3. Mitotic Chromosome Segregation Control

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3.1 Introduction

The somatic division, called mitosis, is characterized by equal distribution of parental genome into two daughter cells. Mitosis involves a dramatic reorganization of both nucleus and cytoplasm driven by protein kinase cascades including master controller Cdk1-cyclin B. Mitosis is an ancient eukaryotic event, and some divergence emerged during evolution. Many single cell eukaryotes, including yeast and slime molds, undergo a closed mitosis, in which mitotic spindle formation and chromosome segregation occur within an intact nuclear envelope. However, higher eukaryotes such as animal and plant cells use open mitosis, in which nuclear envelope disassembles before the chromosomes segregate. This review primarily focuses on mitotic chromosome segregation in animal cells and refers to other organisms when regulation is mechanistically conserved. For convenience of discussion, mitotic chromosome dynamics are subdivided into six phases: prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis.

This chapter highlights the research progress made over the past 10 years, which has sought to identify and illustrate specific roles for proteins involved in kinetochore dynamics, kinetochore–spindle interaction, and mitotic checkpoint, which underlie mitotic chromosome segregation control. Table 1 provides a summary of the various proteins that have been implicated in the regulation of chromosome segregation. In the course of this review, relevant earlier studies are briefly discussed and references
given to related review articles. We discuss how centromeres and their kine­
tetochores assemble, how they power mitotic chromosome movements, how, as signaling elements of the mitotic checkpoint, they control cell cy­cle advance during cell division.

**Table 1. Summary of parietal cell proteins implicated in the cell activation pro­cess**

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<th>Name</th>
<th>Yeast homologue</th>
<th>Cellular location</th>
<th>Presumed function</th>
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<td>Survivin</td>
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### 3.2 Mitotic Spindle: An Elaborate Structure for Chromosome Segregation

Chromosome condensation, the landmark event at the onset of prophase, often begins at the nuclear periphery with simultaneous disassembly of nucleolus. In the cytoplasm, the interphase microtubule arrays emanating from a single centrosome is converted into two sets of astral arrays surrounding the duplicated centrosomes. These two asters then separate and migrate over the surface of the nucleus. Later, chromosomes condense into distinct paired threads, termed sister chromatids, which are closely paired along their entire length. Although chromosome condensation was first observed more than a century ago (e.g., Sutton 1903), the molecular mechanism is just beginning to be uncovered.
Mitosis involves a series of complex chromosome movements coordinated by the mitotic spindle. During prometaphase, spindle microtubules nucleated at centrosomes grow and shrink rapidly until they encounter and bind to a kinetochore via a “search and capture” process. If the sister kinetochore captures a spindle microtubule from the same pole, a syntelic attachment is created; if the captured microtubule is from the opposite pole, the attachment is bipolar. In metaphase, sister chromatids with bipolar attachments align in the middle of the spindle to form the metaphase plate. The spindle assembly checkpoint senses the state of chromosome-spindle attachment, delaying cell cycle progression until all pairs of sister chromatids have formed bipolar attachments. It is not yet known how monopolar, syntelic, and bipolar attachments are distinguished, 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, the anaphase-promoting complex is then activated to degrade the cohesin. Chromatids then begin to move toward the opposite poles while maintaining end-on microtubule attachment. Chromosome-to-pole movements during anaphase A and subsequent separation of the poles during anaphase B create two equal and separated sets of sister chromatids. The rate at which this process goes wrong in yeast is on the order of $1 \times 10^{-5}$ errors per chromosome per cell division, demonstrating the high fidelity of the mechanical and regulatory mechanisms that orchestrate chromosome segregation.

3.3 Kinetochore Dynamics in Mitosis

Chromosome movements on the spindle fibers during mitosis is powered and regulated by the kinetochore. The kinetochore is the site for spindle microtubule-centromere association. (In general, centromere refers to the highly repetitive DNA segment that confers centromere function while kinetochore often refers to the proteinaceous structure assembled onto the surface of the centromere.) Structurally, it is composed of four layers: an innermost plate that apparently consists of a specialized layer of chromatin, an interzone, an outer plate that has been argued to consist of tightly packed fibers (e.g., Rattner 1986), and an outermost fuzzy, fibrous corona that is most clearly seen after microtubule disassembly (e.g., Yao et al. 1997). Under electron microscopy, the kinetochore appears as a narrow band of dense chromatin just at the surface of the primary constriction, the inner kinetochore, and a laminar outer kinetochore domain that contains many of the microtubule binding and signal transduction molecules dis-
cussed below. The cohesin protein complex linking two sister kinetochores will be addressed as well.

Although the molecular composition and structure differ markedly respective to the kinetochore among various organisms, biochemical and genetic studies have revealed some common aspects of the organization, which provides a sketch of a simple "core centromere architecture." Chromatin is the key feature and the centromere domain is built on a unique class of nucleosome found only on centromere, in which atypical histone H3, referred to as CENP-A, governs the characteristics of the nucleosome (Smith 2002). CENP-A is a variant of histone H3 with more than 60% sequence identity at the C-terminal histone fold domain. Using CENP-A purified from HeLa cells, Takeyasu and colleagues employed atomic force microscopy to evaluate whether CENP-A can replace histone H3 in an in vitro nucleosome reconstitution assay (Yoda et al. 2000). They found that typical "beads on a string" images obtained from histone H3-organized nucleosomes were similar to those obtained with CENP-A-mediated assemblies. In fact, mononucleosomes isolated by glycerol gradient sedimentation had a relative molecular mass of approximately 200 kDa and were composed of 120–150 bp of DNA and equimolar amounts of CENP-A, and histones H4, H2A, and H2B. Thus, CENP-A forms an octameric complex with histones H4, H2A, and H2B in the presence of DNA, indicating the exchangeability between CENP-A and histone H3.

