the spindle midzone, the CPC recruits the Centralspindlin complex, which is composed of mitotic kinesin-like protein-1 (MKLP1) and male germ cell Rac-GTPase activating protein (MgcRacGAP) [62]. When bound to the Centralspindlin complex, Aurora B phosphorylates MgcRacGAP which induces RhoGAP activity [116]. CPC recruitment of the Centralspindlin complex also leads to recruitment of the Rho-guanine nucleotide exchange factor epithelial cell transforming gene (ECT2). Recruitment of ECT2 and activation of MgcRacGAP ultimately leads to constriction of the cleavage furrow by activation of RhoA, which is a small GTPase that promotes actin polymerization and myosin II activation [117-119].
Figure 1-5. Spatial-gradient model of CPC-mediated mitotic checkpoint activation

A.) Under low inter-kinetochore tension, the CPC is positioned in close proximity to kinetochores where Aurora B can phosphorylate microtubule-binding kinetochore proteins and reduce their affinity for microtubules. The reduced affinity for microtubules results in dissociation of erroneous kinetochore-microtubule interactions and production of the MCC which activates the mitotic checkpoint. B.) When sister kinetochores come under high tension from bi-orientation, kinetochores are physically separated from Aurora B at centromeres and become dephosphorylated, which stabilizes the kinetochore-microtubule interactions. C.) An Aurora B FRET-sensor placed at centromeres and various locations along the kinetochore reveals a gradient of Aurora B activity along kinetochores. Under low tension, a gradual gradient of Aurora B activity emanates from the inner centromere along the kinetochore axis. However, under high tension the Aurora B phosphorylation gradient is steep.
1.8 The Two-histone Model of CPC Localization to Centromeres

Inner centromere localization of the CPC is essential for CPC-mediated mitotic checkpoint activation according to the spatial gradient model. Localization of the CPC to centromeres reportedly intersects with two histone modifications that overlap at inner centromeres: phosphorylated histone H3 at threonine 3 (pH3\textsuperscript{T3}) and phosphorylated histone 2A at threonine 120 (pH2A\textsuperscript{T120}) (Figure 1-6) [120-123]. Haspin kinase generates pH3\textsuperscript{T3}, which is in turn bound by Survivin [120-122]. The BIR domain of Survivin directly interacts with pH3\textsuperscript{T3} to recruit the CPC and this interaction can be regulated by pH, suggesting that the \textit{in vivo} interaction could be regulated by the local environment [124]. The CPC engages in positive feedback to maintain pH3\textsuperscript{T3} levels during mitosis. For instance, Aurora B directly phosphorylates Haspin at multiple residues during mitosis and mutation of these residues impairs pH3\textsuperscript{T3} [125]. Consistent with this observation, inhibiting Aurora B during mitosis leads to a reduction in pH3\textsuperscript{T3} levels. However, the mechanism of Aurora B-mediated regulation of Haspin activity is unknown because mutation of Aurora B phosphorylation sites does not reduce intrinsic kinase activity of Haspin. Aurora B phosphorylation might regulate the release of a Haspin inhibitor or facilitate substrate binding [125]. Dephosphorylation of pH3\textsuperscript{T3} is mediated by the phosphatase PP1γ-Repo-Man complex and Aurora B-mediated phosphorylation negatively regulates PP1γ-Repo-Man activity towards pH3\textsuperscript{T3} [126]. Thus, Aurora B positively regulates pH3\textsuperscript{T3} abundance by promoting Haspin kinase activity toward H3 and negatively regulating removal of pH3\textsuperscript{T3} by phosphorylating PP1γ-Repo-Man.
The second histone mark that dictates CPC-localization to centromeres is generated by the kinetochore-localized kinase Bub1. Kinetochore-bound Bub1 generates pH2A\textsuperscript{T120} at pericentromeric chromatin, which recruits the Shugoshin proteins (Sgo1/2) [122, 125]. Sgo1/2 can bind CDK1-phosphorylated Survivin in yeast and Borealin in humans, which may explain why centromere accumulation of the CPC begins in late prophase when CDK1 activity is highest in the disassembled nucleus [90, 122, 123]. Interestingly, the BIR domain of Survivin can bind the N-terminus of human Sgo1 \textit{in vitro}, suggesting crosstalk between the two-histone pathways [124]. The CPC also indirectly engages in feedback with Bub1 to generate pH2A\textsuperscript{T120}. For example, Aurora B kinase activity is essential for kinetochore targeting and activation of the mitotic kinase Monopolar Spindle 1 (Mps1) [127-132]. Mps1 phosphorylates the kinetochore protein Knl1 at Met-Glu-Leu-Thr (MELT) motifs, which recruits Bub1 [133-140]. In turn, Bub1 generates pH2A\textsuperscript{T120} to recruit Sgo1/2 and consequently the CPC via the Borealin-Sgo1/2 interaction. Additionally, Aurora B phosphorylates ATM which enhances the kinase activity of Bub1 [141].

Whether or not the CPC contacts both modified histones in the same complex has not been formally demonstrated. The role of both pH3\textsuperscript{T3} and pH2A\textsuperscript{T120} in mediating CPC localization to centromeres is controversial; however, given recent reports that Aurora B localization is unaffected when Mps1 is inhibited resulting in reduced pH2A\textsuperscript{T120} and when Aurora B is inhibited resulting in reduced pH3\textsuperscript{T3} [130]. Nevertheless, the CPC concentrates at inner centromeres where pH3\textsuperscript{T3} and pH2A\textsuperscript{T120} overlap and the prevailing model suggests that Aurora B enhances CPC localization to centromeres by engaging in feedback with Haspin and Bub1 to positively regulate pH3\textsuperscript{T3} and pH2A\textsuperscript{T120} abundance.
The work presented in this dissertation expands the two-histone model of CPC localization and provides additional insight into the molecular determinants of centromere localization of the CPC.
**Figure 1-6. Two-Histone Model of CPC localization to centromeres**

The CPC engages feedback with Bub1 and Haspin to generate two histone modifications that mediate CPC localization to inner centromeres: Bub1 generates pH2A\textsuperscript{T120} which recruits Sgo1 and binds the phosphorylated central region of Borealin while Haspin generates pH3\textsuperscript{T3} which binds the BIR domain of Survivin.
1.9 Hypothesis

In the present study we investigate molecular determinants of CPC localization to centromeres and assess whether or not centromere localization of the CPC is critical for mitotic checkpoint activation. In particular, we investigate the contribution of the C-terminus of Borealin in regulating inner centromere localization and function of the CPC. We hypothesize that the C-terminus of Borealin contains a previously uncharacterized centromere targeting domain to facilitate stable association of the CPC at inner centromeres. We also predict that this unknown C-terminal centromere targeting domain is essential for CPC-mediated mitotic checkpoint activation and chromosome segregation. Additionally, we explore the role of both pH3T3 and pH2AT120 in regulating CPC localization and function at inner centromeres. We hypothesize that both pH2AT120 and pH3T3 are essential for inner centromere localization and dynamics of the CPC and that inner centromere localization of the CPC is essential for mitotic-checkpoint activation. Finally, we explore the possibility of direct regulation of Bub1 by Aurora B and hypothesize that Aurora B-mediated phosphorylation regulates Bub1 kinase activity and localization to kinetochores.
Chapter 2

Structure-Function Analysis of Borealin

2.1 DNA binding of Borealin does not correlate with Centromere Localization

The N-terminus of Borealin binds INCENP and Survivin to form a triple-helical bundle and Borealin can recruit Survivin and INCENP to DNA in vitro [75, 76]. We initially hypothesized that the C-terminus of Borealin contained a DNA-binding motif that mediated centromere localization of the CPC. Therefore, we performed DNA-cellulose pull-downs from lysates isolated from taxol-arrested HeLa M cells expressing FLAG-tagged C-terminal truncations to determine what region of Borealin binds DNA. Deleting amino acids 169-280 had no apparent effect on the ability of Borealin to bind DNA (Figure 2-1A&B). However, we observed that deleting amino acids 142-168 of Borealin resulted in ~5-fold decrease in DNA binding affinity compared to longer truncations and full-length Borealin based on Western blotting of DNA-cellulose isolates (Figure 2-1A&B). The calculated net charge of amino acids 142-168 of Borealin is positive (pI ~9.4) at physiological pH (7.4), which is consistent with the ability to bind negatively charged DNA (Figure 2-2A). Amino acids 142-168 of Borealin also contains multiple, conserved lysine and serine residues, which are known to be post-translationally modified by sumoylation and phosphorylation, respectively and these modifications are
known to impair DNA binding [142, 143]. Preliminary wide-field microscopy of
Borealin-FLAG truncations in HeLa M cells, including truncations with a reduced ability
to bind DNA, revealed punctate foci similar to full-length Borealin; however all
Borealin-FLAG truncations appeared to mislocalize INCENP on the whole cell level
(Figure 2-3). Together these observations suggest that the ability of Borealin to bind
DNA does not necessarily correlate with inner centromere localization of Borealin;
however the C-terminus might play a role in maintaining INCENP levels at centromeres.
Figure 2-1. Amino acids 142-168 mediate the interaction between Borealin and DNA
A.) HeLa M cells were transiently transfected with the indicated Borealin-FLAG constructs. Subsequently, cell lysates were subject to DNA-cellulose binding assays and pull-down. “Mock” indicates mock transfected cells. Pull downs were separated via SDS-PAGE and a western blot using anti-FLAG was performed to determine the presence of Borealin-FLAG mutant constructs. B) Quantification of the DNA-cellulose pull-down western blot via densitometric analysis using ImageJ software. Percent bound was calculated as a function of input band intensity.
Figure 2-2. Amino acids 142-168 of human Borealin are net positively charged and harbor post-translational modifications

A.) Predictive protein sequence information of amino acids 142-168 of human Borealin calculated using the Scripps Institute Protein Calculator. B.) Schematic representation of the Borealin primary sequence. Amino acids 142-168 were subject to a multiple sequence alignment using ClustalW2 software. Amino acids harboring known and putative post-translational modifications that potentially regulate DNA interaction are highlighted.
Figure 2-3. Preliminary wide-field fluorescence imaging of Borealin C-terminal truncations

HeLa M cells were transiently transfected with the indicated Borealin-FLAG constructs. INCENP (green), FLAG (red), and DNA (blue) were assessed and analyzed via immunofluorescence microscopy. “Control” indicates mock transfected cells.
2.2 Amino acids 1-110 of Borealin are sufficient for Inner Centromere Localization while the C-terminus of Borealin is required for stable binding.

