POLO-LIKE KINASE CDC5 REGULATES PERICENTROMERIC COHESIN PERSISTENCE

by
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Abstract

The high fidelity duplication and subsequent segregation of chromosomes is a requirement for life. An essential protein complex termed cohesin ensures that replicated sister chromatids properly segregate between the dividing cells. The DNA-tethering activity of this protein complex is regulated by a host of accessory factors, including the enigmatic polo-like kinase (Cdc5 in budding yeast). Evolutionarily conserved, polo-like kinases are thought to control the spatiotemporal regulation of cellular division programs by specific subcellular localization to, and phosphorylation of, their target proteins. In this dissertation I demonstrate that Cdc5 associates with chromosomes at centromeres, co-localizes with cohesin at centromeres and cohesin-associated regions (CARS), and physically interacts with the cohesin complex. Cdc5’s enrichment on chromosomes requires cohesin’s enrichment at the same loci. Intriguingly, both Cdc5 and cohesin’s enrichment at centromeres decrease when chromosomes are properly attached to the mitotic spindle. Finally, I have also generated a novel, auxin-sensitive allele of CDC5 (cdc5-aid), which may provide further insights into the interplay between cohesin and Cdc5: preliminary data suggests that depletion of cdc5-aid leads to cohesin persistence at centromeres, but not at chromosomal arm CARS, late in the cell cycle.

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# Table of Contents

Abstract ............................................................................................................................................... ii

Acknowledgments ................................................................................................................................. iii

Table of Contents ................................................................................................................................... v

List of Tables ........................................................................................................................................... viii

List of Figures ......................................................................................................................................... viii

Chapter I: Polo-Kinases and the Regulation of Cohesin Disassociation

From Chromosomes ............................................................................................................................... 1

1.1 Introduction ...................................................................................................................................... 1

1.2 Cohesin is Important for Many Cell Cycle Activities ................................................................. 4

1.3 Cohesin Activity is Cell Cycle Regulated ...................................................................................... 8

1.4 Overview of the Centromere ........................................................................................................... 11

1.5 Overview of Kinetochore ............................................................................................................... 12

1.6 The Structure of the Kinetochore ................................................................................................. 13

1.7 The Spindle Assembly Checkpoint .............................................................................................. 14

1.8 The Structure of Polo-like Kinases ............................................................................................... 16

1.9 Polo-Like Kinase and the Centromere/Kinetochore ..................................................................... 19

1.10 Polo-Like Kinases and Biorientation ......................................................................................... 20

1.11 Polo-like Kinase Regulation of Cohesin in Yeast and Humans ................................................. 22

1.12 Conclusion ..................................................................................................................................... 26

Chapter II: Polo-Like Kinase Cdc5 Localizes to Cohesin-Associated Regions ............................... 28

2.1 Introduction ..................................................................................................................................... 28

2.2 Results ............................................................................................................................................. 32
List of Tables

Table 1. Yeast Strains .................................................................................................................. 139

Table 2. Plasmids .......................................................................................................................... 141
List of Figures

Figure 1A. Polo kinase Cdc5 localizes to centromeres during mitosis ..............34
Figure 1B. Cdc5 localizes preferentially to centromeres during mitosis ..........37
Figure 1C. Cdc5 localizes to M-phase promoter CLB2, but not G1 promoter CLN2 ....40
Figure 1D. Cdc5 co-occupies CEN3 DNA with Cse4 .................................................42
Figure 1E. Cdc5 pericentromeric association with CEN3 in technical triplicate ..........................................................43
Figure 1F. Cdc5 is enriched near CEN3 DNA in S and M phases ................46
Figure 2A. Polo-like kinase Cdc5 localizes to centromeres via functional kinetochore complexes .................................................................49
Figure 2B. Additional ndc10 alleles disrupt Cdc5 localization at CEN3 ..........51
Figure 3A. Cdc5 localizes to cohesin-associated regions ..........................53
Figure 3B. Cdc5 co-occupies a pericentromeric CAR with Mcd1 ...............55
Figure 3C. Cdc5 co-occupies a chromosomal arm CAR with Mcd1 ..........56
Figure 3D. Cdc5 co-occupies an additional chromosomal arm CAR with Mcd1 ......57
Figure 4A. Cdc5 localization depends on cohesin ........................................59
Figure 4B. mcd1-aid cells grow poorly on auxin-containing media ..........62
Figure 4C. mcd1-aid cells arrest upon auxin treatment .................................62
Figure 4D-E. mcd1-aid disrupts Cdc5 localization near pericentromeric CEN3 DNA ....64
Figure 5. Cdc5 localization to pericentromeric DNA depends on the absence of biorientation .............................................................67
Figure 6A. Cdc5 associates with cohesin in vivo .......................................70
Figure 6B. Cdc5 associates with cohesin subunit Mcd1 .............................71
Figure 6C. Cdc5 associates with cohesin subunit Mcd1 during mitotic arrests ..........74
Figure 6D. The cell cycle association of Cdc5 and Mcd1 ..........................76
Figure 7. Cdc5 and Mcd1 interaction ................................................................. 87
Figure 8A. Generation of a cdc5-aid auxin-inducible degron allele....................... 101
Figure 8B. cdc5-aid cell growth is impaired during auxin treatment .................... 102
Figure 8C. cdc5-aid cell growth is impaired a second yeast strain background .......... 103
Figure 8D. cdc5-aid viability is rescued by expression of extrachromosomal CDC5 .... 105
Figure 9A. Characterization of cdc5-aid cell growth in YNB ................................ 107
Figure 9B. Auxin-treated cdc5-aid cells arrest late in the cell cycle ...................... 109
Figure 9C. Characterization of cdc5-aid cell growth in YPD .............................. 110
Figure 9D. Auxin-treated cdc5-aid cells hold late cell cycle arrest overnight .......... 113
Figure 9E. Quantification of cdc5-aid late cell cycle arrest after auxin treatment .... 115
Figure 9F. Other cdc5 mutants arrest similar to cdc5-aid ................................. 117
Figure 10A. Characterization of Mcd1 chromatin association in cdc5-aid arrest cells... 121
Figure 10B. Mcd1 enrichment near CEN3 DNA is similar in auxin and nocodazole treated cdc5-aid cells ................................................................. 123
Figure 10C. Mcd1 persists at other CEN DNAs during cdc5-aid arrest .................... 124
Figure 10D. Quantification of cdc5-aid late cell cycle arrest after synchronous G1 release ................................................................................................................ 127
Figure 10E-H. Mcd1 persists at CEN3 DNA during late cell cycle arrest in cdc5-aid cells but not other loci ......................................................................................... 129
Chapter I: Polo-Kinases and the Regulation of Cohesin Disassociation

From Chromosomes

1.1 Introduction

An essential aspect of cell division is the organization of chromosomes such that duplicated sister chromatids are held together after DNA replication and until mid-mitosis when chromosomes are separated from each other. Chromosome segregation also requires that each duplicated sister chromatid form an attachment to a bipolar mitotic spindle, which is comprised of two spindle poles/microtubule organizing centers (MTOC) on opposite sides of the dividing cell. Spindle poles, the microtubules that emanate from them, and motor proteins, compromise the chromosome segregation apparatus that mechanically pull chromosomes poleward. Microtubules attach to chromosomes at centromeres, specialized DNA sequences that assemble a large proteinaceous structure (assembly of >60 proteins) called the kinetochore, which is capable of binding to a single microtubule in budding yeast (Biggins, 2013). Proper kinetochore-microtubule attachment of all pairs of sister chromatids to the mitotic spindle is called biorientation. Biorientation is requisite for a dividing cell to initiate chromosome segregation, thereby ensuring the fidelity of chromosome transmission between generations.

In the first portion of this Chapter, I describe the cellular components involved in assembling a chromosome architecture which promotes biorientation, with an emphasis on the central role of cohesin in enabling this architecture, via its association with chromosomes at chromosomal arms and centromeres. I will also discuss cohesin’s cell cycle regulation, as mediated by cohesin accessory factors.
In the second part of this Chapter, I describe polo-like kinases (PLKs) and their general roles as master regulators of mitotic cell division, and then detail the role PLKs play in the regulation of cohesin. Furthermore, I will compare and contrast functional differences in PLKs between budding yeast and metazoans.

1.1.1 Chromosome segregation requires centromere architecture mediated by the essential protein cohesin.

Central to chromosomal biorientation on the mitotic spindle is the formation of chromosome architecture mediated by an essential protein complex call cohesin. Cohesin complexes physically link sister chromatids together, which generates ‘cohesion’ between chromosomes prior to biorientation. When attached to the bipolar spindle, cohesion resists the pulling forces driving the sister chromatids towards opposite spindle poles. During budding yeast mitosis, in cells with bioriented chromosomes, cohesin bound to centromeres can be conceptualized as a subcellular structure described as a ‘cohesin barrel’ (Stephens et al., 2013). The cohesin barrel depicts a three-dimensional arrangement of chromosome-associated cohesin complexes, forming an elongated hollow cylinder that encompasses microtubules of the mitotic spindle. The distribution of cohesin complexes within the cohesin barrel and along chromosome arms suggests that cohesin complexes are engaged in both intra- and inter-chromosomal tethering. In the centromeric domain, spanning ~25 kilobases on either side of the centromere (the pericentric region), cohesin is thought to mediate intrachromosomal DNA interactions among distal DNA sequences on either side of the centromere, where cohesin molecules tether the same strand of DNA to form an extruded centromeric “loop” structure (Yeh et al., 2008). In contrast, cohesin enriched at chromosomal arm sites is used to pair sister
chromatids along the linear axes of DNA molecules, thereby generating sister chromatid cohesion.

The intrachromosomal linkages between DNA sequences on either side of the yeast centromere within the pericentromeric region, necessitates that a DNA loop is formed at the pericentromere, where the 125 base pair centromeric (CEN) DNA and the kinetochore formed at it are contained at the tip of the loop (Yeh et al., 2008; Bloom, 2014). Cohesin within the pericentromeric region enhances the fidelity of chromosome segregation and is hypothesized to create a DNA structure that facilitates biorientation by promoting attachments of sister kinetochores to microtubules from opposite spindle poles (Eckert et al., 2007). The organization of pericentromeric cohesin constrains linear chromosomes, forcing them to adopt a cruciform-shaped structure, as pericentromeric DNA loops jut out perpendicularly in opposite directions, from the axes of the linear pair of chromosomes. The cruciform DNA is proposed to create a back-to-back sister kinetochore geometry, which biases the chromosome for biorientation by rendering the kinetochore-microtubule attachment sites at centromeres sterically accessible to microtubules originating from opposing spindle poles. In the back-to-back configuration, once a single kinetochore-microtubule attachment to a microtubule emanating from one pole is made to a first sister kinetochore, the second sister kinetochore becomes aligned facing towards the opposite second pole, thereby favoring an attachment to the second pole. Thus cohesin-imposed chromosome architecture creates an intrinsic bias of paired sister chromatids for biorientation.
1.2 Cohesin is Important for Many Cell Cycle Activities

1.2.1 General functions of cohesin. In addition to playing an essential role in biorientation during cell division, cohesin complexes are involved in a myriad of other processes including chromosome condensation, regulation of gene expression and homology-directed DNA repair (Sjogren & Nasmyth, 2001), as well as meiotic chromosome segregation (reference), meiotic recombination (Brar et al., 2009) and large scale organization of chromatin via modulation of topologically adjusted domains (Pombo & Dillon, 2015). In budding yeast, the ribosomal DNA (rDNA) locus constitutes a special chromatin domain consisting of many tandem rDNA gene repeats domain, and is organized by cohesin (Laloraya, Guacci, & Koshland 2000). Cohesin may also be involved in silencing the retrotransposition of yeast Ty1 elements (Ho et al., 2015). At metazoan enhancers, a general transcriptional co-cofactor called a mediator can recruit the cohesin complex (Kagey et al., 2010). The association of cohesin with a mediator is thought to form a DNA loop, which would bring distal transcription factors into close proximity to promoters bound by RNA polymerase II, where it can regulate the transcription apparatus. While these roles of cohesin are important for understanding cohesin, the emphasis of this study will be on the role of cohesin in the mitotic cell division cycle.

1.2.2 The Structure and genomic localization of the cohesin holocomplex:

SMC family super structure and conservation. Structural Maintenance of Chromosomes (SMC) family complexes are a conserved family of DNA-binding proteins that mediate DNA linkages; the best-characterized examples of SMC complexes are cohesin and condensin, which are required for chromosomal transmission in eukaryotes
At their core, all SMC complexes contain an SMC-SMC dimer; cohesin and condensin are comprised of Smc1/Smc3 and Smc2/Smc4, respectively. Other SMC complexes include the bacterial MukB/MukB homodimer and the DNA repair Smc5/Smc6 complex (Losada, Hirano & Hirano, 1998; Fujjoka et al. 2002; Jeppsson et al., 2014). In the prokaryotic *Escherichia coli*, the loss of MukB function leads to defects in the partitioning of replicated chromosomes; this observation underscores the evolutionary importance of SMC complexes (Niki et al., 1992; Losada & Hirano, 2005, 2007).

Individual SMC proteins are large (>140kDa) molecules that fold back on themselves such that their N and C termini form a globular head domain while a hinge domain is formed at the point of folding (Nasmyth & Haering, 2009). A long, antiparallel coiled-coil region separates the head and hinge domains (Nasmyth & Haering, 2009). The hinge domain is named as such because it mediates SMC-SMC interaction by forming a V-shaped heterodimer (Haering et al., 2002). SMC dimerization is critical for cohesin complex interaction with DNA (Hirano & Hirano, 2002). The head domains of each SMC subunit can also bind to form a large 35nm ring complex that has been proposed to encircle DNA (Zhang & Pati, 2009). Each SMC subunit head domain contains ATP-binding cassettes of the ABC family that, when bound to the other SMC subunit, forms two functional ATPase domains. The ATP hydrolysis activities of Smc1 and Smc3 are thought to regulate the opening and closing of the SMC ring hinge domain, which allows DNA entry and exit and hence, cohesin association with chromosomes (Arumgam et al., 2003; Weitzer et al., 2003). At least one of these ATPase domains can be tuned to adjust
cohesin’s DNA-tethering activity (Camdre et al., 2015) and ATPase is also required for cohesin acetylation (discussed more below) (Ladurner et al., 2014).

In addition to the Smc1-Smc3 dimer, the core cohesin complex is comprised of additional subunits including Mitotic chromosome determinant 1/Sister chromatid cohesion 1 (Mcd1/Scc1), and Scc3 (Guacci, 1999; Toth et al., 1999; Haering et al., 2002). Pds5 and Wpl1 further associate with the cohesin holocomplex, although often in substoichiometric amounts (Losada et al., 1998; Kuleminza et al., 2012).

Within the cohesin ring complex, Mcd1 binds to and bridges the head domains of Smc3 and Smc1, with its N and C-termini respectively, thereby securing the topology of a tripartite cohesin ring structure. Mcd1 is a member of the kleisin family (Schleiffer et al., 2003); in budding yeast the kleisin subunits of cohesin are Mcd1 and Recombination 8 (Rec8), variants expressed specifically in mitosis and meiosis, respectively. Across the kleisin family, the N- and C-termini share protein sequence homology (Schleiffer et al., 2003). The middle “linker” region of Mcd1 also contains a conserved region that binds Scc3 (Orgil et al., 2014), an interaction essential for viability and other regulatory aspects of cohesin (Roig et al., 2014; Orgil et al., 2014).

The molecular mechanisms by which cohesin complexes tether DNA molecules are disputed. Experimental observations support various models, although it is generally believed that cohesin complex forms a ring-like structure that interacts with either one or two DNA fibers (Haering, Löwe, Hochwagen, & Nasmyth, 2002; Gruber, Haering & Nasmyth, 2003; Eng et al., 2015). Much study has gone into the mechanisms of opening and closing of the putative cohesin ring complex, how DNA tethering activity of cohesins
are established and maintained, and the detailed molecular nature of the cohesin ring complex (see below).

1.2.3 **Cohesin association with the yeast genome.** The distribution of genomic sites of cohesin association throughout the yeast genome has been extensively mapped and these sites are termed cohesin associated regions, or CARs (Eckert, Gravdahl, & Megee, 2007; Glynn et al., 2004; Laloraya et al., 2000; Rossio et al., 2010). In budding yeast, CARs are rich in adenine and thymine and are spaced approximately ~10 kilobases apart on average (Glynn et al., 2004; Laloraya, Guacci, & Koshland, 2000; Lengronne et al., 2004). Laloraya, Guacci, and Koshland (2000) reported that CARs are on average 0.8 to 1 kilobase in length. Generally, CARs are found on the same chromosomal locations during meiosis (Glynn et al., 2004; Kiburz et al., 2005).

In budding yeast, a 50-60 kilobase pericentromeric region that centers at and surrounds the 125bp centromere is highly enriched for cohesin (Eckert et al., 2007; Glynn et al., 2004; Kiburz et al., 2002; Weber et al., 2005). The increased scale of cohesin association near centromeres relative to the rest of the genome suggests its importance in this region. The magnitude of pericentromeric cohesin association as assayed quantitatively by chromatin immunoprecipitation (ChIP) is roughly three-fold higher than elsewhere in the genome chromosomal arm CARs (Eckert et al., 2007; Glynn et al., 2004; Laloraya et al., 2000; Bloom, 2014). Furthermore, the width of cohesin peaks as measured by cohesin ChIP appears greater in the pericentromeric region than at arms CARs. Cohesin enrichment at pericentromeric regions is dependent on the biorientation status of chromosomes (Eckert et al., 2007), and cohesin complexes can increase dynamically with pericentromeric DNA pre-anaphase depending on the tension status of
centromeres (Ocampo-Hafalla et al., 2007), unlike at other CAR sites engaged in sister chromatid cohesion, where cohesin association with DNA remains unchanged (Eckert et al., 2007; Ocampo-Hafalla et al., 2007; Fernius et al., 2013). The magnitude of pericentromeric DNA association of depends on DNA fork replication factor (Fernius et al., 2009), as well the Ctf19 complex (Fernius et al., 2013).

1.3 Cohesin Activity is Cell Cycle Regulated

The association of cohesin with chromosomes is a cell cycle-regulated process comprised of chromosome binding/loading, cohesion establishment, maintainence, cleavage and dissociation. At the G1/S boundary of the cell cycle of budding yeast, cohesin binds to chromosomal DNA and associates with chromosomal DNA via association with the cohesin-loading complex comprised of Scc2/Scc4 (Ciosk et al., 2000). Cohesin and its loading complex are mutually dependent for their association with DNA, and both are present at CARs (Kogut et al., 2009). The loading complex is also required for increased magnitude of cohesin association at pericentromeric DNA (Fernius et al., 2013). One proposed mechanism for cohesin loading involves the opening of the cohesin ring, which requires the ATPase activity of the SMC head domains, and encircles the DNA fiber (Chan et al., 2012). One possibility is that cohesin loading transduces the force of ATP hydrolysis to mediate establishment of cohesion, however the mechanism of cohesin loading remains an area of ongoing study (Hu et al., 2011; Ladurner et al., 2014).

After initial binding to DNA, cohesin is toggled from a non-tethering to a tethering state during S-phase, and involves the conversion of chromosome-bound cohesin complexes to cohesive complexes actively engaging in linking replicated sister
chromosomes. During cohesion establishment, cohesin is acetylated on its Smc3 subunit by the activity of Eco1 acetyltransferase. Importantly, Smc3 acetyl-null mutants are inviable and fail to establish sister chromatid cohesion (Ben-Shahar et al., 2008; Unal et al., 2008). Smc3 acetylation (and hence the activity of Eco1) is restricted to S-phase, thereby limiting the establishment of sister chromatid cohesion to S-phase (Lyons & Morgan, 2011).

The cohesin subunit Scc3 contains a conserved domain that is known to interact with the cohesin-loading complex. This domain is also required for Scc3 interaction with Mcd1, as well as cohesin association with chromosomes and the process of cohesion (Orgil et al., 2015). Interestingly, the cohesin-loading complex can also interact with cohesin complexes independent of Scc3, albeit with reduced efficiency (Orgil et al., 2015).

In budding yeast, the establishment of chromosome biorientation is coupled to concurrent centromeric DNA replication (Natusme et al., 2013) and the establishment of centromeric cohesion during S-phase (Goshima & Yanagida, 2000). The kinetochore recruits replication licensing factors Synthetically Lethal with Dpb11-1 3 (Sld3)-Sld7, to early firing origins near the centromere that initiates centromeric DNA replication during early S-phase (Natusme et al., 2013), and also recruits the cohesin loading complex (Fernius et al., 2013). Cohesin and its loading complex are mutually dependent on one another for their association with centromeres, and once cohesin is loaded, it spreads away from the centromeres along the adjacent pericentromeric DNA (Fernius et al., 2013; Hu et al., 2011). Mutations in cohesin subunits that render them unable to hydrolyze ATP allow cohesin to load at the centromere region, but not to translocate away from the
centromere (Hu et al., 2011). Thus, the partially duplicated sister centromeres establish pericentromeric cohesion, which leads to subsequent biorientation (Goshima & Yanagida, 2000).

The coupling of biorientation to centromere replication may be one means by which yeast promote accurate chromosome segregation. DNA replication and biorientation are not coupled in higher metazoans, as a distinct G2 phase typically separates S-phase from metaphase. In metazoans, the breakdown of the nuclear envelope during prophase allows growing microtubules emanating from the centrosome to invade and interact with chromosomes, ultimately leading to their binding to kinetochores and to chromosome biorientation. In budding yeast, the spindle pole body (SPB), analogous to the metazoan centrosome, is embedded in the nuclear membrane throughout the cell cycle (Rout & Kilmartin, 1990). Within the nucleus, centromeres remain clustered near the SPB throughout the majority of the cell cycle, including G1 due to their interactions with microtubules that originate from the SPB (Guacci et al., 1994; Jin et al., 1998). The SPB duplicates and separates during S-phase, forming an intranuclear spindle. Thus, the nuclear envelope does not have to break down for the spindle to interact with chromosomes.

In addition to cleavage, budding yeast cohesin complexes are subjected to various post-translation modifications such as the aforementioned acetylation [this needs to be rewarded, not the entire coplex gets cleaved or acetylated]. Small ubiquitin-like modification (SUMO), or SUMOylation has been identified as important in regulation of chromosome segregation via its role in the regulation of Mcd1 during mitosis. Recently, sumoylation has been identified in regulation of the boundary between cohesin
complexes that are found within the pericentromeric region and cohesins involved in sister chromatid cohesion outside the pericentromeric region (MacAleenan et al., 2012; Almedawar et al., 2012; D’Ambrosio & Lavoie, 2014; Stephens & Bloom, 2015). A second important modification is regulation of the cohesin complex by polo-like kinase Cdc5 through phosphorylation of the cohesin subunit Mcd1, which will be discussed at length below.

1.4 Overview of the Centromere

Throughout eukaryotes, the hallmark feature of centromeres is the presence of nucleosomes of unique composition, in which the canonical histone H3 is replaced by the centromere-specific histone H3 variant, Centromere Protein A (CENP-A) (Henikoff & Henikoff, 2012). CENP-A containing nucleosomes establish the base for nucleating the kinetochore, upon which all other kinetochore components are assembled (discussed in more detail below). CENP-A retains an essential role in demarcating the presence of centromeres in budding yeast; however, remarkably, budding yeast and closely related yeast species appear to be exception to this rule of requirement for CENP-A dependent assembly of all other kinetochore proteins. Most higher eukaryotes contain regional centromeres that do necessarily maintain a preference for DNA sequence DNA that span from hundred of thousands of bp to megabases in length. Budding yeast and CENs from closely related fungal species evolved from regional centromeres into a DNA sequence-specific ‘point’ centromeres that are approximately 125 bp in length (Clark and Carbon, XX). The evolutionary quirk in budding yeast of CENP-A not being required for assembly of all other kinetochore proteins can be explained by the presence of centromere-DNA specific binding proteins (CBF3 complex and to a lesser extent Cbf1),
which associate directly with centromere DNA and the CENP-A nucleosome (Meraldi et al., 2006).

In metazoans, CENP-A containing nucleosomes are interspersed with regular nucleosomes along the length of the centromere (Blower, Sullivan & Karpen, 2002). The repeated CENP-A nucleosome may each assemble a single kinetochore subunit into a modular repeat pattern, to which multiple microtubules bind (Zinkowski et al., 1991). In budding yeast, a single centromeric nucleosome assembles one kinetochore capable of binding a single microtubule (Furuyama & Biggins, 2007; Cole, Howard & Clark, 2011; Henikoff & Henikoff, 2012; Henikoff et al., 2014). Thus, it is unclear how representative budding yeast centromere architecture may be of other metazoans (Bloom, 2014); however, the simplicity allows for tractability of centromere studies using ChIP (Meluh et al., 1998), or in vivo epifluorescence microscopy combined with computational modeling (Stephens et al., 2013).