Great progress has been made toward a better understanding of the molecular composition of the kinetochore protein complex mediating the attachment of spindle microtubules to mitotic chromosomes in budding yeast (e.g., He et al. 2000). He and his colleagues show that four major protein complexes comprised of >20 components are central players in outer kinetochore assembly and microtubule binding. The Ctf19 complex and the Ndc80 complexes appear to represent "adaptors" that interact with both core centromere and distal kinetochore or spindle components (Ortiz et al. 1999; Wigge and Kilmartin 2001; Janke et al. 2001). The Dam1p complex (Cheeseman et al. 2001; Janke et al. 2002) (also known as DASH [Li et al. 2002]) may be the central component of microtubule binding activity, with its activity regulated by the yeast Aurora B kinase (Ipl1) (Biggins et al. 1999; Tanaka et al. 2002). Interestingly, Ipl1-dependent phosphorylation of the three Dam1p components is essential for microtubule capture of yeast kinetochore (Cheeseman et al. 2002a), suggesting a critical role of Ipl1 in kinetochore dynamics. While the yeast homologues of INCENP (Slil5) and survivin (Birl) are highly conserved in eukaryotic cells (Bolton et al. 2002), it would be interesting to see whether animal cells use similar components to govern chromosome movements.
Mammalian cell centromere organization is in much more complex than that in the yeasts. For example, human centromeres contain extensive (1500 to >30,000 copies), tandemly repeated arrays of a 171 bp sequence element called α-satellite. Centromere function has been mapped to α-satellite arrays by centromeric deletions, either naturally occurring on the X chromosome (Schueler et al. 2001) or those induced by telomere insertion into the Y (Brown et al. 1994). Centromeric satellite DNAs are not conserved in sequence among metazoans, but share: (1) their presence in very large tandem arrays, and (2) unit repeat lengths that tend toward multiples of the nucleosome repeat length (Henikoff et al. 2001).

CENP-A nucleosomes are bound to α-satellite DNA in human centromeres (Vafa and Sullivan 1997), but these are not uniformly distributed. Stretched chromatin fibers reveal interspersed CENP-A- and histone H3-containing nucleosomes (Blower et al. 2002). These foci may represent kinetochore "subunits" that assemble together to form multiple binding sites for the multiple microtubules that attach to mammalian centromeres (Zinkowski et al. 1991). In fact, our own attempt to dissection this complex structure using proteomic approach combined with in vitro reconstitution supports the notion that kinetochore is assembled by several protein subcomplexes (see below).

The fundamental link of centromere to a distinct histone also supports a role for chromatin structure in centromere determination. CENP-A appears to be at the foundation of the kinetochore assembly process and is required for assembly of most distal kinetochore components examined (Howman et al. 2000; Oegema et al. 2001). CENP-A assembled chromatin does not form a specialized domain of DNA replication (Shelby et al. 2000; Sullivan and Karpen 2001), and its loading is uncoupled from that of the conventional histones (Shelby et al. 1997; Takahashi et al. 2000).

3.4 Determinants of De Novo Centromere Formation

What determines the assembly of centromere? This has been answered, at least in part, by engineering artificial mammalian chromosomes. Minichromosomes were initially formed de novo by a mixture of a large synthetic chromosome 7 α-satellite array, telomeric sequences, a selectable marker, and genomic DNA fragments (Harrington et al. 1997). This produced stably transmitted microchromosomes with functional centromeres containing chromosome 7 α-satellite sequences and typical kinetochore components, but only in extremely rare frequency. Subsequently, a yeast
artificial chromosome (YAC) carrying chromosome 21 α-satellite arrays was retrofitted with telomeres by recombination and introduced into human cells, which yields a much enhanced frequency of microchromosomes containing newly assembled functional centromeres, but only when the YAC contained α-I type satellite arrays, a frequent site marked by the CENP-B, a centromeric DNA-binding protein (Ikeno et al. 1998). This suggested a role for CENP-B in centromere biogenesis, an idea previously dismissed by demonstration that CENP-B null mice are viable and fertile with only mild phenotypic effects on gonad size (Hudson et al. 1998). By engineering two α-I satellite arrays such that they differed only by the presence of functional CENP-B boxes, CENP-B was shown to be necessary for de novo centromere formation, but it functions efficiently only in the context of α-satellite DNA. Since heterochromatin formation is a highly cooperative process driven by a series of mutually reinforcing reactions (Richards and Elgin 2002), it is likely that CENP-B functions in mediating centromeric chromatin modification, as demonstrated by the three CENP-B homologues in *Schizosaccharomyces pombe* (Nakagawa et al. 2002). Given the biochemical feature and complexity of kinetochore in mammalian cells and the availability of genomic database, proteomic analysis will shed light on molecular composition of kinetochore in mammalian cells. Precise delineation of centromere assembly machinery must await molecular dissection of kinetochore composition and reconstitution in vitro using sequentially added purified components.

