

Chapter 2

The Basics of Chromosome Segregation

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2.1 Scope of this Chapter

During cell division, chromosomes carrying thousands of genes are correctly transmitted to daughter cells via a motile apparatus named the mitotic spindle (a schematic outline of the cell (division) cycle is shown in Fig. 2.1). In post-replicative (post S phase) cells, chromosomes comprise duplicated sister chromatids. In the cell cycle stage called mitotic metaphase, all sister chromatid pairs are aligned and bi-oriented to the spindle apparatus. In anaphase, all sister chromatids separate in concert and segregate oppositely along the anaphase spindle (towards the spindle poles/centrosomes) into the two daughter cells (Fig. 2.2). The once-in-a-cell-cycle occurrence of the chromosome-segregation process suggests that this event should be studied with respect to cell cycle control (reviewed in Morgan 2006).

Our current understanding of chromosome segregation was greatly advanced by the discovery of cyclin-dependent protein kinases (CDKs; Doree and Hunt 2002). CDKs promote cell-cycle transitions and are the main engines of the cell cycle (Sánchez and Dynlacht 2005). Mitotic CDKs are inactivated when bound cyclin is degraded by the 26S proteasome through the ubiquitin pathway (Hershko 2005) thereby promoting the transition from metaphase to anaphase. Simultaneously, securin, a key inhibitor of separase, the enzyme whose action triggers chromosome segregation, is degraded by the same mechanism (Yanagida 2000, 2005; Fig. 2.1). Cell cycle control and chromosome segregation are temporally coordinated using the same destruction motif. In meiosis, the sexual reproduction cycle, there are two types of metaphase. In metaphase I, homologous chromosomes are associated, and in anaphase I they segregate without sister chromatid separation (Clarke and Orr-Weaver 2006).

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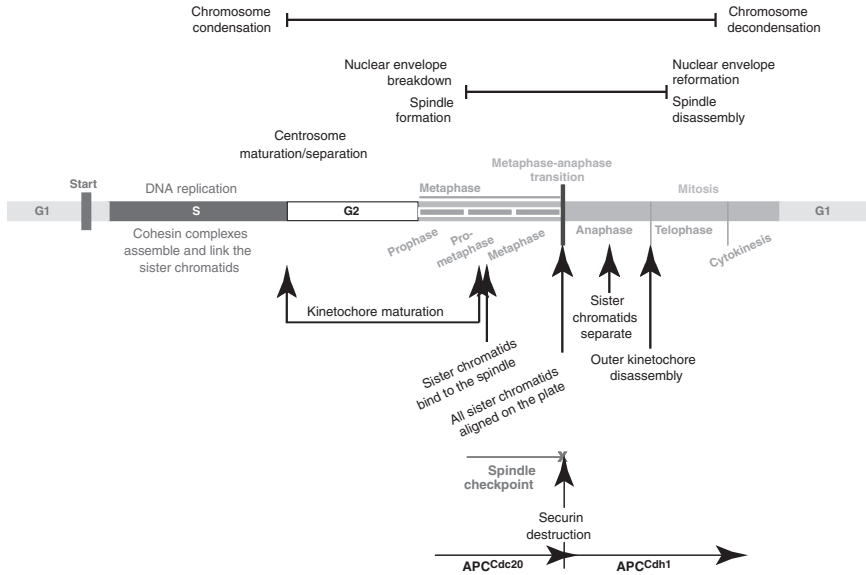


Fig. 2.1 The mitotic cell division, kinetochore and chromosome cycles. Schematic outline of the cell cycle stages (G1: blue, S-phase: red, G2: white, mitosis: green), and of the state/activity of chromosomes, the spindle, kinetochores and sister chromatids (shown in black). The activities of the mitotic spindle checkpoint and the anaphase-promoting complex (APC) are shown in pink and brown, respectively. In metazoan, kinetochores undergo a cycle of maturation and partial disassembly. In the yeasts, kinetochores are tethered to microtubules throughout the cell cycle and shortly detach from the microtubule tips during replication of the centromeric regions in S phase. (See Color Insert)

In metaphase II and anaphase II, chromosomes are segregated, as they are in somatic cells (Morgan 2006).

The chromosome segregation process is elaborate, with checkpoints and error-correction mechanisms, as the transmission of chromosomes requires high fidelity. Errors in chromosome segregation cause aneuploidy, cancer, and various diseases (Epstein 2007, Stallings 2007, Weaver and Cleveland 2007). Of the ~5,000 genes in simple eukaryotes, ~500 genes are presumed to be required for proper chromosome segregation. Two eukaryotic microbes, the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, have proven to be excellent model species for studying chromosome segregation. These organisms are evolutionarily distant, and thus mechanisms that are conserved between them are generally also conserved in vertebrates. Studies on the chromosome behavior of worm, fly, and vertebrates are largely consistent with the notions developed in yeast studies (Oegema et al., 2001, Herzig et al., 2002). In sum, firm evidence suggests that the basic mechanisms underlying eukaryotic chromosome segregation are the same in all eukaryotes as many of the genes involved in the process have been evolutionary conserved (Yanagida 2005).