1.5 Overview of Kinetochore

All eukaryotic centromeres/kinetochores, including budding yeast, have the ability to recruit cohesins (Bloom, 2014). The kinetochore is not only a passive DNA-protein linker to the mitotic spindle; it is a dynamic regulatory platform that senses the status of microtubule-kinetochore attachment/biorientation, and relays this information to the cell cycle via the spindle assembly checkpoint (SAC; discussed below). The kinetochore promotes biorientation by recruitment of various factors to the kinetochore, including proteins of the SAC, motor proteins that facilitate attachment, and kinases such as Aurora B that promote the correction of erroneous kinetochore-microtubule attachments (i.e., two sisters chromatids attached to the same spindle pole; Pinsky et al.,
2006), as well as cohesin. Thus, the recruitment and establishment of pericentromeric cohesin may represent a key factor in the establishment of biorientation at kinetochores.

1.6 The Structure of the Kinetochore

The conserved elements of the kinetochore include two large evolutionary conserved networks of protein, the first of which is the constitutive centromere-associated network (CCAN) and the second is the KNL1-MIS12/MIND-NDC80 (KMN) network (discussed below). While not all individual protein components are conserved in budding yeast, each of the three major CCAN subcomplexes are conserved, suggesting a high degree of functional conservation across eukaryotes (Lampert & Westermann, 2011). The CCAN is a complex of 16 chromatin-proximal proteins that bind to and interface with centromeric nucleosomes and associated DNA (Lampert & Westerman, 2011). In human cells, the composition of the CCAN at the kinetochore itself changes during cell cycle progression (Nagpal et al., 2015); the dynamics of cell cycle composition of the CCAN in budding yeast remains to be characterized. In metazoans, the ectopic localization of CCAN components can lead to recruitment of other kinetochore components, including CENP-A containing nucleosomes, underscoring the importance of the CCAN in the formation of the kinetochore structure (Hori et al., 2012). In budding yeast, the CCAN is also important for recruitment and establishment of cohesin to the pericentromeric region via the Chromosome Transmission Fidelity 19 (Ctf19) subcomplex, as well as maintaining cohesin at centromeres until biorientation is achieved (Eckert et al., 2007; Fernius Marston, 2009; Nerusheva et al., 2014).

The CCAN serves as a scaffold on which the KMN network is assembled (Cheeseman & Desai, 2008). The KMN is a group of three conserved protein complexes
that collaborate to bind microtubules and regulate microtubule dynamics. The KMN network also ensures biorientation by recruiting factors that sense sense tension and components of the spindle assembly checkpoint (SAC), which halt the cell cycle until the establishment of proper kinetochore-microtubule attachments.

1.7 The Spindle Assembly Checkpoint

Biorientation of all chromosomes is required for mitotic progression at the metaphase-to-anaphase transition. The lack of biorientation of a single pair of sister kinetochores delays the cell cycle by a pathway called the spindle assembly checkpoint (SAC) (Musaccio, 2015). All components of the SAC pathway localize to kinetochores, and a functional kinetochore is required for the SAC (Musaccio, 2015). SAC components are thought to sense the lack of kinetochore-microtubule attachment (Musaccio, 2015), as well as the lack of tension forces between sister centromeres (Musaccio, 2015), normally generated by cohesin complexes engaged in sister chromosome cohesion resisting the pulling forces of the bipolar mitotic spindle. The tension-generating spindle forces are imposed by microtubule dynamics and motor proteins, and, in metazoans, cause bioriented chromosomes to congress towards and align at the metaphase plate, a process that prepares them for the ultimate act of segregation in anaphase. Various types of incorrect microtubule-kinetochore attachments can form and must be corrected through in order for proper biorientation to occur and the cell cycle to proceed. Only proper configurations of microtubule-kinetochore attachments become stabilized and lead to a bioriented state. Aurora kinases phosphorylate the NDC80 complex which is responsible for binding directly to microtubules; this phosphorylation destabilizes erroneous kinetochore-microtubules attachments, thereby activating the SAC. Aurora kinase thus
promotes error-correction by the turnover of kinetochore-microtubule attachments. When all chromosomes are bioriented, proper tension across all sister centromeres is achieved, which leads to relief of SAC inhibition. Downregulation of the SAC allows the cell to transition from metaphase to anaphase (Lara-Gonzalez et al., 2012).

The SAC pathway halts the cell cycle by sequestering an activator of the Anaphase Promoting Complex (APC), Cell division cycle 20 (Cdc20); both the APC and Cdc20 are required for the cell to enter anaphase (Cohen-Fix et al., 1996; Shiryama et al., 1998). When bound to Cdc20, the APC functions as an E3 ubiquitin ligase that targets a number of proteins including securin (Precocious disassociation of sisters 1, Pds1, in budding yeast) for ubiquitination and subsequent proteolytic destruction by the proteasome (Cohen-Fix et al., 1996). Securin functions by binding to and sequestering a protease called separase (Extra spindle pole bodies 1, Esp1, in budding yeast) whose chief activity is to enzymatically cleave the kleisin subunit of the cohesin ring complex to allow anaphase onset (Yanamato et al., 1996; Ciosk et al., 1998; Uhlmann et al., 1999; Uhlmann et al., 2000).

During anaphase onset in budding yeast, cleavage of cohesin subunit Mcd1 irreversibly disrupts the topology of the cohesin rings, thereby untethering formerly ‘cohesed’ sister chromatids, and allowing the chromosomes to be pulled towards opposite spindle poles via shortening of microtubules attached to the kinetochores. The poleward movement of the chromosomes is termed anaphase A. During anaphase B, the mitotic spindle elongates but does not lengthen the cell, facilitating the movement of chromosomes away from the plain of cell division.
1.8 Polo-like Kinases

1.8.1 Metazoan polo-like kinases. Polo-like kinase family members are conserved from yeast to humans and are essential for eukaryotic life. The founding family member, polo, was discovered in *Drosophila melanogaster* as mutants that express the eponymous polo phenotype: a circular or wheel-like pattern around the centriole (Sunkel & Glover, 1988). Subsequently, polo-like kinase homologs were identified in other organisms, and polo-like kinase-depleted cells typically give rise to abnormal spindle morphology phenotypes and defects in chromosome segregation (Sumara et al., 2004). The human genome encodes five polo-like kinase variants, the best characterized of which is Plk1. Human Plk1 has essential mitotic roles (Petronczki et al., 2008). Loss of Plk1 leads to early embryonic death in murine models, consistent with its essential role in cell division (Lu et al., 2008). Plk1 localizes to subcellular structures including the centrosome, kinetochore and central spindle and changes location over the course of cell cycle progression (Barr et al., 2004; Achambault et al., 2009; Zitouni et al., 2014). Plk1 is observed at kinetochores as early as prophase, persists until metaphase—although intensity fades upon biorientation, and dissipates altogether at anaphase.

1.8.2 Yeast polo-like kinase Cdc5. In contrast to humans, budding yeast *Saccharomyces cerevisiae* express a single polo-like kinase protein, Cdc5. Cdc5 is essential (Hartwell et al., 1973; Song et al., 2000; Park et al., 2002). The fission yeast *Schizosaccharomyces pombe* and the fruit fly *Drosophila melanogaster* also encode single essential polo-like kinase proteins. It is unclear whether in yeast species a single isoform of polo-like kinase can carry out the diverse array of duties attributed to metazoan polo-like kinases. However, remarkably, much of the function has been
conserved despite millions of years of evolutionary divergence between yeast and human; an ectopically expressed human Plk1 (and Plk3) isoform is sufficient to support the viability of budding yeast cdc5 mutants (Lee et al., 1998).

1.8.3 The Structure of Polo-like Kinases. Polo-like kinases localize to their various targets via a C-terminal phosphobinding domain called the polo-box domain (PBD) (Lowery, Lim, & Yaffe, 2005). To bind to its targets, the conserved PBD typically requires target amino acid residues to be phosphorylated by a priming phosphorylation (Elia, Cantley & Yaffe, 2003; Lowery et al., 2005; Park et al., 2010). The priming phosphorylation is typically performed by cyclin-dependent kinases (Cdk) or polo-like kinases themselves. The PBD recognizes primed serine or threonine residues and grabs onto phosphorylated proteins with a “pincher” domain, thereby tethering the polo-like kinase catalytic domain to its substrate (Lowery et al., 2005). Thereafter, polo-like kinases are capable of engaging in processive phosphorylation by subsequently phosphorylating other residues on the PBD-bound protein (Lowery et al., 2005). The N-terminal serine/threonine kinase domain of target-unbound polo-like kinases is negatively repressed by the PBD. Therefore, PBD-dependent binding liberates and activates the kinase domain, allowing the substrate to be phosphorylated by the kinase (Lee & Erikson, 1997; Mundt et al., 1997; Jang et al., 2002; Elia et al., 2003). In some instances, allosteric inhibition of the polo-like kinase domain can be achieved by the PBD binding its substrate (Kachaner et al., 2015). An activating allosteric phosphorylation can liberate the PBD-binding and promote an active kinase domain (Qian et al., 1999; Carmena et al., 2012; Kachaner et al., 2015), as well as the relocation of polo-like kinase within the cell (Kachaner et al., 2015). Thus, polo-like kinases are regulated by the complex interplay of
PBD-binding to target proteins, self-repression, and allostERIC inhibition and activation, as well as subcellular localization and subsequent redistribution throughout the cell division cycle.

Both the kinase domain and polo-box domain (PBD) of Plk1 are required to complement cdc5-1, a conditional temperature-sensitive (t.s.) allele of CDC5 (Lee et al., 1998). The evolutionary conservation of polo-like kinase structure and function in budding yeast make it an optimal model organism for studying polo-like kinase in mitotic chromosome segregation. Cdc5 contributes to major cell cycle transitions by activating phosphorylation of Cdk and the anaphase-promoting complex late in cell cycle (Charles et al., 1998). Furthermore, Cdc5 contributes to the metaphase-to-anaphase transition, as well as the degradation of cyclin B prior to mitotic exit (Charles et al., 1998). Accordingly, Cdc5 is expressed and localizes to its various subcellular domains in a cell cycle-dependent manner (Lee et al., 1998). Like its metazoan counterparts, Cdc5 localizes to discrete cellular structures including the bud neck and spindle pole bodies, which correspond to the cytokinetic ring/cleavage furrow and centrosomes, respectively, in metazoans (Lee et al., 2005). Cdc5 mRNA and protein expression rise in S-phase and peak in M-phase (Shiryama et al., 1998; Darieva et al., 2006; Snead et al., 2007). Cdc5 requires phosphorylation for maintenance of its protein levels, as well as activation of its kinase activity (Simpson-Lavy & Brandeis, 2011; Ratisma et al., 2011). Simpson-Lavy and Brandeis (2011) suggested that this stabilizing phosphorylation is likely mediated by Cdk1. Like its metazoan homologs, Cdc5 is found in the nucleus in metaphase, where it associates with chromatin and appears to be present at cohesin-associated regions (Yu & Koshland, 2005; Rossio et al., 2010; Botchkarev et al., 2014). Interestingly, Cdc5 may
bind to its own promoter, thereby enhancing its own gene expression in a positive feedback loop (Darieva et al., 2006). During anaphase, Cdc5 promotes its own destruction by phosphorylating and activating the APC E3 ubiquitin ligase complex which ubiquitinates Cdc5 thereby leading to its target destruction by the proteasome via two different APC-recognition motifs present in its protein sequence (Visintin et al., 2008; Arnold et al., 2015).

Polo-like kinases are master regulators of the cell cycle and are best characterized for their roles in mitotic progression and cytokinesis (Archambault & Glover, 2009; Zitouni et al., 2014); they have other roles in interphase, as well as non-cell cycle related functions. During mitosis, polo-like kinases are instrumental in activating Cdk1-cyclin B, in the assembly of the bipolar mitotic spindle, and in the regulation of mitotic exit (Archambault & Glover, 2009; Zitouni et al., 2014). Given their multitude of tasks in mitosis, polo-like kinases phosphorylate a myriad of proteins (Bibi, Parveen, & Rashid, 2013; Santamaria et al., 2011). A fundamental question is how can a master regulator achieve the specificity to coordinate all of these diverse mitotic processes? A primary means is through the regulation of polo-like kinase localization, by binding to different proteins at distinct subcellular structures as mitosis progresses (Archambault & Glover, 2009; Park et al., 2010).

1.9 Polo-Like Kinase and the Centromere/Kinetochore

In budding yeast, the polo-like kinase Cdc5 is not known to localize to the kinetochore, although it has been shown to co-localize with cohesin on the left arm of chromosome six, including sites near centromeres (Rossio et al., 2010). In higher eukaryotes, polo-like kinases are known to localize to kinetochores, where numerous
targets have been characterized (Wong & Fang, 2006; Elowe et al., 2007; Wong & Fang, 2007; von Kim et al., 2014; Schubert et al., 2015). In budding yeast, Cdc5 has been shown to associate with or phosphorylate numerous kinetochore proteins, many of which are highly conserved. These kinetochore-localized components include Cse4 (i.e. metazoan CENP-A), Ndc80, as well as Stu2, and Slk19 (Kang et al., 2006; Kim et al., 2014; Park et al., 2008; Park et al., 2015; Snead et al., 2007). In metazoans, the association of polo-like kinases with kinetochores and their kinase activity at kinetochores increases until biorientation (Lenart et al., 2007; Liu et al., 2012), when the SAC signal is quelled, leading to the onset of anaphase and the cleavage of cohesin by separase. A major question is how the cell perceives chromosome biorientation and relays the signal to cleave and disassociate cohesin. These disassociation processes are poorly understood and merit further study. Cohesin cleavage is discussed below. A likely regulator of these processes is the kinetochore-localized polo-like kinase. Given the colocalization of polo-like kinase with cohesin during mitosis (Rossio et al., 2010), and that cohesin is a known substrate of polo-like kinase phosphorylation (Alexandru et al., 2001; Hornig & Uhlmann, 2004; Hauf et al., 2005), I hypothesized that polo-like kinase Cdc5 may bind and phosphorylate cohesin in the vicinity of the kinetochore, promoting its disassociation from chromatin.

1.10 Polo-Like Kinases and Biorientation

In addition to localizing to centrosomes and centromeres/kinetochores (Archambault & Glover, 2009; Park et al., 2010), polo-like kinases are found on microtubules themselves (Archambault et al., 2008) in the fruit fly. Taken together, these
observations suggest the importance of polo-like kinases in regulating the chromosome segregation apparatus.

However, it is unclear if polo-like kinase-dependent defects are due to disruption of the organization of the mitotic spindle, kinetochore or centromere architecture. In budding yeast, \textit{cdc5} mutants give rise to misaligned spindles, as well as aberrantly formed spindle pole bodies (Park et al., 2008; Ratsima et al., 2011; Snead et al., 2007). In the original \textit{Drosophila polo} mutant, cells arrested with monopolar spindles and chromosomes that were not bioriented (Sunkel & Glover, 1988). However, this mutant was likely a hypomorph and not a true null for polo-like kinase activity. Given the requirements for fully formed spindles for biorientation, it may be difficult to discern whether aberrations in kinetochore function due to polo-like kinase inactivation are directly due to polo-like kinases’ roles at kinetochores, or indirectly due to aberrant formation of mitotic spindles (Sunkel & Glover; 1998; Moutinho-Santos & Sunkel, 2012; Ratsima et al., 2011). Cells that expressed a different polo-like kinase mutant in \textit{Drosophila} appeared to progress further in mitosis and arrested with bipolar spindles and bioriented chromosomes, as well as aberrant kinetochore architecture that included a shortened inter-kinetochore distance and the abnormal displacement of kinetochore proteins (Moutinho-Santos Conde, & Sunkel, 2012). Yeast cells also display variations in \textit{cdc5} mutant arrest phenotypes depending on the mutant allele analyzed (Park et al., 2008; Snead et al., 2007; Ratsima et al., 2011). Commonly observed phenotypes include large budded arrest late in the cell cycle with aberrant spindle morphology. In several instances chromosome segregation defects have been reported, particularly at telomeres (Alexandru et al., 2001; Snead et al., 2007). Ratsima et al. (2011) report differing
phenotypes when the kinase domain or PBD domain are lost; further they report a novel phenotype when the PBD is inhibited such that spindle pole morphology is abnormal and accumulation of 4N DNA contents. Thus, the choice of the experimental ablation method of polo-like kinases may impact the observed phenotypic cell cycle-dependent outcomes, likely due to different degrees of Cdc5 inactivation (Moutinho-Santos et al., 2012; Ratisma et al., 2011).

1.11 Polo-like Kinase Regulation of Cohesin in Yeast and Humans

Polo-like kinases regulate the disassociation of chromosomal cohesin in eukaryotes (Losada et al., 2002; Hauf et al., 2005; Giménez-Abián et al., 2004; Alexandru et al., 2001), and like cohesin, the loss of their function is associated with abnormal chromosome segregation and genome instability (Donaldson et al., 2001; Ratsima et al., 2011; Sunkel & Glover, 1988). In the mitotic cell cycle, cohesin associates with chromosomes during S-phase (Ciosk et al., 2000) and remains present on chromosomes until mitosis, where it is removed in a stepwise temporal fashion and at specific genomic loci. In metazoan cells, at least, two distinct pathways mediate cohesin disassociation from chromosomes: The first pathway is polo-like kinase-dependent; the second is dependent on the proteolytic destruction of the cohesin Scc1/Rad21 (yeast Mcd1) subunit (Losada, 2014). The cohesin ring is phosphorylated by polo-like kinases during prophase and subsequently removed (Hauf et al., 2005; Losada, Hirano, & Hirano, 2002; Giménez-Abián et al., 2004). Little is known about the mechanism of the prophase cohesin removal process other than that cohesin complexes are removed by a separase-independent mechanism, and cohesin disassociation appears limited to the arms of chromosomes (Hauf et al., 2005; Losada et al., 2002; Sumara et al., 2000). The
association of cohesin near centromeres is preserved until the metaphase to anaphase transition, when cohesin is cleaved by separase (Hauf et al., 2001); this destroys the integrity of the cohesin ring, and liberates the embrace of sister chromatids, thereby allowing them to separate. In human cells, polo-like kinases associate with centromeres (or kinetochores), however it is not understood whether they also contribute to cohesin disassociation at anaphase onset within this chromosomal region (Archambault & Glover, 2009; Park et al., 2010). Given their role in prophase in removing chromosomal arms cohesins (Losada et al., 2002; Giménez-Abián et al., 2004; Hauf et al., 2005), it is possible that human polo-like kinases also contribute to the separase-dependent cleavage of cohesin via the phosphorylation of cohesin at centromeres during anaphase. My hypothesis is that cohesin disassociation from DNA and separase cleavage represent distinct phenomena and thus may operate in parallel pathways. However, these phenomena need not be mutually exclusive; at the metaphase-to-anaphase transition cleavage by separase itself may not be sufficient to remove cohesin from DNA, and the activity of polo-like kinase may be required for an additional disassociation step during anaphase, just as they promote the disassociation of cohesin during prophase in metazoans (Losada et al., 2002; Giménez-Abián et al., 2004; Hauf et al., 2005).

In contrast to the stepwise cohesin removal that occurs in metazoan cells, in budding yeast chromosome-associated cohesin is thought to be removed in bulk at the onset of anaphase (Alexandru et al., 2001). The budding yeast polo-like kinase Cdc5 is thought to phosphorylate the Mcd1 subunit of cohesin before cohesin's cleavage by separase at the onset of anaphase (Alexandru et al., 2001; Hornig & Uhlmann, 2004). Hornig and Uhlmann (2004) have demonstrated that the proteolytic cleavage of yeast
cohesin by separase is enhanced \textit{in vitro} by phosphorylation by recombinant human polo-like kinases. The same amino acid residues within the yeast cohesin complex subunit, Mcd1, that are phosphorylated by human Plk1 \textit{in vitro} and are also phosphorylated \textit{in vivo} by a kinase proposed to be Cdc5 (Alexandru et al., 2001; Swaney et al., 2013).

While in the Alexandru et al. (2001) study the electrophoretic mobility shift of Mcd1 was reduced in cells expressing a Cdc5-depletion allele, but not in control cells, this result is a qualitative comparison, and does not directly show that Cdc5 directly phosphorylates Mcd1. Further, Alexandru et al. (2001) did not examine the efficacy of Cdc5 depletion; as such, in this study it remains possible that some lingering Cdc5 protein activity remains in these cells.

Ratisma et al. (2011) demonstrated that the effects of Cdc5 perturbation on Mcd1 depend on the particular \textit{cdc5} mutant employed. A mutant that disrupts PBD function shows little effect on Mcd1 phosphorylation, while in cells expressing a mutation that impairs the kinase domain, little phosphorylation of Mcd1 was observed (Ratisma et al., 2011). Thus, within budding yeast cells, the kinase that phosphorylates Mcd1 is most likely Cdc5 (Alexandru et al., 2001; Hornig & Uhlmann, 2004; Ratisma et al., 2011; Snead et al., 2007). Expression of a mutant \textit{mcd1} allele lacking these phosphorylation sites causes defects in chromosome segregation during mitosis; these defects are more severe when the cohesin cleavage regulatory pathway is also disrupted by loss of \textit{pds1} (Alexandru et al., 2001). Depletion of Cdc5 activity from yeast cells caused slight defects in the timing of chromosome segregation near telomeres but not at centromeres (Alexandru et al., 2001; Snead et al., 2007). Taken together, this collection of
observations implies that polo-like kinases collaborate with separase-dependent cleavage to remove cohesins from chromosomes at anaphase onset.

Due to limits of resolution of the methods previously employed by Alexandru et al. (2001), it is not understood whether budding yeast possesses a two-step cohesin removal that transpires during prophase and then metaphase, at cohesin arms and then at centromeres, respectively. Both cohesin and Cdc5 were shown to be associated with chromatin via low-resolution chromatin spread assays, and co-localized via ChIP on chromosome 6 (Hornig & Uhlmann, 2004; Yu & Koshland, 2005; Rossio et al, 2010).

Given the nature of their enzyme-substrate relationship, and co-localization on chromosomes, my prediction is that the loss of function of either cohesin or Cdc5 may perturb the normal interaction of the other with chromatin. Surprisingly, neither protein appeared to depend on the other when assayed for association in chromosome spreads (Hornig & Uhlmann, 2004). As such, the effects of depletion of cohesin and polo-like kinase on one another may not be observable when using chromatid spreads. This approach is insufficient in determining more subtle changes in aggregate chromatin binding, particularly if the effects specifically occur at some loci but not others, or at different phases of the cell cycle. Thus, one possibility is that polo-like kinases may only localize to a subset of cohesin-associated regions (CARs) and, therefore, regulate a subset of chromosome-associated cohesin complexes. An alternative hypothesis is that, as in human cells, polo-like kinases may regulate cohesin in a stepwise fashion, first at CARs in prophase, and then at centromeres in anaphase. However, differences in cohesin regulation between yeast and humans may also reflect differences in the timing and process of biorientation, requirements for nuclear envelope breakdown, centromere
structure, or the number of microtubules required for biorientation between the two organisms.

Intriguingly, polo-like kinases are not known to regulate centromeric cohesin, despite the presence of polo-like kinases at centromeres in metazoan cells. Thus, there are apparent differences in cohesin regulatory processes between budding yeast and metazoans, both in terms of cell cycle timing, and the roles of separase and polo-like kinases at different chromosomal locations. It remains unclear whether these differences reflect gaps in knowledge regarding how the disassociation of cohesin occurs, or reflect true differences in chromosome segregation between organisms with different life cycles. Further, the role, if any, of polo-like kinases in the regulation of cohesin at centromeres, remains to be determined. In this study, I describe the co-localization of budding yeast polo-like kinase with cohesin on chromosomes, and demonstrate that polo-like kinase also has a role in the disassociation of cohesin from pericentromeric DNA. This study has a broader impact on our understanding of the conservation of regulation of cohesin disassociation among eukaryotes.

1.12 Conclusion

In light of the existing research to date, this study employed budding yeast *Saccharomyces cerevisiae* as a model to uncover the polo-like kinase-dependent regulation of cohesin association with chromosomes during mitosis. My results showed that: (a) Cdc5 colocalizes to the previously identified sites of cohesin association on chromosome arms, consistent with a previous report (Rossio et al., 2010; see Chapter 2), however, it is also found at centromeres, which was not previously identified (Rossio et al., 2010; see Chapter 2); (b) Cdc5 physically interacts with the cohesin subunit Mcd1 *in*
vivo (see Chapter 2); (c) Cdc5 association with chromosomes near centromeres is
cohesin-dependent (see Chapter 2); and (d) the depletion of Cdc5 leads to the persistence
of cohesin near centromeres during anaphase, but not at the distal arm sites examined
(see Chapter 3). These findings led to the following conclusions: (a) the polo-like kinase,
Cdc5, differentially regulates chromosomal arm cohesin and centromere cohesion, (b) the
removal of centromere cohesin complexes is not required for anaphase, and (c) Cdc5
activity is likely required for cohesin disassociation at pericentromeric DNA.