Condensin, a complex of five proteins, is a major constituent of mitotic chromosome, and it appears to play an essential role for chromosome condensation (Hirano 2000). Phosphorylation of two condensin subunits by Cdk1-cyclin B stimulates their entry into the nucleus and association with chromatin as cells enter prophase. Condensin was discovered in a search for proteins that associated with mitotic chromosome using *Xenopus* egg extracts. Depletion of condensin caused defects in mitotic chromosome assembly, suggesting the importance of condensing in centromere formation and/or maturation. While the molecular function of condensin remains to be established, it seems that condensin induces a superhelical twist into DNA molecules (Hirano 2000). Recent studies reveal that the onset of condensation correlates to the phosphorylation of histone H1 by Cdk1-cyclin B and H3 by aurora-B. It has been hypothesized that local chromatin unfolding due to histone phosphorylation allows binding of other factors such as condensing proteins, which condense the chromosome.

To identify the molecular machinery underlying centromeric cohesion, Watanabe and his colleagues using fission yeast meiosis as a
model system (Kitajima et al. 2004). Since meiosis produces haploid germ cells after a pair of specialized nuclear divisions, those authors reasoned that sister chromatids must segregate together during the first meiotic division (meiosis I), which requires that sister chromatid cohesion persists at centromeres. Using genetic assay, they indeed identified Sgo1 (shugoshin), a protector of the centromeric cohesin Rec8 in fission yeast. Moreover, they identified Sgo2, a paralogue of shugoshin in fission yeast, which is required for faithful mitotic chromosome segregation. Both Sgo1 and Sgo2 are well conserved in eukaryotic cells. Interestingly, localization of Sgo1 and Sgo2 at centromeres requires the kinase Bub1, identifying shugoshin as a crucial target for the kinetochore function of Bub1, which validates the role of Bub1 in maintaining chromosome stability during mitosis as mutation of Bub1 cause chromosome instability in human colorectal cell lines (Cahill et al. 1998; see below). These findings provide insights into the evolution of meiosis and kinetochore regulation during mitosis and meiosis. It would be interesting to delineate whether and how Bub1 kinase regulates Sgo1 and Sgo2 in metaphase I and II, respectively, and the precise function of Sgo2 in mitosis.

3.5 Kinetochores Power Chromosome Movements in Mitosis

It has been a century-long challenge and pursuit to understand how cells divide and faithfully transmit chromosomes at each cell division. In a typical somatic cell cycle, chromosomes in prophase initiate condensation, then, upon disassembly of the nuclear envelope and the interphase microtubule array, the fully compacted chromosomes spill into what was the cytoplasm to produce prometaphase. As a nascent mitotic spindle assembles, a dynamic process of repetitive search by unstable microtubules ensues for capture of chromosomes at their kinetochores (Fig. 1; modified). Initial capture is frequently by binding of one kinetochore of a duplicated chromosome pair along the side of a spindle microtubule (e.g., Yao et al. 1997), allowing rapid (up to 1 µm/s) poleward translocation along that microtubule powered by a minus-end directed, kinetochore bound microtubule motor, almost certainly cytoplasmic dynein (Rieder and Alexander 1990). This is followed by attachment of additional microtubules (up to 50 in humans [Rieder 1981] or 7 at mouse kinetochores [Putkey et al. 2002]), motor action at attachment sites, and oscillatory movements coupled to continued growth and shrinkage of those kinetochore bound microtubules.
Subsequent capture by the unattached kinetochore of a microtubule from the opposite spindle pole produces bi-orientation and congression to the cell center in a rapid, discontinuous series of movements, again mediated by kinetochore motors (e.g., Yao et al. 1997). The presence at kinetochores of active plus and minus end motors has been demonstrated, with net direction of movement redirectable in vitro by mitotic phosphorylation (Hyman and Mitchison 1991). In mammals, known functioning kinetochore motors include the kinesin family member CENP-E (e.g., Yao et al. 2000) and cytoplasmic dynein.

Fig. 1. Ultrastructural analyses of kinetochore-associated motor CENP-E and its binding protein TTK, a spindle checkpoint kinase. I CENP-E is a major component of corona fiber of the kinetochore. At metaphase CENP-E extends from the kinetochore outer plate at least 50 nm along spindle microtubules. A Low-magnification view of a metaphase HeLa cell with chromosomes aligned at the equator between the spindle poles (asterisks). B Magnified view of one metaphase chromosome showing that spindle microtubules indeed associate with a kinetochore with a trilaminar structure. Five 10-nm gold particles are located to each sister kinetochore (arrows). Five additional gold particles just to the right of the boxed area represent CENP-E associated with the kinetochore of another chromosome (more clearly seen in adjacent sections). C Higher magnification view shows that CENP-E is located to the corona fibers of the kinetochore. op, outer plate; ip, inner plate; cf, corona fibers. Bars: A 2 μm; B 170 nm; C 70 nm.
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Fig. 1. (continued) II TTK is localized at the developing kinetochore and spindle poles of mitotic HeLa cells. Visualization of TTK is achieved by 10-nm gold conjugated goat antirabbit IgG. a Low magnification of a prometaphase HeLa cells. Asterisks mark the two spindle poles of the bipolar spindle. An apparent mono-oriented chromosome is boxed, and higher magnification is shown in b. b Magnified view of shows that 10 nm particles represent the TTK localization at the kinetochore (arrow) and spindle microtubules adjacent to centrosome (arrowhead). c Enlarged view of b shows a bi-oriented chromosome with 10 nm gold particles deposit onto the kinetochore (arrows). d Higher magnification view of boxed area of b shows that 10 nm gold particles decorate the outer surface of the kinetochore interfaced with spindle microtubule (arrow). In addition, six 10-nm gold particles also mark an apparent protein complex associated with spindle microtubules (arrowhead). e Magnified view of a centrosome shows that a microtubule-associated protein complex decorated by eight 10-nm gold particles (arrow) traffics toward the pole. Several 10-nm gold particles (arrowhead) are also deposited to the pole 300–400 nm away from the centrioles. Bars: a 2 μm; b 500 nm; c–e 150 nm