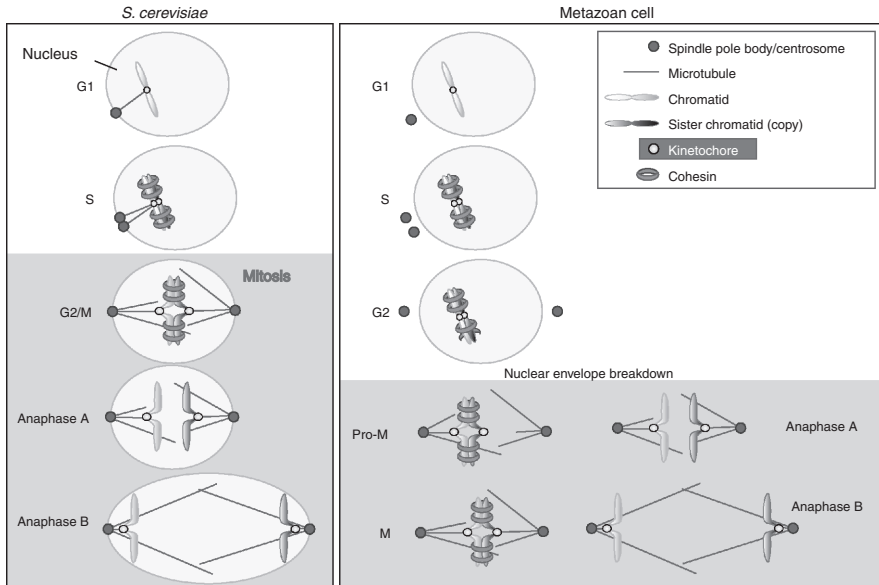


Fig. 2.2 The eukaryotic chromosome cycle. Schematic representation of chromosome replication and segregation in the budding yeast *Saccharomyces cerevisiae* and in a metazoan cell. The processes involved are highly conserved. A notable difference is that the yeast cell cycle is “closed” (occurs inside the nucleus), whereas in higher eukaryotes the cell cycle is “open.” More specifically, chromosomes in higher cells are duplicated inside the nucleus but can only establish contact with the spindle apparatus following the breakdown of the nuclear membrane. Chromosome segregation then takes place in the cytoplasm.

This chapter focuses on the basic mechanisms that underlie the transmission of DNA from one generation to the next, and ensure that this process occurs with the highest fidelity. The main factors involved in chromosome segregation will be discussed and special attention will be given to players whose roles in mitosis are not discussed elsewhere in this book (e.g., condensin, cohesin).

2.2 Gene Identification in Chromosome Segregation is Incomplete

Classic genetic analyses that led to the phenotypic identification of many genes (and processes) involved in chromosome segregation have been recently extended using comprehensive, high-throughput methods. In higher eukaryotes, defects in chromosome segregation are examined using small inhibitory dsRNA oligonucleotides that knockdown individual gene products (versus mutations of particular genes obtained by mutagenesis). The identification of whole suites of genes required for chromosome segregation, however, is far from complete. The redundancy of gene function is the principal reason for our

inability to obtain mutants in genes mediating chromosome segregation. Indeed, certain mutant or RNA interference (RNAi) phenotypes are weak or not apparent when the genes under investigation are functionally redundant. For example, histone genes are multigene systems such that histone mutants are hard to obtain by mutagenizing the wild-type strain. The *S. pombe* histone H2B gene, however, is encoded by a single gene (*htb1*⁺) and conditional-lethal mutants of *htb1* are easily obtained (Maruyama et al., 2006). Systematic approaches for constructing multiple mutants or for applying multiple RNAi will be necessary to substantially increase the number of genes implicated in chromosome segregation. Thus, our current assumption that ~500 genes are required for high-fidelity chromosome segregation likely is an underestimation.

2.3 Basic Versus Quality Control Mechanisms

The physical principals underlying accurate DNA replication are based on the double helical structure of DNA with complementary base pairing. DNA-replicating polymerases and additional proofreading enzyme activities (e.g., DNA-damage repairing proteins) ensure correct replication of DNA. Although there are currently no known central physical principles underlying chromosome segregation, distinctions exist between the basic and quality-control mechanisms of chromosome segregation. The basic mechanisms may comprise a relatively small number of proteins and complexes. However, if these were to function on their own, then the fidelity of segregation would be relatively low. The high-fidelity segregation observed in cells may require the combination of several quality control mechanisms.

Quality-control genes are involved in numerous cell activities, including the cell cycle, signal transduction, macromolecule and metabolite trafficking, organelle formation and segregation, metabolic turnover, ubiquitylation, phosphorylation, sumoylation, and proteolysis. In addition, the spindle with the spindle poles (mitotic centrosomes) and the kinetochore contain a large number of proteins, many of whose functions are not well understood at the molecular level. Some of these proteins may be required for quality control rather than for basic segregation activities, particularly in higher eukaryotes. We predict that many gene functions dispensable for cell division and viability may serve to improve chromosome segregation. Hence, chromosomal abnormalities and human diseases may be due more to abnormalities of quality control genes than to the loss of the basic genes, as the latter would likely lead to cell and embryonic death.

Essentially nothing is currently known about the metabolic regulation of chromosome segregation. Mechanisms that produce cellular energy may be part of the chromosome segregation process and may be crucial for mitotic progression (Pederson 2003). In *S. pombe*, reducing the concentration of glucose in the medium immediately inhibits mitosis. Cell growth in *S. pombe* (e.g., increase in cell length) is abruptly blocked during the G2-mitosis

transition suggesting the presence of a nutrient switch from growth to mitosis. While the defect in energy metabolism may block the entire program of cell cycle progression, including chromosome segregation, mitosis itself requires significant amounts of energy. Entry into and exit from mitosis require phosphorylation and ubiquitin-mediated protein degradation, respectively, processes that consume ATP. Chromosome segregation requires the assembly and dynamic movements of the spindle apparatus, which requires a number of proteins with ATPase and GTPase activities (Morgan 2006).

2.4 Gene Nomenclature for Chromosome Segregation

The nomenclature used for genes involved in chromosome segregation is a serious problem in communicating results obtained in different organisms. Many genes are initially identified through the use of mutants, antibodies, or amino acid sequences of purified proteins and their molecular functions are not known. Thus, many of the gene names do not give functional clues and are difficult to remember. Although similar proteins exist in other organisms, researchers tend to use their own organism's nomenclature, as it is often unclear whether these genes are functionally equivalent to similar genes in other organisms. Indeed, genes with analogous sequences but distinct functions are not uncommon. It is therefore very difficult for researchers in other fields and for newcomers to the field to understand the functions of a particular gene by reading the literature.