In this chapter, I summarized what is known about the role of polo-like kinase in
regulating cohesin association with chromosomes, with an emphasis of polo-like kinase
and its substrate cohesin’s roles during biorientation in mitosis. In Chapter 2, I will
characterize the relationship of Cdc5 with chromatin and show that it is cohesin-
dependent at centromeres. In Chapter 3, I will describe the generation and
characterization a conditional cdc5 allele, and utilize it to show that Cdc5 regulates the
persistence of cohesin at centromeres during anaphase.
Chapter II: Polo-Like Kinase Cdc5 Localizes to Cohesin-Associated Regions

2.1 Introduction

Proper chromosome segregation during mitosis requires that duplicated sister chromatids form attachments with microtubules emanating from opposing spindles poles. This process is called biorientation. Attachments form at the kinetochores, a large assembly of proteins that assemble on centromeric DNA. Kinetochores have two major functions: to mediate the capture of microtubules, and to sense the status of the microtubule attachment. The latter is mediated by aurora kinase (Biggins et al., 1999); lack of proper attachment transmits to the spindle assembly checkpoint (SAC), which delays the separation of chromosomes until the biorientation of all sister kinetochores is achieved (Musacchio, 2015). A failure to establish biorientation prior to chromosome segregation can result in aneuploidy, a condition in which a cell inherits an aberrant chromosome number. Therefore, pathways that promote biorientation are essential for safeguarding the integrity of the genome.

Cohesion facilitates biorientation by mediating the stable pairing of DNA molecules. Two types of cohesion are important for facilitating mitotic chromosome segregation. First, cohesion between duplicated sister chromatids is necessary for their association until chromosome separation. Second, cohesion near the centromere promotes biorientation by imposing a back-to-back sister kinetochore geometry that is favorable for attachment of microtubules, originating from opposite spindle poles (Yeh et al., 2008; Sakuno et al., 2009). Cohesion is mediated by cohesin complexes, which are thought to form a proteinaceous ring that encircles two DNA molecules (Nasmyth & Haering,
Cohesin rings associate with chromosomes at regular intervals along the arms of chromosomes and are prominently enriched at DNA near centromeres (Weber et al., 2004; Glynn et al., 2004). A DNA replication fork-associated factor Csm3 is important for cohesin association at pericentromeric DNA (Fernius & Marston, 2009). In addition to Csm3-dependent pericentromeric cohesin association, the kinetochore, in particular the Ctf19 complex directs increased cohesin to the pericentromeric DNA of chromosomes via association with the cohesin loading complex (Tanaka et al., 1999; Weber et al., 2004; Fernius & Marston, 2009; Fernius et al., 2013). Conversely, cohesin is diminished from pericentromeric DNA when chromosomes become bioriented (Eckert et al., 2007; Ocampo-Hafalla et al., 2007). In budding yeast, the failure to properly remove cohesin from chromosomes during metaphase results in incomplete chromosome segregation, and is associated with inappropriate cohesin persistence late in the cell cycle (Alexandru et al., 2000; Hornig & Uhlmann, 2004). Polo-like kinases (Plks) are integral to the establishment of biorientation and the removal of cohesins (Sumara et al., 2004; Elowe et al., 2007; Matsumura et al., 2007; Watanabe et al., 2009; Salimian et al., 2011; Shao et al., 2015; Losada et al., 2002; Hauf et al., 2005). In metazoans, a two-step cohesin removal process from chromosomes occurs in prophase and anaphase, respectively (Sumara et al., 2000; Sumara et al., 2002). During prophase, cohesin is first removed from the chromosomal arms by a mechanism that involves polo-like kinase 1 (Plk1)-dependent phosphorylation of cohesin, which essentially dissociates the cohesin from the chromosomal arms (Sumara et al., 2000; Sumara et al., 2002; Hauf et al., 2005). The second step, which occurs at the onset of anaphase, involves proteolytic cleavage of cohesin by the enzyme, separase, and the dissolution of cohesin at the centromeres.
(Alexandru et al., 2001). By contrast, budding yeast appear to have a single-step process for the removal of all cohesin, which is similar to the second phase in metazoans as it occurs during anaphase and is separase dependent. During this process, the yeast polo-like kinase Cell division cycle 5 (Cdc5) phosphorylates cohesin, which is thought to enhance bulk cohesin cleavage at anaphase (Alexandru et al., 2001; Hornig & Uhlmann, 2004), which is required for chromosome segregation (Alexandru et al., 2001; Hornig & Uhlmann, 2004). The negative effect of loss of Cdc5 activity on chromosome is exacerbated when regulation of the separase-dependent cleavage pathway is also disrupted (Alexandru et al., 2001).

In budding yeast, it remains unclear whether a separase-independent mechanism exists. If there is a separase-independent mechanism, it will be interesting to determine it requires Cdc5 and whether both pathways collaborate, as they do in metazoans, to remove all cohesins. Furthermore, site-specific removal of cohesin on chromosomes has not been experimentally addressed. How polo-like kinases are directed to the chromosomal sites of cohesin cleavage and where these sites occur on chromosomes is not known. Moreover, how removal of cohesins near centromeres affects biorientation is also not well understood. Further, the upstream signals indicating that biorientation is complete, leading to the removal of pericentromeric cohesin, are unknown.

Polo-like kinases are master regulators of mitotic progression including the orchestration of many facets of chromosome segregation (Barr et al., 2004). Major questions in the field pertain to how so few central control proteins, such as polo-like kinases, can regulate several processes and how these regulators achieve specificity during mitotic progression. Polo-like kinases phosphorylate a host of target proteins.
during multiple phases of the cell cycle (Snead et al., 2007; Lowery et al., 2007). One possible approach of achieving target specificity for promiscuous kinases, such as polo-like kinase, is through differential subcellular localization, which is mediated, at least in part, by binding to its substrates. This occurs via a C-terminal polo-box domain (PBD), a domain that is shared by all members of the polo-like kinase family, and binds to motifs on target proteins, tethering the enzymatic kinase domain to its substrates (Lowery et al., 2005). This binding of the PBD to target proteins typically requires their prior phosphorylation by a priming kinase, frequently cyclin-dependent kinase 1 (Cdk1) – which functions to promote cell cycle transitions (Fabbro et al., 2005; Presinger et al., 2005; Niya et al., 2005). Once bound to its substrates, the polo-like kinase enzyme is activated and can phosphorylate target proteins in its subcellular vicinity, allowing polo-like kinase to modulate mitotic pathways via PBD-dependent binding.

The localization pattern of metazoan polo-like kinase changes over the course of the cell cycle (Arnaud et al., 2004; Barr et al., 2004). Likewise, the expression of the budding yeast polo-like kinase Cdc5 is cell cycle-regulated, and at different points in the cell cycle, Cdc5 is visible at the bud neck, spindle pole bodies, and in the nucleus (Song et al., 2000; Park et al., 2004; Ratisma et al., 2011; Botchkarev et al., 2014). Unlike in metazoan species, Cdc5 has not been previously visualized at kinetochores via microscopy, suggesting a more sensitive methodology to determine whether Cdc5 localizes to kinetochores in *S. cerevisiae* is required. To this end, Id employed chromatin immunoprecipitation (ChIP), a method for mapping protein-DNA interactions on chromosomes in high resolution. ChIP is ideally suited for the study of centromere localization and recruitment in *S. cerevisiae*, due to the highly specific unique DNA
sequence at their point centromeres. The presence of unique centromeric DNA enables the rendering of a highly specific map of the chromosomal addresses of kinetochore-associated proteins (Meluh et al., 1998). By contrast, it is not readily tractable to map the localization of kinetochore proteins to non-unique DNA sequences via ChIP in organisms with repetitive centromeric DNA sequences. Understanding of Cdc5 localization to chromatin in budding yeast may offer insight into how and why polo kinase activity is directed to kinetochores, and how it coordinates the spatiotemporal regulation of cohesins in the vicinity.

In this study I found that the polo-like kinase of budding yeast, Cdc5, localizes to kinetochores and is enriched at pericentromeric DNA, as well as chromosomal cohesin-associated regions (CARs). I further discovered that the centromeric localization of Cdc5 depends on a functional kinetochore, functional cohesin, and the absence of biorientation. My work also shows that Cdc5 associates with the cohesin complex in vivo in a cell cycle-dependent manner. Collectively, this set of observations suggests that cohesin binds to chromatin and recruits its own regulator, the polo-like kinase Cdc5, at centromeres and potentially all CARs.

2.2 Results

2.2.1 Cdc5 accumulates at pericentromeric DNA in a cell-cycle dependent manner. Cdc5 protein peaks in expression and kinase activity at G2/M (Kitada et al., 1993; Snead et al., 2007; Shiryama et al., 1998). Cdc5 association with chromatin was low in synchronous G1 cells and peaks in mitosis, before dropping late in the cell cycle (Darieva et al., 2006). This result suggested that assaying a synchronous population of cells during peak Cdc5 expression, coincident with maximal chromatin-binding capacity,
might increase the likelihood of observing Cdc5 at centromeres. Therefore, to determine if and when Cdc5 might associate with centromeres, the localization of Cdc5 was examined throughout a synchronous cell cycle using ChIP.

Cells expressing an epitope-tagged version of Cdc5, Cdc5-myc, from the endogenous locus, were arrested in G1 using the yeast mating pheromone alpha-factor, and then released synchronously and sampled at regular time intervals throughout the cell cycle. In order to process cells for ChIP, each sample was treated with formaldehyde to form covalent crosslinks between the protein and DNA, the cells were lysed and the chromatin was randomly sheared with sonication into fragments of ~400-600 base pairs. A fraction of sonicated lysate was set aside for normalization as an “input” fraction, and the remainder of the lysate was subjected to immunoprecipitation for myc-epitope tagged Cdc5 and the associated chromatin. Subsequently, DNA from the input and immunoprecipitated fractions were purified and analyzed with PCR at specific regions of chromosomal DNA. Initially, only centromere regions were analyzed for Cdc5 association. Primer pairs for PCR analysis of ChIP samples were designed to flank either side of the CEN DNA sequence, such that the CEN was the designated the midpoint of the amplicon, to amplify pericentromeric sequences within a 200 to 250 base pair range from the midpoint. At every time point, the association of Cdc5 with two centromeres, CEN1 and CEN16, was examined by using semi-quantitative PCR analysis (Figure 1A).
Figure 1A. Polo kinase Cdc5 localizes to centromeres during mitosis. Cells expressing Cdc5-myc (MAY8775) were released from a G1 arrest and sampled at the indicated time points and processed for ChIP with anti-myc antibody. For each time point, Cdc5 ChIP DNA and dilute input DNA were analyzed by PCR and quantified at CEN1 and CEN16. For each time point, the ratio of enrichment was compared to time = 0.
Immediately after a synchronous release from G1, Cdc5 association with centromeres was low, as expected due to the low levels of Cdc5 in this cell cycle stage. Centromere association began to rise about 50 minutes following the release, until it peaked after roughly 80 minutes, and then appeared to decrease until the last time point at 120 minutes, presumably as the cells began to exit mitosis. At the maxima, Cdc5 was roughly 8-fold higher than at the G1 arrest point (time = 0 minutes), and the timing of Cdc5 association with the two centromeres examined was remarkably similar.

Unfortunately, the cell cycle index at each time point was not precisely determined, thus it cannot be determined exactly when maximal Cdc5 association with centromeres occurs during mitosis. Nonetheless, the increase in Cdc5 occupancy at both centromeres at 50 to 60 minutes coincides roughly with the time of mitotic entry after G1 release in budding yeast, as indicated in previous Cdc5 studies (Darieva et al., 2006; Snead et al., 2007). The bell-shaped pattern and peak of centromeric Cdc5 occupancy is consistent with the reported expression pattern of Cdc5 (namely low in G1, high in mitosis and low again late in the cell cycle), cell cycle dependent Cdc5 promoter binding activity, and the well characterized role for Cdc5 as a mitotic regulator that is ultimately destroyed at the end of mitosis (Darieva et al., 2006, Charles et al., 1998; Shiryama et al., 1998; Botchkarev et al., 2013).

Distinguishing the chromosomal region where Cdc5 localizes may provide clues about its recruitment to centromeric DNA. In metazoan cells, when Plk1 is visualized microscopically, it forms distinct foci at each kinetochore, suggesting its location is confined to the centromeric region. However, the size of the associated chromosomal region is not discernible due to the resolution limit of light microscopy. Likewise, the
previous experiment in Figure 1A did not establish the region of the yeast genome that Cdc5 localizes to; formally, Cdc5 could also accumulate at other chromosomal locations, or all chromatin, in a cell cycle-dependent manner as protein levels accumulate during mitosis. A centromere-specific localization pattern might indicate kinetochore-related functions of Cdc5, whereas a more global pattern of chromosome association would implicate Cdc5 in general chromatin-associated functions. Either scenario is possible, given that polo-like kinases have known roles at the kinetochores and in the regulation of cohesin, which exhibit a more general association with chromosomes.

To determine whether Cdc5 centromere-assocation is specific, we compared Cdc5 association with two additional centromeric sequences, centromere 3 (CEN3) and centromere 13 (CEN13) to its association with four centromere-distal regions on the same chromosomes (CHRIII and CHRXIII, Figure 1B). These centromere-distal regions and their approximate chromosomal distances from their respective centromere sequences are listed as follows: CHRIII-1, (19.3 kb); CHRIII-2 (13.3 kb); CHRXIII-1 (2.8 kb) and CHRXIII-2 (3.4 kb). As before, cells were arrested in G1 by treatment with alpha factor, and then released synchronously from the arrest. Cells were sampled every 20 minutes and processed for ChIP. Overall, the temporal patterns of Cdc5 association with centromeres were similar to the experiment depicted in Figure 1A. Immediately following the release from G1 arrest (t = 0 minutes), Cdc5 signal at all regions appeared low. At CEN3 and CEN13, Cdc5 ChIP signal began to rise at 60 minutes and peaked at 100 minutes, after which it began to decline, presumably as cells exited mitosis.
Figure 1B. Cdc5 localizes preferentially to centromeres during mitosis. ChIP was performed as in Figure 1A at the indicated chromosomal loci located at the indicated genetic distances (kb) from the relevant centromere.
In contrast, Cdc5 signal did not accumulate significantly at any of the tested centromere-distal regions relative to its accumulation at CEN locations. These data suggest that Cdc5 enrichment is more prominent at centromeres than centromere-distal chromosomal sites during a normal cell cycle, and may reflect a specificity of Cdc5 for centromeric DNA. Alternatively, Cdc5 may associate less prominently or more transiently at non-centromeric sites but ChIP analysis in cycling cells fails to capture the low affinity, or transient nature of these associations. Another possibility is that Cdc5 fails to localize to non-centromeric sites chosen for this analysis, but may localize to other sites on chromatin. In this case, a more global approach to studying Cdc5 chromosomal association (such as ChIP-seq) would be needed to identify those additional sites in the genome. Nonetheless, these findings are consistent with the prominent cell cycle-dependent kinetochore-specific localization pattern of Plk1 observed in metazoans (Farr et al., 2004; Lee et al., 2005; Zoutini et al., 2014). Thus, it is very likely that the budding yeast polo kinase homolog Cdc5 also associates with the kinetochore. Similarly, Cdc5 is present in the vicinity of centromere 6 (CEN6) during a mitotic arrest (Rossio et al. 2010), although a prominent peak is not observed directly at CEN6 DNA. Taken together, these observations add budding yeast to the list of eukaryotes with a polo-like kinase localized at centromeres in a cell cycle-dependent fashion.

While the overall trend of Cdc5 association over the course of the cell cycle is comparable between the experiments in Figure 1A and Figure 1B, the magnitudes of enrichment are much higher in the second experiment. It is worth noting that the PCR analysis and ChIP conditions were not optimized, and therefore, measurements of signal are not necessarily within the linear range. Consequently, the magnitudes of Cdc5
association as measured by ChIP between independent experiments may not be comparable. In addition, each time point is normalized to the signal at $t = 0$ minutes, thus variations in the Cdc5 signal at this initial point impart undue weight on all other time points in the temporal association curve. Hence, while qualitative trends within a ChIP time course can be inferred, quantitative differences in fold change between experiments may not be biologically meaningful. Further experimental optimization will be required in order to make quantitative inferences.

In human cells, Plk1 localizes to centromeres as early as G1, where localization increases through M-phase, and persists until anaphase (Lee et al., 2008; Barnhart-Dailey, & Foltz, 2004). Similarly, the findings above suggest that budding yeast Cdc5 localizes to centromeric DNA in a cell cycle-dependent manner. However, these data did not precisely delineate the phases of the cell cycle in which Cdc5 binds to centromeres. Furthermore, although it appears that Cdc5 is strictly confined to centromeric DNA, we cannot exclude the possibility that it may associate more broadly with other regions of chromatin, proximal to the centromere. To define the portions of the cell cycle during which Cdc5 localized to $CEN$ DNA, we arrested cells expressing Cdc5-myc at different cell cycle phases using various established treatments and processed them for Cdc5 ChIP. Cells were arrested in G1 (using alpha factor mating pheromone), S-phase (hydroxyurea) and mitosis (nocodazole); asynchronous cells (YPD) were included as a control (Figure 1C). We further sought to determine if Cdc5 cell cycle-dependent association with DNA was confined to centromeres, or localized to other $CEN$-proximal regions. Accordingly, a subset of primers previously used to map cohesin association with a region of chromosome III surrounding $CEN3$ (Weber et al., 2004; Eckert et al., 2007) was
Figure 1C. Cdc5 is enriched near CEN3 DNA in S and M phases. Cells expressing Cdc5-myc (MAY8775) were left untreated (labeled YPD) or arrested in G1 (α-mating pheromone $10^{-8}$M), S-phase (0.2 mM hydroxyurea), or mitosis (15 μg/mL nocodazole) and processed for ChIP. The ratio of ChIP DNA to diluted input DNA was analyzed by PCR across a 60 kilobase pericentromeric region of CEN3, at the indicated chromosome III coordinates. The position of CEN3 is denoted by the black oval.
employed for the analysis of Cdc5 localization. In S-phase arrested cells, three broad peaks of Cdc5 enrichment were observed in the region surrounding CEN3, including a large central plateau of signal that appeared to extend for 30 kilobases with two peaks on either side (Figure 1C). This finding suggests that, initially, Cdc5 localizes to CEN DNA and pericentromeric chromatin at the onset of or during S-phase. However, it should be noted that Cdc5 association with this region in the presence of hydroxyurea (HU) should be repeated before any firm conclusions can be drawn.

In cells arrested in mitosis with nocodazole (NZ), ChIP analysis showed that Cdc5 exhibits a prominent central peak over CEN3, and smaller peaks of Cdc5 enrichment on either side of CEN3 (Figure 1C, NZ treatment). The central peak corresponded to Cse4 association at CEN3 in the same region (Figure 1D). In this analysis, Cdc5 chromosome occupancy near CEN3 appeared to correspond to the previously described sites of cohesin enrichment (Tanaka et al., 1999; Megee et al., 1999; Weber et al., 2004; Eckert et al., 2007). Presumably Cdc5 association with the DNA was detected in G1 arrested cells because Cdc5 is not expressed during this phase in the cell cycle (Figure 1C). Similarly, in asynchronous cells, the Cdc5 signal was below the sensitivity of detection in a non-uniform population of cells (Figure 1C). When triplicate experiments of Cdc5 association with the pericentromeric region of CEN3 are compared, association with CEN3 is very robust, however association with the greater pericenteromeric region outside the central peak located at CEN3 is robust in only 2 of the three experiments (Figure 1E).

The observed changes in spatiotemporal patterns of Cdc5 centromeric localization are consistent with observations of Plk1 in higher eukaryotes. The significance of a broad
Figure 1D. Cdc5 co-occupies CEN3 DNA with Cse4. Cells expressing Cdc5-myc (MAY8775, top) or Cse4-myc (MAY8605, middle) were arrested and processed for ChIP as in Figure 1C, and analyzed at the indicated chromosome III coordinates. Cdc5 and Cse4 ChIP profiles were compared at enhanced resolution near centromere 3 (blue diamonds, Cdc5; red squares, Cse4). Primers for ChIP analysis were used as described in Weber et al. (2004). Black oval denotes the site of CEN3.
Figure 1E. Three technical replicates of Cdc5 in the pericentromeric region of CEN3. Three independent replicate (rep) ChIP samples derived from Cdc5-myc cells (MAY8775) were analyzed for comparison as in Figure 1D. Missing points in red curve represent signal below the threshold of detection likely due to a technical issue and were omitted from the analysis. Black oval denotes the site of CEN3.
distribution of Cdc5 at pericentromeric DNA during S-phase is unclear. Since I was interested primarily in kinetochores, SAC function and the role of Cdc5 polo-like kinase at kinetochores during mitosis, we chose to focus on Cdc5 association in metaphase-arrested cells, which appeared to yield a discrete centromere-specific localization pattern.

Cdc5 localizes in the chromosomal vicinity of CEN6 and co-localizes with Mcd1 across a region of chromosome 6 (Rossio et al., 2010). However, in that study Cdc5 localization to centromeres was not examined in detail, and Cdc5 did not appear to specifically associate with CEN6 DNA as a discrete apex; instead, it exhibited a broad CEN6-proximal distribution in mitotic cells arrested with the fungal-specific microtubule-depolymerizing agent, benomyl. While centromere localization of Cdc5 is not surprising in light of the activity of metazoan homologs of Cdc5 and its putative interactions with yeast kinetochore/pericentromeric-localized proteins, I sought to corroborate the authenticity of this finding. A previous study determined that Cdc5 binds to the promoters of genes expressed in the “cyclin B cluster,” a group of budding yeast genes that are expressed early in mitosis (Spellman et al., 1998), but does not bind to the promoters of genes expressed earlier in G1 (Darieva et al., 2006). Examination of the cell cycle-dependent association of Cdc5 at the promoters of the mitotic cyclin B gene, CLB2, and the G1 cyclin, CLN2 found that Cdc5 binds to the CLB2 promoter via the cell cycle transcription factor complex comprised of Fkh2-Mcm1-Ndd1, but not to the promoter of CLN2 (Darieva et al., 2006). The primer sequences for ChIP analysis of Cdc5 at the promoters of these genes were designed to span the published consensus sites for DNA binding of Mcm1 and Ndd1 (Harbison et al., 2004; Darieva et al., 2006) using the primer design software, Primer3 (Untergasser et al., 2007). ChIP signals at the CLB2 and CLN2
promoters were compared during three different cell cycle arrests in cells tagged with Cdc5-myc, and untagged control cells, as described above.

Minimal Cdc5 association with either *CLB2* or *CLN1* promoters was detected in G1 arrested cells (Figure 1F), consistent with the mitotic expression pattern of Cdc5 and previous data (Darieva et al., 2006). Cdc5 associated strongly with the promoter of the mitotic cyclin *CLB2*, but not with the promoter of G1 cyclin *CLN2*, in both S-phase and mitotic arrested cells. Recapitulation of these published findings suggested that Cdc5 ChIP signal at centromeric DNA sites represented bona fide Cdc5 localization.

A more detailed analysis of large chromosomal regions as opposed to a single locus should reveal more details regarding the association pattern of Cdc5 with distinct chromosomal DNA elements. Therefore, ChIP analysis was expanded to a larger primer set encompassing a 60 kilobase pericentromeric region of *CEN3* (Weber et al., 2004). As a control, we compared the distribution of Cdc5 occupancy with the binding distribution of centromere-specific histone H3 variant Cse4, under the same conditions (Figure 1D). Cse4 is a homolog of CENP-A, histone H3 variant that localizes exclusively to centromeres in all eukaryotes. Both proteins exhibited prominent peaks of chromatin association, centered directly over the *CEN3* sequence, which is the site of kinetochore assembly. As Cdc5 exhibits strong co-localization with a canonical centromere-specific protein, it is very likely that Cdc5 also localizes to the centromere or kinetochore. The peak of Cdc5 association is distributed more broadly than that of Cse4 association, specifically, Cdc5 associated with several thousand kilobases on each side of *CEN3*, while Cse4 association was restricted to the *CEN3* sequence (less than 1 kb on each side). This Cse4 localization pattern agrees with a previously characterized localization to a
Figure 1F. Cdc5 localizes to M-phase the promoter of the M phase expressed gene, *CLB2*, but not G1 the promoter of the G1 expressed gene, *CLN2*. Cells expressing Cdc5-myc (MAY8775) were left untreated (labeled ASYNCH) or arrested in G1 (α-mating pheromone 10⁻⁸M), S-phase (0.2 mM hydroxyurea), or M-phase (15 μg/mL nocodazole) and processed for ChIP at the promoter regions for the cyclin genes *CLB2* or *CLN2*. For each promoter and treatment, the ratio of ChIP DNA to diluted input DNA was plotted as % of Input. * denotes that signal was below the threshold of detection.
single centromere-specific nucleosome, although the ChIP procedure used here is not sufficient to resolve the mapping to single nucleosomes.