3.6 Motors in Chromosome Congression

A long perplexing question is how chromosome congression is precisely achieved. Laser ablation to disconnect the two chromatids or destroy either kinetochore argues that the major force behind the chromosome motility is generated by the leading kinetochore (Khodjakov and Rieder 1996), i.e., the one whose bound microtubules are shortening and whose motors are moving toward the microtubule minus ends. Microinjection of p50 dynamin and a dynein antibody, two soluble dynein inhibitors, disrupts the alignment of kinetochores at metaphase (Sharp et al. 2000). Mutations in the tethers (Rough deal [Rod] and Zeste white 10 [ZW10]) that link dynein to kinetochores attenuate the rate of poleward chromosome movement (Savoian et al. 2000), implicating dynein as a likely primary motor for congression. Depletion of CENP-E using antisense oligonucleotide effect-ed a block of chromosomal alignment at the metaphase plate, indicating the essential role of CENP-E in chromosome congression and/or metaphase alignment (e.g., Yao et al. 2000). Indeed, chromosome alignment is precluded by disruption of CENP-E function in vitro using Xenopus egg extracts (Wood et al. 1997) and antibody injection in mammalian cells (Chan et al. 1997). A final class of kinetochore motor-like component, ex-
emplified by the Kin I subgroup of the kinesin family (MCAK in mammals [Wordeman and Mitchison 1995] and XKCM1 in Xenopus [Walczak et al., 1996]), is a microtubule depolymerase [Desai et al. 1999]. Two most recent studies demonstrate that Aurora B phosphorylates and regulates MCAK activity both in vitro and in vivo (Andrews et al. 2004; Lan et al. 2004). Interestingly, Aurora B kinase activity was required for localization of MCAK to kinetochores, but not to spindle poles. Protein phosphorylation of serine 196 by Aurora B in the neck region of MCAK inhibited its microtubule depolymerization activity, perhaps due to conformational changes. Using phospho-S196 specific antibody, this phosphorylation was shown at centromeres and anaphase spindle midzones in vivo. Addition of phospho-S196 antibodies to cultured cells or in vitro assembled spindle caused defects in chromosome positioning and/or segregation. It remains interesting to see whether Aurora B participates mitotic checkpoint by directing MCAK to depolymerize incorrectly oriented kinetochore microtubules, which allows removing mitotic brake.

Chromosomes also experience forces exerted along the chromosomes arms (polar wind) as a result of spindle microtubule interaction with plus-end directed microtubule motors bound to chromatin (chromokinesins). The first identified, Drosophila Nod, is required for proper alignment of meiotic chromosomes that have not undergone recombination (Afshar et al. 1995). Immunodepleting another (Kid) prevents normal chromosome alignment (Antonio et al. 2000; Funabiki and Murray, 2000), while antibody-induced inhibition of human Kid blocks chromosome oscillations, with chromosome arms atypically extending toward spindle poles during congression (Levesque and Compton 2001). It is generally believed that Kids provide an additional layer of surveillance for chromosome segregation.

Despite greater progress made toward molecular composition of kinetochores, the precise mechanics responsible for chromosome segregation remains elusive. Early biophysical and microscopic studies pointed to the role of dynein in chromosome congression. The ~50 pN force generated during chromosome movement (Alexander and Rieder 1991; Nicklas 1983, 1988) is equivalent to several dynein molecules per kinetochore (~6 pN per motor molecule [Shingyoji et al. 1998]). Since kinetochore is a multimotor protein complex, it is difficult to assign an individual motor for the complex chromosome movement based on evidence available. However, it would be possible and exciting to ascertain individual motor's function in different aspects of chromosome movements using inducible dominant negative mutation and real-time microscopic image.
3.7 Mitotic Complexities: Protein Subcomplexes and Circuitry

In close mitosis system, for example budding yeast, where the intranuclear spindle forms prior to centromere duplication during S phase, nonmotor microtubule-associated proteins appear to provide chief force for microtubule capture, while dynein plays a role in spindle positioning and perhaps maintaining, but not chromosome movement (Yeh et al. 1995). The Dam1p complex interacts physically with central kinetochore proteins of both the Ctf3 while Ndc80 complexes and binds to microtubules directly in vitro (Cheeseman et al. 2001), consistent with a direct role in mediating kinetochore–microtubule attachments. These authors further pursue the molecular regulation of kinetochore–microtubule by identifying Ipl1p targets using a combination of tandem affinity chromatography and mass spectrometry (Cheeseman et al. 2002b). Among 28 proteins recovered by this assay, ten of these phosphorylation proteins are targeted directly by Ipl1p. Their systematic mutational analysis of the Ipl1p phosphorylation sites demonstrated that the essential microtubule binding protein Dam1p is a key Ipl1p target for regulating kinetochore–microtubule attachments in vivo.