A number of protein complexes essential for chromosome segregation, however, have been given common names across organisms. The presence of multiple subunits that all share sequence similarity in different organisms is convincing evidence of the functional similarity of these complexes, such as condensin, cohesin, anaphase-promoting complex (APC/C), and mitotic checkpoint complex (MCC). The use of a common nomenclature for these complexes promotes integrated studies. For example, condensin is a hetero-pentameric complex required for mitotic chromosome architecture. It consists of two subunits belonging to the structural maintenance of chromosome (SMC) ATPase protein family, and three non-SMC components (reviewed in Nasmyth and Haering 2005, Belmont 2006, Hirano 2006). Frog condensin contains XCAP-C (SMC4) and XCAP-E (SMC2), two heterodimeric coiled-coil SMCs and three non-SMC proteins: XCAP-H, -G, and -D2. In *S. cerevisiae*, the dimeric Smc2 and Smc4 associate with three non-SMC subunits, Ycg1, Ycs4, and Brn1. Similarly, two SMC proteins of *S. pombe*, Cut3 and Cut14, form a heterodimer and bind to three non-SMC subunits, Cnd1, Cnd2, and Cnd3 (Nasmyth and Haering 2005, Belmont 2006, Hirano 2006). The sequences of each of these sets of five subunits are similar from fungi to human, indicating that they are functionally conserved. Although different names remain for individual subunits, they are less important than those of complexes.

Complexes required for chromosome segregation are often multifunctional. Condensin (see above) is also required for interphase activities, such as

DNA-damage repair (Heale et al., 2006). Cohesin, the multiprotein complex that holds sister chromatids together following DNA replication, is also required for DNA-damage repair (Ström et al., 2007, Unal et al., 2007, Ball and Yokomori 2008) and developmental transcriptional regulation (Dorsett et al., 2005, Dorsett 2007, Gullerova and Proudfoot 2008, Wendt et al., 2008). The name, usually based on the initially discovered function, might only partially represent the functions mediated by the complex and could be misleading. Therefore, biologists and geneticists should use caution when naming a complex according to its originally discovered function.

The anaphase-promoting complex/cyclosome (APC/C) has an instructive history with regard to the naming. The APC/C was discovered as a complex and called a cyclosome (Sudakin et al., 1995), as it is essential for the degradation of mitotic cyclin *in vitro*. This same complex was also called the APC, as it was defined as an anaphase-promoting complex (King et al., 1995). The APC/C, which contains ~15 subunits (Passmore et al., 2005), is the E3 ubiquitin ligase that poly-ubiquitylates mitotic cyclin and securin for degradation in a destruction-box (DB)-dependent manner (reviewed in Sullivan and Morgan 2007). APC/C activation is inhibited by the spindle assembly checkpoint (also called the spindle checkpoint or mitotic checkpoint; see Chapter 11). Poly-ubiquitylated cyclin and securin are rapidly degraded by the 26S proteasome, leading to the activation of separase, the cleavage of cohesin, the separation of the sister chromatids, and the onset of anaphase (Morgan 2006, Fig. 2.1). Because the abbreviation APC also refers to the frequently cited tumor suppressor protein *adenomatous polyposis coli*, it is currently recommended that the abbreviation APC/C be used to avoid confusion. This distinction has become particularly necessary as the tumor suppressor APC interacts with the plus ends of the microtubules and is implicated in the spindle checkpoint (Draviam et al., 2006). While the APC/C regulates the exit of mitosis in dividing cells (Sullivan and Morgan 2007), it is also abundant in non-dividing cells such as neurons and muscles (van Roessel et al., 2004, Zarnescu and Moses 2004). The APC/C seems to have a postmitotic role at *Drosophila* neuromuscular synapses: in neurons, the APC/C controls synaptic size, and in muscles, it regulates synaptic transmission (van Roessel et al., 2004). The roles of the APC/C in non-dividing differentiated cells are elusive, but clearly different from its role in mitotic progression and exit. Thus, a new name, particularly one based on a single function, could cause misconceptions concerning the roles of these complexes.

2.5 Basic Mutant Phenotypes

Defects in chromosome segregation are variable but classifiable. Mutations in the subunits of the same complexes often produce similar phenotypes. For example, more than 10 APC/C subunits are essential for mitotic progression and their mutants inhibit degradation of mitotic cyclin and securin, leading to

similar mitotically arrested phenotypes. A typical mutant phenotype is the arrest or delay that occurs with condensed chromosomes and continued activation of mitotic CDK. Poly-ubiquitylation mediated proteolysis by the APC/C and the 26S proteasome is necessary to inactivate mitotic CDK (Sullivan and Morgan 2007). The spindle checkpoint inhibits mitotic progression through APC/C inactivation (Hwang et al., 1998, Kim et al., 1998; see below). A failure to form the proper spindle apparatus or kinetochore structure activates the spindle checkpoint (Rieder et al., 1994, see Chapter 11). Therefore, many genes implicated in the structure and function of the mitotic spindle, the kinetochore, APC/C, and the 26S proteasome, produce similar mitotically arrested or delayed phenotypes. Only detailed phenotypic analyses can reveal molecular differences underlying these similar phenotypes (e.g., aberrantly formed kinetochore, kinetochores misattaching the chromatid pair to the spindle, non-dynamic microtubules, etc).

Another principal mitotic phenotype is the *cut* (*cell untimely torn*) phenotype in which chromosome segregation is physically impaired but cytoplasmic events, such as cytokinesis, are not. Mutants exhibiting such apparently uncoupled phenotypes occur in various genes (reviewed in Yanagida 1998). In *S. pombe*, for example, *cut* mutants are defective in Top2/DNA topoisomerase II, *cut1/separase*, *cut2/securin*, *cut3* and *cut14/condensin* subunits, etc. Events following anaphase appear to take place in which only a portion of the chromosomes, such as the centromere/kinetochore, is separated and segregated and moves to the spindle poles, while the bulk of the chromosomes remains associated and stuck near the spindle equator. The mitotic checkpoint is not activated so that mitotic cell cycle progression is not inhibited in these mutant cells (Yanagida 1998). The reason for this failure is unclear.

Another characteristic mitotic phenotype represents unequal chromosome segregation, which can be visualized by the sizes of the nuclear chromatin or the actual number of chromosomes in the daughter nuclei. In *S. pombe*, almost all mutants defective in essential centromere-binding proteins that are bound to the central core domain of centromeres (see below) show the phenotype of large and small daughter nuclei (Takahashi et al., 1994). Whether these mutants (many of them are called *mis* mutants) are defective in spindle checkpoint control remains to be determined. There are other missegregation phenotypes, such as aneuploidy or ploidy changes. Changes in chromosome number are important diagnostic features of cancer and other diseases, and are due to a wide variety of causes.