ChIP analysis of cells arrested with nocodazole was performed to examine the reproducibility of the pattern of Cdc5 association with centromeres in metaphase-arrested cells (Figure 1E). All Cdc5 ChIP replicate samples exhibited highly similar pericentromeric distributions in a 50 to 60 kilobase region near CEN3. Based on these findings, nocodazole arrest was chosen as the default condition for further Cdc5 ChIP studies at centromeres, as mitosis is likely when the ChIP signal of Cdc5 is maximal at DNA. Importantly, the cell cycle dependent enrichment of Cdc5 at kinetochores was independently verified by the Basrai lab at NIH (Mishra et al., 2016) and is consistent with a previous study (Rossio et al. 2010).

2.2.2 Cdc5 localization depends on a functional kinetochore. Budding yeast kinetochore assembly is hierarchical; the recruitment of all other known kinetochore proteins depends on the binding of the CBF3 complex to CEN DNA, where it nucleates the middle and outer kinetochore assembly (Jiang et al., 1993; Przewloka & Glover, 2009). Targeting of Cdc5 to centromere 3 may rely on interactions with the various subcomplexes that assemble into a functional kinetochore. Alternatively, Cdc5 may associate with CEN DNA itself, CEN-proximal chromatin proteins such as histones, or centromeric DNA origin complexes such as the replication-licensing factor Dbf4-Cdc7, which is known to bind Cdc5 (Chen & Weinrich, 2010). To examine these possibilities, we tested whether Cdc5 requires a functional kinetochore to associate with centromeric DNA. To this end, I performed ChIP analysis of Cdc5 in a yeast strain carrying a temperature sensitive Cbf2 complex, specifically, the ndc10-I allele (Goh & Kilmartin,
1993; McAinsh et al., 2003), which prevents assembly of kinetochore components at non-permissive temperatures. Cdc5 association with centromeric DNA was then assayed by ChIP in ndc10-1 cells at both permissive and non-permissive temperatures. Cells carrying the wild type NCD10 (blue) or ndc10-1 (green) that showed Cdc5 localization to CEN DNA at both permissive and non-permissive temperatures (Figure 2A) comparable to Cdc5 the magnitude of occupancy levels observed previously (Figure 1D). However, Cdc5 localization was perturbed in mutant cells at the non-permissive temperature (Figure 2A, purple), despite showing normal association at the permissive temperature (Figure 2A). These data suggest that Cdc5-enrichment at centromeric DNA is dependent on a functional kinetochore. Interestingly, in NDC10 expressing cells, Cdc5 enrichment is greatly enhanced at the pericentromeric DNA at 37°C (red curve) relative to 26°C (blue curve). This may reflect increased efficiency of protein-DNA crosslinking during the formaldehyde fixation step of ChIP analysis, rather than increased Cdc5 binding at elevated temperatures (e.g. see Figure 2A& 4B, Cdc5 X-linking 26°C vs. 37°C, wt vs mutant). Furthermore, it should be noted that all samples were treated with nocodazole, but as ndc10-1 expressing cells lack a functional spindle assembly checkpoint, it is possible that these cells have progressed into later cell cycle phases (Poddar et al., 2004), thus differences in Cdc5 association with kinetochores in the ndc10-1 mutant may be due to comparison of different cell cycle phases.

To corroborate the effects of ndc10-1 on Cdc5 localization to kinetochores, I employed cells that express ndc10-2 (Kopski & Huffaker, 1997) or ndc10-42 (Doheny et al., 1993) temperature-sensitive alleles that are capable of mounting a spindle checkpoint response (Doheny et al. 1993). These strains also exhibit reduced levels of centromeric
Figure 2A. Polo-like kinase Cdc5 localizes to centromeres via functional kinetochore complexes. Cdc5-myc cells expressing the temperature sensitive (ts) allele ndc10-1 (MAY8990) or NDC10 (MAY8775) were arrested with 15 μg/mL nocodazole in mitosis until >95% large budded (~2.5 hours) at the permissive and non-permissive temperatures, processed for ChIP, and analyzed as in Figure 1C.
Cdc5 in ChIP assays (Figure 2B), supporting the model that Cdc5 requires a functional kinetochore for centromeric association, rather than differences being due to the cell cycle phase. However, as these experiments did not include NDC10 cells in the same experimental cohort, we cannot rule out the apparent defect in Cdc5 binding being the resulting technical issue, as there was no positive control.

Taken together, these data suggest that Cdc5 depends on a functional kinetochore for localization to centromeric DNA.

2.2.3 Cdc5 localization to the pericentromeric region depends on cohesion.

Another mechanism of targeting Cdc5 to chromatin is through cohesin. Several lines of evidence, along with the above findings, suggest that Cdc5 association may be cohesin-dependent. Cdc5, like other polo-kinases, is known to bind or localize to the target proteins it regulates, including nuclear proteins such as Cdc14 phosphatase and the transcription factor Ndd1 in budding yeast (Darieva et al., 2006; Botchkarev et al., 2014). In budding yeast, the chromosome-bound SMC kleisin subunits, Mcd1, and its meiotic homolog Rec8, are known to be phosphorylated by Cdc5. During mitosis, phosphorylation of Mcd1 renders it more susceptible to cleavage via separase (Alexandru et al., 2001; Hornig & Uhlmann, 2004), thereby facilitating mitotic cohesion disassociation. Cdc5 is known to be present in the nucleus (Botchkarev et al., 2014) where it localizes to chromatin (Alexandru et al., 2001; Hornig & Uhlmann, 2004; Rossio et al., 2010; Darieva et al., 2006; Hu & Koshland, 2005) and associates with cell cycle promoters during mitosis (Darieva et al., 2006). Furthermore, it appears to exhibit co-occupancy with the Mcd1 subunit on chromosome six at most CAR sites (Rossio et al., 2010), but their co-occupancy is not as apparent in the region immediately near CEN6.
Figure 2B. Additional ndc10 alleles disrupt Cdc5 localization at CEN3. Cdc5-myc cells expressing the ts alleles ndc10-2 (MAY8471) or ndc10-42 (MAY8473) were grown in the presence of nocodazole at the permissive (26°C; blue, ndc10-2; green, ndc10-42) and non-permissive (37°C; red, ndc10-2; purple, ndc10-42) temperatures, processed for ChIP as in Figure 1B and analyzed at indicated for centromere of chromosome III proximal loci, where CEN3 = 0 bases.
Nonetheless, given these findings in conjunction with the observations in the figures above, a strong possibility is that Cdc5 localizes to cohesion-associated regions.

In addition to this mapping on chromosome VI, pericentromeric distribution of Mcd1 has been previously mapped on chromosome III (Weber et al., 2004) and genome-wide distributions of Mcd1 and Smc3 subunits of the cohesin complex have also been reported (Glynn et al., 2004). Like Cdc5 recruitment to the centromere (see above), pericentromeric cohesin enrichment also depends on a functional kinetochore subunit Ncd10, as well as other kinetochore-associated proteins (Weber et al., 2004; Eckert et al., 2007; Ng & Biggins, 2009). In particular, members of the conserved kinetochore-associated Ctf19 complex are required for pericentromeric cohesin recruitment (Eckert et al., 2007; Ng & Biggins, 2009; Fernius et al., 2009). The pattern of Cdc5 localization at the pericentromeric region of CEN3 resembles that of published Mcd1 localization data, in mitotically arrested cells (Weber et al., 2004; Eckert et al. 2007). To examine this, an epitope-tagged Mcd1-myc expressing strain was generated; subsequently, Mcd1-myc cells were arrested in mitosis with nocodazole, and ChIP was performed to determine the association of Mcd1 with the pericentromeric region of CEN3. An overlay of Cdc5-myc and Mcd1-myc ChIP localization data at the pericentromeric region of CEN3 underscores the similar localization pattern of the two proteins (Figure 3A). Both proteins exhibit prominent localization immediately over the centromere, although the ChIP signal is much weaker for Cdc5. Perhaps this is due to the fact that cohesin binds DNA directly, whereas Cdc5 likely binds to chromatin-associated proteins, and consequently crosslinks less efficiently. Alternatively, this may reflect differences in the concentrations of Cdc5 and Mcd1 near centromeres, although this is not ascertainable by comparison of ChIP
Figure 3A. Cdc5 localizes to cohesin-associated regions. Cells expressing Cdc5-myc (MAY8775, blue squares) or Mcd1-myc (MAY8776, red diamonds) were grown and processed for ChIP and analyzed as in Figure 1E&F. In order to compare relative enrichment of each protein around the centromere, both proteins were plotted together (panel 3). In this panel, Mcd1 enrichment is plotted on the left Y-axis and Cdc5 enrichment is plotted on the right Y-axis (bottom panel). Black oval denotes the site of CEN3.
patterns of two different proteins with potentially different crosslinking and/or immunoprecipitation efficiencies.

Mcd1 enrichment distributes more broadly over this central portion of the pericentromeric region, with peaks on either side of the centromere and a low ChIP signal immediately at CEN3. This low signal or centromeric ‘cohesin hole’ is presumably where the kinetochore occupies the central-most CEN DNA, possibly occluding cohesin occupancy, or masking the cohesin epitope used for ChIP. Moreover, this phenomenon was also observed in many other cohesin occupancy experiments in budding yeast (Verzijlbergen et al., 2014; Hinshaw et al., 2015). In contrast, Cdc5 displays a single, discrete peak of enrichment at CEN3 DNA, suggesting that Cdc5 association is confined to this central-most CEN DNA region, most likely through interactions with kinetochore proteins. This data further suggests that Cdc5 localizes to more distal pericentromeric CAR sites, on either side of the CEN, that are also occupied by Mcd1, but the levels of Cdc5 at distal pericentromeric regions are lower than at CEN DNA. However, due to the primer sets used, which limited the resolution, it is not possible to precisely map Cdc5 and cohesin local maxima that may occur within the analyzed regions.

Mcd1 occupancy has been previously characterized in detail at many chromosomal CAR sites (Laloraya et al., 2000; Weber, 2004; Kogut et al., 2009). We therefore performed fine-scale ChIP analysis for Cdc5 associated with the CAR sites immediately proximal to CEN3 (Figure 3B), and two distal CAR sites on chromosome XII, the rDNA locus and GAL2 locus (Figure 3C and 3D). At each of these CAR sites, we found peaks of Cdc5 signal that closely coincided with the peaks of signal for either my experimental or published Mcd1 ChIP data, demonstrating that Cdc5 is present at all CAR sites tested. A
Figure 3B. ChIP analysis of Cdc5-myc cells (MAY8775) at indicated chromosome III coordinates as in 3A. The plotted Mcd1-HA (red) ChIP data was derived from Laloraya et al. (2000) with a peak maxima at ~100.9 kb from left arm of CHRIII, which defines a unique cohesin-associated region (CAR). Both proteins were plotted on the same Y-axis scale.
Figure 3C. ChIP analysis of Cdc5-myc (MAY8775) and Mcd1-myc (MAY8776) cells at indicated chromosome XII coordinates as in 3A. This CAR site was previously described in Kogut et al., 2009.
Figure 3D. ChIP analysis of Cdc5-myc (MAY8775) and Mcd1-myc (MAY8776) cells at indicated chromosome XII coordinates as in 3A. This CAR site was previously described in Laloraya et al., 2000. Both proteins were plotted on the same Y-axis scale.
cells using ChIP coupled with microarrays, demonstrated similar findings (Rossio et al., 2010), that Cdc5 and Mcd1 align with similar patterns along the left arm of chromosome 6, as well as to CEN6, suggesting Cdc5 and Mcd1 co-localize to the same regions of DNA. These findings, in combination with my ChIP analyses, highly suggest that Cdc5 may associate with all CAR sites genome-wide, and localize with a similar pattern as the cohesin complex.

2.2.4 Association of Cdc5 with centromeres is cohesin-dependent. The co-localization of Cdc5 with Mcd1 at CAR sites and centromeres suggests that Cdc5 may associate with chromatin in a cohesin-dependent manner. To test this hypothesis, I employed a temperature-sensitive allele of the cohesin subunit Mcd1, mcd1-1, which at non-permissive temperatures prevents formation of cohesin complexes and causes existing complexes to dissociate (Guacci et al., 1997; Heidinger-Pauli et al., 2008). Therefore, if Cdc5 is dependent on cohesin for association with chromatin, we should observe its loss at non-permissive temperatures in mcd1-1 expressing cells. Accordingly, we compared Cdc5 association with a pericentromeric region near CEN3 at the permissive and non-permissive temperatures using ChIP with mcd1-1 cells treated with nocodazole (Figure 4A). Cdc5 no longer associated with the pericentromeric region of CEN3 at the non-permissive temperature in mcd1-1 cells, as opposed to the strong Cdc5 association with this region in mcd1-1 cells at the permissive temperature, and the control cells at both temperatures (Figure 4A). At the non-permissive temperature in mcd1-1 cells, Cdc5 chromatin association was barely detectable across the 60 kilobase CEN3 pericentromeric region. A slight peak of Cdc5 ChIP signal persisted at CEN3 at the non-permissive temperature (Figure 4A, red curve) but the significance of this is not clear; it
Figure 4A. Cdc5 localization depends on cohesin. Cdc5-myc cells containing the t.s. mutation mcd1-1 (MAY8851) were grown at the permissive (blue) or non-permissive temperature (red) and processed for ChIP and analyzed as in 1E. The black oval denotes CEN3.
remains possible that Cdc5 localizes to centromeres by a second mechanism that is not
dependent on cohesin. Alternatively Mcd1 may not have completely dissociated from
chromatin under the experimental conditions. A caveat to interpreting the Cdc5
localization as cohesin-dependent in this experiment is that the cell cycle index was not
quantified: if nocodazole treatment at the non-permissive temperature was inefficient,
Cdc5 association with chromatin would be undetectable, as cells might not arrest in
mitosis (Figure 4A). However, loss of functional Mcd1 activates the SAC, which may
reinforce nocodazole-mediated SAC arrest (Indejian & Murray, 2005; Shimogawa et al.,
2009).

Interestingly, even at the permissive temperature, Cdc5 association with the
pericentromeric CEN3 region was altered in mcd1-1 cells, as compared to Cdc5 in MCD1
cells in the following ways: (1) the strength of the association (normalized to the input)
was several fold higher across the region in MCD1 cells, compared with the mutant allele
at either permissive or non-permissive temperatures (Compare 2A to Figure 1D, top
panel), and (2) Cdc5 appeared to distribute differently in mutant cells than in MCD1
expressing cells in the region near the centromere (Compare 2A to Figure 1D). Moreover,
the pattern of Cdc5 association in mutant mcd1 cells contained a depression near CEN3,
whereas in wild-type MCD1-expressing cells, Cdc5 forms a single isolated peak in this
region with CEN3 at the apex. We note that this pattern is reminiscent of cohesin ChIP
signal at this region (Weber et al., 2004; Eckert et al., 2007); however, as Cdc5 was only
assayed once, experimentally in mcd1-1 strains at a 60-kilobase resolution, it is premature
to draw definitive conclusions. Nonetheless, if this pericentromeric pattern is
reproducible, this data suggests that the \textit{mcd1-1} mutant cells display an altered Cdc5 association with the pericentromeric DNA even at permissive temperatures, hinting at a physical interaction between cohesin and Cdc5 on chromatin.

Elevated temperatures enhance the efficiency of formaldehyde-crosslinking of proteins bound to DNA (see above), rendering quantitative comparison of the distribution of protein on chromatin at two different temperatures problematic when assayed via ChIP. Additionally, temperature-sensitive alleles can be hypomorphs, meaning they do not guarantee complete loss of activity/function of a given protein. Therefore, I sought to confirm the Mcd1-dependent chromatin association of Cdc5 through an alternative method that did not depend on the temperature-sensitive mutant \textit{mcd1-1} that potentially alters the properties of cohesin (Eng et al., 2014). For this, I generated a conditional auxin-inducible degron version of Mcd1, \textit{mcd1-aid}. Upon addition of the small molecule auxin, the aid-tagged protein is degraded via the ubiquitination pathway (Kanemaki et al., 2009; see Chapter 3 for further description). In the aid system the ectopically expressed OsTir1 functions as an adapter to the auxin-inducible degron thus both components must be present to achieve degradation. Cells containing OsTir1 and wild type MCD1 were thus able to grow normally on auxin. Cells expressing OsTir1 and \textit{mcd1-aid} failed to grow on media containing auxin, as did the control cells expressing OsTir1 and \textit{cdc16-aid}, an essential subunit of the APC); these data are consistent with the lethality of association with loss of function of the essential Mcd1 and Cdc16 proteins (Figure 4C). Cells expressing \textit{mcd1-aid} were cultured and upon treatment with auxin, arrested in mitosis with large buds and a nucleus that traverses the bud neck, indicative of
Figure 4B. Cdc5-myc cells expressing mcd1-aid OsTir1 (MAY8981), MCD1 (MAY589) or OsTir1 MCD1 were grown YPD plates containing auxin (IAA, 4 mM). Cells expressing cdc16-aid OsTir1 (MAY8988) were included as controls.

Figure 4C. Cdc5-myc cells expressing mcd1-aid OsTir1 (MAY8981, left) or smc3-aid OsTir1 (MAY8982, right) were cultured in yeast rich media in the presence of auxin (IAA, 4 mM) for 3 hours, stained with DAPI and imaged with epifluorescence microscopy.
incomplete nuclear segregation (Figure 4C), consistent with loss of cohesin phenotypes (Guacci et al., 1007). The levels of mcd1-aid protein depletion remain to be determined however mcd1-aid depletion was sufficient to induce inhibition of growth and the expected cell cycle delay. Cells expressing smc3-aid also arrested with large buds and unsegregated DNA, demonstrating that auxin-mediated depletion of other cohesin subunits produces similar phenotypes. To determine whether the loss of Mcd1-aid affects the ability of Cdc5 to localize to chromatin, ChIP analysis of Cdc5-myc was performed in mcd1-aid expressing cells, 2.5 hours after release from a G1 arrest, in media containing nocodazole, auxin, or auxin plus nocodazole. In mcd1-aid expressing cells treated with nocodazole only, Cdc5 appeared to localize normally to pericentromeric chromatin (Figure 4D), as expected. However, in cells treated with auxin or auxin plus nocodazole, the Cdc5 signal was diminished at the pericentromeric region to levels comparable to those seen in cells expressing an untagged Cdc5 (Figure 4D). A more fine-scale ChIP analysis of Cdc5 at a 2-kilobase region surrounding CEN3 further illustrates this finding, suggesting that Cdc5 is unable to localize properly to the pericentromeric region in cells depleted of Mcd1 (Figure 4E). To rule out a possible contribution of auxin itself to lack of Cdc5-pericentromere association, a useful control would have been auxin treated mcd1-aid cells lacking the TIR1 ligase. Combining the findings with mcd1-1 and mcd1-aid alleles strongly suggests that Cdc5 association at pericentromeric regions is dependent on cohesin. Importantly, the cohesin dependence cycle Cdc5 at kinetochores was independently verified by the Basrai lab at NIH (Mishra et al., 2016).
Figure 4D. Cdc5-myc cells expressing mcd1-aid (MAY8981) were cultured in yeast rich media in the presence of nocodazole only (15 μg/mL nocodazole), auxin (4mM IAA), auxin plus nocodazole (4mM IAA and 15 μg/mL nocodazole), and processed for ChIP as the indicated located near CEN3, as in 2C. Untagged cells arrested in nocodazole were used as a control.

Figure 4E. ChIP samples from E were analyzed at indicated loci near CEN3.
**2.2.5 Cdc5 associates with pericentromeric DNA exclusively in the absence of biorientation.** Cohesin exhibits biorientation-dependent association with pericentromeric DNA prior to the onset of anaphase. Therefore, I hypothesized that like cohesin, polo-like kinase Cdc5 may exhibit biorientation/tension-dependent binding to pericentromeric DNA.

To this end, I used the same protocol used previously to demonstrate that cohesin is enriched at pericentromeric DNA of chromosomes that are not bioriented (lack tension), whereas cohesin levels remain low (or are absent) on the pericentromeric DNA of bioriented chromosomes (Eckert et al. 2007). Using an allele of *CDC16*, which encodes an essential subunit of a ubiquitin ligase, the Anaphase Promoting Complex (APC), a homogenous population of metaphase-arrested cells with either bioriented, or not-bioriented chromosomes were generated (Eckert et al., 2007). Cells that express the temperature sensitive allele *cdc16-1* arrested in mitosis with bioriented chromosomes at non-permissive temperatures, as these cells cannot form functional APC complexes, which are required for the degradation of securin and hence the onset of anaphase (See Chapter 1). In the Eckert et al. (2007) experiment, one population of cells was arrested by inactivating Cdc16 only (namely growing *cdc16-1* cells at the non-permissive temperature), and the other was arrested in a *cdc16-1* arrest in the presence of nocodazole. The former condition yielded bioriented chromosomes, whereas in the latter spindle disassembly resulted in loss of kinetochore-microtubule interactions and hence loss of biorientation. We used *cdc16-1* cells to assay Cdc5 localization at a 60 kilobase region of *CEN3* using ChIP at a non-permissive temperature, in the presence or absence of nocodazole (Figure 5). In *cdc16-1* cells arrested without nocodazole, very little Cdc5...
enrichment was observed, whereas addition of nocodazole shows enrichment of Cdc5 (Figure 5). One possible interpretation of these data is that binding of microtubules to CEN DNA occludes Cdc5 and cohesin occupancy from centromeric DNA. Another explanation is that Cdc5 and cohesin are actively removed or slide away from bioriented centromere DNA, depending on the state of interkinetochore tension. These data suggest that Cdc5 is present at pericentromeric DNA in the absence of bioriented chromosomes, but absent at pericentromeric DNA of chromosomes that are bioriented. While direct conclusions cannot be made, an indirect comparison of Cdc5 enrichment in cdc16-1 cells arrested at a high temperature in nocodazole (Figure 5), to CDC16 cells arrested with nocodazole (Figure 1E) in different experiments, shows that Cdc5 is more prominently enriched in cdc16-1 cells at CEN3 (Figure 5). One possibility is that Cdc5 crosslinks more strongly at high temperatures (Figure 5); alternatively, Cdc5 may be more highly enriched due to the mutant cdc16-1 background. A control experiment performed in CDC16 cells at a high temperature should resolve this matter. It is possible that high temperatures, the genetic background or the stage of cell cycle arrest imposed by cdc16-1 arrest, contributed to prominent Cdc5 enrichment. Nonetheless, Cdc5 recruitment to kinetochores appears to be dependent on the biorientation status of chromosomes at high temperatures in cdc16-1 cells. Given that Cdc5 association with this region depends on cohesin (Figure 4), and cohesin also displays a biorientation-dependent pattern of centromeric localization, these findings suggest that Cdc5 association may be governed by its partnership with cohesin. While polo kinases are observed to localize to metazoan centromeres in similar fashion, the underlying mechanistic basis for this phenomenon remains elusive.
Figure 5. Cdc5 localization to pericentromeric DNA depends on the absence of biorientation. Cdc5-myc cells containing the temperature sensitive mutation cdc16-1 (MAY8931) were grown at the permissive (blue) or non-permissive temperature (red) and processed for ChIP and analyzed as in 1E.
2.2.6 Cdc5 associates with cohesin in vivo. Cohesin at pericentromeric DNA is regulated by proteins such as Sgo1, which co-localize with cohesin on pericentromeric DNA, such as Cdc5 (Kiburz et al., 2005; Rossio et al., 2010), yet their activities oppose one another: Sgo1 promotes pericentromeric cohesin association, while polo-like kinases such as Cdc5 promote disassociation. How these proteins can regulate opposing activities remains unclear, but for Cdc5, and potentially Sgo1, a likely mechanism of chromatin localization is through direct binding to one or more subunits of the cohesin complex.

Cohesins are conserved substrates of polo-like kinases (Alexandru et al., 2001; Hornig & Uhlmann, 2004; Losada et al., 2002; Sumara et al., 2002; Hauf et al., 2005), however direct binding of polo-like kinases to cohesin has not been characterized in detail (Ho et al., 2002). Large-scale protein-protein interaction screens, utilizing mass spectrometry to identify proteins, indicate that cohesin enriches for Cdc5 (Ho et al., 2002). Given my findings showing the co-localization of Mcd1 and Cdc5 on chromatin (Figure 3), the Cdc5-dependent phosphorylation of the Mcd1 cohesin subunit (Alexandru et al., 2000; Hornig & Uhlmann, 2004), and the dependence of Cdc5 on functional cohesin for chromosomal localization (Figure 4), I hypothesized that Cdc5 associates with the cohesin complex in vivo.