A group of microtubule plus-end binding proteins (e.g., the EB1 protein family and CLIP-170 in mammals) might also be involved in mediating interactions between microtubules and kinetochores. The yeast EB1 homologue BIM1 localizes to the plus ends of cytoplasmic microtubules and increases dynamic instability (Timauer et al. 1999) while removal of EB1 nails down the spindle microtubule stability (Rogers et al. 2002). During congression, EB1 is found at the ends of kinetochore of a chromatid pair which are bound to microtubules that are growing, but not at those that are shrinking (Timauer et al. 2002). EB1 interacts (Su et al. 1995) with the human adenomatous polyposis coli (APC) tumor suppressor protein. Adenomatous polyposis coli also binds to and stabilizes microtubules (Zumbrunn et al. 2001), localizes to the ends of microtubules embedded in kinetochores, forms a complex with mitotic checkpoint proteins Bub1 and Bub3, and is a substrate for both of the Bub1 and BubR1 kinases in vitro (Kaplan et al. 2001). As truncations of the APC gene are found in most colorectal tumors, Fodd and colleagues (2001) reasoned that mutations in APC might be responsible for chromosomal instability and examined mouse ES cells homozygous for Min (multiple intestinal neoplasia) or Apc1638T alleles. They show that Apc mutant ES cells display extensive chromosome and spindle aberrations, providing genetic evidence for a role of APC in chromosome segregation. Consistent with this, APC accumu-
lates at the kinetochore during mitosis. *Apc* mutant cells form mitotic spindles with an abundance of microtubules that inefficiently connect with kinetochores. This phenotype is recapitulated by the induced expression of a 253-amino-acid carboxy-terminal fragment of APC in microsatellite unstable colorectal cancer cells. One possible explanation for these observations is that EB1/APC complex may be one of the nonmotor linker(s) that connect microtubule attachment and the spindle checkpoint signaling machinery on the kinetochore.

Although there has been a recent explosion in the identification of budding yeast kinetochore components, the physical interactions that underlie kinetochore function remain obscure, in particular to kinetochore of mammals, where open mitosis governs cell duplication. To better understand how mammalian cell kinetochores attach to microtubules and how this attachment is regulated, we sought to characterize the kinetochore composition of human cells using a combination of affinity chromatography and mass spectrometric analyses. Potential protein–protein interactions among kinetochore proteins were assessed using yeast hybrid screen and epitope tagging. Our current analyses provide a draft map for kinetochore protein subcomplexes at the kinetochore of human cells (Fig. 2). Further examination of these interactions in living cells will delineate protein–protein interaction circuitry at the kinetochore and consolidate these interactions into mitotic regulation, and elucidate how aberrant protein–protein interactions cause chromosome instability phenotype.

### 3.8 Anaphase Movements Driven by Motors and Flux

During anaphase identical sister chromatids separate and move towards opposite poles of the mitotic spindle. In the mitotic spindle, kinetochore microtubules have their plus ends embedded in the kinetochore and their minus ends at the spindle pole, revealed by photobleaching fluorescent microtubules (Gorbsky et al. 1987) and confirmed using fluorescence photoactivation of tubulin assembled into kinetochore bound microtubules (Mitchison and Salmon 1992), flux represents continuous addition of tubulin subunits at kinetochores, coupled to disassembly at the poles driven by plus-end directed, pole bound microtubule motors presumably pulling on kinetochore microtubules and sliding them poleward. The major mechanism for chromosome movement in anaphase in vertebrate somatic cells is motor-powered kinetochore movement coupled to microtubule disassembly at the kinetochore (e.g., Mitchison and Salmon 1992; Walters et al. 1996). Early studies show that poleward flux makes a relatively minor
Fig. 2. Schematic drawing of hypothetical composition of kinetochore subcomplexes. Information presented here is derived from published studies and our own unpublished observation (pull-down assay, yeast genetic screen, and computational analyses).

collection with chromosome-to-pole movement three to eight times faster than flux. Yeast appears to lack microtubule depolymerization at poles and poleward microtubule flux during anaphase (Mallavarapu et al.)
In Xenopus egg extracts, however, anaphase A movement occurs at rates similar to poleward spindle microtubule flux (Desai et al. 1998), consistent with flux as the predominant mechanism. Elsewhere, the situation is controversial: fluorescent speckle microscopy has been used to claim a dominant role for flux (Maddox et al. 2002) in syncytial Drosophila embryos, while other efforts have found that dynein inhibitors disrupt chromatid-to-pole movement during anaphase A (Savoian et al. 2000; Sharp et al. 2000).

Rogers and his colleagues have recently shown (2004) that two functionally distinct microtubule-destabilizing KinI kinesin enzymes are responsible for anaphase chromosome motion in Drosophila. One of them, KLP59C, is required to depolymerize kinetochore microtubules at their kinetochore-associated plus ends, thereby contributing to chromatid motility through a Pac-Man-based mechanism. The other, KLP10A, is required to depolymerize microtubules at their pole-associated minus ends, thereby moving chromatids by means of poleward flux. One question remain unanswered is how microtubules maintain their attachment to kinetochores and spindle poles while undergoing polymerization and depolymerization. Also, do other motile motors at the kinetochore (e.g., dynein and CENP-E) contribute to the process?