2.6 Simple Analogies of the Chromosome Segregation Process

In order to better understand the complex chemo-mechanical processes that underlie the chromosome segregation process, simple but useful analogies can be proposed.

2.6.1 Cooking Analogy

The period of chromosome segregation in anaphase is short (in the order of minutes and comparable to the “meal time”). However, many steps must occur prior to chromosome segregation. The term “chromosome cooking” refers to the long and careful preparatory steps (in human cells in the order of hours) that culminate in anaphase. The chromosome-cooking phase is under the control of the cell cycle. As in cooking, significant changes occur in the chromosome structures during the preparatory period. Cellular structures implicated in chromosome segregation either form (e.g., maturation of the kinetochore) or are greatly altered (e.g., spindle dynamics) or even disappear (e.g., cleavage/removal of cohesin complexes).

2.6.2 Festival Analogy

Chromosome segregation and, in general, mitosis resemble a “festival.” Indeed, festivals typically occur on a seasonal basis and do not last very long. Similarly, chromosome segregation and mitosis are the shortest of the four cell cycle stages: G1, G2, S, and M (G, S, and M stand for gap, DNA synthesis, and mitosis, respectively; Fig. 2.1). The spindle apparatus and the kinetochores appear only during mitosis for the movement of the chromosomes (Fig. 2.1). A great deal of energy is used to push mitosis toward the festival of segregation and completion of cell division. Of note, like human festival participants and supporters, many of the mitotic molecules do not live just for mitotic events, but have additional functions during the non-festive interphase. For example, DNA topoisomerase II (Top2) is required during interphase (G1, G2, and S) for replication and transcription as a housekeeping enzyme (reviewed in Larsen et al., 2003). It becomes essential during mitosis for the final condensation and chromosome segregation, perhaps because of its ATP-driven enzymatic ability of catenation–decatenation (reviewed in Bates and Maxwell 2007).

The cohesin subunit Rad21/Scc1/Mcd1 was initially determined to be involved in DNA damage repair (Birkenbihl and Subramani 1992) but is also cleaved to allow sister chromatid separation in anaphase (Tomonaga et al., 2000, Sonoda et al., 2001).

Condensin and separase–securin are required for DNA damage repair (Nagao et al., 2004, Heale et al., 2006) as well as for proper chromosome segregation. These mitotic complexes also have roles in the ordinary interphase stages of the cell cycle.

2.6.3 Freight Train Analogy

Chromosomes are like freight trains carrying thousands of genes: the centromere-associated kinetochore acts as the “locomotive,” a powered vehicle. The

shortest freight train may carry only a single gene but nevertheless needs a locomotive. Indeed, *S. cerevisiae* minichromosomes may contain only a short piece of centromeric DNA, the replication origin, and a single marker gene (Clarke and Carbon 1980). *S. pombe* minichromosomes, even those containing a single marker gene, require a much longer centromeric DNA that contains replication origins for proper segregation (Niwa et al., 1987). In the freight train analogy, the locomotive vehicle is far more important than the cargo vehicles so that enigmas in chromosome segregation may be more efficiently understood through investigating the segregation mechanisms of sister kinetochores. This analogy is oversimplified and may not address interesting problems such as the segregation of telomeres, rDNAs, and the non-coding DNA sequences in the arm regions of chromosomes. It is a useful analogy, however, for understanding the problems of chromosome segregation. Centromeres/kinetochores are very complicated molecular machines consisting of hundreds of proteins. The process of sister kinetochore segregation itself raises many difficult questions, nearly equivalent to the number of questions raised regarding the control of chromosome segregation.

2.6.4 Glue-Cohesion Analogy

Postreplicative chromosome DNAs in eukaryotic cells are held together by “glue” until metaphase. The presence of glue on the chromosomes is a marker of postreplicative chromosomes and should not be present on the chromosomes in anaphase. In postreplicative (G2 stage) haploid cells, there is one set of glued (paired) sister chromatids, whereas in prereplicative (G1 stage) diploid cells, there are two sets of non-glued single duplex chromosomes. The latter cells, which contain the same amount of chromosomal DNA, can be distinguished by the presence of the glue. The currently accepted terminology is cohesion rather than glue, and the cohesin complex, which is hypothesized to form a large ring that embraces the sister chromatids, is thought to be the sole link present between sister chromatids (reviewed in Nasmyth and Haering 2005). Other glues, however, may be present, and may include proteins (chromatid binding through protein–protein interaction) or RNA that exists between duplicated DNAs or any topologic bond of DNA, or may even consist of non-covalent macromolecular bonds.

2.6.5 Cleansing Analogy

In interphase cells, numerous proteins and RNAs are associated with the chromosomes and participate in various DNA metabolic activities, such as replication, transcription, recombination, damage repair, etc. In mitosis, prior to anaphase, such components bound to interphase chromosomes are thought

to be removed as they may inhibit the segregation mechanism or reduce the fidelity of segregation. This removal process summons an analogy to cleansing. If sister chromatids are catenated, anaphase cannot proceed properly until the obstacles are removed. The interphase components, which remain present in metaphase, must be cleaned up or degraded in anaphase. Certain proteins essential for segregation may actually function in cleansing processes rather than in chromatid separation and segregation. Little is known about the molecular cleansing machinery. Top2 is a potential candidate as it may abolish catenation and topological entanglement prior to anaphase. Polo kinase or separase may also have a role in removing cohesin from the chromosomes. Many components might be involved in such presumed cleansing functions or be the targets of the cleansing molecular machines, as so many components are bound to interphase chromosomes.

2.6.6 *Chromosome–Corpse Analogy*

In this analogy, the spindle apparatus is dynamic and lively, but mitotic chromosomes are condensed and gene expression is thought to be minimal in mitosis. Investigators first interested in mitosis half-century ago studied how the spindle moved and worked, and thought the chromosomes looked like corpses carried by the spindle. This analogy of mitotic chromosomes as corpses was proposed long before the discovery of many mitotic components that dynamically regulate the structure and behavior of chromosomes in mitosis (see p. 212, Mazia 1961).