To obtain better resolution of Borealin truncations at inner centromeres, confocal immunofluorescence microscopy was performed on taxol-arrested HeLa M cells expressing FLAG-tagged Borealin truncations. Full-length Borealin localized to inner centromeres between Hec1 stained kinetochores with little to no detectable cytoplasmic staining (Figure 2-4A&B). Similarly, both Borealin1-221 and Borealin1-110 displayed punctate foci between Hec1 stained kinetochores; however, truncated forms of Borealin showed elevated cytoplasmic staining compared to full-length Borealin (Figure 2-4A&B). We also performed immunofluorescence for the CPC subunit INCENP to assess co-localization of Borealin truncations with the CPC. Interestingly, the level of INCENP on chromosomes was reduced by ~60% in cells overexpressing either Borealin1-221 or Borealin1-110 compared to full-length Borealin (Figure 2-5A&B). To better understand the elevated cytoplasmic staining of Borealin truncations and the reduced INCENP levels upon truncation overexpression, we measured centromere binding dynamics of GFP-tagged Borealin in nocodazole-arrested mitotic cells. Full-length Borealin-GFP recovered to ~40% of pre-bleach levels within 60 seconds with a rate-constant of ~0.020 sec\(^{-1}\) (Figure 2-6A-C). Borealin1-110 recovered to ~90% of pre-bleach levels with a rate-constant of approximately 0.068 sec\(^{-1}\) (Figure 2-6A-C). Similarly, Borealin1-221 recovered to ~90% of pre-bleach levels with a rate-constant of 0.056 sec\(^{-1}\) (Figure 2-6A-C). A Borealin mutant in which its dimerization domain was fused to amino acids 1-110 (Borealin110+D.D.) recovered to ~90% of pre-bleach levels with a rate-constant of 0.054 sec\(^{-1}\) (Figure 2-6A-C). Together, these observations suggest that although the N–
terminus of Borealin is sufficient for localization to inner centromeres, the C-terminus suppresses dynamic exchange to maintain stable CPC binding at this location. Furthermore, because Borealin_{1-221} and Borealin_{1-110} behave similarly, the N-terminus likely plays a dominant role in localization under these conditions.
Figure 2-4. Amino acids 1-110 of Borealin are sufficient for localization to inner centromeres

A.) Maximum projections of taxol-arrested HeLa M cells after transient transfection with the indicated FLAG-tagged Borealin truncations. Immunofluorescence was performed using antibodies against FLAG (red) and Hec1 (green), as a kinetochore marker. B.) Close-up images and line scans of individual sister kinetochores/centromeres from a single plane are to the right.
Figure 2-5. Expression of Borealin C-terminal truncations reduces INCENP levels at centromeres

A.) Maximum projections from cells treated as in A except that immunofluorescence was performed using antibodies against FLAG and INCENP. B.) Quantification of the relative level of INCENP intensity in DNA area of maximum projections. Greater than 30 cells were quantified. Bars represent standard deviation.
Figure 2-6. FRAP analysis of Borealin C-terminal truncations
A.) Representative still images of GFP foci from FRAP experiments in nocodazole-arrested HeLa M cells after transient transfection with indicated GFP-tagged Borealin truncations. B.) Post-recovery kinetics of GFP-tagged Borealin constructs measured by FRAP. More than eight centromeres per condition were averaged. Bars represent standard deviation. C) The average time-constant values of FRAP curves from B (see methods).
2.3 The C-terminus of Borealin is required for CPC function

Defects in CPC function can lead to errors in chromosome segregation and cell division, ultimately resulting in polyploidization [144]. Because all Borealin truncations could be detected at centromeres, we tested whether these mutants retained CPC function during mitosis. Cells were transfected with Borealin shRNA along with shRNA-resistant Borealin C-terminal truncations and analyzed by western blotting to confirm knockdown and by flow cytometry to determine DNA content. As expected, Borealin shRNA reduced Borealin protein levels below a detectable level and Borealin truncation mutants migrated at their predicted molecular weights (Figure 2-7A&B). Borealin shRNA alone increased the percentage of polyploid cells to ~13% compared to ~1% in control cells, indicating impaired CPC function (Figure 2-8A). Expression of full-length Borealin reduced the incidence of polyploidy to ~3.5%, while expression of any Borealin truncation partially rescued the incidence of polyploidy to ~8% (Figure 2-8A). To determine whether the persistence of polyploidy in cells expressing Borealin truncations was due to errors in mitotic progression we measured the frequency of mitotic phases in cells expressing Borealin shRNA, Borealin truncations, and H2BGFP as a screenable marker. Borealin shRNA alone increased the percentage of prometaphase cells to ~95% compared to ~20% in control cells, indicating impaired mitotic progression and defective CPC function (Figure 2-8B). Co-expression of full-length Borealin with Borealin shRNA reduced the percentage of prometaphase cells to 60% (Figure 2-8B). Co-expression of any Borealin C-terminal truncation with Borealin shRNA only partially rescued the incidence of prometaphase cells to ~75% (Figure 2-8B). Therefore, the partial function observed with the truncations we have analyzed maps mainly to the N-terminus rather than the central
region. Also, the extreme C-terminal region containing the dimerization domain is essential for full binding and function of the CPC at centromeres.
Figure 2-7. Western blot analysis of Borealin knockdown and Borealin truncation protein expression
A.) Western blot showing level of Borealin knockdown in HeLa M cells after transient transfection with pBabePURO, as a selection marker, and either empty pSUPER (Control) or pSUPER-Borealin shRNA. Antibodies against endogenous Borealin and Actin were used. B.) Cells were treated as in A except plasmids encoding FLAG-tagged Borealin truncations were included. Antibodies against FLAG and Actin were used.
Figure 2-8. Borealin truncations partially restore endogenous Borealin knockdown

A.) The percentage of cells with greater than 4N DNA content (Polyploid) in cells treated as in Figure 2-7 except cells were fixed and analyzed by flow cytometry after selection.

B.) The frequency of mitotic phases in H2BGFP expressing HeLa M cells after co-transfecting plasmids encoding H2BGFP, Borealin shRNA, and Borealin truncations. Three days post-transfection cells were visualized by fluorescence and phase contrast microscopy. Bars from A and B represent standard error from three independent experiments.
2.4 Borealin Dimerization regulates CPC abundance at Centromeres

Despite localizing to centromeres, Borealin\textsubscript{1-221} exchanges rapidly which likely reduces steady state levels of centromere INCENP (Figure 2-4). Borealin contains a dimerization domain from amino acids 207 to 280 whose structure has been solved [93]. Although a dimerization-defective Borealin mutant reduces pCENP-A\textsuperscript{Ser7} levels, the role of dimerization in regulating CPC localization is unknown [76, 93]. Therefore, we created FKBP fusions using Borealin\textsubscript{1-221} or Borealin\textsubscript{1-110} where dimerization of FKBP fusion proteins can be induced using the small molecule AP20187 [145]. Borealin-FKBP fusion proteins were expressed in taxol-arrested HeLa cells, which were then cultured in the presence or absence of the homo-dimerizing agent AP20187. Borealin-FKBP fusions migrated to the predicted molecular weight, as determined by western blotting, and adding AP20187 to HeLa M cells expressing the Borealin-FKBP fusions did not alter the total levels of the fusion proteins (Figure 2-9). Immunofluorescence was performed to assess localization of HA-tagged Borealin-FKBP fusions and Survivin as a measure of CPC localization. Similar to FLAG-tagged Borealin truncations, expression of Borealin\textsubscript{1-221}-FKBP-HA or Borealin\textsubscript{1-110}-FKBP-HA reduced Survivin levels in the absence of AP20187 (Figure 2-10A&B). Under these conditions, undimerized Borealin\textsubscript{1-110}-FKBP-HA was more difficult to detect at centromeres compared to Borealin\textsubscript{1-221}-FKBP-HA (Figure 2-10A&B). Inducing dimerization before or after mitotic entry increased centromere localization of both Borealin\textsubscript{1-221}-FKBP-HA and Borealin\textsubscript{1-110}-FKBP-HA compared to the undimerized proteins (Figure 2-10A&B). Additionally, inducing dimerization rescued centromeric Survivin localization in both mutants (Figure 2-
10A&B). Borealin localization and Survivin rescue was more efficient using dimerized Borealin\textsubscript{1-221} versus Borealin\textsubscript{1-110}. Therefore, CPC dimerization enhances its localization to the centromere, while the central region of Borealin is required to maximize CPC at the centromere.
Figure 2-9. Expression of Borealin truncation FKBP fusion proteins
The indicated Borealin-FKBP fusions were expressed in HeLa M cells. After AP20187 treatment cells were harvested and lysates were separated by SDS-PAGE. Proteins were transferred to PVDF membrane and HA-tagged FKBP fusions were detected using an antibody to HA. Actin was probed for as a loading control.
Figure 2-10. Borealin dimerization regulates CPC abundance at centromeres

A.) Schematic of experimental design and maximum intensity projections of taxol-arrested HeLa M cells expressing HA-tagged Borealin-FKBP fusion proteins in the absence (-) or presence (+PRE, +POST) of the homo-dimerizing agent AP20187. Immunofluorescence was performed using antibodies against HA (red) and Survivin (green). B.) Quantitation of Survivin signal intensity standard deviation from A. Bars represent standard deviation. Asterisk indicates $p<0.05$ from a type-two, two-tailed Student’s t-test relative to untransfected cells (control).
Chapter 3

Analysis of the Two-histone Model of CPC localization

3.1 Inner centromere localization of the CPC is regulated by Haspin kinase activity

CPC localization to centromeres overlaps with two histone modifications, pH3$^{T3}$ and pH2A$^{T120}$, which in turn are regulated by Aurora B, Mps1, Bub1, and Haspin [121, 122, 125]. Interestingly, centromere localization of Aurora B was not affected in cells exposed to reversine, a potent inhibitor of Mps1, when cells were previously arrested in mitosis with nocodazole [129]. Furthermore, Borealin truncations lacking the central region that binds Sgo1/2 share similar phenotypes in all most assays presented thus far.