### 3.9 Signaling Cascade for Spatial-temporal Control of Chromosome Segregation

To assure accurate segregation, the mitotic checkpoint (also known as the spindle assembly checkpoint) acts to block entry into anaphase until both kinetochores of every duplicated chromatid pair have attached correctly to spindle microtubules. It has been proposed that unattached kinetochores and/or those not under microtubule-exerted tension are the central signaling elements that produce a "wait anaphase" signal. By filming mitoses, it was initially found that anaphase ensues about 20 min after the last kinetochore attaches to the spindle (Rieder et al. 1994) and that by repeated detachment of a meiotic chromosome from a spindle by manipulation with a microneedle delayed anaphase indefinitely (Li and Nicklas 1995). That it was an unattached kinetochore that was responsible came from the seminal demonstration that laser ablation of the last unattached kinetochore produces anaphase onset within about 15 min (Rieder et al. 1995). A kinetochore-dependent wait anaphase signal is also suggested in budding yeast: blocking centromere assembly (by destruction of the Cbf3 component
Ndc10) eliminates mitotic delay in the presence of microtubule assembly inhibitors (Gardner et al. 2001).

Yeast genetic screen initially identified seven components of the mitotic checkpoint, Mad1–Mad3 (Mitotic arrest defective) (Li and Murray 1991), Bub1–Bub3 (Budding uninhibited by benomyl) (Hoyt et al. 1991), and Mps1, a kinase that is also essential for spindle pole body duplication (Weiss and Winey 1996). There are vertebrate homologues of all of these except Bub2. As initially demonstrated for Mad2 (Chen et al. 1996; Li and Benezra 1996), other mitotic checkpoint proteins have now been identified to bind to and act at unattached kinetochores including Mad1 (Chen et al. 1998), Bub1 (Taylor and McKeon 1997), Bub3 (Taylor et al. 1998), BubR1 (the mammalian Mad3) (Taylor et al. 1998), and Mps1 (Abrieu et al. 2001). In mammals, there are several additional components involving mitotic checkpoint. For example, loss of the kinetochore-associated microtubule motor protein CENP-E, a binding partner of BubR1, the checkpoint cannot be established or maintained Xenopus egg extracts (Abrieu et al. 2000), HeLa cells (Yao et al. 2000), in mice (Putkey et al. 2002). Most recently, Cleveland and his colleagues show that single unattached kinetochores due to depletion of CENP-E cannot block entry into anaphase but result in aneuploidy in 25% of divisions in primary mouse fibroblasts in vitro and in 95% of regenerating hepatocytes of knock-out mice. Significantly, they further demonstrate that CENP-E binds to and directly stimulates the kinase activity of purified BubR1 in vitro. Thus, CENP-E is required for enhancing recruitment of its binding partner BubR1 to each unattached kinetochore and for stimulating BubR1 kinase activity, implicating it as an essential amplifier of a basal mitotic checkpoint signal. It would be interesting to further establish the inter-relationship of BubR1–CENP-E and provide structural view as how CENP-E association enhances BubR1 activity in vitro and whether this holds in living cells.

Although the nature of the direct molecular interaction(s) between checkpoint proteins and kinetochores and the inter-relationship among checkpoint proteins has not been determined, the basic scheme of the signaling cascade has been established. Mad2 is recruited to unattached kinetochores in a complex with Mad1 (Chen et al. 1998). BubR1 and Bub1, both kinases, are required for generation and then rapid release from kinetochores of one or more inhibitors of Cdc20 (Fizzy in flies [Dawson et al. 1993], p55 in mammals [Kallio et al. 1998] or Slp1p in fission yeast [Kim et al. 1998]). Interestingly, recent studies show that TTK (human homologue of yeast MPS1) interacts with CENP-E, a mitotic kinesin located to corona fiber of kinetochore (Zhang et al. 2002). To elucidate the molecular function of TTK1, Dou and his colleagues (2003) conducted un-
trastructural studies and revealed its dynamic distribution profile. TTK is present at the nuclear pore adjacent complex of interphase HeLa cells. Upon nuclear envelope fragmentation, TTK targets to the outermost region of the developing kinetochores of mono-orient chromosome as well as to spindle poles. After stable attachment, throughout chromosome congression. TTK is a constituent of the corona fibers, extending up to 90 nm away from the kinetochore outer plate. Upon metaphase alignment, TTK departs from the kinetochore and migrates toward the centrosomes. Taken together, this evidence strongly supports a model in which TTK functions in spindle checkpoint signaling cascades at both kinetochore and centrosome, which were supported by several independent studies (Liu et al. 2003; Fisk et al. 2003).

In an attempt to elucidate the signaling cascade of Mad1, Lou and his colleagues (2004) carried out yeast genetic screen to identify Nek2A as a potential binding partner. Chromosome segregation in mitosis is orchestrated by protein kinase signaling cascades. Although it was established that Nek2A is a centrosome-associated protein kinase. Like Mad1, Nek2A is localized to HeLa cell kinetochore of mitotic cells. Significantly, elimination of Nek2A by siRNA does not arrest cells in mitosis but causes aberrant premature chromosome segregation. Nek2A is required for Mad2 but not Mad1, Bub1 and Hec1 to associate with kinetochores. Moreover, loss of Nek2A impairs mitotic checkpoint signaling in response to spindle damage by nocodazole, which effected mitotic escape and led to generation of cells with multiple nuclei. These studies demonstrate that Nek2A is a kinetochore-associated protein kinase essential for faithful chromosome segregation. The dynamic distribution of Nek2A at kinetochore and centrosome provide another example of checkpoint molecular dynamics linking between kinetochore and centrosome.