In reality, *de novo* RNA transcription during mitosis has been scarcely investigated that so little is known about how the transcriptional machinery acts on the chromosomes from early to late mitosis.

2.7 Centromere and Kinetochore

The centromere and kinetochore that assembles on it orchestrate chromosome segregation in eukaryotes. The use of the artificial minichromosome first constructed by Clarke and Carbon (1980) was immensely helpful for analyzing the segregation mechanism and led to identification of the functional centromeric DNA sequence (Chapters 1 and 3) and kinetochore proteins (Chapter 6). The size of the minimal functional centromere, determined using the *S. cerevisiae* circular minichromosome, is rather small (~125 bp). A large number of centromere/kinetochore proteins interact with this small centromere region to ensure correct segregation (Chapter 6). The linear minichromosomes of *S. cerevisiae* (Murray and Szostak 1983), which are faithfully transmitted during cell division, attach to the telomeric sequences at their ends and are much longer than the circular minichromosomes.

The centromere DNAs of *S. pombe* are 300–1,000 times longer than those of *S. cerevisiae* (Takahashi et al., 1992). Fly and higher eukaryotic centromeres are longer (up to 7 Mb) and more complex (Chapter 3). The centromeric regions of *S. pombe*, identified based on the mitotic stability of artificial linear and circular minichromosomes (Clarke et al., 1986, Nakaseko et al., 1986) have two functionally distinct domains. The central domains (*ctr* and *imr*) are roughly constant in length (~15 kb), while the outer heterochromatic repeat region (*otr*) that surrounds the central domain varies in length (20~100 kb). The central domains and a small portion of the outer region are necessary for correct mitotic segregation of minichromosomes (Chapter 3). Known kinetochore proteins such as CENP-A, Mis6, Mis12, Mis18, and Mad2 (Hayashi et al., 2004) are bound to the central regions of the *S. pombe* centromere, while the *otr* regions contain heterochromatin and small inhibitory RNA transcribed in the *otr* outer region (Volpe et al., 2002). Cohesin and aurora kinase are also enriched in the *otr* region. The *otr*-like heterochromatin is also present in the mating-type and telomeric regions (Grewal and Klar 1997), and is thus not specific to the centromere (Chapter 3).

2.8 Basics of Centromere-Kinetochore Proteins

The highly diverged centromeric DNA sequences of different organisms first seemed to eliminate any chance of obtaining a unified concept for the centromere (Chapters 1 and 7). When the first proteins binding to the centromere were identified (e.g., CENP-A/Cse4/CID/Cnp1, CENP-C/Mif2), their evolutionary conservation from fungi to humans was a pleasant surprise (Chapter 7). Centromeric DNA together with a number of centromere-binding proteins constitute the centromeric chromatin, which underlies the kinetochore, the proteinaceous structure that attaches each sister centromere to the spindle microtubules. Kinetochore formation is initiated by CENP-A/Cse4/CID/Cmnp1 and occurs in a complex, epigenetically regulated (Chapter 10) and hierarchical manner from more than 100 proteins (Chapter 6). Kinetochore components are classified based either on their location within the kinetochore (e.g., inner centromere-bound proteins versus outer microtubule-binding components), their activity at the kinetochore (structural versus enzymatic components; e.g., epigenetic factors) or on their stability (non-dynamic or dynamic, e.g., spindle checkpoint proteins). Human kinetochores start to assemble (mature) on centromeric chromatin at the initiation of prometaphase, following nuclear envelope breakdown (Fig. 6.1, Chapter 6). The formation of the kinetochore and its subsequent binding to the spindle is monitored by the spindle checkpoint (Chapter 11). Following binding to the spindle and mediation of sister chromatid segregation (via outer kinetochore motor proteins and spindle depolymerization) the outer kinetochore components leave the centromere. In many cases, these components become degraded following epigenetic marking (e.g., ubiquitylation, Chapter 10).

2.9 Generation of Force Required to Segregate Separated Chromatids Towards the Poles

How the spindle generates the force required to bring the separated chromatids toward the opposing spindle poles in anaphase and whether that force is regulated are important questions (also see Chapter 8). Although there are, as yet, no firm answers to these questions, some experimental results allow for speculation. Legendary experiments using grasshopper spermatocytes (Nicklas 1983) indicated that the force needed for pulling chromosomes is in the range of 50 pN per kinetochore microtubule, though the precise value remains to be determined (Rieder et al., 1986). Factors that depolymerize microtubules may generate the spindle force. Is chromosome a heavy cargo for a microtubule to carry? The average weight of a single *S. pombe* chromosome is roughly 30 femtograms equivalent to $\sim 50 T_{\text{even}}$ bacteriophages or 10,000 ribosome particles. The weight of the average human chromosome is 1 picogram, ~ 30 times greater than that of a fission yeast chromosome. As 2–3 kinetochore microtubules are bound to each kinetochore in *S. pombe* and 20–30 are bound to each kinetochore in mammalian cells, the chromosome mass pulled by a single kinetochore microtubule in fission yeast and human cells may be around 15–50 femtograms. Nicklas (1983) stated that the spindle apparatus and kinetochore microtubules are powerful enough to generate 10^4 times the force required for free movement of the chromosomes, and showed that a single microtubule can move a newt chromosome, which is much bigger than a human chromosome (Nicklas and Kubai 1985). The force required for the anaphase spindle to separate the chromosomes is thus rather small relative to the maximal force that the spindle can produce. The maximal spindle force may never be used in normal chromosome segregation. Alternatively, such force may be required very briefly to overcome the built-in sister chromatid-linking in metaphase and even anaphase chromosomes (e.g., Baumann et al., 2007). There may be force-sensitive association and dissociation reactions between sister chromatids or sister kinetochores.

The pulling and/or pushing forces (the latter is also called the polar ejection force (Rieder et al., 1986, Salmon 1989) or -more poetically- the polar wind (Carpenter 1991)) of the spindle are created in prometaphase to metaphase spindles through the dynamic properties of microtubules and/or motors. There is a common belief among investigators that the forces are generated primarily by microtubule dynamics that occur in a spontaneous as well as in a regulated manner, and that these forces are spatially and temporally controlled directly by microtubule interacting proteins (Chapter 8). Little is known, however, about how these forces are exerted on kinetochores and microtubules, which produce their sequential changes during mitotic progression under the surveillance of the spindle checkpoint.

