Regulation of Chromosome Attachment and Dynamics by

*Saccharomyces cerevisiae* Kinetochores

by

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Submitted to the Department of Biology on October 31, 2005
In Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy in Biology

Abstract

Kinetochores are large, multi-protein complexes that bind centromeric DNA to the microtubules of the mitotic spindle and mediate chromosome movement throughout the cell cycle. The proteins that regulate both force generation at kinetochores as well as and the cell-cycle-dependent changes in kinetochore architecture are largely unknown. The relative simplicity of centromere specification and kinetochore-microtubule attachment make *Saccharomyces cerevisiae* an attractive model organism for investigations into kinetochore-microtubule attachment and regulation.

We used a combination of cell biology and biochemistry to study the roles of the four nuclear kinesin motor proteins at budding yeast kinetochores. We discovered that each of the four nuclear kinesins localizes to kinetochores. Three of these, Cin8p, Kip1p, and Kip3p are present at mature chromosome-microtubule attachments in metaphase. Cin8p and Kip1p align metaphase chromosomes into the characteristic bilobed configuration that is analogous to the metaphase plate in higher eukaryotes. Kip3p regulates microtubule dynamics throughout the cell cycle and regulates poleward movement during anaphase. Kar3p, the final nuclear kinesin, is recruited specifically to detached kinetochores. In addition, we have discovered that kinetochore-microtubule attachments alter during the cell cycle indicating that kinetochore function is temporally regulated.

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Acknowledgements

I would like to thank Peter for the opportunity to join his lab. Your enthusiasm and excitement have always been contagious. I have also learned a great deal from your way of looking at problems as mechanical systems and from your desire to embrace new technology. In addition to the many thanks for scientific manners, I am forever grateful for your help and support throughout what was a somewhat stressful pregnancy and during the transition back to work with an infant. Your advice (and sense of humor) helped me transition from floundering graduate student to more efficient and more confident scientist and mother.

I would like to thank Frank Solomon both for his help as a member of my Thesis Committee and for his support during some serious frustrations in my graduate career. Thank you for reminding me to follow my heart and for listening to my complaints, concerns, hopes, and fears. Knowing that you were there to turn to made my problems more bearable.

I would like to thank Angelika Amon for her help on my Thesis Committee during most of my graduate career. You incredible enthusiasm and insights were invaluable.

I would like to thank Steve Bell, Steve Harrison, Graham Walker, and Frank Solomon for their time, comments, and insights at my Thesis Defense and in writing and preparing my thesis.

I would like to thank the more senior grad students in the Sorger lab during my time in the lab: Max Dobles, Aurora Burds Connor, Emily Gillett, Chris Espelin, and Dan Rines. You were all so wonderful at welcoming me to the lab, teaching me techniques, lending a hand (or a sympathetic ear), and answering patiently the myriad of questions I had about everything under the sun. Working with you and learning from you was a pleasure.

I would like to thank all members of the Sorger lab, past and present, for their support and enthusiasm. You made coming to lab every day a pleasure and I will miss the camaraderie of the lab very much.

I would like to thank my parents and siblings for their love and support over the years. You have always encouraged me to follow my dreams and for that I am eternally grateful.

Finally, I would like to thank Dave and Ben for making everything in life more wonderful. Dave, you believed in me even when I was losing faith and encouraged me in good times and in bad. Thanks especially for your patience with me through the ups and downs. I know this degree was hard work for you as well and I appreciate all that you have done. I could not have done this without your love and support. Ben, you weren’t born yet when I started this but you changed my whole world when you were. Thank you for keeping me focused on what really matters. Our family is more than I ever hoped for. I love you both so much.
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CHAPTER ONE

Structure, Function, and Regulation of Budding Yeast Kinetochores

This chapter is adapted with permission from McAinsh et al. 2003.

1.1 Introduction

Nearly a century ago Boveri and Sutton postulated that chromosome segregation from mother to daughter cells was the basis of heredity (Boveri, 1907). Since then, errors in chromosome segregation have been shown to have dire consequences. Misseggregation during meiosis in gametogenesis can lead to chromosomal abnormalities such as Down, Klinefelter, Prader-Willi and Angelman syndromes, and are a leading cause of infertility (Bittel and Butler, 2005; Jiang et al., 2004; Lowe et al., 2001; Tempest et al., 2004). Errors during mitosis can cause chromosome instability and cancer (for review see (Draviam et al., 2004; Storchova and Pellman, 2004). Therefore, a detailed knowledge of the mechanisms driving chromosome segregation is vital for understanding and preventing these problems.

At the molecular level chromosome segregation is an extremely complex process. The mitotic spindle, consisting of arrays of microtubule fibers emanating from two organizing centers, called centrosomes in mammals (or spindle pole bodies (SPBs) in yeast), forms a dynamic scaffold that organizes chromosomes and positions the axis of division. After DNA replication, sister chromatids must establish attachments to microtubules from opposite SPBs. Kinetochores are protein complexes that tether centromeric DNA to spindle microtubules, forming a bridge between the sister chromatids and the spindle. Once all sister chromatid pairs form mature bipolar attachments, the cell progresses into anaphase and the sisters irreversibly separate and segregate into the new cells.

One major question in chromosome segregation is how the kinetochore regulates chromosome movement. Throughout mitosis, the chromosomes oscillate, microtubules grow and shrink, and the spindle elongates and matures. Microtubule dynamics are highly regulated; more than 13 microtubule associated proteins (MAPs), including kinesin motors, bind to kinetochores...
to regulate chromosome movement and spindle organization. However, the contributions of many individual MAPs and motors are still unknown. I have used the budding yeast *Saccharomyces cerevisiae* as a model system to investigate the role of kinesins at kinetochores in regulating chromosome movement and attachment. I have also investigated the cell cycle regulation of attachment. In this chapter, I present an overview of spindle architecture and dynamics during mitosis, the mechanisms of chromosome movement, kinetochore composition and assembly, and the role of kinetochores in responding to lesions in tension and attachment.

1.2 Overview of Mitosis

Mitosis consists of a series of stereotypical chromosome movements within the complex mechanical system of microtubules, centrosomes, and other factors that constitute the mitotic spindle. Live-cell analysis performed during the 1980s and 1990s in animal cells established the critical features of chromosome and spindle dynamics during the three primary mitotic phases: prometaphase, metaphase, and anaphase. During prometaphase, the chromosomes condense, the nuclear envelope breaks down, and chromosomes attach to microtubules. Attachment occurs via a “search and capture” process whereby microtubules nucleated at centrosomes grow and shrink rapidly until they encounter and bind to a kinetochore (Kirschner and Mitchison, 1986). If the sister kinetochore then captures a microtubule from the same pole, a syntelic attachment is created; if the captured microtubule is from the opposite pole, the attachment is bipolar. Sister chromatids are held together from S-phase through metaphase by cohesin. The cohesin complex – composed of four subunits, Scc1p/Mcd1, Scc3p, Smc1p, and Smc3 – loads onto DNA during replication (Carson and Christman, 2001; Uhlmann, 2004). In the absence of cohesion, such as in temperature-sensitive alleles of Scc1p, the spindle elongates and chromosomes segregate.
prematurely. This early elongation demonstrates that cohesin exerts inward forces that oppose
the pulling from the kinetochore microtubules (k-MTs) and that this balance of force is vital to
proper spindle assembly and morphology (Figure 1.1; (Guacci et al., 1997b; Michaelis et al.,
1997).

During metaphase, sister chromatids with mature bipolar attachments congress toward
the center of the spindle to form the metaphase plate. When all chromosomes have made mature
bipolar attachments, tension is generated across the spindle. The number of microtubules
attached to mammalian kinetochores varies in response to tension and mature bipolar
attachments recruit 15–30 microtubules per kinetochore (Brinkley and Cartwright, 1971; Rieder,
1982). A surveillance mechanism, called the spindle checkpoint, monitors the state of
chromosome-microtubule attachment, delaying cell cycle progression until all pairs of sister
chromatids have formed bipolar attachments (reviewed in (Amon, 1999). It is not yet known how
bipolar attachments are distinguished from monopolar and syntenic attachments, but only bipolar
attachments are stable and give rise to tension across paired sister kinetochores. When all pairs of
sister chromatids have made stable bipolar attachments to microtubules, the mitotic checkpoint is
silenced, and the Anaphase Promoting Complex (APC) is activated, irreversibly driving the cells
into anaphase (Peters, 2002). APC then targets the securin Pds1p for degradation (Yamamoto et
al., 1996a; Yamamoto et al., 1996b). Pds1p degradation activates the separase Esp1p, a protease
that cleaves the Scc1p subunit of cohesin, allowing the sisters to separate (Ciosk et al., 1998).
During anaphase, chromatids move poleward while maintaining end-on microtubule
attachments. Chromosome-to-pole movements during anaphase A, and separation of the poles
during anaphase B, create two equal and separated sets of sister chromatids.
1.2.1 Yeast Mitosis

In contrast to higher eukaryotes, *S. cerevisiae* undergo a closed mitosis and possess a relatively simple spindle in which the spindle pole bodies (SPBs) remain embedded in the nuclear envelope and chromosomes are bound to microtubules throughout the cell cycle (Figure 1.1A; and (Adams and Kilmartin, 2000). This results in close association between chromosomes and SPBs from telophase through prometaphase in budding yeast (Guacci et al., 1997a; Jin et al., 2000). In G1, a short linear array of microtubules emanates from the unduplicated SPB. Chromosomes are bound to these microtubules via their kinetochores (Figure 1.1A). During S-phase, the SPB duplicates and a short bipolar spindle forms (<1.0μm). Chromosomes replicate and the new sister must attach to the opposite SPB. It is believed that early in spindle assembly, sister chromatids frequently bind microtubules emanating from the same SPB, creating a syntelic attachment (Figure 1.1B) that must be dissolved for mature bipolar attachments to form (Tanaka et al., 2002).

The budding yeast spindle elongates steadily throughout metaphase, achieving a length of about 2.5 μm prior to the onset of anaphase. The metaphase spindle contains two classes of intranuclear microtubules: those that bind to kinetochores (k-MTs) and those that project into the spindle midzone to overlap with microtubules from the opposite pole (polar; p-MTs) (Figure 1.1C). In contrast to animal cells, each *S. cerevisiae* centromere binds to a single k-MT (Winey et al., 1995). A fully assembled metaphase spindle contains approximately 32 k-MTs and 8 p-MTs (O’Toole et al., 1999; Winey et al., 1995). A small number of SPB-nucleated astral microtubules (a-MTs) project from the SPB toward the plasma membrane where they bind to cortical attachment sites to generate forces that position the nucleus to the bud neck at anaphase (Pearson and Bloom, 2004). The k-MTs, p-MTs, and a-MTs form a coupled mechanical system
Figure 1.1: Cell cycle in budding yeast. (A) Chromosomes have end-on attachments to microtubules during G1. (B) After duplication of chromosomes and SPBs, sister chromatids can form syntelic attachments, where both sisters are attached to the same pole. (C) During metaphase, the nucleus is positioned into the bud neck by the a-MTs. The spindle elongates via the crosslinking of the p-MTs at the spindle midzone. Bipolar attachments are formed and tension is generated across the kinetochores. (D) Once all chromosomes make bipolar attachments the cell proceeds into anaphase and the chromosomes are pulled to the SPBs. (E) Cohesin opposes the force applied to kinetochores by the k-MTs resulting in tension at kinetochores and transient separations. (F) Representative image of the bilobed distribution of kinetochores in metaphase in cells coexpressing the kinetochore protein Ndc80p-GFP with the SPB protein Spc42p-CFP.
linked by SPBs, sister kinetochores, kinesins, microtubule associated proteins (MAPs), and cohesin (Figure 1.1C).

Unlike metazoans, budding yeast chromosomes do not undergo congression per se. However, *S. cerevisiae* kinetochores do adopt a highly characteristic bilobed metaphase configuration analogous to the metaphase plate (Figure 1.1F; (Goshima and Yanagida, 2000; He et al., 2000). Typically, kinetochores form two lobes that lie on either side of the spindle midzone that are separated by roughly half the distance between the SPBs (Figure 1.1F). This unexpected localization pattern, similar to the two foci of separated SPBs, initially caused some confusion in the literature and several proteins originally designated as SPB components were subsequently shown to localize to kinetochores. The bilobed localization pattern is the result of chromosome oscillation and transient sister separations caused by tension across centromeres (described below) and it changes subtly on a time scale of seconds as the extent of overlap among kinetochores varies (He et al., 2000). However, once metaphase connections are established, chromosomes cross between lobes only rarely (Pearson et al., 2004).

### 1.2.2 Dynamics

One striking feature of mitosis in all eukaryotes is that chromosomes and microtubules are continuously in motion both before and after bipolar attachment. Throughout prometaphase and metaphase sister chromatid pairs move back and forth along the spindle axis, a behavior known as directional instability (Rieder and Salmon, 1998). Electron microscopy (EM) reveals that metaphase microtubule attachments are “end-on” with the extreme plus-ends of spindle microtubules embedded in kinetochores (McEwen et al., 1997). Thus, chromosome movement is coupled to microtubule polymerization at one kinetochore and microtubule depolymerization at
its sister. The rates of polymerization and depolymerization are not always matched, leading to
tension across sister kinetochores that can transiently separate them (Goshima and Yanagida,
2000; He et al., 2001; Tanaka et al., 2000). The amount of tension varies on a time scale of
seconds and is sufficient to physically stretch centromeric chromatin (Shelby et al., 1996). The
coupling of directional instability to microtubule dynamics suggests that kinetochores regulate
both microtubule binding and regulate plus-end dynamics to control chromosome attachment and
movement along the spindle.

Examination of the movement of individual centromeres demonstrated that paired sister
chromatids in *S. cerevisiae* separate transiently toward opposite ends of the spindle prior to the
onset of anaphase (Goshima and Yanagida, 2000; He et al., 2000; Tanaka et al., 2000). These
transient separations pull the sisters apart for up to several minutes and cause centromeric
chromatin to become stretched, but do not involve cohesin degradation. Transient sister
separation is observed in chromatin up to 10kb away from the centromere and appears to involve
a towards-the-pole pulling force mediated by kinetochores, and a countervailing adhesive force
dependent on cohesin (Figure 1.1B). Similar transient separation of centromeric chromatin
occurs in animal cells, but in yeast, the magnitude of these separations (up to 1 μm) is much
greater relative to the small size of the spindle (about 2 μm) (He et al., 2000; Shelby et al., 1996;
Tanaka et al., 2000). The existence of transient sister separation raises a number of interesting
issues about sister chromatid cohesion. Centromeres contain the highest levels of cohesin of any
region in the genome (Blat and Kleckner, 1999; Tanaka et al., 1999) and it is not obvious how
stretching and sister cohesion coexist. Nor is it clear how the 20kb domain of elastic chromatin
surrounding centromeres is defined. Changes in the pulling forces applied to pairs of sister
chromatids that have achieved bipolar attachment would be expected to cause centromeric
chromatin to undergo cycles of stretching and compaction. There is evidence suggesting that the cohesin complex forms a ring around DNA rather than binding as rungs on a ladder (Gruber et al., 2003). If this is the case, it is tempting to imagine the cohesin ring sliding along the DNA away from centromeres in response to tension, allowing for the transient separation of sister centromeres (Figure 1.1E; (Nasmyth, 2005). Thus, it seems very likely that transient sister separation in yeast is a consequence of directional instability in chromosome movement and can serve as a readout of the forces at the kinetochore-microtubule interface.

1.3 Microtubule Attachment and Force Generation

Chromosome movement is dependent on attachment to the spindle and on microtubule dynamics. Depolymerizing microtubules can drive chromosome movement in vitro (Coue et al., 1991). Addition of kinetochores to microtubules in vitro alters their dynamics (Hunt and McIntosh, 1998). Therefore, kinetochores can drive chromosome movement by regulating the dynamic instability of microtubules. In the next section, I describe the basic biochemistry of microtubules and the way it is harnessed to generate forces within the mitotic spindle. In Section 1.4.3, I also describe many of the kinetochore components that regulate microtubule dynamics.

1.3.1 Biochemistry of Microtubules

Microtubules are polymers of α and β tubulin heterodimers. αβ dimers self-assemble into long hollow tubes 25nm in diameter, composed of 12-15 protofilaments (Amos and Klug, 1974). Microtubules are asymmetrical, with α-tubulin exposed at the more stable minus-end and β-tubulin at the more dynamic plus-ends. Microtubules intermittently switch between growth and shrinkage, a process known as dynamic instability (Desai and Mitchison, 1997). Microtubule
motion is characterized by four different types of movement: growth, shrinkage, catastrophe, and rescue. “Growth” and “shrinkage” refer to steady addition or loss of tubulin subunits, respectively. “Catastrophe” describes the rapid depolymerization of the microtubule and “rescue” describes the subsequent switch back to growth. Both α and β-tubulin bind GTP, however, only β-tubulin hydrolyzes and exchanges GTP and GDP (Mitchison and Kirschner, 1984). Only GTP-bound αβ-tubulin dimers incorporate into the growing microtubule lattice. Therefore, newly added subunits are GTP bound while previously added subunits are GDP bound. The GDP-tubulin has a >1000 fold higher off rate from microtubule ends than GTP-tubulin (Walker et al., 1988). Therefore, the GTP-tubulin stabilizes the growing ends and loss of the GTP “cap” could explain the rapid depolymerization of microtubules undergoing catastrophe.

GDP-microtubule protofilaments have inherent curvature that is constrained in microtubules, storing potential energy in the microtubule lattice in the form of steric strain. This energy is released upon depolymerization where the protofilaments curve away from the microtubule fiber. The energy released upon microtubule depolymerization can be harnessed to exert force on other systems including transporting kinetochores and their attached chromosomes toward the poles (Coue et al., 1991; Koshland et al., 1988).

The rates of growth, shrinkage, rescue, and catastrophe are much different in vivo than in vitro indicating that microtubule dynamics are highly regulated in the cell (Heald and Nogales, 2002). Many proteins can alter microtubule dynamics, including the +TIP family of MAPs, and kinesins (described in Section 1.4.3). In vitro studies of microtubule biochemistry show that GTP state and plus-end status can be recognized and selectively bound by these proteins (Akhmanova and Hoogenraad, 2005). Therefore, microtubule dynamics are highly regulated in vivo by a number of MAPs and motors that fine tune and harness their energy.
1.3.2 Force Generation in the Mitotic Spindle

An important goal of mitosis research is determining the origins and magnitudes of the forces that move sister chromatids. Experiments in animal cells show that both ATP-dependent sliding of kinesins along microtubules and GTP-dependent turnover of microtubules are capable of generating sufficient force to move chromosomes (Koshland et al., 1988; Maney et al., 2000). *In vitro*, kinesins can couple the energy of ATP hydrolysis and microtubule depolymerization to cause movement of chromosomes (Heald and Walczak, 1999). The relative importance of these two processes is unknown, but is currently a subject of significant debate.