The recruitment of Mad2 at kinetochores has often been taken as a measure of ongoing checkpoint signal generation (and release). However, inhibition of ZW10/Rod yields an inactive checkpoint despite prominent Mad2 binding at kinetochores (Chan et al. 2000). Diminution of Hec1, the homologue of yeast Ndc80, on the other hand, yields a chronically activated checkpoint with no Mad2 bound to kinetochores (Martin-Lluesma et al. 2002). Thus, it is now abundantly clear that the steady-state level of Mad2 bound to kinetochores is not a faithful reporter for checkpoint activation or inactivation.

Another candidate for spindle checkpoint reporters is BubR1, which directly binds Cdc20 and APC/C elements Cdc16, Cdc27, and APC7 (Chan et al. 1999; Wu et al. 2000) and by doing so can block Cdc20 activation of APC/C for mitotic substrates. The inhibitory activity has been argued to be BubR1 alone without a contribution of Mad2 (Tang et al.
2001) or in a complex (named MCC) that apparently contains stoichiometric amounts of BubR1, Bub3, Mad2, and Cdc20 (Sudakin et al. 2001). An equivalent quaternary complex containing Mad3 (the yeast BubR1), Bub3, Mad2, and Cdc20 has also been observed in budding yeast (Fraschini et al. 2001; Hardwick et al. 2000). Further, the much higher APC/C inhibitory activity in vitro of MCC (>3000-fold more potent than tetrameric Mad2) (Sudakin et al. 2001) would make this complex an attractive candidate for a diffusible inhibitory signal were it not that it (and its yeast counterpart) is formed in a kinetochore-independent manner (Sudakin et al. 2001; Fraschini et al. 2001). This has led to the suggestion that the kinetochore contribution may modify APC/C itself to increase its affinity for MCC (Sudakin et al. 2001). However, equally plausible are models with spatial signal amplification, for example, MCC gradient near kinetochores, thereby producing a high concentration of an inhibitor to saturate available Cdc20. In fact, such intracellular gradients can be evaluated using a FRET assay.

3.10 Silencing the Checkpoint Signal: Attachment and Tension

There is a continuing debate as to whether the mitotic checkpoint is silenced by microtubule attachment (Rieder et al. 1995) or by the tension exerted between bioriented kinetochore pairs after attachment (Li and Nicklas 1995) and whether activities of subsets of the known components are selectively silenced by one or the other (Skoufias et al. 2001; Zhou et al. 2002).

McIntosh initially proposed the tension model under which the mechanical tension generated by poleward-directed forces acting on both kinetochores of a bioriented chromatid pair turns off checkpoint signaling (McIntosh 1991). Compelling evidence for a tension requirement initially emerged using praying mantis spermatocytes in meiosis I, which have a Y chromosome and two genetically different X chromosomes. Although the Y is supposed to pair with both X chromosomes, occasionally it pairs only with one, thus yielding a mono-oriented X. When this occurs, anaphase onset is delayed by up to 9 hr but can be triggered to initiate almost immediately by application of mechanical tension applied across the mono-oriented kinetochore by use of a force-calibrated microneedle (Li and Nicklas 1995).

This is a general finding in meiosis: eliminating tension between homologous chromosomes by preventing recombination delayed anaphase onset in yeast, presumably from checkpoint activation. This de-
lay was eliminated by genetically allowing sister kinetochores to inappropriately separate during meiosis I, thereby allowing each homologue to biorient in the absence of recombination and restore tension across the sisters (Shonn et al. 2000). Similarly, in cells that enter mitosis without a prior round of DNA replication, the unreplicated chromatids attach to spindle microtubules, but no tension can be developed and the mitotic checkpoint is chronically activated (Stern and Murray 2001).

However, in the initial demonstration that kinetochores are the signaling elements for the checkpoint, destruction of the last unattached kinetochore eliminated checkpoint signaling despite lack of tension on the kinetochore of the sister chromatid (Rieder et al. 1995). Similarly, in maize, satisfying the checkpoint requires tension in meiosis, but in mitosis, attachment is sufficient (Yu et al. 1999). Furthermore, in PtK1 cells, loss of Mad2 recruitment to kinetochores depends on microtubule attachment, not tension (Waters et al. 1998). Similarly, very low doses of the microtubule inhibitor vinblastine produce loss of tension and kinetochore bound Mad2 and a ‘checkpoint’ arrest that, unlike the case after inhibition of spindle assembly (Gorbsky et al. 1998), is insensitive to inactivation with Mad2 antibody injection (Skoufias et al. 2001).

This has led to the proposal that there are two branches of checkpoint signaling and silencing. One, in which the signal released is an activated, inhibitory form of Mad2, is silenced by microtubule attachment, while the other, presumably involving conversion of BubR1 into a Cdc20 inhibitor, is silenced by tension. The challenge ahead is to distinguish these two possibilities and illustrate the checkpoint signaling regulation.