I first determined whether Cdc5 co-purifies with the core members of the cohesin complex: Mcd1, Smc1, Smc3 and Scc3 (Michaelis et al., 1997; Toth et al., 1999). Accordingly, four strains over-expressing tandem affinity purification (TAP) tags for each of the core cohesin complex members, along with a myc-tagged Cdc5, were tested by arresting them in nocodazole and processing them for co-immunoprecipitation via the TAP tag. Cells that did not contain a TAP-tag, and cells expressing TAP-tagged Sgo1, a
protein known to associate with pericentromeric chromatin and regulate cohesin, were also included in this experimental panel as controls. Cdc5 was able to associate with each of the TAP-tagged subunits of the cohesin complex, consistent with previous results (Ho et al., 2002), but Cdc5 was not precipitated with TAP-tagged Sgo1 or a strain lacking a TAP tag (Figure 6A). Although not indicative of a direct interaction with Mcd1, Cdc5 is associated with Mcd1 and the other cohesin subunits, allowing for Cdc5 kinase activity to be near its substrates at the onset of anaphase.

Although preliminary, it is interesting that Sgo1, which is thought to protect cohesin from phosphorylation by polo kinase, does not detectably associate with Cdc5 (Marston, 2015). This suggests that binding of Cdc5 and Sgo1 with the cohesin complex and Mcd1 is mutually exclusive. Understanding this possible antagonistic relationship may be critical to elucidating the role of Cdc5 at pericentromeric cohesin.

Given the phosphorylation of Mcd1 by human polo-like kinase in vitro (Alexandru et al., 2000), and the inference that yeast Cdc5 accomplishes the same task in vivo, it seemed most likely that Cdc5 interacts with the Mcd1 subunit of cohesin. We thus tested whether Mcd1 and Cdc5 associate in vivo using co-immunoprecipitation during a nocodazole-induced mitotic arrest in cells where MCD1 was expressed from its endogenous promoter. Cells expressing epitope tagged versions of Cdc5-HA and/or Mcd1-myc were subjected to co-immunoprecipitation analysis. When Mcd1-myc is immunoprecipitated in the strain containing both epitope tagged proteins, a single HA epitope containing protein consistent with the size of Cdc5-HA co-purified with it, but not in cells containing either a single epitope tagged protein or in untagged control cells (Figure 6B). Furthermore, the enrichment of Cdc5-HA was dependent on the presence of
Figure 6A. Cdc5 associates with cohesin in vivo. Cells with Cdc5-myc expressed from the endogenous locus (MAY8775) containing vectors expressing MCD1-TAP (MAB3551), SMC1-TAP (MAB3555), SMC3-TAP (MAB3556), SCC3-TAP (MAB3776), or SGO1-TAP (MAB3549) expressed from the GAL1-10 promoter, or containing an empty vector (pRS426) were cultured to early log phase in media containing 2% raffinose. To induce expression of each construct and promote Cdc5-myc expression, 2% galactose and nocodazole (15 μg/mL) were added to the media for 3 hours before processing cells for immunoprecipitation of TAP. Input (IN) and cognate immunoprecipitated (IP) samples were analyzed by western blot analysis with anti-TAP (top panel) or anti-myc (bottom panel) antibodies.
Figure 6B. Cells expressing Mcd1-myc and Cdc5-HA (MAY8750), Mcd1-myc only (MAY8776), Cdc5-HA only (MAY8364), or untagged cells (MAY1606) were arrested in 15 μg/mL nocodazole and processed for immunoprecipitation with anti-myc. An additional sample derived from cells expressing Mcd1-myc and Cdc5-HA was subjected to mock immunoprecipitation the absence of anti-myc (rightmost lane). Input (left panel) and cognate immunoprecipitated (right panel) samples were analyzed by western blot analysis with anti-myc (TOP) or anti-HA (bottom) antibodies.
an anti-myc antibody specific to the epitope tagged Mcd1-myc (Figure 6B, lane 9). In a reciprocal experiment, Cdc5-HA was immunoprecipitated and a protein with a myc epitope tag consistent with the size of Mcd1-myc was co-purified in cells containing Cdc5-HA and Mcd1-myc, but not in control cells. One caveat to this co-immunoprecipitation experiment is that Cdc5-HA does not appear in the input lanes. It is highly improbable that Cdc5 could be present in the immunoprecipitated fraction of a lysate, but not the input/total fraction when they originate from the same source. In fact, in a more saturated image, one of the missing Cdc5 bands does appear, suggesting that technical problems arose during this experiment. The relatively low expression of Cdc5 may make its detection more susceptible to issues such as poor cell lysis, protein degradation, and poor protein transfer during western blotting. Nonetheless, these data suggest that Cdc5 and Mcd1 maintain a robust interaction in vivo regardless of the primary protein immunoprecipitation.

We next asked whether the presence or absence of biorientation during mitotic arrest might alter the physical association of Cdc5 with Mcd1. To this end cells expressing Mcd1-myc and a temperature sensitive allele cdc16-1 were generated. Cdc16 is an essential component of the APC and required for the metaphase-to-anaphase transition; cdc16-1 cells at the non-permissive temperature, cells arrest at this boundary with bioriented chromosomes. Thus, this allele can use to obtain a population of cells with bioriented chromosomes.

Cells expressing Mcd1-myc and cdc16-1 were grown at the non-permissive temperature in the presence or absence of nocodazole at the non-permissive temperature
and subjected to immunoprecipitation analysis for Mcd1-myc. Cells grown at the permissive temperatures showed no Cdc5 association with Mcd1, consistent with the cells being in various stages of the cell cycle. In contrast, Cdc5 and Mcd1 co-immunoprecipitated in cdc16-1 cells that treated with nocodazole (Figure 6C, lane 6). When grown at non-permissive temperatures, Cdc5 co-immunoprecipitated with Mcd1 regardless of the presence of nocodazole (Figure 6C, lanes 7 and 8). This result is interesting in light of the findings that there is less Cdc5 at pericentromeric DNA on bioriented chromosomes. It remains possible that the majority of Cdc5 is associated with non-pericentromeric cohesin at CAR sites (see Figure 3), and is not affected by the biorientation status of chromosomes (Eckert et al., 2007). Thus, this assay may not be sufficiently sensitive to detect putative changes in Cdc5-Mcd1 interaction at pericentromeric chromatin, which may constitute a small subset of all the DNA bound protein. Other possibilities are that there is no biorientation dependent change in Cdc5-cohesin interaction, or that Cdc5 and Mcd1 associate independent of chromatin in the cytoplasm or nucleoplasm.

This is consistent with previous findings (Weber et al., 2004; Glynn et al., 2004; Eckert et al., 2007; Ocampo-Hafalla et al., 2007) that a mitotic arrest enriches for the Cdc5-Mcd1 association due to the prominent expression of both proteins in this cell cycle phase. Collectively, the findings from the co-immunoprecipitation experiments above suggest that Mcd1 and Cdc5 associate, either directly or through interactions with another subunit of the cohesin complex or cohesin-associated regulator. Given the previous evidence of Cdc5 association with the cohesin complex (Ho et al., 2002) and likely Cdc5-dependent phosphorylation of Mcd1, the simplest explanation is a direct Cdc5-Mcd1
Figure 6C. Cdc5 associates with cohesin subunit Mcd1 during mitotic arrests. Cells expressing the t.s. mutation cdc16-1 and Mcd1-myc (MAY8569) were grown in the presence or absence of nocodazole at the permissive and non-permissive temperatures and processed for immunoprecipitation of myc as in Figure 6A. Input (left) and cognate immunoprecipitated (right) samples were analyzed by western blot analysis with anti-myc (top) or anti-Cdc5 antibodies.
interaction, however at this time we cannot firmly draw this conclusion (Alexandru et al., 2001; Hornig & Uhlmann, 2004). The Cdc5-cohesin interaction finding was independently confirmed by another laboratory with another cohesin subunit (submitted manuscript, Mishra et al., 2016).

I further characterized the interaction between Mcd1 and Cdc5 by defining the phase of cell cycle in which they interact. To address this question, I grew cells expressing Mcd1-TAP from the endogenous MCD1 promoter to log-phase, and arrested them in nocodazole (mitosis), hydroxyurea (S-phase) or alpha factor (G1), or left them untreated (i.e. asynchronous). These cells were then processed for immunoprecipitation of Mcd1-TAP and analyzed for co-immunoprecipitation of Cdc5 (Figure 6D). Neither Mcd1 nor Cdc5 were expressed during an alpha factor induced arrest (in G1), and hence no interaction was detected (Figure 6D). Cdc5 strongly co-purified with Mcd1 in both the S-phase and nocodazole arrested cells (Figure 6D), and Cdc5 associated with Mcd1 in asynchronous cells, albeit at lower levels, consistent with their cell cycle dependent expression. Mcd1 is not detect in the asynchronous IP lane due to technical errors. These co-IP data, combined with the Cdc5 ChIP observations, suggest that Cdc5 associates with cohesin soon after it is loaded onto chromatin in S-phase, and remains associated with cohesin at least until the metaphase to anaphase transition.

2.3 Discussion

The findings in this study add budding yeast Cdc5 to the family of polo-like kinases that localize to mitotic centromeres. This study expands the known chromosome localization pattern of polo-like kinases in a eukaryotic genome, demonstrating that Cdc5
Figure 6D. The cell cycle association of Cdc5 and Mcd1. Cells expressing Mcd1-TAP (MA8952) were arrested in G1 (α), S-phase (HU) or M-phase (NZ), or left untreated (asynch.) and processed for immunoprecipitation of TAP. Input (IN) and cognate immunoprecipitated (IP) samples were analyzed by western blot analysis with anti-CDC5 and anti-TAP antibodies.
co-localizes to pericentromeric chromatin, as well as to all genomic cohesin-associated regions (CARs) examined, in a cell cycle dependent manner (Figures 1-2). These data further provide evidence for a physical interaction of Cdc5 with the cohesin complex in vivo (Figure 6), and demonstrates that Cdc5 localization to CARs is cohesin-dependent (Figure 4). These data are consistent with the known characteristics of polo-like kinases, which bind to and phosphorylate their substrates.

The association of cohesins with chromosomes contributes to sister chromatid cohesion and biorientation; both processes are critical for error-free chromosome segregation. Thus, the finding that a known regulator of cohesin association with chromosomes, polo-like kinase Cdc5, occupies the same chromosomal DNA regions adds another piece of evidence towards understanding the mechanism of cohesin regulation, and the role Cdc5 plays in this. Spatiotemporal regulation of Cdc5 recruitment to distinct chromosomal sites during the cell cycle may underlie how this master regulator achieves specific cell cycle-dependent functions.

Cdc5 arrives at centromeres as early as S-phase and peaks in mitosis, before waning (Figure 1). The arrival of Cdc5 is similar to that of cohesin deposition on chromatin, which typically occurs in S-phase and is removed at the onset of anaphase (Michaelis et al., 1997; Ciosk et al., 2000). Here, we find that Cdc5 physically associates with cohesin as early as S-phase and this association increases in mitosis (Figure 6). Additionally, these findings suggest that Cdc5 recruitment to chromatin depends on the presence of a functional cohesin complex, at all locations analyzed (Figure 4), suggesting a cohesin-dependent mechanism for Cdc5 recruitment to chromatin. While cohesins are known substrates of polo-like kinase, a role for cohesin in the subcellular targeting of
polo-like kinase Cdc5 to CAR sites has not been previously reported in yeast. In fact, Hornig and Uhlmann (2004) observed that depletion of cohesin does not affect Cdc5 presence or association with chromatin in budding yeast nuclei (Hornig & Uhlmann, 2004). However, the experimental assays in that study were limited to bulk chromatin association and did not address Cdc5 localization to specific chromosome loci. While untested, it remains likely that global chromosomal disruption of cohesin binding affects Cdc5 occupancy at all CAR sites in the genome. Whether cohesin also depends on Cdc5 for initial chromosome association is unknown. Furthermore, whether Cdc5 and cohesin arrive on chromosomes separately, or at the same time is not known. Cdc5 recruitment to centromeres and cohesin may occur concurrently with cohesin deposition on chromosomes, similar to the chromosomal association of cohesin and its loading complex, which are known to be mutually dependent (Fernius et al., 2013). Alternatively, Cdc5 may arrive at CAR sites after cohesin deposition. It also remains possible that Cdc5 arrives at distinct CARs at different times in the cell cycle, as only a subset of CARs was examined in this study. Deciphering which mitotic events trigger the recruitment of Cdc5 to CAR sites will be important for understanding how Cdc5-mediated regulation of cohesin is achieved.

**2.3.1 Analysis of Cdc5 chromosome occupancy.** The Cdc5 ChIP data suggest localization to three distinct chromosomal pools: kinetochores/centromeres (CEN DNA sequence), pericentromeric chromatin, and chromosomal arm cohesin-associated regions (CARS). The kinetochore/centromere pool is present at the DNA sequence immediately at centromere DNA. Pericentromeric CARs are limited to a 50 to 60-kilobase region near centromeres where cohesin is prominently enriched on chromosomes that are not
bioriented but is absent when cells have bioriented chromosomes. Cdc5 also occupies chromosomal arm CARs outside of this pericentromeric region; these CARs mediate sister chromatid cohesion and are likely involved in regulation of gene expression (Lengronne et al., 2004; Losada, 2014; Bloom, 2015).

Due to the technical resolution issues with ChIP, the resolution of protein association/binding to DNA is limited by the random fragment shear size of the DNA. Since any fragment that contains centromeric sequences will likely also contain pericentrometic sequences, both will come down in the IP even if only one of them is bound to the protein of interest. Thus with ChIP, it is not possible to separate CEN DNA from pericentromeric DNA, in order to pare down binding sites to CEN DNA or pericentromeric DNA. Newer variations of ChIP such as ChIP-exo (Rhee et al., 2012) that incorporate exonuclease treatment of ChIP DNA prior to immunoprecipitation, may allow for an enhanced resolution of DNA-binding/association up to a single nucleotide resolution. However, we provide evidence that localization of Cdc5 to all three locations exhibits cohesin-dependence. Centromere-associated Cdc5 depends on the presence of a functional kinetochore, which may also reflect the dependence of cohesin localization on a functional kinetochore (Weber et al., 2004; Eckert et al., 2007; Fernius & Marston, 2009; Fernius et al., 2013).

Comparison of the chromosomal occupancy profiles of the cohesin subunit Mcd1 and Cdc5 at centromere 3 (CEN3) DNA sequence suggests that Cdc5 is present directly at the centromere, whereas cohesin may be excluded from direct CEN association but interacts closely with chromatin on either side of the CEN (Figure 3). Cdc5's chromatin enrichment forms a single discrete apex immediately at CEN3 DNA (Figure 1D, 1F and
Figure 5). Comparison of this Cdc5 peak with Mcd1 enrichment and specialized centromere-specific histone variant, Cse4 enrichment (Figure 3A and 1E, right panel, respectively), suggests that Cdc5 occupancy is broader than Cse4, but narrower than Mcd1 enrichment. At kinetochores, some proteins are present in more than one copy and others may distribute radially from the CEN3 along DNA, which may contribute to differential widths of occupancy at CEN DNA. The use of techniques such as ChIP-exo could help resolve the true range of the occupancy for each protein at centromeres. However, it is also possible that differences in the range of enrichment between Cdc5 and Cse4 could be due to technical differences in sample preparation. It is important to note that absolute height, width, and position of the peaks of Cdc5, Cse4 and Mcd1 association, may not be reflected by the ChIP profile as the primer pairs do not distribute evenly along the DNA, and the resolution is limited by technical processing during ChIP.

Notably Cdc5’s localization pattern to CEN and pericentromeric region exhibit some variance between experiments. When analyzed with ChIP, many other kinetochore and kinetochore-associated proteins including the CTF19 complex components, as well as Bub1, Mad1 and Mad2 exhibit similar CEN-specific localization (Owens et al., 2010; Gilett & Sorger, 2004). Similarly Mif2, Cbf1, Ndc10, Cep3 and Cse4 exhibit CEN-limited localization when analyzed by ChIP, while canonical histone H3 does not (Cohen et al., 2008). By contrast, Mcd1 typically exhibits peaks immediately adjacent to the CEN3 DNA, with reduced occupancy observed at the CEN itself (Eckert et al., 2007). This observation suggests cohesin is sterically excluded from CEN DNA by the kinetochore proteins, but surrounds it on the other side. This ‘cohesin hole’ has been
observed in other studies, most clearly using a high-resolution ChIP-seq method, to analyze cohesin association with chromatin (Hinshaw et al., 2015).

In contrast to Cdc5 localization at the centromere, Cdc5 occupancy at pericentromeric sites reveals co-localization with sites of cohesin enrichment (Figure 3A). Cdc5’s association with chromosomes in the pericentromeric region closely resembles Mcd1’s occupancy. We find evidence of Cdc5 peaks on either side of the CEN sequence, with minor peaks 4 kb to the left, and 6 kb to the right, as well as prominent peaks 12 kb to the left, and 17 kb to the right of the CEN DNA. Each of these peaks has more robust Mcd1 occupancy in the same region. This may be an artifact of crosslinking efficiency, or it is representative of actually binding affinities. The symmetry displayed by the peaks of Cdc5 and Mcd1 enrichment around CEN3 is interesting, as it may support the hypothesis of a pericentromeric loop of DNA (Yeh et al., 2008; Verzijlbergen et al., 2014; Hinshaw et al., 2015). Cohesin near CENs is proposed to tether DNA via intramolecular connections such that it forms a large loop with CEN at its apex (Yeh et al., 2008; Bloom et al., 2014). Moving out from CEN3 in either direction, each reflective pair of Cdc5 and Mcd1 peaks may represent an intramolecular tethering DNA loops on either side of the CEN, as well as a larger pericentromeric DNA loop (Yeh et al., 2008; Lawrimore et al., 2015; Bloom, 2015). The formation of larger pericentromeric loops appears to depend on the activity of Smt4, a desumoylating enzyme (Stephens et al., 2014). CARs on either side of CEN5 (Hinshaw et al., 2010) and CEN6 (Rossio et al., 2010), but within the pericentromeric region, also appear to exhibit reflective symmetry. Additionally, what specifies proteins to pericentromeric sites and how cohesin and Cdc5 are directed and maintained at these sites is not understood. A prediction that follows
from the pericentromeric loop model is that perturbing one site might affect its loop-forming partner.

Cdc5 is also found along chromosomal arms, outside of the pericentromeric region (Figure 4). The cohesin outside of the 50-60 kilobase pericentromeric regions of yeast chromosomes is thought to engage in intermolecular sister chromatid cohesion, and regulation of gene expression (Bloom, 2014; Losada, 2014). It is not known what factors mediate the boundary between pericentromeric and chromosomal arm cohesion. This boundary is important to delineate pericentromeric cohesin engaged in intrachromosomal cohesion involved in biorientation from arm cohesin engage in interchromosomal cohesion involved in sister chromatid cohesion (Stephens, 2015; Bloom, 2014). Notably, one of the chromosomal arm sites analyzed is found in the rDNA locus (Figure 3D), which may have separate cohesive properties, as this DNA is organized distinctly from other chromatin (Johzuka et al., 2005; St-Pierre et al., 2009). Despite potentially different roles played by the underlying cohesin, Cdc5 appears to bind to and likely regulate nearly all chromosomal cohesin.

Although my data shows that functional kinetochores and cohesin complexes are required for Cdc5 association with CEN and pericentromeric regions, I did not examine how these regulate Cdc5 binding along the chromosomal arms. Given that functional kinetochore are not required for cohesin localization outside of the CEN and pericentromeric region, it seems unlikely that Cdc5 association would changed in kinetochore mutants compared to wild type cells outside of the pericenteromeric region (Eckert et al., 2007). Conversely, it is expect that depletion of cohesin would affect Cdc5 localiziation at all CARs.
The simplest model to explain Cdc5 association with CARs is that all genomic recruitment of Cdc5 depends on cohesin. Disruption of the kinetochore specifically disrupts pericentromeric cohesin (Eckert et al., 2007), which in turn, may lead to reduced or abolished Cdc5 localization in the same region. The observation that loss of functional cohesin abolishes Cdc5 localization at centromeres is interesting, because it is not well understood how cohesin may contribute to the structure of the kinetochore. One possibility is that functional cohesin is required for normal kinetochore architecture and assembly, and thus disruption of cohesin leads to loss of Cdc5 recruitment at centromeres, with global disruption of cohesin being epistatic to any kinetochore-specific phenotypes related to Cdc5 recruitment.

Alternatively and perhaps not mutually exclusive, Cdc5 may be recruited to centromeres by a kinetochore-dependent mechanism via the Ndc10 kinetochore subunit, which is a centromere-specific DNA binding protein in budding yeast. Accordingly, several kinetochore-dependent factors have been hypothesized to contribute to polo-like kinase kinetochore recruitment in metazoans (Elowe et al., 2007; Yeh et al., 2013), with defects in polo-like kinase localization being a secondary defect of improper kinetochore assembly. Therefore, determining the mechanism of polo-like kinase recruitment and which, if any, kinetochore proteins recruit polo-like kinase, directly or indirectly, is essential for understanding this regulation.

In budding yeast a handful of conserved kinetochore proteins have been found to associate with Cdc5, including Cse4, Ndc80, Mad3, Stu2, and Slk19 (Rancati et al., 2005; Snead et al., 2007; Park et al, 2008; Rahal et al., 2008), each of which has a critical role in chromosome segregation (see Chapter 1). Many or all of these proteins contain
potential Cdc5 consensus phosphorylation motifs and PBD-binding motifs. Snead et al. (2007) demonstrated the PBD of Cdc5 interacts with Cse4 in pull down experiments. The kinetochore assembles hierarchically, and the stepwise genetic dissection of Cdc5 localization to the kinetochore can be accomplished by depletion of specific kinetochore proteins and subcomplexes, to decipher at which stage Cdc5 is recruited and/or which subcomplex is required for the recruitment. Additionally, a distinct kinetochore-mediated pathway of cohesin loading at centromeres via the CTF19 complex was uncovered recently (Ng & Biggins, 2009; Fernius and Marston, 2009; Fernius et al., 2013). Cohesin initially loads at centromeres and is thought to spread outward radially across pericentromeric DNA (Hu et al., 2011; Fernius et al., 2013). Thus, Cdc5 may be directed to kinetochores and pericentromeric chromatin through the same recruitment pathways that direct pericentromeric cohesin.

My findings suggest that Cdc5 may possess multiple, distinct pathways of recruitment to chromatin. Thus, the chromosomal occupancy profiles observed in this study, particularly at the pericentromeric region of CEN3 (Figure 1D-1F) may reflect the summation of these independent phenomena. Cohesin, the chromatin-bound substrate of Cdc5, is recruited to chromosomes by at least two pathways relevant to mitosis: one at chromosomal arm CARs and one at pericentromeric chromatin (Laloraya et al., 2000; Eckert at al., 2007; Ng et al., 2009; Fernius, 2009, 2013; Kogut et al., 2009). Our data supports a model whereby Cdc5 recruitment to both classes of cohesin enrichment sites is directly cohesin-dependent. Additionally, we also provide evidence that Cdc5 may also be recruited to chromatin by a kinetochore-specific localization via the Ndc10
kinetochore subunit (versus pericentromeric localization) that may not be directly cohesin-dependent (Figure 2).

**2.3.2 Cdc5 regulated dissociation of cohesion.** The polo-like kinase-dependent phosphorylation of cohesins is required for the disassociation of cohesins in metazoans, and ensures proper chromosome segregation in budding yeast (Alexandru et al., 2001; Hornig & Uhlmann, 2004). In yeast, cohesin displays a cell cycle–dependent electrophoretic gel mobility shift, suggesting the phosphorylation of subunit Mcd1 in metaphase or near the onset of anaphase (Alexandru et al., 2001). Mcd1 is phosphorylated by human Plk1 *in vitro* at amino acid sites that, when mutated, alter cohesin cleavage and chromosomal association patterns *in vivo*. After phosphorylation *in vivo*, subsequent Mcd1 cleavage is thought to be important for cohesin removal, and is enhanced by Plk1 phosphorylation *in vitro* (Alexandru et al., 2001; Hornig & Uhlmann, 2004). Given the importance of the timely removal of cohesin and co-localization with Cdc5 (Figure 3), it is highly likely that cohesin and Cdc5 interact while chromatin-bound. Here, we show that Cdc5 physically associates with cohesin complexes.