Four principles of general significance are emerging from studies of microtubule attachment and force generation in yeast. First, multiple proteins mediate microtubule attachment to kinetochores. Second, mutations in different microtubule attachment factors give rise to diverse defects in chromosome movement, suggesting that the process of forming a mature attachment to a kinetochore is quite complex. Third, many of the proteins involved in kinetochore-microtubule attachment also localize to other microtubule-based structures in the cell. Finally, chromosome-microtubule attachment must be regulated so that errors such as syntelic attachment can be corrected.

Motor- and polymerization-dependent processes have the potential to generate force in at least four spindle locations: the plus-ends of k-MTs embedded in kinetochores, the minus-ends of k-MTs embedded in spindle poles (traction fiber forces; (Hays and Salmon, 1990), along chromosome arms via interactions with non-kinetochore microtubules (polar ejection forces; (Rieder et al., 1986) and at cortical tips. In higher eukaryotes, all of these mechanisms are utilized. After nuclear envelope break down, the chromosomes must form new attachments to the
spindle. Initially, the chromosomes form lateral attachments and are carried by dynein to the poles (Echeverri et al., 1996; Rieder and Alexander, 1990; Sharp et al., 2000; Vaisberg et al., 1993). Dynein is highly concentrated at unattached kinetochores, but relocalizes to the spindle as kinetochores develop a full complement of k-MTs (Banks and Heald, 2001; Hoffman et al., 2001; King et al., 2000).

Chromokinesins, which bind directly to the chromosome arms, function in chromosome condensation, metaphase alignment, spindle organization, and cytokinesis (Mazumdar and Misteli, 2005). While motors are actively positioning the chromosomes, the spindle exerts force on the chromosomes. Spindle microtubules undergo treadmilling, a process in which the centrosomes embedded minus-ends depolymerize, while the kinetochore embedded plus-ends polymerize. This pull on the microtubule makes up a substantial fraction of force generation at kinetochores. Therefore, both the chromosomes and the spindles are constantly in motion, and a careful balance of plus and minus-end microtubule regulatory elements is likely required for proper spindle function during mitosis.

The relative importance of these processes varies among different organisms, but EM and live-cell studies suggest that polar ejection forces are unlikely to play a major role in yeast (O'Toole et al., 1999). Photobleaching experiments detect no recovery of microtubules at the minus-ends but swift recovery at the plus-ends, suggesting that there is little or no traction fiber force (Maddox et al., 2000). Thus, our working hypothesis is that chromosome movement in *S. cerevisiae* is powered largely, if not exclusively, by kinetochores and processes occurring at the plus-ends of k-MTs.

Although much remains to be learned about the directions and magnitudes of the forces operating on chromosomes in *S. cerevisiae* and animal cells, it is helpful to make some
comparisons between simple and complex eukaryotes. First, it is clear that the generation and stabilization of bipolar kinetochore-microtubule attachment is a complex process that occupies much of mitosis. In animal cells, both sisters kinetochores must capture microtubules, whereas in yeast, it is thought that kinetochores remain bound to microtubules throughout the cell cycle. Capture is presumably necessary in yeast only after centromeres are duplicated and a new kinetochore assembles. In both yeast and animal cells, syntelic and monopolar attachments are eliminated and cell cycle progression is delayed by the spindle checkpoint until all sets of chromatids have achieved bipolar attachment. Attached chromosomes exhibit directional instability (stochastic movement along the spindle axis), and tension is generated across pairs of sister kinetochores (He et al., 2000; McEwen et al., 1997). It is tempting to consider the conserved features of chromosome segregation – bipolar attachment, error correction, tension generation, checkpoint control, and directional instability — to be the most fundamental aspects of the process.

1.4 Kinetochore Assembly

In the last few years significant progress has been made in identifying the protein components of yeast kinetochores and determining their overall architecture. In 1995 there were five known and five suspected kinetochore proteins (Hyman and Sorger, 1995). Yeast kinetochores were thought to comprise a set of DNA binding proteins and a kinesin: Kar3p (Hyman et al., 1992). We now know that budding yeast kinetochores contain at least 50 proteins that are organized in at least three layers: DNA binding, microtubule binding, and a “linker” layer of proteins that bind to neither the DNA nor the microtubules (Figure 1.4). About half of these proteins are essential and many have close mammalian homologs (Kitagawa and Hieter,
The HEC1/Ndc80 complex, which has four subunits, exhibits strong sequence and structural conservation and human HEC1 protein can complement the yeast Ndc80 deletion (described below) (Ciferri et al., 2005; Wei et al., 2005; Wigge and Kilmartin, 2001; Zheng et al., 1999). Conversely, the specialized histone H3, Cse4p can functionally substitute for its homolog CENP-A in human cells, overcoming the arrest caused by RNAi depletion of CENP-A (Wieland et al., 2004). Thus, important aspects of kinetochore architecture have been conserved from yeast to humans despite the significant differences in mechanisms of centromere specification.

In the following sections, I provide a brief description of selected kinetochore proteins complexes before proposing a model for kinetochore assembly. Many kinetochore proteins form discrete subcomplexes that can be isolated from cells and are thought to represent assembly intermediates (De Wulf et al., 2003). Whenever possible, I have grouped kinetochore subunits together by multi-protein complex or by shared function (e.g. “kinesins”). In lieu of a better convention, I name complexes after the best-described component (e.g. “The Ndc80 Complex”). Very few kinetochore subunits have been studied biochemically, but I have indicated those whose functions can be inferred by sequence homology or phenotype. In many cases I suggest that proteins may be “linkers” (blue in Figures 1.3&1.4) between DNA binding (pink) and microtubule binding components (green). However, this designation is a reflection of the preliminary state of functional studies rather than a true assignment of function. Although the exact order of assembly is still unknown I have ordered the complexes from those thought to be more centromere proximal to the microtubule binding proteins.
1.4.1 Specifying Centromere Location.

The first step in kinetochore attachment is assembling the core kinetochore complex onto centromeric DNA. *S. cerevisiae* was the first organism in which centromeres were cloned and sequenced. A 125 bp centromeric DNA (CEN) sequence, present in a single copy on each chromosome, is necessary and sufficient for accurate segregation during meiosis and mitosis (Clarke and Carbon, 1980; Cottarel et al., 1989). Comparison of centromeres from different *S. cerevisiae* chromosomes reveals that they have three conserved elements: CDEI, CDEII, and CDEIII (Fitzgerald-Hayes et al., 1982). CDEI and CDEIII are imperfect palindromes and are bound in a sequence-specific manner by the CBF1 and CBF3 protein complexes, respectively (Cai and Davis, 1990; Lechner and Carbon, 1991; Ng et al., 1986). The sequence of CDEII varies but is always about 85 bp long and highly A-T rich (Clarke and Carbon, 1980). *CBF1* is not essential for viability, though its deletion increases chromosome loss (Cai and Davis, 1990). CBF3 is composed of four essential proteins, Ndc10p, Ctf13p, Cep3p, and Skp1p and has a molecular weight of 240 kDa (Connelly and Hieter, 1996; Goh and Kilmartin, 1993; Lechner and Carbon, 1991; Strunnikov et al., 1995). The assembly of CBF3 is reasonably well understood and involves a series of regulated steps that are described in Figure 1.2. CBF3 is required for the association of all other known kinetochore proteins with centromeric DNA and loss of function mutations in CBF3 proteins disrupt kinetochore-microtubule attachments *in vitro* and *in vivo* (He et al., 2001; Sorger et al., 1994). The CBF3 component Ndc10p is the only protein known to bind CDEII *in vitro*, but other proteins have been suggested to be CDEII-interactors *in vivo* (Espelin et al., 2003).

Centromeres from closely related yeasts such as *K. lactis* are similar in size and organization to those in *S. cerevisiae* (Heus et al., 1990), but centromeres in other organisms are
Figure 1.2: Steps Involved in Activation and Assembly of the CBF3 Complex. (A) Skp1p binds to the F-box of Ctf13p, an event that requires the chaperone Hsp90p to correctly fold Ctf13p. (B) Ctf13p is activated by Skp1p-dependent phosphorylation and the transient interaction of Sgt1p with Skp1p. (C) Activated Ctf13p associates with Cep3p to form a complex competent in Ndc10p association and CEN binding. (D) Skp1p-Ctf13p-Cep3p is in equilibrium with free Skp1p-Ctf13p which can be degraded following Skp1p mediated ubiquitination by SCF and degradation via the 26S proteosome. (E) The CBF3 core complex binds to conserved bases in CDEIII. Ctf13p, Cep3p and Ndc10p contact DNA in the major groove. Inset shows the bases that can be cross-linked to CBF3 subunits. An extended CBF3 complex containing an extra Ndc10p dimer also assembles centromeric DNA by making direct contact with bases proximal to the CDEIII core. Ndc10p also appears to bind to CDEII. In vitro this binding occurs in the absence of other CBF3 subunits. Model based on (Espelin et al., 1997; Espelin et al., 2003; Kaplan et al., 1997; Rodrigo-Brenni et al., 2004; Russell et al., 1999; Stemmann et al., 2002)
much larger and contain long stretches of repetitive DNA. In fission yeast, for example, the centromeric region spans 40–100 Kb of DNA, in humans, centromeres are 1–40 Mb long, and in worms there is no specific centromeric region and kinetochores assemble along the length of chromosomes (Albertson and Thomson, 1982; Bloom, 1993). Thus far, it has not been possible to identify sequence-specific DNA binding proteins in complex kinetochores that are analogous to budding yeast CBF1 and CBF3. Rather, specification of more complex centromeres appears to be linked closely to the formation of chromatin that contains the specialized histone H3 CENP-A (or its homologues). Moreover, it is widely thought that an epigenetic rather than a sequence-specific mechanism is responsible for determining the location of CENP-A containing heterochromatin in organisms with complex centromeres (Choo, 2001). The pathogenic yeast *Candida albicans* have no detectable point centromeres and no similarity between centromeres of different chromosomes. Despite this they recruit CENP-A to their centromeres (Sanyal et al., 2004; Sanyal and Carbon, 2002).

Until recently it was understood that point centromeres evolutionarily preceeded their more complex counterparts. However, sequence analysis from multiple families of fungi demonstrates that point centromeres exist in only a small subset of yeast, suggesting that point centromeres are a specialization of regional centromeres rather than a precursor (E. Rheinbay, personal communication). The fungus *E. cuniculi*, which possesses the smallest number of kinetochore components and is believed to contain the most primitive kinetochores, has no detectable point centromeres supporting the notion that regional centromeres evolved first.

Like animal centromeres, *S. cerevisiae* centromeres contain heterochromatin. Budding yeast have a CENP-A homologue, Cse4p, that is essential for chromosome segregation (Stoler et al., 1995). One model suggests that *CSE4*-containing nucleosomes bind to CDEI and CDEII
DNA and form the scaffold onto which other kinetochore proteins assemble (Figure 1.3A; (Keith et al., 1999; Meluh et al., 1998). However, it is clear that CBF3 and not Cse4p is the primary determinant of kinetochore location in *S. cerevisiae*: centromere binding by Cse4p *in vivo* requires functional CBF3 but the reverse is not true (Measday et al., 2002; Ortiz et al., 1999). Whereas all known kinetochore proteins require CBF3 for centromere association, only subsets are known to require Cse4p (Measday et al., 2002). Current models for Cse4p function are based on *in vivo* crosslinking studies, which have limited resolution (Meluh et al., 1998). Thus, the data are equally consistent with Cse4p-containing nucleosomes forming the phased chromatin array that lies on either side of the core kinetochore complex (Figure 1.3B). In this speculative model, Cse4p would perform an essential function in centromere specification but one that is secondary to CBF3.

### 1.4.2 Linker Complexes

**The Ctf19 Complex.** The Ctf19 complex, also called the COMA complex, contains at least four stably associated subunits, Ctf19p, Okp1p, Mcm21p, and Ame1p, though some studies suggest that it might contain additional subunits (Cheeseman et al., 2002; De Wulf et al., 2003; Ortiz et al., 1999). *CTF19* and *MCM21* are non-essential whereas *OKP1* and *AME1* are required for cell growth. The biochemical functions of the Ctf19 complex are not know, but Ctf19p has been shown by immunoprecipitation and yeast two-hybrid experiments to interact with the CBF3 complex (Ortiz et al., 1999). It is unclear whether this interaction is direct or indirect, but Ctf19p may bind directly to CBF3.
Figure 1.3: Kinetochore Assembly and Architecture. (A) Nucleosome-centric model and (B) CBF3-centric model of kinetochore assembly (C) Logical organization of the DNA-microtubule bridge that forms at centromeres.
THE CTF3 COMPLEX. The composition of the Ctf3 complex is not yet entirely defined, but immunoprecipitation experiments suggest that it contains at least three subunits, Ctf3p, Mcm22p, and Mcm16p, all of which are non-essential and localize exclusively to kinetochores (Measday et al., 2002). Ctf3p has been conserved through evolution and the *S. pombe* homolog of Ctf3p, Mis6+ is required for the recruitment of the fission yeast Cse4p histone (Cnp1+) to centromeric DNA (Takahashi et al., 2000). However, the reciprocal is found in budding yeast and human cells where Ctf3p/hMis6 requires Cse4p/CENP-A for CEN association (Goshima et al., 2003; Measday et al., 2002). The current thinking is that the Ctf3 complex may participate in some aspect of centromeric chromatin assembly.

THE NDC80 COMPLEX. The Ndc80 Complex contains four protein subunits, Ndc80p, Nuf2p, Spc24p, and Spc25p, all of which are essential and localize exclusively to kinetochores (He et al., 2001; Janke et al., 2001; Wigge and Kilmartin, 2001). Mutations in yeast Ndc80 components result in complete detachment of chromosomes from microtubules. Loss of *SPC24* and *SPC25* function causes microtubule detachment and also inactivates the spindle assembly checkpoint (He et al., 2001; Janke et al., 2001), suggesting a role for the Ndc80 Complex in the recruitment of spindle checkpoint proteins to kinetochores. The Ndc80 Complex also appears to be required for kinetochore binding by the Dam1 complex and the MAP Stu2p, suggesting a linker role for the Ndc80 Complex in bridging DNA and microtubule-binding components of the kinetochore (He et al., 2001; Janke et al., 2002). Budding yeast Ndc80 Complex forms a rod-like structure with globular domains in each end (Wei et al., 2005). The human Ndc80 Complex also appears to be extremely elongated (Ciferri et al., 2005). Ndc80p and Nuf2p are evolutionarily well conserved and are required for chromosome segregation in human cells, budding yeast, *Xenopus*
and other organisms (DeLuca et al., 2002; Martin-Lluesma et al., 2002; McCleland et al., 2003; Wigge and Kilmartin, 2001; Zheng et al., 1999). Spc24p and Spc25p homologs are also present in higher eukaryotes but their sequences are less conserved (Bharadwaj et al., 2004; McCleland et al., 2004). The Ndc80 Complex appears to be functionally conserved since RNAi depletion of Spc25 leads to aberrant mitosis and chromosome detachment in humans and *Xenopus*, and Hec1/Ndc80 and Nuf2 are required for microtubule attachment (Bharadwaj et al., 2004; DeLuca et al., 2005; McCleland et al., 2004). This complex is unique as it is the only complex other than the CBF3 complex whose mutation causes complete loss of chromosome-microtubule attachment.

**The Mtw1 Complex.** The Mtw1 Complex contains at least four essential protein subunits including Mtw1p, a protein initially identified as a homolog of the *S. pombe* Mis12+ kinetochore protein (Goshima and Yanagida, 2000). Mtw1p-GFP localizes to kinetochores by imaging, and *mtw1* mutations result in a loss of tension across sister kinetochores (Goshima and Yanagida, 2000). Yeast Mtw1p mutants have some unattached chromosomes and it has been proposed that Mtw1p aids in establishing bipolar attachments of kinetochores (Pinsky et al., 2003), but Mtw1’s biochemical function remains unknown. *DSN1, NNF1* and *NSL1* exhibit genetic or two-hybrid interactions with Mtw1p and are stably associated with Mtw1p in solution in 1:1:1:1 stoichiometry (De Wulf et al., 2003; Euskirchen, 2002; Nekrasov et al., 2003; Westermann et al., 2003). The Mtw1p homologs in fission yeast and humans, Mis12, localize to kinetochores. Depletion of these proteins causes detachment of chromosomes from the spindle (Goshima et al., 2003; Obuse et al., 2004). This suggests that Mtw1p function is conserved through evolution. While the Mtw1p protein may act as a linker between chromosomes and microtubules, no
biochemical function is known. Intriguingly, human Mtw1/Mis12 requires a heterochromatin protein (HP1) for kinetochore recruitment, suggesting a larger interplay between heterochromatin and kinetochore function (Obuse et al., 2004). This heterochromatin dependency has yet to be addressed in budding yeast.

THE SPC105 COMPLEX. The Spc105 Complex contains two subunits: Spc105p and YDR532p (De Wulf et al., 2003; Nekrasov et al., 2003). Both Spc105 and YDR532p are essential, and mutations in Spc105 lead to defects in chromosome segregation (Nekrasov et al., 2003). Spc105 was originally purified with Mtw1p and the two complexes are thought to be spatially close within the kinetochore structure. An Spc105 homolog is also present in fission yeast. The biochemical activity of the Spc105 complex is unknown but this complex is required for recruitment of a subset of kinetochore MAPs, indicating that it fine tunes microtubule attachment or regulation (P. De Wulf, personal communication).

1.4.3 Microtubule Associated Proteins

THE DAM1 COMPLEX. The Dam1 Complex binds to microtubules in vitro and contains 12 essential subunits (Figure 1.4). Dam1p localizes to both kinetochores and spindle microtubules in vivo and dam1 mutants arrest as large budded cells with shortened or broken spindles (Cheeseman et al., 2002; Cheeseman et al., 2001; Enquist-Newman et al., 2001; Janke et al., 2002; Li et al., 2002). The Dam1 Complex is required for both the establishment and the maintenance of bipolar microtubule attachment. By live-cell analysis, dam1-1 and dam1-11 mutants exhibit monopolar attachment and directional instability centered on a single spindle pole. Elegant experiments by Janke et al. (2002) showed that when cohesin has been inactivated
in cells bearing a mutation in the Dam1 Complex member Spc34p, individual sisters segregate almost equally between the two spindle poles (as opposed to being monopolar). This finding implies that both sets of sister kinetochores in spc34 cells can capture microtubules, but the attachments are not strong enough to counteract the pulling forces generated by bipolar attachment. Thus, the chromosomes can be pulled off the microtubules (Janke et al., 2002).