To further investigate the role of pH2A$^{T120}$ and pH3$^{T3}$ in CPC localization we used Aurora B, Mps1, or Haspin kinase inhibitors to correlate pH2A$^{T120}$ and pH3$^{T3}$ levels with CPC localization. Both pH3$^{T3}$ and pH2A$^{T120}$ are abundant in nocodazole-arrested cells (Figure 3-1A&B). Compared to control cells, inhibiting Aurora B with the small molecule ZM447439 (ZM) reduced pH3$^{T3}$ levels by ~70% and pH2A$^{T120}$ by ~90% indicating that Aurora B kinase activity is indeed required for maintaining both histone marks during mitosis (Figure 3-1A&B). Inhibiting Mps1 with the small molecule Reversine (Rev) had no effect on pH3$^{T3}$ abundance and reduced pH2A$^{T120}$ by ~90% indicating that
Mps1 kinase activity plays a major role in maintaining pH2A$^{T120}$, but not pH3$^{T3}$ (Figure 3-1A&B). Finally, inhibiting Haspin kinase with the small molecule 5-Iodotubercine (5Itu) reduced pH3$^{T3}$ by ~95% and pH2A$^{T120}$ by ~75% indicating that Haspin kinase activity is essential for maintaining both histone modifications (Figure 3-1A&B). Borealin, Aurora B, and Survivin remained localized to centromeres after inhibiting Aurora B with ZM447439 despite a 90% reduction of pH2A$^{T120}$ levels and ~70% reduction in pH3$^{T3}$ levels (Figure 3-2A-E, Fig. 17). Inhibiting Mps1 with Reversine also had no apparent effect on inner centromere localization of the CPC despite a ~90% reduction in pH2A$^{T120}$ with no significant change in pH3$^{T3}$ levels (Figure 3-2A-E, Figure 3-1). On the other hand, inhibiting Haspin with 5Itu reduced the amount of each CPC subunit at inner centromeres by ~60% (Figure 3-2A-E, Figure 3-1). Interestingly, inhibiting Haspin kinase revealed a sub-population of the CPC that was detected near kinetochores in two-distinct pools as indicated by detailed confocal imaging (Figure 3-2A-D). To confirm that kinase inhibition did not affect the protein abundance of the CPC, particularly in 5Itu, we assessed CPC levels by Western blotting. Inhibiting Aurora B, Mps1, or Haspin kinase had no apparent effect on CPC protein levels (Figure 3-3A). To exclude the idea that mislocalization of the CPC after inhibiting Haspin was a result of the inability for the CPC to form a complex, Borealin immunoprecipitation was performed in the presence of 5Itu and western blotting performed to confirm the presence of Survivin. Indeed, the interaction between Borealin and Survivin was retained in 5Itu (Figure 3-3B).

The kinetochore-proximal pool of the CPC revealed after Haspin inhibition is similar to the recently reported localization pattern of the pH2A$^{T120}$ [122, 146]. Therefore, we assessed the levels and distribution of pH2A$^{T120}$ after inhibiting Aurora B,
Mps1, or Haspin to determine if this is also true in HeLa M cells. In control cells, pH2AT120 displayed a two-peak bow-tie pattern that emanated from kinetochores, which is consistent with previous studies (Figure 3-4A)[146]. As expected from our histone westerns, inhibiting Aurora B and Mps1 significantly reduced the two pH2AT120 peaks, while inhibiting Haspin had a less severe effect (Figure 3-4A). Immunofluorescence was also performed in nocodazole-arrested A8GFP#24 cells to assess both pH2AT120 and Borealin-GFP localization in the same cells after inhibiting Aurora B, Mps1 and Haspin. A8GFP#24 cells were used due to the availability of monoclonal antibodies to GFP, but not Borealin. As expected, Borealin-GFP localized between sister kinetochores despite a major reduction in pH2AT120 when Aurora B or Mps1 was inhibited (Figure 3-4B). Borealin-GFP at inner centromeres was significantly reduced after inhibiting Haspin and the residual pool of Borealin formed two peaks which co-localized with pH2AT120 (Figure 3-4B). These results suggest that, although inner centromere localization appears to be independent of pH2AT120, pH2AT120 may regulate a kinetochore-proximal pool of the CPC independent of the Haspin-regulated inner centromere pool.

To determine whether pH2AT120 and/or pH3T3 played a more subtle role in CPC localization, FRAP was performed in nocodazole-arrested cells stably expressing full-length Borealin-GFP (A8GFP#24) in the presence or absence of Aurora B, Mps1, or Haspin inhibitors. Inhibiting Aurora B, Mps1, or Haspin kinase had a similar effect in A8GFP#24 cells compared to HeLa M cells (Figure 3-5A). As expected from previous studies, Borealin-GFP exchanged slowly at centromeres in control cells (DMSO), recovering to ~40% of pre-bleach levels within a 60 second time-window and a rate constant of 0.02 sec⁻¹ (Figure 3-5B-D) [88]. Inhibiting Aurora B with ZM or Mps1 with
Reversine did not significantly change the kinetics of Borealin-GFP recovery at centromeres (Fig. 3-5B-D). On the other hand, inhibiting Haspin with 5Itu significantly increased the post-bleach recovery kinetics of Borealin-GFP, which recovered ~85% of pre-bleach levels with a rate-constant of 0.05 sec\(^{-1}\) in a 60 second time-window (Figure 3-5B-D). These results suggest that stable association of Borealin with inner centromeres is regulated by Haspin kinase activity, but relatively unaffected by Aurora B or Mps1 inhibitors when cells have entered mitosis.
Figure 3-1. Analysis of pH2A\textsuperscript{T120} and pH3\textsuperscript{T3} after Aurora B, Mps1, or Haspin inhibition

A.) Western blot analysis of histone extractions from nocodazole-arrested HeLa M cells following treatment with MG132 (to prevent mitotic block) and ZM447439 (ZM), reversine (Rev), or 5Iodotubericine (5Itu). Asynchronously growing cells were used as a negative control (Asynch). Antibodies against pH2A\textsuperscript{T120} and pH3\textsuperscript{T3} were used. Ponceau S staining served as loading control. B.) Quantification of band intensities from three independent western blots prepared as in A. Bars represent standard error.
Figure 3-2. Analysis of CPC localization after inhibiting Aurora B, Mps1, or Haspin
A-C.) Maximum projections of HeLa M cells treated as in A. Immunofluorescence was performed using antibodies to either Borealin, Aurora B, or Survivin and Hec1. Hoechst was used to visualize DNA. Close-up images and line scans of individual sister kinetochores/centromeres from a single plane are to the right. D.) Quantification of the relative level of Borealin or Aurora B intensity in DNA area of max projections from A and B, respectively. Greater than 30 cells were quantified. Bars represent standard deviation.
Figure 3-3. Analysis of CPC protein levels and complex formation after treatment with kinase inhibitors  

A.) Western blot analysis of INCENP, Borealin, and Survivin from nocodazole-arrested HeLa M cells following treatment with MG132 (to prevent mitotic block) and ZM447439 (ZM), reversine (Rev), or 5Iodotubericine (5Itu). Actin was probed as a loading control.  

B.) Western blot of Borealin immunoprecipitates from cells treated as in A except only 5Itu was used.

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**Figure 3-3. Analysis of CPC protein levels and complex formation after treatment with kinase inhibitors**

A.) Western blot analysis of INCENP, Borealin, and Survivin from nocodazole-arrested HeLa M cells following treatment with MG132 (to prevent mitotic block) and ZM447439 (ZM), reversine (Rev), or 5Iodotubericine (5Itu). Actin was probed as a loading control.  

B.) Western blot of Borealin immunoprecipitates from cells treated as in A except only 5Itu was used.
Figure 3-4. Analysis of pH2A<sup>T120</sup> distribution after inhibiting Aurora B, Mps1, or Haspin

A.) Maximum projections of HeLa M cells treated as in Figure 18. Immunofluorescence was performed using antibodies to pH2A<sup>T120</sup> (red) and Hec1 (green). Hoechst was used to visualize DNA. Close-up images and line scans of individual sister kinetochores/centromeres from a single plane are to the right.