Our main focus here lies on the regulated behavior of spindle and kinetochore microtubules and whether these properties abruptly change in anaphase.

Again, there is little data relating to this question. The rate of chromosome movement during anaphase A in *S. pombe* and HeLa cells is $\sim 1\text{--}3\ \mu\text{m}/\text{min}$ ($16\text{--}50\ \text{nm}/\text{s}$). In *S. pombe*, the path of anaphase chromosome movement is $\sim 1\ \mu\text{m}$; thus, anaphase A is completed within 1 min. In HeLa cells, anaphase A chromosomes move $\sim 5\ \mu\text{m}$ within 2 min ($2.5\ \mu\text{m}/\text{min}$, $\sim 40\ \text{nm}/\text{s}$). Thus, the duration of anaphase A is rather brief. Anaphase A movement of chromosomes in living yeast and HeLa cells is smooth and steady, although when examined at high resolution in PtK1 cells the movements remain oscillatory, even during anaphase (Salmon, E.D., personal communication). Note, however, that kinetochore microtubule shortening is 10 times slower than the maximum shortening speed of cytoplasmic or isolated microtubules, which is $30\ \mu\text{m}/\text{min}$ (Mitchison and Kirschner 1984).

Microtubule- and kinetochore-interacting proteins that critically regulate the dynamic properties to shorten kinetochore and spindle microtubules during metaphase–anaphase progression have not yet been established. Certain microtubule-binding proteins such as Dis1/Stu2/XMAP215/ch-TOG are microtubule polymerizers (Brouhard et al., 2008), while others including kinesin-13 family members MCAK/XKCM1, depolymerize microtubules (Walczak et al., 1996, Helenius et al., 2006). The activity of the latter is suppressed by tau and, to a lesser extent, by XMAP215 (Noetzel et al., 2005). In anaphase A, only kinetochore microtubules are shortened, whereas the pole-to-pole microtubules that are interdigitated at the middle of the spindle begin to elongate in anaphase B. In addition to these two classes of microtubules, the aster microtubules radiating from the spindle poles and their plus ends associating with the cellular cortex determine the positions of the spindle apparatus within the cell (for details, see Chapter 8).

2.10 Key Players in Chromosome Segregation

A few of the key players in chromosome segregation, including CENP-A, cohesin, condensin, components that are required for mitotic checkpoint and anaphase, are described below.

2.10.1 CENP-A and Its Recruitment Factors

The presence of centromere-specific histone CENP-A/CID/Cse4/Cnp1, which replaces canonical histone H3 at centromeric nucleosomes (Earnshaw and Rothfield 1985, Valdivia and Brinkley 1985, Palmer et al., 1991), suggests that the centromere chromatin is specific, but its nature is not well understood. In *S. pombe*, the centromere region containing Cnp1 (hereafter referred to as CENP-A) has a smeared micrococcal digestion pattern, while the surrounding heterochromatic regions show regular digestion patterns, but this difference is not due solely

to the presence of CENP-A (Takahashi et al., 2000). As CENP-A and its family members are present in all functional centromeres, CENP-A is an appropriate marker for centromere identity in eukaryotic organisms (Chapter 7). Understanding how CENP-A is recruited to the centromere and how CENP-A containing nucleosomes form are important questions (for details, see Chapter 6).

A surprising finding that was recently reported is that a centromere-specific nucleosome core in *S. cerevisiae* lacks the canonical histone H2A-H2B dimer, which is replaced by Scm3, a non-histone protein that interacts with *S. cerevisiae* CENP-A/Cse4 (Mizuguchi et al., 2007). Scm3, conserved across fungi, stoichiometrically binds to H4-CENP-A *in vitro*, and forms the centromere specific “nucleosome” containing CENP-A/Cse4. These results suggest that centromeric histones of *S. cerevisiae* do not form octamer nucleosomes, but instead non-histone Scm3 may serve to assemble and maintain CENP-A-H4 at the centromeres in *S. cerevisiae*. Whether the same type of centromeric chromatin forms in other organisms remains to be determined. Scm3 may also be a recruitment factor rather than the replacement of histone H2A and H2B (for details, see Chapter 6).

The recruitment of CENP-A to the centromere requires multiple protein complexes (for a discussion in detail, see Chapter 6). In *S. pombe*, Mis16 and Mis18 are the most upstream factors for centromeric loading of CENP-A, whereas Mis6 (and its partner proteins) is located downstream (Hayashi et al., 2004). In their absence, CENP-A is not recruited to the centromere. In human cells, homologues of the above CENP-A-loading proteins exist. RbAp46/48, similar to Mis16, may be a chaperone for histone H4 and is implicated in chromatin assembly through histone H4 acetylation (Fujita et al., 2007). In the greater part of the cell cycle, centromeric acetylation of H4 is minimal, while acetylation may occur during mitosis. A striking property of Mis18 is its cell cycle-dependent localization at the centromere: Mis18 is absent from mitotic kinetochores, but is located transiently at the centromere from telophase to early G1. This localization is essential for subsequent recruitment of CENP-A (Hayashi et al., 2004). The Mis18–Mis16/RbAp complex seems to “prime” the pre-existing centromeric chromatin in the telophase-G1 phase for the loading of newly made CENP-A (Fujita et al., 2007). Priming is related to protein acetylation, as trichostatin A, an inhibitor of histone deacetylases, suppresses the loss of recruitment factors. Once primed, newly synthesized CENP-A is recruited onto centromeres with flexible timing prior to mitosis. The histone acetylation–deacetylation cycle seems to strongly affect CENP-A loading (Fujita et al., 2007, see also Chapter 10).