Consistent with this idea, chromatid pairs in dam1 cells are observed to jump occasionally from one SPB to the other, establishing frequent but unstable monopolar attachments first with one SPB and then another. Further live-cell analysis demonstrates that dam1 mutants display a range of attachment phenotypes — including some that are unattached (Dorn, 2005; He et al., 2001).

Copurification of 10 Dam1 Complex subunits revealed that they form a 210 kDa heterodecamer that is globular in free form but forms rings or helices when incubated with microtubules (estimated 10–15 heterodecamers/ring) (Miranda et al., 2005). The Dam1 rings appear to stabilize microtubules, aid in microtubule polymerization, and bind preferentially to GTP-tubulin in vitro (Westermann et al., 2005). The Dam1 Complex requires microtubules for association with kinetochores (Li et al., 2002). These data suggest that the Dam1 Complex ring acts as a collar around the microtubule to which CEN assembled kinetochore proteins attach. Interestingly, there are no known homologs of Dam1p in higher eukaryotes. This suggests that higher eukaryotes, which have multiple microtubule attachments per kinetochore, utilize a different tethering mechanism. However, it is also possible that structural homologs exist that have not yet been identified by sequence homology.

**THE +TIPS.** +TIPS are a class of microtubule associated protein, so named because they associate specifically with the plus-ends of microtubules (Akhmanova and Hoogenraad, 2005; Maekawa
and Schiebel, 2004). There are many +TIP families that function in microtubule regulation during mitosis; some differ in their method of plus-end association. There are at least eight distinct +TIP proteins at vertebrate kinetochores, all of which have budding yeast homologs. However, several of these MAPs are found only in the cytoplasm in S. cerevisiae and, therefore, the number of +TIPs at budding yeast kinetochores is likely much lower (Akhmanova and Hoogenraad, 2005). Thus far there are only two +TIPs – Stu2p and Bik1p – that have confirmed kinetochore localization, but there are three others that may localize to kinetochores.

**Stu2p.** The +TIP Stu2 has homologs in all major kingdoms, including XMAP215 in *Xenopus* and ch-TOG1 from humans (Gard et al., 2004; Wang and Huffaker, 1997). Stu2p is localized in budding yeast to kinetochores and cortical tips, two sites of plus-end microtubule dynamics, and perhaps to the spindle midzone as well (He et al., 2001). The TOG/XMAP215 family of proteins appears to stabilize microtubules (Gard and Kirschner, 1987; Tournebize et al., 2000; Vasquez et al., 1994). Surprisingly, Stu2p destabilizes microtubules *in vitro* through direct plus-end binding (Van Breugel et al., 2003). However, live-cell microscopy shows that Stu2p is transported from newly captured kinetochores to the plus-ends of the attached microtubules, and its arrival at the tip coincides with microtubule rescue (Tanaka et al., 2005). Therefore, Stu2p may actually stabilize microtubules *in vivo*. In *stu2* cells, chromosomes make bipolar attachments, but no transient sister separation is observed and the velocity of chromosome oscillations is reduced compared to wildtype cells (He et al., 2001). Taken together these data demonstrate that Stu2p regulates microtubule dynamics.
BIK1p. The +TIP protein Bik1p localizes to kinetochores as well as to cortical attachments sites where it may function with Kar9p to capture the plus-end of a-MTs (Berlin et al., 1990; He et al., 2001). The deletion of Bik1p has no obvious phenotype in haploid or diploid cells, but Bik1p becomes essential in polyploid cells where it contributes to the generation of tension across sister kinetochores (Lin et al., 2001). The Bik1p homolog CLIP-170 selectively binds and stabilizes the tips of growing microtubules in vitro (Perez et al., 1999). Bik1p, in contrast, is held at both growing and shrinking a-MT plus-ends by the kinesin motor Kip2p (Carvalho et al., 2004). Since Kip2p is present only in the cytosol, other components must tether Bik1p to kinetochores. Bik1p does not localize to kinetochores that have become detached from microtubules (Tanaka et al., 2005) Bik1p and CLIP-170 are not as conserved as Cse4 and CENP-A as Bik1p can localize to kinetochores but cannot substitute for CLIP-170 function in human cells (Wieland et al., 2004).

OTHER POSSIBLE KINETOCHORE +TIPS. Bim1p is another +TIP protein whose homologs (EB1 and Mal3) have been shown to associate with kinetochores in mammalian cells and in fission yeast (Kerres et al., 2004; Tirnauer et al., 2002). Xenopus EB1 stabilizes microtubules and depletion of EB1 in human cells leads to a decrease in tension at kinetochores (Draviam, 2005; Tirnauer et al., 1999) but their role in budding yeast remains unknown.

Stu1p and Pac1p are +TIP members of the MAST/Orbit and LIS1 families of MAPs, respectively. Both MAST and LIS1 function at kinetochores in higher eukaryotes (Akhmanova and Hoogenraad, 2005; Sharp, 2002). Stu1p is essential in budding yeast and localizes to the spindle midzone; stul mutants undergo spindle collapse (Yin et al., 2002). STU1 has genetic interactions with DAM1 and CIN8 (Jones et al., 1999; Yin et al., 2002). It is currently unknown
whether Stu1p associates with budding yeast kinetochores. PAC1 has genetic interactions with many members of the kinetochore and cortical tip including BIM1 and CIN8 (Tong et al., 2004).

KINESINS. Kinesins are motor proteins that harness the chemical energy of ATP hydrolysis and convert it to mechanical force, most often in transporting cargo along microtubules (Asbury, 2005). Most kinesins are dimers that consist of a coiled-coil stalk connecting a cargo-binding domain to globular heads. Each head is a catalytically active ATPase whose affinity for microtubules depends on its bound nucleotide. Kinesin moves in 8nm steps along microtubule protofilaments hydrolyzing one ATP per step. The exact mechanism of movement is still under intense debate, but it appears that kinesin may walk by alternately swinging one “leg” past the other. The many different kinesins subfamilies have diverse activities. Some kinesins function as true motors translocating cargo toward the plus or minus-ends of microtubules. Others specifically destabilize either plus-or minus-ends (for table see (Lawrence et al., 2004)). Differences in kinesin directionality are due to changes in the neck region which reorient the motor heads (Goldstein and Philp, 1999). Microtubule destabilization is caused by changes in the motor heads causing them to exert pulling force on the microtubule protofilaments and encouraging them to curve away from the microtubule polymer (Desai et al., 1999). While the roles for several kinesins in higher eukaryotes are known, the functions of kinesins at budding yeast kinetochores are less well established. In Chapter 2, I describe my efforts to determine the complement of kinesin motors at budding yeast kinetochores and elucidate their functions.
1.4.4 Kinetochore Architecture

The budding yeast kinetochore has been estimated to be at least 5MDa in mass and between 85-100nm in length (De Wulf et al., 2003; Dorn, 2005). The large size and complexity of kinetochores leads to the question of how such a structure might assemble. They could resemble highly structured organelles such as ribosomes or SPBs that are composed of one or two large, stable, multi-protein complexes that assemble in solution and bind – intact – to centromeric DNA. An alternative view is that kinetochores resemble eukaryotic transcriptional enhancers in being composed of many discrete complexes that assemble on DNA either by contacting DNA bases directly or by binding to proteins that are themselves in contact with DNA. The available evidence strongly favors the latter possibility and the existence of a multi-layer structure (Figure 1.3C). Yeast kinetochores appear to be composed of at least eight and perhaps as many as twenty protein complexes each containing up to twelve different components (Figure 1.4). These kinetochore complexes can be isolated as stable species by velocity sedimentation and sizing chromatography (De Wulf et al., 2003; Nekrasov et al., 2003).

If kinetochores contain distinct “layers,” then one would expect a hierarchical set of interdependencies among different kinetochore complexes. This is exactly what is observed. In the first step of kinetochore assembly, shortly after DNA replication, CBF3 binds to DNA and presumably initiates the formation of Cse4-containing centromeric chromatin. DNA-bound CBF3 is required for the Ndc80 complex to bind to centromeres in vivo, but the reciprocal is not true (He et al., 2001). The microtubule-binding Dml Complex and Stu2p require both CBF3 and Ndc80p, but, once again, the reciprocal is not true (Enquist-Newman et al., 2001; He et al., 2001; Janke et al., 2002; Jones et al., 1999). Thus, we can develop a preliminary model in which CBF3 is bound to centromeric DNA, the Ndc80 Complex associates with DNA-bound CBF3,
Figure 1.4
Figure 1.4: Speculative model for the organization of known kinetochore components.

Protein complexes are drawn approximately to scale based on hydrodynamic analysis. The assignment of functions to various proteins is preliminary, except in the case of CBF3 and CBF1 proteins. For references see text, except for Plc1p (Lin et al., 2000), Sli15/Lpl1p/Bir1p (Cheeseman et al., 2002), Hir1/Cac1p (Sharp et al., 2002), Mif2p (Meluh and Koshland, 1997), and Slk19p (Zeng et al., 1999).
and the Dam1 Complex and Stu2p associate with Ndc80 Complex proteins. Significantly, this model is based on a set of genetic interdependencies and we do not know whether CBF3, Ndc80, and Dam1 complexes interact directly. However, if this model is correct, we might expect proteins within a layer to exhibit independent association with centromeric DNA. Once again, this seems to be true. The Ndc80 Complex requires CBF3 for centromere association, as does the Ctf19 Complex, but neither appears to be dependent on the other (He et al., 2001; Janke et al., 2001; Ortiz et al., 1999).

By way of comparison, the mammalian interferon beta promoter is a prototypical eukaryotic enhancer in which activators (NF-κB, ATF-Jun, etc.) and architectural proteins (e.g. HMG(I)) bind in a sequence specific manner to cis-acting regulatory sequences (Struhl, 2001). The similarity between enhancers and kinetochores suggests possible models for the function of Cse4p-containing centromeres in yeast. In enhancers, nucleosomes are excluded from the core of the enhancer but bind on either side — a possible model for the organization of centromeric chromatin. DNA-bound activators then mediate the stepwise recruitment of a set of multiprotein complexes that remodel surrounding chromatin and recruit additional complexes involved in transcriptional transactivation. Similarly, it appears that some, but not all, kinetochore assembly is Cse4p dependent: the Ctf19 and Ctf3 complexes require CSE4 function for centromere association in vivo but, based on functional evidence, the Ndc80 Complex does not (Gardner et al., 2001; McCleland et al., 2003; Measday et al., 2002). Additional support for the ordering of complexes comes from the observation that components of individual complexes appear to copurify (albeit in sub-stoichiometric amounts) with neighboring complexes but not distant ones. Therefore, copurifying proteins can give an indication of nearby complexes (De Wulf et al., 2003; Nekrasov et al., 2003; Westermann et al., 2003).
Preliminary data suggest that the assembly of kinetochores, like the assembly of enhancers, is temporally regulated. Ip1p, Sl15p and Ndc10p relocalize from the kinetochores to the spindle midzone at the metaphase-to-anaphase transition (Buvelot et al., 2003), as does Slk19p, which is cleaved at the metaphase-to-anaphase transition by Esp1p. The cleaved N-terminal fragment of Slk19p is recruited from the kinetochore to the spindle midzone where it is thought to stabilize the anaphase spindle (Sullivan et al., 2001). These so-called “passenger proteins” are found in many organisms (Vagnarelli and Earnshaw, 2004). In addition, subcomplex purification has shown that some of these complexes can be found in intermediate forms, which indicates that their composition could change during the cell cycle (De Wulf et al., 2003). Moreover, we do not know whether all centromeres assemble identical kinetochore complexes, or if yeast kinetochores assemble and disassemble depending on the state of microtubule attachment during cell cycle progression. It is also unknown how phosphorylation by cyclin/CDKs and other cell cycle modulators affects kinetochore function.

Kinetochores probably do not fully assemble on centromeric DNA prior to the establishment of microtubule attachment. Whereas Bik1p and Ctf19p are recruited to kinetochores independently of microtubules, the Dam1 Complex is not kinetochore-bound following microtubule depolymerization by nocodazole. In normally dividing cells, Dam1p is found on both spindle microtubules and kinetochores (Hyland et al., 1999; Li et al., 2002; Lin et al., 2001). Stu2p is also present on spindles and kinetochores, and remains on intranuclear microtubules even when kinetochores are inactivated using an ndc10 mutation (He et al., 2001). Therefore, some proteins that associate with centromeres in vivo probably bind microtubule plus ends in the absence of kinetochore attachment. It is interesting to consider the possibility that unattached kinetochores may not capture microtubules directly, but may instead associate with a
preformed set of plus-end binding proteins. The Dam1 ring makes an appealing candidate for a capture structure on microtubules. One can imagine how centromere assembled kinetochore structures might grab and hold on to the ring.

1.4.5 Complexity of MAP and Motor Localization

A significant fraction of kinetochore MAPs and motors localize to structures other than kinetochores and have functions distinct from chromosome-microtubule attachment. In particular, several microtubule binding proteins present at kinetochores are also found at sites of cortical attachment. In addition to binding to kinetochores, Bik1p and Kip3p localize to cortical attachment sites; Stu2p localizes to the spindle, SPBs, and cortical attachments, and Dam1p localizes along the spindle (He et al., 2001; Hofmann et al., 1998; Jones et al., 2001; Kosco et al., 2001; Lin et al., 2001). Moreover, Stu2p and Kip3p are known to play roles in nuclear positioning and Cin8p and Kip1p have important functions in spindle assembly (Cottingham and Hoyt, 1997; Kosco et al., 2001). These findings suggest a “mix and match” reuse of microtubule binding and motor proteins in the spindle and at kinetochores. One striking example of this is the colocalization of many MAPs and motors at kinetochores and cortical attachments sites at the plus-ends of a-MTs. Both involve the capture of microtubule plus-ends and they may have important structural similarities.

One consequence of the localization of MAPs and motors to multiple structures in the cell is that it introduces considerable complexity in determining protein function. Available evidence suggests that the roles of spindle components are determined as much by their location as by their intrinsic biochemical activity. To prove this point, it will probably be necessary to generate specific mutant alleles that disrupt recruitment to one structure but not to another. A
second issue arises from the tight mechanical coupling within the spindle. The disruption of one component in the spindle has the potential to affect, via microtubule linkages, other elements that are spatially distant. Thus, even when proteins are present on only one structure, their inactivation can affect multiple spindle activities. With 16 kinetochore-mediated pole-to-pole connections in yeast cells and perhaps 4–8 p-MTs, it is easy to imagine that spindle stability is quite dependent on kinetochore function. Thus the interdependence of kinetochore function and other cell cycle processes is becoming ever more complicated. As previously mentioned, spindle dynamics appear to be coregulated with kinetochores at the metaphase-anaphase transition (Higuchi and Uhlmann, 2005). Genetic evidence also links kinetochore function to processes as diverse as the Rac, Ras, and PKA pathways, and chromatid cohesion (Li et al., 2005; Mayer et al., 2004). Therefore, dissecting out specific mechanisms and functions of these proteins will be a challenge.

1.5 Regulation of Microtubule Attachment

During metaphase, chromosomes must form attachments to microtubules emanating from opposite poles. These mature, bipolar attachments have two main characteristics: the kinetochores are bound by microtubules, and tension is generated across the kinetochores. Errors in tension or attachment are sensed by the cell and regulated by two different mechanisms. The Aurora B/Ipl1p kinase is required for the resolution of syntelic attachments and acts early in the cell cycle to release inappropriate kinetochore-microtubule connections and allow the sisters to establish bipolar attachments (Tanaka, 2002). The spindle checkpoint recognizes lesions in attachment and generates a “wait anaphase” signal until the lesions are resolved (Gillett and Sorger, 2001). Although the function of the spindle checkpoint is well established, it is still
unclear as to whether the checkpoint arrests only in response to detachment, or if it recognizes lesions in tension as well.

1.5.1 Role of Aurora B/Ipl1p

Syntelic attachments are common early in the cell cycle and must be corrected to allow the formation of bipolar attachments. These lesions are recognized by the conserved Aurora-B type kinase Ipl1p, and its partner protein Sli15p (whose human homolog is INCENP). *ipl1* and *sli15* mutants fail to make bipolar attachments and, instead, create monopolar links roughly similar to those seen in *dam1* mutants (He et al., 2001; Tanaka et al., 2002). However, unlike *dam1* mutants, if *ipl1* mutants are allowed to establish bipolar attachments at the permissive temperature, and then shifted to the restrictive temperature, bipolarity is maintained. This suggests that Ipl1p/Sli15p is required for the establishment – but not the maintenance – of bipolar attachment (Tanaka et al., 2002). Ipl1p/Sli15p responds to the absence of tension in monopolar or syntelic attachments and resolves them by stimulating microtubule detachment, thereby freeing kinetochores to attach to the opposite SPB (Tanaka et al., 2002).

In higher eukaryotes, removal of improper attachments from kinetochores is mediated by the kinesin MCAK, a homolog of budding yeast Kip3p. Aurora-B from *Xenopus* and mammals regulates the localization of MCAK to kinetochores as well as its depolymerization activity. Phosphorylation of MCAK by Aurora B decreases its destabilization activity in vitro and mutation of the phosphorylation sites leads to defects in spindle structure and chromosome attachment in vivo (Andrews et al., 2004; Gorbsky, 2004; Lan et al., 2004). The current model suggests that tension regulates the interaction of Aurora B and MCAK by controlling their colocalization (Li et al., 2005). In the absence of bipolar attachments, sister centromeres are
closely situated and MCAK and Aurora-B signals completely overlap. When chromosome achieve bipolar attachments, Aurora-B remains at kinetochores while MCAK relocalizes to the inner centromere, where it is believed to be more accessible to type I phosphatases that oppose Aurora B function (Andrews et al., 2004; Hsu et al., 2000; Murnion et al., 2001). This suggests a mechanism whereby unstable attachments cause microtubule depolymerization and aid in the release of improper attachments. Once bipolar attachment occurs, the forces become more balanced leading to oscillation rather than detachment. This mechanism reinforces the importance of microtubule dynamics, and kinesins, in regulation of cell cycle events. Currently the regulation of Kip3p by Ipl1p is unknown; however, these data suggest that Ipl1p may regulate Kip3p function in budding yeast.