Polo kinase-like kinase mediated regulation of SMC complexes is not merely limited to mitotic cohesin, and also includes meiotic cohesin and mitotic condensin (Chapter 1). Cdc5 binds to and phosphorylates the meiotic paralog of Mcd1, Rec8, however it is not required for separase-mediated cleavage of Rec8 (Brar et al., 2006; Katis et al., 2010; Attner et al., 2013). Although we do not show evidence of Mcd1 direct binding to, or phosphorylation by, Cdc5, to my knowledge, there are no reported examples of Cdc5, or any polo-like kinases, binding to a protein complex member, and phosphorylating another member within the same complex. Binding by Cdc5 PBD may
ready cohesin for subsequent phosphorylation of Mcd1 by Cdc5 which may be required to specify/signal the timing of separase-mediated cleavage of a particular pool of chromosome-associated cohesins, or globally for all cohesin removal from chromosomes. Outside of mitosis, cohesin is recruited to chromosomes to mediate DNA repair and modulates gene regulation (Unal et al., 2004; Strom et al., 2004). It remains to be determined whether polo-like kinases contribute to the chromosomal removal of these non-mitotic cohesin complexes as well. Conversely, it remains possible that Cdc5 binds to cohesin in vivo, in a manner not dependent on prior substrate phosphorylation, although to date only several examples of this non-canonical phenomenon have been reported for any polo-like kinase, including Dbf4 in budding yeast (Chen & Weinrich, 2010). Interestingly, Ratsima et al. (2011) report that mutation of Cdc5 PBD, does not significantly affect Mcd1 phosphorylation, while mutation of the kinase domain diminishes it significantly. Although these cells progress through mitosis, they show significant genomic instability, indicating that Cdc5 regulation of mitosis is aberrant. This suggests that while Cdc5 does not require PBD-dependent targeting for normal phosphorylation of cohesin complexes, it is required for regulating mitosis as a whole.

It is unknown which cohesin subunit(s) mediate Cdc5-cohesin interaction. Further, the priming kinase(s) that may mediate Cdc5-cohesin association are currently unknown. One possibility is that another mitotic kinase primes Mcd1 for cohesin binding; alternatively, Cdc5 may prime its own binding, as polo-like kinases have been demonstrated to create their own self-tethering site (Kang et al., 2015). Mcd1 contains nine different motifs that partially match the polo-box recognition sequence, although it does not contain a perfect match (Figure 7). In vitro, recombinant Plk1 phosphorylates at
Figure 7. Cdc5 and Mcd1 interaction. (A) The Mcd1 amino acid sequence and previously identified Plk1 in vivo phosphorylation sites (red), and separase proteolytic cleavage sites (underlined) (Alexandru et al., 2001). (B) A list of putative Cdc5 phosphorylation motifs with the Mcd1 amino acid sequence that match the regular expression: [D/E]-X-X-[S/T*]-X-X-[D/E] derived from Nakajima et al. (2003). (C) A list of putative Cdc5 polo-box domain (PBD) binding sites within the Mcd1 amino acid sequence that match the regular expression: [S/T]-[S/T] or [S/T]-P derived from Elia et al. (2003a).
least one of these sites, and phosphorylation of this site is detected in living yeast cells (Alexandru et al., 2001; Ghaemmaghami et al., 2002). These observations suggest a model in which polo-like kinases may engage in self-directed priming of their targets (in this case, Mcd1), followed by binding and processive phosphorylation of their bound target protein (Lowry et al., 2005). Interestingly, two potential PBD-binding sites (S175 and S183) are located on either end of a string of 9 amino acids SLEVGRFFS (Figure 7) that contains the separase cleavage site (between RR), and both serines are phosphorylated in vitro by human Plk1. When S175 of these sites is disrupted in conjunction with an analogous serine 263 at a second separase-cleavage site, the regulation of cohesin is disrupted (Alexandru et al., 2001). However, the mutation of these two sites is not as severe as the loss of Cdc5, suggesting that Cdc5 may have additional roles in phosphorylating the cohesin complex that contributes to its regulation. Serine 175 is also phosphorylated in vivo, underscoring its importance (Alexandru et al., 2001; Ghaemmaghami et al., 2003). One prediction is that disruption of Cdc5 binding to cohesin may phenocopy disruption of Mcd1 phosphorylation. Site-directed mutagenesis of sites on Mcd1 predicted to be bound by the polo-box domain might abolish Cdc5 binding to Mcd1 thereby disrupting the regulation of cohesin.

**2.3.4 A model for Cdc5 association with chromosomes and future directions.**

The data above suggest a model where Cdc5 binds directly to the Mcd1 or another subunit of the cohesin complex, however the result of this association remains to be confirm by recombinant in vitro binding assays. Mcd1 is first phosphorylated about 75 minutes after release from G1 (Alexandru et al., 2001), putatively by Cdc5. Mcd1's electrophoretic mobility further shifts higher at 120 min, near the onset of anaphase.
Cohesin cleavage was first observed at 135 minutes. Thus, Cdc5 may first phosphorylate Mcd1 upon its initial interaction with cohesin, associate with Mcd1 and associate with cohesin via PBD binding to one or more phosphosites and phosphorylate Mcd1 again prior to the onset of anaphase after it is no longer protected by Sgo1. This would be consistent with the processive phosphorylation behavior described in other polo-like kinases. Ultimately this phosphorylation leads to the cleavage or removal from chromosomes of cohesin. One caveat to this model is the assumption that gel electrophoretic mobility is solely due to phosphorylation via Cdc5 as other modifications to Mcd1 that may lead to a gel shift including sumoylation have been reported (Almedawar et al., 2012; McAleenan et al., 2012).

It is unknown whether Cdc5 binding to cohesin may be PBD-dependent, or if it requires prior phosphorylation of cohesin by Cdc5 or other cell cycle kinases. Ratisma et al. (2010) report that loss of the Cdc5 PBD domain does not significantly affect Mcd1 phosphorylation, while mutation of the Cdc5 kinase domain nearly abolishes it. Thus, localization of Cdc5 via the PBD may not be required for regulation of cohesin, however the kinase activity itself is important. Hornig and Uhlmann (2004) demonstrated that mutation of 10 putative phosphosites increases the rate of chromosome loss. It is possible that phosphorylation of Mcd1 in cells where Cdc5 is lacking the PBD causes Cdc5 to remain unaffected in its ability to phosphorylate Mcd1, or that other kinases with overlapping specificity act redundantly, if Cdc5 is unable to localize properly. Alexandru et al. (2001) note that phosphorylation of Mcd1 by Cdc5 is independent of Cdk1, suggesting that Cdk1-dependent phosphorylation is not required for Mcd1-Cdc5 interaction. Another possible candidate kinase for Mcd1 phosphorylation is Mps1, which
has significant overlap of target specificity with polo-like kinase in human cells (Espeut et al., 2015), however there is no evidence for this overlap in budding yeast.

It remains unclear how Cdc5 is recruited to all chromosomal sites of cohesin association by binding directly to cohesin, and what its function is at those sites. Interestingly, Cdc5 is also subject to changes in association with centromeres and pericentromeric CARs upon the presence of chromosome biorientation (Figure 5), a phenomenon consistent with changes in cohesin occupancy in the presence or absence of biorientation (Eckert et al., 2007; Ocampo-Hafalla, 2007). How cohesin at pericentromeric chromatin, but not at chromosomal arm CARs, is removed prior to the onset of anaphase and separase activity in cdc16-1 cells is not known. One possibility is that Cdc5 contributes to cohesin removal in this pericentromeric region once successful chromosome biorientation is achieved. Cohesin removal, specifically at the pericentromeric region, may promote anaphase or strengthen kinetochore-microtubule attachments. For example, little is known about how microtubule attachments to kinetochores are converted from lateral to end-on attachments. While speculative, one possibility is that cohesin removal via polo-like kinase dependent mechanisms is important in this process. The role of Cdc5 in regulating specific chromosome sites will be examined in Chapter 3.

2.4 Materials and Methods

The yeast strains and plasmids used in this study are listed in Tables 2-1 and 2-2, respectively.

2.4.1 Strain Construction. Cdc5 was C-terminally tagged using single-step gene disruption as described in Longtine et al. (1998), and Nishimura et al. (2009). PCR
amplification of constructs for transformation and integration was performed using high fidelity Phusion Taq DNA polymerase (NEB). Transformation of PCR amplicons or plasmids was performed as described in Geitz and Woods, (2002). Successful integration of epitope tag sequences was verified by PCR using primers complementary to genomic sequences external to the integrating cassettes. Verification primers were designed using Primer3 (Untergasser et al., 2007).

The Δbar1 strains were made using plasmid pBW1120 as described in Reneke et al. (1998). Δbar1 strains were confirmed by testing for alpha factor sensitivity. OsTir1 strains were made by digesting plasmid pNHK53 (Nishimura et al., 2009) with SruI and transforming the linearized DNA into various strains, resulting in URA3::OsTir1 cells. PCR primers internal to the OsTir construct were used to validate the presence of OsTir1. mcd1-1 strains were made by digesting pVG257 with AgeI and transforming linearized DNA into MAY8775, resulting in the MCD1::URA3-mcd1-1 construct. To generate the mcd1-1 allele via homologous recombination, counterselection against URA3 was performed by streaking cells on plates containing 5-fluoroorotic acid, and selecting for cells with positive growth. The mcd1-1 strains were confirmed by absence of growth at 37°C. TAP-tagged strains were derived from yeast ORF collection provided by GE Life Sciences (Gelperin et al., 2005). The construct for TAP-tagging, pBS1479 is available from Euroscarf and is described in Puig et al. (2001).

2.4.2 Strain Growth and Media. Rich (YP) yeast media was made as described in Sherman et al., (1983). Auxin-containing agarose plates were made by adding indole acetic acid (IAA, Sigma) from a 10 mg/ml stock in ethanol to autoclaved media to a final
of 8 mM. Nocodazole was added to liquid media from a 5 mg/ml stock in DMSO to a final concentration of 15 μg/mL.

2.4.3 Chromatin immunoprecipitation. ChIP was performed as described in Harbison et al. (2004) with the following modifications: after the bead washing, samples were re-suspended in Tris-EDTA, 1% SDS and reversal of crosslinks and elution were performed simultaneously at 95°C for 20 min. After crosslink reversal, DNA was purified using ChIP DNA Clean and Concentrator (Zymo).

2.4.4 ChIP Analysis. PCR was performed as described in Harbison et al. (2004) with the following modifications. One microliter of ChIP DNA and/or input DNA (diluted 1:40) was input into a 20 microliter PCR reaction. Primers for ChIP analysis were designed using Primer3, except where noted otherwise (Untergasser et al., 2007). Crimson Taq DNA Polymerase was used for DNA amplification (NEB). One half of the PCR samples were loaded onto a 3% Agarose (Sigma) gel containing ethidium bromide (Sigma), and quantified using a G-Box imager and gel imaging software from Syngene. Signal intensities of ChIP DNA were normalized to Input DNA to obtain % of input ratios, which were plotted versus the corresponding chromosomal coordinates of the midpoint of the amplicon. Chromosomal coordinates were obtained from Saccharomyces Genome Database, or the indicate loci. Primers for analysis of pericentromeric CEN3, are described by Weber et al. (2004). Primers for CARs at the rDNA locus on CHRXII and CARC1 on CHRII are described in Laloraya et al. (2000). Primers for CAR at GAL2 are described in Kogut et al. (2009).

2.4.5 DIC and Immunofluorescence Microscopy. Prior to chromatin immunoprecipitation or immunoprecipitation experiments, cells were staged for cell
cycle arrest using DIC with a 100X objective lens on a Zeiss Axioskop microscope. Cells were fixed and permeabilized with 70% ethanol, treated with 4', 6-diamidino-2-phenylindole to final concentration of 1 ng/ml in Phosphate Buffered Saline and imaged with epifluorescence microscopy on a Zeiss Axiovert 200 using a 100X objective.

2.4.6 Western blotting. Cells were harvested and subjected to lysis by bead beating in co-IP buffer as in Heidinger-Pauli et al. (2008), and extracts were clarified using centrifugation. Samples were diluted with an equivalent volume of 2X Laemmli buffer and boiled for 5 min prior to SDS-PAGE using Mini-protean gels (Biorad). Proteins were transferred to nitrocellulose membranes using the iBlot system. The membrane was blocked in Tris-buffered saline containing an additional 0.1% Triton and 5% dry milk powder, before blotting with the indicated antibodies at a dilution of 1:10,000.

2.4.7 Co-immunoprecipitation. Cells were staged for cell cycle arrest and subjected to co-immunoprecipitation as described in Heidinger-Pauli et al. (2009).
Chapter III: \textit{cdc5-aid} Cells Arrest With Persistent Cohesin at Centromeres

3.1 Introduction

The faithful cleavage of cohesin complexes and their dissociation from chromosomes are processes critical to chromosome segregation. Cleavage at anaphase onset is carried out by separase (Uhlmann, 1999; Uhlman, 2000) in a process that is regulated by the polo-like kinase family, in budding yeast and human cells (Alexandru et al., 2001; Hornig & Uhlmann, 2004; Hauf et al., 2005). However, there are fundamental differences in cohesin chromosomal regulatory pathways between yeast and human, and it remains unclear whether cohesin cleavage and disassociation are molecular events coupled by polo-like kinases, or perhaps parallel processes for achieving cohesin removal from chromosomes.

In budding yeast, research from Nasymyth’s and Uhlmann’s laboratories led them to propose that polo-like kinase Cdc5 phosphorylates the Mcd1 subunit of cohesin, which promotes bulk cohesin cleavage and separation of chromosomes at anaphase (Uhlmann et al., 2000; Alexandru et al., 2001; Hornig & Uhlmann, 2004). Conversely, in human cells, cohesin is removed in a stepwise fashion during mitosis, first from chromosome arms in prophase, and then from centromeres at the onset of anaphase; these differences in cohesin regulation raise multiple questions with respect to the mechanism of mitotic cohesin removal. First, it is unclear why this process occurs during two spatiotemporally distinct steps in humans, while it appears to occur in a single step in budding yeast. Second, it is not known whether in human cells, polo-like kinases are involved in separase-mediated cleavage of cohesin at centromeres at the onset of anaphase, or are limited to prophase-dependent cohesin removal at chromosomal arms. Third, it is not
known whether the yeast polo-like kinase, Cdc5, participates in cohesin disassociation at all cohesin-associated regions (CARs) at anaphase, or is limited to either chromosomal arms or centromeres. Answering these questions will help elucidate whether polo-like kinase and separase act synergistically at anaphase, or operate by redundant pathways to remove cohesin complexes from chromosomes. These questions can be answered in part by determining the chromosomal sites where Cdc5 acts on cohesin. By employing previous fine-scale maps of cohesin occupancy in the yeast genome, in combination with yeast genetics, the chromosomal sites where Cdc5 regulates cohesin disassociation can be determined.

In budding yeast, the depletion of Cdc5 has effects on the timing and completion of chromosome segregation (Alexandru et al., 2001; Snead et al., 2007). However, it is important to keep in mind that polo-like kinases also affect mitotic spindle formation, which could in turn, affect these events, or cohesin regulation itself (Discussed in Chapter 1). During unperturbed mitosis, Cdc5 does not appear to be required for biorientation or the initiation of chromosome separation, as evidenced by the timely splitting of centromeres and nuclear DNA masses (Alexandru et al., 2001) observed in Cdc5-depleted cells. However, Alexandru et al. (2001) report a significant lag in the separation of telomeres, and incomplete segregation of nuclear DNA often spreading across the bud neck, suggesting that incomplete cohesin cleavage could cause these effects.

Accordingly, loss of Cdc5 leads to persistent cohesin in anaphase as compared to control cells (Figure 10), and cohesin persistence appears to overlap with a centromere-specific binding protein Ndc10 (Alexandru, 2010). In mutant cell division cycle 15 (cdc15) cells that presumably arrest later in the cell cycle than cdc5-arrested cells in teleophase (Bardin
et al., 2003), cohesin persistence was significantly decreased, although not altogether absent (Alexandru et al., 2001).

Another study (Snead et al., 2007), reported a slight delay in the timing of centromeres, but did not report on telomere separation or cohesin persistence when employing a Cdc5 analog-sensitive allele (cdc5-as), which specifically inhibited the kinase activity of Cdc5 in the presence of the analog. A delay in late anaphase progression (i.e., delayed timing and incomplete nuclear segregation, as well as anaphase spindles) is also observed in cdc5-as cells, as was a defect in Mcd1 phosphorylation (Snead et al., 2007).

Taken together, these studies suggest that polo-like kinase Cdc5-dependent regulation of Mcd1 is required for proper chromosome segregation, and that the absence of Cdc5 activities gives rise to cohesin persistence. However, it remains difficult to determine whether the effects observed are directly due to inhibition of polo-kinase dependent regulation, or some other aspect of cell cycle misregulation caused by the polo-like kinase defect. For instance, it is difficult to compare the results of the timing of centromere separation between the Alexandru et al. (2001) and Snead et al. (2007) studies, as timing experiments were each performed once, suggesting that any differences between findings may be marginal, thus impossible to compare. Further, neither study showed the efficacy of depletion (Alexandru et al., 2001) or kinase inhibition (Snead et al., 2007). Ratsima et al. (2011) suggested that cells can survive on very low levels of essential kinases. Thus, small amounts of polo-like kinase activity may have remained in the cells, in both experiments. It is unclear whether, Cdc5 inhibition was fully responsible for the observed changes in electrophoretic mobility (Alexandru et al., 2001), other
modifiers of cohesin kleisin subunits such as sumoylation are also contributing to these changes (Almedawar et al., 2012; Stephens, 2015), or whether these modifications are directly linked to cohesin-cleavage via separase in vivo. Lastly, it remains unclear whether the cohesin persistence observed in Cdc5 mutants represents a defect in cohesin regulation, or whether it is a normal aspect of anaphase due to lower amounts of cohesin on chromosomes later in the cell cycle (Alexandru et al., 2001; Hornig & Uhlmann, 2004).

Given this collection of findings, I set out to understand the molecular mechanisms by which Cdc5 regulates cohesin dissociation from chromosomal DNA in S. cerevisiae. As in other organisms, the yeast polo-like kinase Cdc5 is essential for viability. Therefore, I created a potent conditional allele of the CDC5 gene, which allowed me to deplete Cdc5 protein in an inducible and cell cycle-specific manner. In the experiments described below, we create and characterize a novel auxin-inducible degron allele cdc5-aid. Characterization of cdc5-aid expressing cells revealed that they do not grow in the presence of auxin; further, these cells exhibited a late cell cycle arrest phenotypes, recapitulating the phenotypes found using other previously described cdc5 alleles (Lee et al., 1998; Park et al., 2009; Ratsima et al., 2010; Uhlmann et al., 2001). These observations suggest that cdc5-aid depletion is efficient and can act as a potent means for the study of Cdc5 function. Accordingly, the cdc5-aid allele generated in this study was shared with Walters et al. (2014) where it was employed to study effects of Cdc5 on nuclear morphology, suggesting the usefulness of this allele in characterizing novel cdc5 phenotypes. In this study, I found that in cdc5-aid cells treated with auxin, cohesin persisted at pericentromeric DNA late in the cell cycle, but disassociates at
chromosomal arm CAR DNA. This finding implies that Cdc5 differentially regulates cohesin at chromosomal arms and centromeres.

3.2 Results

3.2.1 Generation and characterization of a \( cdc5\)-\( aid \) allele: Introduction to the auxin-inducible conditional degradation system. In order to understand how Cdc5 regulates cohesin, it was necessary to generate an allele that inactivates Cdc5. It has previously been shown that inactivation of Cdc5 and other polo-like kinases gives rise to a range of phenotypes, depending on the method of depletion used, and may give rise to partial or incomplete activation and arrest points, at various stages of the cell cycle (Alexandru et al., 2001; Moutinho-Santos et al., 2011; Park et al., 2008; Ratsima et al., 2011; Ratsima et al., 2016). Due to the essential nature of Cdc5, loss-of-function studies must be conducted with a conditional allele. Accordingly, previous alleles were problematic for various reasons. For instance, a temperature-sensitive allele may not give rise to total inactivation of the protein. Many alleles may also not allow for precise cell cycle control of depletion. Generation of a conditional temperature-sensitive (t.s.) Cdc5 allele, using an existing t.s. degron system was problematic (data not shown), partially due to the function of this degron system in the normal degradation of the byproducts of cohesin cleavage (Rao et al., 2001; Dohmen & Varshavsky, 2005). Thus, an alternative approach was required to deplete Cdc5 from cells.

Towards this end, I generated a conditional allele of Cdc5 that allows for inducible, temporally controlled degradation of Cdc5 using the auxin-inducible degron (AID) system (Nishumora et al., 2009). The AID system accomplishes specific protein degradation by exploiting the E3 ubiquitin ligase activity of the Skp, Cullin, F-box-
containing (SCF) complex. Encoded by the rice plant *Oryza sativa* (Os), Os Transport Inhibitor Response (OsTir1) contains an F-box protein adapter that can bind and form a specific SCF E3 ligase. When the small molecule plant hormone auxin/indole acetic acid (IAA) is present, OsTir1-SCF can recognize and bind to transcriptional repressors of auxin indole acetic acid/auxin (IAA/Aux) responsive genes (Dharmasiri et al., 2005), which contain an auxin-inducible degron (AID) protein sequence. The AID-tagged protein is then polyubiquitinated by OsTir1-SCF and targeted to the proteasome for degradation.

While the auxin response pathway is limited to plants, other eukaryotes contain the SCF ubiquitin machinery (Nishimura et al., 2009). Nishimura et al. (2009) exploited the conservation of SCF by moving the auxin response system to co-opt the endogenous SCF machinery in other organisms, including yeast, for conditional protein degradation mediated by the addition of auxin (Nishimura et al., 2009). To this end, OsTir1 under the control of a yeast promoter can be directly integrated into the yeast genome (Nishimura et al., 2009). Further, DNA encoding the AID protein sequence derived from the IAA-responsive transcription factor can be integrated at the 3’ end of a gene of interest within the yeast genome. Thus, when the gene of interest is expressed as a protein, it contains the ectopically appended auxin responsive domain, which can be subjected to conditional degradation (Nishimura et al., 2009; Liscum & Reed, 2002). During experimentation, the protein of interest can be specifically targeted for degradation in a controlled manner by addition of auxin to the cell media.

### 3.2.2 Generation and growth characterization of a *cdc5-aid* allele.

*CDC5* is an essential gene, thus, if auxin-mediated degradation of *cdc5-aid* protein is effective, *cdc5-*
aid cells (MAY8976) should be rendered slow growing or inviable. To test this assertion, cdc5-aid OsTir1 strain MAY8976, a parent strain expressing CDC5 OsTir1 (MAY8975) and a control strain lacking OsTir1 CDC5 (MAY0589) the Hoyt laboratory’s wild-type strain, were assayed for growth by streaking cells on YPD plates containing the auxin IAA (Figure 8A). Cells expressing cdc5-aid OsTir1 (MAY8976) grew very poorly on auxin-containing YPD media, while the wild type CDC5 (MAY0589) and CDC5 OsTir1 expressing cells (MAY8975) appeared to grow normally (Figure 8A). Other aid-tagged essential genes demonstrate the robustness of aid method (Figure 8A). In liquid culture, cdc5-aid OsTir1 cells (MAY8976) exhibited a faster, sigmoidal growth pattern (as compared to untreated cells) in yeast rich media before their growth reach a plateau (Figure 8B), but grew much more slowly in an approximate linear fashion, in the presence of auxin, never achieving a growth plateau (Figure 8B). These observations suggest that cdc5-aid strains also grow very poorly in YPD liquid culture upon the addition of IAA (Figure 8B); the increase in optical density may reflect an increase in cell volume in the absence of cell division due cdc5 arrest. The poor growth of cdc5-aid OsTir1 cells in the MAY0589 derived strain, in the presence of auxin, was reproduced in cdc5-aid OsTir1 cells (MAY8972) grown in the presence of auxin in the Nishimura et al. (2009) strain derived from W303; suggesting the robustness of cdc5-aid depletion in multiple yeast strain backgrounds (Figure 8C). Despite these growth assays, it remains possible that in cells expressing Cdc5-aid protein, the aid-tag affects cellular growth, as compared to Cdc5 protein expressing cells, and should be carefully tested in the future.
Figure 8A. Generation of a cdc5-aid auxin-inducible degron allele. Control cells MAY0589 (WT) and MAY8975 (OsTir1) and cdc5-aid OsTIR1 (MAY8976) cells were streaked on YPD media containing 4 mM auxin IAA and grown for 2 days at 30°C before imaging. Cells from a panel of AID degron alleles (see Table 3-1 for genotypes) were also assayed for growth sensitivity on auxin. Red underlined text denotes that cells contain the essential gene of interest tagged with AID, as well as expressing OsTir1.
Figure 8B. *cdc5-aid* cell growth is impaired during auxin treatment. Cells expressing *cdc5-aid* OsTir1 (MAY8976) were inoculated into YPD in the presence (green & purple) or absence (red & blue) of auxin and cultured for the indicated times. To determine the effects of exogenous tryptophan (top right), which is similar to auxin IAA molecularly (top left), cells were grown in the presence (red) or absence (purple) of exogenous tryptophan. A control containing media-only (light blue) for growth containing media only was also cultured for comparison to a baseline of no growth. The absorbance of OD$_{600}$, a measure of cell growth, was monitored at the indicated regular intervals.
Figure 8C. *cdc5-aid* cell growth is impaired a second yeast strain background. Cells from BWY5323 (BY25598 from Nishimura et al., 2009) a strain expressing OsTir1, and derivative strain expressing OsTir1 *cdc5-aid* (MAY8972) were streaked on YPD media containing 4 mM auxin IAA and grown for 3 days at 30°C. Cells expressing *OsTir1 pan1-aid* (BWY5394) were also added as a control for growth on auxin containing media. The *pan1-aid* strain was previously described in Bradford et al. (2015).
In parallel with the generation of cdc5-aid, a panel of other aid-tagged genes (all essential for viability) were constructed and tested for auxin-sensitive growth (Figure 8A). These strains were created to mediate arrest in metaphase (cdc16-aid, ndc80-aid) or anaphase (cdc14-aid, cdc15-aid), or to deplete cohesin function (mdc1-aid, smc3-aid), to study Cdc5 and Mcd1 function. The genotypes of these strains can be found in Table 3-1. All degron strains grew poorly on media containing auxin, indicating the potentially useful nature of the AID system in eliminating distinct, essential proteins in related pathways (Figure 8A).