2.10.2 Cohesin for Cohesion, DNA-Break Repair, and Transcriptional Regulation

Cohesin, which consists of two SMC (Smc1 and Smc3) ATPases and two non-SMC subunits (reviewed in Nasmyth 2005a, Nasmyth and Haering 2005), is

required for the formation of sister chromatid cohesion, mitotic and meiotic chromosome segregation, and the repair of double-strand breaks (Guacci et al., 1997, Ciosk et al., 1998, Uhlmann et al., 1999). In addition, cohesin is involved in developmental transcriptional regulation (Dorsett et al., 2005, Dorsett 2007, Gullerova and Proudfoot 2008, Wendt et al., 2008). In the normal cell division cell cycle, cohesin is associated with the chromosome during replication, supposedly to hold sister chromatids together, and removed from the chromosome during mitosis. The cohesin complex is highly dynamic, as it is associated with postreplicative chromosomes by a single DNA break to create the cohesion necessary for DNA repair (Ström et al., 2007, Unal et al., 2007, Ball and Yokomori 2008).

Cohesin is removed from the chromosome in a two-step manner. The first step is phosphorylation by polo-like kinase and the second step is proteolysis by separase. Polo is the principal kinase that regulates cohesin, but other kinases are also involved under different physiologic conditions. In the absence of cohesin, sister chromatids separate prematurely, the mitotic checkpoint is activated, and chromosome segregation is greatly delayed and abnormal (reviewed in Nasmyth and Haering 2005, Belmont 2006, Hirano 2006). Loading of cohesin to the chromosome is intimately linked to DNA replication and requires evolutionarily conserved recruitment factors, including deposition factors (adherins) Scc2 and Scc4, helicases, DNA polymerases and the Ctf7–RFC–PCNA complex, which pairs cohesin complexes together, thereby linking the sister chromatids (reviewed in Skibbens 2005). A defect in the human gene responsible for loading cohesin (the Scc2 homologue named NIPBL) causes the Cornelia de Lange syndrome (Krantz et al., 2004, Tonkin et al., 2004, reviewed in Dorsett 2004), a congenital disorder associated with delays in physical development and mental retardation, among other abnormalities.

2.10.3 Condensin for Condensation, Segregation, and DNA-Damage Repair

Condensin consists of two SMC ATPases (Smc2, Smc4) and three non-SMC subunits (reviewed in Nasmyth and Haering 2005, Belmont 2006, Hirano 2006). None of the subunits is shared with cohesin. Condensin can induce positive supercoiling of DNA in the presence of ATP (Kimura and Hirano 1997). The heterodimeric SMC complex has the ability to re-anneal single strand DNAs in the absence of ATP (Sutani and Yanagida 1997). Although the requirement for condensin in both mitotic condensation and chromosome segregation is well established (Hirano and Mitchison 1994, Hudson et al., 2003), the actual molecular role of condensin is not understood (Gassmann et al., 2004, Hirano 2005). The interphase chromosome is already 1,000 to 2,000-fold compacted, and further mitotic condensation of the chromosome is only ~5-fold. As condensin does not seem to have any role in interphase chromosome compaction,

the actual role of condensin in chromosome condensation *per se* should not be overestimated. Instead, condensin may function during prepreparation of the sister chromatids in the metaphase chromosome. Condensin appears to increase the integrity of individual sister chromatids in metaphase chromosomes to maintain the “state of being whole” against the pulling force exerted on the kinetochores (Oliveira et al., 2005, Gerlich et al., 2006). This integrity increase may result from being “condensed” in mitosis. If condensin is absent, the integrity is lost so that only the kinetochore portions may be pulled out and moved towards the opposite poles, leaving the bulk of the remaining sister chromatids in the middle of the nuclei. In vertebrates, there are two kinds of condensin complexes (I and II), and condensin II is enriched at kinetochores (Ono et al., 2003). These two condensins have distinct tasks for increasing the integrity of the kinetochores and the arms of chromosomes in vertebrate cells (reviewed in Hirano 2005, Belmont 2006).

In *S. pombe*, the amount of condensin associated with the interphase chromosome is low. Condensin is needed for DNA damage repair at the intra S-phase checkpoint (Aono et al., 2002). Condensin is also required for DNA repair in vertebrates (Heale et al., 2006). Upon entry into mitosis, the accumulation of condensin at mitotic chromosomes occurs in multiple steps, and this accumulation at kinetochores and rDNAs appears to be necessary for normal chromosome segregation (Nakazawa et al., 2008). First, condensin is mobilized into the nucleus through Cdc2 phosphorylation of an SMC subunit with the aid of importin α . In addition, Ark1/Aurora B kinase and Survivin/Bir1/Cut17, a subunit of the Aurora B kinase chromosomal passenger complex, are required for condensin to locate on the mitotic chromosome docking sites. Further, certain centromere- and rDNA-binding proteins are necessary to dock condensin at both the kinetochores and rDNAs. Several proteins that are necessary to form the chromatin architecture of the kinetochores and rDNAs are required for condensin to accumulate specifically at these sites. The mechanism of condensin accumulation at the kinetochores may be conserved, as human condensin II fails to accumulate at kinetochores in cells treated with RNAi for the same CENP (Nakazawa et al., 2008).

2.10.4 Components Required for the Mitotic Checkpoint

Proper attachment/alignment of the mitotic chromosomes and a correct timing of sister chromatid separation are assured by the mitotic checkpoint (spindle checkpoint; reviewed in Musacchio and Salmon 2007, Chapter 11). Checkpoint proteins called mitotic arrest deficient proteins (MADs) and budding uninhibited by benzimidazole proteins (BUBs) are necessary for establishing the mitotic checkpoint. They are conserved from fungi to human, and are temporarily recruited to the kinetochores. The failure of checkpoint proteins to locate at kinetochores leads to chromosome misalignment and/or premature sister

chromatid separation, thus raising the question of how checkpoint proteins control the progression of mitosis. Checkpoint proteins are linked to the APC/C complex by Cdc20/Slp1/Fizzy, which directly interacts with APC/C. Mad2 and Mad3/BubR1 are physically bound to Cdc20 and are thought to inactivate APC/C through this interaction, suggesting that the activation of APC/C requires the inactivation of Mad2 (Musacchio and Salmon 2007, Chapter 11).