Phosphorylation of other Ipl1p substrates, including Ndc10p, Cse4p, Ndc80p, the Dam1 Complex proteins Dam1p, Spc34p, and Ask1p may also play a role in removing tensionless attachments in budding yeast (Biggins et al., 1999; Buvelot et al., 2003; Cheeseman et al., 2002; Shang et al., 2003; Tanaka, 2002). Dam1p phosphorylation may be important for its regulation since phosphorylation site mutations phenocopy the chromosome segregation defects in ipl1-2 mutants. In addition, at semi-restrictive temperatures, the ipl1-2 mutation can be suppressed by serine-to-aspartate mutations that mimic Dam1p phosphorylation (Cheeseman et al., 2002). Interestingly, Dam1p is not recruited to kinetochores in an ipl1-2 mutant, a phenotype that is also rescued by mimicking Dam1p phosphorylation. The current model is that the phosphorylation of Dam1p by Ipl1p downregulates Dam1p activity and promotes bipolar attachment early in the cell cycle (Cheeseman et al., 2002). The role of phosphorylation in the regulation of the other Ipl1p substrates is unknown. Thus, Dam1p and other proteins may be phosphorylated to decrease their affinity for microtubules, thereby favoring chromosome detachment.
1.5.2 The Spindle Checkpoint

Not all attachment errors are sensed and corrected by Aurora B/Ipl1p. Therefore, the spindle checkpoint is required to block the metaphase-to-anaphase transition until all chromosomes have attained bipolar attachment to the mitotic spindle. In budding yeast, this arrest depends on the protein products of the \textit{MAD}, \textit{BUB}, and \textit{MPS1} genes (Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996). The spindle checkpoint is extremely sensitive. Studies in PtK1 cells demonstrate that a single unattached kinetochore is sufficient to engage the spindle checkpoint (Ault et al., 1991; Ault and Rieder, 1992). The spindle checkpoint is essential in higher eukaryotes where each chromosome must form a novel attachment in every cell cycle. All metazoan checkpoint proteins are localized to kinetochores in prometaphase cells, and several of them are released as chromosomes become attached to microtubules. In budding yeast the checkpoint is non-essential but Bub1p and Bub3p associate with kinetochores until metaphase, when they are displaced (Gillett et al., 2004; Hoyt et al., 1991; Li and Murray, 1991). Bub1p is also recruited to unattached kinetochores later in the cell cycle. Mad2p, in contrast, is recruited only to unattached kinetochores following microtubule depolymerization or kinetochore disruption, but not to kinetochores in a normal cell cycle (Gillett et al., 2004; Iouk et al., 2002).

Kinetochore are required for checkpoint function. The complete inactivation of CBF3 leads to an abrogation of checkpoint control (Gardner et al., 2001; Goh and Kilmartin, 1993) and prevents Mad and Bub proteins from associating with kinetochores \textit{in vivo}. However, hypomorphic mutations in CBF3 subunits engage the checkpoint (Doheny et al., 1993). Thus, the checkpoint is sensitive to partially inactive kinetochores but if no kinetochores are present, then
checkpoint signaling complexes cannot form. A similar phenomenon is observed in DNA replication: hypomorphic mutations in replication factors allow origins to form and engage the DNA replication checkpoint, but complete loss-of-function mutations cause cells to skip DNA synthesis, bypass the checkpoint, and undergo a haploid mitosis (Kelly et al., 1993; Piatti et al., 1995). It remains unknown how checkpoint proteins associate with kinetochores and sense attachment problems, but two-hybrid interactions have been detected between yeast Spc25p and Mad1p and between human Ndc80 and Mad1p (Martin-Lluesma et al., 2002; Newman et al., 2000). Moreover, recruitment of Bub1p and Bub3p to centromeres requires some but not all members of the Ndc80p Complex, implicating this complex in checkpoint protein recruitment (Gillett et al., 2004).

Kinesin motors also function in the spindle checkpoint. In mammalian cells, the BubR1 kinase, similar to Mad3p in budding yeast, binds the kinesin CENP-E (Chan et al., 1999; Yao et al., 2000). Depletion of BubR1 by RNAi decreases the amount of CENP-E at kinetochores indicating that CENP-E is recruited to kinetochores by BubR1 (Johnson et al., 2004). CENP-E stimulates the kinase activity of BubR1, and α-CENP-E antibodies that mimic MT binding lead to downregulation of BubR1 activity (Mao et al., 2003). Therefore kinesin activity regulates the spindle checkpoint in mammalian cells through interaction with BubR1. CENP-E has no clear homolog in budding yeast; however, these data indicate that kinesins can regulate checkpoint activity in addition to their roles in mediating chromosome attachments and dynamics.

The loss of kinetochore-microtubule attachment is sufficient to activate the spindle checkpoint, but it is unclear if loss of tension alone can activate the spindle checkpoint. In praying mantid spermatocytes, application of tension to an unpaired X chromosome by pulling with a glass needle leads to the initiation of anaphase approximately one hour later (Li and
Nicklas, 1995), indicating that establishment of tension can satisfy the checkpoint. Evidence that lack of tension activates the checkpoint in budding yeast comes from \textit{cdc6} mutants, which enter mitosis with unreplicated chromosomes. In these cells, kinetochores form monopolar attachments but have a spindle-checkpoint dependent delay in anaphase onset indicating that lack of tension is sufficient to engage the checkpoint (Stern and Murray, 2001). However, there are several caveats to these tension experiments. First, the application of tension stabilizes microtubules and it is possible that pulling on chromosomes leads to subsequent recruitment of microtubules (King and Nicklas, 2000). Therefore, the checkpoint could be silenced by microtubules that attach to kinetochores after the application of tension. Second, little is known about the cell cycle regulation of kinetochores and it is possible that kinetochores mature after replication. Therefore, the \textit{cdc6} mutants may have an abnormal metaphase kinetochore structure, which might activate the spindle checkpoint regardless of tension. Distinguishing tension from attachment sensing is also complicated by Ipl1p, which detaches chromosomes not under tension (Tanaka et al., 2002). These detached chromosomes may be required to signal the spindle checkpoint.

Furthermore, evidence from human cells indicates that loss of tension alone does not engage the checkpoint. Depletion of the MAPs EB1 and Adenomos Polyposis Coli lead to decreases in interkinetochore distance signifying loss of tension. However, these cells do not signal the checkpoint, and instead, proceed into anaphase with segregation errors (Draviam, 2005). Studies from budding yeast also suggest that tension may not signal the checkpoint. Mutations in the +TIP Stu2p cannot exert tension on the spindles and engage the spindle checkpoint. However, visualization of chromosomes in these cells demonstrates that detached chromosomes are present and that they recruit high levels of checkpoint proteins. These data indicate that detached chromosomes, rather than tensionless chromosomes, are signaling the
spindle checkpoint. Thus, tension and attachment are extremely interconnected and convincingly discriminating between the two is currently a major challenge in the field.

1.6 Summary:

Kinetochores are large, complex structures whose function is vital to the fidelity of chromosome segregation. Although much is known about kinetochore structure and function much still remains obscure. Two fundamental aspects in kinetochore function are the kinetochore-mediated regulation of k-MT dynamics and chromosome attachment and the temporal regulation of kinetochore activity. Therefore, during my thesis research I have focused on kinetochore regulation of microtubule dynamics and attachment. Kinesins function in many diverse and important aspects of chromosome segregation from attachment and regulation of movement to regulation of spindle checkpoint activity. In Chapter 2, I present the results of my extensive investigation into the roles of the four nuclear budding yeast kinesins. In Chapter 3, I present preliminary data exploring the alterations in chromosome-microtubule attachment during the cell cycle. These studies have advanced the field of kinetochore biology and suggest even more avenues for future investigation that are detailed in Chapter 4.
References


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CHAPTER TWO

Functional Analysis of Kinesins at Budding Yeast Kinetochores

I have executed and designed all the experiments in this chapter with the exception of G1 imaging of Kar3 (Figure 2.9) which was performed by research technician Greg Jelson.
2.1 Abstract

Accurate chromosome segregation during mitosis requires biorientation of sister chromatids on the microtubules of the mitotic spindle. Chromosome-microtubule binding is mediated by kinetochores, multiprotein structures that assemble on centromeric (CEN) DNA. The simple CENs of budding yeast are among the best understood, but the roles of kinesin motor proteins have yet to be determined despite evidence of their importance in higher eukaryotes. Here we show that all four nuclear kinesins in *Saccharomyces cerevisiae* localize to kinetochores and function in three distinct processes. Kip1p and Cin8p, Kinesin-5/BimC family members, cluster kinetochores into their characteristic bilobed metaphase configuration. Kip3p, a Kinesin-8/KinI kinesin, synchronizes poleward kinetochore movement during anaphase A. The Kinesin-14 motor Kar3p, appears to function at the subset of kinetochores that become detached from spindle microtubules. These data demonstrate roles for structurally diverse motors in the complex processes of chromosome segregation and reveal important similarities and intriguing differences between higher and lower eukaryotes.

2.2 Introduction

Kinetochores are multi-protein complexes that assemble on centromeric (CEN) DNA and attach chromosomes to spindle microtubules (Mitchison and Salmon, 2001). Kinetochore-microtubule attachments generate the forces required for sister chromatid biorientation during metaphase and toward-the-pole movement during anaphase (Maiato et al., 2004). Evidence from a variety of organisms suggests that regulation of microtubule dynamics by kinetochores is critical to both of these processes, and that multiple motor and non-motor microtubule associated proteins (MAPs) are involved (Kline-Smith et al., 2005). The
comparative simplicity of budding yeast centromeres makes *Saccharomyces cerevisiae* an attractive organism in which to undertake a thorough study of this aspect of kinetochore biology (McAinsh et al., 2003).

*S. cerevisiae* has six kinesins and a single dynein heavy chain (for review see Hildebrandt and Hoyt, 2000) but only the four nuclear kinesins – Cin8p, Kip1p, Kip3p, and Kar3p – are potential kinetochore subunits. Yeast nuclear kinesins belong to different subfamilies with distinct directionality, structure and function. Cin8p and Kip1p are members of the Kinesin-5 family of plus-end directed motors (BimC motors; Dagenbach and Endow, 2004) that form homotetramers active in crosslinking parallel and antiparallel microtubules (Gordon and Roof, 1999; Kapitein et al., 2005). Cin8p and Kip1p function in spindle assembly and in other microtubule-based processes (Hildebrandt and Hoyt, 2000). *cin8A* mutants are viable at 25°C but have high rates of chromosome loss and undergo frequent spindle collapse (Hoyt et al., 1992); at 37°C, *cin8A* cells are dead. *cin8A* and *kip1A* are synthetically lethal and KIP1 over-expression suppresses the spindle collapse phenotype of *cin8A*, though *kip1A* does not cause elevated chromosome loss. *cin8A* (but not *kip1A*) is synthetically lethal with *mad2A* (Geiser et al., 1997) presumably because checkpoint-mediated cell cycle delay is required for *cin8A* cells to complete mitosis successfully. Overall, these data show that Kip1p and Cin8p are functionally redundant (Hoyt et al., 1992; Roof et al., 1992) but that Cin8p plays the larger role under normal circumstances.

Kip3p is closely related to the Kinesin-13 family (Kin1 kinesins; Lawrence et al., 2004; Moore and Wordeman, 2004; Severin et al., 2001) that includes the kinetochore motors MCAK in mammals, XKCM1 in *X. laevis*, KLP10A, KLP59C, KLP59D in *D. melanogaster*, and Klp5 and Klp6 in *S. pombe* (Desai et al., 1999; Maney et al., 2001; West et al., 2001). Kinesin-13
motors destabilize microtubule protofilaments causing microtubule-depolymerization primarily at plus-ends (Niederstrasser et al., 2002). Drosophila KLP10A and KLP59C mediate the disassembly of microtubules from the plus and minus ends respectively (Rogers et al., 2004). S. cerevisiae kip3Δ cells are resistant to the microtubule-depolymerizing drug benomyl, consistent with a role for Kip3p in microtubule destabilization in yeast (Cottingham and Hoyt, 1997). Although required for chromosome movement during anaphase in all organisms examined to date, Kinesin-13 motors are also thought to function during metaphase to correct improper kinetochore-microtubule attachment and to align chromatid pairs at the metaphase plate (for review see Moore and Wordeman, 2004). Thus, functions of Kinesin-13 motors in vivo include kinetochore-microtubule (kMT) attachment during metaphase and kMT depolymerization during anaphase.

Kar3p, the fourth nuclear motor in budding yeast, is a minus-end directed Kinesin-14 family member that localizes to spindle pole bodies (SPBs) and the tips of cortical microtubules. Kar3p destabilizes microtubule minus-ends in vitro (Endow et al., 1994; Meluh and Rose, 1990) and has been found at low levels in biochemical preparations of the CBF3 centromere-binding complex (Hyman et al., 1992). Like Cin8p (He et al., 2001), Kar3p associates with CEN DNA when assayed by chromatin immunoprecipitation (ChIP; Tanaka et al., 2005). Kar3p is involved in the sliding of minichromosomes with a GAL-regulated CEN laterally along microtubules under circumstances in which newly induced kinetochores are captured by microtubules. Endogenous S. cerevisiae chromosomes are bound to microtubules throughout the cell cycle however, making it unclear whether Kar3p functions at kinetochores during normal cell division.

Functional analysis of nuclear kinesins in budding yeast is complicated by their involvement in multiple mitotic processes either individually or in combination. This multiplicity
of function creates complex loss-of-function phenotypes. To begin to understand kinesin functions specifically at kinetochores, we have applied a series of fixed and live-cell assays that focus on kinetochore biology. We find that all four *S. cerevisiae* nuclear kinesins localize to kinetochores and perform three distinct functions: Cin8p and Kip1p are required for correct alignment and clustering of kinetochores on the metaphase spindle; Kip3p is required for coordinated movement of chromatids to spindle poles at anaphase; and Kar3p appears to function specifically at a subset of kinetochores on which microtubule attachments are slow to form. Thus, while nuclear kinesins in budding yeast are best known as essential players in spindle assembly, they also have important roles in ensuring the accurate attachment of kinetochores to microtubules.

2.3 Results

2.3.1 Localization Patterns of Kinesins During the Cell Cycle

To determine whether Cin8p, Kip1p, Kip3p, and Kar3p localize to kinetochores, we applied three criteria previously used in the analysis of other kinetochore proteins (He et al., 2001). First, GFP-tagged kinesins were examined in fixed cells and localization patterns compared to patterns for known kinetochore proteins; second, tagged kinesins were tested for *CEN* association by ChIP; third, the role of CBF3 in *CEN* binding of kinesins was examined by using a temperature sensitive mutation (*ndc10-I*) in a subunit of CBF3. CBF3 is an essential four-protein complex required for initiating kinetochore assembly and for the recruitment of all known kinetochore proteins to *CEN* DNA (Goh and Kilmartin, 1993; He et al., 2001; Lechner and Carbon, 1991). Ndc10p-dependent localization to kinetochores and association with *CEN* DNA are diagnostic of kinetochore proteins.
To image motor proteins, kinesins were fused at their C-termini to GFP and integrated into endogenous loci in a strain containing Spc42p-CFP labeled SPBs. Genetic tests established that the tagged motors were biologically active (see materials and methods). In early mitosis, kinetochore proteins localize to a single focus spanning the short (<1 μm) distance between the spindle poles. Subsequently, at spindle lengths of 1.0 to 1.2 μm, kinetochores resolve into two distinct foci lying between the SPBs. This bilobed pattern is analogous to the metaphase plate in metazoans and is maintained until anaphase, at which time chromatids move toward the poles and become tightly associated with SPBs (Figure 2.1A; He et al., 2000). When metaphase cells were examined by 3D-deconvolution microscopy, Cin8p-GFP, Kip1p-GFP, and Kip3p-GFP were found to have bilobed localization patterns similar to that of Ndc80p-GFP, a well-characterized kinetochore protein (Figure 2.1A; top panel). In addition, these kinesins also decorated interpolar microtubules during metaphase (data not shown). In anaphase, Cin8p-GFP and Kip3p-GFP were found at the spindle midzone and Kip1p-GFP localized to faint puncta along pole to pole microtubules (pMTs) whereas Ndc80p-GFP was visible only near kinetochores (Figure 2.1A, bottom panel; and He et al., 2001). Biochemical experiments have shown that the majority of Kip1p is degraded at the metaphase to anaphase transition (Gordon and Roof, 2001) implying that the Kip1p-GFP visible in anaphase cells represents a small fraction of undegraded microtubule-bound protein. Overall, imaging data suggest that Cin8p, Kip1p, and Kip3p localize to kinetochores as well as to other microtubule-based structures. As further demonstration of this point, we established that the bilobed localization of Cin8p, Kip1p, and Kip3p was lost in ndc10-1 cells but that GFP fluorescence along spindle microtubules was maintained (Figure 2.1C). Cin8p-GFP localized close to the poles in ndc10-1 cells, whereas Kip1p-GFP was found along the spindle (data not shown and Figure 2.1C) and Kip3p-GFP was
Figure 2.1: Localization of GFP-tagged kinesins in wildtype cells. (A) Typical 2D projections of 3D images of metaphase (top panel) and anaphase (bottom panel) cells co-expressing the SPB marker Spc42p-CFP (red) and Cin8p-GFP, Kip1p-GFP, Kip3p-GFP, Kar3p-GFP or Ndc80p-GFP (green). Surface plots beneath each image depict fluorescence signal intensity distribution in arbitrary units for CFP (red) and GFP (green). The intensity distributions were generated from 2D maximum intensity projections of 3D image stacks. (B) Representative 2D projections of 3D images of G1 cells co-expressing Kip3p-GFP, Kar3p-GFP or Ndc80p-GFP (green) with Spc42p-CFP (red). (C) Images of cells expressing Cin8p-GFP, Kip1p-GFP, and Kip3p-GFP (green) with Spc42p-CFP (red) in ndc10-1 cells. Strains were grown to mid-log phase at 25°C and shifted to 37°C for 3 hr prior to analysis. Spindles elongate abnormally in many ndc10-1 cells, creating metaphase cells with spindles lengths typical of anaphase (1.2-2.0μm).
concentrated at the spindle midzone. We interpret these data to mean that, in the absence of CBF3, the association of Cin8p, Kip1p, and Kip3p with kinetochores was disrupted whereas localization to other microtubule-based structures was retained.