We also observed similar auxin-dependent growth deficits in yeast containing a previously validated pan1-aid allele (BWY5394), which results in degradation of an essential gene involved in endocytosis and actin dynamics (Bradford et al., 2015). To ascertain whether cells containing cdc5-aid grow poorly on auxin due to specific inhibition of Cdc5, cdc5-aid expressing cells were transformed with plasmids expressing pRS-CDC5 or an empty vector control (Figure 8D, data was provided courtesy of Kate Bradford). The plasmid pRS-CDC5 was not able to complement pan1-aid (data not shown). The growth of these cells was compared on media in the presence or absence of the auxin molecule 1-Naphthaleneacetic acid (NAA), a synthetic plant hormone. Previously, IAA was used (Figure 8A-C), and will be used interchangeably with “auxin” hereafter, for the remainder of this study. Cells expressing cdc5-aid and containing pRS-CDC5 plasmid or an empty vector control both grew on media lacking auxin NAA (Figure 8D, left); however, only cells containing the plasmid expressing CDC5 grew on media containing auxin NAA (Figure 8D, right). This demonstrates that expression of ectopic CDC5 is sufficient to complement the growth inhibition of cdc5-aid cells in the
Figure 8D. cdc5-aid viability is rescued by expression of extrachromosomal CDC5. Cells expressing cdc5-aid (MAY8972) and pRS426-CDC5 or empty control vector were streaked on YNB–leucine (-leu) dropout media containing additional 1 mM auxin NAA or nothing and grown for 3 days at 30°C for 3 days before plates were imaged. Data was provided courtesy of Kate Bradford.
presence of auxin, suggesting that the poor growth of \textit{cdc5-aid} cells in the presence of auxin is due to the loss of Cdc5. Additionally, these findings also suggest that multiple auxin compounds can be employed effectively with the \textit{cdc5-aid} system, at least as it pertains to cell growth.

\textbf{3.2.3 Characterization of a \textit{cdc5-aid} allele.} Given that the AID system was developed only in recent years, there was little published information or consensus on standard conditions for auxin-mediated protein depletion, which led us to optimize conditions in our yeast background. To optimize the response of \textit{cdc5-aid} cells to auxin, the sensitivity of \textit{cdc5-aid} cells to a range of auxin concentrations was examined during growth in liquid media YNB, a form of minimal yeast media. Liquid YNB was initially chosen over rich media in optimization studies, as it contains a defined and limited number of components, thus reducing the chance that an unknown component in rich media would interfere with auxin sensitivity or efficacy. Auxin is known to be labile, and we sought to avoid any possibility of interfering with auxin uptake into cells, sequestration of auxin in the media, or chemical interference with auxin chemical structure, due to adverse chemical reactions with media components. We chose to use the auxin molecule indole-3-acetic acid (IAA), a naturally occurring auxin, and the most abundant type of auxin in plants, as it was more readily soluble, and hence easier to work with experimentally. I next determined the range of auxin sensitivity of \textit{cdc5-aid} expressing cells in YNB during log-phase growth (Figure 9A). Cells expressing OsTir1 \textit{cdc5-aid}, or their cognate parent strain containing only OsTir1, were grown to log-phase and diluted back to early log-phase, then subjected to 2-fold serial dilutions of IAA, ranging from 8 mM to 0.5 mM, and grown for 3 hours. The range of auxin concentrations
Figure 9A. Characterization of cdc5-aid cell growth in YNB. Cells expressing cdc5-aid (MAY8976) or control cells (MAY8975) were grown to log-phase in YNB + glucose and diluted to OD$_{600}$ = 0.25 before the addition of auxin IAA in the carrier ethanol at the serial dilution indicated and cultured for 3 hours before taking a terminal OD$_{600}$ reading. To compare the growth in the presence or absence of auxin, the OD$_{600}$ for each dilution of auxin was plotted as indicated.
tested was based on previous studies (Nishimura et al. 2009; Walters et al., 2014; Bradford et al., 2015). In both cdc5-aid and control cells, a negative relationship between the concentration of auxin and the rate of cellular growth was observed (Figure 9A). At the highest concentrations of 4-8 mM, control cells failed to significantly increase their optical density (Figure 9A), and effectively appeared dead when examined by light microscopy. In contrast, cdc5-aid cells treated with 2 mM auxin appeared uniformly arrested at a late stage in the cell cycle with large buds, and mononucleate or binucleate DNA masses when examined by DIC or epifluorescence microscopy, in conjunction with DAPI staining (Figure 9B). This is in contrast to control cells, which did not appear to accumulate in any particular phase of the cell cycle, despite a slowing down in growth rates (Figure 9B). Encouragingly, the terminal arrest of cdc5-aid phenocopies those observed in a variety of other cdc5 mutants (Hartwell et al., 1970; Lee et al., 1998; Park et al., 2010; Alexandru et al., 2001; Snead et al., 2007).

3.2.4 Characterization in liquid YNB media. The negative effects of auxin concentration on cell growth in CDC5 and cdc5-aid cells in YNB were undesirable for conducting further assays (Figure 9A). In Chapter 2, each of the Cdc5 ChIP assays (in which cells are CDC5+) were performed in YPD; thus, this is the most relevant condition for comparing cdc5-aid cells to CDC5 cells during auxin treatment. We next assayed growth in standard yeast rich media, in which all previous Cdc5 experiments were conducted (Figure 9C). The growth of OsTir1 cdc5-aid and OsTir1 CDC5 cells was assayed at a range of auxin concentrations. There was very little difference in growth between the cdc5-aid and the control strain, except for at the higher concentrations of
Figure 9B. Auxin-treated cdc5-aid cells arrest late in the cell cycle. To assess the bud and nuclear morphology of cells expressing cdc5-AID (MAY8976, panel 1) or control cells (MAY8975, panel 2) during auxin treatment, cells were grown in the presence of 2 mM IAA for 3 hours. Cells were stained with DAPI and imaged by DIC and epifluorescence microscopy. Two representative images are depicted from the cdc5-aid strain (left) while one representative image is depicted on the right from CDC5 cells.
**Figure 9C.** Characterization of *cdc5-aid* cell growth in YPD. To assess their growth in YPD, *cdc5-aid* (MAY8976, red) and control (MAY8975, blue) cells were grown as in Figure 9A, except that YPD media was used instead of YNB.
auxin, between 2-8 mM, suggesting that the effects of auxin on Cdc5 may only be observable at high concentrations in YPD. This was suboptimal because ideally, auxin should have no effect on $CDC5$ cell growth, and a drastic effect on $cdc5$-aid cells. However, optical density is not a great measure of cell proliferation because arrested cells continue to increase in size isotropically, which increases the optical density, while not increasing the cell numbers. Even at the highest auxin concentration, both strains more than doubled from their initial optical density (Figure 9C). This is in contrast to the much more severe concentration-dependent effect of auxin on cell growth in the minimal media YNB, possibly because YNB contains fewer components that might interfere with the effective auxin concentration, rendering the cells more sensitive (Figure 9A). At the 4 mM auxin concentration, $CDC5$ cells did not appear to be significantly affected by auxin, as compared to lower concentrations of auxins, while the growth of $cdc5$-aid cells was inhibited by auxin. As a result of these observations, I chose the 4 mM auxin as the default experimental condition for YPD growth experiments, as I wanted to maximize auxin-mediated Cdc5-aid depletion and the severity of the resulting phenotype, while minimizing auxin-dependent growth. In the future, a complete growth analysis would include time courses of growth assays, in the presence and absence of auxin (similar to Figure 8B), using OsTir1 $CDC5$ and $cdc5$-aid cells, as well as OsTir1- $CDC5$ and $cdc5$-aid cells.

3.2.5 Characterization of $cdc5$-aid allele in liquid YPD. To perform ChIP assays of a cell-cycle dependent process, a highly uniform arrest of a population of cells is preferable, although can be technically challenging to achieve. I was concerned that $cdc5$-aid cells arrested with auxin may ultimately adapt to auxin treatment, resulting in
phenotypic slippage such as rebudding, or cohesin reloading. To determine if the phenotype we observed in cdc5-aid cells upon acute auxin treatment was a terminal arrest, we grew cdc5-aid OsTir1 and OsTir1 only cells overnight (12 hours) in YPD, in the presence or absence of auxin, and then stained cells with DAPI and imaged them with DIC/epifluorescence microscopy (Figure 9D). Examination of OsTir1 cdc5-aid cells treated with auxin revealed that the majority of cells arrested with large buds, an enlarged dumbbell shape, and in most cases, exhibited two nuclei that were distributed equally between the mother and daughter cell, but had not fully separated to opposite poles (Figure 9D). Further these cells contained no small buds. By contrast cells in each of the control condition appear to distributed in all phases of the cell cycle as they contained small, medium, large and unbudded cell. Thus as the auxin phenotypes appeared limited to cdc5-aid cells in auxin treatment it suggesting that auxin imposed a very strong and long-lasting phenotype (at least 12 hours) on these cells relative to the duration of their cell cycle. In contrast, cdc5-aid cells not treated with auxin, and cells expressing only OsTir1, grown with or without auxin, appeared to be normally sized and asynchronous (Figure 9D). This is supported by liquid growth assays in which cdc5-aid cells arrest and fail to grow, while control cells grow normally, suggesting that auxin mediates a high-quality terminal arrest phenotype (data not shown). In some cdc5-aid cells, it appeared that chromatin bridges stretched between the two partially segregated DNA masses (data not shown).

Alexandru et al. (2001) report a similar “butterfly” nuclear phenotype in their cdc5 cells. Finally, in cdc5-aid cells, the nuclear masses often appeared diagonal to the hypothetical, normally aligned spindle axis that traverses between the mother and
Figure 9D. Auxin-treated cdc5-aid cells hold late cell cycle arrest overnight. To assess the stability of their terminal arrest phenotype during 4 mM IAA (auxin) treatment cdc5-aid OsTIR1 (MAY8976; right) and control cells CDC5 OsTIR1 (MAY8975; left) were grown in YPD in the presence (+IAA, bottom) or absence (-IAA, top). Cells were grown 12 hours total, stained with DAPI and imaged with epifluorescence and DIC microscopy.
daughter cells at the bud neck; the nuclear masses were often distributed off-axis, on one side of the bud neck in the mother cell, and the opposite side of the bud neck in the daughter cell. This phenotype is reminiscent of the misaligned spindle phenotype observed in *cdc5-as* cells (Snead et al., 2007), and the misaligned spindles in *cdc5* cells likely give rise to misaligned nuclear masses during mitosis. In the future, it would be useful to quantify both phenotypes to determine if they co-occur in *cdc5* cells. By contrast, the cells from the cultures in the control conditions did not appear to display a bias for a particular phase of the cell cycle, budding, or nuclear morphology phenotype (Figure 9D). These control conditions were not quantified, however, and a comparison of *cdc5-aid* cells to these control cells is needed.

**3.2.6 Quantification of cell cycle index during *cdc5-aid* arrest.** In order to characterize the *cdc5-aid* arrest after treatment with auxin the cell cycle index of *cdc5-aid* cells in the presence of auxin was determined. This quantification was performed in *cdc5-aid* cells that also expressed Mcd1-13myc, as the ultimate goal was to assay Mcd1 chromatin association in the absence of Cdc5. To this end, *cdc5-aid* cells expressing Mcd1-13myc bar1 were treated with auxin for three or four hours; 3-hour auxin treatment resulted in cells arrested with >80% anaphase nuclei, and were approximately 90% large budded (Figure 9E). In alpha factor treated cells, the majority of cells (>95%) were arrested in G1 due to the hypersensitivity of the bar1 strain to alpha mating pheromone (data not shown). After four hours of auxin treatment, the percentage of anaphase cells decreased slightly (73%), resulting in a corresponding increase in multibudded or unbudded cells (Figure 9E). This suggested that auxin treatment for longer than 3 hours would not significantly improve the uniformity of anaphase arrest in
Figure 9E. Quantification of \textit{cdc5-aid} auxin-mediated cell cycle arrest. To compare the quality of synchronous anaphase arrest after 3 (blue) or 4 (red) hours, auxin treated \textit{bar1} \textit{cdc5-aid MCD1-13MYC} cells (MAY8980) were fixed, stained with DAPI, imaged and scored for cell cycle index (N=300 cells) and nuclear morphology at each time point and binned into the indicated morphology categories.
One possibility is that cdc5-aid depletion is ‘leaky’ allowing for gradual phenotypic slippage to occur; alternatively, cells may hit a mid-anaphase checkpoint (Yang et al., 1997) due to lack of Cdc5, and then ultimately adapt to that checkpoint over time. However it is unlikely that cdc5 cells exit mitosis as Cdc5 is required for the mitotic exit network (MEN) (reference), a pathway that must be executed in order for cells to pass thru M-phase and into G1. It is possible that shorter arrest times or altering media conditions, might improve the efficiency of the arrest, and hence the percentage of cdc5-aid cells in anaphase. Nonetheless, we chose cdc5-aid cells arrested with auxin for 3 hours, for further ChIP analysis of the Mcd1 genome association.

3.2.7 Similarity of cdc5-aid with other cdc5 mutants. To compare the cell cycle distribution of cdc5-aid cells with temperature-sensitive cdc5-99 and temperature-sensitive cdc5-deg mutants, we examined the DNA using DAPI staining in conjunction with epifluorescence microscopy (Figure 9F; St.-Pierre et al., 2009). All cdc5 mutants employed in this study typically arrest as large budded cells with two separated nuclear masses, a phenotype that is indicative of late mitosis. Temperature-sensitive cdc5-1I cells (Park et al., 2008) and analog-sensitive cdc5-as cells (Snead et al., 2007), also displayed similar phenotypes. Thus, the phenotype of the cdc5-aid OsTir1 mutant in the presence of auxin, is similar to previously characterized conditions that lead to Cdc5 inactivation (Alexandru et al., 2001; St. Pierre et al., 2009; Ratsima et al., 2011; Lee et al., 1998; Park et al., 2008).

3.2.8 Analysis of Cdc5-aid protein degradation. We next attempted to validate the degradation of the Cdc5-aid protein upon auxin-treatment. Due to technical issues, efforts to perform immunoblotting with anti-Cdc5 antibody were not successful.
Figure 9F. Other cdc5 mutants arrest similar to cdc5-aid. To examine the arrest phenotypes of other cdc5 mutants, temperature-sensitive mutant cdc5-99 (MAY8915, left) or temperature-sensitive cdc5-degron (cdc5-deg), (MAY8803, right) cells were grown for 3 hours in YPD at the non-permissive temperature 37°C, and imaged as in Figure 9B. MAY8915 was previously described in St-Pierre et al., 2009. MAY8803 was generated as described in Dohmen & Varshavsky, 2005.
However, Walters et al. (2014) demonstrated that Cdc5-aid is depleted in auxin, in an OsTir1-dependent manner.

With the generation of a *cdc5-aid* allele, we were able to recapitulate phenotypes typical of *cdc5* depletion alleles including anaphase arrest, which was identified in the original temperature sensitive *cdc5-1* allele from Hartwell’s screen, in which *cdc5* was initially discovered (Hartwell et al., 1973; Lee et al., 1998). This is very strong evidence that *cdc5-aid* is efficiently depleted upon addition of auxin. Additionally, this *cdc5-aid* allele was used to confirm a novel Cdc5-mediated phenotype, a defect in normal nuclear flare morphology, thereby demonstrating the utility of a *cdc5-aid* degron system (Walters et al., 2014). Given the cellular phenotype observed in *cdc5-aid* cells, these data that *cdc5-aid* is efficiently depleted in an auxin-dependent manner. Therefore, *cdc5-aid* could be used for assessing the effects of Cdc5-depletion on cohesin chromosomal association, and the spindle assembly checkpoint (SAC) activation and bypass, as well as other Cdc5-mediated pathways in including kinetochore-dependency relationships.

### 3.2.9 Cohesin persists on pericentromeric chromatin during anaphase in *cdc5-aid* arrested cells.

Depletion of Cdc5 leads to an overall decrease in bulk cohesin cleavage by separase, and cells arrest with some cohesin persisting on their chromosomes (Alexandru et al., 2001; Hornig & Uhlmann, 2004). The locations on the chromosomes where Cdc5-dependent phosphorylation of cohesin and separase-mediated cohesin cleavage occur remain uncharacterized. These regulatory events may occur simultaneously on chromosomes, occur at distinct loci, or vary within the cell cycle. The *cdc5-aid* strain described above provides a system for investigating cohesin localization across the genome, after Cdc5 depletion, and in a cell cycle-specific fashion.
In Chapter 2, we demonstrated that Cdc5 localizes to pericentromeric chromatin in a cohesin-dependent fashion, and we predict that Cdc5 most likely associates with all chromosomal cohesin-associated regions (CARs), in a fashion that depends on functional cohesin complexes. Given the evidence that Cdc5 binds to mitotic cohesin (Chapter 2; Ho et al., 2002) and phosphorylates Mcd1 (Alexandru et al., 2001; Hornig & Uhlman, 2004), I hypothesized that Cdc5 localizes to cohesin to regulate cohesin cleavage and/or removal from chromosomes. Therefore, we next asked what effect cdc5-aid depletion had on the association of cohesin complexes with chromatin. In particular, I sought to determine where cohesin bound to the genome in Cdc5-depleted cells. To this end, cdc5-aid OsTir1 bar1 MCD1-myc cells (MAY8979) were arrested with auxin to deplete Cdc5-aid protein, and processed for ChIP analysis of the cohesin subunit Mcd1-myc. The best experimental approach to assay Mcd1 in cdc5-aid cells was not previously known; thus, three different technical variations of the same experiment were performed as described below. Despite technical variations in generating these data, these three experiments each corroborate the hypothesis that cdc5-aid depletion results in cohesin persistence near centromeres, during anaphase.

In the first experiment, the objective was to maximize the uniformity of cdc5-aid arrested cells to ensure that our assay best modeled the effects of Cdc5 activity loss in cells. In poorly arrested populations of cells, the distribution of cells in different phases of the cell cycle could confound our analysis due to the potential for underlying changes in cell cycle-dependent cohesin association with chromosomes. Therefore, two cdc5-aid OsTIR1 MCD1 bar1 cultures were treated with auxin for three or four hours, with the intention of using the cells with the most uniform arrest characteristics for ChIP. Before
ChIP analysis, an aliquot of cells from each arrest was scored for cell cycle index via DAPI staining, by epifluorescence and DIC microscopy, as described above (Figure 9E).

Mcd1-myc ChIP DNA from cdc5-aid cells treated for three hours with auxin was analyzed for Mcd1 distribution at a ~60 kilobase pair region surrounding CEN3, and was compared to ChIP DNA from cdc5-aid cells arrested in G1 with alpha factor, a cell cycle phase that serves negative control for Mcd1 presence on chromosomes. Cells arrested with auxin for 3 hours were arrested in >80% anaphase and >90% large budded. Cells arrest with auxin were >95% unbudded due to the sensitivity of bar1 cells to alpha factor. Mcd1 was enriched on chromatin at the pericentromeric region of CEN3 during anaphase arrest (blue, Figure 10A), as compared to G1 arrest (red, Figure 10A). Mcd1 enrichment appeared most prominent just proximal to CEN3, but also appears to be associated with pericentromeric CAR sites. Notably, the distribution of Mcd1 chromatin association resembles that from the ChIP experiments of cohesin subunits during metaphase, as reported in Chapter 2 (Compared with Figure 3A), as well as findings from other groups (Eckert et al. 2007; Weber et al., 2004). Since auxin-treated cdc5-aid OsTir1 cells accumulate >90% anaphase (Figure 9E) these data suggest a disruption in the removal of pericentromeric cohesin at the metaphase to anaphase transition, but possibly not in the dissolution of cohesion as the DNA as able to segregate and form two separate DNA masses. This finding further suggests that Cdc5 may contribute to the timely removal of cohesin from pericentromeric regions during mitosis.

To determine whether metaphase-arrested and anaphase-arrested cells display similar amounts of cohesin at centromeres, cdc5-aid, OsTir1 bar1 MCD1-myc cells
Figure 10A. Characterization of Mcd1 chromatin association in cdc5-aid arrested cells. MAY8980 bar1 cdc5-aid MCD1-13MYC cells from a 3 hour auxin-arrested time point (blue) in Figure 9E or a 3 hour G1-arrested sample (red) were processed for Mcd1-myc ChIP and analyzed for pericentromeric DNA association at CEN3 as in Figures 1E and 1F.
MAY8980) were synchronized in G1, using alpha factor, and released into the cell cycle in the presence of auxin only, nocodazole only, or auxin and nocodazole (Figures 10B and 10C). Each sample was processed for ChIP 2.5 hours after the G1 release, when the cells were >90% large budded in all treatment conditions. Nocodazole arrests cells as large budded in metaphase, with a single unsegregated nucleus, whereas cdc5-aid cells treated with auxin arrest as large budded in anaphase with two partially segregated nuclei (Figure 9, panel1). However, because the cell cycle distribution of these cells was not quantified, it remains possible that these cultures contain a mixed population of cells. Cells expressing cdc5-aid, in which Mcd1 was untagged, were included as negative controls for Mcd1-myc pericentromeric association (Figures 10B and 10C).

In this experiment, ChIP analysis was performed using a subset of pericentromeric sites located throughout the CEN3 region (Figure 10B). Similar amounts of Mcd1 association with the pericentromeric region were observed in all cdc5-aid cells (Figure 10B), consistent with the pronounced cohesin enrichment previously noted (Chapter 2; Figure 3A; Weber et al., 2004; Eckert et al., 2007; Kogut et al., 2009). In the nocodazole only condition, high Mcd1 association with the pericentromeric region was observed, as expected, due to the mitotic arrest in which chromosomes are not bi-oriented. Cohesin was present at comparable levels in the auxin only and nocodazole only conditions, presumably because cohesin persists into anaphase arrest upon Cdc5 depletion. Finally, cohesin was also present at similar levels within this region during auxin and nocodazole treatment presumably because nocodazole blocks these cells in M-phase before cohesin has been remove chromosomes.
Figure 10B. enrichment near CEN3 DNA is similar in auxin and nocodazole treated cdc5-aid cells. To compare the pericentromeric association of Mcd1-myc in synchronous cycling bar1 cdc5-aid MCD1-13myc (MAY8980) cells treated with nocodazole (green), auxin (blue), or auxin plus nocodazole (red) were release from G1 with the appropriate treatment, processed for ChIP and analyzed for Mcd1 association. Negative control cells with untagged Mcd1 were grown synchronously or and processed in parallel (purple).
Figure 10C. Mcd1 persists at other CEN DNAs during cdc5-aid arrest. To compare the association of Mcd1 at additional centromeric loci, ChIP samples from 10B were analyzed at the indicate centromeres (CENs) by PCR. An untagged strain and CDC5 strain were included as negative and positive controls respectively for Mcd1-myc CEN occupancy.
However, these results are difficult to interpret because the cell cycle index was not quantified prior to the ChIP experiments. In the nocodazole and auxin treatment, since there is no detectable difference between the nocodazole only and auxin only treatments at centromeres, the same number of cells was used in all experiments, and nocodazole presumably arrests cells earlier than the auxin-treatment. Therefore, the simplest explanation is that nocodazole blocks cells at metaphase, giving rise to pronounced cohesin enrichment at pericentromeric DNA. While these data may suggest that loss of Cdc5 leads to similar cohesin persistence at centromeres in the various treatments, these findings are inconclusive. Notably, cohesin association is comparable in cells subject to treatment with nocodazole or auxin, on the three other centromeres analyzed, suggesting anaphase persistence of cohesin occurs similarly on all chromosomes (Figure 10C). Taken together, along with the findings depicted in Figure 10A, these data suggest that anaphase persistence of cohesin near centromeres is a robust phenotype displayed on all chromosomes in cdc5-aid arrested cells.