B.) A8GFP#24 cells stably expressing Borealin-GFP were treated as in A. Immunofluorescence was performed using antibodies against GFP (green) and pH2A<sup>T120</sup> (red). Close-up images and line scans of individual sister kinetochores/centromeres from a single plane are to the right.
Figure 3-5. FRAP analysis of Borealin-GFP after inhibiting Aurora B, Mps1, or Haspin

A.) Western blot analysis of histone extractions from nocodazole-arrested A8GFP#24 cells following treatment with MG132 (to prevent mitotic block) and ZM447439 (ZM), reversine (Rev), or 5Iodotubericine (5Itu). B) Representative still images of single centromeres from A8GFP#24 cells stably expressing Borealin-GFP treated as in A. C.) Post-recovery kinetics of Borealin-GFP under the indicated conditions. More than eight centromeres per condition were averaged in a single experiment. Bars represent standard deviation. D.) The average time-constant values of FRAP curves from G (see methods)
3.2 Haspin inhibition reveals a kinetochore-proximal pool of CPC

Previous studies have indicated that the CPC is reduced upon Aurora B or Mps1 kinase inhibition, while others have not shown such an effect [125, 130, 147]. A major difference between these observations is whether Aurora B or Mps1 were inhibited before or after mitotic entry. Thus, we directly compared Borealin localization in cells exposed to Aurora B, Mps1, or Haspin inhibitors either before or after mitotic entry. As before, inhibiting Haspin kinase after mitotic entry (+POST) mislocalized Borealin from inner centromeres, while inhibiting Aurora B or Mps1 had no effect (Figure 3-6). On the other hand, inhibiting either Aurora B or Mps1 prior to mitotic entry (+PRE) led to increased chromosome arm staining of Borealin and lower, but detectable levels of Borealin at inner centromeres (Figure 3-6). As mentioned previously, punctate staining of Borealin was also observed near kinetochores in 5Itu-treated cells (Figure 3-6). Careful analysis of control cells also revealed low levels of Borealin near kinetochores, although this pool is difficult to discern due to high levels of inner centromere staining (Figure 3-6; DMSO). Together these data suggests that Haspin kinase is a master regulator of CPC inner centromere localization, while Aurora B and Mps1 merely regulate maximal accumulation of the CPC at inner centromeres, presumably during early mitosis, but not after cells have been arrested in mitosis for prolonged periods.
Figure 3-6. Effects of Aurora B, Mps1, and Haspin inhibitors added before or after mitosis
Schematic of experimental design and maximum projection images of nocodazole-arrested HeLa M cells treated with the indicated inhibitors either before (+PRE) or after (+POST) mitotic entry. Immunofluorescence was performed using antibodies against endogenous Borealin and Hec1. Close-up images and line scans of individual sister kinetochores/centromeres from a single plane are at the bottom.
Chapter 4

Analysis of Bub1-mediated localization of the CPC near kinetochores

4.1 Bub1 regulates the kinetochore-proximal pool of the CPC

Aurora B and Mps1 co-regulate Bub1 localization to kinetochores [128, 129, 148]. Inhibiting Haspin mislocalizes the CPC from inner centromeres, which may be responsible for the partial reduction in pH2A$^{T120}$ (Figures 3-1, 3-2, 3-4). Mislocalization of the inner centromere pool of the CPC also revealed a kinetochore-proximal pool of the CPC that co-localizes with pH2A$^{T120}$ (Figure 3-4). To further test the role of pH2A$^{T120}$ in targeting CPC to a kinetochore-proximal location, we constitutively tethered Bub1 to the kinetochore by expressing a Mis12-Bub1 fusion. In this manner, the feedback loop responsible for Bub1 retention would be bypassed allowing Bub1 and pH2A$^{T120}$ to persist despite reducing Aurora B at the centromere after inhibiting Haspin. As a control, cells were transfected with a GFP-Bub1 fusion. HeLa M cells were transfected with Bub1 constructs, blocked in mitosis with taxol and analyzed by immunofluorescence. Expression of neither GFP-Bub1 nor LAP-Mis12-Bub1 rescued inner centromere localization of the CPC after Haspin inhibition (Figure 4-1A&B). However, the residual kinetochore-proximal pool of Borealin was increased in cells expressing LAP-Mis12-Bub1 after inhibiting Haspin (Figure 4-1A&B). Thus, Bub1 and possibly pH2A$^{T120}$...
recruit Borealin to this kinetochore-proximal location.

Because expression of Mis12-Bub1 increased levels of Borealin near kinetochores, we predicted that increasing levels of the CPC near kinetochore would influence mitotic timing owing to the kinetochore-microtubule destabilizing activity of Aurora B. Therefore, we monitored the length of mitosis in cells expressing GFP-Bub1 and cells expressing LAP-Mis12-Bub1. Compared to untransfected cells which spent ~40 minutes in mitosis, cells expressing GFP-Bub1 increased the length of mitosis to ~50 minutes and cells expressing LAP-Mis12-Bub1 increased the length of mitosis to ~60 minutes (Figure 4-2). Thus, tethering Bub1 to kinetochores increases the length of mitosis, presumably by increasing CPC levels near kinetochores.
Figure 4-1. Tethering Bub1 to kinetochores increases Borealin abundance after inhibiting Haspin

A.) Maximum projections of taxol-arrested HeLa M cells expressing either GFP-Bub1 or LAP-Mis12-Bub1 following exposure to 5Itu for 90 minutes. B) Quantification of Borealin and GFP signal intensity at individual sister kinetochores/centromeres in 5Itu-treated cells expressing either GFP-Bub1 or LAP-Mis12Bub1 as in A. Signal intensities from 30 sister kinetochores/centromeres from 6 cells were quantified. Bars represent standard deviation.
Figure 4-2. Tethering Bub1 to kinetochores lengthens the duration of mitosis
Cells expressing GFP-Bub1 or LAP-Mis12-Bub1 were monitored by time-lapse phase and fluorescence microscopy. The duration of mitosis was measured by phase contrast visualization of nuclear envelope breakdown (NEB) to anaphase onset as determined by segregation of metaphase chromosomes to opposing spindle poles.
4.2 Borealin dimerization is essential for localization near the kinetochore

Depletion of pH3\textsuperscript{T3} by inhibiting Haspin revealed a kinetochore proximal pool of Borealin that co-localizes pH2A\textsuperscript{T120} and is regulated by Bub1. To investigate the mode of binding we tested the effect of inducible dimerization on this pool. Immunofluorescence was performed using antibodies to HA to detect Borealin-FKBP fusions along with kinetochore protein Hec1. Taxol-arrested HeLa M cells expressing Borealin\textsubscript{1-221}-FKBP-HA or Borealin\textsubscript{1-110}-FKBP-HA were incubated in the presence or absence of the dimerizing agent AP20187 after inhibiting Haspin. Unlike full length Borealin-GFP, neither Borealin\textsubscript{1-221}-FKBP-HA nor Borealin\textsubscript{1-110}-FKBP-HA localized near kinetochores in Haspin-inhibited cells in the absence of the dimerizer AP20187 (Figure 4-3). In the presence of AP20187, Borealin\textsubscript{1-221}-FKBP-HA, but not Borealin\textsubscript{1-110}-FKBP-HA, was detectable as punctate foci that co-localized with Hec1 indicating that the central region and dimerization are essential for CPC localization to the kinetochore (Figure 4-3).

Experiments with Borealin-GFP suggest that Borealin co-localizes with pH2A\textsuperscript{T120} in 5Itu-treated cells (Figure 3-4). We repeated this co-localization experiment using the inducible FKBP-dimerization system. As before, undimerized Borealin\textsubscript{1-221}-FKBP was dispersed in the cytoplasm after 5Itu treatment (Figure 4-4). Interestingly, this truncated protein also dispersed the residual pH2A\textsuperscript{T120} in 5Itu-treated cells (Figure 4-4). Adding AP20187 rescued both punctate Borealin\textsubscript{1-221}-FKBP and pH2A\textsuperscript{T120} staining (Figure 4-4). We also tested the effect of combined treatment with 5Itu and either reversine or ZM447439 on localization of the dimerized Borealin\textsubscript{1-221}-FKBP. Similarly to our results with FLAG-tagged proteins, adding either reversine or ZM447439 appeared to uncouple
Borealin localization from pH2A$^{T120}$, in that Borealin was still partially punctate, while the histone modification was dispersed (Figure 4-4). Overall, these observations suggest that Borealin must be dimerized to be recruited to a kinetochore-proximal location. When either Mps1 or Aurora B are inhibited, Borealin localization appears to be uncoupled from pH2A$^{T120}$ (Figure 4-4).
Figure 4-3. Borealin dimerization is essential for localization near the kinetochore
Schematic of experimental design and maximum projection images of taxol-arrested HeLa M cells expressing the indicated Borealin-FKBP fusion proteins in the absence (-) or presence (+AP) of the homo-dimerizing agent AP20187. Immunofluorescence was performed using antibodies against HA (red) and Hec1 (green). Close-up images of individual sister kinetochores/centromeres from a single plane are below
Figure 4-4. Dimerization of Borealin is essential for feedback with Bub1 at kinetochores
Schematic of experimental design and maximum projection images of taxol-arrested HeLa M cells expressing Borealin1-221-FKBP after inhibiting Haspin with 5Itu for 90 minutes. Following 5Itu treatment, cells were then exposed to either ZM447439 (ZM) or reversine (Rev) for 1 hour. Dimerization was induced prior to upon release from thymidine block (+AP). Immunofluorescence was performed for HA (red), pH2A$^{T120}$ (blue), and Hec1 (green)
4.3 Checkpoint function of a kinetochore-proximal CPC pool.