In contrast to Cin8p-GFP, Kip1p-GFP, and Kip3p-GFP, Kar3p-GFP was found primarily along the nuclear face of SPBs and did not appreciably co-localize with Ndc80p-CFP (Figures 2.1A & 2.2A-B). Localization of Kar3p-GFP to SPBs in living cells confirms previous immuno-EM data (Zeng et al., 1999). However, faint Kar3p-GFP foci were also visible along the spindle in about 30% of early (<1.5 μm spindles) and 20% of late (1.5-2.5 μm spindles) metaphase cells (Figures 2.2C&E). These Kar3p-GFP foci were rarely if ever seen during anaphase (Figure 2.2E) and did not adopt the bilobed pattern typical of core kinetochore proteins. To test the idea that Kar3p might associate specifically with detached or partially attached kinetochores (which are more abundant early in mitosis), cells were arrested in α-factor and released into the microtubule poison nocodazole. Most kinetochores in nocodazole-treated cells migrate to SPBs, apparently by following shrinking microtubules (Gillett et al., 2004), but a subset becomes detached, moves farther from the SPBs and recruits high levels of Bub1p, Mad1p, and Mad2p checkpoint proteins (Gillett et al., 2004). In nocodazole-treated cells we observed that the majority of the Kar3p-GFP signal remained associated with SPBs (visualized with Spc42p-CFP) but in 75% of cells one or more faint foci were visible distal to the SPBs (Figure 2.2D). These fainter, more distant Kar3p-GFP foci colocalized with Ndc80p-CFP (Figure 2.2D bottom). Based on our previous analysis (Gillett et al., 2004), the Kar3p-GFP signal distant from SPBs almost certainly represents kinetochores that have detached from microtubules (Figures 2.2D-E). Quantitation of SPB distal Kar3p-GFP foci showed that they represented 8% ± 2.5% of the total GFP signals; implying efficient recruitment of Kar3p to detached kinetochores. We conclude that Kar3p becomes bound
Figure 2.2: Kar3p-GFP recruitment to improperly attached kinetochores. (A-B) Images representative of the majority of cells that have only two foci. (A) Wildtype cells expressing Kar3p-GFP (green) and the Spc42p-CFP (red). Surface plots as described in Figure 2.1. (B) Images of Kar3p-GFP (green) coexpressed with kinetochore protein Ndc80p-CFP (red). (C) Typical images of the early metaphase (spindle length <1.5 \mu m; top panel) or late metaphase (spindles 1.5-2.5 \mu m; bottom panel) cells in which additional Kar3p-GFP foci are present. Spc42p-CFP labeled in red to show location of SPBs. Peak intensity areas of additional foci are indicated by arrows. (D) Representative images of cells released from \alpha-factor arrest into nocodazole for 2 hours. (top panel) Cells coexpressing Kar3p-GFP (green) with & Spc42p-CFP(red). (bottom panel) Cells coexpressing Kar3p-GFP (green) & Ndc80p-CFP (red). (E) Percentage of cells containing Kar3p-GFP foci in addition to those overlapping the SPBs as scored in early metaphase (spindle length <1.5 \mu m), late metaphase (spindle length 1.5 -2.5 \mu m), anaphase (spindle length <2.5 \mu m) or nocodazole (treated as described in D).
to detached or improperly attached kinetochores but not to the majority of kinetochores in an unperturbed cell. Instead, most Kar3p is bound to SPBs in vegetatively growing cells.

2.3.2 Association of Cin8p, Kip1p, and Kip3p with Kinetochores by ChIP

Cin8p and Kar3p have previously been shown to associate with CEN DNA by ChIP (He et al., 2001; Tanaka et al., 2005) and we therefore concentrated on the analysis of Kip1p and Kip3p. Kip1p-GFP and Kip3p-GFP were observed to crosslink efficiently by ChIP to a 200bp region centered on CENIV but not to equal length fragments lying 400bp upstream and downstream (Figure 2.3A). As additional specificity controls, we showed that GFP-tagged kinesins did not crosslink appreciably with the URA3 locus, IP of untagged kinesins yielded a negative ChIP signal at CENIV, and shifting ndc10-1 cells carrying Kip1p-GFP, Kip3p-GFP, or Cin8p-GFP to 37°C for 3hrs lowered ChIP signals 10-fold or more (Figure 2.3B & He et al., 2001). Overall, these data show that Cin8p, Kip1p, and Kip3p associate specifically with CEN DNA in a CBF3-dependent manner.

2.3.3 Recruitment of Kinesins to Kinetochores

To investigate how Cin8p, Kip1p, and Kip3p are recruited to kinetochores, ChIP was performed in ndc80-1 and spc25-7 cells. Nde80p and Spc25p are components of the Nde80 Complex, a multi-protein “linker” that bridges the DNA and microtubule-binding components of kinetochores (McAinsh et al., 2003). Kinetochores partially disassemble in nde80-1 and spc25-7 mutants and chromosomes dissociate from spindle microtubules (He et al., 2001; Janke et al., 2002). We observed a ~4-fold drop in the CEN-specific ChIP signal for Cin8p-GFP and Kip1p-GFP in nde80-1 and spc25-7 cells (Figures 2.3C-D). In contrast Kip3p-GFP showed wildtype
Figure 2.3: Dependence of kinesin-CEN crosslinking by ChiP on core kinetochore components. (A-D) ChiP of Kip1p-GFP, Kip3p-GFP and Cin8p-GFP to CEN IV or flanking DNA as assayed in (A) wildtype, (B) ndc10-1 (C) spc25-7 or (D) ndc80-1 cells. Asynchronous cultures were grown to mid-log phase at 25°C and in panels B-D, shifted to 37°C for 3 hr prior to analysis. Results in (A) are expressed as the ratio of the %IP from the arm regions to the %IP of the CEN. Results in (B-D) are expressed as a ratio of the %IP in the mutant to the %IP of the wildtype strain.
levels of CEN-binding in *ndc80-1* and *spc25-7* cells (Figures 2.3C-D). Because a functional Ndc80 Complex is required for kinetochores to bind to microtubules, we can conclude that Kip3p is recruited to CEN DNA in an microtubule-independent fashion, implying that Kip3p is a core kinetochore protein, as are Kinesin-13 family members in higher eukaryotes (Wordeman and Mitchison, 1995). Data are more ambiguous for Cin8p and Kip1p: the motors could either require microtubules for CEN association, or the Ndc80 Complex could be directly involved in Cin8p and Kip1p recruitment to kinetochores.

### 2.3.4 Cin8p and Kip1p Organize Kinetochores During Metaphase

To begin to investigate the functions of kinetochore-bound motors we asked whether mutations in kinesins would alter the localization of core kinetochore proteins such as Ndc80p and Mtw1p. The bilobed distribution of these proteins represents the average position of kinetochores during metaphase, and is therefore a sensitive readout of microtubule attachment and chromosome congression (He et al., 2000; Pearson et al., 2004). We examined the localization of Ndc80p-GFP or Mtw1p-GFP in cells carrying Spc42p-CFP tagged SPBs and *cin8Δ, kip1Δ, or cin8F467Akip1Δ* mutations. Because *cin8Δ kip1Δ* double deletions are inviable (Hoyt et al., 1992; Roof et al., 1992) we examined the effects of impairing both Kinesin-5 motors by combining *kip1Δ* with the temperature sensitive *cin8F467A* mutation (which is defective in microtubule binding; Gheber et al., 1999). To obtain mitotic cells for imaging, cells were synchronized in α-factor and released into fresh medium. In wildtype and *kip1Δ* cells, bilobed metaphase configuration was visible in cells 60–75 min after release (Figure 2.4A). In *cin8Δ* and *cin8F467Akip1Δ* cells, however, spindle assembly was delayed to a variable extent (Gheber et al., 1999; Hoyt et al., 1992; Roof et al., 1992; Saunders and Hoyt, 1992) and we therefore
Figure 2.4: Localization of Ndc80-GFP kinetochore protein in wildtype and kinesin mutants. (A-E) Typical 2D projections of 3D images of Ndc80p-GFP (green) and Spc42p-CFP (red) in (A) wildtype, (B) cin8Δ, (C) kip1Δ, (D) kip3Δ, and (E) cin8Δkip1Δ cells. Arrow in top panel of (B) marks the location of a kinetochore that appears to have detached from spindle microtubules. Spindles in cin8Δkip1Δ cells rarely reach normal metaphase lengths and the top panel of (E) shows a metaphase cell with an abnormally short spindle (<1.0 μm) whereas the bottom panel shows a representative of the 10% of cells that reach wildtype spindle length (>1.5 μm). (F-G) GFP-Tub1p (green) and Spc42p-CFP (red) in (F) wildtype and (G) cin8Δ cells. (H) Percentage of metaphase cells with atypical Ndc80p-GFP foci as scored in wildtype, cin8Δ, kip1Δ, and kip3Δ cells with spindle lengths 1.2-2 μm. 100% of cin8Δkip1Δ cells had gross defects in Ndc80p-GFP localization but the interpretation of these images is complicated by the severity of the spindle assembly defect in these cells. (I) Percentage of atypical Ndc80p-GFP foci in metaphase cin8Δ cells as a function of time after α-factor release: 45-90 min post release is designated as "early" and 105-120 min as "late."
focused on the 20-30% of \textit{cin8}\textDelta\textit{A} cells at T= 60–105 min with metaphase spindles that were at least 1.2 \(\mu\)m long. When these cells were examined, more than 60% contained supernumerary Ndc80p-GFP foci along the spindle axis or had abnormally diffuse GFP lobes ("declustering"); Figures 2.4B&H). The same phenotype was observed in cells in which kinetochores were labeled with Mtwlp-GFP (data not shown). In \textit{cin8F467Akip1\Delta} cells, Ndc80p-GFP and Mtwlp-GFP localization patterns were altered to a greater extent, although only a subset of cells could be scored due to spindle collapse. However, in the 5-10% of cells that formed metaphase-length bipolar spindles, kinetochores were distributed all over the spindle and 10-15 partially resolved foci were visible (Figure 2.4E). In contrast, kinetochore distribution was only slightly altered in \textit{kip1\Delta} and \textit{kip3\Delta} single mutants (Figures 2.4C-D&H). Taken together, these data suggest that Cin8p is involved in establishing or maintaining the normal metaphase configuration of yeast chromosomes, perhaps by bundling kMTs. \textit{kip1\Delta} alone does not disrupt kinetochore localization to a significant extent but the severity of the \textit{cin8F467Akip1\Delta} phenotype suggests that Cin8p and Kip1p work together to cluster kinetochores, as they do during spindle assembly.

Because \textit{cin8}\textDelta\textit{A} mutants are known to have unstable mitotic spindles, one concern with the localization data described above is that declustering might be a simple consequence of failing to form a spindle. To explore this possibility, spindle morphology was compared in wildtype and mutant cells using GFP-Tublp (\(\alpha\)-tubulin; Straight et al., 1997). In wildtype cells, microtubules were visible as a thick bar with a slight increase in intensity near the SPBs, reflecting the termination of many kMTs near the spindle poles (Figure 2.4F; Maddox et al., 2000). kMTs are particularly prominent in budding yeast because they outnumber pMTs (Winey et al., 1995). GFP-Tublp morphology was similar in \textit{cin8}\textDelta\textit{A} cells, indicating that bipolar spindles had formed, although the GFP-Tublp signal was less highly concentrated near SPBs (Figure 2.4G). This is
precisely what one would expect if pMTs were correctly assembled but kMTs mislocalized due to defects in congression. We conclude from these data that gross defects in spindle morphology are not responsible for the disruption of kinetochore clustering in cin8Δ cells.

We were also concerned that kinetochore declustering might be a consequence of spindle collapse and regrowth. Were this the case, we would expect the mutant phenotype to be more severe as mitosis progressed and spindles had time to undergo multiple cycles of collapse and regrowth. However, when metaphase declustering was measured in cin8Δ cells as a function of time after α-factor release, the fraction of cells with Ndc80p-GFP declustering early (T=60–75) and late (T=90–105) was similar (Figure 2.4I). We therefore conclude that declustering was not a consequence of altered mitotic timing in cin8Δ cells or of rounds of spindle collapse and regrowth.

A third possible explanation for kinetochore declustering was the presence of large numbers of unattached or improperly attached chromosomes. To investigate this possibility, we assayed the degree of transient separation in α-factor synchronized cells carrying a CENIV-proximal TetO/TetR-GFP tag, and Spc42p-GFP labeled SPBs (Ciosk et al., 1998; He et al., 2000; Straight et al., 1996). Transient separation arises when kMTs pull sister kinetochores in opposite directions (Goshima and Yanagida, 2000; He et al., 2000; Tanaka et al., 2000) and represents an in vivo measure of force generation. No significant decrease in transient separation was observed in cin8Δ and kip1Δ single mutants relative to wildtype cells (Figure 2.5A). Quantitation was difficult in cin8F467Akip1Δ cells due to their very short spindles, but live-cell imaging revealed transient separations qualitatively similar to those seen in wildtype (Figures 2.5B-C). While kip3Δ cells and kip1Δkip3Δ cells both exhibited wildtype levels of transient separation, cin8Δkip3Δ cells had a statistically significant 30% decrease (Figure 2.5A). Overall
Figure 2.5: Measurements of transient separation in kinesin mutants.
(A) Percentage of metaphase cells undergoing transient sister separation in synchronized cultures of wildtype, cin8Δ, kip1Δ, kip3Δ, cin8Δkip3Δ, and kip1Δkip3Δ. Transient separation was determined in cells tagged near CENIV with TetO/TetR and coexpressing Spc42p-CFP as a reference for the SPBs (spindle length 1.5-2.5 μm at T=75 and T=90 after release from α-factor). (B) Geometry of 3D live-cell tracking experiments. Chromatids are labeled using the TetO/TetR-GFP system and compared to GFP-tagged SPBs. Distances are measured between each sister chromatid and the same reference SPB (d1 - black and d2 - green) allowing for visualization of chromatid separation. The spindle length (d3 - red) is also plotted to show cell cycle progression. (C) Chromatid trajectories in a representative cin8F467Akip1Δ cell showing occurrences of transient separations; yellow fill denotes separations. Transient separations highlighted in yellow.
these data show that chromosome-microtubule attachment is not severely disrupted by the
deletion of individual kinesins but that *CIN8* and *KIP3* might work together in force generation
during metaphase. Moreover, with respect to declustering in *cin8Δ* cells, we conclude that it is
not simply a consequence of defective kinetochore-microtubule attachment. Instead, Kip1p and
Cin8p appear to have a specific role in maintaining the metaphase configuration of budding yeast
kinetochores, and thus to function in the process of sister chromatid “congression.”

2.3.5 Kip3p Regulates Anaphase A Movement

Since many Kinesin-13 motors play a role in the movement of chromatids poleward we
asked whether *KIP3* deletion would alter the anaphase movement of budding yeast kinetochores.
We observed sister chromatid disjunction to be complete in wildtype cells with 2.0-4.0 μm
spindles spanning the bud neck (a morphological marker of anaphase) as evidenced by the
appearance of two bright puncta of Ndc80p-GFP or Mtw1p-GFP immediately adjacent to the
SPB. The puncta represent clusters of disjoined kinetochores (Figure 2.6A). In ~20-25% of these
cells, particularly those very early in anaphase, an extra focus of Ndc80p-GFP or Mtw1p-GFP
was visible away from the SPBs (Figure 2.6A and data not shown). In contrast, *kip3Δ* cells with
2.0–4.0 μm spindles had multiple supernumerary kinetochore foci, typically two to five (Figures
2.6B&C), and they persisted for longer. The number of supernumerary GFP foci in *kip3Δ* cells
fell as anaphase progressed and none were visible when spindles had reached their maximum
anaphase length of 7-10 μm, indicating that all chromatids had eventually moved to the poles
(Figure 2.6C-E). We propose that supernumerary Ndc80p-GFP and Mtw1p-GFP foci represent
lagging chromosomes.
Figure 2.6: Localization of kinetochore foci during anaphase.

(A-B) Representative images of Ndc80p-GFP (green) and Spc42p-CFP (red) in (A) wildtype, and (B) kip3Δ cells during anaphase. Arrow along bottom denotes time progression during anaphase (C-E) Quantitation of additional anaphase kinetochore foci throughout anaphase by spindle length. (F) Anaphase localization of Ndc80p-GFP and Spc42p-CFP in kip3Δmad2Δ anaphase cells.
An alternative explanation for supernumerary kinetochore foci is that *kip3Δ* cells with abnormal metaphase spindle morphology are transiently delayed in metaphase by the spindle checkpoint. However when checkpoint-dependent cell cycle arrest was abolished by deleting *MAD2* (Li and Murray, 1991), the number of lagging chromosomes was similar to that in *kip3Δ* cells (Figure 2.6F). Thus supernumerary Ndc80p-GFP or Mtw1p-GFP foci in *kip3Δ* cells are unlikely to arise from malorientation of chromatid pairs during an extended metaphase. Moreover, the observation that anaphase movement is not so delayed as to prevent chromatid disjunction prior to cytokinesis supports previous data showing that chromosome loss rates are normal in *kip3Δ* cells (DeZwaan et al., 1997). Taken together our findings argue that in the absence of Kip3p, synchronous movement of chromatids toward the spindle poles is disrupted.