We next sought to determine if cohesin persistence during anaphase in cdc5-aid arrested cells was limited to pericentromeric chromatin and centromeres, or was also observed at other chromosomal CAR sites. Before performing the ChIP analysis, the cell growth protocol was slightly modified by addition of alpha factor after the initial G1 release, to block cells from re-entering the next mitosis. This experimental modification should eliminate the possibility that cdc5-aid cells could re-enter the cell cycle and reload cohesin onto the DNA. After the initial G1 release from alpha factor synchrony, cells were released into YPD-only, or auxin-only or auxin plus nocodazole treatments (Figures 10D-10H). In this experiment, the YPD-only control cells should pass through mitosis
unperturbed, and arrest again in G1, a phase of the cell cycle in which Mcd1 is not expressed and thus should be absent from chromatin; Mcd1 ChIP signal should be low in this condition. Cells treated with nocodazole or auxin should arrest in metaphase and anaphase, respectively, therefore eliminating mitotic re-entry and subsequent cohesin loading, as a possible source of elevated pericentromeric cohesin. In the event of slippage from nocodazole or cdc5-aid mediated arrest, the cells should encounter a second alpha factor imposed block in G1, before the next cell cycle when Mcd1 could be reloaded, thereby contributing a minimal ChIP signal, at most.

Before ChIP, each of the four samples was scored for cell cycle index via DAPI staining in conjunction with epifluorescence and DIC microscopy (Figure 10D). As expected, the majority (~90%) of cdc5-aid cells treated with YPD only plus additional alpha factor at 65 min exhibited G1 arrest morphology (shmooed cells), typical of cells blocked with the alpha factor. In each of the other conditions, we noticed a significant increase in the fraction of cdc5-aid cells (~20-30 %) in G1, compared to previous experiments where the alpha factor was not added. We reasoned that the majority of these G1 arrested cells probably failed to release from the alpha factor arrest initially, because the background strain used for these ChIP experiments is deficient for Bar1, a secreted extracellular protease that normally degrades alpha factor. Cells deficient for Bar1 are much more sensitive to alpha factor, making it technically easier to arrest a large number of these cells in G1, but also harder to achieve a complete release upon alpha factor washout; this allowed us to collect the large number of synchronized cells required for ChIP experiments, but at the cost of having some cells remain in G1. However, as we do not expect Mcd1 to be present in G1, and hence to contribute to a chromatin association
Figure 10D. To compare the quality of synchronous arrest of cdc5-aid cells treated with auxin (aux), nocodazole (NZ) or nothing (YPD) were grown as in Figure 10B. Each culture was subjected to additional alpha factor treatment to block them from re-entry into the next cell cycle. For each culture, the cell cycle index of the terminal time point was quantified as in 9E.
signal, this population of cells is likely not to be a problem. Moreover, they constitute a similar proportion of the cells in each of the auxin or nocodazole-treated samples, so they can effectively be regarded as background in all samples. I further noted that some of the nocodazole-only treated cells escaped into anaphase, suggesting nocodazole arrest may have been problematic. One way to prevent this in the future is to re-add microtubule depolymerizing agents to the cell culture. However, the majority of cdc5-aid cells treated with auxin appeared to be arrested in anaphase, with partially segregated nuclei. As this was the most important experimental condition, I continued with ChIP analysis.

Mcd1 was analyzed at 4 separate genomic loci known to recruit cohesin and Cdc5: a ~60 kilobase pair pericentromeric region of CEN3 (Figure 3A; Figure 10E; Weber et al., 2004; Eckert et al., 2007; Kogut et al., 2009), a CAR site at the rDNA locus on the chromosomal arm of CHRXII (Figure 3D; Figure 10F; Laloraya et al., 2000), a CAR site near GAL2 on the chromosomal arm of CHRXII (Figure 3C, Figure 10G; Kogut et al., 2009), and a CAR site on the chromosomal arm CHRIII (Figure 10H; Eckert et al., 2007). As expected, all chromosomal sites in the nocodazole-treated cells exhibited prominent peaks of Mcd1 enrichment due to arrest of the major of cells in metaphase prior to the time in cell cycle of cohesin removal from chromosomes. In cells that were grown in YPD only, I observed very low amounts of Mcd1 at all regions, consistent with the idea that Mcd1 is absent from chromatin in G1 (Figures 10E-10H). In auxin-treated cells, Mcd1 was found to be at levels comparable to nocodazole arrest near centromere 3, as was previously observed in Figure 10B. However, within this pericentromeric region of CEN3, Mcd1 appeared less prominent in regions more distal from CEN3 in auxin-treated cells, as compared to nocodazole-arrested cells. It is unclear why this result is
Figure 10E-H. Mcd1 persists at CEN3 DNA but not at other loci during late cell cycle arrest in cdc5-aid cells. To compare Mcd1-myc association in cdc5-aid cells during mitotic arrest (NZ, blue), auxin arrest (red, Aux) or no treatment (YPD), cells from Figure 10D were processed for ChIP and analyzed at the chromosomal regions indicated (E-H). The YPD only treatment was omitted from panel F. (F) Mcd1 does not persist at chromosome 12 arm CAR DNA during late cell cycle arrest in cdc5-aid cells. (G) Mcd1 does not persist at second chromosome 12 arm CAR DNA during late cell cycle arrest in cdc5-aid cells. (H) Mcd1 does not persist at chromosome 3 arm CAR DNA during late cell cycle arrest in cdc5-aid cells.
different from the results depicted in figure 10B, but it may have to do with the differences in growth conditions, or analyses of a large chromosome region, where more points for an analysis distribute over a larger dynamic range during gel imaging. At each of the other three CAR sites on the chromosome arms assayed for Mcd1 association, cohesin association appeared low in auxin-treated cells, suggesting cohesin had been removed from these regions. This is consistent with chromosomes being separated during cdc5-aid arrest due to loss of sister chromatid cohesion. Importantly, this observation was independently validated in temperature-sensitive cdc5-99 cells, suggesting that it is not an artifact that is specific to the cdc5-aid cells, our strain background, or the AID system (data not shown; Mishra et al., 2016, in submission). Taken together, these data suggest that cohesin association is differentially regulated at chromosomal arm CARs and at pericentromERIC regions, and that loss of Cdc5 leads to cohesin persistence during anaphase on centromeres.

3.3 Discussion

In the work described above, I developed a potent allele for the conditional depletion of Cdc5 using the auxin-inducible degron (AID) system. I further characterized the phenotypes of cells expressing cdc5-aid and demonstrated that they arrest at late stages of the cell cycle, a phenotype which is characteristic of other mutant cdc5 cells under non-permissive conditions (Hartwell et al., 1970; Alexandru et al., 2001; Snead et al., 2007; Shirayama et al., 1998; Park et al., 2008; Lee et al., 1998). Furthermore, an expanded toolkit of AID alleles was developed to study Cdc5 localization. These alleles were generated to study Cdc5 function with respect to cohesin, the kinetochore, and the cell cycle.
The findings in this chapter expand on the discovery that Cdc5 localizes to sites of cohesin occupancy in the yeast genome, presented in Chapter 2. As Cdc5 is a known regulator of the cohesin complex, and in particular the Mcd1 subunit in budding yeast, the cdc5-aid allele was developed to determine how depletion of Cdc5 affects the regulation of cohesin on chromosomes. I show that during cdc5-aid arrest, the Mcd1 cohesin persists late into the cell cycle at pericentromeric DNA (Figure 10). Cohesin persistence in anaphase is limited to pericentromeric DNA; cohesin normally present at all cohesin-associated regions (CARs) during early mitosis, is not found at those same sites in anaphase (Figure 10; Weber et al., 2004). Cohesin persistence at pericentromeric DNA is a novel and previously uncharacterized phenotype, and has many implications for how cohesin is regulated and disassociated from chromosomes in a Cdc5-dependent fashion. Importantly, Mishra et al. (2016) recapitulated the key findings in this study; they employed a different Cdc5 depletion method, and assayed another cohesin subunit (Manuscript in Submission). The similarity of their findings strengthens the significance of this study. Below, I will discuss the implications of these findings, as well as future experimental approaches to address them.

3.3.1 Requirement for Cdc5 in removal of cohesin from different chromosomal loci. The finding that the cohesin subunit, Mcd1, persists on pericentromeric DNA and at other centromeres is interesting in that cohesin is thought to be cleaved, and hence removed from chromosomes, at the onset of anaphase (Alexandru et al., 2001; Hornig & Uhlmann, 2004). One possibility is that the cohesin persistence phenomenon is a normal physiological aspect of anaphase that happens to be captured during a cdc5-mediated arrest, late in the cell cycle. If this possibility is true, then other
conditions or mutants that allow assaying of cells in anaphase may show that cells not normally possess cohesin at pericentromeric DNA. This prediction could be tested by assaying the association of cohesin in \textit{cdc5-aid} cells over the course of a single cell cycle, in the presence or absence of auxin, to determine whether Cdc5 itself affects cohesin association with pericentromeric DNA, beyond imposing an anaphase arrest when depleted. One way to make this experiment more tractable might be to allow cohesin to first maximally load onto chromosomes via nocodazole arrest in \textit{cdc5-aid} cells, and then assay cohesin association after nocodazole washout and resumption of the cell cycle, in the presence or absence of auxin. If cohesin persistence is the result of \textit{cdc5-aid} depletion, then cohesin can be expected to persist in auxin-treated cells, but not in untreated cells, or to a lesser extent in the untreated cells. One caveat to interpreting these types of temporal experiments is that cells depleted of Cdc5, an essential cell cycle regulator, may lag in mitotic progression and hence, may be delayed in the progression of other mitotic processes. In fact, \textit{cdc5} temperature-sensitive cells are known to regrow spindles more slowly, indicating that interpretation of this type of experiment, which is dependent on timing, may be problematic (Park et al., 2008).

Alternatively, cohesin persistence could be assayed in other cell cycle mutants that arrest late in the cell cycle. As \textit{cdc14} and \textit{cdc15} depletion both give rise to late cell cycle arrest, aid alleles of both proteins were generated. Initial characterization suggests that when treated with auxin, \textit{cdc14-aid} and \textit{cdc15-aid} strains predominantly arrest late in the cell cycle, similar to previously characterized mutants. One caveat to employing these alleles is that Cdc14 and Cdc15 are essential conserved regulators of the cell cycle and may regulate cohesin in unknown ways. The finding that Cdc5 is required for cohesin
persistence in anaphase could have many interesting implications for further study of the Cdc5-Mcd1 regulatory interaction. Alternatively, the phenomenon of cohesin persistence on pericentromeric DNA during an unperturbed cell cycle would also be interesting, and merits further experimental analysis.

3.3.2 Regulation of cohesin association by Cdc5. A strong possibility is that cdc5-mediated arrest is directly responsible for cohesin persistence at centromeres during anaphase, given that Cdc5 is known to associate with and phosphorylate cohesin; thereby, directly impacting cohesin cleavage and disassociation from chromosomes (Chapter 2; Alexandru et. al, 2001; Hornig & Uhlmann, 2004). Accordingly, depletion of Cdc5 activity is associated with a decrease in Mcd1 electrophoretic mobility (Alexandru et al., 2001; Ratsima et al., 2011). As cohesin persistence in cdc5-aid arrested cells is observed at pericentromeric DNA, but not at chromosomal arm CARs, these findings imply that Cdc5 activity, mostly likely through direct binding and phosphorylation of cohesin, is required for the disassociation of cohesin complexes from pericentromeric DNA, but not from chromosome arms. One possibility is that Cdc5 phosphorylation of cohesin is required for all cohesin disassociation, however, this process occurs more slowly at centromeres than at chromosomal arms, thereby allowing chromosomes to separate partially, as observed by Alexandru et al. (2001). Their study further showed that only a fraction of cohesin is phosphorylated and cleaved, and these events appear to occur later in the cell cycle (Alexandru et al., 2001). Alternatively, these findings may imply that Cdc5 is not essential for the disassociation of cohesin complexes from chromosomal arm CARs, and that cohesin disassociation at arm CAR sites occur by a separate mechanism. Alternatively, cohesin dissociates more slowly at pericentromeric chromatin than it does
at chromosomal arm CARs and persists near centromeres into anaphase. This prediction is supported by the ability of chromosomes to initiate segregation in various cells depleted of Cdc5 (Figures 9B, 9D and 9E; Alexandru et al., 2001; Snead et al., 2007; Ratsima et al. 2011), suggesting that some cohesin is removed from chromosomal sites, thereby enabling nuclei to segregate into two masses. However, it does not preclude the possibility that Cdc5 is involved in cohesin regulation at CARs, given its co-localization with cohesin at CARs (Chapter 2; Rossio et al., 2010). Previous assays of Cdc5-dependent regulation of cohesin were limited to assaying total cellular cohesin, rather than cohesin at specific sites in the genome; this may explain why Alexandru et al. (2001) arrived at their conclusion that Cdc5 regulates cohesin more broadly at chromosomes. Conversely, the assays conducted in my study can discern the outcomes of cohesin regulation at specific chromosomal loci.

During mitotic arrest, cohesin is present at all CAR sites and pericentromeric DNA (Glynn et al., 2004), yet it appears to only be present at pericentromeric DNA and centromeres, during anaphase (Figure 10); the caveat here is that it was not technically feasible to assay cohesin in a genome-wide manner in this study. The findings above (Figure 10) appear to indicate that stepwise cohesin disassociation occurs in budding yeast as it does in humans. The order of cohesin disassociation, first at chromosomal arms and then at centromeres, appears to be the same in both budding yeast and human cells, at least in the cdc5-aid depletion mutant. Paradoxically, the opposite appears to be true in temperature-sensitive cdc16-1 arrested cells (Eckert et al., 2007), which arrested in metaphase with bioriented chromosomes, where cohesin is missing near centromeres but remains present at CAR sites. One possibility is that their observation is due to the
inaccessibility of cohesin at centromeres when microtubules are bound to the kinetochores. Alternatively, successful biorientation may allow for this cohesin to be removed from centromeres in a Cdc5-dependent manner in cdc16-1 cells, a process that cannot occur in cdc5-aid cells. Accordingly, polo-like kinases are involved in biorientation pathways in higher eukaryotes. Despite the disparities in cohesin disassociation in cdc5 and cdc16 mutants, it is puzzling that the requirement for polo-like kinase activity in the stepwise process appears to be reversed in cdc5-aid, in that polo kinases are required for cohesin disassociation in prophase at chromosomal arms in humans, and appear to not be essential for cohesin disassociation in chromosomal arms, which occurs earlier than cohesin disassociation at centromeres in cdc5-aid arrested cells. It is unknown whether polo kinase activity is also required for cohesin disassociation near centromeres in human cells, or whether this activity is limited to being separase-dependent. To answer these questions, it will be necessary to examine the disassociation of cohesin at centromeres and chromosomal arms in wild-type cells during an unperturbed cell cycle.

Understanding when and where Cdc5-dependent phosphorylation of cohesin occurs may be important for unlocking the mechanisms of a Cdc5-dependent cohesin disassociation pathway from chromosomes. Going forward, it will be necessary to address whether depletion with cdc5-aid leads to changes in cohesin cleavage or the phosphorylation of cohesin. Furthermore, while technically challenging, it may be possible to address where on chromosomes Cdc5 phosphorylates cohesin, and during what phase of the cell cycle phosphorylation of cohesin occurs, using phospho-specific antibodies. Another interesting possibility is that cohesin cleavage by separase and
cohesin disassociation are distinct events, mechanistically and temporally. Thus, when cleavage or phosphorylation of cohesin occurs, a second regulatory event may be required for the disassociation of cohesin, or to stimulate the rate of cohesin disassociation. In budding yeast cells, separase may act globally on all cohesin or at specific chromosomal sites. It is possible that in \textit{cdc5-aid} cells, cohesin cleavage via separase occurs at centromeres; however, cohesin disassociation from this region may require extra assistance from polo-like kinase, thereby explaining the cohesin persistence phenomenon. Assaying cohesin cleavage in \textit{cdc5-aid} cells will further shed light on these mechanisms. Similar to the phosphorylation of cohesin, to best of my knowledge little is known regarding when and where separase acts on chromosomal cohesin and what recruits it to cohesin in a timely manner. It may be possible to directly assay separase association with chromosomes, to ascertain where cohesin cleavage occurs, and at what points in the cell cycle. One possibility is that like polo-like kinase, separase associates with all CARs during mitosis. A second possibility is that the activity of separase on different CARs, including pericentromeric DNA, depends on the timing of its arrival at those sites, which may be distinct for cohesin at chromosomal arm CARs and pericentromeric cohesin. In this event, the findings in Figure 10 would imply that separase first acts on cohesin at chromosomal arm CARs, and then at pericentromeric cohesin during late anaphase or does not act at pericentromeric cohesin at all. A final possibility is that Cdc5 is required for the disassociation of cohesins at centromeres involved in intrachromosomal formation of the cohesin barrel, such that that they are not engaged in interchromosomal cohesion per se; these cohesins may be more dependent
on Cdc5 for their removal than cohesins engaged in sister chromatid cohesion, hence their persistence into anaphase when Cdc5 is activity is diminished.

As mentioned above, it is unclear why *cdc5-aid* cells arrest with bioriented chromosomes during *cdc16* arrest with low or absent pericentromeric cohesin mitosis (Eckert et al., 2007), but arrest with an accumulation of pericentromeric cohesin during anaphase (Figure 10). A possible explanation is that cohesin association with pericentromeric chromatin is dispensable after chromosome biorientation, which leads to its disassociation and removal from this region. The findings in Figure 10 imply that Cdc5 is required for pericentromeric cohesin persistence and thus, may be responsible for pericentromeric cohesin removal, after biorientation but before the onset of anaphase, and before a return to G1. If these predictions are true, then cohesin may persist at pericentromeric DNA in cells arrested with *cdc16* and *cdc5-aid*, as *cdc16* depletion will block the cells before anaphase, and *cdc5-aid* depletion will prevent pericentromeric cohesin removal. This prediction is testable with readily available mutant alleles and may offer important insights into the timing of action of Cdc5-dependent regulation of cohesin disassociation. Conversely, employing *cdc14* or *cdc15* mutants in conjunction with *cdc5-aid* to assay pericentromeric cohesin persistence may allow the time of action of Cdc5 regulation of pericentromeric cohesin, to be ordered. However, as a master mitotic regulator, Cdc5 may interact with these genes in unanticipated ways and thereby affect the observed cohesin persistence. For instance, Cdc5 is known to bind and regulate Cdc14 a cell cycle regulated phosphatase involved in anaphase progression (Rahal et al., 2008).
3.4 Materials and Methods

3.4.1 ChIP and western blotting. These techniques were performed as previously described in Chapter 2.

3.4.2 Construction of auxin-inducible degron strains. To develop an AID system for depleting Cdc5 and other degrons, the two components had to be integrated into the MAY589 yeast strain, which is a derivative of S288C. First, a plasmid containing the F-box protein pADH1-OsTir1 was integrated into the genome at the \textit{URA3} locus of MAY589 lacking \textit{BAR1}. The promoter of the constitutively expressed metabolic enzyme, alcohol dehydrogenase (\textit{ADH1}) in budding yeast is p\textit{ADH1}. Subsequently, the \textit{cdc5-aid-kanMX} construct was integrated at the endogenous \textit{CDC5} locus, as described in Nishimura et al. (2009). After positive selection for transformants containing the resistance marker linked to the C-terminus of \textit{CDC5}, positive strains were identified via colony PCR. Cells expressing \textit{cdc5-99} were provided courtesy of Damien D’Amours and are described in St-Pierre et al. (2009). Cells expressing temperature-sensitive (t.s) \textit{cdc5-deg} (MAY8803) were constructed as described in Dohmen and Varshavsky (2005). Cells expressing temperature-sensitive \textit{cdc5-11} (MAY8282) were described in Park et al. (2008).

3.4.3 Auxin-containing media. All media was made as previously described in Chapter 2. IAA is commercially available (Sigma-Aldrich); due to its photosensitivity and labile nature, it was solubilized in 100% ethanol and stored at -20°C, in a light blocking container. Plates containing auxin, auxin was added to autoclaved sterile media after cooling to touch, before pouring the plates. Plates were stored at 4°C in the dark.
Fresh IAA in ethanol was added to yeast liquid media immediately before use. In control conditions, the equivalent volume of ethanol was added.

3.4.4 Alpha factor arrest and release, and cell cycle time courses. Alpha factor mating pheromone, a short peptide with the amino acid sequence WHWLQLKPGQPMY was synthesized and purified by reverse-phase HPLC by the Johns Hopkins Synthesis and Sequencing facility. Purified lyophilized alpha factor peptide was stored at -80°C. Alpha factor powder was solubilized in sterile water to 66,600X for bar1 strains stock concentration (10^{-3} M) and stored at -20°C. To conduct cell cycle release experiments, bar1 cells growing asynchronously in log-phase were arrested with 1X alpha factor for 2.5 to 3 hours at 30°C. The efficiency of alpha factor-induced G1 arrest was confirmed by DIC microscopy, by ascertaining the frequency of unbudded, shmooed yeast. To release cells from G1 synchrony, cells were washed three times with fresh YPD containing Pronase E (Sigma-Aldrich P-6911). After the final wash during time course experiments, cells were resuspended in fresh media, and sampled at time point 0 minutes, and again at the indicated time points. Where relevant, alpha factor was re-added to cycling cells at 1X after 65 minutes, to block cells at the next G1 phase, and prevent re-entry into a second cell cycle.
## Yeast Strains

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Table 2

*Plasmids*

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<td>Lee Lab</td>
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References


Mitotic cyclin destruction machinery in S. cerevisiae. *Current Biology, 8*(9), 497–507.


Curriculum Vitae

David B. Reynolds
306 E 32nd St. Apt. M, Baltimore, MD 21218 | 617-835-9078 | dreynolds@jhu.edu

Summary

Experienced molecular biologist with over a decade of developing, optimizing and conducting biological assays. Expert at chromatin immunoprecipitation and have performed hundreds of experiments on numerous proteins to characterize their association with the genome. Employs a diverse repertoire of skills in molecular biology, microbiology, cell biology, and genetics as well as proficiency in computer programming. Thrives off energy of others. Looking to harness the combination of my passion for biology and analytical skills to solve challenging real-world problems in a highly collaborative, dynamic environment.

Research Experience

Carnegie Institute for Science, Department of Embryology, Baltimore, MD
2016- Current
Research Technician
- Optimizing ChIP-seq in murine model to characterize the epigenetic program of muscle stem cells during quiescence and differentiation.

Johns Hopkins University, Department of Biology, Baltimore, MD
2006-2016
PhD Researcher
- Characterized novel interaction of a cell cycle regulator Cdc5 with pericentromeric DNA and cohesin.
- Harnessed auxin-inducible degron system for depletion of essential cell cycle proteins and employed cdc5-aid allele to discover a novel cohesin phenotype.
- Invented a ChIP-based assay to measure microtubule-kinetochore association.
- Managed and trained laboratory personnel; developed projects for laboratory personnel.
- Acted as lab manager and procured laboratory materials/reagents and performed general laboratory maintenance.
- Teaching assistant for Genetics and Cell Biology laboratory courses and general courses.

Whitehead Institute for Biomedical Research, MIT, Cambridge, MA
2002-2006
Research Technician
- Developed a high-throughput chromatin immunoprecipitation on chip (ChIP-on-chip) protocol.
- Mapped genome-wide transcriptional regulatory networks.
- Collaborated on a genome-wide study of centromeric cohesion during meiosis.
- Investigated interactions of signal transduction pathways with transcription on a genome-wide scale.

Skills

- Tissue culture, chromatin immunoprecipitation, ChIP-on-chip PCR, qPCR/real-time PCR, tissue/cell culture, DNA/RNA purification, co-immunoprecipitation, microarray, protein purification, microscopy, cloning, transformation, western blot/immunodetection, southern blot, yeast genetics, sequencing, ligation, DNA sequence analysis, oligo design, electrophoresis, cryopreservation, reagent preparation, procurement, ordering and stocking supplies, autoclaving, sterile technique
- Familiar with: siRNA, CRISPR/Cas9 gene editing, next-Generation Sequencing
- JAVA, C, C++, Python, Linux/UNIX, relational databases, MS Word, MS Excel, MS Powerpoint, FileMaker Pro,
Education

Johns Hopkins University, Baltimore, MD
Doctor of Philosophy: Biology, Defense Scheduled: June 16, 2016
Master of Arts: Biology, May 21, 2015

University of Rochester, Rochester, NY
Bachelor of Science: Molecular Genetics, 2002

Publications/Patents