The role of CPC in error correction is thought to rely on its inner centromere localization, providing a spatially restricted gradient of Aurora B phosphorylation [103]. On the other hand, studies in yeast suggest that the CPC may contribute to error correction even when displaced from the inner centromere [109]. Mitotic arrest induced by the microtubule depolymerizing drug nocodazole is much more efficiently abrogated by Aurora B inhibitors compared to the Haspin inhibitor 5Itu [147]. Additionally, we have characterized a previously unknown sub-population of the CPC near kinetochores, independent of the inner centromere pool. Therefore, we tested whether or not inner centromere localization of the CPC was required for a taxol-induced mitotic arrest and whether augmenting the kinetochore proximal pool had any effect on CPC-mediated checkpoint activation in taxol after inhibiting Haspin. Control cells or cells transfected with LAP-Mis12-Bub1 were synchronized into a taxol-induced mitotic arrest followed by treatment with 5Itu to inhibit Haspin and mislocalize the inner centromere pool of the CPC. The percentage of cells remaining in mitosis 15 hours after 5Itu treatment were quantified. We predicted that mislocalization of the CPC after 5Itu treatment would decrease the percentage of cells in mitosis and that cells expressing Mis12-Bub would not recover the percentage of mitotic cells after inhibiting Haspin due to the lack of CPC at the inner centromere. Surprisingly, inhibiting Haspin with 5Itu had no effect on the percentage of cells arrested in mitosis after inhibiting Haspin in control cells or cells expressing LAP-Mis12-Bub1 (Figure 4-5). On the other hand, inhibiting Aurora B with ZM447439 significantly decreased the percentage of cells in mitosis, resulting in an increase in cells that escape mitotic arrest (Figure 4-5). This suggests that inner
centromere localization of the CPC is not strictly required for maintaining a taxol-induced mitotic arrest.

Mislocalization of the CPC after inhibiting Haspin did not abrogate a taxol-induced mitotic arrest suggesting that either cytosolic CPC or the kinetochore-proximal pool of the CPC is sufficient to mediate checkpoint activation (Figure 4-5). Expression of monomeric Borealin1-221-FKBP impaired feedback with Bub1 to generate pH2A$^{T120}$, resulting in CPC displacement from the kinetochore-proximal pool (Figure 4-4). Therefore, we tested the hypothesis that residual CPC recruited to the kinetochore-proximal pool in Haspin-inhibited cells contributes to continuous error correction in taxol leading to persistent mitotic arrest. We used monomeric Borealin1-221-GFP to displace CPC from the kinetochore-proximal pool in cells simultaneously exposed to 5Itu to remove the inner centromere pool. In this manner we could test whether the kinetochore proximal pool contributes to the persistent taxol arrest observed in cells exposed to 5Itu. Cells expressing full-length Borealin-GFP or Borealin1-221-GFP were synchronized into taxol and the mitotic index and fraction of cells that escaped mitosis were monitored by time-lapse microscopy following exposure to 5Itu. Inhibiting Haspin in cells expressing full-length Borealin-GFP reduced the mitotic index to $\sim$35% compared to $\sim$49% in DMSO treated controls (Figure 4-6). Also, $\sim$25% of cells escaped mitosis after 5Itu treatment compared to $\sim$7% escape in DMSO treated cells. Inhibiting Haspin in cells expressing Borealin1-221-GFP decreased the mitotic index to $\sim$20% compared to $\sim$41% in DMSO treated cells. Moreover, $\sim$40% of Borealin1-221-GFP expressing cells escaped mitosis in 5Itu compared to $\sim$12% escape in DMSO controls (Figure 4-6). Importantly, in the presence of 5Itu, overexpressing Borealin1-221-GFP weakened the mitotic arrest
compared to cells overexpressing full-length Borealin. Overall, these results suggest that the kinetochore-proximal CPC pool plays an important role in maintaining the mitotic arrest induced by taxol.
Figure 4-5. Inhibiting Haspin does not abrogate a taxol-induced mitotic arrest
Percentage of untransfected or LAP-Mis12-Bub1 expressing cells remaining in mitosis (%Mitosis) or having escaped mitosis (%Escape) by 15-hours after the indicated treatments. Briefly, cells were transfected and synchronized into S-phase by 24-hour treatment with 2mM thymidine. Following synchronization, cells were released into taxol for 16 hours and time-lapse imaging was performed immediately after DMSO, 5Itu, or ZM. Bars represent standard error.
Figure 4-6. Checkpoint function of a kinetochore-proximal CPC pool

Percentage of Borealin-GFP expressing cells remaining in mitosis or having escaped mitosis by 15-hours after 5I ту treatment. Cells were treated as in A except that the indicated Borealin-GFP constructs were used. Bars represent standard error from three independent transfections. Asterisks indicate p<0.05 from two-tailed Student’s t-test.
Chapter 5

Analysis of Aurora B-mediated regulation of Bub1

5.1 Aurora B regulates Bub1 kinase activity independent of kinetochore targeting and ATM-mediated activation.

Aurora B and Mps1 co-regulate Bub1 localization to kinetochores and inhibiting Aurora B reduces pH2A\(^{T120}\) [128, 129, 148]. Therefore, we tested whether tethering Bub1 to the kinetochore rescued pH2A\(^{T120}\) after inhibiting Aurora B. HeLa M cells were transfected with LAP-Mis12-Bub1 and synchronized into a nocodazole-induced mitotic arrest followed by treatment with MG132 (to prevent mitotic exit) and DMSO (control) or ZM447439 to inhibit Aurora B. Immunofluorescence was performed for GFP to detect transfected cells and pH2A\(^{T120}\) to assess Bub1 kinase activity at kinetochores. Inhibiting Aurora B with ZM447439 significantly reduced pH2A\(^{T120}\) in cells expressing LAP-Mis12-Bub1, indicating that Aurora B plays a more complex role in regulating pH2A\(^{T120}\) than merely localizing Bub1 to kinetochores through Mps1 (Figure 5-1).

ATM regulates Bub1 kinase activity during mitosis and Aurora B phosphorylates and activates ATM [141]. Therefore, we tested the relative contribution of Aurora B and ATM in regulating pH2A\(^{T120}\) abundance using small molecule inhibitors of Aurora B and ATM. HeLa M cells were synchronized into a nocodazole-induced mitotic arrest
followed by treatment with MG132 (to prevent mitotic exit) and DMSO (control), ZM447439 to inhibit Aurora B, or caffeine to inhibit ATM. Western blotting was performed for pH2A$^{T120}$ to determine the relative levels of pH2A$^{T120}$ after kinase inhibition. Inhibiting Aurora B with ZM447439 significantly reduced pH2A$^{T120}$ levels, while inhibiting ATM with caffeine had a less severe effect (Figure 5-2A). Additionally, inhibiting both Aurora B with ZM447439 and ATM with caffeine reduced pH2A$^{T120}$ to levels similar to ZM447439 alone (Figure 5-2A). We also tested whether a chemically distinct ATM inhibitor (KU55933) had the same effect as caffeine. Similarly, inhibiting ATM alone with KU55933 led to a smaller reduction in pH2A$^{T120}$ compared to inhibiting Aurora B alone with ZM447439 (Figure 5-2B). Again, inhibiting both Aurora B and ATM led to a similar reduction in pH2A$^{T120}$ as inhibiting Aurora B alone. Together, these results suggest that Aurora b might regulate pH2A$^{T120}$ independent of ATM-mediated activation of Bub1. To test whether Aurora B regulates pH2A$^{T120}$ independent of ATM, we examined pH2A$^{T120}$ levels in ATM-null human fibroblasts by immunofluorescence. Inhibiting Aurora B with ZM447439 in ATM-null cells reduced pH2A$^{T120}$ levels indicating that Aurora B regulates Bub1 independent of ATM (Figure 5-3).
Figure 5.1. Tethering Bub1 to kinetochores does not rescue pH2AT120 when Aurora B is inhibited

A.) Maximum projections of nocodazole-arrested HeLa M cells expressing LAP-Mis12-Bub1 after treatment with MG132 (to prevent mitotic exit) and DMSO (control) or ZM447439 to inhibit Aurora B. Immunofluorescence was performed for GFP (green) and pH2A\textsuperscript{T120} (magenta) B.) Quantification of pH2A\textsuperscript{T120} levels at individual centromeres in cells from A. Five centromeres from 10 cells were quantified for a total of 50 centromeres. pH2A\textsuperscript{T120} levels were normalized against control levels. Bars represent standard deviation. Asterisk indicates p<0.001 from a type two, two-tailed Student’s t-test.
Figure 5-2. The effects of inhibiting Aurora B and ATM on pH2A$^{T120}$ levels

A.) Western blots of histones extracted from taxol-arrested HeLa M cells treated with MG132 (to prevent mitotic exit) and ZM447439, Reversine, or Caffeine to inhibit Aurora B, Mps1, or ATM, respectively. B.) Cells were treated as in A except that ZM447439 and KU55933 (ATM inh.) were used.
Figure 5-3. Inhibiting Aurora B reduces pH2A^{T120} in ATM-null cells
A.) Maximum projection images of taxol-arrested PEB5 cells (ATM-null) treated with MG132 (to prevent mitotic exit) and DMSO (control) ZM447439 (ZM) or Reversine to inhibit Aurora B or Mps1, respectively. Immunofluorescence was performed using antibodies to pH2A^{T120} (red) and DNA (blue) was stained with Hoechst 33342. B.) Quantification of pH2A^{T120} intensity levels in 50 cells from each cell. Bars represent standard deviation.
5.2 Aurora B phosphorylates Bub1 \textit{in vitro} and may directly regulate Bub1 \textit{in vivo}

Because Aurora B appeared to play a more direct role in regulating Bub1-mediated pH2A\textsuperscript{T120}, we wondered whether Bub1 could be a substrate of Aurora B. Bub1 contains 13 serine or threonine residues that conform to the loose Aurora B consensus sequence ([K/L]-X-[R/T]) (Figure 32). Seven of the 13 putative Aurora B sites are conserved from mice to humans and 5 of the 13 putative Aurora B phosphorylation sites were identified as phosphorylated \textit{in vivo} in high throughput phospho-proteomic screens (Phosphositeplus, PhosphoELM) (Figure 5-4). Therefore, we tested whether Bub1 was a substrate of Aurora B using recombinant GST-tagged Bub1 fragments and recombinant Aurora B \textit{in vitro} kinase assays. Indeed, the N-terminus and central region of Bub1 were phosphorylated \textit{in vitro} by Aurora B (Figure 5-5). Thus, Bub1 is a substrate of Aurora B.