To obtain further evidence for lagging chromatids in *kip3Δ* cells, we filmed anaphase in live cells carrying TetO/TetR-GFP-tagged *CENIV* and Spc42p-GFP-tagged SPBs. In wildtype cells, *CEN*-proximal GFP tags were briefly stretched into a line along the spindle axis. Such “hyperstretching” presumably reflects increased pulling forces on *CEN* DNA prior to the dissolution of sister chromatid cohesion leading to unraveling of chromatin ultrastructure. After 15–45 sec (mean 25 sec) of hyperstrectching, individual TetO/TetR tags resolved in two compact dots (Figures 2.7A & C-E) and moved rapidly to within 0.4 μm of the SPBs, where they remained for the duration of anaphase (Figure 2.7E). In ~15% of *kip3Δ* cells (n=13) one chromatid made a swift movement toward the pole but then paused for several minutes before finally moving all the way to the pole (Figures 2.8A&D with pause highlighted in yellow). In other *kip3Δ* cells, chromosomes remained hyperstretched for significantly longer than in wildtype (30–100 sec, mean 70 sec; Figures 2.7B&D), implying imposition of pulling forces prior to the dissolution of sister cohesion. The accumulation of lagging chromatids demonstrates
Figure 2.7: Live cell analysis of *kip3Δ* defects in anaphase. (A-B) Consecutive frames from representative movies of (A) wildtype and (B) *kip3Δ* cells demonstrating preanaphase "hyperstretching." Numbers on bottom left of each image indicate seconds prior to anaphase A completion. (C) Percent of anaphase cells where "hyperstretching" occurs prior to anaphase A completion. (D) Average time in seconds of "super-stretching". Error bars are standard error of the mean (SEM). (E) Average difference in chromosome to pole distance after anaphase A completion.
**Figure 2.8: Live cell analysis of a lagging chromatid in kip3Δ cells.** (A) Consecutive frames from representative movies of kip3Δ cells containing a lagging chromosome. White arrows denote locations of TetO/TetR-GFP tags and yellow arrows denote SPBs (B) Geometry of 3D live-cell tracking showing distances between labeled chromatids and the nearest SPB (d1and d4) and spindle length (d3). Chromatid trajectories in (C) wildtype and (D) kip3Δ cells; yellow fill denotes a pause in which one chromatid remained ca. 1.5 μm from the nearest SPB for 100 sec. Note the difference in scales for d1 and d4 v. d3. Times for A,C and D displayed relative to anaphase onset which is set to t=0.
failure of kip3Δ kinetochores to complete poleward movement in a timely fashion; the existence of hyperstretching suggests abnormally early imposition of anaphase A forces. Thus, Kip3p may function both to mediate kMT depolymerization and to coordinate pulling forces with the metaphase-anaphase transition.

2.3.6 Kip3p Regulates microtubule length and dynamics in G1 and α-factor arrested cells

The involvement of Kip3p in chromosome movement during anaphase suggested that it might regulate microtubule dynamics. To measure directly the effects of kinesin mutations on chromosome movement we used fast-acquisition live-cell imaging coupled with machine vision tools. Our software makes it possible to track TetO/TetR-tagged CENs with great precision and to extract rates of microtubule growth, shrinkage, rescue, and catastrophe (Dom, 2005). Currently, accurate tracking is possible only within the simple monopolar spindle geometry of G1 cells. Cin8p and Kip1p are not present in G1 and we therefore focused on analyzing Kip3p and Kar3p (Gordon and Roof, 2001; Hildebrandt and Hoyt, 2001). Kip3p deletion led to a statistically significant decrease in mean microtubule growth (p < 10^{-3}) and shrinkage speeds (p < 10^{-3}) implying a role for Kip3p in the regulation of G1 microtubule dynamics (Figure 2.9A). In contrast, kar3Δ did not affect either of these key parameters to a significant degree. As a second means to show that Kip3p alters kMT behavior, we examined the distribution of Ndc80p-GFP or Mtw1p-GFP in α-factor-arrested cells. After 2 hr in α-factor, Ndc80p-GFP or Mtw1p-GFP foci in wildtype cells averaged 0.8 μm from SPBs and were rarely > 1.5 μm away (Figures 2.9B-D); in kip3Δ cells under identical conditions, kinetochore foci averaged 1.2 μm from SPBs and were often as far as 2.5 μm away (Figures 2.9B-D). Taken together, these data show that Kip3p is involved in regulating the dynamics and mean lengths of kMTs in G1 and α-factor arrested cells.
Figure 2.9: Analysis of chromosome dynamics and microtubule length in G1 and α-factor-arrested cells. (A) Comparison of microtubule growth, shrinkage, catastrophe, and rescue in wildtype, kar3Δ, and kip3Δ cells as determined using automated tracking methods (Dorn, 2005). (B) Representative images in α-factor arrested cells of Ndc80p-GFP localization (green) in wildtype or kip3Δ cells relative to Spc42p-CFP reference (red). (C) Quantitation of data in B showing average distance of kinetochore foci to SPBs (in μm). Error bars are SEM from biological repeats. (D) Distribution of data in C.
2.4 Discussion

2.4.1 Cin8p, Kip1p, and Kip3p Localize to Kinetochores

In this paper we determine which of the six kinesin motor proteins in *S. cerevisiae* localize to kinetochores and analyze the functions of kinetochore kinesins using fixed and live-cell microscopy. Because *S. cerevisiae* has a closed mitosis, only the four nuclear kinesins Cin8p and Kip1p (Kinesin-5/Bim-C family members), Kip3p (a Kinesin-13/KinI motor) and Kar3p (a minus-end directed Kinesin-14) have the potential to bind to kinetochores. ChIP has previously established that Cin8p and Kar3p associate with *CEN* and we show that this is also true of Kip1p and Kip3p, the two remaining nuclear motors. Live and fixed-cell imaging shows that kinetochores are one of the primary structures to which Kip1p-, Cin8p-, and Kip3p-GFP are localized during mitosis in normally growing cells. However, association with other structures is observed in cells lacking active kinetochores (as a consequence of a mutation in the core *CEN*-binding complex CBF3) consistent with previous data showing that kinesins play important roles in spindle assembly.

How are kinesins recruited to kinetochores? In the case of Kip3p, it appears that the motor binds directly to core kinetochore components: Kip3p remains *CEN*-bound in *ndc80-1* and *spc25-7* mutants despite the dissociation of chromosomes from microtubules. In this respect, Kip3p is similar to the human Kinesin-13/KinI motor MCAK, which is a component of the inner kinetochore (Wordeman and Mitchison, 1995). The finding that *CEN*-binding by Cin8p and Kip1p is partially but not entirely dependent on *NDC80* and *SPC25* is ambiguous with respect to the role of microtubule attachment, but it seems likely that both motors require microtubules to associate with kinetochores. Other yeast kinetochore proteins, including members of the microtubule-binding Dam1/DASH complex require microtubules for kinetochore association.
(McAinsh et al., 2003; Miranda et al., 2005; Westermann et al., 2005) as do plus-end MAPs such as CLIP-170 in higher eukaryotes (Maiato et al., 2004). It therefore seems that microtubule-binding kinetochore proteins in *S. cerevisiae* fall into two classes: those that are recruited directly by core kinetochore proteins and those that bind to, or are transported to, microtubule plus ends and then associate with kinetochores. These two classes of k-MAPs must then dock together to form a fully functional kinetochore-microtubule attachment site.

In contrast to Kip1p, Kip3p and Cin8p, which mainly localize to kinetochores, Kar3p-GFP is found primarily on the nuclear face of SPBs. It has been suggested that Kar3p might be a kinetochore motor, based on its co-purification with CBF3 (Hyman et al., 1992) and genetic interaction with other motors (Hildebrandt and Hoyt, 2000). However, previous immuno-EM data (Zeng et al., 1999) are consistent with our live-cell imaging in showing Kar3p to be primarily SPB bound. Low levels of Kar3p can be detected at a subset of kinetochores early in mitosis and higher levels on kinetochores that are detached from microtubules by nocodazole-treatment. Kar3p has recently been implicated in lateral microtubule sliding of newly captured kinetochores formed *de novo* on GAL-regulated CENs (Tanaka et al., 2005). However, kinetochores normally remain microtubule-bound throughout the cell cycle (Dorn, 2005), and microtubule capture is probably important only during a brief period in S phase. This may explain both the low levels of Kar3p on kinetochores under normal conditions and the absence of elevated chromosome loss in *kar3Δ* cells. Moreover, while Kar3p may function in *de novo* microtubule-kinetochore attachment, we have not been able to detect a kinetochore function for Kar3p in cells under normal growth conditions. Deletion of *KAR3* has been shown to partially suppress the lethality of *cin8Δkip1Δ* double mutants (Saunders and Hoyt, 1992) and kinetochore
localization does not explain this phenotypic suppression. Instead, it must reflect functions for Kar3p, Cin8p and Kip1p in processes other than kinetochore-microtubule interaction.

2.4.2 Organization of metaphase chromosomes by Cin8p and Kip1p

Our data show that Cin8p, and to a lesser extent Kip1p, are involved in the generation or stabilization of the distinctive bilobed kinetochore clusters found in budding yeast from mid to late metaphase. The disruption of bilobed clustering in cin8Δ mutants does not appear to reflect gross disorganization of the spindle, dramatic increases in the number of detached chromosomes or changes in the fraction of transiently separated sister CENs. Instead, we speculate that Cin8p and Kip1p, like other Kinesin-5 motors that can crosslink parallel and anti-parallel microtubules (Gordon and Roof, 1999), are involved in crosslinking kMTs. Because S. cerevisiae CENs associate with a single microtubule, kinetochore-bound Cin8p and Kip1p must crosslink microtubules from different kinetochores. Metaphase sister kinetochores can transiently separate by 0.5 μm or more and therefore it seems unlikely that Cin8p and Kip1p are able to crosslink sisters; instead, we propose that crosslinking involves kMTs from different chromatids, though not necessarily kMTs emanating from the same pole (see figure 2.10B). If Cin8p and Kip1p, like human Eg5, can remain attached to microtubule plus ends (Kapitein et al., 2005), the motors may link kinetochores together directly. The net effect of microtubule and kinetochore crosslinking would be to create kMT bundles in which the polymerization of multiple microtubules was coupled, perhaps explaining the requirement for Cin8p/Kip1p in forming bilobed kinetochore clusters. The significance of clustering is suggested by the appearance of detached kinetochores in cin8Δ mutants and an elevated rate of chromosome loss. It is interesting in this regard that higher eukaryotes contain kinetochore fibers made up of 20 or more microtubules. Bundles of
A. (I) single unattached kinetochore (S phase) (II) both kinetochores unattached (nocodazole) (III) both kinetochores properly attached

B. (I) With Cin8p - Kinetochore bundling (II) Without Cin8p - Kinetochores mislocalized

C. (I) With Kip3p - synchronous anaphase movement (II) Without Kip3p - asynchronous anaphase movement

Figure 2.10
Figure 2.10. Models of kinesin functions at budding yeast kinetochores. (A) Kar3p is recruited to (I) improperly attached or (II) unattached kinetochores but not (III) bioriented kinetochores as well as to SPBs. In contrast, Cin8p, Kip1p and Kip3p are present on bioriented kinetochores and on spindle microtubules. (B) Model for Cin8p/Kip1p-mediated bundling of kinetochores. (C) Model depicting the role of Kip3p at microtubule plus ends in synchronizing the movement of chromatids toward spindle poles during anaphase A.
yeast kMTs created by Cin8p and Kip1p may therefore resemble the multi-stranded kinetochore fibers found in higher cells, except that, multiple chromatids would be involved in the yeast microtubule bundles. Further analysis of Cin8p and Kip1p function during metaphase will require deeper understanding of the forces that generate the bilobed configuration of yeast kinetochores, an effort that is currently underway in several labs.

2.4.3 Kip3p and Anaphase Chromosome Movement

Live and fixed cell imaging of *kip3Δ* cells reveals abnormally asynchronous sister chromatid separation during anaphase. A subset of chromatids in *kip3Δ* cells lags behind the majority and is found arrayed along spindle microtubules when the bulk of disjoined sisters have already arrived at the spindle poles. Surprisingly, a second subset of *kip3Δ* chromatids exhibits the opposite behavior: prolonged centromere hyperstretching. Transient sister separation and chromosome stretching are observed in wildtype cells, but coordinated dissolution of sister cohesion and poleward movement generate only a brief period of *CEN* hyperstretching at anaphase A onset. In *kip3Δ* cells stretching is greater in magnitude and duration. Hyperstretching presumably reflects the initiation of poleward movement prior to the complete degradation of cohesin. However, despite these problems early in anaphase, all chromatids in *kip3Δ* cells are disjoined correctly by the end of anaphase B, consistent with a normal rate of chromosome loss in *kip3Δ* mutants (DeZwaan et al., 1997). The simultaneous generation of lagging and hyperstretched chromatids in *kip3Δ* cells can be understood if Kip3p plays a role in ensuring the synchronicity of poleward movement, presumably by coupling plus-end microtubule depolymerization to the release of tension on sisters following cohesion degradation. In Drosophila, a similar role has been proposed for Kinesin-13 motors in triggering timely
destabilization of microtubules (Rogers et al., 2004). Kip3p function does not appear to be restricted to anaphase however, because kinetochore dynamics during G1 and microtubule length in α-factor are altered in kip3Δ cells. Moreover, Cin8p and Kip3p function together during metaphase to generate pulling forces on kinetochores, as evidenced by the 30% decrease in transient sister separation observed in cin8Δkip3Δ double mutants. Overall we conclude that budding yeast Kip3p, like Kinesin-13/KinI motors in higher eukaryotes, plays an important role in the timely and efficient depolymerization of kMTs during anaphase, and probably also during other phases of the cell cycle.

2.4.4 Conclusions

We have established that all four nuclear kinesins localize to mitotic kinetochores in S. cerevisiae, implying a surprising degree of complexity in kinetochore-microtubule interaction, During normal cell division Cin8p, Kip1p, and Kip3p are found at high levels on most, if not all, kinetochores whereas Kar3p is found transiently only on a subset of maloriented or unattached kinetochores. The absence of Kar3p from the majority of metaphase chromatids suggests that kinetochores do not normally move poleward along the sides of microtubules, though such motion may by observed during microtubule capture by newly assembled ectopic minichromosomes (Tanaka et al., 2005). Instead, it appears that in yeast, as in other organisms, the primary way in which kinetochores move is by binding to microtubule plus-ends and then altering their dynamics. Our data suggest that Kip3p is the motor involved in this regulation. Among our most striking observations is that Cin8p and Kip1p, are important in organizing the bilobed metaphase configuration of yeast kinetochores. No precedent exists for this in higher cells, but we speculate that kinetochores with a single bound microtubule, such as those in S.
*cerevisiae*, present mechanical problems not found in complex kinetochores that bind multiple microtubules. Perhaps by bundling 16 or so kMTs (the number bound to one pole in a haploid) in *S. cerevisiae* cells create a structure similar to a kinetochore fiber in higher cells, thereby strengthening microtubule attachment.
Materials and Methods

Yeast Strains and Manipulations

Strains in this study were derived from W303 or S288C. GFP-tagged proteins were constructed as described (Gillett et al., 2004) and integrated into the genome so as to replace the endogenous wild type copy. Because loss-of-function phenotypes for individual motor deletions are subtle, GFP-tagged kinesins were tested for function in strains carrying deletions in other motors exhibiting synthetic lethality and the resulting compound mutants tested for viability and growth: Kip1p-GFP was examined in cin8Δ cells; Cin8p-GFP in kip1Δ cells; Kar3p-GFP in kip3Δ cells and Kip3p-GFP in kar3Δ cells. In all cases, compound mutants were viable with growth rates indistinguishable from wild-type. KanMX deletion strains were constructed by amplifying the deleted gene of interest from ATCC deletion strains by PCR using primers 500bp upstream and downstream of the deleted gene. PCR products were then transformed into fresh cells and correct integrants confirmed by PCR. pFA6a-HisMX6 deletion strains were made as described in (Longtine et al., 1998) using primers with at least 50bp of homologous sequence

Microscopy Analysis

Image acquisition and processing were performed as described previously (Gillett et al., 2004) on an Applied Precision Deltavision RT microscope with 100X 1.4NA optics and CoolSnap camera. Fixed cells were prepared by treatment with 2% formaldehyde for 2 to 5 min followed by 0.1M phosphate buffer (pH6.6) for at least 10 minutes prior to microscopy analysis. Live cells were grown in SD media for several hrs and then resuspended in fresh media prior to imaging either at room temperature or at 30°C.

ChIP
Temperature-sensitive strains and wildtype controls were grown at 37°C for 3 hrs before
crosslinking; protocol described in (Megee et al., 1999) with minor modifications as described
(Gillett et al., 2004). Untagged strains served as a control. To establish the linearity of the ChIP
assay, serial dilutions of immunoprecipitated DNA or total DNA were used as substrates for
PCR amplification of amplify 200bp CENIV or fragments 200bp fragments lying 400bp
upstream or downstream. The ChIP signal was determined as a ratio of CENIV DNA recovered
by IP to CENIV in the total DNA preparation. ChIP data is presented as a ratio of signals for
mutant vs. wildtype strains or CENIV vs. flanking DNA.
Acknowledgements

We thank J. Dorn for aid with statistical analyses and image processing, G. Jelion for microscopy and image analysis and members of the Sorger lab for critical readings of the manuscript. J.D.T. was supported by a fellowship from the HHMI. This work was funded by NIH grants GM51464 and GM64524 to PKS.

Abbreviations list

*CEN* – centromere
MAP – microtubule associated protein
ChIP – chromatin immunoprecipitation
SPB – spindle pole body
pMTs – pole-to-pole microtubules
kMTs – kinetochore microtubules
References


CHAPTER THREE

Cell Cycle Regulation of Kinetochore-Microtubule Attachment

I planned and executed all experiments in this chapter.
3.1 Abstract

Attachment of centromeric DNA to microtubules is vital to the fidelity of chromosome segregation. This connection is mediated by the kinetochore, a large, multi-protein complex bridging chromosomes to microtubules. Although the identity of proteins mediating attachment is becoming clearer, the dynamics and regulation of kinetochore-microtubule attachment during the cell cycle are still poorly understood. In this work, I demonstrate the existence of a period early in the cell cycle during which treatment with the microtubule depolymerizing drug nocodazole results in an increase in chromosome detachment. Interestingly, nocodazole addition after this period causes only minor detachment, indicating that kinetochore-microtubule attachments are not uniform throughout mitosis. In addition, I show that in populations of cells exhibiting little detectable chromosome detachment, metaphase arrest is still robust. This indicates that loss of attachment may not be required to activate the spindle checkpoint.

3.2 Introduction

Proper chromosome segregation requires that sister chromatids become attached to opposite poles of the mitotic spindle and maintain this attachment during complex changes in force and microtubule dynamics. Attachment occurs via kinetochores, large protein structures that assemble onto centromeres and tether them to the plus-ends of microtubules. During G1, each kinetochore is attached to a single kinetochore-microtubule (k-MT) emanating from the spindle pole body (SPB) (Winey et al., 1995). As the cell progresses into S-phase, the chromosomes become replicated and the new sister chromatid must become attached to a microtubule emanating from a newly replicated SPB. These attachments are not rigid. Instead the kinetochores must remain attached to the dynamic k-MTs as they undergo frequent catastrophe and rescue events (Maiato et al., 2004). The cohesin complex, which holds together newly
formed sister chromatids, opposes the forces generated by the k-MTs pulling toward the pole, resulting in the generation of tension across the kinetochores (Tanaka et al., 2000). In higher eukaryotes, an increase in tension leads to larger inter-kinetochore distances and a higher number of microtubules per attachment (King and Nicklas, 2000; Shelby et al., 1996). In budding yeast, tension across kinetochores results in the transient separation of the sisters and stretching of the centromeric chromatin (Goshima and Yanagida, 2000; He et al., 2000; Tanaka et al., 2000). Additional force across sister kinetochores is generated by the elongation and antiparallel sliding of the interpolar microtubules of the mitotic spindle (p-MTs). Subsequently, at anaphase onset, cohesin is cleaved and the kinetochores remain attached to the rapidly depolymerizing k-MTs.