Currently there are two mechanistic models of the spindle checkpoint. First, the two-state model is based on biochemical identification of the *in vitro* inhibitor of mitotic APC/C, which was purified from HeLa cells, and is called the mitotic checkpoint complex (MCC). The MCC contains BubR1 (similar to Mad3 in fungi), Bub3, Cdc20, and Mad2 in near equal stoichiometry (Sudakin et al., 2001, Sudakin and Yen 2004, Chapter 11). The MCC inhibition of APC/C is 3,000-fold greater than that of recombinant Mad2, which also inhibits APC/C *in vitro*. MCC is present and unexpectedly active in interphase cells. Only APC/C isolated from mitotic cells, however, is sensitive to inhibition by MCC. The majority of APC/C in mitotic lysates is associated with the MCC, which likely contributes to the lag in ubiquitin ligase activity. The preformed interphase pool of MCC may allow for the rapid inhibition of APC/C when cells enter mitosis. This inhibition by MCC is independent of the kinetochores, and the proposed role of unaligned kinetochores is to sensitize APC/C to MCC-mediated inhibition. In the original model, the MCC is stable across the cell cycle, but may dissociate upon exit from the checkpoint, suggesting that MCC is dynamically regulated (Braunstein et al., 2007).

Second, an alternative model proposes that Mad1-Mad2 at kinetochores acts as a template to change the conformation of another molecule of Mad2 (discussed in Nasmyth 2005b). This templated change in Mad2 conformation is proposed as a mechanism for the amplification of the “wait anaphase” signal (DeAntoni et al., 2005). In this model, Mad2 acts to sequester Cdc20 to halt anaphase. Mad2 is recruited to the kinetochores in prometaphase, and is activated with the help of Mad1 and binds to Cdc20. Mad2 has two conformers, a closed form that is bound to its kinetochore receptor Mad1 or its target in the checkpoint Cdc20 and an open form that is not bound to these ligands. A closed conformer of Mad2 constitutively bound to Mad1 is the kinetochore receptor for cytosolic open Mad2. In this model, the interaction between the open and closed forms of Mad2 is essential to sustain the spindle assembly checkpoint. The closed Mad2 bound to Mad1 is proposed to represent a template for the conversion from the open Mad2 to closed Mad2 bound to Cdc20 (discussed in Mapelli and Musacchio 2007). This predicts a mechanism for cytosolic propagation of the spindle checkpoint signal away from the kinetochores. The cause of the loss of this ability of Mad2 is p31Comet (Cmt2; Habu et al., 2002), which is required for exit from the spindle checkpoint. p31Comet associates with Mad2 and blocks Mad2 activation through structural mimicry (Mapelli et al., 2007, Yang et al., 2007).

What is the role of kinetochores in the mitotic checkpoint? The HEC1/Ndc80 and Blinkin/Spc105/Spc7/KNL-1 kinetochore complexes are involved

in spindle checkpoint activity (Martin-Lluesma et al., 2002, Gillett et al., 2004, Kiyomitsu et al., 2007). The HEC1 complex has not yet been shown to directly interact with components of the spindle checkpoint. Recently, Blinkin was revealed to interact via two-hybrid analysis with BubR1 and Bub1 (Kiyomitsu et al., 2007). The depletion of both HEC1 and Blinkin abolishes the kinetochore localization of the spindle checkpoint, causing premature entry into anaphase and chromosome misalignment (discussed in Chapter 11). How both kinetochore complexes dovetail with the two-state and/or the template model of the checkpoint remains to be determined.

2.10.5 Components Required for Anaphase

The current prevailing concept for the mechanism triggering anaphase onset is the silencing of the mitotic checkpoint, which allows for the activation of APC/C, which destroys mitotic cyclin and securin to promote anaphase (reviewed in Sullivan and Morgan 2007). The loss of securin or the inactivation of CDK activates separase, which removes cohesin from chromosome. In human cells, CDK1/cyclin B phosphorylation inhibits separase activation. CDK1/cyclin B1 and securin interact with separase in a mutually exclusive manner (Gorr et al., 2005). In addition, type 2A phosphatase (PP2A) containing the B56 subunit is bound to separase and regulates the timing of separase activation (Holland et al., 2007). Securin is a chaperone and inhibitor of separase, and a part of its structure may mimic the Rad21 cleavage site recognized by separase. The chaperonic role of securin/Cut2 may be regulated by phosphorylation under different stress conditions. High stress induces more phosphorylation and stabilizes securin. In *S. cerevisiae*, Pds1/securin is the target of the Rad53/Chk2 damage checkpoint protein to arrest the cell cycle for repair (Cohen-Fix and Koshland 1997).

In addition to its role as a site-specific protease that cleaves Rad21/Mcd1, separase seems to have various other roles. In *S. pombe*, separase/Cut1 is required for normal duplication and separation of the spindle pole bodies (SPBs, equivalent to the centrosome). It binds to interphase microtubules, metaphase and extending anaphase mitotic spindles, and to the centrosome-equivalent of SPBs (Nakamura et al., 2002). The protease-dead carboxy fragment can strongly affect SPB positioning. Separase is also required for duplication of the centrosome (Tsou and Stearns 2006). A single centrosome that contains a pair of centrioles duplicates once before mitosis. During duplication, new centrioles form orthogonally to existing ones and remain engaged with those centrioles until late mitosis or early G1 (Nigg 2007). Centriole disengagement appears to require separase, and the disengagement is hypothesized to control centriole duplication in the next cell cycle. The involvement of separase in both centriole disengagement and sister chromatid separation would prevent premature centriole disengagement before anaphase onset, and thereby inhibit the formation of multipolar spindles as well as genomic instability.

2.11 Future Prospects

Studies on chromosome segregation began to have a real cellular basis after the discovery of CDKs in 1988, and a significant impact after finding the connection between cell cycle control and chromosome segregation through the shared destruction mechanisms of poly-ubiquitylated cyclin and securin. Importantly, the research area is still expanding. Indeed, a PubMed search for chromosome segregation hit 4,135 original papers; half of which were published since 2002. We hope that the fundamental issues discussed here will inspire young researchers interested in chromosome and cellular biology. Their contributions during the next 10 years promises the discovery of novel genes and gene functions that underlie chromosome segregation and ensure the fidelity of the process in various organisms.

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