To this point our data indicate that Bub1 is a substrate of Aurora B and that Aurora B regulates Bub1 kinase activity independent of kinetochore targeting and ATM-mediated kinase activation. Therefore, we hypothesize that Aurora B directly regulates Bub1 kinase activity. To test this idea \textit{in vivo}, we mutated all 13 putative Aurora B phosphorylation sites in Bub1 (GFP-Bub1\textsuperscript{13A}) and assessed pH2A\textsuperscript{T120} levels in cells expressing this mutant. We predicted that mere overexpression of GFP-Bub1\textsuperscript{13A} would impair pH2A\textsuperscript{T120} levels in a dominant-negative manner. Compared to cells expressing wild-type Bub1 (GFP-Bub1\textsuperscript{WT}), pH2A\textsuperscript{T120} levels appeared similar in cells expressing GFP-Bub1\textsuperscript{13A} (Figure 5-6). We also tested whether expressing GFP-Bub1\textsuperscript{13A} led to overt defects in mitotic timing compared to GFP-Bub1\textsuperscript{WT} by measuring the length of mitosis in cells expressing either GFP-Bub1\textsuperscript{WT} or GFP-Bub1\textsuperscript{13A}. Compared to untransfected cells
which spent roughly 60 minutes in mitosis, cells expressing GFP-Bub1\textsuperscript{WT} spent roughly 90 minutes in mitosis (Figure 5-7). On the other hand, cells expressing GFP-Bub1\textsuperscript{13A} spent ~65 minutes in mitosis, similar to untransfected controls (Figure 5-7). Thus, GFP-Bub1\textsuperscript{13A} does not retain the ability to increase the length of mitosis when overexpressed, compared to GFP-Bub1\textsuperscript{WT} indicating that the mutated residues are important for increasing the length of mitosis when Bub1 is overexpressed.
Figure 5-4. Bub1 contains multiple putative Aurora B phosphorylation sites
Schematic representation of the primary sequence of Bub1. Known domains are indicated in red. Arrows indicate the positions of serine or threonine residues that conform to the Aurora B consensus motif ([K/L]-X-[R/T]). Green stars represent residues that have been identified in high-throughput phospho-proteomic screens. Blue stars represent residues conserved from mice to humans that were identified in high throughput phospho-proteomic screens.
Figure 5-5. **Aurora B phosphorylates the N-terminus and central region of Bub1 *in vitro***

A.) Schematic showing the relative size and number of amino acids of GST-Bub1 fragments in used for *in vitro* kinase assays. B.) Autoradiograph of GST-Bub1 fragments after *in vitro* kinase assays with recombinant Aurora A and INCENP. C.) Western blot using antibodies to GST to detect GST-Bub1 fragments.
Figure 5-6. Mutating putative Aurora B phosphorylation sites in Bub1 has no effect on pH2A\textsuperscript{T120} levels when overexpressed
3D projections of nocodazole-arrested HeLa M cells expressing GFP-Bub1\textsuperscript{WT} or GFP-Bub1\textsuperscript{13A}. Immunofluorescence was performed using antibodies to GFP (green) and pH2AT120 (red). DNA (blue) was stained with Hoechst33342.
Figure 5-7. Expression of GFP-Bub1 13A does not increase the length of mitosis when overexpressed

Cells expressing GFP-Bub1 WT or GFP-Bub1 13A were monitored by time-lapse phase and fluorescence microscopy to determine the length of time spent in mitosis. Phase contrast was used to determine when nuclear envelope breakdown and anaphase onset were initiated. Bars represent standard deviation.
Chapter 6

Discussion

The CPC has long been recognized as a master regulator of mitotic events, including chromosome segregation and mitotic checkpoint activation. The molecular determinants of CPC localization to centromeres have only recently been described in mechanistic detail and whether or not inner centromere localization of the CPC is essential for the mitotic function of the CPC is currently under debate. Together, our data provides new insight into CPC localization and function during mitosis.

Consistent with the recently described two-histone model of CPC localization, we provide evidence that the CPC localizes to a major sub-population at inner centromeres that is regulated primarily by Haspin kinase and a minor sub-population at near kinetochores that may be regulated by the Bub1-pH2A\textsuperscript{T120} pathway. The CPC localizes to inner centromeres and a minor pool can be detected at near kinetochores by immunofluorescence when both histone marks are most abundant. Inner centromere abundance of the CPC and Borealin dynamics are most significantly impaired when Haspin is inhibited and both histone marks are reduced. However, CPC abundance and Borealin dynamics are largely unaffected under conditions where only pH2A\textsuperscript{T120} at
pericentromeres is reduced ~90%. Surprisingly, inhibiting Aurora B reduced both pH2A_T120 and pH3_T3, but had no effect on Borealin dynamics and CPC abundance at inner centromeres. Aurora B has a reported role in inhibiting CPC localization by phosphorylating Survivin, which is restrained during mitosis, and CPC levels might be maintained due to lack of Survivin phosphorylation when Aurora B is inhibited. Alternatively, it is possible that the residual pH2A_T120 and/or pH3_T3 present after inhibiting Aurora B is sufficient to maintain CPC abundance at inner centromeres. Finally, a minor pool of CPC is retained near kinetochores when Haspin is inhibited and the kinetochore-proximal of CPC is augmented by tethering Bub1 to kinetochores. Thus, we conclude that the CPC exists in two distinct, but not necessarily mutually exclusive, sub-populations at inner centromeres and near kinetochores.

We also provide evidence that dimerization of the CPC via Borealin is essential for inner centromere and pericentromere localization of the CPC. Firstly, the N-terminus of Borealin can be detected, but exchanges rapidly at inner centromeres and overexpression of the N-terminus of Borealin reduces the abundance of the CPC at inner centromeres, which is rescued by induced dimerization. We interpret these results to mean that Borealin is shuttled to the centromere via interaction with INCENP and Survivin via pH3_T3. If Borealin is a monomer then the CPC binds pH3_T3, via Survivin, with low affinity and consequently exchanges rapidly; thus, preventing accumulation of the CPC at inner centromeres. On the other hand, if Borealin dimerizes, then the CPC potentially engages multiple pH3_T3 and/or pH2A_T120 binding sites; thus, suppressing dynamic exchange of Borealin and enabling accumulation of the CPC at inner centromeres. In addition to dimerization, however, the central region of Borealin that...
binds Sgo1 is essential for CPC localization near kinetochores. In this case, dimerization of Borealin could facilitate multiple interactions with Sgo1, which would enable localization to near kinetochores. Altogether, we conclude that Borealin dimerization is essential for inner centromere localization of the CPC, independent of the central region that binds Sgo1; however the central region and dimerization of Borealin are essential for localization and function of the CPC near kinetochores.

Combining our analysis of Borealin and the CPC with the two-histone model of CPC localization we can attempt to explain why dimerization of the CPC might facilitate stable inner centromere binding. The two-histone model suggests that the CPC contacts both pH3T3 via Survivin and pH2AT120 via the Sgo1-Borealin interaction. Because dimerization of Borealin is critical for maintaining inner centromere abundance of the CPC and suppressing Borealin dynamics we initially speculated that dimerization simply doubles the number of contacts with pH3T3 and pH2AT120. However, induced dimerization of the N-terminus of Borealin was sufficient to rescue inner centromere abundance of the CPC and reducing pH2AT120 had no effect on the dynamics of Borealin at and localization of the CPC to inner centromeres. Thus, the interaction with Sgo1 and consequently pH2AT120 might not be required for inner centromere abundance. Survivin can bind a Sgo1 peptide that resembles pH3T3 and recently Liu et al. provided evidence that Sgo1 localizes to inner centromeres under low tension scenarios, such as taxol or nocodazole, by binding cohesin. Thus, dimerization of the CPC could double the interactions between Survivin-pH3T3 and Survivin-Sgo1, or by enabling binding with unknown inner centromere receptors, such as cohesin.
If we extend the two-histone model to the kinetochore-proximal sub-population of the CPC, again, dimerization of Borealin is predicted to double the number of contacts with pH3\textsuperscript{T3} and pH2A\textsuperscript{T120}. However, kinetochore-proximal localization of the CPC persists after inhibiting Haspin. Thus, pH3\textsuperscript{T3} may not contribute to localization of the CPC near kinetochores. Sgo1 seems like a putative receptor of the CPC at pericentromeres because dimerization and the central region of Borealin are both essential for localization of the CPC near kinetochores. However, induced-dimerization enables persistent localization of our Borealin truncation even after pH2A\textsuperscript{T120} is reduced when Aurora B or Mps1 is inhibited. Furthermore, Sgo1 reportedly localizes to inner centromeres rather than kinetochores under low tension conditions; thus, Sgo1 might not mediate localization of the CPC near kinetochores through Borealin. If Survivin and Borealin are not binding pH3\textsuperscript{T3} and Sgo1, respectively, then the receptor(s) of the CPC near kinetochores are unknown.

We also provide evidence that the kinetochore-proximal pool of CPC can regulate mitotic checkpoint activation. Borealin truncations that cannot bind near kinetochores transiently interact with inner centromeres and only partially rescue endogenous Borealin knockdown, suggesting that complex formation is sufficient to confer only partial checkpoint activity. HeLa M cells maintain a taxol-induced checkpoint arrest under conditions where the CPC is mislocalized from inner centromeres, but not near kinetochores, suggesting that the kinetochore-proximal and/or cytosolic CPC can mediate mitotic checkpoint activation. Furthermore, mislocalization of the inner centromere pool of the CPC with 5Itu has the most significant effect on a taxol-induced mitotic arrest in cells expressing a Borealin truncation that disrupts localization and function of the CPC.
near kinetochores, compared to full-length Borealin. Thus, we conclude that, at the very least, both the major and minor pools of CPC at inner centromeres and near kinetochores, respectively, contribute to mitotic checkpoint activation.