Despite the fact that budding yeast kinetochores are attached to only a single, dynamic microtubule, kinetochore-microtubule attachments are extremely stable. Many kinetochore mutants weaken these chromosome-microtubule attachments resulting in chromosome missegregation or arrest by a surveillance mechanism known as the spindle checkpoint (for review see McAinsh et al., 2003; Taylor et al., 2004). However, with the exception of mutations in the CBF3 Complex, which completely abrogate kinetochore assembly, only mutations in the Ndc80 Complex have been shown to cause detachment of all kinetochores from the spindle microtubules (He et al., 2001). This suggests that microtubule attachment is accomplished through the interaction of many proteins with a high degree of partial redundancy.

The spindle checkpoint is a surveillance mechanism that monitors kinetochore-microtubule attachment to protect cells from chromosome loss. Lesions in attachment trigger the spindle checkpoint to generate a “wait anaphase” signal. This checkpoint signal results in sequestration of Cdc20p, the activator for the ubiquitin ligase Anaphase Promoting Complex (APC) (Visintin et al., 1997). Mad1-3p, Bub1p, and Bub3p respond to spindle defects and
sequester Cdc20p, and all these proteins are essential for checkpoint function (for review see Gillett and Sorger, 2001).

Treatment of higher eukaryotic cells with the microtubule depolymerizing drug nocodazole causes spindle collapse and kinetochore detachment from microtubules resulting in sister chromatid pairs floating freely throughout the cell. Activation of the spindle checkpoint prevents further mitotic progress. Although nocodazole is routinely used in budding yeast to detach chromosomes and arrest cells in mitosis, nocodazole addition results in limited detachment of chromosomes from microtubules (Gillett et al., 2004). Instead, treatment of asynchronous cells with nocodazole leads to p-MT and k-MT collapse, with the majority of kinetochores colocalizing with SPBs by microscopy. Only a subset of cells contain chromosomes distant from those clustered at the SPBs, and only these foci recruit the spindle checkpoint proteins Mad1p, Mad2p, and Bub1p (Gillett et al., 2004). These data indicate that not all chromosomes have equal attachment strengths.

I have utilized this observation to probe kinetochore-microtubule attachments during the cell cycle by differential timing of nocodazole treatment. I observed a window early in the cell cycle during which chromosomes were significantly more sensitive to nocodazole. When cells are exposed to nocodazole after this period, the overall amount of detachment was extremely low. Surprisingly, even in populations with low chromosome detachment, cells established an extremely robust metaphase arrest, indicating that arrest did not depend on detached chromosomes. These results indicate that chromosome-microtubule attachments vary with cell cycle progression and provide a powerful new assay to detect subtle defects in chromosome-microtubule attachment.
3.3 Results

3.3.1 Nocodazole Treatment Causes Limited Chromosome Detachment

To evaluate the strengths of chromosome-microtubule attachments, I observed the effect of nocodazole on asynchronous cultures of *S. cerevisiae* coexpressing the kinetochore protein Ndc80p-GFP and the SPB protein Spc42p-CFP. Prior to the addition of nocodazole, Ndc80p-GFP exhibited the prototypical bilobed kinetochore distribution between the SPBs, indicating that all chromosomes were properly attached. After treatment with nocodazole, many cells were found to contain faint Ndc80p-GFP foci distant from the chromosomes (Figure 3.1A).

Chromosomes distant from SPBs recruit high concentrations of the spindle checkpoint proteins Bub1p, Mad1p and Mad2p, and are believed to be detached from the mitotic spindle (Gillett et al., 2004). The percentage of cells with additional Ndc80p-GFP foci increased from 0% before the addition of nocodazole (n=451) to 31% after 1 hour in nocodazole (n=179) and 49% after 2 hours (n=151)(Figure 1B). Of the cells with extra foci, 80% had only a single focus far from the SPBs (n=74). In all cells the majority of Ndc80p-GFP colocalized with the SPBs. The low percentage of kinetochores distal to the SPBs is in stark contrast to nocodazole treatment in ndc80 mutants. In these cells, numerous kinetochore foci (visualized with Mtw1p-GFP) are visible throughout the nucleus, indicating that most of the kinetochores have lost their attachments (Figure 3.1C). Because chromosomes are held together by cohesin until anaphase onset, we believe that each Ndc80p focus is a single pair of sister chromatids. Therefore, we conclude that in the vast majority of nocodazole-treated cells, only a single chromosome pair became detached. These data suggest that k-MTs depolymerize in nocodazole, and though chromosome-microtubule attachments in budding yeast are very strong and the majority remained attached to chromosomes, a small percentage become detached.
Figure 3.1: Chromosome detachment in asynchronous populations. (A) Representative image from asynchronous cells treated for two hours with nocodazole coexpressing the kinetochore marker Ndc80p-GFP (green) and the SPB marker Spc42p-CFP (red). White arrows indicate collapsed SPBs. Yellow arrow indicates detached chromosome. (B) Percentage of asynchronous cells with at least one detached kinetochore as a function of time. Nocodazole was added just after the 0 minute time point. (C) Representative image of Mtw1p localization (green) in ndc80-1 cells incubated at 37°C for 2 hours in nocodazole.
3.3.2 Response to Nocodazole Varies During the Cell Cycle

It remained unclear why some, but not all, cells exhibited detached chromosomes. I postulated that there might be a window of sensitivity during which the attachments were less mature. During S-phase, kinetochores are presumed to be displaced from the DNA in order to allow replication of centromeric DNA. I speculated that this displacement, albeit transient, might increase the sensitivity to nocodazole. To test this hypothesis I arrested cells coexpressing Ndc80p-GFP and Spc42p-CFP with α-factor and then released half of the culture directly into nocodazole (early addition) and released the other half into fresh medium for one hour before nocodazole addition (late addition) to allow progression through S phase. Time points for microscopy were taken every hour after α-factor arrest and scored by microscopy for the proximity of the Ndc80p-GFP foci to the SPBs. Before release from α-factor, all chromosomes were in close proximity to the SPBs (Figure 3.2A). In cells released directly into nocodazole, 53% of cells had at least one detached chromosome after one hour (n=299), and this rose to 78% of cells after 2 hours (n=232)(Figure 3.2A). The cells with late addition of nocodazole showed significantly different results. One hour after nocodazole addition, only 3.5% of cells exhibited any detached chromosomes (n=172). After 2 hours in nocodazole only 8% of cells had detached chromosomes (n=120)(Figure 3.2B). These data indicate that there is a period early in the cell cycle – perhaps S-phase – during which kinetochore-microtubule attachments are sensitive to nocodazole, and that exposure to nocodazole after this period leads to collapse of kinetochore-microtubules but not the detachment of chromosomes from the spindle.
Figure 3.2: Cell-cycle-dependence of nocodazole sensitivity. (A) Percentage of cells with attached and detached chromosomes after α-factor release into nocodazole. x-axis indicates time after α-factor on top and time after nocodazole addition on the bottom. (B) Percentage of cells with attached and detached chromosomes before and after nocodazole treatment. Nocodazole added 60 minutes after α-factor release. x-axis indicates time after α-factor on top and time after nocodazole addition on the bottom. In both graphs the dotted line indicates time of nocodazole addition.
3.3.3 Checkpoint arrest does not depend on timing of nocodazole addition

It has been suggested that the spindle checkpoint might require detached chromosomes to generate the “wait anaphase” signal (Gillett et al., 2004). Therefore I tested whether the checkpoint mediated arrest was as robust in cells with late nocodazole treatment as in asynchronous cells. The extremely low levels of chromosome detachment observed in cells with late nocodazole treatment suggested that checkpoint proteins might be recruited in just this small subset of cells. If the checkpoint only senses detached kinetochores, then the checkpoint should only be activated in the >10% of these cells with detached chromosomes, and just those few cells should arrest. The remaining 90% of cells should proceed into anaphase unencumbered. However, FACS analysis demonstrated that both “early” and “late” addition of nocodazole leads to robust arrest with 2N DNA content lasting for at least 2½ hours after addition of nocodazole (Figures 3.3A&B). Therefore, based on our observations, activation of the checkpoint and metaphase arrest are not solely dependent on the presence of unattached kinetochores. However, it remains a distinct possibility that the attached, but collapsed, kinetochores might represent incomplete attachments that might activate the spindle checkpoint without recruiting levels of checkpoint proteins visible by microscopy.

3.4 Discussion

3.4.1 Kinetochores are Sensitive to Nocodazole Early in the Cell Cycle

It is commonly presumed that nocodazole eliminates microtubule attachments through depolymerization in yeast, as in humans cells. Here I show evidence that, contrary to these general assumptions, the majority of kinetochore attachments survive nocodazole treatment. Only half of asynchronous cells treated with nocodazole have detectable detached chromosomes. Because chromosome loss in nocodazole was non-uniform, I suspected that there might be cell
Figure 3.3: FACS analysis of early and late nocodazole addition. (A) FACS plots of cultures to which nocodazole was added immediately upon release from α-factor. (B) FACS plots of cultures to which nocodazole was added immediately after the 60-minute time point.
cycle dependent weaknesses in chromosome attachment. Further investigation uncovered such a cell cycle dependent weakness of kinetochore-microtubule attachment; nearly 80% of cells released directly into nocodazole exhibit detached chromosomes after 2 hours, whereas those cells allowed to enter the cell cycle for one hour in the absence of nocodazole had less than 10% detachment after two hours in nocodazole. Notably, even in cells with visibly detached chromosomes, the majority of chromosomes were closely associated with the SPBs, suggesting that they maintained chromosome-microtubule attachment.

As DNA replication proceeds, all bound proteins are presumed to be removed from the DNA for the replication machinery to pass through. Therefore, kinetochore complexes and their attached microtubules become dissociated from centromeric DNA, at least briefly, during S-phase. It is possible that under wildtype conditions, microtubule plus-ends might retain partial kinetochore binding, stabilizing the microtubules and keeping them near the centromeres until the DNA-recruited kinetochore proteins reassemble following replication. Addition of nocodazole might depolymerize these microtubules, thereby removing the prebound microtubules from the vicinity of the centromeres and making reattachment by the sisters much more difficult (Figure 3.4A). This model would explain the sensitivity to early but not late nocodazole addition.

However, if all kinetochores are released from the centromere during S-phase, why are most chromosomes still attached after early release into nocodazole? When treated with nocodazole after α-factor release, the chromosomes collapse to the poles and the mitotic spindle never elongates. Therefore, chromosomes are likely in close proximity to the duplicated SPBs during S-phase and when kinetochore dissociate, the microtubules cannot depolymerize far away
Figure 3.4: Models for nocodazole response. (A) Model for S-phase progression. Before replication kinetochores are attached to a microtubule. (top panel) During S-phase, the CEN binding kinetochore components transiently dissociate from the DNA but the microtubule remains nearby, stabilized by the microtubule associated proteins (MAPs). Once the replication fork has passed, the proximity of the microtubule allows it to be recaptured by the remainder of the kinetochore. (bottom panel) In the presence of nocodazole, the CEN binding kinetochore components still dissociate from DNA but the microtubule depolymerizes away from the DNA, making recapture difficult. After replication the CEN-binding kinetochore components bind the centromere, but the microtubule cannot be captured. (B) Model for kinetochore recapture in nocodazole. Upon addition of nocodazole both kinetochore-microtubules and interpolar microtubules collapse. Therefore all chromosomes are nearby the SPBs. If short microtubules can be nucleated, these can capture chromosomes that lose attachments forming either (I) syntelic or (II) bipolar attachments. Either attachment is sufficient to sequester the chromosomes near the SPBs.
from the centromeres. In addition, the two SPBs are in close proximity to the sister chromatids. Therefore, the nucleation of short microtubules from either SPB might capture the nearby centromeres by raising the effective concentration of microtubules and centromeres (Figure 4.4B).

The intermediate level of detachment in nocodazole suggests that nocodazole treatment might be an excellent assay for detecting proteins that make small contributions to attachment or for factors that are loaded during S-phase. It is possible that many kinetochore proteins play small redundant roles in chromosome attachment, and because of their redundancy, cause no obvious defects in normal cell cycles. Nocodazole treatment of these mutant cells might then increase the number of detached chromosomes. Similarly, this assay could be used to assess the ability of various kinetochore mutants to form novel attachments after chromosome detachment. To this end, one could release cells from α-factor into nocodazole and quantitate the detached chromosomes. Subsequently, nocodazole could be washed out and one could quantitate the ability of the cells to reattach the detached chromosomes.

3.4.2 The Spindle Checkpoint Signals in the Absence of Detached Chromosomes

Although a great deal is known about the spindle checkpoint, there are still many open questions. Notably, debate exists regarding the types of lesions sensed by the checkpoint; specifically, whether the checkpoint can directly sense tension defects or whether detachments are required for checkpoint activation (Biggins and Walczak, 2003). Studies from cdc6 mutant cells indicate that unreplicated chromosomes that have made monopolar attachments signal the checkpoint indicating that cells can respond to tension (Stern and Murray, 2001). However, these
data are not conclusive and there is also significant evidence that the spindle checkpoint cannot recognize tension alone (Draviam, 2005; Gillett et al., 2004).

I have found that only a low percentage of cells treated with nocodazole after S-phase have kinetochores distant from the SPBs. Previous studies found that only these chromosomes recruited checkpoint proteins (Gillett et al., 2004). Taken together, these data suggest that the majority of chromosomes in such cultures are attached to microtubules. However, these cells robustly arrest, indicating that detached chromosomes are not required to signal the checkpoint. It is tempting to consider the possibility that loss of tension triggers the checkpoint in these cells. However, there are several caveats to this conclusion. First, the Ipl1p kinase is functional in these cells and could be causing the transient detachment of chromosomes in response to the loss of tension. Also, it is possible that some of the kinetochores have only monopolar attachments, sequestering them to the SPBs, and that the unattached sisters are signaling the checkpoint without recruiting high concentrations of the checkpoint proteins. Low levels of checkpoint proteins could go undetected by microscopy. Further investigation is required to distinguish between checkpoint response to tension and attachment.
References:


CHAPTER FOUR

Conclusions and Future Directions
4.1 Conclusions

Accurate chromosome segregation is vital to the genetic stability of all organisms. Proper segregation requires that every sister chromatid pair establishes a bipolar attachment to the mitotic spindle, whereby each sister binds a microtubule extending from an opposing pole. This attachment is mediated by the kinetochore, a multi-protein complex that bridges centromeric DNA to the microtubules.

When I began my thesis research (2000), the complexity of the kinetochore was not yet evident. Many kinetochore proteins required for assembling the kinetochore structure were still being discovered. It was also just becoming clear that kinetochores do not simply form attachments — they also regulate the microtubules to which they are attached (He et al., 2001). In addition, the dynamic nature of chromosome movement and its interrelation with the mitotic spindle were not yet fully appreciated. The main cause of chromosome movement was thought to be the transport of chromosomes as cargo along microtubules by motor proteins. However, it is now apparent that kinetochores are not simply static attachment factors, but instead are active regulators of microtubule dynamics and chromosome movement. It is also clear that the coupling of kinetochores to the microtubule plus-ends, and the concurrent regulation of microtubule dynamics, generate a significant fraction of chromosome movement.

This thesis presents data that advances our understanding of the role of the kinesin motors in the regulation of budding yeast chromosome movement and organization, as well as chromosome-microtubule attachment. Using fluorescence microscopy and chromatin immunoprecipitation, I show that three of the four nuclear kinesins in budding yeast are present at kinetochores with bipolar attachments: Cin8p, Kip1p, and Kip3p. The Kinesin-5 motors Cin8p and Kip1p function redundantly to align chromosomes during metaphase into a bilobed
distribution – the typical metaphase kinetochore localization pattern that is analogous to the metaphase plate of higher eukaryotes. I propose that these motors crosslink k-MTs and bundle centromeres. The plus-end binding microtubule-destabilizing kinesin, Kip3p, regulates microtubule dynamics throughout the cell cycle and is required for synchronous chromosome movement during anaphase A. The minus-end directed motor, Kar3p, localizes specifically to improperly or unattached kinetochores, but not to bioriented kinetochores during the cell cycle. Through careful study of asynchronous and synchronous cultures treated with nocodazole, I have discovered that kinetochore-microtubule attachments are not uniform throughout the cell cycle and that nocodazole does not detach the majority of chromosomes. I also have preliminary data suggesting that cells can sustain a robust spindle checkpoint arrest in the absence of detached chromosomes. Together these findings represent a significant advancement in kinetochore biology.

4.1.1 Kinesins Localize to Budding Yeast Kinetochores

It was originally hypothesized that motors were responsible for the majority of chromosome movement in yeast (McIntosh et al., 2002). Motors were thought to bind chromosomes as cargo and walk them along microtubules. According to this model, plus-end motors carried chromosomes to the metaphase plate while minus-end directed motors transported chromosomes poleward during anaphase. Further examination of both yeast and higher eukaryotic chromosome movement has shown that microtubule dynamics are regulated by MAPs such as the +TIPs, and are responsible for a considerable fraction of force regulation in the mitotic spindle (for review see Akhmanova and Hoogenraad, 2005). Further, once captured, kinetochores maintain an end-on attachment, calling into question the model of chromosomes as cargo. When I began my research, the role of the motor proteins in budding yeast had yet to be
determined and their roles in higher eukaryotes were still debated. Therefore, I set out to
determine the complement of motors at budding yeast kinetochores. I found that all four nuclear
kinesins function at budding yeast kinetochores. The plus-end directed motors Cin8p, Kip1p, and
Kip3p localize to mature kinetochores, whereas the minus-end directed motor Kar3p localizes to
kinetochores with attachment defects, but not to kinetochores that have established bipolar
attachments. The presence of only plus-end directed motors during anaphase rules out the model
of motors walking chromosomes as cargo toward the poles. Instead their presence supports the
model whereby kinetochores remain attached to the depolymerizing plus-ends of microtubules.