3.11 Mitotic Checkpoint Defects Promote Aneuploidy and Tumorigenesis

The mitotic checkpoint in budding yeast, where the intranuclear spindle forms quickly after centromeres are duplicated in S, is a real checkpoint activated only to arrest mitosis in the relatively rare instances when attachment is delayed. However, in most other higher organisms such as mammals, it becomes an essential cell cycle control pathway activated at every mitosis/meiosis immediately upon nuclear envelope disassembly. Loss of Mad2, Bub3, or Rael in mice is lethal early, with cells accumulating mitotic errors and undergoing apoptosis by embryonic day 5 or 6 (Dobles et al. 2000; Kalitsis et al. 2000; Babu et al. 2003). Similarly, in Drosophila, loss of Bub1 causes chromosome mis-segregation and lethality (Basu et al. 1999). Microinjection of antibodies to Mad2 yields premature
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anaphase onset and chromosome mis-segregation (Gorbsky et al. 1998). Haplo-insufficiency in Mad2 provokes late onset, self-limiting lung tumors (Michel et al. 2001). Elimination of Nek2A affects insufficient targeting and/or retention of Mad2 to the kinetochore and causes premature chromosome segregation in cultured cells, which displays chromosome instability phenotype (Lou et al. 2004). Reduction in Bub3 or Rael generates aneuploidy in vitro and a sharply increased susceptibility to chemically induced tumorigenesis (Babu et al. 2003), suggesting the checkpoint control is essential for mitotic regulation. While the primary mission of the checkpoint is to prevent such errors in chromosome segregation, a hallmark of human tumor progression (Hartwell and Kastan 1994), it would be of great interests to illustrate how perturbation of kinetochore protein–protein interaction effects chromosome instability.

3.12 The Epigenetic Regulation of Centromere Structure and Function

Human centromere formation involves the assembly of the mitotic kinetochore onto chromosomal locations that contain the interphase prekinetochore. In budding yeast, centromere DNA alone can nucleate centromere formation de novo, centromeres in *S. pombe* depend strongly, and those of metazoan cells primarily, on epigenetic factors rather than DNA sequence for activity. Three lines of evidence support this statement. First, centromere sequences are evolving at an unusually high rate, coevolving with their essential partner CENP-A, and show no obvious sequence conservation that links divergent species or even different chromosomes in the case of *Drosophila* (Henikoff et al. 2001). Second, centromere DNA sequences by themselves are unable to specify centromere function: stable dicentric chromosomes have been found in which one centromere has been silenced with no obvious rearrangement of centromere DNA (Sullivan and Willard 1998). Third, acquisition of centromere function has been found on certain rearranged chromosomes lacking an endogenous centromere.

Syndromes of disordered "chromatin remodeling" are unique in medicine because they arise from a general deregulation of DNA transcription caused by mutations in genes encoding enzymes which mediate changes in chromatin structure. Chromatin is the packaged form of DNA in the eukaryotic cell. It consists almost entirely of repeating units, called nucleosomes, in which short segments of DNA are wrapped tightly around a disk-like structure comprising two subunits of each of the histone proteins H2A, H2B, H3 and H4. Histone proteins are covalently modified by a
number of different adducts (i.e., acetylation and phosphorylation) that regulate the tightness of the DNA–histone interactions. Mutations in genes encoding enzymes that mediate chromatin structure can result in a loss of proper regulation of chromatin structure, which in turn can result in deregulation of gene transcription and inappropriate protein expression. Significantly, there are several diseases whose defects in chromatin remodeling are tied to chromosome instability (e.g., Rett syndrome (RS); immunodeficiency-centromeric instability–facial anomalies syndrome). Molecular elucidation of these regulatory elements will not only provide insights into our understanding of centromere assembly but shed light on pathogenesis of the aforementioned diseases.

The tumor suppressor gene RASSF1A is frequently silenced in lung cancer and other sporadic tumors as a result of hypermethylation of a CpG island in its promoter. However, the precise mechanism by which RASSF1A functions in cell cycle regulation and tumor suppression has remained unknown. Song and his colleague show that RASSF1A regulates the stability of mitotic cyclins and the timing of mitotic progression by interacting with Cdc20, an activator of the APC/C, resulting in the inhibition of APC/C activity. Although RASSF1A does not contribute to either the Mad2-dependent spindle assembly checkpoint or the function of Emi1, depletion of RASSF1A by RNA interference accelerated the mitotic cyclin degradation and mitotic progression as a result of premature APC activation. It also caused a cell division defect characterized by centrosome abnormalities and multipolar spindles. Thus, these findings link epigenetic regulation of spindle protein activity to mitotic progression.

3.13 Conclusions

It becomes increasingly clear that the centromere and its associated kinetochore are much more than simple attachment sites for spindle microtubules. Mammalian centromeres are much more complex than initially imagined, representing repeated assemblies of the simple, one-nucleosome centromeres in budding yeast. Central to genetic inheritance, in almost all examples known they are also epigenetically determined and regulated. In addition, kinetochore regulators include active components in microtubule capture, stabilization, and in powering chromosome movements essential for faithful segregation. More than that, they are the signaling elements for controlling cell cycle advance through mitosis by as yet identified protein–protein interaction circuitry.
3.14 Future Directions

In the next few years, we can expect proteomic and biochemical analyses to generate a complete list of kinetochore components and subcomplexes, encompassing perhaps 150–180 proteins, and interaction circuitry about the modes and roles of these proteins in orchestrating sophisticated chromosome dynamics in dividing cells. Real-time studies will also provide a framework of kinetochore architecture and dynamics at various stages of cell cycle. Given the spatial-temporal trait of protein–protein interactions, we believe that functional studies will require the development of high-throughput image robotics for studying real-time protein dynamics in live-cell chromosome movements in wild-type and “mutant” cells with high precision. It would be exciting and challenging task to consolidate the protein–protein interaction informatics from different genetic and biochemical background into a model for kinetochore dynamics in mitotic chromosome segregation. It will be also necessary to develop new in vitro assays to reconstitute and evaluate the interactions of kinetochore complexes to mimic kinetochore dynamics of mitosis. A combination of proteomic, genetic, biochemical and biophotonic analyses with computational modeling will enable us to consolidate the mechanistic view of heredity envisioned more than 100 years ago.

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