We also provide preliminary evidence that Aurora B directly regulates Bub1 at kinetochores to generate pH2A\(^{T120}\). Firstly, tethering Bub1 to kinetochores fails to rescue pH2A\(^{T120}\) when Aurora B is inhibited indicating that Aurora B plays a more complex role in regulating Bub1 at kinetochores than simply regulating Bub1 localization via Mps1. Secondly, we show that Aurora B regulates pH2A\(^{T120}\) abundance in the absence of ATM, which has been reported to phosphorylate an activate Bub1 to generate pH2A\(^{T120}\). Testing whether or not tethering Bub1 to kinetochores in ATM-null cells rescues pH2A\(^{T120}\) would be the most direct method of confirming that Aurora B indeed regulates pH2A\(^{T120}\) abundance independent of ATM-mediated activation of Bub1 and localizing Bub1 to kinetochores. We also show that Aurora B directly phosphorylates the central region and N-terminus of Bub1. Mutation of putative Aurora B sites in Bub1 does not affect pH2A\(^{T120}\) levels when GFP-Bub1\(^{13A}\) is overexpressed; however depletion of endogenous Bub1 would be the most desirable method of determining whether or not mutation of these sites effects. Because GFP-Bub1\(^{13A}\) could be detected at kinetochores there is no reason to suspect that mutating these residues affects interactions with known binding partners, all of which mediate kinetochore localization. Additionally, testing whether mutation of putative Aurora B sites on Bub1 using \textit{in vitro} kinase assays with immunoprecipitates of Bub1\(^{WT}\) and Bub1\(^{13A}\) would be another method of determining whether these residues regulate Bub1 kinase activity. Overexpressing GFP-Bub1\(^{13A}\) does not increase the length of mitosis when overexpressed suggesting that the mutated sites,
which may be phosphorylated by Aurora B, might mediate the increase in mitotic timing. Again, testing whether mutation of these sites has any effect on the mitotic checkpoint or chromosome segregation would be most desirable in the absence of endogenous Bub1. Altogether, our data supports the hypothesis that Aurora B directly regulates Bub1.
Chapter 7

Materials and Methods

Cell culture conditions, synchronization, and drug treatments: Cells were grown in Dulbecco’s minimal essential medium (Gibco) with penicillin/streptomycin and 10% fetal bovine serum in a humidified atmosphere of 10% CO₂ at 37° C. All experiments were performed with HeLa M cells, a sub-line of HeLa [149]. FRAP experiments in Figures 2-6 and 3-5 were performed using a HeLa M clone stably transfected with Borealin-GFP. Synchronization of cells was performed by treating cells with 2mM thymidine (Sigma Aldrich) for 24 hours followed by three PBS washes to release cells from S-phase block. Subsequently, cells were treated with either 3.3μM nocodazole (Sigma Aldrich) or 0.5μM taxol (Cayman Chemical) to arrest cells in mitosis. 14 hours post-release from thymidine block cells were treated with the proteasome inhibitor MG132 (Cayman Chemical) at a concentration of 20μM to prevent mitotic exit followed by treatment with kinase inhibitors for 90 minutes. Cells were then fixed for immunofluorescence or harvested for western blot analysis where indicated. Hydroxyurea was used at a concentration of 2mM. Kinase inhibitors were used at previously determined effective concentrations: 2.5μM ZM447439 (AstraZeneca), 2μM reversine...
(Calbiochem), 2μM 5-iodotubericidin (Santa Cruz), 10μM Ro31 (Cayman Chemical), 10μM Chelerythrine chloride (Cayman Chemical). Cell selection in Figure 2-8 was accomplished using puromycin at 1μg/μL (Invitrogen). In Borealin-FKBP experiments the small molecule AP20187 was used at 500nM (Clonetech). Okadaic acid was used at a concentration of 200nM.

**Construction of Borealin truncation mutants, Borealin fusions, GFP-Bub1, and GST-Bub1 fragments:** Borealin truncation mutants were constructed by amplifying the corresponding regions of a cDNA encoding human Borealin by PCR. The PCR products encoding a FLAG-tag at the C-terminus were TOPO cloned into pcDNA 3.2 (Invitrogen) [89, 94]. Borealin-GFP truncations were created by amplifying the appropriate reading frames from pcDNA3.2 plasmids containing Borealin cDNAs. The PCR products were TOPO-cloned into pENTER-D-TOPO and subsequently Gateway-cloned into pcDNA-DEST47. An internal deletion of Borealin was constructed by amplifying regions of Borealin cDNA corresponding to amino acids 1-110 and 221-280 with SpeI restriction sites at the 3’ and 5’ ends. The PCR products were digested with SpeI followed by gel purification and ligation. The ligated product was Gateway cloned into pcDNA-DEST47 (Invitrogen). Borealin-FKBP fusions were constructed by amplifying regions of Borealin cDNA corresponding to amino acids 2-110 or 2-221 with XbaI and SpeI restriction sites at the 3’ and 5’ ends, respectively. The PCR products were digested with SpeI and XbaI followed by gel purification and ligation into XbaI-digested pC4-Fv1E (ARIAD Pharmaceuticals). GFP-Bub1 was created by amplifying the Bub1 cDNA from a clone of Bub1 (gift from S.T. Liu), TOPO cloning the PCR product into pENTR-D-TOPO, and
Gateway cloning into pcDNA-DEST47. GST-Bub1 fragments were created by amplifying the regions of Bub1 cDNA corresponding to amino acids 1-332 (Bub1 N), amino acids 333-789 (Bub1 M), and amino acids 790-1085 (Bub1 C). PCR products were TOPO cloned into pENTR-D-TOPO and Gateway-cloned into pDEST15. To note, GST-Bub1 C<sup>K821R</sup> was created via site-directed mutagenesis (Strategene Multi-site kit) of the pENTR-Bub1C entry clone before recombination into pDEST15. All constructs were confirmed by sequencing.

**DNA Binding Assay:** DNA binding assays were performed by transfecting HeLa M cells with Borealin-FLAG tagged truncations and cell were lysed in Cell Lysis Buffer (1XPBS, 10% glycerol, 0.5%NP-40) containing protease and phosphatase inhibitors for 30 minutes on ice and centrifuged at 16.1xg for 20 minutes at 4°C to obtain the soluble proteins. DNA-cellulose beads (Sigma) were washed with Cell Lysis Buffer and 4mg of DNA-cellulose beads were incubated with 2.5mg of cell lysates from mitotic Borealin-FLAG mutant-expressing cells and incubated at 4°C for 16-18 hours with constant rocking. After incubation with lysates, DNA-cellulose beads were spun at 1.5xg and suspended in Cell Lysis Buffer three times then suspended in 2X Laemmli buffer (2% SDS, 100mM Tris, 0.05% BPB, 30% glycerol). DNA bound proteins and input lysates were separated on a 15% acrylamide SDS-PAGE gel, transferred to polyvinylidifluoride (PVDF) membranes (Millipore) and analyzed by western blotting using anti-FLAG antibodies (Sigma).
**Transient transfections:** Transient transfections were carried out by transfecting HeLa M cells using Expressfect (Denville Scientific Inc), XtremeGene9 (Roche), or polyethylenimine (PEI) at a ratio of 3:1 (μL transfection reagent/μg DNA). A typical transfection in a 6-well plate was performed by suspending 2μg plasmid DNA in 500uL serum-free DMEM and adding 6μL transfection reagent. The DNA-polymer complexes were briefly vortexed and incubated for 20 minutes at room temperature before being added to each well. Transfected cell were incubated at least 12 hours at 37°C before any downstream assay. Experiments were scaled up or down accordingly.

**Western blotting:** Cells lysates were obtained by suspending cells in RIPA buffer (50mM Tris pH 7.5, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) supplemented with protease inhibitors (1μg/ml aprotinin, 2 μg/ml leupeptin 1μg/ml pepstatin) and phosphatase inhibitors (1 mM sodium fluoride and 1 mM sodium vanadate) and incubating on ice for 20 minutes followed by centrifugation at 16.1xg for 20 minutes at 4°C to isolate the soluble fraction. Western blotting was performed by resolving cell lysates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% acrylamide at an acrylamide: bisacrylamide ratio of 37.5:1). Following SDS-PAGE, proteins were transferred to PVDF membranes (Millipore). Subsequently, membranes were blocked in buffer containing of 0.05% (v/v) Tween and 5% (w/v) non-fat dry milk in PBS. After blocking, membranes were incubated with the appropriate antibodies, which were diluted in blocking buffer, at either room temp for 1.5 hours or at 4°C for 16-18 hours. Subsequently, membranes were washed three times in PBST and incubated in HRP-conjugated secondary antibodies (Bio-Rad, 1:10,000) for 45
minutes. After three PBST washes, the resulting signal was detected by enhanced chemiluminescence (BioRad).