Deletion of KAR3 slows transport of newly captured kinetochores to the poles (Tanaka
et al., 2005). It has been proposed that Kar3p might function at kinetochores throughout the cell
cycle. However, I have found that Kar3p rarely colocalizes with kinetochores during unperturbed
cell cycles. When treated with nocodazole, Kar3p colocalizes with kinetochores - indicating that
it is only recruited to detached kinetochores. Furthermore, G1 chromosome dynamics in kar3
mutants demonstrate that Kar3p plays little or no role in regulating chromosome motion in G1.
Therefore, I hypothesize that Kar3p is recruited only to immature or detached kinetochores and
that it functions to return detached chromosomes to the SPBs. It is important to note that, unlike
with mature attachments, captured chromosomes move laterally along microtubules. Therefore
Kar3p may transport chromosomes as cargo to the poles before they establish bipolar
attachments.

This role for Kar3p is analogous to the role of dynein in higher eukaryotes. Dynein aids
in the capture of kinetochores and transports them laterally along microtubules toward the pole.
Dynein dissociates from kinetochores and moves to the spindle as chromosomes establish mature
attachments. Therefore, despite the substitution of a different motor type, I believe that the mechanism of chromosome capture has been conserved through evolution.

One important focus for future research is the process of establishing end-on attachments after recruitment to microtubule minus-ends. The microtubules along which newly captured kinetochores are transported, are preferentially stabilized at the plus-ends by the +TIP proteins: Stu2p and Biklp (Tanaka et al., 2005). This suggests that the microtubule to which a captured chromosome forms its lateral attachment might not be the one to which it makes its mature attachment. There are several models to explain how microtubules could transition from a lateral to a mature attachment. Kar3p could walk captured kinetochores toward the SPB where chromosomes could be efficiently captured by the high concentration of searching microtubules. This idea is appealing in that it does not require any additional protein function. A second attractive model proposes that the Ip1lp kinase responds to tensionless kinetochores once they are returned to the poles by detaching them from the microtubule as it does for syntelic attachments (Tanaka et al., 2005). Another possibility is that the minus-end depolymerizing activity of Kar3p preferentially depolymerizes the microtubule from the minus-end after transporting the chromosome to the SPB. This third model is in keeping with the known biochemical activity of Kar3p, which depolymerizes stabilized microtubules preferentially from the minus-ends (Endow et al., 1994). However, no evidence of treadmilling has been observed in budding yeast and, therefore, the minus-ends of budding yeast microtubules are thought to be nondynamic (Maddox et al., 2000). Also, kinetochores and chromosomes are bulky and may be sterically inhibited from reaching the extreme minus-end of the microtubule. Current data suggest that one of the first two models are correct — Kar3p walks chromosomes to the SPB embedded minus-ends of microtubules, where they are captured either with or without the help of
AuroraB/Ipl1p. However, further experiments will be required to distinguish between these models.

4.1.2 Differential Recruitment of Kinesins to Kinetochores

Kinesins are recruited to kinetochores by different subcomplexes. While all three metaphase motors require the CBF3 Complex in order to associate with centromeric DNA, only Cin8p and Kip1p also require the Ndc80 Complex. Because kinetochores dissociate from microtubules in the absence of the Ndc80 Complex, dependence on this complex implies that microtubule binding is also required for association with centromeres. Along with other studies of kinetochore architecture, these data indicate that not all kinetochore proteins assemble directly onto DNA. Instead, I hypothesize that only a subset of proteins – consisting mainly of DNA binding proteins and linkers – assemble directly onto DNA. The majority of the plus-end microtubule associated proteins and the ring structure formed by the Dam1p Complex, combine to form a plus-end substructure. Mature kinetochore attachments are established by docking of the centromere-assembled subcomplex with the plus-end subcomplex (Figure 4.1A).

Unlike the other MAPs and motors, Kip3p does not require the Ndc80 Complex for centromere association. This indicates that Kip3p may be recruited to the centromere-associated subcomplex of kinetochores rather than the microtubule plus-ends, mimicking the localization of the Kip3p homolog MCAK to the inner kinetochore region in higher eukaryotes (Wordeman and Mitchison, 1995). I hypothesize that Kip3p may colocalize with the ends of microtubules embedded in kinetochores, allowing the protein to directly regulate the microtubule plus-ends (Figure 4.1B).
Figure 4.1: Model for kinetochore architecture. (A) Most microtubule associated proteins (MAPs-yellow), including the Dam1 Complex are recruited to microtubules, while the centromere binding proteins (pink) and linker complexes (blue) and a few kinetochore recruited MAPs (including Kip3p) assemble onto centromeric DNA. The centromere binding component then captures the microtubule via the associated complexes forming the mature functional kinetochore. (B) Model for differential recruitment of kinesins to kinetochores.
It remains unclear, however, how Kar3p is specifically recruited to detached or improperly attached kinetochores. One hypothesis is that recruitment of Kar3p to kinetochores may involve the spindle checkpoint protein Bub1p. Bub1p localization mirrors that of Kar3p, with the exception that a much lower percentage of prometaphase cells show Kar3p localization than show Bub1p localization (Gillett et al., 2004). However, Kar3p localization to unattached kinetochores disappears in ndc80-1 mutants as assayed by chromatin immunoprecipitation and microscopy, whereas Bub1p does not require Ndc80p for kinetochore localization (though it does require the Ndc80 Complex members Spc24p and Spc25p) (Gillett et al., 2004 and P. De Wulf personal communication). The mammalian checkpoint protein, BubR1, recruits the kinesin CENP-E to kinetochores and CENP-E, in turn, modulates BubR1 activity (Mao et al., 2003; Yao et al., 2000). Thus, it is tempting to speculate that Kar3p and Bub1p may interact in a similar manner to CENP-E and BubR1. Experiments are currently underway to clarify the mechanism of Kar3p recruitment to detached kinetochores in yeast.

4.1.3 Cin8p and Kip1p Regulate the Establishment of the Budding Yeast Metaphase Plate

Cin8p and Kip1p are closely related kinesins that form homotetramers (Gordon and Roof, 1999). The higher eukaryotic homolog Eg5 can crosslink and walk along parallel and antiparallel microtubules in vitro (Kapitein et al., 2005). While budding yeast do not form the stereotypical metaphase plates of higher eukaryotes, they do have an analogous localization pattern – the bilobed kinetochore distribution (He et al., 2000). I have found that mutation of Cin8p and Kip1p in budding yeast leads to defects in chromosome alignment during metaphase. Based on biochemical data, I hypothesize that Cin8p and Kip1p crosslink adjacent kinetochores during metaphase. This crosslinking of multiple centromeres could coordinate chromosome movement.
and stabilize the spindle (Figure 4.2). Bundling kinetochores and microtubules might also strengthen chromosome-microtubule attachments, and could explain the high rate of chromosome loss in \textit{cin8} mutants.

While budding yeast kinetochores are bound to a single microtubule via an end-on attachment, higher eukaryotes recruit an average of 20–25 microtubules per kinetochore (Brinkley and Cartwright, 1971; Rieder, 1982; Winey et al., 1995). However, some evidence suggests that the regional centromeres could be composed of multiple individual centromere units. These units are specified by the specialized histone CENP-A and interspersed with regions of non-centromeric histone H3 (Vafa and Sullivan, 1997; Zinkowski et al., 1991). Each individual centromere could recruit a single microtubule. In this case, mammalian kinetochores could be composed of dozens of interconnected point-centromere-like modules. I speculate that the crosslinking of adjacent centromeres in budding yeast is analogous to the bundling of centromere modules in higher eukaryotes. These data suggest that centromere organization and microtubule binding may be more evolutionarily conserved than previously believed.

4.1.4 Kinesins function in regulating chromosome dynamics

I have discovered that Kip3p regulates microtubule dynamics throughout the cell cycle. Loss of Kip3p causes alterations in G1 microtubule dynamics. In \(\alpha\)-factor, k-MTs are longer in \textit{kip3}\(\Delta\) cells than in wildtype cells, indicating a loss of depolymerizing ability. In metaphase, \textit{kip3}\(\Delta\textit{cin8}\(\Delta\) double mutants have decreased transient separation, indicating that Cin8p and Kip3p regulate microtubule dynamics during metaphase. In the absence of Kip3p, cells initiate anaphase asynchronously, suggesting that Kip3p is a switch-like signal regulating microtubule dynamics. In wildtype cells, anaphase A is extremely fast, and microtubules depolymerize
Figure 4.2: Models for kinetochore architecture. Cin8p and Kip1p bundle separate centromeres together to form linked units from individual kinetochores (green).
without pausing until they reach the SPBs. In contrast, in some kip3Δ mutant cells chromosomes move toward the SPBs but then pause before completing anaphase A. Other kip3Δ mutant cells destabilize their chromosomes too early (before cohesin is cleaved) as evidenced by the duration of preanaphase stretching. Therefore, Kip3p does not simply act as a microtubule-destabilizing factor, but instead it acts as a complex modulator of microtubule dynamics. The premature anaphase A movement in kip3Δ mutant cells suggests that Kip3p may actually stabilize k-MTs during metaphase. The change in Kip3p activity from stabilization to destabilization of microtubules suggests that Kip3p itself is regulated in a cell cycle dependent manner.

This leads to the important question of the mechanism of Kip3p regulation. The most likely mechanism is phosphoregulation. Kip3p is a phosphoprotein that can be phosphorylated by the Cdc28p kinase \textit{in vitro} (Sullivan et al., 2001; Ubersax et al., 2003). Dephosphorylation by the Cdc14p phosphatase regulates spindle dynamics at the metaphase-to-anaphase transition and is a good candidate for Kip3p regulation. It has been suggested, however, that Kip3p is not a substrate of Cdc14p (Sullivan et al., 2001). Evidence from higher eukaryotes demonstrates that the Kip3p homolog, MCAK, is phosphorylated by the Aurora B/Ipl1p kinase \textit{in vitro} and that phosphorylation of MCAK reduces its ability to destabilize microtubules and alters its localization (Andrews et al., 2004; Gorbsky, 2004; Lan et al., 2004). Therefore, Kip3p may also be a substrate of Ipl1p in \textit{S. cerevisiae}.

Phosphorylation of Kip3p by Ipl1p should occur early in the cell cycle, perhaps early in metaphase after kinetochore capture. Since Ipl1p moves from kinetochores to the spindle midzone at the metaphase-to-anaphase transition, this could allow for the dephosphorylation of Kip3p. Dephosphorylation may then act to increase MT depolymerization. In higher eukaryotes, Aurora B activity is opposed by type 1 protein phosphotase (Gorbsky, 2004). In budding yeast,
the type 1 phosphatase Glc7p, localizes to kinetochores and opposes Ipl1p function \textit{in vivo},
though its substrates are unknown (Francisco et al., 1994). Therefore, Glc7p might
dephosphorylate Kip3p, as well as other Ipl1p targets, to regulate kinetochore-microtubule
attachment microtubule dynamics during metaphase and anaphase and perhaps in repairing
syntelic attachments early in metaphase as well. The regulation of Kip3p function is an important
question that remains to be addressed.

4.1.5 Cell Cycle Regulation of the Kinetochore

Unlike higher eukaryotes where chromosomes condense and must become attached to the
mitotic spindle following nuclear envelope break down, budding yeast kinetochores remain
attached to microtubules throughout the cell cycle (Dom, 2005). Although kinetochores remain
attached to microtubules, it is unknown if this attachment is uniform or if kinetochore-
microtubule attachments change or mature as the cell cycle progresses. I found that when k-MTs
proceed into the cell cycle in the presence of nocodazole, a large percentage of cells lose some
kinetochore attachment. However, when cells are allowed to proceed past S-phase in the absence
of nocodazole, they lose a significantly smaller percentage of chromosomes. These results
suggest that chromosomes proceeding through S-phase in the presence of nocodazole are unable
to establish mature attachments. It is initially tempting to assume that the detached chromosomes
we observe dissociate during S-phase. One model is that kinetochores dissociate briefly from the
centromeres during replication. In the absence of nocodazole, the microtubules may remain near
the DNA, stabilized by the microtubule-associated proteins of the kinetochore. The addition of
nocodazole at this point would depolymerize the microtubules, resulting in spatial separation
between centromeres and the microtubules. However, this explanation may be too simplistic or
may address only part of the process since chromosome detachment continues 2 hours after nocodazole addition whereas DNA replication is complete after the first hour.

Another possible explanation for differences in kinetochore-microtubule attachment strengths is cell-cycle-dependent changes in kinetochore architecture and regulation. Kinetochore composition is not uniform throughout the cell cycle. Instead, there is mounting evidence that the kinetochore is highly regulated during the cell cycle both by differential recruitment of subunits and by posttranslational modifications – especially phosphorylation. At least three separate phosphorylation pathways function at kinetochores during the cell cycle. The cyclin dependent kinases phosphorylate many kinetochore proteins, including Ipl1p and Kip3p (Ubersax et al., 2003). Ipl1p in turn, has been shown to phosphorylate many kinetochore proteins, presumably in response to inappropriate microtubule attachments (Cheeseman et al., 2002; Hsu et al., 2000; Shang et al., 2003). Finally the spindle checkpoint kinases, Bub1p and Mps1p, are likely to phosphorylate kinetochore proteins and/or kinetochore regulatory proteins (Gillett and Sorger, 2001). Dephosphorylation of kinetochore components has also been shown to regulate their function and localization (Higuchi and Uhlmann, 2005). Investigation of subunit composition demonstrates that the make-up of the linker COMA Complex changes during the cell cycle (De Wulf et al., 2003). Cell cycle regulation of protein stability also regulates kinetochore function. Cin8p and Kip1p are degraded at mitotic exit and anaphase onset respectively, and do not associate with kinetochores until S-phase (Gordon and Roof, 2001; Hildebrandt and Hoyt, 2001). Therefore, it is evident that cell cycle regulation plays a significant role in kinetochore regulation, and that a complete understanding of cell-cycle-dependent regulation will be required for understanding kinetochore function.
4.1.6 Triggers for the Spindle Checkpoint

Although the data are preliminary, it appears that populations of cells can establish and maintain a robust spindle checkpoint arrest in response to nocodazole even when less than 10% of these cells have a single detached kinetochore. Since spindle checkpoint proteins are only detectable by microscopy at unattached kinetochores, these data suggest that the checkpoint is responding to a signal other than detachment. Several issues could account for this observation. Most simply, the chromosomes that have collapsed back to the SPBs could be recruiting low levels of checkpoint proteins to an unattached sister in a monopolarly attached chromosome pair. Similarly, the checkpoint could be responding to syntelic or bipolar attachments that are lacking tension. In these cases, spindle checkpoint proteins would be directly recruited to chromosomes with subtle defects. It is also possible that transient detachments caused by Ipl1p-mediated detachments from microtubules signal the checkpoint. Another option is that nocodazole treatment might alter kinetochore structure itself (distinct from mutation in kinetochore components), and that displaced kinetochore proteins are engaging the spindle checkpoint in the absence of checkpoint recruitment to kinetochores. Circumstantial evidence supporting this last idea comes from the observation that some kinetochore proteins lose CEN association in nocodazole treatment – but not in mutants of the Ndc80 complex. This indicates that nocodazole treatment alters kinetochores differently than detachment by kinetochore mutants (J. Tytell unpublished observations). Further experiments will be required to distinguish between these mechanisms.
4.1.7 Modeling of the Budding Yeast Mitotic Spindle

Many motor and non-motor MAPs function in higher eukaryotes to regulate spindle and chromosome dynamics at both microtubule plus- and minus-ends. The extreme complexity of spindle regulation in higher eukaryotes has made dissecting kinetochore-specific functions of microtubule binding proteins extremely difficult. Although complicated, budding yeast kinetochores are much easier to study because the minus-ends of microtubules appear to be embedded in the SPBs and nondynamic, suggesting that all changes in chromosome movement are regulated from the kinetochore-attached plus-ends (Maddox et al., 2000). Therefore, in collaboration with the Danuser lab at Scripps Research Institute, we have undertaken experiments to study chromosome dynamics with high levels of precision. Tandem arrays of Tet operator regions are integrated into a single chromosome near the centromere in cells expressing the Tet repressor (TetR) and the SPB component Spc42p tagged to GFP.

We then acquire live-cell movies with images taken every second. The SPBs and centromeres are tracked using sophisticated algorithms to measurements chromosome and SPB position in three dimensions over time. From these data we acquire rates of microtubule dynamics (Dorn, 2005; Thomann et al., 2002). Computational tracking allows us to acquire a much larger number of accurate data points than would ever be possible to measure by hand. This allows for statistically significant changes to be calculated from live-cell movies. Our technique has proven successful in measuring G1 dynamics in which distinctions could be made between different mutant phenotypes (Dorn, 2005).

Measurement of microtubule dynamics using this technique suggest that the standard descriptors of microtubule dynamics do not adequately describe true microtubule motion. Therefore, we are evaluating more sophisticated methods for modeling microtubule dynamics to
find more a more comprehensive set of descriptors (Jaqaman, 2005). These show that chromosome motion in attached kinetochores at a given time point is linked to the previous time point, whereas motion of unattached kinetochores is not. We believe that these improved descriptors will aid in the analysis of kinetochore mutants. We plan to combine these modeling approaches with more traditional techniques — including localization studies and biochemistry — to fully explore the mechanisms of the budding yeast kinetochore.

4.2 Summary

In this thesis I have evaluated the mechanisms of chromosome movement and dynamics. The goal of my research has been to increase the understanding of basic cellular processes as it relates to chromosome segregation with the hope of providing insight into the treatment and prevention of human disease. I have discovered that all four \textit{S. cerevisiae} nuclear kinesins function at kinetochores. Two, Cin8p and Kip1p, are involved in organizing centromeres into the budding yeast equivalent of a metaphase plate. Another, Kip3p, is involved in regulating the coordinated onset of anaphase at the metaphase-to-anaphase transition, and in regulating microtubule dynamics during the cell cycle. I have found that Kar3p does not localize to mature kinetochores, but that it is found at detached kinetochores and in a small subset of immature spindles. This suggests that Kar3p is recruited only to improperly attached kinetochores. I have also discovered that the commonly used drug nocodazole, does not detach the majority of kinetochores as previously believed. However, it does increase chromosome detachment when administered at a discrete period early in the cell cycle. Moreover, regardless of when nocodazole is added, or whether chromosomes appear to be detached, the spindle checkpoint is activated. This indicates that the spindle checkpoint may be sensitive to lesions other than
detachment. These findings represent a significant advance in the field of kinetochore biology and will hopefully serve as a building block for further research.
References


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