**Immunofluorescence Microscopy:** For immunofluorescence microscopy, cells were grown on coverslips followed by transient transfection where indicated. Following synchronization and drug treatment, where indicated, cells were fixed with 2% formaldehyde/PBS for 15 minutes at room temperature then permeabilized with Lysis Wash (150mM NaCl, 10 mM Tris (pH 7.5), 0.1% Triton-X-100 (v/v) and 0.1% BSA (w/v)) for 20 minutes at room temperature. Fixed cells were blocked with PBS containing 0.1% BSA (PBSP) for 16 hours at 4°C followed by incubation with the appropriate antibodies, which were dissolved in PBSP, at either 37°C for 45 minutes, room temperature for 1.5 hours, or 4°C for 16-18 hours. Coverslips were then washed with PBS and subsequently incubated with fluorophore conjugated secondary antibodies at a dilution of 1:1000 in PBSP. Coverslips were washed with PBS and DNA was stained using Hoechst 33342 diluted in PBS at 1:400 for 15 minutes at room temperature. Finally, coverslips were mounted using Vectashield (Vector Laboratories) and analyzed using a Leica SP8 confocal microscope or an Olympus IX-81 microscope using a 40X, NA 0.50 UPlanFLN objective at 2x2 binning, where indicated.

**Flow Cytometry and Analysis of Mitotic Progression:** For analysis of DNA content by flow cytometry, cells were transfected with pBabePURO, pSUPER-Borealin-shRNA or pSUPER empty (control), and plasmids encoding FLAG-tagged Borealin truncations.
Following three days of selection in 1μg/μL puromycin, cells were collected by centrifugation at 2000×g, for 7 min at 4°C and resuspended in PBS [150]. Cells were fixed in 70% ethanol at -20°C for 16-18 hours. Fixed cells were collected by centrifugation, resuspended in PBS, and stained with 0.5mg/ml of propidium iodide along with 5μg/ml of RNAse A for 30 min. Cells were then analyzed by flow cytometry using CellQuest and WinMDI software. Ten thousand cells were analyzed for each sample. For experiments analyzing mitotic progression in Figure 2-8, cells were transfected as above; however, pBabePURO was replaced with a plasmid containing H2BGFP and puromycin selection was omitted. Three days post-transfection, live cells were viewed by fluorescence microscopy and the frequency of mitotic stages quantified in a blinded manner.

**Time-lapse microscopy**: Cells were transfected and cultured for at least 12 hours before synchronization, where indicated. Prior to imaging, cells were cultured in 10% FBS penicillin/streptomycin Ham’s F12 (Thermo Scientific) imaging media supplemented with 20mM HEPES pH 7.4 buffer for at least 12 hours. Cells were then transferred to an Olympus IX-81 microscope with a stage top incubator to incubate at 37°C for 1 hour to equilibrate before imaging. For experiments using GFP-tagged Borealin or Mis12-Bub1 in Figures 4-5, 4-6, and 5-7, respectively, bright-field and fluorescence images were obtained of GFP-expressing cells prior to time-lapse imaging using a 40X, NA 0.50 UPlanFLN objective at 2x2 binning. Subsequently, bright-field images were taken at 10 minute intervals over the course of the experiment.
Histone extraction: For western blot analysis of histone modifications, cell pellets from 10cm plates (~5x10^6 HeLa M cells) were obtained after synchronization and drug treatment and histones were extracted as previously described [151]. Cell pellets were lysed in Nuclear Prep Buffer (10mM Tris pH 7.6, 150mM NaCl, 1.5mM MgCl₂, and 0.65% NP40) supplemented with protease and phosphatase inhibitors for 30 minutes on ice then spun at 1500xg for 10 min at 4°C. Pelleted nuclei were then suspended in RSB Buffer (10mM NaCl, 10mM Tris-HCl pH 7.5, 3mM MgCl₂, and 0.4N sulfuric acid) supplemented with protease and phosphatase inhibitors and incubated on ice for 30 minutes then spun at 16.1xg for 20 minutes at 4°C. Trichloroacetic acid was added to the supernatant at 18% and incubated on ice for 30 min then spun at maximum speed for 30 min at 4°C. Pelleted histones were washed with acetone:HCl (99.5%:0.5% v/v) then with 100% acetone followed by air drying and suspension in H₂O. Histones were resolved on a 12.5% acrylamide SDS-PAGE gel and transferred to PVDF membranes. Prior to western blotting, the membrane was stained with Ponceau solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) to control for loading.

Fluorescence Recovery after Photobleaching (FRAP): For FRAP studies, cells were plated on glass bottom 35mm dishes (World Precision Instruments) followed by transfection and synchronization into mitosis. During imaging, cells were maintained at 37°C at 5% CO₂ in a Tokai Hit Stage Top Incubator. Photobleaching and four-dimensional confocal microscopy were performed on a Leica SP8 system using the 100X oil objective. GFP was excited with a 488nm-Argon (power varying from 0.05 to 0.1% at 70% maximum power). Individual centromeres were imaged by successive acquisition
of 2.5μm volumes at 0.5μm per step along the Z axis at a zoom factor of 10. Centromeres were bleached by 5 iterations of a full power laser and recovery was monitored every 2.616 seconds for approximately 2 minutes post-bleaching. Fluorescence intensities of bleached centromeres were acquired from the Leica AF software. After background subtraction, the mean intensity of the region prior to photobleaching was set at 100% and relative intensities at each time point were averaged for greater than eight centromeres from individual cells (averaged data set). Fit curves were generated by minimizing the chi-squared value of the averaged data set and a fit curve by adjusting parameters C, A, and k (time-constant) in the following equation using the Microsoft Excel Solver function:

\[ I(t) = C + A(1 - \exp(-kt)) \]

Average time-constants were obtained by fitting individual FRAP curves then averaging the time-constants for each condition.

**Immunoprecipitation:** For immunoprecipitation of endogenous Borealin, pelleted cells were lysed in Immunoprecipitation Buffer (50mM Tris pH8.0, 400mM NaCl, 0.5% NP-40, 0.1% D.O.C., 30ug/mL RNAse A, 80U/mL DNAse I) supplemented with protease and phosphatase inhibitors. 500 μg of lysate was incubated with rabbit anti-Borealin at a final volume of 500μL at 4°C for 16-18 hours. Subsequently, 15uL of Protein-A sepharose beads, which were previously blocked in 1mg/mL BSA/Immunoprecipitation Buffer and washed with three times with immunoprecipitation buffer, was added to lysates containing anti-Borealin and incubated for 2 hours at 4°C. Immune complexes were then pelleted by centrifugation at 1.5xg for 3 minutes and suspended in
Immunoprecipitation Buffer three times followed by centrifugation at 16.1xg and suspension in 2X Laemmli buffer (2% SDS, 100mM Tris, 0.05% BPB, 30% glycerol). For immunoprecipitation of endogenous Bub1 or Knl1, cells were lysed in RIPA buffer. 500 μg of lysate was incubated with either rabbit anti-Bub1 (gift from Dr. ST Liu) or rabbit anti-Knl1 (Millipore) at a final volume of 500μL for 4 hours at 4°C and 16 hours at 4°C, respectively. Subsequently, 15uL of Protein-A sepharose beads, which were previously blocked in RIPA buffer containing 1mg/mL BSA and washed with three times with RIPA buffer, was added to lysates containing anti-Bub1 or anti-Knl1 and incubated for 2 hours at 4°C. Immune complexes were then pelleted by centrifugation at 1.5xg for 3 minutes and suspended in RIPA buffer three times followed by suspension in 2X Laemmli buffer (2% SDS, 100mM Tris, 0.05% BPB, 30% glycerol).

**Recombinant protein production:** For recombinant protein production of GST-Bub1 fragments, BL21-(DE3)-RIPL bacterial cells were transformed with pDEST15 plasmids containing the appropriate Bub1 fragment cDNA. Positive clones were grown overnight at 37°C. Cultures were inoculated at 1:50 until they reached an OD<sub>600</sub> of 0.5 units then protein production was induced with 1mM IPTG for 4 hours at room temperature. Cell pellets were obtained by centrifugation at 16.1xg for 5 minutes at 4°C. Pelleted bacteria were suspended in Bacterial Lysis Buffer (50mM Tris pH7.5, 150mM NaCl, 5% glycerol, 0.1% Triton-x, 1mM DTT) supplemented with protease and phosphatase inhibitors at 5mL/g bacteria and subsequently sonicated at 30% for 20 seconds on, 40 seconds off for seven iterations. Lysates were centrifuged at 16.1xg for 20 minutes at 4°C to obtain the soluble fraction followed by incubation with GSH-sepharose beads for 4 hours at 4°C.
GST-Bub1-bound beads were then centrifuged at 1.5xg for 3 minutes and suspended in Bacterial Lysis Buffer three times. GST-Bub1 fragments were eluted from beads by incubation with reduced 25 mM glutathione at pH 8.8 and reconstituted in Kinase Buffer (50mM Tris pH 7.5, 10mM MgCl₂).

**In vitro kinase assays:** For *in vitro* kinase assays, recombinant GST-Bub1 fragments and His-Aurora B and His-INCENP<sup>822-919</sup> were incubated in Kinase Buffer (50mM Tris pH7.5, 10mM MgCl₂) containing <sup>32</sup>P-γ-ATP at 37°C for 30 minutes. Reactions were quenched with 2X Laemmli Buffer (2% SDS, 100mM Tris, 0.05% BPB, 30% glycerol) and boiled for 5 minutes. Proteins were resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were exposed to film under an intensifying screen to visualize incorporation of <sup>32</sup>P by means of phosphorylation via auto-radiation. Western blotting was performed on the same membrane to confirm the presence and relative abundance of GST-tagged Bub1 fragments using rabbit anti-GST antibodies (Thermo Scientific).

**Site-directed Mutagenesis:** Site-directed mutagenesis was performed using the Stratagene Multi-site-directed mutagenesis kit. Briefly, 30-40 nucleotide primers with a melting temperature of roughly 70°C were generated against the appropriate cDNA sequence containing the codon to be mutated with the appropriate mutation in the primer. The site directed reaction was performed using the appropriate primers and plasmid to be mutated per the manufacturer's instructions. Mutations were confirmed by sequencing.
References


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